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Sébastien Alfonso

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR
DE L'UNIVERSITÉ DE MONTPELLIER

En écologie et biodiversité

École doctorale GAIA

Laboratoire Adaptation et Adaptabilité des Animaux et des Systèmes, Ifremer (UMR MARBEC)
Laboratoire Ressources Halieutiques de La Rochelle, Ifremer

Interactions entre traits de personnalité des téléostéens, environnement et polluants

Présentée par Sébastien Alfonso
le 29 novembre 2018

Sous la direction de Marie-Laure Bégout

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Introduction générale

1. L'environnement, une source de stress

1.1. Le stress et les facteurs de stress

1.1.1. Le stress

L'environnement est défini comme l'ensemble des facteurs biotiques et abiotiques qui entourent un individu ou une espèce (Fischesser et Dupuis-Tate, 1996). Les facteurs abiotiques représentent l'ensemble des paramètres physico-chimiques caractérisant un écosystème et les facteurs biotiques représentent l'ensemble des interactions intra- ou interspécifiques entre les organismes vivants (Fischesser et Dupuis-Tate, 1996). Les téléostéens, évoluant dans un environnement aquatique (eau douce, salée ou saumâtre), les facteurs abiotiques directeurs des performances biologiques (*sensu*: Fry, 1947, 1971) sont principalement liés à la qualité de l'eau (température, concentration en oxygène, dioxyde de carbone, pH, turbidité, illumination, présence de polluants, *etc.*). Concernant les facteurs biotiques, les téléostéens sont soumis aux mêmes facteurs que les espèces évoluant dans un environnement terrestre. On peut par exemple noter les différentes interactions intraspécifiques liées aux activités de nourrissage, la compétition pour un territoire ou pour l'accès à la reproduction. Les interactions interspécifiques comme la prédation ou la compétition font également partie des facteurs biotiques auxquels un téléostéen est confronté dans son environnement (**Figure 1**).

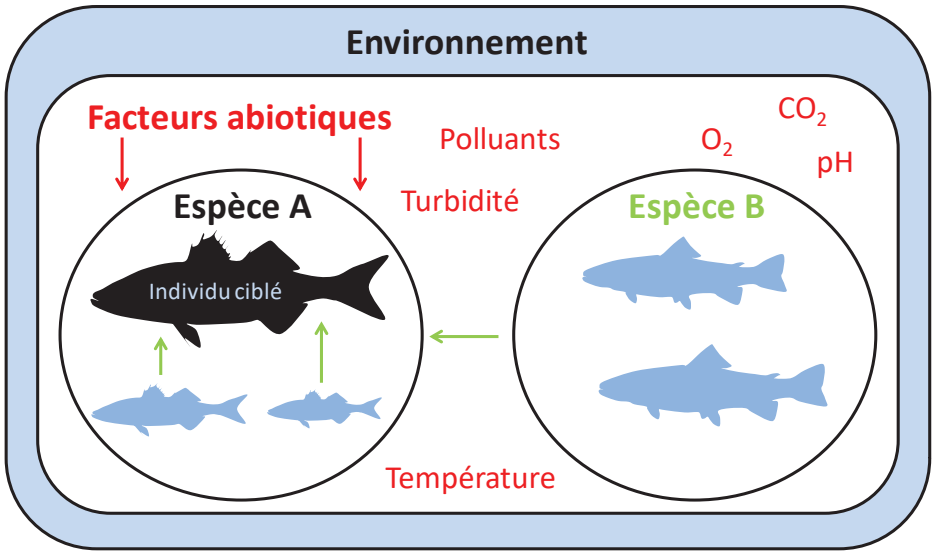

Figure 1. L'environnement, incluant les facteurs biotiques et abiotiques, et leur action sur l'individu/l'espèce cible. La couleur verte se réfère aux facteurs biotiques et la couleur rouge aux facteurs abiotiques. D'après Fischesser et Dupuis-Tate (1996).

L'environnement, à travers les facteurs biotiques et abiotiques qui le caractérisent, exerce une pression sur l'individu. En réponse à cette pression, une cascade d'événements intervient au sein de l'organisme afin de maintenir ses constantes physiologiques stables. Cette notion de stabilité physiologique de l'organisme est appelée l'homéostasie (Korte et al., 2005). Des variations de l'environnement peuvent venir perturber cette stabilité physiologique de l'organisme et entraîner un stress pour l'individu qui doit revenir à son état de stabilité. Le stress a été défini pour la première fois en 1950 comme une réponse non spécifique de l'organisme à toutes les pressions exercées sur celui-ci (Selye, 1950). Depuis, le concept de stress a évolué et reçu de multiples définitions:

- Un état produit par un facteur environnemental qui étend la réponse adaptative d'un individu au-delà de sa plage normale (Brett, 1958).
- La somme des réponses physiologiques qui apparaissent lorsque l'individu tente de rétablir son homéostasie (Wedemeyer et Mcleay, 1981).
- L'altération d'une ou de plusieurs variables physiologiques au point que la survie peut être altérée (Bayne, 1985).
- Le stress est un état causé par un facteur de stress, ou stresseur, qui dévie d'un état normal où l'homéostasie est maintenue (Barton et Iwama, 1991).
- Un état homéostatique menacé qui est rétabli par une suite complexe de réponses adaptatives (Chrousos, 1998).
- Le stress est une condition dans laquelle une demande environnementale dépasse les capacités normales de régulation de l'organisme (Koolhaas et al., 2011).

Toutes ces définitions sont relativement proches et se rejoignent sur de nombreux points. **Le stress** peut donc être défini comme la cascade d'événements physiologiques mis en place par l'individu afin de maintenir son homéostasie suite à la variation de facteurs environnementaux, appelées facteurs de stress. L'ensemble des processus de maintien d'une certaine stabilité par le changement (*stability through change*) permettant à l'individu de maintenir son d'homéostasie à travers des mécanismes physiologiques, est appelé allostasie (McEwen et Wingfield, 2003).

Ces concepts de stress, d'homéostasie et d'allostasie sont tous intégrés dans celui de « bien-être » (Veissier et Boissy, 2007). En effet le bien-être est défini comme un état d'harmonie entre l'animal et son environnement, aboutissant à la complète santé mentale et physique (Broom, 1993). Broom (1987) propose de partir du concept de *coping*, littéralement «je fais

avec», qu'il utilise au sens de **l'adaptation à l'environnement**, en relation avec l'homéostasie. Si l'individu puise dans ses réserves pour s'adapter, alors le bien-être est faible et inversement. Cela correspond en physiologie à des modifications internes en réponse à des *stimuli* externes. Dans un état de bien-être, l'individu peut s'épanouir et prospérer. Face à des conditions environnementales plus contraignantes ou changeantes, l'individu peut tout de même maintenir son état de bien-être en s'adaptant grâce aux ajustements comportementaux et/ou physiologiques pertinents (allostasie). En revanche, lorsque le poids des facteurs environnementaux est trop important pour l'individu, il ne peut pas s'adapter et se retrouve en situation de souffrance. Lors de cette situation de souffrance, la stabilité physiologique de l'individu (homéostasie) ne peut être maintenue, de même que sa santé physique et mentale, et cela peut entrainer l'apparition de pathologies pouvant déboucher sur une altération de la croissance, de la reproduction et de la survie (Veissier et al., 2007).

Lorsqu'on considère en particulier la dimension mentale, alors le bien-être est considéré comme une expérience subjective de l'animal, un état mental correspondant à l'absence d'émotions négatives (peur, faim, soif, douleur, *etc.*) et vraisemblablement à la présence d'émotions positives (confort, plaisir; Dawkins (1983); Duncan (2005)). Ici, le terme « expérience subjective » renvoie à **l'individualité de la perception du stress**, et donc la réponse sera étroitement liée à la personnalité de l'individu définie ci-après en détail. C'est pourquoi l'étude des **traits de personnalité** peut aider à mieux comprendre le **registre adaptatif** des animaux et donc le potentiel de bien-être individuel.

1.1.2. Les facteurs de stress

Chez les téléostéens, les facteurs de stress peuvent être séparés en 3 types : le stress « chimique », le stress « physique » et le stress « perçu » (Barton (2002); **Figure 2**). Le stress chimique regroupe différents facteurs abiotiques tel que la qualité d'eau (concentration en oxygène, pH, concentration en polluants, acidité, *etc.*). Le stress physique fait surtout référence au transfert et à la manipulation des individus, et intervient principalement en milieu expérimental ou artificiel. Le dernier type de stress est le stress perçu qui est principalement lié aux facteurs biotiques de l'environnement comme la présence de prédateur ou les différents *stimuli* entrainant des réponses physiologiques (*i.e.* des modifications internes en réponse à des stimuli externes). Ces réactions ne sont déclenchées que si l'animal perçoit la situation comme une menace pour lui (Mason, 1971), il peut s'agir par exemple de l'anticipation de la survenue d'un désagrément telle que la présence de prédateur.

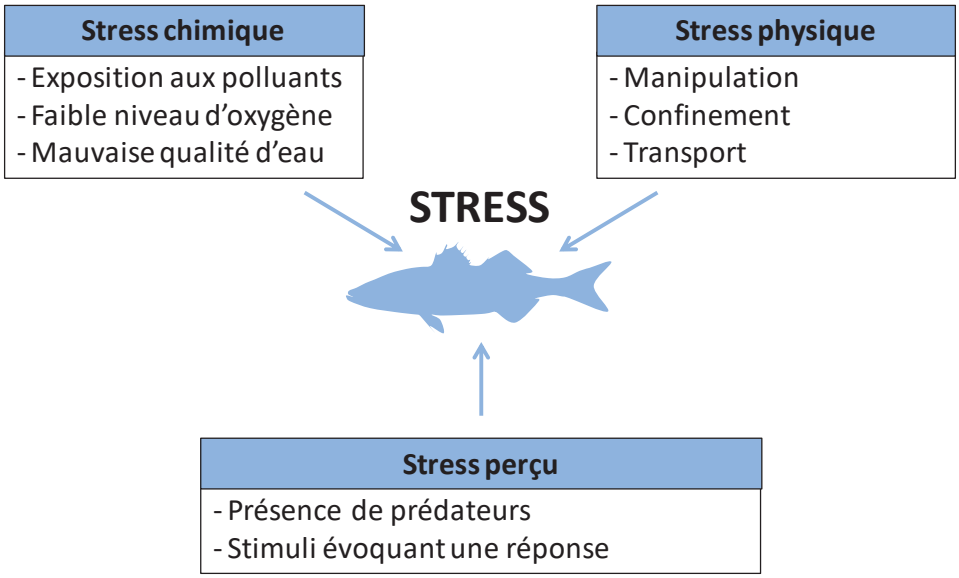

Figure 2. Les différents facteurs de stress. D'après Barton (2002).

Un autre facteur de stress, non présenté par Barton (2002) est introduit par Galhardo et Oliveira (2009), il s'agit du « stress psychologique ». Le stress psychologique pourrait être considéré comme intermédiaire entre stress perçu et stress physique. Chez les mammifères, les facteurs de stress psychologiques se distinguent des facteurs de stress physique du fait qu'il ne doivent pas débuter du corps mais débuter dans l'esprit (Lovallo, 2005). Cependant pour être perçus comme tels par les téléostéens, il faut supposer que ces derniers arrivent à percevoir et évaluer l'importance de ces facteurs dans un premier temps et dans un deuxième temps, que les mécanismes d'adaptation soient évalués mentalement afin que l'individu puisse y faire face (Galhardo et Oliveira, 2009). Ces dernières années, les scientifiques se sont questionnés sur le fait que les téléostéens possédaient ou non des capacités psychologiques suffisantes pour l'évaluation mentale de situations stressantes. De ce fait, ce facteur de stress est bien moins souvent abordé (Galhardo et Oliveira, 2009; Rose, 2002, 2007) bien que de nouvelles preuves semblent démontrer que les téléostéens sont, eux aussi, dotés d'une conscience (Chetroo et al., 2004a, b; Le Neindre et al., 2018; Rey et al., 2015; Sneddon, 2003, 2004). Les facteurs de stress psychologiques tels que définis par Galhardo et Oliveira (2009) seraient donc principalement l'environnement social, incluant la formation de hiérarchie et la compétition pour l'accès aux territoires et aux ressources, croisant ainsi également avec la définition de facteurs de stress perçus.

Que ce soit en milieu naturel ou en milieu contrôlé (élevage ou expérimentation animale), les individus sont soumis à différents facteurs de stress chimiques. La concentration en oxygène (O_2), en dioxyde de carbone (CO_2), en azote ammoniacal, le pH ou la salinité font partie des facteurs principaux permettant de qualifier une eau. Le réchauffement climatique planétaire actuel, qui entraine une diminution des concentrations en O_2 et une élévation du niveau de CO_2 dans les océans, est une source de stress potentielle pour les téléostéens qui alerte les scientifiques du monde entier (Mccarthy et Houlihan, 1996; Mohseni et al., 2003; Root et al., 2003). Outre le réchauffement climatique, la présence et la concentration de nombreux polluants dans les écosystèmes aquatiques est également au cœur des préoccupations de notre société (Jones et de Voogt, 1999; Lecomte et al., 2017; Rios et al., 2007; Robinson et al., 2017).

Les sections suivantes **1.2 et 1.3** visent à présenter les différents facteurs de stress étudiés dans le cadre de cette thèse et la pertinence de leur étude en fonction du contexte actuel.

1.2. Les polluants organiques persistants

Au cours du siècle dernier, plusieurs milliers de produits chimiques ont été inventés, et leur production a été multipliée par 400 entre les années 1930 et 2000 (Tordjman, 2009). Parmi ces polluants massivement produits et utilisés lors du 20ème siècle, il y a une famille de produits chimiques qui sont persistants dans l'environnement et partagent des propriétés communes: les polluants organiques persistants (POP; Jones et de Voogt, 1999). Ces polluants sont hydrophobes, lipophiles, pas ou peu métabolisés par les organismes et ont un long temps de demi-vie dans l'environnement (Gray, 2002; Jones et de Voogt, 1999; Luo et al., 2007). Par conséquence, ils peuvent s'accumuler dans les différents compartiments de l'environnement et persister sur de longues périodes (Jones et de Voogt, 1999).

Parmi ces polluants organiques persistants, on peut distinguer *e.g.* des pesticides (DDT), des cosmétiques, des produits chimiques industriels tels que les polychlorobiphényles (PCB) et les polybromodiphényléthers (PBDE).

Les polychlorobiphényles (PCB) sont composés d'un noyau biphényle pouvant accueillir 1 à 10 atomes de chlore (**Figure 3.A**). Deux cent neuf congénères de PCB existent en fonction du nombre et de la position des atomes de chlore sur le noyau biphényle (McFarland et Clarke, 1989). En raison de leurs propriétés chimiques et de leur forte stabilité, ces molécules ont été

utilisées dans plusieurs domaines industriels dans des systèmes fermés en tant que fluides hydrauliques dans les transformateurs ou les condensateurs (Breivik et al., 2002a). Les PCB étaient également utilisés dans des systèmes ouverts en tant que bactéricides et insecticides, en tant qu'additifs, stabilisants ou agents ignifuges.

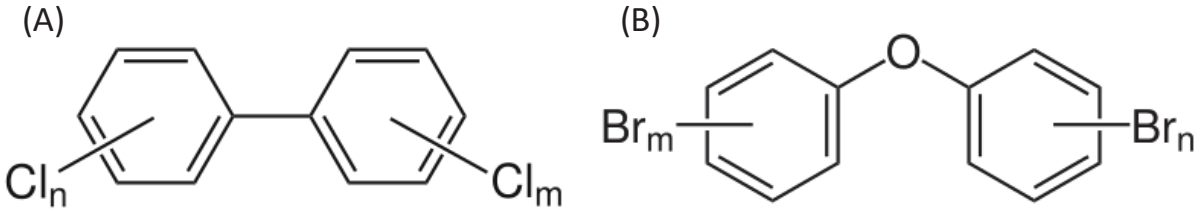

Figure 3. (A) Structure d'une molécule de PCB. D'après McFarland et Clarke (1989); **(B)** Structure d'une molécule de PBDE. D'après Rahman et al. (2001).

Les polybromodiphényléthers (PBDE) sont des molécules composées d'un noyau diphényléther pouvant contenir de 1 à 10 atomes de brome (**Figure 3.B**). Comme pour les PCB, il existe 209 congénères de PBDE en fonction du nombre et de la position des atomes de brome (Rahman et al., 2001). Les propriétés de ces molécules, y compris leur stabilité, les rendent très utilisés comme ignifugeants dans une large gamme de produits industriels mais également dans des produits de la vie courante tels que les équipements électroniques, les matériaux de construction, le revêtement de véhicules ou le traitement du bois (Darnerud et al., 2001; Rahman et al., 2001).

Les PCB et les PBDE sont produits et utilisés sous la forme de mélanges techniques (*e.g.* pour les PCB, les aroclor, les pyralènes et pour les PBDE, le pentaBDE). La composition de ces mélanges techniques pouvait varier en proportion relative de différents congénères. Par exemple pour l'aroclor1254, la concentration de CB-126 pouvait varier de 0.2 à 3.2 $mg.g^{-1}$ et pour le pentaBDE la proportion de BDE-47 variait entre 38 et 42% (Kodavanti et al., 2001; La Guardia et al., 2006). Ceci explique la grande variabilité des concentrations et proportions relatives des différents congénères de PCB et de PBDE dans l'environnement.

Après de nombreuses alertes sur leur toxicité, la production et l'usage des PCB ont été régulés depuis les années 1970 (Breivik et al., 2002b) et depuis les années 2000 pour les PBDE (Kemmlein et al., 2009). Cette régulation, bien que nécessaire, intervient assez tardivement car les PCB et les PBDE avaient déjà eu le temps de se retrouver dans l'environnement principalement *via* le lessivage des sols, le rejet des eaux usées et les retombés atmosphériques (Breivik et al., 2002a; Darnerud et al., 2001; de Wit, 2002). A cause de leur

persistance, ils sont toujours détectés dans l'environnement et notamment dans les écosystèmes aquatiques (de Wit, 2002; Muir et al., 2003; Shaw et Kannan, 2009). Les PCB et les PBDE sont toujours retrouvés dans les sédiments marins autour du globe (Eljarrat et Barcelo, 2004; Eljarrat et al., 2004; La Guardia et al., 2007; Luo et al., 2007; Robinson et al., 2017). Chez les vertébrés, l'exposition à ces polluants se fait majoritairement par exposition alimentaire, qui est la principale voie d'entrée des PCB et PBDE dans l'organisme (Muir et al., 2003; Nyman et al., 2002).

Le risque associé à l'exposition à des congénères uniques de PCB ou de PBDE commence à être bien connu et considéré comme un facteur de stress par la communauté scientifique. Les effets d'une exposition aux PCB et PBDE sur le comportement et la physiologie des téléostéens seront développés dans la **section 3.1**.

1.3. Les conditions environnementales en système d'élevage

Comme évoqué précédemment, la concentration en oxygène (O_2), en dioxyde de carbone (CO_2), le pH, la concentration en azote ammoniacal et la salinité font partie des principaux facteurs permettant de caractériser une eau vis-à-vis des téléostéens. En milieu naturel, ces facteurs sont soumis aux fluctuations des conditions environnementales. En milieu contrôlé, comme son nom l'indique, il incombe aux personnes en charge de l'élevage ou de l'expérimentation, grâce aux infrastructures dédiées, de maintenir les individus dans des conditions proches de leur *preferendum* afin de leur garantir un état de bien-être.

L'augmentation de la demande pour les produits de la mer et la diminution simultanée des pêcheries naturelles a conduit à une expansion importante de l'aquaculture au cours des dernières décennies (Conte, 2004). L'aquaculture existe sous de multiples formes, l'éleveur peut être fortement dépendant du milieu naturel (cages en mer, pisciculture d'étang) ou maitriser totalement les facteurs du milieu (circuit fermé avec eau recirculée). La production de téléostéens, issue de l'aquaculture, a augmenté d'environ 20 millions de tonnes entre 2009 et 2014 pour atteindre 73,8 millions de tonnes en 2014 (FAO, 2016). Représentant seulement 38 % de la ressource exploitée totale (aquaculture et capture) en 2009, la production liée à l'aquaculture représente plus de 44% en 2014 (**Figure 4.A, B**).

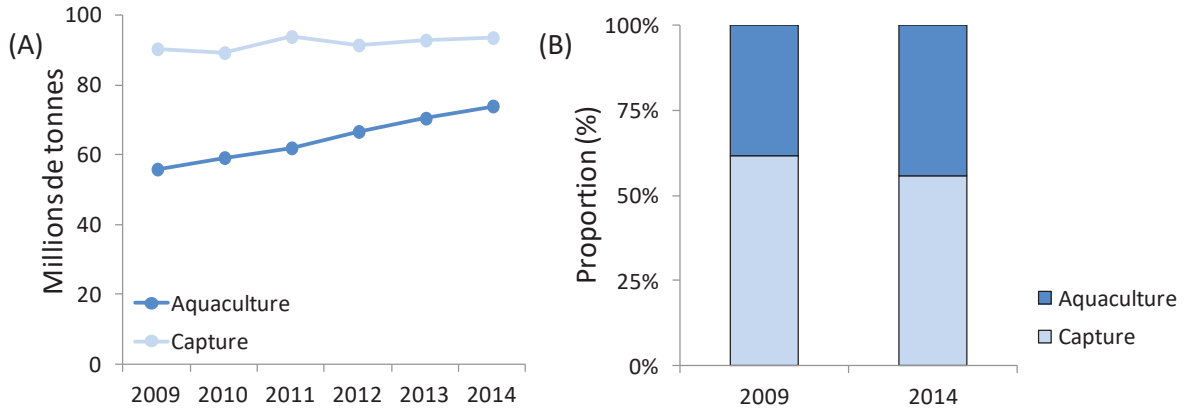

Figure 4. (A) Evolution de la production de téléostéens issus de l'aquaculture ou des captures de 2009 à 2004. D'après FAO (2016); **(B)** Evolution de la proportion des téléostéens issus de l'aquaculture ou des captures entre 2009 et 2014. D'après FAO (2016).

L'aquaculture est maintenant reconnue comme une importante industrie de production alimentaire. Ainsi, de même que pour l'industrie alimentaire terrestre, les préoccupations concernant la durabilité, les problèmes environnementaux et le bien-être animal en aquaculture sont en constante augmentation (Ashley, 2007; Conte, 2004; Hixson, 2014; Martins et al., 2010, 2012). Parmi ces préoccupations, celles liées au bien-être, notamment les variations de la qualité d'eau en milieu contrôlé (circuit fermé), sont celles qui nous intéressent dans le cadre de cette thèse.

1.3.1. La qualité de l'eau en milieu d'élevage

Dans des conditions d'aquaculture en milieu fermé, la recherche d'une forte productivité, couplée aux contraintes économiques, limitent parfois la présence de personnel et l'apport d'eau neuve. Cela conduit donc à des variations des facteurs de qualité de l'eau tels que la température, l'oxygène (O_2), le dioxyde de carbone (CO_2), le pH, et/ou l'azote ammoniacal total (AAT ou TAN en anglais) dans les élevages. Ces variations, lorsqu'elles atteignent un certain seuil de concentration et/ou de durée, en fonction des *preferendum* de l'espèce, peuvent donc devenir un stress pour les téléostéens, altérer leur état de bien être, pour finalement avoir des conséquences économiques négatives pour les éleveurs (Conte, 2004).

L'oxygène est particulièrement important car il joue un rôle vital dans les processus de respiration et dans le métabolisme énergétique des organismes aérobies, incluant les téléostéens. Des variations, diminution mais aussi augmentation, trop importantes en oxygène peuvent donc constituer un stress chimique pour l'organisme (Berschick et al., 1987; Chabot et Claireaux, 2008; Diaz et Breitburg, 2009; Espmark et Baeverfjord, 2009).

L'AAT est la somme de l'ion ammonium (NH_4^+) et de l'ammoniac (NH_3) présents dans le milieu. L'azote ammoniacal est plus toxique sous sa forme non-ionisée (NH_3) que sous sa forme ionique (NH_4^+; Camargo et Alonso (2006)). La présence d'AAT dans les élevages est principalement due au catabolisme des protéines inclues dans les aliments et/ou à l'excès de nourrissage *i.e.* le gaspillage (Person-Le Ruyet et Boeuf, 1998). De faibles apports d'eau neuve peuvent donc induire le stockage d'AAT, ainsi que l'accumulation de nitrites et nitrates après transformation de l'AAT par les bactéries du système d'élevage, et constituer une source de stress potentielle pour les téléostéens (Dosdat et al., 2003; Gao et al., 2017; Lemarié et al., 2004; Person-Le Ruyet et al., 1995; Shingles et al., 2001).

La concentration en CO_2, le pH ou bien encore la quantité de matière en suspension sont également des facteurs très importants suivis par l'éleveur et pouvant également constituer une source de stress chez les téléostéens (Borch et al., 1993; Colson et al., 2015; Sadoul et al., 2017b).

1.3.2. Les manipulations

En plus des stress chimiques présentés dans la section précédente auxquels les téléostéens sont soumis en condition d'aquaculture, ils sont également exposés à de nombreux stress physique. En effet, le stress physique intervient en milieu contrôlé lorsque l'individu est confronté à la manipulation par l'homme (Barton, 2002). En aquaculture, la manipulation est réalisée pour permettre la mesure de véritables d'intérêts comme la croissance ou bien le transfert et/ou le tri des cheptels entre bassins. Elle peut aussi être couplée à un isolement social de l'individu pour cause de risques pathologiques ou de blessures par exemple. Les manipulations peuvent entrainer des blessures chez l'individu et constituer un stress physique mais également avoir des conséquences psychologiques, bien que non catégorisées comme telles par Galhardo et Oliveira (2009), et poser questions en terme de bien-être animal (Ashley, 2007). En effet, l'introduction dans un nouvel environnement, l'isolement social ou le confinement, peuvent être une source très importante de stress chez les téléostéens (Auperin et al., 1997; Rotllant et al., 2003; Shams et al., 2015; Strange, 1992; Young et Cech, 1993).

Même si les manipulations ne constituent pas un facteur de stress dont il était prévu d'étudier les réponses durant ma thèse, elles sont souvent nécessaires à la mise en place d'expérimentations. La réponse des individus à cette source de stress ne constituera donc pas un objet d'étude à part entière mais sera abordée et discutée au cours de cette thèse.

La section suivante vise à présenter plus en détail les différentes réponses physiologiques et comportementales mises en œuvre par les téléostéens pour faire face à divers facteurs de stress et maintenir un état homéostatique compatible avec leurs performances biologiques et/ou leur bien-être.

2. La régulation du stress, des mécanismes communs mais une variabilité interindividuelle

2.1. La régulation moléculaire du stress

Les mécanismes endocriniens liés à la régulation du stress sont fortement conservés chez les vertébrés (Galhardo et al., 2011; Martins et al., 2011a; Øverli et al., 2005; Schjolden et Winberg, 2007). Chez les téléostéens, suite à un stress, le système nerveux central (SNC) active en parallèle l'axe hypothalamus-pituitaire-interrénale (HPI) et le système nerveux sympathique (SNS) aboutissant à la libération d'hormones (Barton, 2002; Sumpter, 1997; Winberg et Nilsson, 1993). Ces hormones vont avoir un rôle de médiateurs afin de permettre à l'individu de faire face et de s'adapter au facteur de stress auquel il est soumis (Barton, 2002).

2.1.1. L'axe hypothalamus-pituitaire-interrénale

L'axe HPI est impliqué dans la réponse au stress avec la production d'hormones glucocorticoïdes et principalement le cortisol chez les téléostéens (Sadoul et Vijayan, 2016; Schreck et Tort, 2016). Brièvement, un stress induit la production de corticolibérine (hormone cortico-releasing, CRH) par l'hypothalamus qui va ensuite entraîner la production d'hormone adreno-cortico-tropin (ACTH) par la glande pituitaire pour finalement aboutir à la libération de cortisol par la glande interrénale (Sumpter, 1997; Wendelaar Bonga, 1997; **Figure 5**).

Ensuite, les récepteurs à glucocorticoïdes (*gr1 et gr2*) et minéralocorticoïdes (*mr*), sont retrouvés dans de nombreux tissus chez les téléostéens (Faught et al., 2016) et connus pour être activés en présence de cortisol (Bury et al., 2003; Colombe et al., 2000; Kiilerich et al., 2018a). Ils sont impliqués dans le rétrocontrôle négatif du cortisol (Alderman et al., 2012; Bernier et al., 2009; Johansen et al., 2011) bien que les mécanismes associés soient encore à déterminer (Kiilerich et al., 2018b; Prunet et al., 2006).

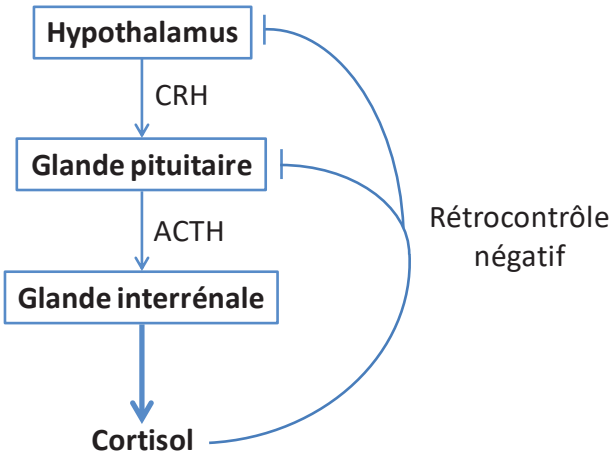

Figure 5. Régulation de l'axe hypothalamus-pituitaire-interrénale (HPI). D'après Wendelaar Bonga (1997).

2.1.2. Les systèmes monoaminergiques

D'un autre côté, l'activation du système nerveux sympathique va déclencher la production de catécholamines telles que la dopamine (DA) et de la norépinephrine ou noradrénaline (NE) (Øverli et al., 2007; Reid et al., 1998; Winberg et Nilsson, 1993). Ces deux catécholamines sont synthétisées à partir de tyrosine (TYR). Premièrement, TYR est hydroxylée en 3,4-dihydroxyphenylalanine (L-DOPA), puis décarboxylée pour former la dopamine. La norépinephrine est alors produite par hydroxylation de la dopamine (Winberg et Nilsson, 1993). Les métabolites majeurs de la dopamine et de la norépinephrine sont respectivement 5-dihydroxyphenylacetic (DOPAC) et 3-methoxy-4-hydroxyphenylglycol (MPHG ; **Figure 6**).

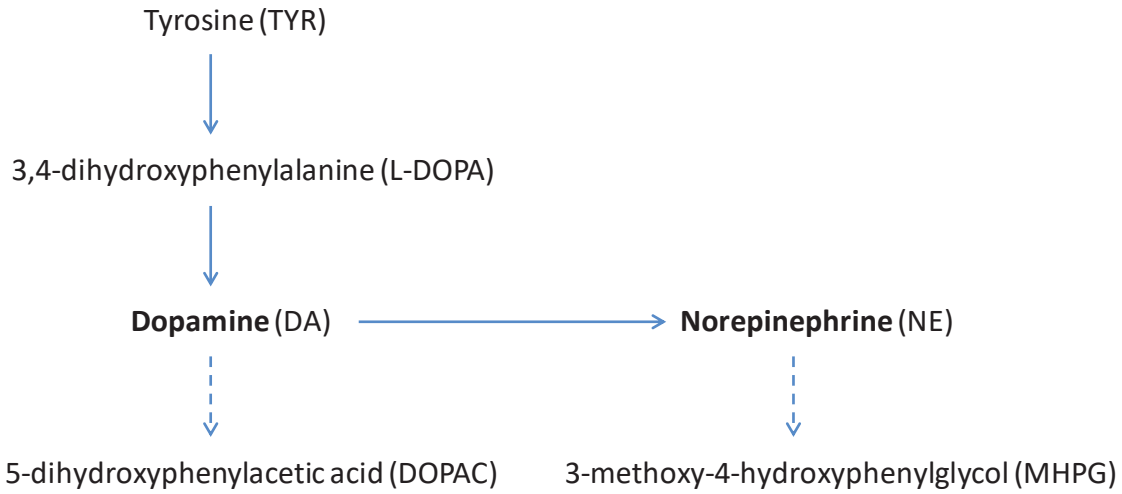

Figure 6. Voie de biosynthèse de la dopamine (DA) et de la norépinephrine (NE). Les flèches pleines représentent les différentes étapes de la synthèse de la sérotonine et les flèches hachurées représentent la métabolisation de la dopamine et de la norépinephrine. D'après Winberg et Nilsson (1993).

En plus de la réponse du système nerveux sympathique, le système sérotonergique amenant à la synthèse de la sérotonine (5-HT) apparait également très important dans la régulation du stress (Gesto et al., 2013; Øverli et al., 2007; Winberg et Nilsson, 1993). La sérotonine est synthétisée à partir du tryptophane (TRP). Brièvement, TRP est hydroxylé en 5-hydroxytryptophan (5-HTP). Ensuite, 5-HTP est décarboxylé en sérotonine (5-hydroxytryptamine, 5-HT) dont le métabolite majeur est l'acide 5-hydroxyinoleacetic (5-HIAA, Winberg et Nilsson (1993); **Figure 7**).

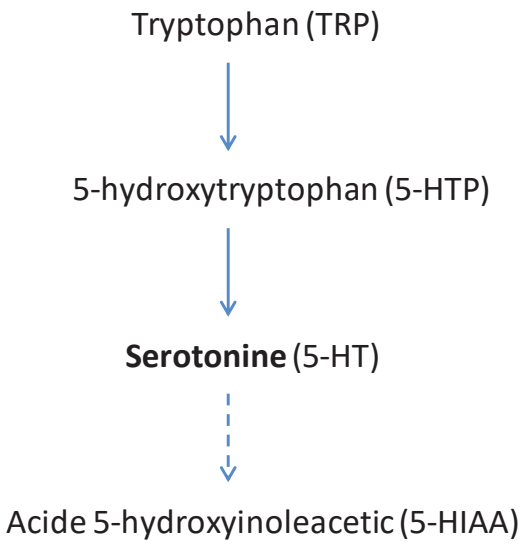

Figure 7. Voie de biosynthèse de la sérotonine (5-HT). Les flèches pleines représentent les différentes étapes de la synthèse de la sérotonine et les flèches hachurées représentent la métabolisation de la sérotonine. D'après Winberg et Nilsson (1993).

Ces monoamines sont produites dans le cerveau et libérées ensuite vers le système cardiovasculaire (Lillesaar, 2011; Reid et al., 1998). Que ce soit pour la dopamine, la norépinephrine ou la sérotonine, le rapport entre la concentration dans les tissus cérébraux et leur métabolite principal est utilisé respectivement comme indicateur de l'activité dopaminergique, noradrénergique et sérotoninergique de l'individu (Gesto et al., 2013; Øverli et al., 2001; Shannon et al., 1986).

La libération de ces différentes hormones présentées précédemment (cortisol, dopamine, norépinephrine et sérotonine) va constituer la réponse primaire de l'individu face au stress (Mazeaud et al., 1977; Reid et al., 1998; Sumpter, 1997; Wendelaar Bonga, 1997). La production et la libération de ces hormones va ensuite entraîner un ajustement des réponses moléculaires et physiologiques de l'individu. Brièvement, l'individu va mobiliser l'énergie disponible *via* la dégradation des réserves glucidiques et lipidiques et l'oxygénation

musculaire afin de faire face au stress. Ces ajustements afin de maintenir l'homéostasie sont appelées les réponses secondaires (Iwama et al., 2004; Pickering, 1981). Les conséquences d'un stress sur les réponses primaires et secondaires d'un individu sont largement dépendantes de la durée et de l'intensité du stress (Fatira et al., 2014). En conséquence, l'énergie allouée par l'individu pour y faire face ne pourra plus être allouée aux performances biologiques de l'individu comme par exemple la croissance (Sadoul et Vijayan, 2016). De ce fait, à intensité égale, un stress chronique sera plus préjudiciable qu'un stress aigu pour les performances biologiques de l'individu devant constamment allouer de l'énergie pour maintenir son état d'homéostasie. Enfin, les réponses tertiaires interviennent, elles se réfèrent aux adaptations comportementales mises en place et incluent également des altérations de performances avec des répercussions sur les traits d'histoire de vie (survie, croissance, reproduction) suite à un stress (Barton, 2002; Wedemeyer et al., 1990).

La figure suivante résume les **sections 2.1.1** et **2.1.2** et illustre les réponses primaires, secondaires et tertiaires d'un individu suite à un stress (**Figure 8**).

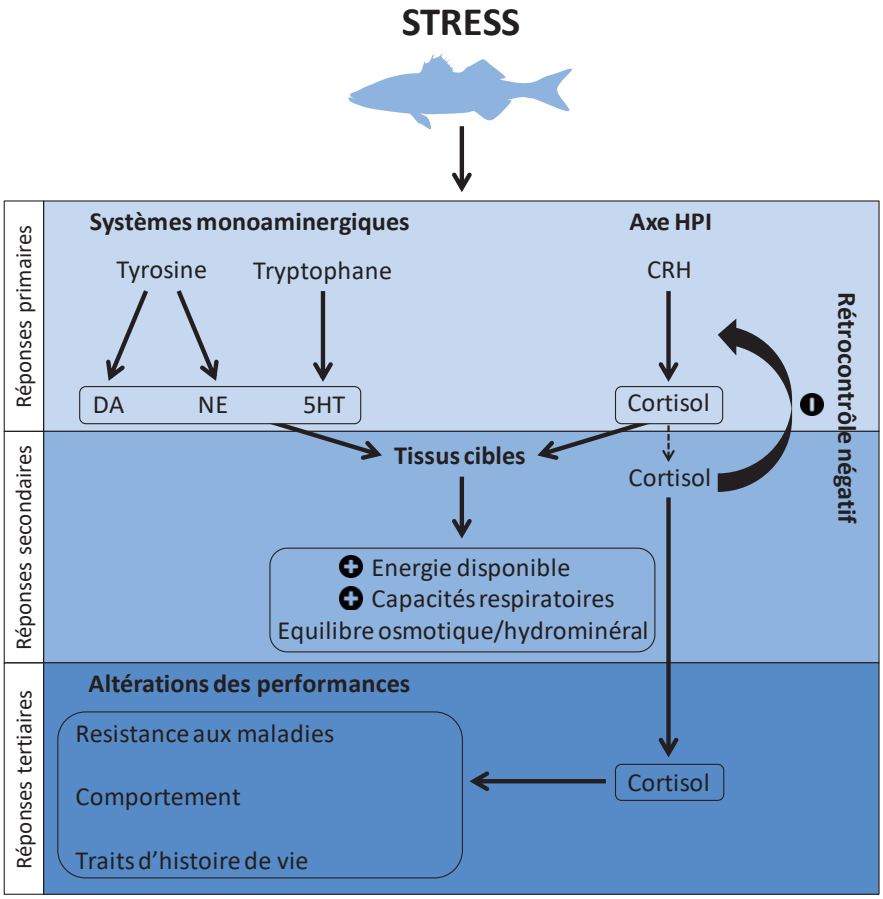

Figure 8. Les réponses primaires, secondaires et tertiaires d'un individu suite à un stress. D'après Barton (2002).

2.1.3. L'activité et la plasticité neuronale

Les réponses physiologiques et comportementales intervenant chez l'individu suite à un stress semblent aussi être accompagnées de changement dans l'activité et la plasticité neuronale du système nerveux central (Johansen et al., 2012; Sorensen et al., 2011; Sorensen et al., 2013).

Chez les téléostéens, différents marqueurs sont connus pour être impliqués dans l'activation et la différentiation des neurones. Par exemple, les facteurs de transcription *c-fos* et *egr1* renseignent tous deux sur l'activité neuronale (Burmeister et Fernald, 2005; Duncan et al., 1996). Le gène *pcna* (codant un facteur important pour la réplication de l'ADN) est considéré comme un marqueur de l'activité proliératrice (Eisch et Metyam, 2007; Metyam et al., 2007). Enfin, le facteur de transcription *neurod* est impliqué dans la différentiation neuronale et la survie des nouveaux neurones immatures (Korzh et al., 1998; Steiner et al., 2006).

Même si d'autres mécanismes existent, des études suggèrent que le stress et les hormones corticostéroïdes, incluant le cortisol, impactent le comportement de la manière suivante : un stress aigu va stimuler la réactivité comportementale alors qu'un stress chronique aura tendance à l'inhiber (Haller et al., 1997; Øverli et al., 2002; Seti et al., 1996). De la même manière, un stress aigu est connu pour stimuler la prolifération et la différentiation des cellules neuronales alors qu'un stress chronique a tendance à l'inhiber (Chen et al., 2015; Joels et al., 2006; Johansen et al., 2012; Sorensen et al., 2013; **Figure 9**).

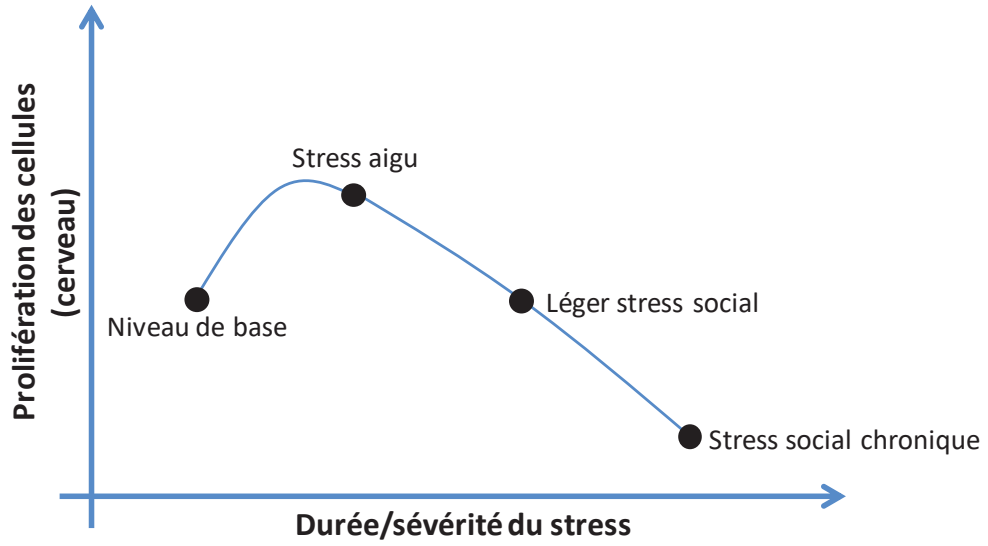

Figure 9. Effet diphasique du stress en fonction de la durée/sévérité sur la prolifération de cellules cérébrales. D'après Sorensen et al. (2013).

Cette relation a été démontrée par plusieurs études menées chez la truite arc-en-ciel (*Oncorhynchus mykiss*). Un stress aigu va augmenter l'expression de *pcna* dans le cerveau des individus (Johansen et al., 2012). Un léger stress social ne va pas causer de modification de l'expression de *pcna* dans le cerveau des truites alors qu'un stress social chronique va conduire à une diminution drastique du niveau de *pcna* dans le cerveau (Sorensen et al., 2011, 2012).

Les indicateurs de stress tels que le cortisol, la dopamine, la norépinephrine et la sérotonine, les gènes impliqués dans la libération et la régulation du cortisol (*crf*, *gr*, *mr*), ainsi que les marqueurs d'activité et de plasticité neuronale, sont donc des variables importantes à mesurer pour étudier la réponse individuelle face au stress. Comme évoqué brièvement dans la **section 2.1.2**, le comportement fait partie des réponses tertiaires au stress et c'est donc, à ce titre, un indicateur de stress pertinent et intégrateur chez les téléostéens (Martins et al., 2012), et sera détaillé dans la suite de cette introduction.

2.2. Le comportement

Le comportement est défini comme l' « ensemble des processus de décision par lesquels les individus ajustent leur état et leur situation par rapport aux variations biotiques et abiotiques du milieu » (Danchin et al., 2005). Konrad Lorenz, Nikolaas Tinbergen et Karl von Frisch sont les premiers à mettre en lumière l'étude du comportement animal, l'éthologie, grâce à leurs travaux sur l'organisation et le déclenchement de séquences comportementales et sociales. Leurs découvertes, notamment sur la danse des abeilles ou la notion d'empreinte, les conduiront au prix Nobel de physiologie et de médecine en 1973.

Depuis, l'éthologie est devenue une discipline présente dans de nombreux domaines de recherche incluant la physiologie, l'écologie, la génétique, la neuroscience, l'écotoxicologie et l'aquaculture (Ferrari et al., 2014a, 2016; Geffroy et al., 2015, 2018; Kalueff et al., 2016; Réale et al., 2007; Thornqvist et al., 2018; Vignet et al., 2015, 2014b; Vindas et al., 2017). Ces connections entre l'éthologie et les autres domaines de recherche ont permis une avancée significative de notre compréhension des relations entre environnement, physiologie et comportement. Dans cette partie, nous allons discuter brièvement d'une partie de ces avancés, notamment sur les concepts liés au comportement et les mécanismes physiologiques identifiés comme sous-jacents et/ou prédictifs.

2.2.1. La personnalité animale

Ce concept de personnalité, transposé aux animaux, est issu de la psychologie humaine. En effet chez l'homme, la personnalité est décrite comme « l'ensemble des traits physiques et moraux par lesquels une personne est différente des autres » (Larousse, 2017). La personnalité est caractérisée par 5 axes: les 5 grands facteurs (*Big factor model*) selon la célèbre théorie de John (1990). Ces 5 grands facteurs sont l'extraversion, le névrosisme, l'agréabilité, la consciencioisité et l'ouverture (**Tableau 1**).

Plus tard, Gosling et John (1999) ont mis en évidence une analogie entre 3 traits de personnalité décrits chez l'homme et des traits décrits chez 12 espèces animales. L'axe de l'extraversion, du névrosisme et de l'agréabilité semblent être commun chez toutes ces espèces. Gosling est le premier à parler de personnalité chez les animaux non humains (Gosling, 2001) mais basculera ensuite vers le terme tempérament par souci d'éviter l'anthropomorphisme (Jones et Gosling, 2005; Vazire et Gosling, 2004).

Tableau 1. Synthèse des 5 grands facteurs de la personnalité chez l'homme. D'après John (1990).

Axe de la personnalité	Exemple de facettes impliquées dans l'axe
Extraversion vs Introversion	Socialité, activité, émotions positives
Névrosisme vs Stabilité émotionnelle	Anxiété, dépression, vulnérabilité au stress
Agréabilité vs Antagonisme	Compassion, coopération, manque d'agressivité
Consciencioisité vs Impulsivité	Autodiscipline, respect des obligations, organisation
Ouverture vs fermeture à une expérience	Imagination, créativité, curiosité

Encore récemment, le terme tempérament est parfois préféré au terme personnalité pour signifier la même chose, donnant lieu à des incompréhensions dans la communauté scientifique (Réale et al., 2007). La situation s'éclaircit en 2007 lorsque la personnalité (ou tempérament) est définie par Réale et al. (2007). Selon ces auteurs, la personnalité correspond aux « différences comportementales interindividuelles, cohérentes dans le temps et dans différents contextes ». Par exemple, en milieu naturel, si deux individus sont divergents pour un comportement X lors de la recherche de nourriture, ils doivent également diverger pour ce comportement X dans la recherche de partenaire.

En laboratoire, de la même façon, ces deux individus doivent présenter les mêmes différences comportementales qu'ils soient testés en groupe *vs.* isolé, dans un test A ou un test B pour que cela soit considéré comme un trait de personnalité.

La personnalité est alors caractérisée, à l'instar de celle de l'homme, par 5 axes, ou *continuum*, qui sont l'audace, l'activité, l'agressivité, l'exploration et la socialité (Réale et al., 2007). Chaque axe va maintenant être décrit ainsi que des outils de caractérisation adaptés aux téléostéens.

- **Axe 1: l'audace**

L'audace est l'axe de la personnalité le plus étudié. Elle se définit généralement par la propension d'un individu à prendre des risques (Réale et al., 2007). Plus un individu a tendance à prendre des risques, plus il sera considéré comme audacieux. Au contraire, un individu moins enclin à prendre des risques sera considéré comme timide. Différents types de tests sont utilisés pour mesurer l'audace d'un individu. Le test de prise de risque est le plus utilisé et peut être réalisé en individuel ou en groupe. Le ou les individus sont transférés dans une zone refuge (le plus souvent un abri sombre), et après une période d'habituation, on mesure la latence que met l'individu à quitter cette zone refuge et prendre le risque de découvrir une zone ouverte, éclairée et inconnue (Brown, 2005; Castanheira et al., 2013; Ferrari et al., 2015; Rey et al., 2013; **Figure 10**). Le test de néophobie, qui vise à mesurer la propension d'un individu à découvrir un nouvel objet, permet également de mesurer l'audace chez les téléostéens (Castanheira et al., 2017; Millot et al., 2009).

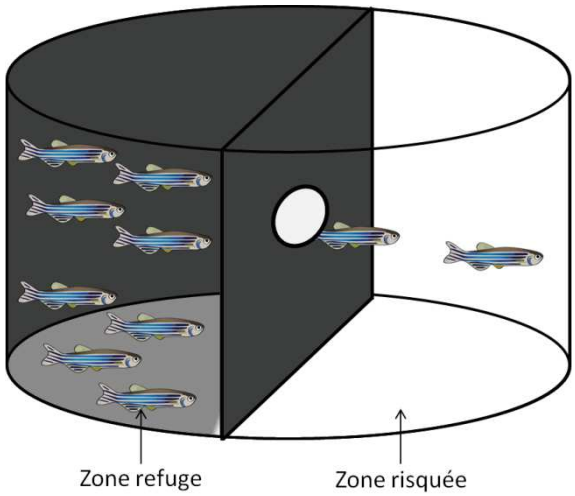

Figure 10. Test de prise de risque. Un groupe d'individus est transféré dans une zone refuge et a accès à une zone ouverte risquée. Représentation adaptée des travaux de Ferrari et al. (2015).

- **Axe 2: l'activité**

Comme l'audace, l'activité est un axe très important de la personnalité d'un individu. Elle est caractérisée par la quantité de déplacement qu'effectue l'individu sur une période donnée (Quinn et Cresswell, 2005; Réale et al., 2007). C'est un axe très important du fait que l'activité d'un individu est généralement corrélée avec le taux métabolique (Killen et al., 2012; Martins et al., 2011b). La mesure de l'activité peut se faire dans un test d'*open field* (OFT). La surface de la zone d'étude d'un OFT est généralement vide; l'individu est alors placé au milieu et peut se déplacer librement sur toute la surface de l'arène de test où son activité locomotrice est mesurée (Archard et Braithwaite, 2011; Schnorr et al., 2012). Une zone de départ, pouvant également servir de refuge, peut être ajoutée à l'OFT classique. Cela permet, en plus de la mesure de l'activité locomotrice de l'individu, de mesurer le temps d'émergence du refuge et un second marqueur d'activité qui est le nombre de transitions entre la zone ouverte et la zone refuge (Benhaim et al., 2017; Ferrari et al., 2014a; **Figure 11**).

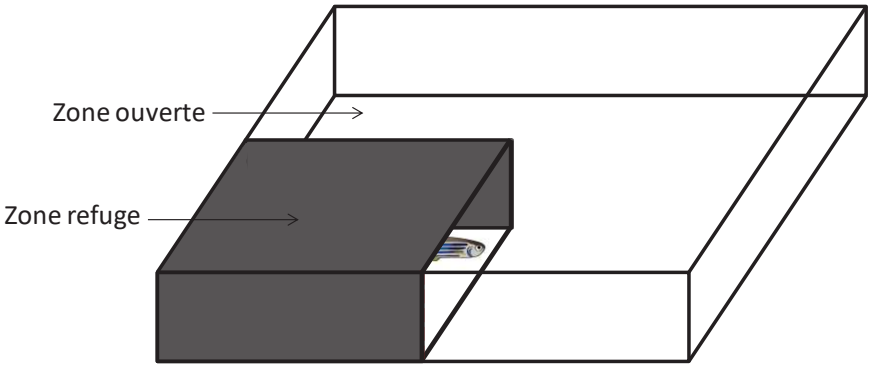

Figure 11. Test d'*open field* avec un refuge. Un individu seul est transféré dans une zone refuge et a accès à une zone ouverte. Son activité, ainsi que d'autres composantes (émergence, positionnement, nombre de transitions entre la zone ouverte et le refuge), peuvent être mesurées dans la zone ouverte. Représentation adaptée des travaux de Ferrari et al. (2014a).

- **Axe 3: l'agressivité**

L'axe de agressivité est défini généralement par le nombre d'interactions agonistiques d'un individu envers les autres (Réale et al., 2007). Un individu agressif aura tendance à interagir avec ces congénères afin de maintenir sa domination alors qu'un individu peu agressif interagira peu. Il existe différentes méthodes pour quantifier le niveau d'agressivité d'un individu qui permettent d'obtenir des informations complémentaires (Way et al., 2015). Ces tests reposent en général sur la quantification des interactions agonistiques (poursuite, attaque, comportement spécifique) d'un individu par rapport à d'autres (Bertucci et al., 2013; Verbeek et al., 1996).

L'individu testé est isolé du groupe et transféré plusieurs jours dans un aquarium. Après cette période d'adaptation à son nouvel environnement, il devient alors résident et on observe alors les différentes interactions agonistiques de l'individu testé face à un intrus (Bertucci et al., 2013). Une autre méthode répandue afin de mesurer l'agressivité d'un individu est de le confronter à sa propre image dans un miroir et ensuite quantifier les interactions agonistiques de l'individu face à son reflet (Rey et al., 2013; **Figure 12**).

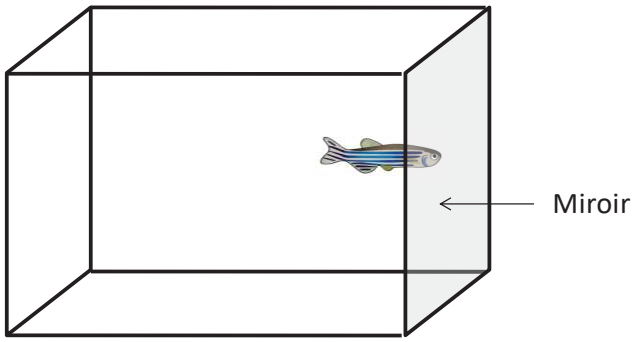

Figure 12. Test du miroir. Un miroir est disposé sur une paroi de l'aquarium et reflète l'individu qui perçoit son reflet comme un conspécifique et interagit avec son image. Représentation adaptée des travaux de Rey et al. (2013).

L'avantage de cette méthode est que l'individu testé est confronté à un individu (son reflet) qui possède les mêmes caractéristiques phénotypiques que lui. En revanche, il n'y a pas de vainqueur/vaincu, étant donné que le reflet effectue les mêmes comportements que l'individu testé. Cela n'entraîne donc pas les mêmes réponses moléculaires liées à la victoire ou à la défaite contrairement à une interaction agonistique réelle avec un autre individu (Oliveira et al., 2005, 2009; Teles et al., 2015).

- **Axe 4: l'exploration**

L'axe de l'exploration définit la manière dont un individu explore son environnement, identifiant des explorateurs rapides et superficiels et des explorateurs lents et attentifs (Réale et al 2007). Afin d'évaluer le niveau d'exploration, le comportement de l'individu peut être mesuré dans un labyrinthe en Z (Chapman et al., 2010). Différentes variables telles que le nombre de zones du labyrinthe explorées, le temps passé dans ces différentes zones ou la latence pour arriver au bout du labyrinthe, sont fréquemment utilisées pour quantifier les capacités d'exploration d'un individu (Chapman et al., 2010; Vignet et al., 2014b; **Figure 13**).

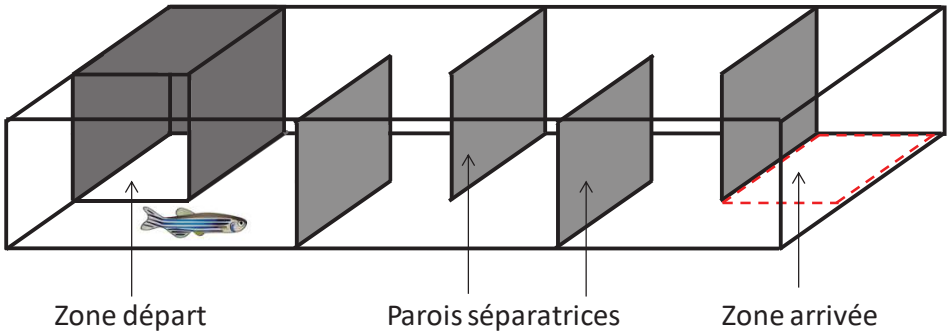

Figure 13. Labyrinthe en Z consiste en une zone départ où l'individu est transféré et acclimaté au début du test. La zone d'arrivée, entravée par des parois, marque la fin du labyrinthe. Représentation adaptée des travaux de Chapman et al. (2010).

- **Axe 5: la socialité**

L'axe de la socialité représente la propension d'un individu à plutôt rechercher ou éviter la présence de conspécifiques (Cote et Clobert, 2007; Réale et al., 2007). Un individu plus social aura tendance à chercher la présence d'autres individus et l'inverse pour un individu asocial. Afin de mesurer la socialité d'un individu, il est généralement confronté à un test de choix entre une zone présentant plusieurs congénères et une zone vide (ou avec un seul congénère) (Kaidanovich-Beilin et al., 2011; Pritchard et al., 2001; Yang et al., 2001; **Figure 14**). Plus l'individu passe de temps dans la zone proche des conspécifiques, plus il est considéré comme social.

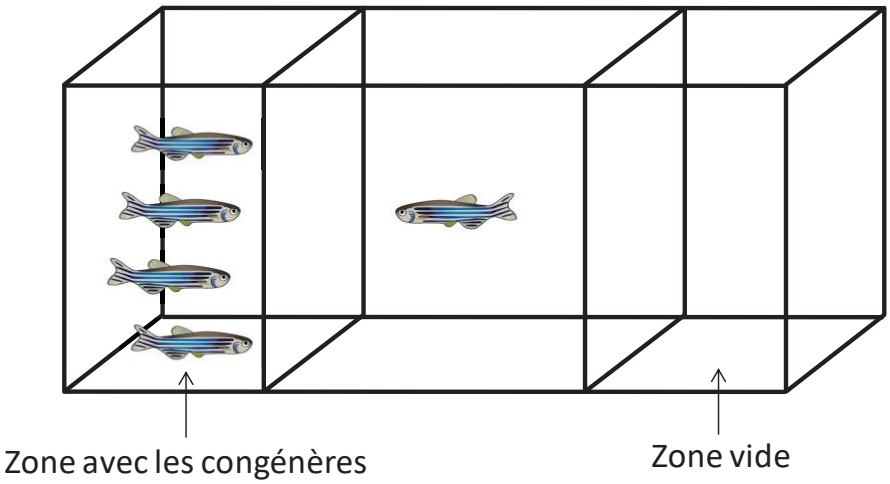

Figure 14. Test de choix congénères *vs*. zone vide. L'individu est placé seul dans une zone ouverte. La proximité de l'individu test avec les congénères est mesurée. Représentation adaptée des travaux de Pritchard et al. (2001).

Les différents tests présentés précédemment visent chacun à évaluer un axe de la personnalité, ils peuvent également permettre de mesurer des variables rattachées à d'autres axes. Par exemple l'audace peut être mesurée dans un test de prise de risque mais aussi dans un test d'*open field* ou un labyrinthe en Z en mesurant la latence mise par l'individu pour quitter la zone refuge. Cela peut notamment permettre de mesurer le seuil de performance d'un individu pour un axe de la personnalité donnée dans différents contextes et donc de vérifier la stabilité de la personnalité en fonction du contexte (Réale et al., 2007).

- **Quid de l'anxiété ?**

Comme cela a été évoqué précédemment, un axe des 5 grands facteurs de la personnalité chez l'homme est l'axe du névrosisme et est décrit par l'anxiété, la dépression et la vulnérabilité au stress (John, 1990). En revanche, chez l'animal, aucun axe de la personnalité ne décrit ce trait important. Pourtant, en parallèle de l'étude de la personnalité, l'anxiété est une caractéristique que l'on sait mesurer chez les téléostéens aussi bien chez les larves qu'aux stades ultérieurs (Egan et al., 2009; Levin et al., 2007; Schnorr et al., 2012). Le plus souvent il s'agit de mesurer les réponses comportementales des téléostéens suivant l'introduction dans un nouvel environnement. La réponse première consiste à s'immobiliser près du fond ou des bords, puis les individus explorent ensuite l'ensemble de l'environnement nouveau. Ce positionnement près du fond ou des bords (thigmotaxie), loin de la surface, fait écho au comportement anti-prédateur très répandu chez les téléostéens (Marcucella et Abramson, 1978). La spécificité de ces réponses liées à l'anxiété (*anxiety-like behaviour*) a été validée en utilisant des molécules anxiolytiques ou anxiogènes. Dans un test de nouvel environnement, un individu exposé à des molécules anxiolytiques aura tendance à rester dans la zone haute de l'aquarium alors qu'il restera plutôt dans la zone basse si il est exposé à des molécules anxiogènes (Egan et al., 2009; Levin et al., 2007; **Figure 15.A**). De la même façon lors d'un test *open field*, un individu exposé à des molécules anxiolytiques aura tendance à rester dans la zone centrale de l'aquarium alors qu'il restera plutôt dans la zone périphérique (thigmotaxie) s'il est exposé à des molécules anxiogènes (Schnorr et al., 2012; **Figure 15.B**). D'après ces résultats, ces comportements consistant à rester près du fond ou des bords de l'aquarium ont pu être définis comme liés au niveau d'anxiété de l'individu. Certains de ces tests ont été ensuite adaptés pour mesurer le niveau d'anxiété des téléostéens en réponse à différents facteurs de stress (Benhaim et al., 2017; Ferrari et al., 2014a; Glazer et al., 2016; Macaulay et al., 2015; Vignet et al., 2014b).

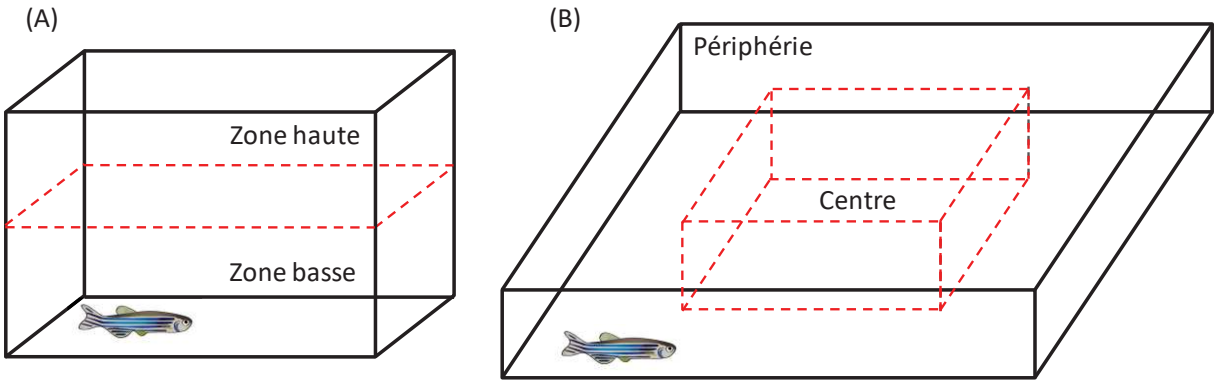

Figure 15. (A) Test du nouvel environnement. L'individu est placé seul dans un nouvel environnement. Le positionnement dans la zone haute ou basse de l'aquarium est suivi. Représentation adaptée des travaux d'Egan et al. (2009); **(B)** Test *open field*. L'individu est placé seul dans une zone ouverte. Le positionnement dans la zone centrale ou périphérique de l'aquarium est mesuré. Représentation adaptée des travaux de Ferrari et al., (2014a).

Pour l'ensemble de ces tests dits de personnalité, et avec les précautions nécessaires, il est permis de transposer le comportement observé en laboratoire au milieu naturel et de faire des hypothèses quant aux capacités de l'individu à survivre et se reproduire, *i.e.* les effets sur les traits d'histoire de vie. Par exemple, un individu audacieux et/ou peu anxieux peut s'exposer plus facilement aux prédateurs lors de sa recherche alimentaire. A l'opposé, un individu timide et/ou plus anxieux sera moins exposé aux prédateurs mais la recherche de nourriture ou de partenaire pourra s'avérer plus complexe (Conrad et al., 2011). D'autres comportements, comme les interactions sociales ou la dispersion, peuvent être également liés à la personnalité de l'individu.

2.2.2. Le syndrome comportemental

Différentes mesures comportementales reliées aux différents axes de la personnalité, peuvent être corrélés les uns aux autres (Bell, 2007; Castanheira et al., 2017; Sih et al., 2004). Par exemple, chez l'épinoche (*Gasterosteus aculeatus*), les individus les plus audacieux étaient également les plus agressifs lors de la défense de leur territoire (Huntingford, 1976). Depuis, de nombreuses études ont mis en évidence une corrélation positive entre les différents axes de la personnalité, notamment entre l'audace, l'agressivité et l'activité (Geffroy et al., 2015; Sih et Bell, 2008). Le terme de syndrome comportemental est alors utilisé pour définir ce type d'observations. Il définit une suite de comportements corrélés entre eux dans des contextes multiples (Sih et al., 2004). Le concept de syndrome comportemental est représenté **figure 16**.

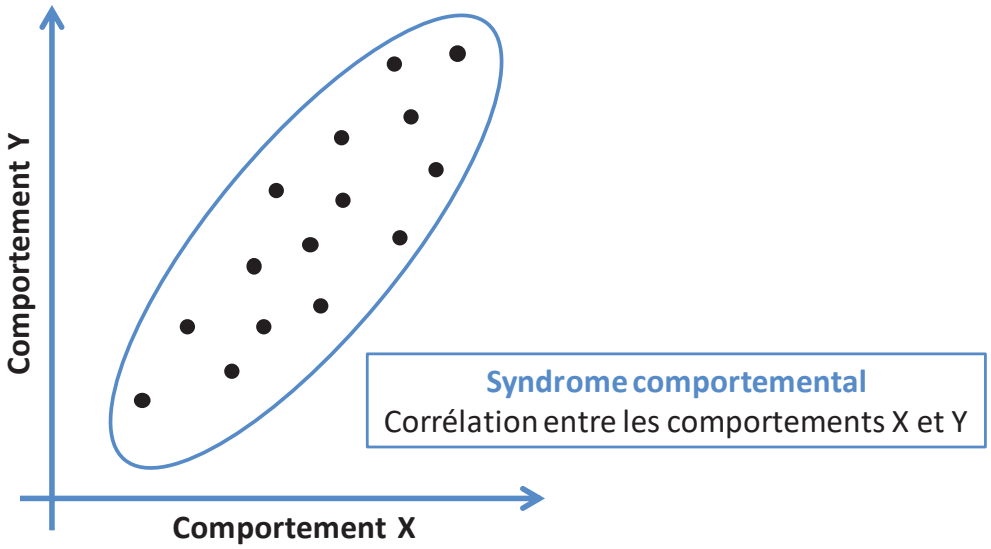

Figure 16. Illustration de la notion de syndrome comportemental. Corrélation entre les réponses comportementales (*e.g.* différentes variables, différents contextes ou différents tests) de plusieurs individus d'une population. D'après Bell (2007).

2.2.3. La plasticité comportementale

Les différents concepts présentés dans les **sections 2.2.1** et **2.2.2** font tous intervenir le concept de stabilité des réponses comportementales dans le temps et dans différents contextes. Opposé au concept de stabilité comportementale, il existe le concept de plasticité comportementale qui stipule qu'un individu exprime un comportement particulier en fonction de l'environnement ou du contexte (Snell-Rood, 2012).

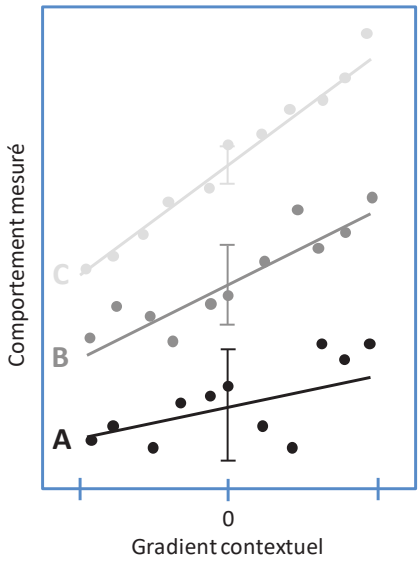

Figure 17. Représentation hypothétique de 3 individus (Noir, gris foncé et gris clair) dont le comportement a été mesuré 11 fois selon un gradient contextuel. D'après Highcock et Carter (2014).

Ce concept de plasticité comportementale est illustré **figure 17**. Cette figure représente 3 individus (A, Noir ; B, gris foncé et C, gris clair) dont le comportement a été mesuré 11 fois selon un gradient contextuel (Highcock et Carter, 2014).

Ce gradient contextuel peut être lié à la variation des facteurs physico-chimiques différents, le risque de prédation, la quantité de nourriture disponible ou différents contextes de vie différents comme la reproduction ou la recherche de nourriture. Dans cet exemple, les pentes des lignes correspondent aux plasticités individuelles pour le comportement étudié, les ordonnées à l'origine (le 0 du gradient contextuel) représente la réponse comportementale correspondant à l'environnement moyen et les barres d'erreurs représentent les variabilités intraindividuelles. Cet ensemble définit des normes de réaction pour chaque individu. L'individu A possède de faibles valeurs du comportement étudié, une faible plasticité comportementale et une grande variabilité intraindividuelle. L'individu C possède, quant à lui, de hautes valeurs du comportement mesuré, une grande plasticité comportementale et une faible variabilité intraindividuelle alors que l'individu B a des valeurs moyennes pour le comportement étudié, de même pour sa plasticité et sa variabilité. Cette illustration montre que les différences de comportement entre les individus sont toujours présentes quelque soit le gradient contextuel mais que les individus présentent également une certaine variabilité et une plasticité plus ou moins importante dans leur réponse comportementale.

L'exemple ci-dessous illustre, quant à lui, un type de covariation des normes de réaction individuelles que l'on appelle aussi *scénario* de réponse ou stratégie. La **figure 18** suivante représente différents *scenarii* de covariations pour 4 individus matérialisés par des couleurs différentes.

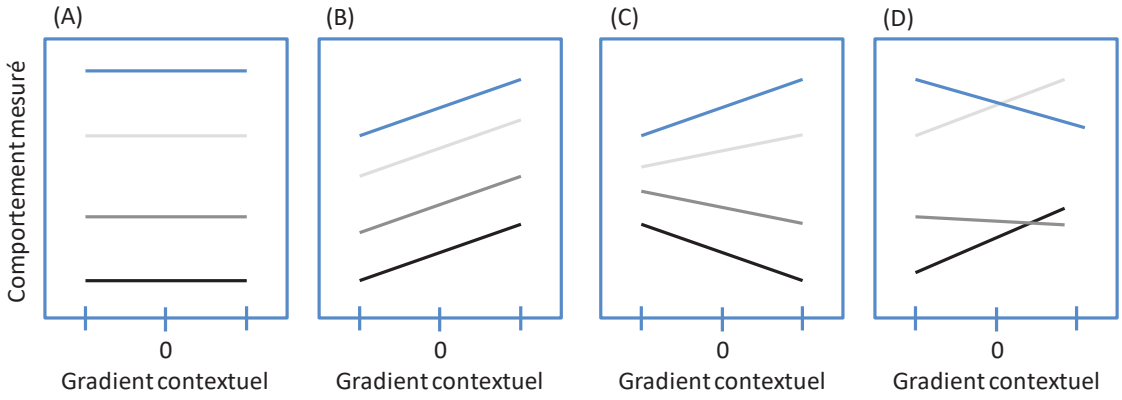

Figure 18. Représentation hypothétique de 4 individus (Noir, gris foncé, gris clair et bleu) dont le comportement a été mesuré selon un gradient contextuel selon 4 différents *scenarii* de réponses (A, B, C et D). D'après Dingemanse et al. (2010).

Sur la **figure 18.A**, les individus expriment des comportements différents mais aucune plasticité comportementale n'est observée en fonction du gradient contextuel (pentes nulles). En revanche, sur la **figure 18.B**, la plasticité comportementale est présente chez les individus qui adaptent leur comportement en fonction du gradient contextuel. Leurs réponses dans un environnement moyen diffèrent, par contre ils présentent la même plasticité comportementale (les pentes sont similaires). Sur la **figure 18.C**, Les individus diffèrent les uns des autres pour leurs réponses dans un environnement moyen différent et leur plasticité comportementale. On peut toutefois noter une covariance positive entre ordonnée à l'origine et pente. Pour finir, sur la **figure 18.D**, les 4 individus diffèrent entre eux pour le comportement dans un environnement moyen, l'évolution de la réponse selon le gradient contextuel et il n'y a pas de covariance. Ces différents *scénarii* permettent donc d'illustrer d'une part la stabilité du comportement (**Figure 18.A**), ainsi que les différents scénarii de la plasticité comportementale qui peuvent intervenir avec ou sans covariation avec l'environnement (**Figure 18.B-D**).

Selon la théorie de la sélection naturelle, une faible plasticité comportementale pourrait apparaître comme peu adaptative (Darwin, 1859). En effet, un phénotype offrant une plus grande plasticité comportementale devrait être avantagé et donc ceux caractérisés par une faible plasticité devrait disparaitre.

La sélection « fréquence-dépendante » stipule que les bénéfices apportés par le comportement d'un individu vont être dépendant du comportement des autres individus (Maynard Smith, 1982). Par exemple, dans un environnement stable avec des ressources limitées, un individu agressif va être plus compétitif dans l'acquisition des ressources. Ces individus agressifs vont être avantagés par rapport aux autres, se reproduiront plus facilement et donc pourront d'avantage transmettre leurs gènes à leur descendance. Cependant, le nombre d'individus de ce phénotype « agressif » va augmenter avec le temps jusqu'à devenir un désavantage. Les individus sélectionnés pour le phénotype agressif vont se battre entre eux et se blesser, ce qui devient un handicap pour la survie et la reproduction. Les gains associés à cette stratégie vont alors devenir inférieurs aux coûts lorsque le nombre d'individus adoptant la même stratégie va augmenter jusqu'à un certain seuil redonnant ainsi l'avantage à la stratégie opposée.

Cette théorie permet d'expliquer d'une part la présence de plasticité comportementale dans la mesure où l'individu adapte son comportement en fonction des facteurs biotiques et abiotiques et, d'autre part, la présence de plusieurs stratégies comportementales au sein d'une même population. La section suivante vise à présenter la théorie des *coping styles* et donc la coexistence de deux stratégies différentes (stratégie proactive *vs.* réactive) au sein d'une même population.

2.3. La théorie des *coping styles*

Les différents comportements corrélés entre eux (syndrome comportemental) peuvent également être corrélés à des réponses physiologiques. Le « style d'adaptation » au stress (*coping style*) décrit cette suite de comportements corrélés entre eux et aux réponses physiologiques cohérente en fonction du temps et du contexte (Koolhaas et al., 1999).

Face à un stress, la réponse de l'individu peut être plus ou moins active ou passive. Ces types de réponses active ou passive ont été employés pour expliquer les deux types de réponses adoptés afin de maintenir l'homéostasie: combat/fuite (*fight or flight*, réponse active) (Cannon, 1915) ou immobilité/ se cacher (*freeze et hide*, réponse passive; Engel et Schmale, (1972). Ce concept bimodale de la réponse individuelle a été plus tard repris pour caractériser des individus plutôt proactifs (réponse active) et des individus plutôt réactifs (réponse passive) et qui face au stress présentent, outre des réponses comportementales divergentes, des réponses physiologiques également différentes (Koolhaas et al., 1999). Dans une population, les individus se positionnent selon un *continuum* entre les deux réponses extrêmes que constituent les proactifs et les réactifs (**Figure 19)**.

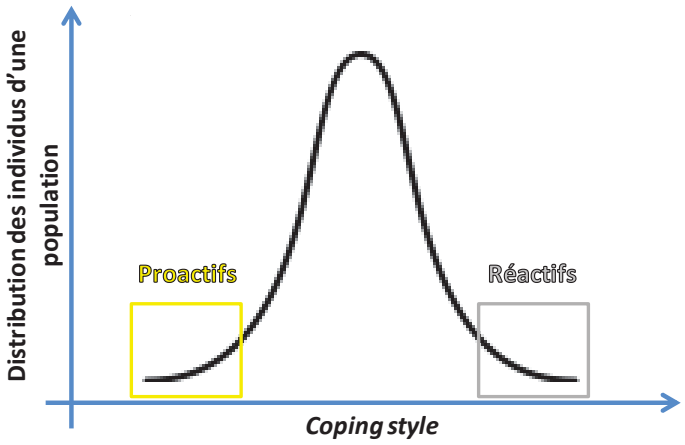

Figure 19. Distribution théorique des individus au sein d'une population en fonction de leur *coping style*. La proportion d'individus proactifs est caractérisée par un rectangle jaune et la proportion d'individus réactifs est catégorisée par un rectangle gris.

Cependant, la plupart des études ayant amené à la théorie des *coping styles* ont été réalisées chez des individus sélectionnés par rapport à une faible ou forte réactivité de l'axe HPA chez la souris (*Mus musculus)* et de l'axe HPI chez la truite arc-en-ciel (Koolhaas et al., 1999; Øverli et al., 2007). C'est pour cette raison que la plupart des études présente le *coping style* de manière bimodale mais il est important de noter que dans des populations naturelles ou expérimentales, la plupart des réponses individuelles au stress est comprise entre ces deux réponses extrêmes.

D'un point de vue comportemental, les individus proactifs sont généralement plus audacieux (Castanheira et al., 2013; Ferrari et al., 2015), plus agressifs et dominants (Øverli et al., 2004a, b) et sont plutôt explorateurs rapides (Millot et al., 2009; Øverli et al., 2006). Les individus proactifs ont tendance à développer des routines comportementales contrairement aux individus réactifs qui vont être plus plastiques (Bolhuis et al., 2004; Ruiz-Gomez et al., 2008). D'un point de vue physiologique, les individus proactifs sont caractérisés par une faible réactivité de l'axe HPI (Øverli et al., 2007; Silva et al., 2010), ainsi qu'une faible réactivité des systèmes monoaminergiques (Øverli et al., 2001, 2007) et une plasticité neuronale plus faible comparée aux individus réactifs (Øverli et Sorensen, 2016), notamment face au stress.

Ces différentes stratégies face à un stress, proactive ou réactive, peuvent potentiellement représenter l'étendue des capacités d'adaptation face aux fluctuations de l'environnement. Ces *coping styles* constituent donc un domaine d'étude intéressant pour les problématiques liés au bien-être en aquaculture (Castanheira et al., 2017) mais également dans le domaine de l'écologie (Conrad et al., 2011; Sih et al., 2012).

Dans le cadre de cette thèse, nous allons nous intéresser d'une part à la mise en place de la personnalité, sa cohérence entre les différents traits de personnalités (syndrome comportemental) et sa cohérence entre les différents stades de vie de l'individu (**Chapitre 1**), ainsi que des mécanismes physiologiques associés aux différences interindividuelles chez les téléostéens (**Chapitre2**).

Le **tableau 2** récapitule les différences comportementales et physiologiques observées chez les téléostéens en fonction de leur *coping style* et le **tableau 3** récapitule les définitions associées aux différents concepts comportementaux présentés dans cette section

Tableau 2. Synthèse des divergences comportementales et physiologiques observées entre les individus proactifs et réactifs chez les téléostéens. D'après Castanheira et al. (2017).

Comportement / Physiologie	Proactifs	Réactifs	Références
Audace	+	-	Ferrari et al. (2015) Millot et al. (2009)
Agressivité	+	-	Øverli et al. (2004)
Exploration	+	-	Mackenzie et al. (2009) Øverli et al. (2006)
Activité	+	-	Mas-Munoz et al. (2011)
Socialité	-	+	Ferrari (2014)
Flexibilité comportementale	-	+	Bolhuis et al. (2004) Ruiz-Gomez et al. (2011)
Taux métabolique standard	+	-	Killen et al. (2011) Mackenzie et al. (2009)
Plasticité neuronale	-	+	Sorensen et al. (2013)
Activité axe HPI	-	+	Silva et al. (2010)
Système monoaminergiques	-	+	Øverli et al. (2001)

Tableau 3. Synthèse de la terminologie et définitions associées aux différents concepts comportementaux évoqués. D'après Castanheira et al. (2017).

Terminologie	Définition	Références
Personnalité (tempérament)	Stabilité d'un comportement au cours du temps et du contexte	Réale et al. (2007)
Syndrome comportemental	Une suite de comportements corrélés dans des contextes multiples	Sih et al. (2004)
Coping style	Une suite de comportements corrélés aux réponses physiologiques et stables dans le temps et entre contextes	Koolhaas et al. (1999)

3. L'individu face aux facteurs de stress de l'environnement

L'objectif de cette partie est de présenter brièvement les réponses physiologiques et comportementales des téléostéens déjà connues face aux facteurs de stress étudiés dans cette thèse. Dans un premier temps, les effets des PCB et des PBDE seront présentés (**section 3.1**), puis dans un second temps les effets des variations de la qualité de l'eau seront exposés dans un contexte d'aquaculture (**section 3.2**).

3.1. Cas d'étude d'un stress chimique : les PCB et les PBDE à dose environnementale

Du fait de leur toxicité avérée, les réponses physiologiques et comportementales d'un individu soumis à une exposition aux PCB **ou** aux PBDE ont été très étudiées ces dernières années.
Dans la plupart des cas, la caractérisation des réponses physiologiques et comportementales après une exposition à des PCB ou des PBDE portait sur l'effet d'un seul congénère de PCB ou de PBDE à des concentrations assez élevées (*e.g.* Eddins et al., 2010; Klüver et al., 2015; Truong et al., 2014). Ce type d'étude est indispensable pour identifier les réponses mais leur pertinence environnementale est très limitée puisque comme on l'a vu les PCB et les PBDE étaient utilisés sous forme de mélanges complexes. Un état de l'art non exhaustif sera fait dans cette section, pour présenter les principales altérations observées chez les téléostéens suite à une exposition à des PCB ou des PBDE.

3.1.1. Les réponses physiologiques

En raison de la grande variété structurale des 209 congénères de PCB et de PBDE, il n'est pas possible de généraliser les effets observés pour un congénère à tous les autres. Par exemple, certains congénères de PCB (CB-77, CB-81, CB-126 et CB-169), sans substitution en position ortho, adoptent une conformation coplanaire et leur structure est très proche de celle de la dioxine. En conséquence ils partagent les mêmes modes d'action par l'intermédiaire du récepteur aryl hydrocarbon (AhR; Sorg (2014)). En raison d'analogies structurales, certains congénères de PCB et de PBDE agissent comme des perturbateurs endocriniens capables d'affecter la synthèse, le transport et l'excrétion d'hormones (Legler et Brouwer, 2003; Mills et Chichester, 2005; Yang et al., 2005). Des altérations de la réponse physiologique des téléostéens au stress ont été démontré que ce soit pour les PCB ou les PBDE (Gauthier et al., 2018; Hontela et al., 1992; Wang et al., 2015).

Chez l'ombre chevalier (*Salvelinus alpinus*), en condition de jeûne, il a été démontré qu'une exposition à un mélange commercial de PCB (Aroclor 1254) entraînait une sous expression du récepteur aux glucocorticoïdes (*gr*) dans le cerveau et causait une diminution du taux de cortisol plasmatique (Aluru et al., 2004; Jorgensen et al., 2002). De même que pour l'axe HPI, les systèmes monoaminergiques peuvent être altérés suite à une exposition à ces POP. Par l'exemple, une exposition à un congénère de PBDE chez les larves, le BDE-71, perturbe le système dopaminergique, notamment en diminuant la concentration en dopamine dans l'ensemble du corps des larves par le biais d'une sous-expression de gènes impliqués dans le développement des neurones dopaminergiques (Wang et al., 2015). Cette neurotoxicité des PCB et des PBDE a été mise en évidence depuis de nombreuses années (Eriksson et Fredriksson, 1998; He et al., 2011; Kimura-Kuroda et al., 2007). Par exemple, l'exposition à un congénère de PBDE, le BDE-47 est capable de causer chez le poisson zèbre une diminution de l'activité de *c-fos* dans le cerveau, un marqueur d'activité neuronale (Zheng et al., 2017).

De plus, grâce à leurs propriétés lipophiles, les PCB et les PBDE s'accumulent dans les œufs (Daouk et al., 2011; He et al., 2011; Nyholm et al., 2008; Yu et al., 2011). Cela permet la transmission verticale de ces polluants d'une génération exposée à la suivante, causant ainsi une exposition très précoce de la génération suivante. Cette exposition peut également entraîner des altérations physiologiques et comportementales chez la descendance (Chen et al., 2012b; He et al., 2011; Péan et al., 2013). En plus de des conséquences liées au transfert des polluants vers les œufs, les effets physiologiques ou comportementaux observés chez la descendance peuvent être la conséquence indirecte du stress maternel causé par l'exposition (Talge et al., 2007). En plus de ces deux modes d'actions, des perturbations des mécanismes épigénétiques peuvent également expliquer les altérations observées chez la descendance et également les effets observés dans des générations non exposées de manière directe ou indirecte (Skinner, 2014a, b).

En effet, les perturbations épigénétiques peuvent modifient de manière persistante dans le temps l'expression de gènes sans modification des séquences génomiques. Certaines modifications épigénétiques peuvent être transmises à la génération suivante et sont à la base d'effets transgénérationnels. La méthylation de l'ADN, est un des mécanismes épigénétiques, impliquant des ADN methyltransférases (DNMT) dans lesquelles certaines cytosines vont être modifiées après ajout d'un groupement méthyle (Aluru, 2017; **Figure 20**).

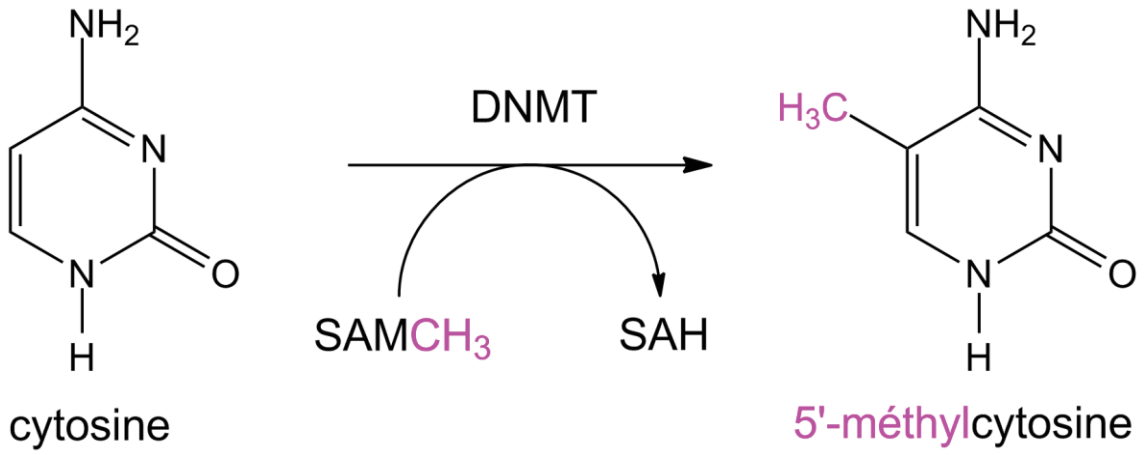

Figure 20. Méthylation d'une base de l'ADN la cytosine en 5'-méthylcytosine sous l'action d'une DNMT. Source : https://planet-vie.ens.fr/article/2277/epigenetique-cancer.

L'exposition à des polluants tels que le bisphénol A ou le TCDD (dioxine) sont capables de perturber l'expression des gènes codant pour ces DNMT et donc les profils de méthylation (Aluru et al., 2015; Laing et al., 2016; Liu et al., 2016). En revanche, aucune étude n'a été menée sur les effets des PCB et PBDE sur des possibles altérations des mécanismes épigénétiques pouvant expliquer une transmission des perturbations à la descendance.

3.1.2. Les réponses comportementales

En écotoxicologie, le comportement des individus est très souvent mesuré à l'aide de tests standardisés permettant à la fois une certaine calibration entre les laboratoires et une analyse haut débit (Cachat et al., 2010; Kalueff et al., 2016; Rihel et al., 2010; Schnorr et al., 2012). Le test de nouvel environnement, présenté dans la **section 2.2.1**, permettant de mesurer le niveau d'anxiété des téléostéens est très utilisé chez des juvéniles ou des adultes (Egan et al., 2009; Levin et al., 2007; Macaulay et al., 2015; Vignet et al., 2015). Chez les larves, l'étude de la réponse photomotrice (LPMR) est un test très répandu (Chen et al., 2012a, b; Macaulay et al., 2015; Péan et al., 2013; Vignet et al., 2015). Le test LPMR est une méthode standardisée consistant à étudier l'activité locomotrice des larves, disposées individuellement dans les puits d'une plaque multipuits, en réponse à un changement brusque de lumière vers obscurité et *vice versa* (**Figure 21.A, B**). En réponse à ce changement brusque entre la lumière et l'obscurité, les larves vont exprimer une augmentation normale de leur activité. Un changement d'activité anormal (hypo- ou hyper-activité) en réponse à ce changement est couramment utilisé par les écotoxicologues afin de quantifier et qualifier des perturbations comportementales liées à l'exposition des individus à des polluants. Outre sa pertinence, ce test permet un criblage haut débit de molécules (Ali et al., 2012).

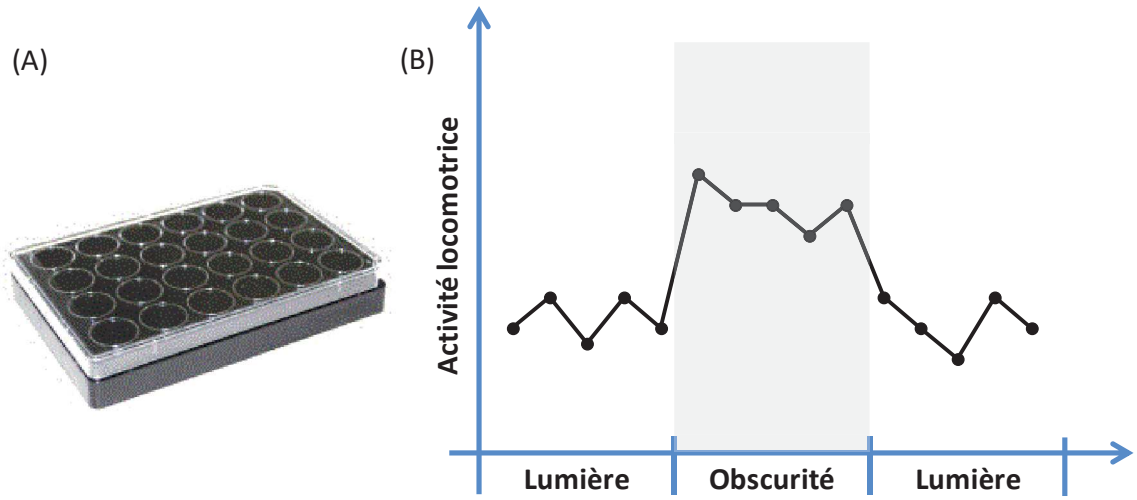

Figure 21. (A) Plaque 24 puits utilisée pour le test LPMR; **(B)** Evolution de l'activité des larves durant un test LPMR. D'après Vignet et al. (2015).

Une exposition à des congénères uniques de PCB ou PBDE est connue pour entrainer des altérations comportementales lors du test de LPMR ou du nouvel environnement. Par exemple, une augmentation de l'activité locomotrice en réponse au changement brusque lumière/obscurité a été observée chez des F1, descendants de la première génération (F0), après exposition chronique des parents au BDE-209 (He et al., 2011) ou d'un mélange de PCB (Péan et al., 2013) et également après une exposition embryo-larvaire de BDE-71 (Chen et al., 2010, 2012a). Suite à une exposition embryo-larvaire de BDE-47, les larves expriment cette fois ci une réduction de leur activité en réponse à la transition lumière-obscurité durant le test LPMR (Chen et al., 2012c). Il s'avère donc que les effets des PCB et des PBDE sur le comportement locomoteur des larves dépendent des congères utilisés, ainsi que de la période et de la durée d'exposition.

De plus, il a également été démontré qu'une exposition à un congénère simple de PCB, le CB-126, ou à un mélange technique (Aroclor 1254, mélange technique riche en CB-126) induit un temps passé au fond du bassin supérieur des individus exposés comparés aux témoins lors d'un test de nouvel environnement ce qui traduit un niveau d'anxiété plus élevé (Glazer et al., 2016; Gonzalez et al., 2016). De la même façon, des individus exposés à un métabolite du BDE-47, le 6-OH-BDE-47, expriment un niveau d'anxiété plus élevé lors du test du nouvel environnement (Macaulay et al., 2015).

Les traits de personnalité, ainsi que les différents concepts tels que les syndromes comportementaux ou le *coping style* évoqués dans la **partie 2**, sont toutefois rarement appréhendés dans le domaine de l'écotoxicologie.

Récemment, Montiglio et Royauté (2014) soulignaient le fait que l'effet des contaminants sur le comportement était négligé par la communauté scientifique. Dans cette revue, les auteurs listent seulement 16 études portant sur l'effet de contaminants sur les variations du comportement (**Tableau 1** dans Montiglio et Royauté (2014)). Parmi ces études, certaines s'intéressent aux comportements importants pour la survie et la reproduction d'une espèce comme par exemple la recherche de nourriture, la migration, les réponses de fuites ou les comportements de court. Mais certaines études font également part d'altérations de la personnalité chez les animaux, notamment chez les téléostéens, suite à l'exposition à des contaminants. Par exemple, une exposition à un perturbateur endocrinien (Ethinyl oestradiol) diminue le niveau d'agressivité de l'épinoche envers des conspécifiques (Bell, 2001) mais peut également augmenter le niveau d'activité et d'audace en présence de prédateurs (Bell, 2004) rendant donc l'espèce plus vulnérable. D'autres contaminants, connus pour leurs propriétés d'inhibition sélective de la recapture de la sérotonine (SSRI), molécules actives des anti-dépresseurs, peuvent également provoquer des altérations de la personnalité chez de nombreuses espèces de téléostéens. Par exemple, la fluoxetine diminue le niveau d'audace et d'exploration chez le combattant (*Betta splendens*; Dzieweczynski et al., 2016a, b). Au contraire, le citalopram augmente le niveau d'audace chez l'épinoche (Kellner et al., 2016). Pour finir concernant les POP, Péan et al. (2013) ont démontré qu'une exposition à un mélange de PCB de la génération F0 entraîne une augmentation de l'activité chez les larves F1 suggérant que les PCB sont eux aussi capables d'entrainer une altération des traits de personnalité, du moins de l'activité.

Comme expliqué dans la **section 2.2.1**, les traits de personnalité sont censés être cohérent dans le temps et le contexte. De plus, ces traits de personnalité sont généralement corrélés entre eux pour former un syndrome comportemental (**section 2.2.2**). Cependant il a été démontré qu'une exposition à un insecticide (organophosphate) était capable, d'une part, d'altérer cette cohérence d'un trait de personnalité en fonction du contexte chez une araignée sauteuse (*Eris militaris*; Royauté et al., 2015) mais également d'altérer la relations entre les différents traits de personnalité formant un syndrome comportemental (Royauté et al., 2014).

Un panel des altérations des traits de la personnalité suivant l'exposition à différents contaminants chez les téléostéens est présentée dans le **tableau 4**.

Tableau 4. Altérations répertoriées des traits de personnalité suite à l'exposition à des contaminants de différentes classes chez les téléostéens. D'après Montiglio et Royauté (2014).

Polluant	Classe du polluant	Exposition	Espèce	Trait de personnalité	Variations	Références
Perturbateur endocrinien	Ethinyl oestradiol	Exposition passive	Epinoche	Agressivité	-	Bell (2001)
				Activité Audace	+ +	Bell (2004)
				Socialité	-	Reyhanian et al. (2011)
	17β-trenbolone		Gambusie	Activité Exploration	+ +	Bertram et al. (2018)
SSRI*	Escitalopram		Poisson zèbre	Audace	+	Nielsen et al. (2018)
	Fluoxetine		Combattant	Audace Exploration	- -	Dzieweczynski et al. (2016a, b)
	Citalopram		Epinoche	Activité Audace	+ +	Kellner et al. (2016)
Hydrocarbones aromatiques polycycliques	Mélange de HAP	Ingestion	Poisson zèbre	Exploration Audace	- -	Vignet et al. (2014b)
POP	Mélange de PCB	Ingestion (F0)	Poisson zèbre	Activité (F1)	+	Péan et al. (2013)

***SSRI :** Inhibiteur sélectif de la recapture de la sérotonine

Malgré le peu d'études sur le sujet, il apparait tout de même que les contaminants peuvent altérer l'expression et la cohérence des traits de personnalité chez les individus exposés (Montiglio et Royauté, 2014). D'après les exemples reportés dans le **tableau 4**, il apparait également que ces altérations sont dépendantes de la classe du contaminant et donc de ses propriétés chimiques. Les traits de personnalité sont essentiels dans la vie de l'individu régissant les interactions entre individus d'une même espèce, la relation proie-prédateur ou les comportements liés à la dispersion ou la recherche de nourriture. Une altération des traits de personnalité peut donc avoir des conséquences directes sur les traits d'histoire de vie des individus (Réale et al., 2010).

Au vue de ce panorama des réponses physiologiques et comportementales suivant une exposition à des POP, un mélange complexe de PCB et de PBDE (représentatif de l'environnement) a été utilisé pour exposer des animaux et explorer diverses réponses comportementales dont les traits de personnalité (**Chapitres 3** et **4** présentés ci-après).

3.2. Cas d'étude d'un stress physico-chimique en système d'élevage

Des variations des facteurs du milieu telles que la concentration en O_2, le CO_2 ou la concentration en azote ammoniacal peuvent être des facteurs de stress pour les téléostéens. Ces facteurs induisent des altérations physiologiques et comportementales qui peuvent avoir des conséquences sur les performances de croissance et de reproduction, une limitation du bien-être, ce qui peut avoir un coût important pour les éleveurs en aquaculture. Un état de l'art non exhaustif sera fait dans cette section, pour présenter les différentes altérations physiologiques et comportementales connues chez les téléostéens suite à des variations de qualité d'eau, incluant surtout des variations du niveau d'O_2 et d'azote ammoniacal.

3.2.1. Les réponses physiologiques

- **Hypoxie**

Comme évoqué précédemment, l'oxygène joue un rôle vital dans les processus de respiration et dans le métabolisme énergétique des organismes aérobies, incluant les téléostéens. Une diminution trop importante de sa concentration dans l'eau est un facteur de stress pour ces derniers. Par exemple, une exposition à une hypoxie sévère durant 90 minutes (25 % de saturation en O_2), entraîne l'activation de l'axe HPI et des systèmes dopaminergique et noradrénergique (augmentation des concentrations de cortisol plasmatique, dopamine et noradrénaline) chez la truite-arc-en-ciel (van Raaij et al., 1996).

- **Hyperoxie**

De même que pour des conditions d'hypoxie, des conditions hyperoxiques peuvent entrainer des réponses physiologiques primaires même si ce stress est moins documenté dans la littérature. Il a notamment été démontré chez le saumon atlantique, qu'une exposition prolongée à des niveaux d'O_2 élevés (150%) pouvait perturber la réponse de l'axe HPI et causer une élévation du taux de cortisol plasmatique (Espmark et Baeverfjord, 2009).

- **Azote ammoniacal**

De fortes concentrations en azote ammoniacal dans l'eau peuvent entraîner des changements dans la concentration en ammonium dans le plasma chez le bar européen (Lemarié et al., 2004) capable de perturber la balance ionique et de causer des stress oxydatifs (Sinha et al., 2012, 2014). Chez les téléostéens en général, des concentrations élevés en NH_4^+ dans l'eau déplacent K^+ et dépolarisent les neurones, ce qui va entraîner l'activation excessive des récepteurs de type glutamate (NMDA), conduisant à un afflux excessif de Ca^{2+} qui peut causer la mort des cellules dans le système nerveux central (Retall et Tsui, 2002).

La réponse de l'axe HPI peut être déclenchée suite à une exposition chronique à de fortes concentrations d'AAT dans l'eau. En effet, une exposition chronique de deux semaines à des concentration d'AAT de 0,15 mg/L entraine une élévation du taux de cortisol plasmatique chez le saumon atlantique (Knoph et Olsen, 1994).

3.2.2. Les réponses comportementales

- **Hypoxie**

D'un point de vue comportemental, des conditions hypoxiques entraînent une respiration des individus à la surface de l'eau car elle est en contact avec l'atmosphère qui est riche en O_2 (Kramer et McClure, 1982). Des conditions hypoxiques altèrent aussi l'activité locomotrice des individus. En fonction de l'espèce étudiée, de la concentration en O_2 et/ou de la durée d'exposition, les téléostéens expriment une diminution ou une augmentation de leur activité (Chapman et Mckenzie, 2009). Par exemple, une exposition à une hypoxie modérée (entre 80 et 20% de saturation en O_2) va induire une diminution d'activité locomotrice chez la sole commune (*Solea solea*; Dalla Via et al. (1998)) alors que le passage d'une hypoxie modérée vers une hypoxie sévère (0,5 mg/L d'O_2) va, quant à elle, induire une augmentation de l'activité de nage chez la lingue (*Urophycis chuss;* Bejda et al. (1987)). De plus, il a également été démontré que des conditions d'hypoxie sévère (20 % saturation en O_2) peuvent entraîner une augmentation de la dispersion des individus au sein d'un banc de Hareng (*Clupea Harengus*; Domenici et al. (2002)).

- **Hyperoxie**

Encore une fois, moins documentées que les réponses comportementales exprimées par l'individu face à des conditions d'hypoxie, des conditions hyperoxiques peuvent altérer le comportement des téléostéens. Par exemple, il a été démontré que des conditions d'hyperoxie augmentent le taux de ventilation du Gobie céphalote (*Gobius cobitis*; Berschick et al. (1987)). Chez le saumon atlantique, des conditions d'hyperoxie (150-175 % O_2) prolongées peuvent entrainer une diminution de la vitesse de nage (Espmark et Baeverfjord, 2009).

- **Azote ammoniacal**

Suite à une exposition à de fortes concentrations d'azote ammoniacal dans l'eau, la concentration en ammonium dans le sang des téléostéens augmentent, ce qui peut causer une diminution de la vitesse de nage critique et un plus faible taux métabolique actif chez la truite (Shingles et al., 2001). Les carpes (*Cyprinus carpio*), exposées à des concentrations allant de 1 à 2 mg/L d'ammonium non ionisé (ANI ou IUA-N en anglais) devenaient plus actives et exprimaient des pertes de l'équilibre et des convulsions (Abd Kadhim et Al-Faragi, 2018). Outre des échanges oraux entre scientifiques sur des observations faîtes sans réelles quantifications, les effets d'une exposition à de hautes valeurs d'azote ammoniacal sur les téléostéens restent rares.

D'autres facteurs comme la concentration en CO_2 dans l'eau, le pH ou la quantité de matière en suspension permettent de caractériser la qualité de l'eau et peuvent altérer des réponses physiologiques et comportementales des individus (Borch et al., 1993; Colson et al., 2015; Sadoul et al., 2017b).

Dans le cadre de cette thèse, nous nous intéresserons à la mise en place d'indicateurs comportementaux de bien-être suite à une exposition ponctuelle à des qualités d'eau dégradées (hypoxie, hyperoxie et forte concentration d'azote ammoniacal) dans le **chapitre 5**. Puis dans le **chapitre 6**, nous nous intéresserons aux réponses comportementales des téléostéens face à des conditions d'hypoxie en fonction de leur personnalité.

4. Présentation de la thèse

4.1. Inscription de la thèse au sein de projets de recherche

Cette thèse s'inscrit dans deux projets distincts que je vais présenter brièvement dans les sections suivantes :

- ANR Fish'N'POPs (ANR-13-CESA-020)
- ERANET-ANIHWA Win-Fish (ANR-14-ANWA-0008)

4.1.1. ANR Fish'N'POPs

Le projet Fish'N'POPs est un projet national financé par l'agence nationale de la recherche (ANR-13-CESA-020) regroupant 5 partenaires :

- Institut Français de Recherche pour l'Exploitation de la Mer Et de ses Ressources (Ifremer)
- Centre National de la Recherche Scientifique (CNRS-UMPC)
- Institut National de la Recherche Agronomique (Inra)
- Ecole Nationale Vétérinaire et de l'Alimentation (Oniris)
- Université du Havre

L'objectif du projet Fish'N'POPs est d'étudier les effets d'un mélange réaliste de congénères de PCB et de PBDE sur le développement, la reproduction et le comportement de la sole commune (*Solea solea*) et du poisson zèbre (*Danio rerio*), ainsi que sur les aptitudes de la descendance. Le projet s'intéresse également au devenir des congénères au sein des organismes chez les individus exposés, ainsi que chez la descendance.

Ce projet comporte 7 « Work package » (WP) que je vais vous présenter brièvement. Mon travail de thèse s'inscrit dans cinq d'entre eux : les WP1, WP4, WP5, WP6 et WP7.

- **WP1**: « Exposition des téléostéens »

Le WP1 est consacrée à la production d'aliment contaminé, ainsi que l'exposition chronique des individus par l'alimentation.

- **WP2**: « Statut endocrinien »

Le WP2 s'intéresse aux possibles altérations du système endocrinien chez les téléostéens exposés au mélange de polluants notamment en mesurant les hormones stéroïdiennes, le cortisol, ainsi que les marqueurs moléculaires associés.

- **WP3**: « Mécanismes moléculaires et aspects fonctionnels de la reproduction »

Le WP3 s'intéresse à l'ovogenèse et la spermatogénèse avec des analyses transcriptomiques et aussi une approche histologique.

- **WP4**: « Performances reproductrices »

Le WP4 examine le succès reproducteur des individus exposés, ainsi que la qualité et la quantité des œufs produits (nombre, taux de fécondation).

- **WP5**: « Développement des larves et performances physiologiques des individus exposés et de la descendance »

Le WP5 étudie d'une part les aspects liés aux traits d'histoire de vie de l'individu (survie, croissance). D'autre part, il s'intéresse au comportement des téléostéens exposés à différents stades de vie (larve, adulte) ainsi que celui de leur descendance jusqu'à la génération F4 afin d'étudier les mécanismes sous-jacents de possibles altérations comportementales.

- **WP6**: « Devenir des polluants »

Le WP6 s'intéresse au devenir des PCB et des PBDE chez les adultes au cours de la maturation des gonades et évalue le transfert de ces POP dans les œufs, les larves et les juvéniles de la génération suivante.

- **WP7**: « Modélisation du devenir et des effets des POP à l'échelle de la population »

Le WP7 intègre toutes les connaissances acquises lors des précédents WP dans le but de développer des modèles de dynamique bioénergétique, démographique et d'évolution des populations.

4.1.2. ERANET-ANIHWA Win-fish

Le projet Win-Fish est un projet européen ANIHWA (Era-Net Animal Health et Welfare) financé par l'agence nationale de la recherche (ANR-14-ANWA-0008) et regroupant 6 partenaires :

- Technical University of Denmark (DTU) – Danemark [Coordination]
- Institut Français de Recherche pour l'Exploitation de la Mer Et de ses Ressources (Ifremer) – France
- Institute of Agrifood Research et Technology (IRTA) – Espagne
- Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) – Italie
- University of Uppsala – Suède
- Technical University of Madrid (UPM) – Espagne

L'objectif du projet Win-Fish est de mieux comprendre les facteurs régissant l' « individualité » et les interactions entre individualité et conditions d'élevage pour tendre à améliorer les performances d'élevage. Les espèces étudiées dans le cadre de ce projet sont le bar européen (*Dicentrarchus labrax*), Daurade royale (*Sparus aurata*), la truite arc-en-ciel, le poisson zèbre et le saumon atlantique (*Salmo salar*). Ce projet comporte 5 WP que je vais vous présenter. Mon travail de thèse s'inscrit dans trois d'entre eux : les WP1, WP2 et WP3.

- **WP1**: « Criblage de la variabilité individuelle chez les espèces ciblées »

Le WP1 s'intéresse aux méthodes de crible du *coping style* chez les différentes espèces étudiées dans le cadre du projet. Les tests de prise de risque et d'évitement de l'hypoxie seront envisagés, ainsi qu'une harmonisation dans le type de test entre les différentes espèces.

- **WP2** : « Validation des indicateurs de bien être »

Le WP2 concerne la validation d'indicateurs comportementaux et physiologiques du bien être chez les téléostéens aussi bien pour des individus que pour des groupes. Ce WP s'intéresse au bar européen, à la truite arc en ciel, ainsi que la dorade royale. Des conditions stressantes, telles que des conditions d'hypoxie, d'hyperoxie, d'hypercapnie ou des variations de la concentration en azote ammoniacal dans le milieu sont envisagées pour répondre aux objectifs de ce WP. Le comportement des différentes espèces sera étudié. Des prélèvements de cerveaux et de sang seront également envisagés afin de valider des indicateurs comportementaux et physiologiques de bien-être.

- **WP3**: « *Coping-styles* et méthodes d'élevage »

Le WP3 s'intéresse aux réponses comportementales et physiologiques des téléostéens élevés dans de nouveaux systèmes d'élevage en fonction de leur *coping style*. Plus précisément, ce WP étudie les réponses du bar européen et de la dorade royale en fonction de la qualité d'eau au sein de systèmes en eau recirculée ainsi que de la densité. Des prélèvements de cerveaux et de sang seront également envisagés afin de mesurer les réponses physiologiques des téléostéens face à ces différentes conditions d'élevage.

- **WP4** : « Marqueurs moléculaires et génétique liés à l'agression »

Le WP4 concerne la mise en place de marqueurs moléculaires et génétiques correspondant à une faible agressivité. Quatre lignées de poisson zèbre ont été sélectionnées en fonction de leur agressivité et de leur audace. Ces lignées vont permettre de suivre les marqueurs neuroendocriniens et génétiques liées à une faible agressivité. L'objectif sera de transférer ces marqueurs au saumon atlantique.

- **WP5**: « Management et coordination »

Le WP5 concerne le management et la coordination du projet sous la responsabilité de Manuel Gesto (DTU, Danemark).

4.2. Les modèles biologiques

De nombreuses espèces de téléostéens, d'eau douce ou marine, sont étudiées dans le domaine de l'écotoxicologie et de l'aquaculture.

En écotoxicologie, le médaka japonais (*Oryzias latipes*; Farwell et al., 2006; Hong et al., 2015, 2010; Le Bihanic et al., 2014; McElroy et al., 2006; Padilla et al., 2009), la truite arc en ciel (Eterson et al., 1996; Sadoul et al., 2017a, 2018b; Thomas et al., 2018), le bar européen (Barboza et al., 2018; Danion et al., 2011a, b; Loizeau et al., 2001; Lorin-Nebel et al., 2014; Peda et al., 2016) et surtout le poisson zèbre (Ali et al., 2012; Cano-Nicolau et al., 2016; Daouk et al., 2011; Knecht et al., 2017; Péan et al., 2013; Vignet et al., 2014a, b) en font par exemple partie.

En aquaculture, les espèces majoritairement étudiées sont la truite arc en ciel (Bermejo-Poza et al., 2015, 2016, 2017; Øverli et al., 1998, 2005; Ruiz-Gomez et al., 2008; Schjolden et al., 2005), le bar européen (Alix et al., 2017; Benhaïm et al., 2012, 2013a, b; Ferrari et al., 2014a,

b 2016; Maugars et al., 2018; Millot et al., 2010, 2011; Samaras et al., 2016), le saumon atlantique (Cubitt et al., 2008; Davidson et al., 2017; Fore et al., 2016; Liu et al., 2017; Overton et al., 2018), le tilapia du nile (*Oreochromis niloticus;* Benhaim et al., 2017; Martins et al., 2011c; Saravanan et al., 2012; Xu et al., 2006) ou encore la daurade royale (Etrew et al., 2004; Koven et al., 2003; Ortuño et al., 2002; Paspatis et al., 2000).

Dans le cadre du projet Fish'N'POPs, mes travaux se sont concentrés sur le poisson zèbre comme modèle d'étude. Son cycle de vie rapide et maitrisé, la transparence de ses œufs, l'accès à de nombreuses lignées, le séquençage de son génome ainsi que des expériences comportementales éprouvées en font un modèle d'étude parfait pour mener à bien des études d'écotoxicologie et notamment celles transgénérationnelles.

Dans le cadre de Win-Fish, l'Ifremer avait en charge les études sur le bar européen comme modèle d'étude. Son importance dans l'aquaculture européenne (148 000 tonnes de bar produites en 2014; FAO (2016)), sa sensibilité au stress, ainsi que des méthodes d'analyses comportementales développées récemment au sein de l'Ifremer en font un modèle d'étude parfait pour répondre aux objectifs du projet.

Les **sections 4.2.1** et **4.2.2** traiteront brièvement de la position systématique, des aspects morphologiques et biologiques, ainsi que les différentes lignées utilisées dans le cadre de cette thèse respectivement pour le poisson zèbre et le bar européen.

4.2.1. Le poisson zèbre, *Danio rerio*

- **Position systématique**

Le poisson zèbre, *Danio rerio*, est un téléostéen de la famille des cyprinidés. Sa position systématique est la suivante :

Super-classe: Ostéichthyens

Classe: Actinoptérygiens

Sous-classe: Neopterygiens

Super-ordre: Téléostéens

Ordre: Cypriniformes

Famille: Cyprinidae

Genre: *Danio*

- **Morphologie**

Le poisson zèbre possède un corps fusiforme orné de teintes vives, métalliques et brillantes et possède une seule nageoire dorsale. Cinq bandes bleues longitudinales ornent son corps sur toute sa longueur. Sa bouche est, quant à elle, plutôt petite et orientée vers le haut ; deux petits barbillons dépassent à la commissure des mâchoires mais sont souvent invisibles car plaqués sur la peau.

Figure 22. Morphologie du poisson zèbre, *Danio rerio.*
Source: http://denigma.de/annotations/species/.

A l'âge adulte, le poisson zèbre présente un dimorphisme sexuel externe. La femelle a un ventre plus bombé que le mâle. Ce dernier possède une couleur jaune plus marquée (**Figure 22**).

- **Répartition géographique**

L'aire de répartition du poisson zèbre est relativement limitée; elle s'étend du nord-est de l'Inde, jusqu'au Bengladesh et au Népal (**Figure 23**).

Figure 23. Aire de répartition du poisson zèbre, *Danio rerio*. D'après Spence et al. (2008).

- **Biologie et développement**

Le poisson zèbre vit en banc dans des eaux peu profondes et à des débits faibles. Il est souvent associé aux rizières où il régule les populations de moustiques présentes dans ces milieux humides en mangeant leurs œufs (Spence et al., 2008). Il est omnivore, et peut manger du zooplancton, des insectes, du phytoplancton, des algues filamenteuses, des plantes vasculaires, des spores mais aussi ses propres œufs et ses juvéniles (Spence et al., 2008).

Le poisson zèbre devient mature en seulement 3-4 mois et mesure au plus 3 à 4 cm à l'âge adulte. Il peut vivre jusqu'à 4 ans et présente une reproduction saisonnière lors de la période de mousson d'été. Les œufs éclosent en seulement 2-3 jours et les larves mesurent alors 3,5 mm. Le stade larvaire dure environ 21 jours (selon la température d'élevage) pendant lesquels la larve grandira jusqu'à atteindre 7 mm. La bouche s'ouvre à 4-5 jours et 4 bandes de pigments noirs apparaissent. Une réponse optique est observée à 72 heures et le système visuel est fonctionnel à partir de 4 jours (Nüsslein-Volhard et Dahm, 2002).

- **Provenance des individus utilisés**

Les poissons zèbres utilisés dans le cadre de cette thèse sont principalement de lignée TU (ZFIN ID: ZDB-GENO-990623-3). J'ai également travaillé sur une lignée transgénique dans laquelle le gène codant la GFP (protéine fluorescente verte) est exprimée sous le contrôle du promoteur de *neurogenin1* (*Tg(-8.4neurog1:gfp*); ZFIN ID: ZDB-GENO-030904-3). Ces différentes lignées sont toutes originaires de l'« European Zebrafish Ressources center » (EZRC, Karlsruhe, Germany) et maintenues à la plateforme d'Ecophysiologie des téléostéens de la station Ifremer de L'Houmeau (PEP - https://wwz.ifremer.fr/pep/).

4.2.2. Le bar européen, *Dicentrarchus labrax*

- **Position systématique**

Le bar européen, *Dicentrarchus labrax*, est un téléostéen de la famille des Moronidés. Sa position systématique est la suivante :

Super-classe: Ostéichthyens

Classe: Actinoptérygiens

Sous-classe: Neopterygiens

Super-ordre: Téléostéens

Ordre: Perciformes

Sous-ordre: Percoidei

Famille: Moronidae

Genre: *Dicentrarchus*

- **Morphologie**

Le bar européen possède un corps fusiforme de couleur argenté composé de petites écailles (62 à 74 sur la ligne latérale). Il est possible d'apercevoir une teinte bleuâtre sur le dos ou jaunâtre sur le ventre de certains individus. Le bar européen est doté de deux nageoires dorsales distinctes, la première possédant 8 à 10 épines alors que la deuxième ne possède qu'une seule épine et 12 à 13 rayons mous. La nageoire anale est, quant à elle, dotée de 3 épines et 10 à 12 rayons mous. Les opercules exhibent une tache noire diffuse et possèdent 2 épines plates tandis que les pré-opercules possèdent de grandes épines dirigées vers l'avant sur le bord inférieur (**Figure 24**). Le bar a une bouche terminale, modérément protractile, avec des dents vomériennes en une bande formant un croissant sans extension sur la ligne médiane de la voûte de la bouche.

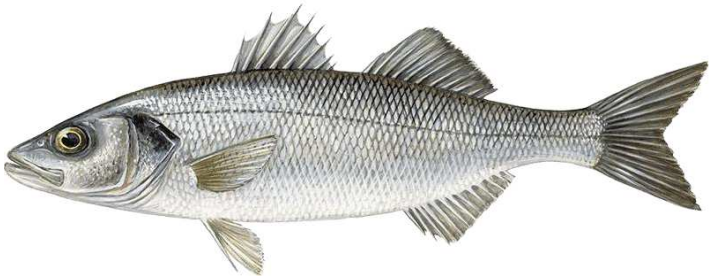

Figure 24. Morphologie du bar européen, *Dicentrarchus labrax.*
Source: https://youngsseafood.co.uk/species/sea-bass/.

Il n'y a pas de dimorphisme sexuel externe chez cette espèce. La distinction entre mâle et femelle se fait donc au niveau interne après observation des gonades. Celles des femelles sont de forme cylindrique et de couleur jaune orangée alors que celle des mâles sont plutôt de forme triangulaire et de couleur blanchâtre.

- **Répartition géographique**

L'aire de répartition du bar s'étend de l'Atlantique nord-est, des côtes du Maroc, au sud de la Norvège incluant la mer d'Irlande, la mer du Nord, la mer Baltique, ainsi que la mer Méditerranée et la mer Noire (**Figure 25**).

Figure 25. Aire de répartition du bar européen, *Dicentrarchus labrax.* D'après https://www.fishbase.de.

- **Biologie et développement**

Les adultes ont des comportements démersaux. Ils peuvent se retrouver jusqu'à des profondeurs d'une centaine de mètres et peuvent aller jusqu'à 80 km des côtes, mais ils sont plus communs dans les eaux littorales peu profondes. Le bar européen est une espèce euryhaline (5 à 28°C) et eurytherme (3 à 38‰). Ils sont capables de fréquenter les eaux côtières intérieures, les estuaires et les lagunes saumâtres. Certains spécimens ont été observés en embouchure de rivières dans des eaux presque douces. Ils migrent en eaux profondes l'hiver et la période de fraie a lieu au printemps. Les œufs sont de petite taille (1,02 à 1,39 mm; Kennedy et Fitzmaurice (1972)) et éclosent entre deux et 5 jours. Les larves mesurent environ 4 mm à l'éclosion (Barnabé, 1976).

Les jeunes vivent en banc et ont un comportement grégaire plus prononcé que les adultes (Barnabé, 1980). Des variations de croissance sont observées tout au long de leur aire de répartition, avec des croissances supérieures aux latitudes sud. Ainsi, à l'âge de cinq ans, la taille moyenne des femelles est de 54 cm en Méditerranée contre 35 cm en Irlande. Celle des mâles est de 48 cm en Méditerranée contre 33 cm en Irlande. Le bar peut vivre jusqu'à 15 ans en milieu naturel. Il existe une différence de croissance entre les sexes dès un âge précoce, *i.e.,* les femelles sont plus grandes que les mâles (Ferrari et al., 2014b). Le bar est une espèce prédatrice opportuniste et très active, se nourrissant de crevettes, crustacés et mollusques, ainsi que d'autres téléostéens. Les juvéniles se nourrissent aussi d'invertébrés alors que les adultes sont plutôt piscivores (Whitehead et al., 1986). Les jeunes comme les adultes chassent en bancs, capturant toutes les espèces saisonnières abondantes dans leurs zones de répartitions.

- **Provenance des individus utilisés**

Les bars utilisés dans le cadre de cette thèse sont issus d'un croisement entre des mâles et des femelles de souches méditerranée-ouest sauvages après 3 générations de domestication réalisé le 27 février 2016 à la station Ifremer de Palavas et élevés selon les standards de l'espèce (Chatain, 1994).

4.3. Objectifs et structure de la thèse

Les objectifs de cette thèse sont les suivants:

(1) Apporter une meilleure compréhension de la **mise en place de la personnalité**, la relation entre **les différents traits de personnalité**, leur **cohérence** à long terme et des **réponses physiologiques associées** aux différentes personnalités chez les téléostéens dans le cadre de la théorie des *coping styles*.

(2) Déterminer les **réponses physiologiques et comportementales** associées à **différents facteurs de stress chimique** (exposition chronique à un mélange de PCB et de PBDE ; variation de la qualité d'eau).

Cette thèse est structurée en trois parties. La première partie vise à répondre à l'objectif **(1)** et comporte deux chapitres:

Chapitre 1: Afin d'étudier la **mise en place de la personnalité**, la relation entre **les différents traits de personnalité**, et leur **cohérence** au cours des différents stades de vie, deux axes de la personnalité (l'audace et l'activité) et l'anxiété ont été mesurés chez les mêmes individus entre deux âges différents chez le poisson zèbre (expérience 1: entre 7 et 21 jours post fécondation, jpf et expérience 2: entre 21 et 60 jpf). Les trois âges choisis permettent de définir différents stade de vie : larves (7 jpf : larves, 21 jpf : transition larves/juvéniles et 60 jpf : juvéniles). Cette expérience a été possible grâce à une méthode de marquage non-invasive (alizarine red S qui permet de marquer les parties calciques d'un organisme) et novatrice dans un tel contexte.

Chapitre 2: Afin d'étudier la **mise en place des comportements** et leur **cohérence** au cours du temps et de différents contextes, l'audace a été mesurée à 227 jpf une première fois à l'aide d'un test de prise de risque en groupe puis une seconde fois à 749 jpf à l'aide d'un test en milieu ouvert (*Open field*) chez le bar européen. Ce test en milieu ouvert, contenant un refuge, a permis de mesurer l'audace des téléostéens pour sortir de la zone refuge mais également permis de quantifier leur comportement dans la zone ouverte. Afin d'étudier les **réponses physiologiques associées** aux comportements mesurés, le sang a été prélevé suite au test pour doser le cortisol. De plus, le cerveau a également été prélevé pour analyser l'expression de gènes impliqués dans la régulation du stress (*mr ,gr, crf*), l'activité (*egr1*) et la plasticité neuronale (*pcna, neurod1, neurod2*), ainsi que pour doser la concentration en monoamines (DA, NE, 5HT).

La deuxième partie de cette thèse vise à répondre en partie à l'objectif **(2)**, étudier les **réponses physiologiques et comportementales** associées à **une exposition chronique à un mélange de PCB et PBDE**. Cette partie comporte également deux chapitres:

Chapitre 3: Pour répondre aux objectifs de cette partie, les poissons zèbres ont été nourris chroniquement à un mélange de PCB et PBDE représentatifs de conditions environnementales (estuaire de Seine) à partir de 5 jpf jusqu'à la reproduction. Pour étudier les **réponses comportementales**, l'anxiété a été mesurée chez les adultes de la génération parentale exposée (F0) à l'aide d'un test de nouvel environnement et chez la descendance jusqu'à la génération F3. De même la réponse photomotrice des larves a été mesurée à 5 jpf de la génération F1 à F4. Ensuite pour étudier les **mécanismes physiologiques** sous jacents, des individus ont été prélevés à 1 jpf pour étudier l'expression de gènes codant pour des enzymes impliqués dans la méthylation de l'ADN (*dnmt*s) et à 5 jpf pour étudier l'expression d'un marqueur d'activité neuronale (*c-fos*). Pour finir, la maturation de l'habenula, qui est une structure du diencéphale susceptible d'expliquer un état d'anxiété, a été étudiée à 4 jpf au sein de la lignée (*Tg(-8.4neurog1:gfp*).

Chapitre 4: Le protocole d'exposition aux PCB et PBDE est le même que dans le chapitre 3. Pour étudier les **syndromes comportementaux** et leurs altérations possibles par ce facteur de stress, nous avons mesuré l'audace à l'aide d'un test de prise de risque en groupe, les capacités d'exploration dans un labyrinthe en Z, la socialité dans un test de choix et le niveau d'activité et d'anxiété lors du test de nouvel environnement. Ces différents tests ont été réalisés à l'âge adulte chez la génération exposée aux polluants F0 et les générations suivantes (F1 et F2). Pour étudier les **mécanismes physiologiques**, une prise de sang a été réalisée le jour suivant la dernière expérience comportementale afin de doser le cortisol. Ce même jour, le cerveau des individus a également été prélevé afin de mener une analyse transcriptomique et identifier des réseaux de gènes pertinents. L'analyse bioinformatique de ces données n'a malheureusement pas pu être finalisée dans le temps de cette thèse.

La troisième et dernière partie vise aussi à répondre à l'objectif **(2)**, étudier les **réponses physiologiques et comportementales** suite à des **variations de la qualité d'eau**. Cette partie comporte également deux chapitres:

Chapitre 5: Pour répondre aux objectifs de cette partie, des bars ont été challengés en situation d'hypoxie (20 % O_2), d'hyperoxie (200% O_2) et de forte concentration d'azote ammoniacal (18 mg/L) dans un nouvel environnement en groupe de 10 durant 2 heures. Leur thigmotaxie, leur activité, ainsi que la cohésion du groupe ont été mesurées au cours du temps dans un test d'*open field*. Cette première étape a permis d'évaluer leurs **réponses comportementales** en condition de stress. Suite à ce test, le sang d'un sous échantillon des individus testés a été prélevé afin de doser le taux de cortisol plasmatique pour relier les réponses comportementales aux **réponses physiologiques**. Un test de nouvel environnement individuel a été réalisé sur un autre sous échantillon avec une qualité d'eau standard pour étudier les réponses comportementales lors de la récupération suivant le test d'*open field* réalisé en condition de stress.

Chapitre 6: Pour finir, l'audace des bars a été mesurée trois fois à l'aide d'un test de prise de risque en groupe. Les individus ont été ensuite testés dans un *open field* en groupe de 8 en condition d'hypoxie progressive afin d'étudier les **réponses comportementales en fonction de leur niveau d'audace**. Un dernier test de prise de risque a ensuite été réalisé 34 jours après ce challenge afin d'étudier l'impact de cette exposition aigue à l'hypoxie sur la personnalité. Pour finir, le cerveau des individus a été prélevé et une analyse transcriptomique est actuellement en cours.

Les indicateurs physiologiques et comportementaux mesurés lors des différents chapitres en fonction des différentes conditions expérimentales, ainsi que les tests comportementaux associés sont récapitulés dans le **tableau 5**.

Tableau 5. Récapitulatif des différents chapitres de la thèse en fonction des conditions expérimentales, des indicateurs comportementaux et physiologiques étudiés, ainsi que des tests comportementaux associés. Les indicateurs en italique sont en cours d'analyse.

Chapitre	Condition expérimentale	Indicateurs: Comportement/ Physiologie	Test comportemental
Chapitre 1	Temps et contexte	Syndrome comportemental: audace, activité, anxiété	Test de prise de risque en groupe Nouvel environnement
Chapitre 2	Temps et contexte	Audace et activité	Test de prise de risque en groupe *Open field* avec refuge
		Réactivité de l'axe HPI Activité et plasticité neuronale Concentrations en monoamines	
Chapitre 3	Exposition chronique à un mélange de PCB et PBDE	Anxiété	Nouvel environnement
		Activité neuronale Mécanismes épigénétiques	
Chapitre 4		Syndrome comportemental: audace, activité, exploration, socialité, et anxiété	Test de prise de risque en groupe Labyrinthe en Z Test de choix Nouvel environnement
		Réactivité de l'axe HPI *Analyse transcriptomique*	
Chapitre 5	Exposition à des conditions hypoxiques, hyperoxiques et à de fortes concentrations d'ammonium	Activité, cohésion du groupe et anxiété	*Open field* Nouvel environnement
		Réactivité de l'axe HPI	
Chapitre 6	Exposition progressive à des conditions hypoxiques	Audace Activité, cohésion du groupe et anxiété en fonction du niveau d'audace	Test de prise de risque en groupe *Open field*
		Analyse transcriptomique	

Partie 1

Le *coping style* chez les téléostéens

Chapitre 1

Zebrafish behavioural syndrome is consistent at different ages from larvae to juveniles but not over time

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Manuscript en préparation

Chapitre 1 – Présentation

Contexte:

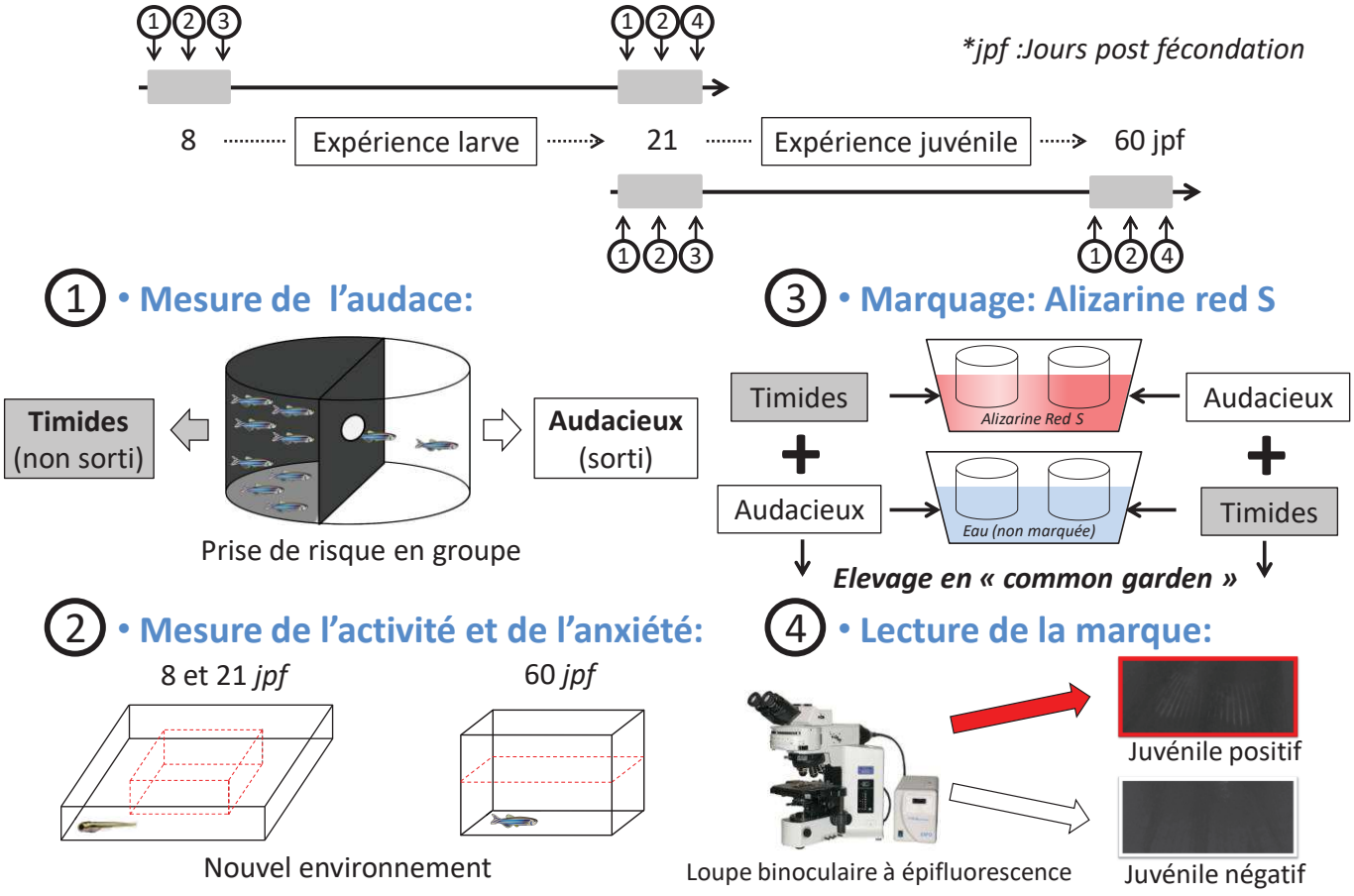
Les *coping styles* sont définis comme une suite de comportements corrélés entre eux (**syndrome comportemental**) et corrélés aux réponses physiologiques, stable dans le temps et dans différents contextes. Si la cohérence du *coping style* entre différents contextes est étudiée à l'âge adulte, très peu d'études portent sur les jeunes stades, notamment le stade larvaire. De plus, les études visant à suivre la cohérence du comportement entre le stade larvaire et les autres stades sont très rares.
Pourtant, de nouvelles connaissances sur cette cohérence entre les différents contextes et les stades de vie du poisson zèbre, qui est un modèle très utilisé dans la recherche, pourraient permettre d'une part, d'expliquer la variabilité observée au sein d'une même lignée et, d'autre part, de diminuer la variabilité des données liées à l'individualité (*coping style*).

Questions:

(1) Existe-t-il un syndrome comportemental chez le poisson zèbre à 8, 21 et 60 jours ?
(2) Ce syndrome comportemental est-il cohérent au cours des différents stade de vie de l'individu ?

Méthode:

Deux expériences ont été menées en parallèle pour suivre la **mise en place** et la **cohérence** d'un possible **syndrome comportemental** entre deux âges (**expérience 1 larve**: entre 7 et 21 jpf et **expérience 2 juvénile**: entre 21 et 60 jpf). Au premier âge de chaque expérience (**larve** ou **juvénile**), l'**audace** des individus a été mesurée dans un test de prise de risque en groupe, puis l'**activité** et l'**anxiété** dans un nouvel environnement. Les individus (audacieux ou timides) ont ensuite été aléatoirement marqués avec l'alizarine red S, maintenus en *common garden* avec des individus non marqués (inversement timides ou audacieux), jusqu'au deuxième âge de l'expérience correspondante où les mêmes comportements ont été mesurés une deuxième fois.



Zebrafish behavioural syndrome is consistent at different ages from larvae to juveniles but not over time

In preparation

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Abstract

Coping style is defined as a set of individual physiological and behavioural differences consistent across time and context. In zebrafish, as well as in many other animals, several covariations were established between behavioural, physiological and molecular responses. There is, however, a lack of study addressing consistency across time starting at larval stage onwards. The aim of our study was to bring a better understanding of behavioural consistency across context and time in zebrafish from larval to juvenile stages. Fish boldness was determined at three ages (8, 21 and 60 days *post* fertilization, dpf) using risk taking test to identify bold (proactive) and shy (reactive) individuals. A second test, either open field test or novel tank challenge, was then performed to evaluate consistency across context and to define a behavioural syndrome. Thereafter, groups of fish (either bold or shy) were bathed in alizarin red S solution for later identification. Fish were challenged again at the following age to evaluate behavioural consistency across time. Our results showed behavioural consistency across context with bold fish being more active and expressing higher thigmotaxis whatever the age. A lack of behavioural consistency was however observed across time between larval (8 dpf) and juvenile stage (60 dpf) suggesting behavioural plasticity.

1. Introduction

Coping style is defined as a coherent set of individual physiological and behavioural traits consistent across time and context, *i.e.* in different times and situations (Koolhaas et al., 1999). The existence of coping styles was demonstrated in many animal species, including fish, and is described as a *continuum* between two extremes phenotypes which can be called proactive and reactive (Castanheira et al., 2017; Ferrari et al., 2015; Øverli et al., 2007; Tudorache et al., 2013). Temperament (Réale et al., 2007), personality (Gostling, 2001; Réale et al., 2010) or behavioural syndrome (Sih et al., 2004) are terminology related to coping style as defined Koolhaas et al. (1999). In fish, proactive individuals are usually bolder (Huntingford et al., 2010; Øverli et al., 2006), more aggressive (Castanheira et al., 2013; Øverli et al., 2004), more active (Øverli et al., 2002) and explore their environment faster than reactive ones (Ferrari et al., 2015; Millot et al., 2009).

Behavioural consistency across time and context was shown in adult fish including zebrafish (Rey et al., 2013; Toms and Echevarria, 2014). As examples, boldness, aggression, fear and exploration, measured in 5 distinct contexts, were consistent across contexts and within a period of 7 days (Toms and Echevarria, 2014). Rey et al. (2013) also showed that boldness, measured in risk-taking test, was positively correlated with aggressive behaviour and strongly consistent across 10 months in adults. In most cases, however, including those mentioned above, coping styles were studied in juveniles or adults and consistency across time evaluated through repeated assays, generally after a short time interval but only within a single stage. Nevertheless, coping styles were also monitored at other stages but only occasionally at early stages, *i.e.* larval stages in fish. In zebrafish 8 days post fertilization (dpf) larvae, Tudorache et al. (2015) showed that swimming behaviour was different depending on emergence order from a sheltered compartment. Early emerging larvae expressed lower velocity and travelled less distance than later emerging ones. Moreover, early emerging larvae expressed higher cortisol level in response to netting stress but reached baseline level faster than late emerging ones which is consistent with a proactive coping style. Pasquet et al. (2016) demonstrated that Northern pike (*Esox lucius*) larvae exhibited consistency in behavioural responses across context in maze and novel object tests. More active larvae visited a higher number of zones in the maze and spent more time near the novel object than others which indicated that coping styles were present and consistent across context. Such cross context consistency is contradictory to what was observed in mangrove killifish in which it was lacking before the juvenile stage at 61 dph (days post hatching, Edenbrow and Croft (2013)).

Ontogenic studies evaluating coping style consistency across stages are even scarcer. Coping style consistency between early (likely larval) and later stages was evaluated in mangrove killifish and nine-spine sticklebacks and authors concluded to a lack of consistency (Edenbrow and Croft, 2013; Herczeg et al., 2013). In both studies, however, individuals were kept isolated to allow for individual identification. This rearing method can introduce several biases such as divergence in physico-chemical variables, unintentional differences in feeding (especially with such small individuals). In addition, in some fish species, social isolation was shown to induce stress (*e.g.* in zebrafish, Pagnussat et al. (2013)).

An option to circumvent these limitations is to use batch marking of larvae. Indeed, this method allows a common garden rearing of fish characterized for their coping style. Such marking can be accomplished using vital staining. Alizarin red S ($C_{14}H_7NaO_7S$), is a fluorescent dye that can label calcium-containing tissue and therefore has been used to monitor skeletal mineralization *in vivo* in zebrafish (Bensimon-Brito et al., 2016; DeLaurier et al., 2010; Tu and Johnson, 2011). Recently, the use of alizarin red S was developed for mass marking at early stage in several fish species showing no particular mortality or growth delay (Caraguel et al., 2015; Lu et al., 2015; Stanczak et al., 2015). Larvae mass marking procedure can be performed by ingestion of *Artemia salina* previously bathed into alizarin red S solution (Stanczak et al., 2015) or directly by bathing larvae into alizarin red S solution (Caraguel et al., 2015; Lu et al., 2015). Therefore, alizarin red S seems to be a promising non-invasive tool to mark larvae and monitor behavioural consistency in zebrafish across life stages. Such information is indeed essential to understand how development and/or environment may shape behaviour and coping style.

With the aim of bringing new knowledge which could benefit zebrafish welfare in husbandry, characterise strains or explain variability observed within a strain (Castanheira et al., 2017; Réale et al., 2007; van den Bos et al., 2017), objectives of this study were first to monitor behavioural consistency of coping styles across context in zebrafish at larval (8 or 21 dpf) and juvenile stage (60 dpf). For this purpose, fish were screened as proactive or reactive using risk-taking test and thereafter challenged in open field test or novel tank diving test at these three ages. Second, behavioural consistency of coping styles across time was monitored by comparing fish behavioural responses between ages (*i.e.,* between 8 and 21, and between 21 and 60 dpf) using alizarin red S bath marking for group identification.

2. Material and methods

This study was conducted under the approval of the Animal Care Committee of Poitou-138 Charentes # 84 COMETHEA (France) under project authorization number CE2012-23 and followed the recommendations of Directive 2010/63/EU.

2.1. Fish rearing

The study was conducted on zebrafish TU strain (ZFIN ID: ZDBGENO-990623-3) kept at the Fish Ecophysiology Platform (PEP - http://wwz.ifremer.fr/pep_eng) and originated from the European Zebrafish Resources Center (EZRC, Karlsruhe, Germany).

To provide larvae for behavioural experiments, eggs were obtained by random pairwise mating using adults from our stock at around 180 dpf. Briefly, one male and one female were placed together in the evening before eggs were required in spawning boxes (AquaSchwarz, Germany). Eggs were collected in the following morning and the fertilization rate was assessed within 2 h of collection. Spawns above 80% fertilization rate were kept for experiments and placed in Petri dishes filled with 30 mL of the isotonic medium E3 (1 L: 17.2 g NaCl, 0.76 g HCl, 2.9 g $CaCl_2$. 2 H_2O, 4.9 $MgSO_4$.7 H_2O) and methylene blue (250 $\mu L.L^{-1}$) and incubated at 28 °C under a 14h/10h dark/light cycle. The day after, 10 eggs from 6 different spawns were mixed, in order to avoid spawn-effects, to provide 60 embryos for one Petri dish. Multiple Petri dishes were prepared depending on the number of replicates needed. At 72 hours *post* fertilization (hpf), after hatching, chorions were removed from the Petri dishes, and larvae were returned to the incubator until 5 dpf. They were then transferred from Petri dishes to 1 L tanks. Initial behavioural tests and alizarin red S marking were performed at 8 and 21 dpf. After these first characterization and marking steps, larvae from both coping styles were kept in common garden (see below for details). At 8 dpf, larvae were reared in 1L tank (24 larvae per tank, n=12 per coping style), at 21 dpf, larvae were reared in 10 L tanks in the rearing system (20 larvae per tank, n=10 per coping style), in both case under a 14h/10h dark/light cycle. Physical water conditions were maintained constant during the experiment: temperature at 27±1°C, conductivity 300±50 $\mu S.cm^{-1}$ and pH 7.5±0.5 using a mixture of reverse osmosis water and tap water, both being initially treated with charcoal and sediments filters. Ammonia, nitrites, and nitrates were monitored weekly and remained within recommended ranges (Lawrence, 2007). Fish were fed three times a day, *i.e.* morning and evening with pellets and at midday with freshly hatched *Artemia salina*. Food pellet size was adapted to fish age and mouth size. From 5 dpf to the end of experiment, fish were fed

sequentially with SDS 100 µm, 200 µm, and 300 µm (Special Diet Service; Dietex international).

2.2. Experimental procedure to monitor behavioural consistency across context and time

Two experiments were performed covering larval stage (Larval experiment) and a juvenile stage (Juvenile experiment) and the succession of steps is described **Figure 1**. In both cases, the first step was a risk-taking test performed in groups to measure boldness and thus determine coping style after which individuals were kept isolated overnight. This was performed at 8 dpf (Larval experiment) and at 21 dpf (Juvenile experiment) after which individuals were kept isolated overnight. The day after, a second test was performed to evaluate coping style consistency across context. Thereafter fish were stained using alizarin red S and fish of both coping styles further reared in common garden to avoid possible rearing bias. Later, at 21 dpf for larval experiment and 60 dpf for juvenile experiment, fish were tested again to evaluate coping style consistency across time at population level and alizarin red S was read to obtain initial coping style. The whole experiment was repeated (see details below) with marking of either bold or shy individuals in order to take possible effect of marking into account.

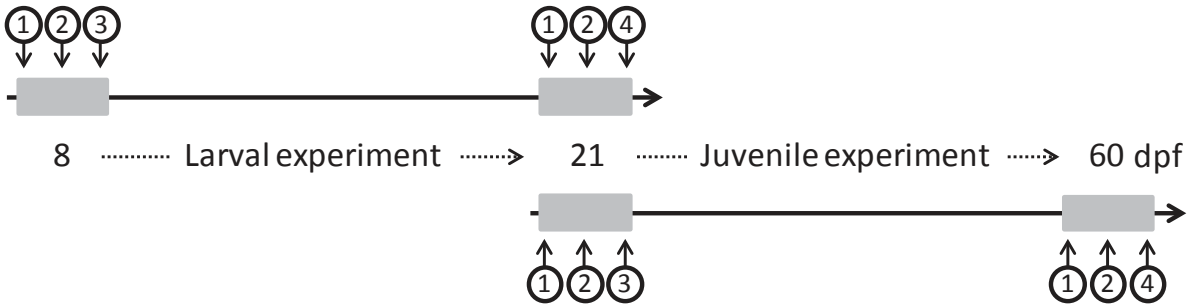

Figure 1. Graphical protocol used to monitor behavioural consistency across context and time in fish larval (Larval experiment) and juvenile (Juvenile experiment) stages. ① Group risk-taking test for coping style screening; ② Individual behavioural characterization using either open field test (at 8 and 21 dpf) or novel tank diving test (at 60 dpf); ③Marking using alizarin red S; ④ Lecture of alizarin red S staining.

2.3. Behavioural experiments

For larval behavioural experiments (8 or 21 dpf), videos were recorded using DanioVision (Noldus, The Netherlands). For juvenile behavioural experiments, videos were recorded with an analogue camera ICD-48E (Ikegami) and a 2.1-13.5 lens (Fujinon) linked to a computer

with an acquisition card and EthoVision XT 10.1 software (Noldus, The Netherlands). Data extraction and analyses were performed using EthoVision XT 10.1 software.

All behavioural experiments were performed in dedicated rooms at 27 °C, with a photoperiod synchronized with the rearing room, *i.e.* 14/10 (light/dark). After each experiment, fish water was changed and fish were fed once a day at the end of the experimental day. Behavioural experiments are detailed in the following sections.

Boldness screening using group risk-taking tests

The day before conducting the test, groups of fish were transferred to the experimental room for acclimatization. Group of fish were introduced into a sheltered compartment. After an acclimatization period of 15 minutes, a door was open allowing fish to exit the sheltered compartment to enter a risky enlightened compartment for a period of 10 minutes. Risk-taking tests were performed using 22 groups (n=330 larvae) at 8 dpf and 4 groups (n=60 larvae) at 21 dpf for the larval experiment. For the juvenile experiment, risk-taking tests were performed using 7 groups (n=105 larvae) at 21 dpf and 5 groups (n=50 fish) at 60 dpf. Depending on fish age, three different risk-taking devices were used and are described below.

At 8 dpf, the device was made of two Petri dishes (3.3cm diameter x 1cm height) connected to one another by a passage tube (0.5cm diameter x 0.6cm height, Tudorache et al. (2015)). One Petri dish was covered of black tape while the other one was enlighten. At 21 dpf, the device was composed of a dark covered compartment (4 cm x 7 cm x 2.5 cm height) connected to another enlighten one (6.5 cm x 7 cm x 2.5 cm height) by an opening situated at the centre of a face compartment. At 60 dpf, the device was composed of a dark covered compartment (8 cm x 10 cm x 11 cm height) connected to another enlighten one (15 cm x 10 cm x 11 cm height) by an opening (2.5 cm in diameter) situated at the centre of a face compartment (Rey et al., 2013).

Whatever the age, at the end of the experiment (10 min after door opened) or if half of the fish had left the sheltered area before the end, the connection between the two compartments was closed. Larvae (8 or 21 dpf) were gently caught using a 3 mL pipette (with a cut wide opening) and placed individually into Petri dish (3.3 cm diameter x 2.5cm height). At 60 dpf fish were netted and placed individually in a 1 L tank (AquaSchwarz, Germany) before further behavioural experiments. Fish that left from the sheltered compartment were considered as bold and fish staying in the covered compartment were considered as shy.

Proportions of bold and shy fish (%) were calculated for each group and age.

Individual open field test and novel tank diving test

The day after risk-taking test, fish were tested individually to evaluate consistency across context and identify a behavioural syndrome by measuring activity, thigmotaxis or vertical positioning (8 dpf: n=48 bold, n=48 shy; 21dpf: n=50 bold, n=55 shy; 60 dpf: n=24 bold, n=23 shy). Depending on fish age, three different devices and different experimental procedures were used, either open field test or novel tank diving test were performed and are described below.

At 8 dpf, the open field test was adapted from Tudorache et al. (2015). Larvae were individually transferred into one well of a 24-well plate (Krystal 24, opaque wall and clear bottom microplate) and placed into DanioVision. After an acclimatization period of 5 minutes in obscurity, the light was switched on and video was recorded in top view for 10 minutes. Open field arena was virtually separated into two equal volume areas (centre and periphery) according to Schnorr et al. (2012). Distance travelled (cm) and proportion of time spent in periphery area (%) were calculated to evaluate activity and thigmotaxis respectively. At 21 dpf, open field test was adapted from Ahmad and Richardson (2013). Larvae were individually transferred to Petri dish (3.3 cm diameter x 1 cm height) containing 5 mL of E3 medium and placed into DanioVision. Video was recorded in top view for 10 minutes. Open field arena was virtually separated into two equal volume areas (centre and periphery). Distance travelled (cm) and proportion of time spent in periphery area (%) were recorded to evaluate activity and thigmotaxis respectively. At 60 dpf, a novel tank diving test adapted from Egan et al.(Egan et al., 2009) was used. Fish were individually transferred into a novel tank (trapezoid 1.5 L tank, Aquatic Habitats) and video was recorded in side view for 4 minutes. Novel tank arena was virtually separated into two equal volume areas (top and bottom) according to Egan et al. (2009). Distance travelled (cm) and proportion of time spent in bottom area (%) were recorded to evaluate activity and bottom dwelling respectively.

2.4. Vital staining using alizarin red S, reading procedure and LPMR test

Marking procedure

Alizarin red S ($C_{14}H_7NaO_7S$) is a fluorochrome, certified by the Biological stain commission for *in vivo* marking. In order to validate the marking procedure performed after Bensimon-Brito et al. (2016), we first verified that marking had no effect on either development, survival

or larval behaviour. For this purpose, we compared these variables between control and alizarine red S bathed larvae. Triplicates of larvae (3x40 larvae) were formed for each treatment, with one at 8 and one at 21 dpf. Treated larvae were bathed into 1 L system water using alizarin red S (Sigma Aldrich, #A5533) solution at 0.01% buffered using sodium hydroxide (50 µL of NaOH at 0.5M) during 150 minutes. After the bathing period, larvae were bathed in clean water twice before reintroduction into the rearing tank. Larvae marked at 8 dpf were then observed at 21 dpf and larvae marked at 21 dpf were observed at 60 dpf to check the efficiency of the marking protocol. The control groups underwent the same manipulation except the exposure and were reared in separate tanks under the same conditions. Survival and staining persistence were monitored during and at the end of the test period.

Reading procedure

Larvae or juveniles were anaesthetized (50 µl of a benzocaine stock solution (50 $g.L^{-1}$ in 100 % ethanol); Benzocaine Sigma, Saint-Louis, USA) for vital staining reading using a stereo microscope (Olympus SZX9) under green fluorescent light (510–550 nm, X-Cite, 120Q EXFO). Images were captured using a camera (DFX31AU03, The Imaging Sources, Germany) and IC Capture 2.4 (The Imaging Sources, Germany).

Monitoring of larval behaviour after alizarin red S marking

During the initial phase of this work, we ascertained that marking procedure produced no behavioural effects in larvae. This was performed using the larval photomotor response test (LPMR; Schnorr et al. (2012); Vignet et al. (2015)). The day after alizarin red S marking (*i.e.* 9 dpf), larvae were transferred individually in wells of 24-well plates (Krystal 24, opaque wall and clear bottom microplate) for acclimatization and plates transferred to the challenge room. Equal number of larvae from each treatment (n=30 control and n=30 marked) were introduced in each plate to avoid potential trial effect. Plates were successively placed into a DanioVision (Noldus, The Netherlands). After a first 10-min acclimatization period in the Daniovision, the LPMR started including the following 5-min steps: Light on–1 (LON1, 70 lux), Light off (LOFF, <1 lux) and Light on–2 (LON2, 70 lux) with constant infra-red light maintained during video recording. Distance travelled (cm) was recorded and summed for each 5-min step.

Application in coping-style experiment

After the behavioural characterisation (at 8 or 21 dpf), groups of larvae - either bold or shy - were marked using alizarine red S using the procedure described above. Thus, either bold or shy marked larvae (8dpf: n=12, 21dpf: n=10) were reared in common garden with unmarked larvae of the opposite coping-style (8dpf: n=12, 21dpf: n=10) until the next test (see **Figure 1**). At least, five replicates (n=3 with bold marked, n=2 with shy marked) were evaluated at each age.

2.5 Statistical analyses

Statistical analyses were performed using R 3.1.0 software (R core team, 2013). All statistical analyses were carried out at 95 % level of significance. Values are represented as mean ± SEM.

To confirm the harmlessness of vital staining, survival was compared between bathed and controls larvae using log-rank Mantel-Cox test. For LPMR, distance travelled were compared between treatments (Control *vs.* Marked) with a repeated-measures ANOVA (with 3 periods, *i.e.* LON-1, LOFF, LON-2) and a Tukey HSD *post-hoc* test.

First, the proportion of fish that left the covered area (bold) during risk-taking tests [here called Context 1] were compared between ages using chi-square test followed by pairwise multiple comparisons. P-values were adjusted using Holm method.

Second, principal component analysis (PCA) was performed within each age and for open field tests or novel tank diving test [here called Context 2:] using ade4 package (Dray and Dufour, 2007) on the variables informing about activity, thigmotaxis and vertical positioning (*i.e.,* distance travelled (cm) and proportion of time spent in periphery area (%) at 8 and 21 dpf; distance travelled (cm) and proportion of time spent in bottom area (%) at 60 dpf). Individual PC scores were downloaded for the first axis (PCA axis 1) at each age and later called individual behavioural scores. In order to validate consistency across contexts [Context 1 x Context 2], Wilcoxon rank sum test was performed on individual behavioural scores between coping styles (bold and shy according to the risk-taking of the corresponding age) at each age of the two experiments.

Finally, exact binomial test was performed to evaluate behavioural consistency in risk taking test between two ages (*i.e.,* 8-21 dpf and 21-60 dpf) with random theoretical probability

within each coping style (p=0.5, i.e. the equal probability of being characterized as proactive or reactive in the second test). Chi-square test was also performed to compare behavioural consistency in risk taking between coping styles within experiments.

3. Results

3.1. Validation of alizarin red S marking procedure

In test groups, 100 % of larvae bathed into alizarin red S 0.01 % during 150 minutes at 8 dpf and 21 dpf presented visible marks in the otolith and calcified tissues of the caudal peduncle at 21 and 60 dpf respectively (**Figure 2.A,B**). Survival of stained groups was above 80% and identical to that of control groups (Mantel Cox: p=0.23 and p=0.94 for marking at 8 and 21 dpf respectively).

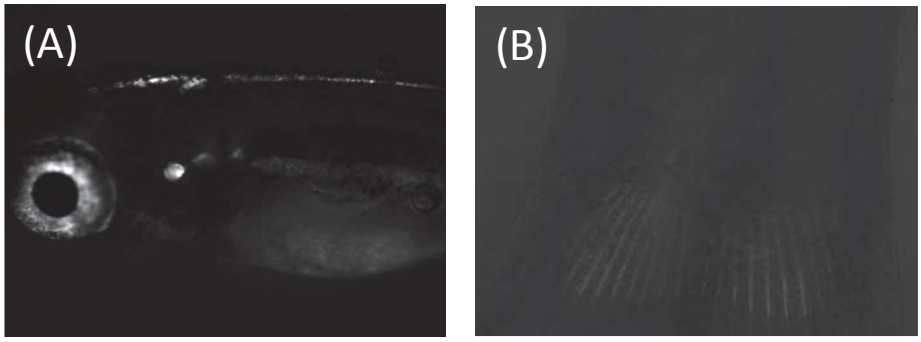

Figure 2. Example of alizarin red S marks observed anaesthetised individuals in **(A)** otolith at 21 dpf and **(B)** in caudal peduncle rays at 60 dpf.

As expected for a LPMR test, the distance travelled was higher during the LOFF period compared to LON1 and LON2 periods regardless of the treatment (Control vs. marked) (repeated measures ANOVA, F=72.5, p<0.001) and there was no effect of treatment (F=0.4, p=0.5) or interaction between treatment and periods (F=0.1, p=0.9; **Figure 3**).

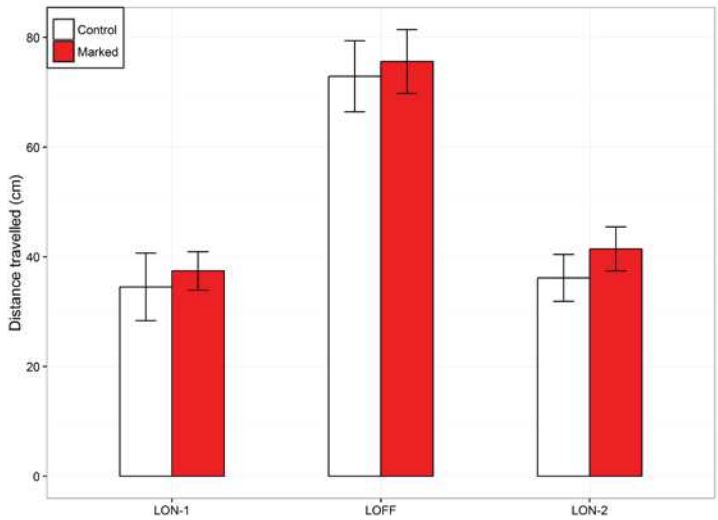

Figure 3. Distance travelled during each 5-min steps (LON-1, LOFF, LON-2) of the LPMR performed at 9 dpf for Control (n=30, white bars) and Marked larvae (n=30, red bars).

3.2. Risk-taking test

The proportion of bold fish, individuals which left from the covered compartment during risk-taking test, was significantly different across ages (χ^2=62.7, df=3, p<0.001). Pairwise comparison indicated that proportion of bold fish was lower at 8 dpf (14.5%) than at 21 dpf (35 %) during the larval experiment (p<0.001). Proportion of bold fish at 21 dpf did not differ between larval and juvenile experiments (35% in larval experiment and 48% in juvenile experiment; p=0.47) and proportion of bold fish from juvenile experiment did not differ between 21 and 60 dpf (48% at both ages; p=1; **Figure 4**).

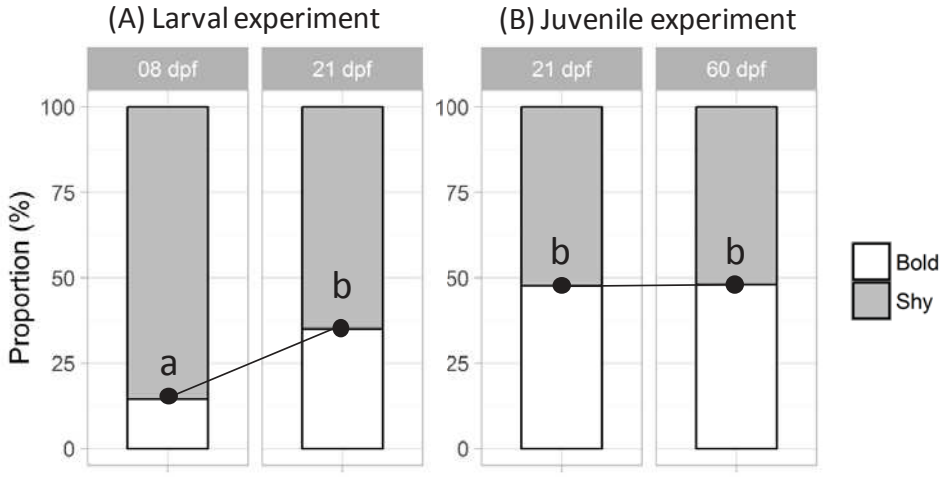

Figure 4. Proportion of bold (white bars) and shy (dark grey bars) individuals during risk-taking test for **(A)** Larval experiment (8 dpf: n=48 bold, n=282 shy; 21 dpf: n=21 bold, n=39 shy) and **(B)** Juvenile experiment (21 dpf: n=50 bold, n=55 shy; 60 dpf: n=24 bold, n=26 shy). Pairwise comparison of proportions: different letters indicate significant difference for proportions of proactive fish across ages.

3.3. Behavioural consistency across context

Since we observed no differences in survival and behavioural responses whether bold or fish groups were marked at 8 or 21 dpf with alizarine red S (not shown), data were treated together.

Individual behavioural score (PCA axis 1) explained 69 and 75 % of the variability observed in behavioural responses at 8 and 21 dpf during larval experiment. Behavioural score explained 70 and 58 % of the variability observed in behavioural responses at 21 and 60 dpf during juvenile experiment. Behavioural score was equally explained by distance travelled (cm) and proportion of time spent in periphery/bottom area (%) for each age. Individuals with high behavioural score expressed higher activity and higher thigmotaxis than individuals with low behavioural score values (**Table 1**).

Table 1. Loadings of behavioural variables for the PCA axis 1 (Behavioural score) performed during larval experiment (8 and 21 dpf) and juvenile experiment (21 and 60 dpf).

	Larval experiment		Juvenile experiment	
Variables in PCA axis 1	8 dpf	21 dpf	21 dpf	60 dpf
Distance travelled (cm)	0.83	0.87	0.84	0.76
Proportion of time (%) spent in periphery (8 & 21 dpf) or bottom area (60 dpf)	0.83	0.87	0.84	0.76

Behavioural score was significantly higher in bold fish than in shy ones at 8 dpf (W=1435, p=0.04) and a trend was observed at 21 dpf (W=468, p=0.09) during larval experiment. During juvenile experiment, bold fish had higher behavioural score than shy ones at both 21 dpf and 60 dpf (W=1046, p=0.04 and W=374, p=0.04 respectively; **Figure 5**). It indicated that bold and shy fish characterized using risk-taking test differed in their behavioural responses during open field test or novel tank diving test. Bold fish displayed a higher activity and higher thigmotaxis than shy ones both during larval and juvenile experiment except a trend for 21 dpf during larval experiment.

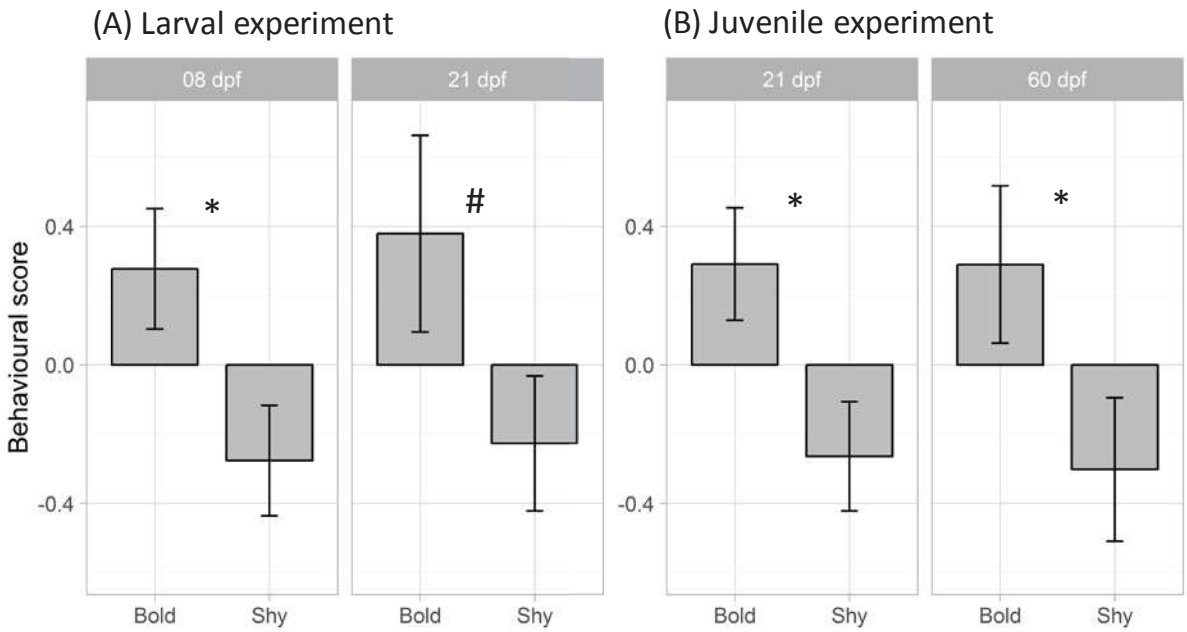

Figure 5. Individual behavioural score of bold and shy fish for **(A)** Larval experiment (8 dpf: n=48 bold, n=48 shy; 21 dpf: n=21 bold, n=35 shy) and **(B)** Juvenile experiment (21 dpf: n=50 bold, n=55 shy; 60 dpf: n=24 bold, n=23 shy). Wilcoxon sum rank test between coping styles within age #: $p<0.10$ and *: $p<0.05$.

3.4. Behavioural consistency across ages

Approximately 33 % of proactive and 61 % of reactive fish expressed behavioural consistency during risk taking test between 8 and 21 dpf. Between 21 and 60 dpf, 41 % of proactive and 61 % of reactive fish expressed behavioural consistency (**Figure 6**). Proportion of consistent behaviour did not differ from random theoretical probability in proactive (p=0.12) and reactive fish (p=0.28) between 8 and 21 dpf. Between 21 and 60 dpf, proportion of consistent behaviour also did not differ from random theoretical probability in proactive (p=0.52) and reactive fish (p=0.40). However, reactive fish tended to be more consistent than proactive ones between 8 and 21 dpf (χ^2= 3.5, df=1, p=0.06). This trend disappeared between 21 and 60 dpf (χ^2= 1.1, df=1, p=0.29, **Figure 6**).

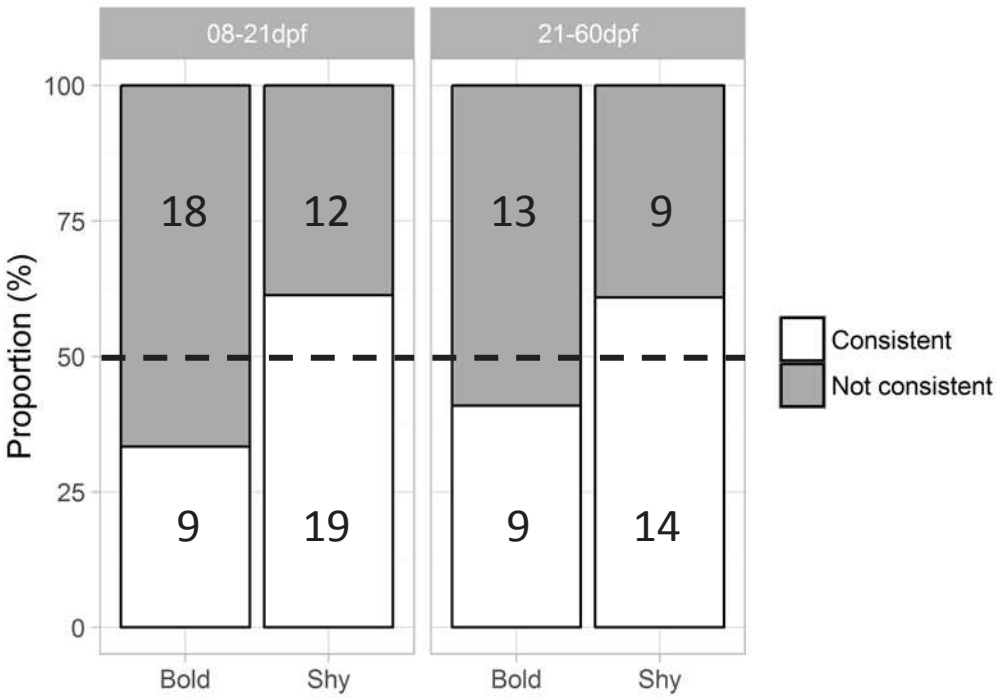

Figure 6. Proportion of fish with consistent (white bars) and non consistent (dark grey bars) coping style during risk-taking test for each age in bold and shy fish (8-21 dpf: n=27 bold, n=31 shy; 21-60 dpf: n=22 bold, n=23 shy). Dashed black line represents random theoretical probability.

4. Discussion

The aim of this study was to monitor coping style ontogeny starting at larval stage in fish. For this purpose we used alizarin red S to mark zebrafish individuals according to their coping style at either 8 dpf (Larval experiment) or 21 dpf (Juvenile experiment). Alizarin red S marks were visible in zebrafish bones and otolith at least 39 days after the marking procedure. As reported in previous studies with other fish species (Caraguel et al., 2015; Lu et al., 2015; Stanczak et al., 2015), alizarin red S marking (at the concentration chosen) did not induce particular mortality or developmental defects in zebrafish. Marking did not induce anxiety-like behaviour in larvae as revealed by LPMR test. These results validate the use of alizarin red S for non-invasive marking suitable for individual or group identification starting at early life stage in fish.

According to definition, coping style is supposed to be consistent over time. In parallel to development, early experience can however affect individuals coping capacity (Koolhaas et al., 1999). In order to evaluate coping style consistency along ontogeny, we took advantage of the possibility to identify group of individuals according to early coping style with alizarin red

S marking. To avoid the influence of marking on the output of the experiments we either marked proactive or reactive individuals. We performed two experiments, the larval experiment starting at 8 dpf and ending at 21 dpf and the juvenile experiment starting at 21 dpf and ending at 60 dpf. In both cases, the first step was an evaluation of boldness. Initial proportion of bold individual was higher in juvenile experiment compared to larval experiment. In larval experiment, the proportion of larvae exiting the shelter was 14.5% of tested individuals. This result contrasts with previous publication which reported a proportion above 90% of larvae exiting the shelter (Tudorache et al., 2015). This difference may be due to difference in fish strain since TU strain has been used in the present report versus individuals from a cross of AB and TL strains. Indeed, several articles have reported significant behavioural differences between classical zebrafish wild-type strains such as AB and TL (van den Bos et al., 2017) including at early stages as shown for AB and TU at larval stage (Vignet et al., 2013). Another explanation may be the lighting level of the room. Indeed the higher the lightning difference between sheltered area and open area, the higher the level of boldness required. Such difference in response according to lightning level in a behavioural test has already been shown with zebrafish larvae (Schnorr et al., 2012). In juvenile experiment, a proportion of 48% of individuals were considered as bold which is a bit higher compared to proportion determined in zebrafish adults (26%) by Rey et al. (2013).

In both experiments, the screening test was followed the day after by a second different test to evaluate consistency across context and to determine a behavioural syndrome. In both larval and juvenile experiments, bold fish expressed higher activity and higher thigmotaxis during open field test or in novel tank diving test respectively. A weaker behavioural consistency across context was however observed at 21 dpf in the larval experiment suggesting that risk taking was less discriminant (higher proportion of 'false' bold) in this case. This also could be attributed to the previous experience and manipulation of the fish at 8 dpf or lower sample size at this experimental age. Such behavioural syndrome, a higher activity in bold individuals, is indeed well described in literature including fish (Castanheira et al., 2017; Geffroy et al., 2015; Moretz et al., 2007; Réale et al., 2010) whereas the link between boldness and thigmotaxis is less documented and sometime controversial (Ariyomo et al., 2013; Burns, 2008; Dahlbom et al., 2011). In our study, bold fish showed a higher thigmotaxis, and as shy individuals are considered to be more plastic than proactive ones (Chapman et al., 2010; Coppens et al., 2010; Ruiz-Gomez et al., 2008), we suggest that shy fish could more easily adapt to open field or novel tank than proactive ones which could

explain a lower thigmotaxis in shy fish. This consistency across contexts allowed us to clearly define exiting fish as proactive or reactive individuals whatever life stage. These results, however, suggest that coping style, or at least the proportion of proactive individuals in a population is not firmly established in larvae.

In both larval and juvenile experiments, the same two tests were repeated 13 days or 40 days after in order to evaluate consistency through time (*i.e.* across ages). Overall, no behavioural consistency was observed between ages both in larval and juvenile experiment for bold and shy fish. This contrasts with a strong behavioural consistency previously reported in zebrafish adults over a period of 10 months (Rey et al., 2013). Our results are however in agreement with other works which analysed time consistency over different ontogenic stages. In nine-spine sticklebacks and starting at 30 dph, feeding behaviour was shown to be consistent within ontogenic stages (juveniles or adults) but not across ages (Herczeg et al., 2013). A similar result was obtained with Eastern mosquitofish which displayed very low personality consistency across ontogeny (Polverino et al., 2016). The same inconsistency of individual behaviour has been shown for three-spine sticklebacks over juveniles, sub-adults and adult stages (Bell and Stamps, 2004). Finally to the best of our knowledge, the only other work including fish larval stage, also concluded to the lack of consistency over time before juvenile stage (61 dph, Edenbrow and Croft (2013)). In addition, in this article, the authors also indicated that there was no cross context consistency before this same age. This is in agreement with the fact that no evidence for personality was found in Eastern mosquitofish 30 days old juvenile versus sub-adults and adults (60 and 120 dph, Polverino et al. (2016)) Regarding these last results, our work lead to different conclusions since we indeed observed consistency across context at all studied stages with a clear behaviour syndrome. This is in agreement with report of the existence of coping style in 8 dpf larvae in zebrafish (Tudorache et al., 2015).

In conclusion, zebrafish boldness measured using risk taking test is lower at 8 dpf than at 21 and 60 dpf. Fish categorized as bold or shy using risk taking test display differential behavioural responses in open field or novel tank diving test at 8, 21 and 60 dpf. Bold fish are more active and show higher thigmotaxis than shy ones whatever the ages starting at least at 8 dpf. No behavioural consistency was however reported between fish ages which suggest behavioural plasticity across life stages. Coping styles are thus present at larval stage but are not fixed for the whole population. The use of alizarin red S to conduct this experiment brings

new perspectives to further study longitudinal evolution of various behavioural traits across life stages.

Acknowledgements

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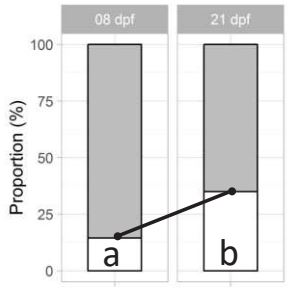
Chapitre 1 – Conclusions

Résultats:

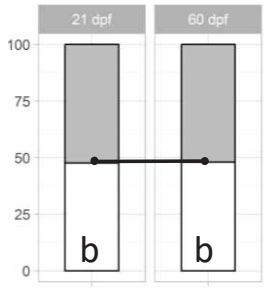
• Audace: test de prise de risque en groupe

• Proportion d'individu audacieux/timide:

• Expérience 1:



• Expérience 2:

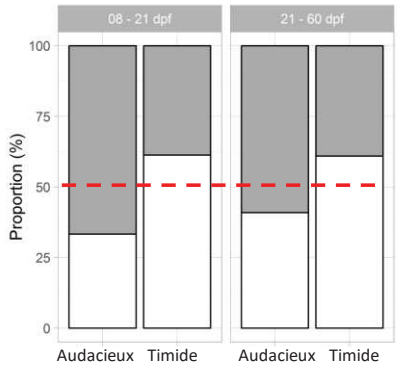


• Légende:

Timides (non sorti)

Audacieux (sorti)

• Cohérence de l'audace entre deux âges:



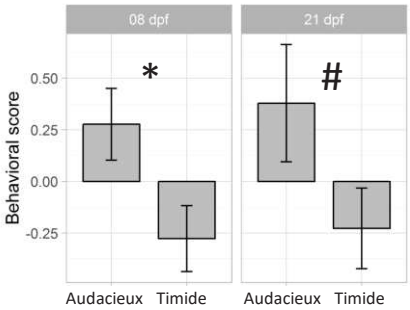
• Légende:

Cohérent

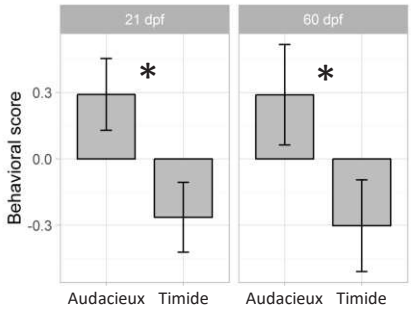
Non cohérent

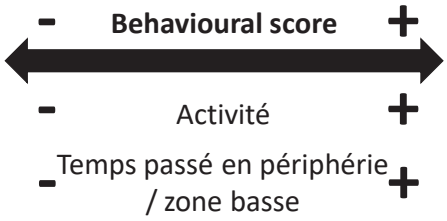
• Syndrome comportemental: nouvel environnement

• Expérience 1:



• Expérience 2:





*: p<0.05 / p=0.09

Points importants:

• La proportion d'audacieux (test de prise de risque) au sein d'une population augmente entre 8 et 21 jours *post fécondation* (*jpf*) pour ensuite devenir stable jusqu'à 60 jpf.

• Les individus audacieux sont plus actifs et montrent davantage de comportements anxieux dans un test de nouvel environnement (thigmotaxie, temps passé en zone basse). Ce syndrome comportemental est retrouvé aux différents âge étudiés.

• L'audace d'un individu semble être un trait de personnalité plastique entre les différents âges, du moins jusqu'à 60 jpf chez le poisson zèbre

➔ En conclusion, les individus présentent le même syndrome comportemental aux différents âges étudiés mais il n'est pas fixé, au moins jusqu'à 60 jpf.

Communications:

• **Société Française d'Etude du Comportement Animal**
Gif-sur-Yvette – 2017 – *Communication affichée*

Chapitre 2

Coping styles in European sea bass: the link between boldness, stress response and neurogenesis

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Physiology and Behavior, in revision

Chapitre 2 – Présentation

Contexte:

Les *coping styles* sont définis comme une suite de comportements corrélés entre eux et corrélés aux réponses physiologiques, stable dans le temps et dans différents contextes. Deux réponses extrêmes sont définies: une réponse active (*proactive*) et une réponse passive (*réactive*). Les individus exprimant des *coping-styles* différents divergent en terme de réalimentation après un stress, de capacités cognitives ou de croissance. Pour cette raison, les *coping styles* peuvent devenir importants dans le domaine de l'aquaculture afin de maximiser les gains de productivité, tout en améliorant les conditions de bien-être des téléostéens. Des études précédentes menées chez le bar européen ont montré la présence de *coping styles* cohérents sur une période mais les mécanismes associés restent encore méconnus.

Questions:

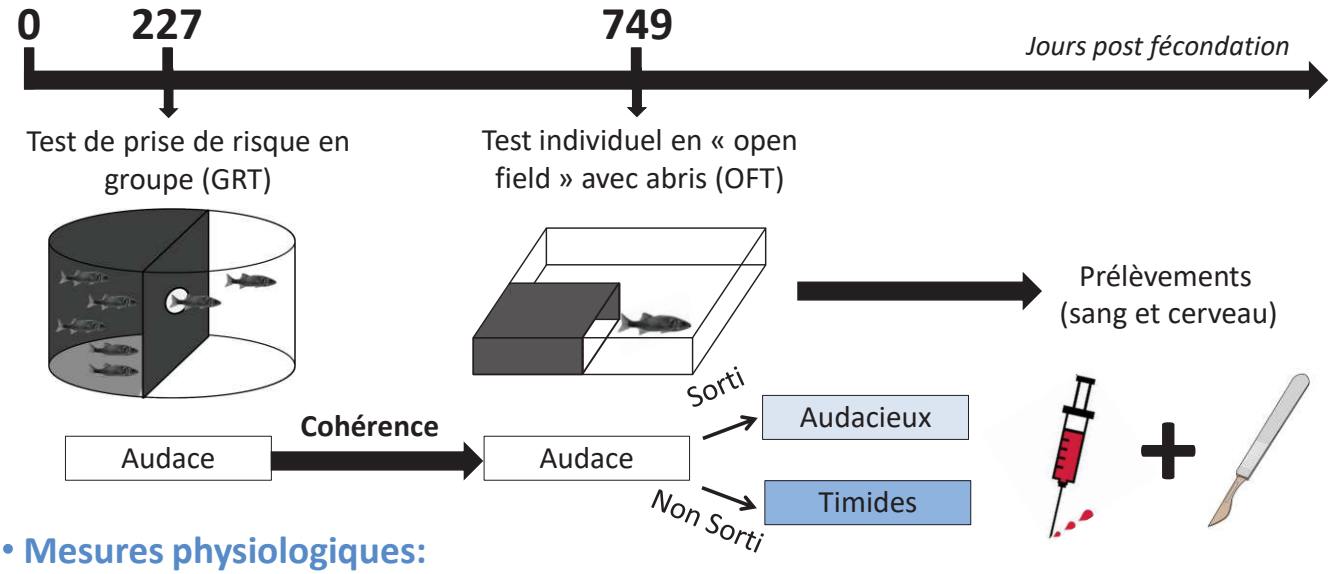
(1) Les *coping styles* chez le bar sont -ils cohérents à long terme et entre contextes ?

(2) Quels sont les mécanismes physiologiques associés à ces différents *coping styles* ?

Méthode:

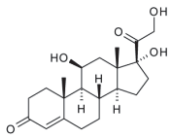
L'**audace** des individus a été mesurée dans un test de prise de risque en groupe (GRT), puis dans en *open field* individuel (OFT) 522 jours après, afin de mesurer la **cohérence** de ce comportement sur une longue période et entre des contextes différents (groupe *vs*. seul). A la fin de l'OFT, les individus ont été prélevés afin de mesurer plusieurs **indicateurs physiologiques** (**cortisol, gènes d'intérêt, monoamines**). Le niveau de ces indicateurs a ensuite été comparé entre les individus catégorisés comme timides ou audacieux lors de l'OFT.

• Mesures comportementales et cohérence au cours du temps:



• Mesures physiologiques:

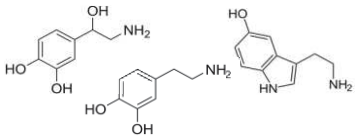
Plasma



Cortisol

Cerveau

Transcription de gènes:
- Régulation du stress (*crf, gr, mr*)
- Activité et plasticité neuronale (*pcna, neurod1, neurod2*) et *egr1*



Concentration en monoamines (dopamine, norépinephrine et sérotonine) et leurs métabolites

Coping styles in European sea bass: the link between boldness, stress response and neurogenesis

In revision in Physiology and Behavior

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Abstract

Coping styles consist of a coherent set of individual physiological and behavioral differences in stress responses that are consistent across time and context. Such consistent inter-individual differences in behavior have already been shown in European sea bass (*Dicentrarchus labrax*), but the associated mechanisms are still poorly understood. Here, we combine physiological measurements with individual behavioral responses in order to characterize coping styles in fish. Fish were tagged and placed in a tank for group risk-taking tests (GRT) at eight months of age to evaluate boldness using the proxy latency of leaving a sheltered area towards an open area. A subsample of these fish were individually challenged 16 months later using an open field test (OFT), in which the boldness was assessed after being placed in a shelter within an open arena. Latency to exit the shelter, time spent in the shelter, and distance travelled were recorded for this purpose. The blood and brain were then collected to evaluate plasma cortisol concentration and neurotransmitter levels (dopamine, norepinephrine, serotonin, and related metabolites), as well as brain transcription of key genes involved in stress axis regulation (*gr1, gr2*, *mr, crf*), neurogenesis (*neurod1, neurod2, pcna*), and neuronal development (*egr1*). Fish acting bolder in the GRT were not necessarily those acting bolder in the OFT, highlighting the relatively low consistency across different types of tests performed with a 16-months interval. There was, however, a significant correlation between stress markers and boldness. Indeed, mRNA levels of *mr, crf, gr2, egr1*, and *neurod2*, as well as norepinephrine levels were higher in shy than bold fish, whereas brain serotonergic activity was lower in shy fish. Overall, our study highlights the fact that boldness was not consistent over time when testing context differed (group *vs.* alone). This suggests that social context play a key role in boldness measurement and that the particular life history of each individual may account in shaping the personality fate of a fish.

1. Introduction

Coping styles have been defined as "a coherent set of individual physiological and behavioral differences in stress responses consistent across time and context" by Koolhaas et al. (1999). They have been described in various animal species, including fish, as a *continuum* between two extreme phenotypes, called proactive and reactive (Castanheira et al., 2017; Ferrari et al., 2015; Øverli et al., 2007; Tudorache et al., 2013). In terms of behavior, proactive fish are bolder, more aggressive, explore their environment faster, and display less flexible behavior than reactive fish (Castanheira et al., 2017; Ferrari et al., 2015; Huntingford et al., 2010; Millot et al., 2009; Øverli et al., 2004b). Moreover, correlations between these behavioral responses across time and context have been established in several fish species (Castanheira et al., 2017; Geffroy et al., 2015; Øverli et al., 2007), as well as between behavior and physiology (Ferrari et al., 2015; Johansen et al., 2012; Killen et al., 2011; MacKenzie et al., 2009; Øverli et al., 2006; Rey et al., 2013; Silva et al., 2010), generally used for defining coping styles since.

Stress can be defined as the cascade of biological events that occur when an organism faces a challenge out of the normal range and attempts to reestablish physiological equilibrium (Barton, 1997). Typical stress responses of fish toward adverse events involve two main neuroendocrine axes, the brain-chromaffin axis and the hypothalamo-pituitary-interrenal (HPI) axis (Barton, 2002; Gesto et al., 2013; Sumpter, 1997). Both axes are important in the primary response to stress (Reid et al., 1998; Wendelaar Bonga, 1997; Winberg et al., 1997), which is activated in various stressful contexts (Gesto et al., 2008; Gesto et al., 2006, 2009; Øverli et al., 2001; Reid et al., 1998; Winberg and Nilsson, 1993) and leads to the release of stress hormones, catecholamines (mostly epinephrine and norepinephrine), and corticosteroids (cortisol in teleost fish) into the systemic circulation. At the central level, several monoaminergic neurotransmitters, such as serotonin (5-HT), dopamine (DA), and norepinephrine (NE), are also known to be involved in the organization of stress responses in vertebrates (Winberg and Nilsson, 1993). Stress induces changes in brain monoaminergic systems, both acutely and chronically, which is especially true for the serotonergic system. For example, serotonergic activity is consistently activated upon both chronic or acute stress exposure in fish (Gesto et al., 2013; Øverli et al., 2001; Winberg et al., 1992). Acute net chasing was for example demonstrated to induce a rapid increase in forebrain serotonergic activity in rainbow trout (Gesto et al., 2013). Similarly, chronic confinement stress (three times a day during four weeks) also induced elevated concentration of 5HIAA and

serotonergic activity in the telencephalon of Artic Charr (Winberg et al., 1992). The concentration of the brain monoamines DA, NE, and 5HT and the concentration of their associated main metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylglycol (MHPG), and hydroxyindoleacetic acid (5HIAA), respectively, are generally used to quantify monoaminergic system activity using the turnover ratio between the monoamine and associated metabolite (Øverli et al., 2001; Shannon et al., 1986; Winberg and Nilsson, 1993). This activity has been suggested to vary between individuals with different behavioral responses (Brelin et al., 2005; Hoglund et al., 2005; Øverli et al., 2001; Winberg and Nilsson, 1993; Winberg and Thornqvist, 2016). Both the stress axis (HPI) and monoaminergic systems (brain-chromaffin) have been shown to be reliable physiological markers correlated with differences in coping styles (Øverli et al., 2004a; Øverli et al., 2004b; Øverli et al., 2001; Sorensen et al., 2013; Vindas et al., 2017a) even if, in some species, physiological markers were not directly linked to behavioral traits related to coping styles (Qu et al., 2018).

The HPI axis, which produces cortisol, its major end-product hormone, acts as an activator of physiological and behavioral responses (Sadoul and Vijayan, 2016; Schreck and Tort, 2016; Sumpter, 1997). Glucocorticoid receptors (Grs) and mineralocorticoid receptor (Mr) bind cortisol (Kiilerich et al., 2018) and are detected in various fish tissues. They are involved in different physiological functions linked for instance to growth, reproduction, immunity and they control cortisol release through a negative feedback (Alderman et al., 2012; Bernier et al., 2009; Faught et al., 2016; Johansen et al., 2011). The production and release of cortisol occurs in interrenal cells, located in the teleost head kidney, and is mainly controlled by adrenocortidotropic hormone (Acth), which is released by the pituitary gland into the blood (Sumpter, 1997). In turn, the release of Acth is mainly controlled by corticotropin releasing-factor (Crf), a neuropeptide produced in the hypothalamus (Doyon et al., 2003; Wendelaar Bonga, 1997). In addition, recent studies suggest that the regulation of stress axis and neurogenesis are closely related (Best et al., 2017; Sadoul et al., 2018; Sorensen et al., 2011) and we previously proposed that fish displaying high sensitivity to stress also exhibit strong neurogenesis in a non-stressful context (Sadoul et al., 2018). Proliferating cell nuclear antigen (Pcna), neurogenic differentiation factors 1 and 2 (Neurod1 and Neurod2), and the early growth response protein (Egr1) are important markers of neurogenesis and neuronal development (Burmeister and Fernald, 2005; Sorensen et al., 2013). While the regulation of stress axes might be an important feature to characterize coping styles in fish, there is still a

paucity of information regarding the differences in neurogenesis between coping styles. Additional data are therefore needed in order to better understand the mechanisms associated to the behavioral responses.

Differences in behavioral and physiological responses to stress may have direct implications on growth, foraging activities, and the resulting fitness of wild fish species (Geffroy and Bardonnet, 2012; Réale et al., 2010; Sadoul and Vijayan, 2016). In the context of aquaculture, better knowledge of individual diversity can help to improve the welfare and management of the fish (Castanheira et al., 2017), as proactive fish sometimes show better feeding motivation and feed efficiency than reactive fish (Castanheira et al., 2017; Martins et al., 2005; Martins et al., 2006; Øverli et al., 2007).

The European sea bass *Dicentrarchus labrax* is a predatory species, widely distributed around Europe and West Africa (FAO, 2012), that has a key ecological role in the marine food chain (Whitehead et al., 1986). It is also one of the most highly farmed fish species in Europe (Tveteras and Nystoyl, 2011). Therefore, additional knowledge of coping styles in European sea bass is required to improve both its management and welfare under conditions of aquaculture (Castanheira et al., 2017; Ferrari et al., 2016a; Ferrari et al., 2015) and to better understand and predict population dynamics (Conrad et al., 2011; Sih et al., 2012).

Here, we had two main objectives. First, we monitored the boldness of the fish, by assessing the behavior of *European sea bass* over time and context testing for behavioral consistency between shy and bold fish. A group risk taking test (GRT) and open field test (OFT) were performed at 227 and 749 days *post* fertilization (dpf), respectively. Second, we aimed to describe the underlying physiological mechanisms associated with boldness. The blood and brains of the fish were sampled after the OFT to measure cortisol concentration in the plasma, the transcription of genes involved in stress regulation (*mr*, *gr1*, *gr2, crf*), neurogenesis (*pcna*, *neurod1*, *neurod2*), and neuronal development (*egr1*) and, finally, the concentration of monoamines (DA, 5HT, NE) and associated metabolites (DOPAC, 5HIAA) in whole brain.

2. Material and methods

Experiments were authorized by ethics committee agreement APAFIS#7098 and all procedures involving animals were in accordance with the ethical standards of the institution and followed the recommendations of Directive 2010/63/EU.

2.1. Fish rearing and pit-tagging

The reproduction and larval rearing of European sea bass, were performed at Ifremer, Palavas-les-flots (France, 34250). Fish were produced from third-generation domesticated West Mediterranean brood stock on February 27, 2016, applying a full factorial mating design combining 10 dams and 50 sires by *in vitro* fertilization. At 223 dpf, ~3000 fish were anesthetized using 200 ppm of benzocaine (benzocaine E1501, Sigma, Saint Louis, MO, USA), measured for body mass (g) and standard length (cm), and pit-tagged in the muscle, just in front of the dorsal fin, with an ISO PIT tag (8 x 1.4 mm) to ensure individual identification.

Fish were maintained until 457 dpf following standard procedures (Chatain, 1994). Thereafter, they were reared in six different 5-m^3 tanks within a recirculating bio-filtered system at a temperature of 20.9 ± 1.3°C, pH = 7.4, and a salinity of 37 PSU under a 10/14 light/dark photoperiod. The fish were fed a standard marine fish aquaculture diet (Neo-grower, Le Gouessant, France) *ad libitum* using a self-feeder.

2.2. Group risk-taking test

The group risk-taking test (GRT) is a standardized test to measure boldness in fish and was adapted from Ferrari et al. (2015). The GRT was performed at 227 dpf with 1,468 fish and the following day, it was repeated with the other 1,468 naive fish (mass = 18.8 ± 1.2 g; standard length = 10.3 ± 1 cm).

The GRT consists of placing all the fish into one half of a 1.5 -m^3 tank, separated in two by an opaque PVC divider (**Figure 1.A**). Fish were gently transferred from their home tank to one half of the experimental tank under light anesthesia (100 ppm Benzocaine, same stock solution as before) between 10:00 and 11:00 am. The side containing the fish was covered with a black opaque curtain to create a sheltered area. Both halves of the tank received water of the same quality, oxygenation, and exchange rate. After an habituation period of 2 h, the door (a circular opening of 12 cm Ø in the centre or the divider) was opened and the fish were left with the possibility to exit the sheltered area, therefore exhibiting a risk-prone behavior reflecting boldness level. The door was equipped with a PIT-tag detection antenna (DORSET, The Netherlands) connected to a control device. The PIT-tag detection antenna allowed the monitoring of fish behavior during the GRT by obtaining the exact exit time (*latency to exit (s)*) of each fish leaving the sheltered area for the first time. As the test lasted for 24 hours, a latency of 1,440 min was assigned to fish that did not exit the sheltered area before the end of

the test. Once the outcome of the GRT was analyzed, the individuals were categorized as bold (fish that exited the shelter) or shy (fish that stayed in the shelter during the entire experiment). They were then transferred back to their home tanks and reared together until the open field test (OFT) was performed, 16 months later.

2.3. Open field test

The open field test (OFT) was adapted from that of Benhaim et al. (2017) and performed at 749 dpf (341 ± 4 g) using a sub-sample of fish (n = 57, half of them bold and the other half shy, based on their GRT categorization). The OFT consists of placing an individual fish in an open and novel arena (75 x 75 cm with a water height of 23 cm, **Figure 1.B**) and tracking its swimming activity. The fish was placed in the closed sheltered area for a habituation period of 5 min. The door was then opened, allowing the fish to explore its novel environment while being video-tracked. Videos were taken with a DMK 31AU03 camera (The Imaging Source, Germany) and the recorded behavior of each fish then analyzed using EthoVision XT 13.1 software (Noldus, The Netherlands) by the same operator (SA). The *latency to exit* the shelter (s) was recorded for each individual. A latency of 1,200 s was assigned to fish that did not exit the shelter at the end of the test. Fish that exited the shelter were categorized as bold, whereas those that did not were categorized as shy. The *total distance travelled* (cm), *number of transitions* between shelter and arena, and the *time spent in the shelter* (s) were also recorded.

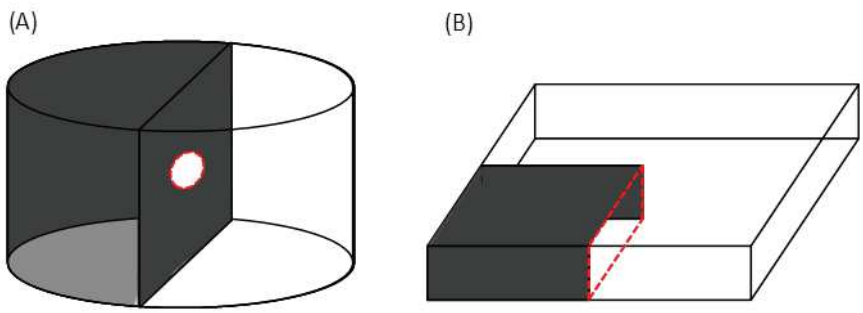

Figure 1. Design of the apparatus for the (A) group risk-taking test (GRT) and (B) open field test (OFT). Dark grey boxes represent the shelter; dashed red lines indicate the door position.

2.4. Blood and brain sampling

Following the OFT, fish were gently removed from the arena and euthanized using 1,500 ppm benzocaine. Blood and brain were sampled without knowing the boldness level of the fish. Blood was sampled from the caudal vessels using lithium heparinized syringes (Sadoul and

Geffroy, 2019). Then, blood was centrifuged (4 min at 13,000 x g) to obtain plasma samples, which were stored at -20°C until analysis. The plasma cortisol concentration was determined by ELISA (RE52061, IBL International, Hamburg, Germany) using a Synergy-HT microplate reader (BioTek Instruments, Winooski, VT, USA) following manufacturer's instructions.

The brain was extracted, divided along the sagittal plane, and placed directly in liquid nitrogen. The left half was used for monoamine analyses and the right half for gene transcription analyses.

2.5. Brain monoamine analyses

The left half of the fish brain was used for HPLC analysis of brain monoamines. Tissues were weighed and immediately homogenized in 1 mL perchloric acid (PCA) solution (0.4 M PCA and 0.1 mM EDTA) with a Sonopuls ultrasonic homogenizer (Bandelin, Germany). Homogenates were then centrifuged for 10 min at 14 000 x g (at 4°C). Supernatants were diluted 20 times in the PCA solution and 20 µL of the dilution injected into the high performance liquid chromatography (HPLC) system.

The diluted supernatants were analyzed high performance liquid chromatography with electrochemical detection (HPLC-EC). The HPLC mobile phase consisted of 73.9 mM NaH_2PO_4, 0.1 mM Na_2EDTA, and 0.58 mM sodium 1-octanesulfonate in deionized water with 15.3% (v/v) methanol. The pH of the mobile phase was adjusted to 3.0 with orthophosphoric acid. The chromatographic separation was performed at 0.9 mL/min with a Reprosil-Pur 120 C18-AQ column (150 x 4 mm, 5 µm particles) (Dr. Maisch, Germany) and electrochemical detection of the separated compounds was performed using an ESA Coulochem II detector. The detection system included a double analytical cell with oxidation potentials set at 0 mV (first electrode) and +340 mV (second electrode). A conditioning cell at + 400 mV was used before the analytical cell to oxidize potential interfering compounds. The rest of the HPLC system consisted of a Shimadzu LC-10AD pump and a Midas autosampler (Spark Holland, The Netherlands). Acquisition and integration of the chromatograms were performed using ClarityTM HPLC software (DataApex Ltd, Czech Republic).

The levels of serotonin (5HT), dopamine (DA), and their main oxidative metabolites 5-hydroxyindoleacetic acid (5HIAA) and 3, 4- dihydroxyphenylacetic acid (DOPAC) were quantified, along with the levels of norepinephrine (NE). The main NE metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG), was below the detection limit. Quantification was carried out by comparing peak areas with those of the corresponding standards. The ratios

between 5HIAA and 5HT and between DOPAC and DA were then calculated as indirect measures of the activity of the serotonergic and dopaminergic neurons, respectively (Winberg and Nilsson, 1993).

2.6. Gene transcription analyses

The right half of the fish brain was used for gene transcription analyses.

Extraction and reverse transcription of total RNA from the brain

Each half-brain (± 200 mg) was mechanically disrupted using a ball mill in 900 µL QIAzol lysis Reagent (Quiagen) in 2 ml RNAse-free tubes. They were then diluted two-fold before starting RNA extraction, following the manufacturer's instructions (RNeasy Plus Universal Mini Kit protocol, Qiagen, recommended initial sample size: 100 mg). The reverse transcription of total RNA to cDNA was performed as described in Geffroy et al. (2016). RNA quantity was assessed by measuring the A260/A280 ratio using the NanoDrop® ND-1000 V3300 Spectrophotometer (Nanodrop Technology Inc., Wilmington, DE, USA). Briefly, an aliquot of each tube was diluted in diethylpyrocarbonate-treated water. Then, 1.5 µg RNA was reverse transcribed using 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (PROMEGA) and 2 µg random hexamers (Promega) in a master-mix buffer and supplemented with 25 U RNase inhibitor (RNasin; Promega) in a final volume of 15 µl. All cDNA were diluted 50 fold in nuclease-free water prior to quantitative real-time PCR (qPCR).

Quantitative real-time PCR

The transcription of genes involved in stress regulation, namely glucocorticoid receptor 1 and 2 (*gr1* and *gr2*), the mineralocorticoid receptor (*mr*), and corticotrophin releasing factor (*crf*) was monitored. We also investigated the expression of genes involved in neurogenesis: neurogenic differentiation factors (*neurod1* and *neurod2*) and proliferating cell nuclear antigen (*pcna*), as well as an early growth response gene (*egr1*), involved in brain development.

Primer sequences were retrieved from the literature (Crespo et al., 2013; Pavlidis et al., 2011; Sadoul et al., 2018) or specifically-designed using the web version of PRIMER 3 (http://primer3.ut.ee; **Table 1**). The qPCR experiments were conducted in a final volume of 1.5 µl, using 1 µl fish brain cDNA and 0.5 µl of an optimized concentration of primers and Fast SYBR Green PCR Master Mix (Applied Biosystems, Life technologies). Distribution of the volume into the plate was performed using a robot (Labcyte Echo525) able to distribute as low as 25 nL by acoustic transfer ensuring reliable repeatability even with small qPCR

volumes. The hot start enzyme was activated for 2 min at 95°C and amplification carried out using the following program: 95°C for 3 s and 60°C for 30 s for 40 cycles. After amplification, a melting curve was obtained using the following protocol: 10 s holding at 55°C followed by sequential 0.05°C increases repeated 80 times. Technical duplicates were run for each sample. A standard curve was generated by mixing all samples and the efficiency (E) for all genes was calculated (*ef1*: 1.98; *l13*: 1.96; *gr1*: 1.90; *gr2*: 2.14; *mr*: 2; *crf*: 1.83; *egr1*: 2; *neurod1*: 1.88; *neurod2*: 1.88; and *pcna*: 1.80).

Housekeeping genes

Ribosomal protein (*l13*) and eukaryotic translation elongation factor 1 alpha (*eef1-alpha*) have both been recommended as housekeeping genes (Bland et al., 2012; Radonic et al., 2004) and were used in this study. The coefficients of variation were below 5% for both *l13* and *eef1-alpha*. The amplification efficiency was 98 and 96 % for *l13* and *eef1-alpha* respectively.

Relative levels of gene transcription were obtained using the following equation with the efficiency (E) of the target gene normalized by the efficiency and the geometric mean of the two housekeeping genes as reference (ref; *l13*and *eef1-alpha*), according to Roche diagnostic (Pfaffl, 2001):

$$Relative\ level\ of\ gene\ transcription = \frac{(E_{ref})^{Ct_{ref}}}{(E_{target})^{Ct_{sample}}}$$

Table 1. GenBank accession numbers and primer sequences for each gene used for gene transcription analyses.

Gene	GenBank accession numbers	Forward primer (5' → 3')	Reverse primer (3' → 5')
mr	JF824641.1	GTTCCACAAAGAGCCCCAAG	AGGAGGACTGGTGGTTGATG
gr1	AY549305[a]	GAGATTTGGCAAGACCTTGACC	ACCACACCAGGCGTACTGA
gr2	AY619996[a]	GACGCAGACCTCCACTACATTC	GCCGTTCATACTCTCAACCAC
crf	JF274994	GCAACGGGGACTCTAACTCT	GTCAGGTCCAGGGATATCGG
neurod1	b*	TTCTCCTTCAGCGTGCACTA	GGTGCGAGTGTCCATCAAAG
neurod2	d*	TGCGTAAAGTGGTTCCATGC	GTCGTGGGTTGGGAGAGTC
pcna	JQ755266[c]	CAGAGCGGCTGGTTGCA	CACCAAAGTGGAGCGAACAA
egr1	d*	AACTCCAGCCTCAGTTCCTC	AGTCAGGAATCATGGGCACA
eef1-alpha	AJ866727.1	AGATGGGCTTGTTCAAGGGA	TACAGTTCCAATACCGCCGA
l13	DT044910.1	TCTGGAGGACTGTCAGGGGCATGC	AGACGCACAATCTTGAGAGCAG

a*: from Pavlidis et al. (2011); b*: from Sadoul et al. (2018); c*: from Crespo et al. (2013); and d*: designed from the European sea bass genome from UCSC.

2.7. Statistical analyses

Statistical analyses were performed using R 3.1.0 software (team, 2013). All statistical analyses were carried out at the 95% level of significance. Values are expressed in the text as the mean ± standard error of the mean (SEM) and illustrated in the figures using boxplots. The central line of the boxplot indicates the median and the boxes the quartiles, with the whiskers covering 95% of the values. Outliers are represented by points. First, the chi-square test was performed to compare the proportion of bold versus shy fish obtained between the GRT and OFT. Then, an exact binomial test was performed to evaluate behavioral consistency between the GRT and OFT by comparing the proportion of consistent behavior with random theoretical probability ($p = 0.5$) for each coping style. The remaining tests (concerning physiology and behavior) were performed on fish screened in the GRT and the OFT respectively.

Normality was verified for other comparisons between coping styles on behavioral and physiological variables using the Shapiro test before applying either the Student's t-test or Wilcoxon rank sum test, depending on whether normality was verified or not. Comparisons between individual coping styles in the *distance travelled*, the *number of transitions* between shelter and OFT arena, and the *time spent in the shelter* were performed using the Wilcoxon rank sum test.

Concerning physiological variables, two individuals were discarded due to an RNA extraction failure (no gene transcription) and therefore analyses were performed on a total of 55 individuals. The Student's t-test was performed to compare plasma cortisol concentrations and monoamine concentrations in the brain (5HT, 5HIAA, 5HIAA/5HT turnover ratio, DA, DOPAC, DOPAC/DA, and NE) between coping styles measured in the OFT. The Wilcoxon rank sum test was performed to compare the transcription of genes (*gr1*, gr2, *mr*, *crf*, *neurod1*, *neurod2* and *pcna*) between coping styles (Student t-test for *egr-1*).

Finally, a principal component analysis (PCA) was performed among fish that exited the shelter with individual continuous variables recorded in OFT (Latency to exit shelter, distance travelled, time spent in shelter and number of transitions) using ade4 package (Dray and Dufour, 2007). Individual PC scores of the two first axes of the PCA were then downloaded. PC scores from axis 2, reflecting coping styles were analyzed using linear regression as in relation to either monoamine concentration or relative level of gene transcription in the brain. Homogeneity of the model residuals was *a posteriori* checked using Shapiro-Wilk.

3. Results

3.1. Behavioral consistency across context and time between the GRT and OFT

During the GRT, 63% of the fish exited the sheltered area within the 24 hours of the test and were designated as bold. Consequently, 37% of the fish were designated as shy in the GRT. In the OFT, 58% of the fish exhibited bold behavior and exited the sheltered compartment *vs.* 42% which exhibited shy behavior and stayed in the sheltered compartment during the 20 min of the test. The proportion of fish with bold/shy behavior was similar between the GRT and OFT ($\chi^2 = 0.51$; $p = 0.48$, **Figure 2A**). However, there was no behavioral consistency across context between the GRT and OFT for either bold or shy fish designated as such during the GRT ($p = 0.28$ and 0.85, respectively). Indeed, 46% of fish designated as shy during the GRT stayed shy in the OFT, whereas the other 54% became bold one year later in the OFT. For fish

designated as bold during the GRT, 61% stayed bold, whereas the other 39% became shy during the OFT (**Figure 2B**).

Table 2. Contingency table showing behavior displayed by the fish during the two tests (GRT and OFT).

		Open field test		
		New shy	New bold	Total
Group risk-taking test	Original shy	12	14	26
	Original bold	12	19	31
	Total	24	33	57

3.2.Open field test

Of the 57 fish screened in the OFT, 58% were categorized as bold. These bold fish actively swam in the tank during the 20 minutes of the test (912.7 ± 60.2 cm; W = 792, $p < 0.001$) and moved between shelter and open field area (2.4 ± 0.4 times; W = 0, $p < 0.001$); whereas the shy fish stayed in the shelter. Although the bold fish exited the shelter and were active in the open arena, most nonetheless spent most of the test time in the shelter (1,000 ± 64 s), but still for less than the shy fish (W = 792, $p < 0.001$, **Figure 2**).

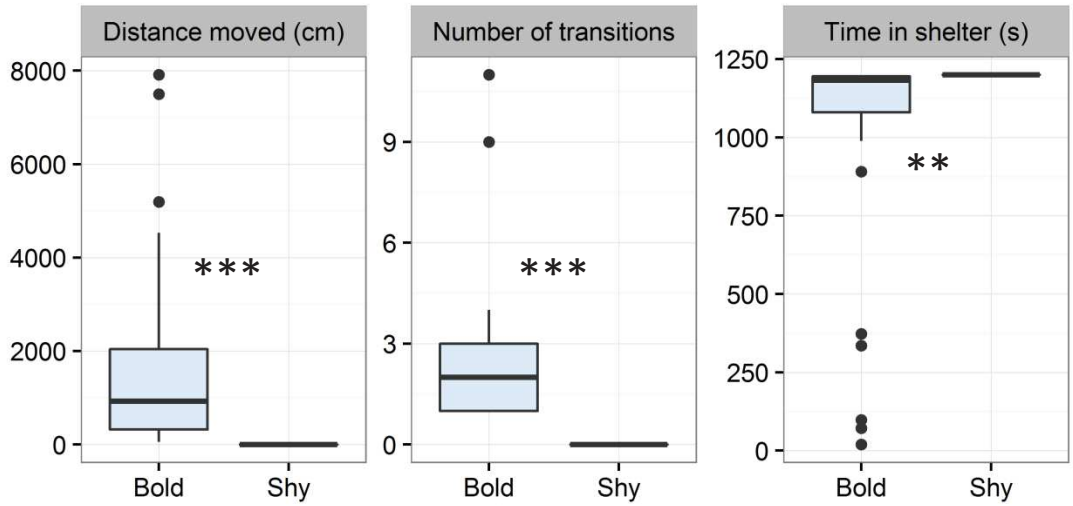

Figure 2. Distance travelled (cm), number of transitions between shelter and arena, and time spent in the shelter (s) during the open field test (OFT) between bold and shy fish categories as assigned in the OFT. Wilcoxon rank sum test: **: $p < 0.01$; ***: $p < 0.001$.

3.3. Plasma cortisol concentration

Plasma cortisol concentrations were similar between bold and shy fish screened in the OFT ($t = -0.10$, $p = 0.92$, data not shown). The mean plasma cortisol concentration in bold fish was 904.3 ± 51.8 ng/mL ($n = 33$) and 912.7 ± 60.2 ng/mL in shy fish ($n = 24$).

3.4. Monoamine concentration in the brain

There was no difference in the concentration of DA or DOPAC in the brain between bold and shy fish screened in the OFT, nor the DOPAC/DA ratio ($t = -0.86$, $p = 0.40$; $t = -0.58$, $p = 0.57$; and $t = -0.61$, $p = 0.54$, respectively). For the serotonergic system, there was no difference in the concentration of 5HT or 5HIAA in the brain between bold and shy fish ($t = 0.45$, $p=0.66$ and $t = -1.15$, $p = 0.25$, respectively). However, shy fish had a significantly lower 5HIAA/5HT ratio in the brain than bold fish ($t = -2.17$, $p = 0.03$). The concentration of NE in the brain was also significantly higher in the shy than bold fish ($t = 2.22$, $p = 0.03$, **Figure 3**). Note that when considering bold and shy fish categories as assigned after the GRT, no difference in monoamine concentration or turnover ratio was detected (**Supplementary material**).

3.5.Gene transcription in the brain

The transcription of *gr1* in the brain did not differ between bold and shy fish screened in the OFT ($W = 312$, $p = 0.32$), whereas *gr2*, *mr* and *crf* were significantly upregulated in the brains of shy fish relative to those of bold fish ($W = 171$, $p = 0.01$, $W = 255$, $p = 0.047$ and $W = 145$, $p < 0.001$, respectively).

The transcription of *neurod1* and *pcna* in the brain did not differ between bold and shy fish ($W = 358$, $p = 0.82$ and $W = 311$, $p = 0.31$, respectively), whereas there was a trend towards the upregulation of *neurod2* in shy fish relative to bold fish ($W = 269$, $p = 0.08$). The transcription of *egr1* was significantly upregulated in the brains of shy fish relative to those of bold fish ($t = -2.70$, $p < 0.01$, **Figure 4**). Note that when considering bold and shy fish categories as assigned after the GRT, no difference in gene transcription was detected (**Supplementary material**).

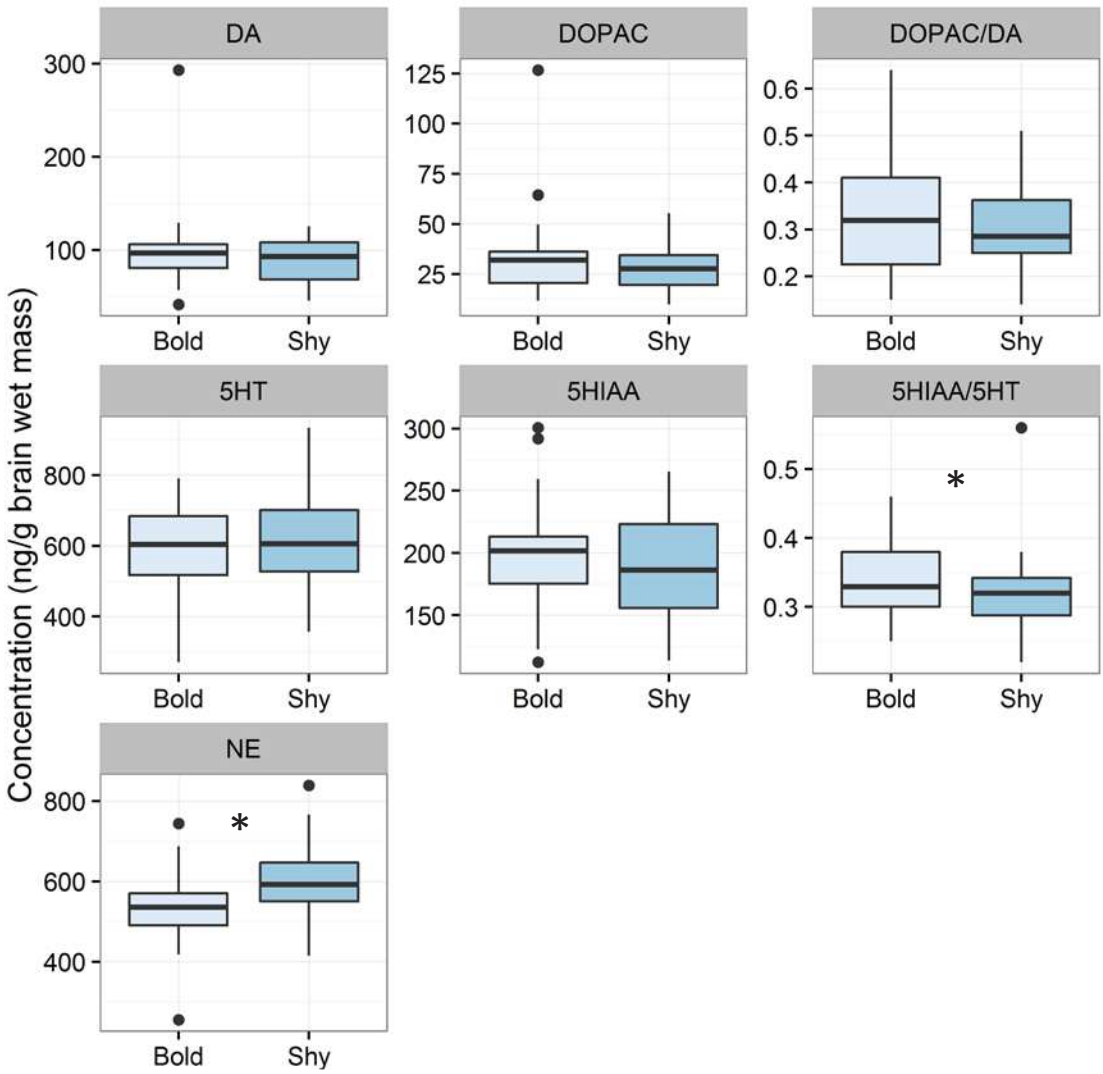

Figure 3. Concentration (ng/g brain wet mass) of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), the DOPAC/DA turnover ratio, serotonin (5HT), 5-hydroxyindoleacetic acid (5HIAA), the 5HIAA/5HT turnover ratio, and norepinephrine (NE) in the brains of bold and shy fish categories as assigned in the open field test (OFT). Student's t-test: *: $p < 0.05$.

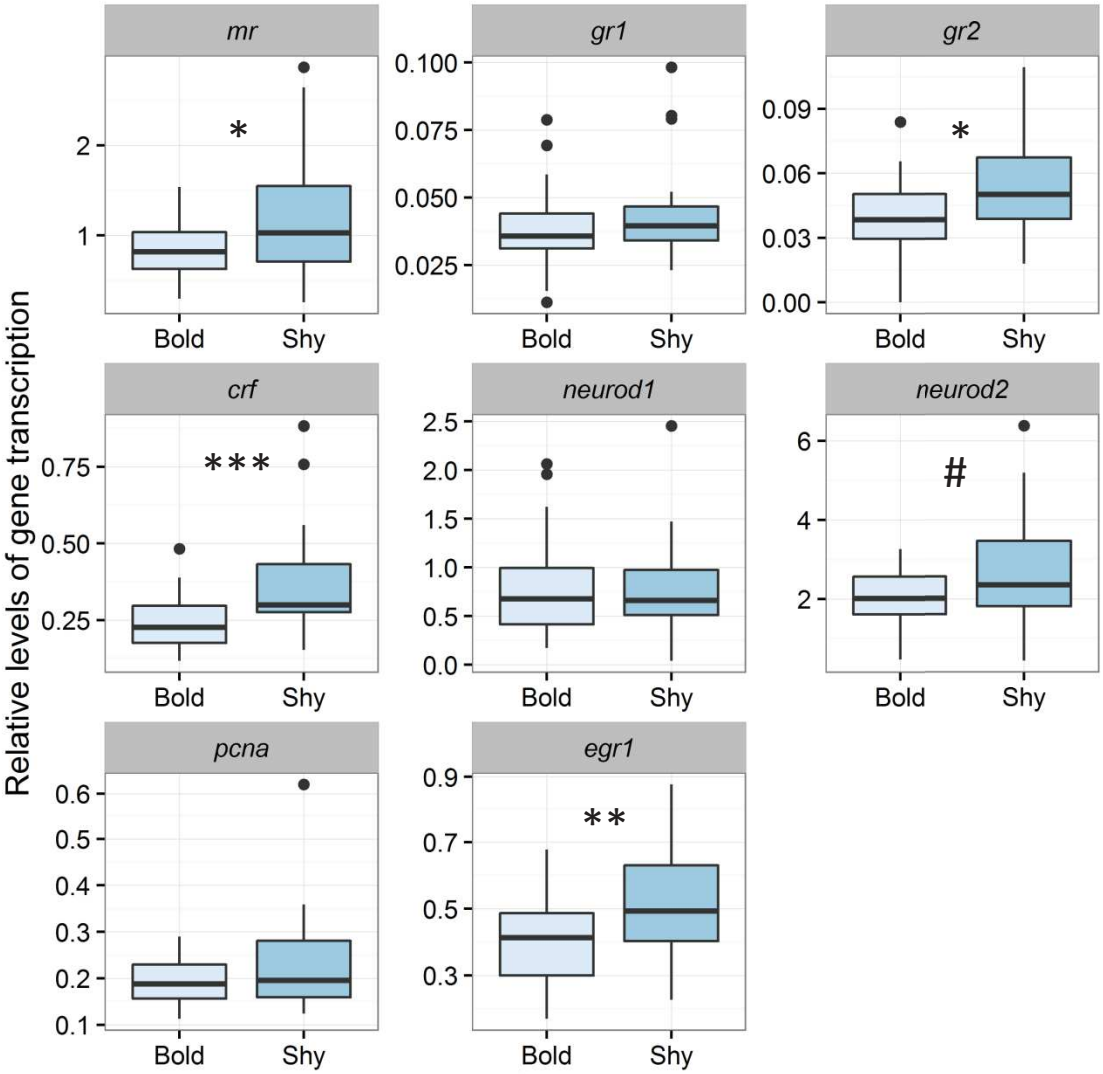

Figure 4. Relative levels of gene transcription of the *mineralocorticoid receptor* (*mr*), *glucocorticoid receptor* 1 (*gr1*), *glucocorticoid receptor* 2 (*gr2*), *corticotropin-releasing factor* (*crf*), *neurogenic differentiation factors* (*neurod1* and *neurod2*), *proliferating cell nuclear antigen* (*pcna*), and *early growth response* (*egr1*) relative to reference genes (*l13* and *eef1-alpha*) in the brains of bold and shy fish categories assigned after the open field test (OFT). Wilcoxon rank sum test (Student's t-test for *egr1*): #: $p < 0.10$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Note that for *gr2*, 6 cDNA samples were lost and are not presented.

3.6.Principal component analysis (PCA)

On one hand, PCA axis 1, which explained 52 % of the data variability observed, was mainly driven by the time spent in shelter, the distance travelled and the number of transitions between shelter and open field arena during the OFT (**Table 3**). On the other hand, PCA axis 2 was mainly driven by the latency to exit the shelter and the number of transitions between shelter and open field arena during the OFT and explained 28 % of the data variability observed (**Table 3**). PCA axis 1 was thus rather representative of activity in the OFT with

individuals displaying high values for PCA axis 1 being more active in the OFT than individuals displaying low values. PCA axis 2 was rather representative of coping styles and individuals displaying high values for PCA axis 2 scores were shyer and moved more between shelter and open field arena than individuals with low values which were bolder and moved less between the two compartments of the OFT. Because of PCA axis 2 being more representative of coping styles, further analyses were performed on PCA axis 2 scores.

Table 3. Loading of behavioral variables (Latency to exit shelter, distance travelled, time spent in shelter and number of transitions between shelter and open field arena during the open field test) for the two PCA axes. Values in bold are the main drivers of the related axis.

Variable contribution	PCA axis 1	PCA axis 2
Latency to exit shelter	0.02	**0.90**
Distance travelled	**0.94**	-0.18
Time spent in shelter	**-0.95**	0.13
Number of transitions	**0.56**	**0.50**
Variability explained (%)	52	28

Regarding monoamines concentration in the brain, concentration of DA was negatively correlated with PCA axis 2 ($F = 5.25$, $p = 0.03$, $R^2 = 0.12$), *i.e.,* bolder fish displayed higher level of DA in the brain but there was no correlation for DOPAC or DOPAC/DA ratio with PCA axis 2 ($F = 0.98$, $p = 0.33$ and $F = 0.002$, $p = 0.96$ respectively). Moreover, 5HT and 5HIAA also were negatively correlated with PCA axis 2 scores ($F = 6.10$, $p = 0.02$, $R^2 = 0.14$ and $F = 7.96$, $p = 0.008$, $R^2 = 0.18$ respectively) but there was no correlations between 5HIAA/5HT ratio and PCA axis 2 ($F = 0.39$, $p = 0.54$). Finally there was no correlation between concentration of NE in the brain and PCA axis 2 scores ($F = 1.4$ $p = 0.25$, **Figure 5**).

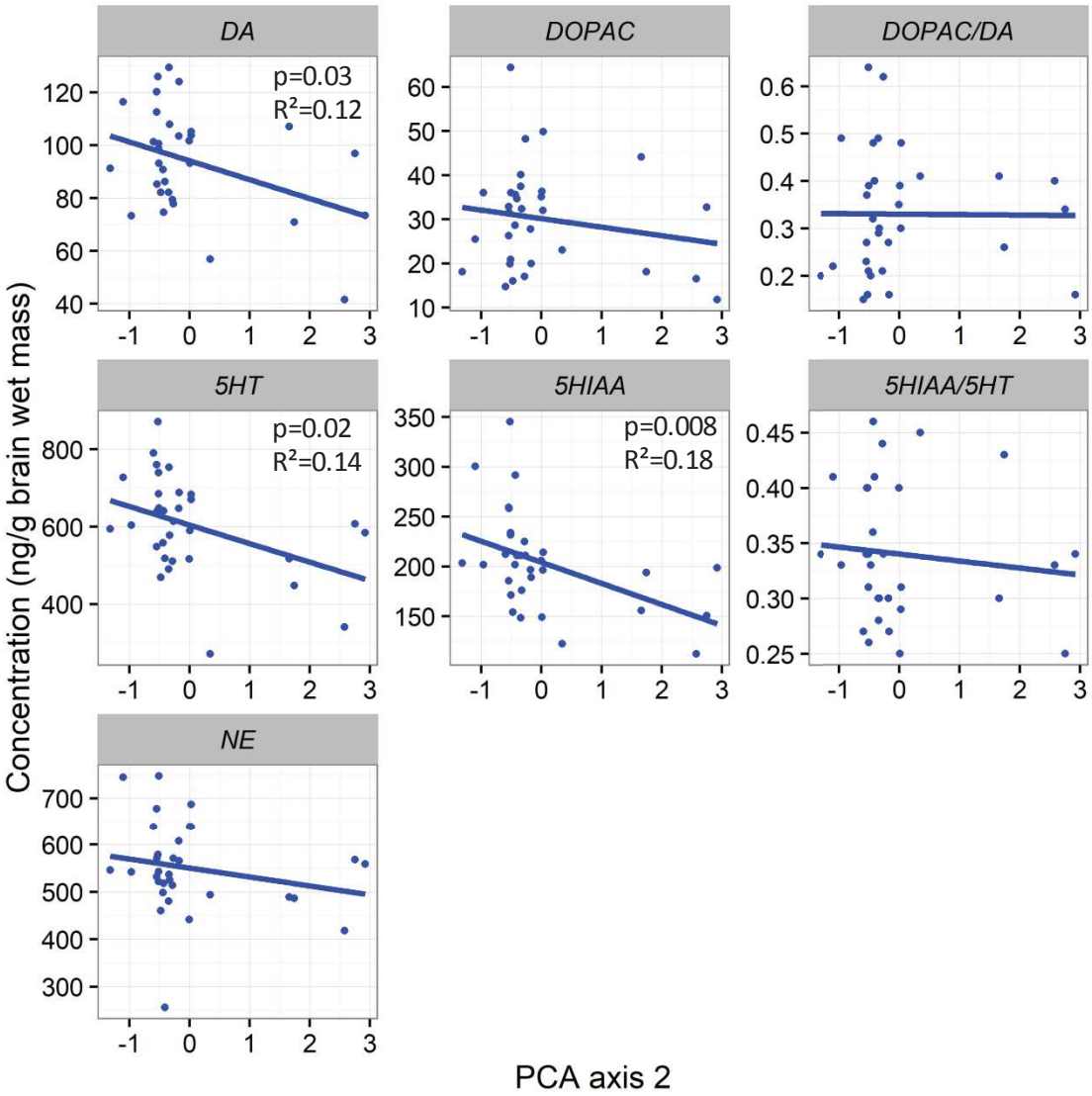

Figure 5. Concentration (ng/ g brain wet mass) of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), the DOPAC/DA turnover ratio, serotonin (5HT), 5-hydroxyindoleacetic acid (5HIAA), the 5HIAA/5HT turnover ratio, and norepinephrine (NE) in the brain in relation to individual PCA axis 2 scores. P-value (p) and the associated R^2 are indicated for each significant linear regression.

Concerning gene transcription in the brain, there was no correlation between the investigated genes and PCA axis 2 scores except a negative trend between relative level of *pcna* and PCA axis 2 scores ($F = 3.99$, $p = 0.07$, $R^2 = 0.08$). All statistical details concerning correlations between the relative levels of other genes are given in the text of the **Supplementary Material** and illustrated in **Figure S1**.

4. Discussion

Here, we evaluated the boldness of European sea bass using two different challenges, which were group risk taking test (GRT) and open field test (OFT) at 227 and 749 dpf, respectively. The proportion of bold/shy fish did not differ between the two tests (approximately 60% of the fish were categorized as bold). The proportion of bold/shy fish however differed from a previous study in European sea bass in which ~80% were identified as shy using a group risk-taking test (Ferrari et al., 2016a). The high proportion of bold fish identified in our study may be attributable to small differences with their protocol and/or the level of fish domestication which could affect boldness (Millot et al., 2009). We did not find any clear behavioral consistency in boldness across context between the GRT and OFT. Indeed, only 50% of fish with shy behavior in the GRT were also categorized as shy in the OFT (522 days between the two tests). The consistency of boldness over time was previously reported in European sea bass when consecutive GRTs were performed 29 days apart (Ferrari et al., 2015). Boldness, as well as other personality traits, is known to be consistent over a short period of time, but can evolve during the life of the animal (Polverino et al., 2016). Moreover, boldness is influenced by many external factors, both biotic and abiotic (Brown et al., 2007; Ferrari et al., 2016b; Frost et al., 2007; Thomson et al., 2012). Abiotic factors, such as temperature, physico-chemical characteristics of the water, or food supply, were kept constant during our experiment. However, some other factors could explain the absence of a clear boldness consistency between the GRT and OFT. The difference in social context between the two tests (group *vs* isolation) could be one and could partly explain the discrepancy between the results, regardless of the time window that separated the two behavioral experiments (Webster et al., 2007). Social context is indeed very important in shaping personality traits in fish (Geffroy et al., 2014) and may have affected our capacity to evaluate boldness in single fish in a social species, such as sea bass, as already mentioned by Ferrari et al. (2015).

Nevertheless, the fish expressed two divergent behavioral responses during the OFT. The majority of fish (58 %) expressed bold trait, with a high level of activity in the OFT arena, whereas shy fish expressed freezing behavior in the shelter. These two different behavioral responses have been largely described in the literature as typical proactive and reactive coping style characteristics (Castanheira et al., 2017; Øverli et al., 2006; Øverli et al., 2007).

Differential stress regulation of the HPI axis and the brain-chromaffin axis are often associated with the opposite behavioral responses expressed by proactive and reactive

individuals (Koolhaas et al., 1999; Øverli et al., 2007; Vindas et al., 2017a; Vindas et al., 2017b). Here, the signal from the dopaminergic system was similar between bold and shy fish. The concentrations of DA and DOPAC and the DA/DOPAC turnover ratio were indeed identical between our two groups, whereas dopaminergic system signaling was found to be different between bold and shy fish in several studies (Koolhaas et al., 2007; Øverli et al., 2001). Possible differences between bold and shy fish could also be masked by the fact that the analyses were performed on whole brain (Backstrom et al., 2011b; Øverli et al., 2001). The boldest fish screened in the OFT nevertheless displayed higher DA concentration in the brain compared to the less bold ones. This is consistent with previous studies suggesting that dopaminergic system may trigger active behavioral responses, including bolder behavior (Thornqvist et al., 2018; Winberg and Nilsson, 1992; Winberg et al., 1991). Concerning the serotonergic system, the 5HT/5HIAA turnover ratio was lower in shy than bold fish following the OFT but the boldest fish displayed both higher levels of 5HT and 5HIAA in the brain than the less bold ones. Similar differences has been previously observed in rainbow trout (*Oncorhynchus mykiss*) following an acute stressor (Øverli et al., 2001); fish with a more reactive coping style showed lower levels of serotonergic activity upon exposure to stress. This supports the view of the shy and bold groups established in the OFT as exhibiting different coping styles, as differences in the serotonergic system could be involved in the determination of the individual coping style (Puglisi-Allegra and Andolina, 2015). Another interpretation is that the differential behavior observed (exploring *vs* freezing in the shelter) might have driven the difference in the serotonergic response. In this case, the bold fish were more active and left the shelter and thus exposed themselves out in the open area, which might be more stressful for them, leading to higher 5HT activity. This alternative interpretation has been considered by Koolhaas et al. (2010) who hypothesized that "it is likely that differential neuroendocrine characteristics are mainly a consequence rather that the cause of behavioral differentiation". Finally, the noradrenergic system is generally less well-documented than the serotonergic and dopaminergic systems in relation to coping styles. However, the higher NE concentrations we measured in the brains of shy fish have been similarly found in previous studies in rainbow trout (Backstrom et al., 2011b; Øverli et al., 2001).

Concerning the HPI axis, plasma cortisol concentrations measured in our study were higher than those previously found in European sea bass (Rotllant et al., 2003; Samaras et al., 2016). It is likely that the social isolation during the OFT is a very strong stressor for European sea

bass, as was shown for rainbow trout (Bernier et al., 2008). Further, there was no difference in the cortisol response between bold and shy fish categorized using the OFT. This may seem surprising, as high cortisol levels have been shown to be predictive of the reactive coping style in sea bass in another context (Ferrari et al., 2015). Nevertheless, it cannot be excluded that the lack of difference may be due to the fact that, once in the OFT, bold fish take more risk and are further stressed by exploring the open field arena whereas shy fish stay in the shelter resulting in different physiological status when blood sample was taken. Moreover, such an absence of a difference in the peak cortisol response was previously reported following an acute stressor in various fish species (Tudorache et al., 2013; Vindas et al., 2017b), but divergences appeared during the recovery phase (Tudorache et al., 2013). The transcription of *gr2*, *mr* and *crf,* which are involved in the regulation of the HPI axis, were higher in shy than bold fish, whereas *gr1* transcription did not differ between these two groups. Elevated *gr2*, *mr* or *crf* transcription in shy fish has also been reported in other fish species and contexts (Backstrom et al., 2011b; Geffroy et al., 2018; Summers et al., 2017; van den Bos et al., 2017). For example, HR rainbow trout (shy) displayed higher levels of *crf* than LR (bold) rainbow trout following confinement stress (Backstrom et al., 2011b). These effects were shown to be modulated by binding proteins reducing the activity of Crf but increasing its longevity (Flik et al., 2006; Samaras et al., 2018). In addition to its effects on the regulation of the HPI axis, Crf can interact with neurotransmission and modulate behavior (Backstrom and Winberg, 2017; Carpenter et al., 2009; Clements et al., 2003; Lowry and Moore, 2006). Overall, Crf interacts with serotonergic and noradrenergic pathways, resulting in an increase in locomotor behavior, including non-ambulatory behavior, and has been reported to cause anxiety (Lowry and Moore, 2006). In the present study, shy fish (which expressed higher levels of *crf* than bold fish) expressed a lower turnover of serotonergic activity and higher NE concentrations. Shy fish also displayed a freezing behavior in the shelter during the OFT. This echoes a previous study showing that intraventricular Crf injections in rats increase the anxiety state of the animal, as well as the turnover ratio between NE and its metabolite DHPG in the brain-stem locus coeruleus (Butler et al., 1990). More recently, it was reported in rainbow trout that Crf-injected fish have higher 5HT and 5 HIAA levels in various brain regions (*i.e.,* the subpallium, raphe, and preoptic hypothalamus) and/or express head-shaking behavior, which is related to anxiety (Backstrom et al., 2011a; Carpenter et al., 2007). Interactions between Crf and both the serotonergic and the noradrenergic system appear promising to further explain the divergent phenotypes observed in fish coping styles (Backstrom et al., 2011a; Carpenter et al., 2007; Lowry and Moore, 2006).

Recent studies suggest that stress axis regulation and neurogenesis are tightly linked (Best et al., 2017; Sadoul et al., 2018; Sorensen et al., 2011). For example, Sadoul et al. (2018) have shown that high levels of *mr* and *gr* in the brain correlate with high levels of *pcna* and *neurod1* in several fish species, including the European sea bass. Here, we also detected a strong and significant correlation (at the individual level) between genes involved in the stress response and those involved in neurogenesis (**supplementary material**), confirming the results of Sadoul et al. (2018). In the present study, we show that the expression of *egr1* and *neurod2*, which are important markers of neurogenesis involved in neural activation and differentiation, respectively (Burmeister and Fernald, 2005; Sorensen et al., 2013), were higher in the brains of shy fish than in those of bold fish (only a trend for *neurod2*). This higher propensity to display strong neurogenesis in shy fish is suggested in the literature (Geffroy et al., 2018; Øverli and Sorensen, 2016; Sorensen et al., 2013; van den Bos et al., 2017) and may be important for learning (Sorensen et al., 2013). This is in accordance with previous results showing that shy fish are more proficient in learning and triggering self-feeders (Benhaim et al., 2017; Ferrari et al., 2014).

Here, both monoamine concentrations and gene transcription analyses were performed on the whole brain, including multiple regions with specific functions and patterns of monoamine synthesis and gene transcription (Øverli et al., 2001; Vindas et al., 2018; Vindas et al., 2017b). Significant effects were observed using the whole brain, and therefore we could expect even more significant differences between groups in specific areas of the brain. Microdissections or dissection of the different areas of the brain would have probably lead to more robust conclusions. A refined analysis would be necessary to better understand the differential pattern of responses in the different brain areas.

Overall, European sea bass characterized by two divergent behavioral phenotypes on the boldness axis, bold and shy, also display two distinct physiological responses which could be reflect the proactive and reactive differentiation, respectively. Altogether, our results suggest that the two coping styles display differences in the HPI axis, the serotonergic and noradrenergic system reactivity, and in neurogenesis. The behavioral responses of a social animal to a challenge in isolation are good markers of its physiological state and capacity to regulate and react to a stressor. More data are needed to verify whether such responses are specific to one time point or if they are indicators of a lifelong coping style.

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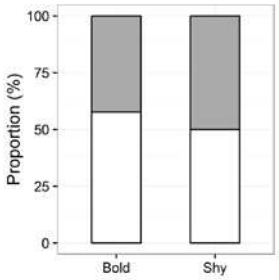
Chapitre 2 – Conclusions

Résultats:

• Cohérence entre GRT et OFT:

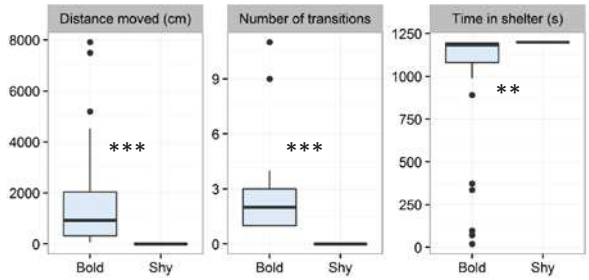


• **Légende:**

Cohérent

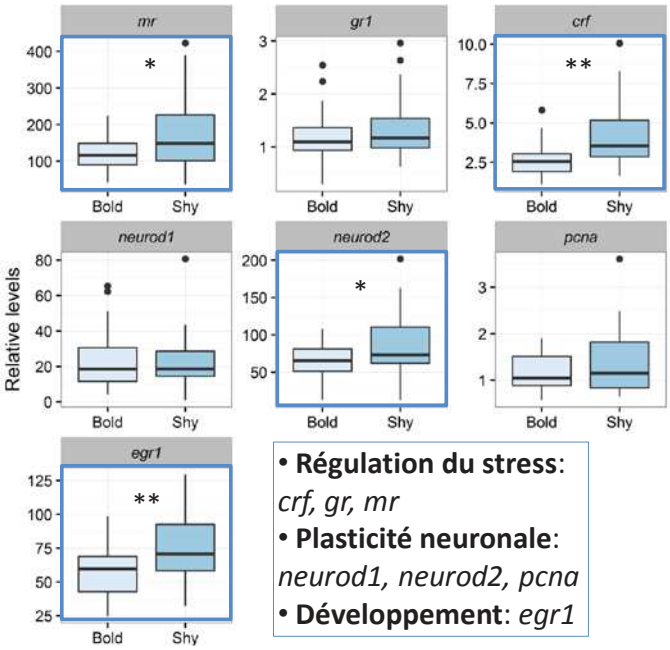
Non cohérent

• Comportement dans OFT:



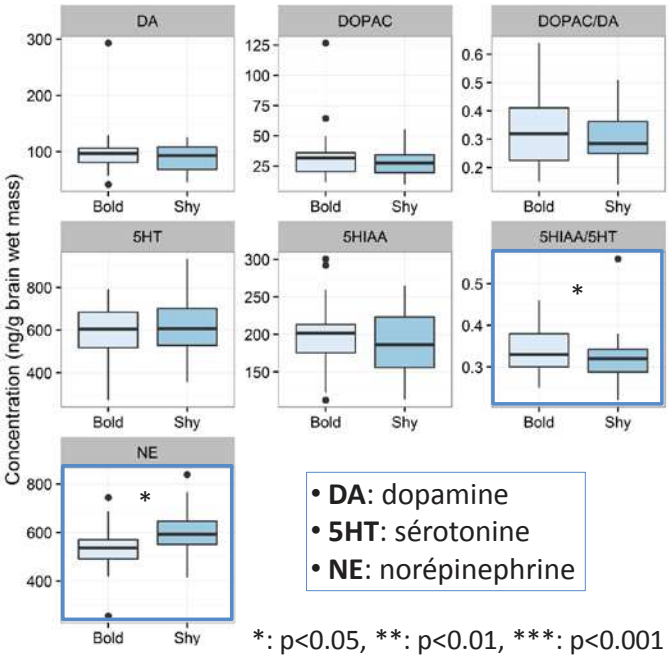
Bold/shy: déterminés dans l'OFT

• Transcription de gènes:



• **Régulation du stress**: *crf, gr, mr*
• **Plasticité neuronale**: *neurod1, neurod2, pcna*
• **Développement**: *egr1*

• Concentration en monoamines:



• **DA**: dopamine
• **5HT**: sérotonine
• **NE**: norépinephrine

*: p<0.05, **: p<0.01, ***: p<0.001

Points importants:

• La cohérence comportementale du *coping style* à long terme et dans des contextes differents (groupe (GRT) *vs*. seul (OFT)) est faible (environ 50 % de cohérence).

• Les *coping styles* extrêmes identifiés dans le second test (OFT) divergent dans leur réponse au stress *via* la régulation de *mr*, *crf*, *neurod2* et *egr1.*
De plus, l'activité du système sérotonergique (5HIAA/5HT) est plus forte chez les individus audacieux alors que la concentration en norépinephrine est plus faible.

➔ Ces différences sur le plan comportemental et physiologique entre ces *coping styles* offrent des possibilités prometteuses, d'une part, pour l'amélioration du bien-être et, d'autre part, pour une meilleure maîtrise des performances biologiques en aquaculture.

Communications:

• **Society for Experimental Biology – Florence – 2018**
Communication orale
• **World Aquaculture Society – Montpellier – 2018**
Communication orale

Supplementary material

Coping styles in European sea bass: the link between boldness, stress response and neurogenesis

In revision in Physiology and Behavior

Material and methods

Statistical analyses

Linear regression analyses were performed between the transcription of neurogenesis genes (*i.e., pcna, neurod1* and *neurod2*) and that of cortisol receptors (*i.e., mr, gr1* and *gr2*).

Results

Monoamine concentration in the brain

Using the GRT initial fish coping style characterization, there was no difference in the concentration of DA or DOPAC in the brain between bold and shy fish, no difference either for the DOPAC/DA ratio ($t = -0.05$ $p = 0.96$; $t = -0.38$, $p = 0.71$; and $t = -0.41$, $p = 0.68$, respectively), or for 5HT, 5HIAA and 5HIAA/HT ($t = 0.17$ $p = 0.87$; $t = -0.06$, $p = 0.95$ and $t = -0.77$, $p = 0.44$, respectively) and concentration of NE in the brain ($t = 0.01$, $p = 0.99$, **data not shown**).

Gene transcription in the brain

Using the GRT initial fish coping style characterization, there was no difference in brain transcription or *mr* ($W = 322$, $p = 0.38$), *gr1* ($W = 408$, $p = 0.59$), *gr2* ($W = 280$, $p = 0.74$), *crf* ($W = 325$, $p = 0.99$), *neurod1* ($W = 405$, $p = 0.62$), *neurod2* ($W = 325$, $p = 0.41$), *pcna* ($W = 398$, $p = 0.71$) and *egr1* ($W = 0.73$, $t = 0.47$) between bold and shy fish.

Principal component analysis (PCA)

There were no correlation between PCA axis 2 and *mr* ($F = 0.16$, $p = 0.69$), *gr1* ($F = 0.23$, $p = 0.64$), *gr2* ($F = 0.22$, $p = 0.64$), *crf* ($F = 0.69$, $p = 0.41$), *neurod1* ($F = 1$, $p = 0.33$), *neurod2* ($F = 1.1$, $p = 0.31$) and *egr1* ($F = 1.11$, $p = 0.3$, **Figure S1**).

Link between stress regulation and neurogenesis

The correlations were significant between the relative level of *mr* and *pcna* transcriptions ($F = 13.18$, $p < 0.001$, $R^2 = 0.18$), between *mr* and *neurod1* ($F = 7.56$, $p = 0.008$, $R^2 = 0.11$) and between *mr* and *neurod2* ($F = 363.8$, $p < 0.001$, $R^2 = 0.87$, **Figure S2.A**).

The correlations were also significant between the relative level of *gr1* and *pcna* transcriptions ($F = 48.28$, $p < 0.001$, $R^2 = 0.47$), between *gr1* and *neurod1* ($F = 20.79$, $p < 0.001$, $R^2 = 0.27$) and between *gr1* and *neurod2* ($F = 30.4$, $p < 0.001$, $R^2 = 0.35$, **Figure S2.B**).

Finally, correlations were significant between relative level of *gr2* and *pcna* transcriptions ($F = 9.90$, $p = 0.002$, $R^2 = 0.16$), between *gr2* and *neurod2* ($F = 41.09$, $p < 0.001$, $R^2 = 0.46$) and between *gr2* and *neurod1* ($F = 0.88$, $p = 0.35$, **Figure S2.C**).

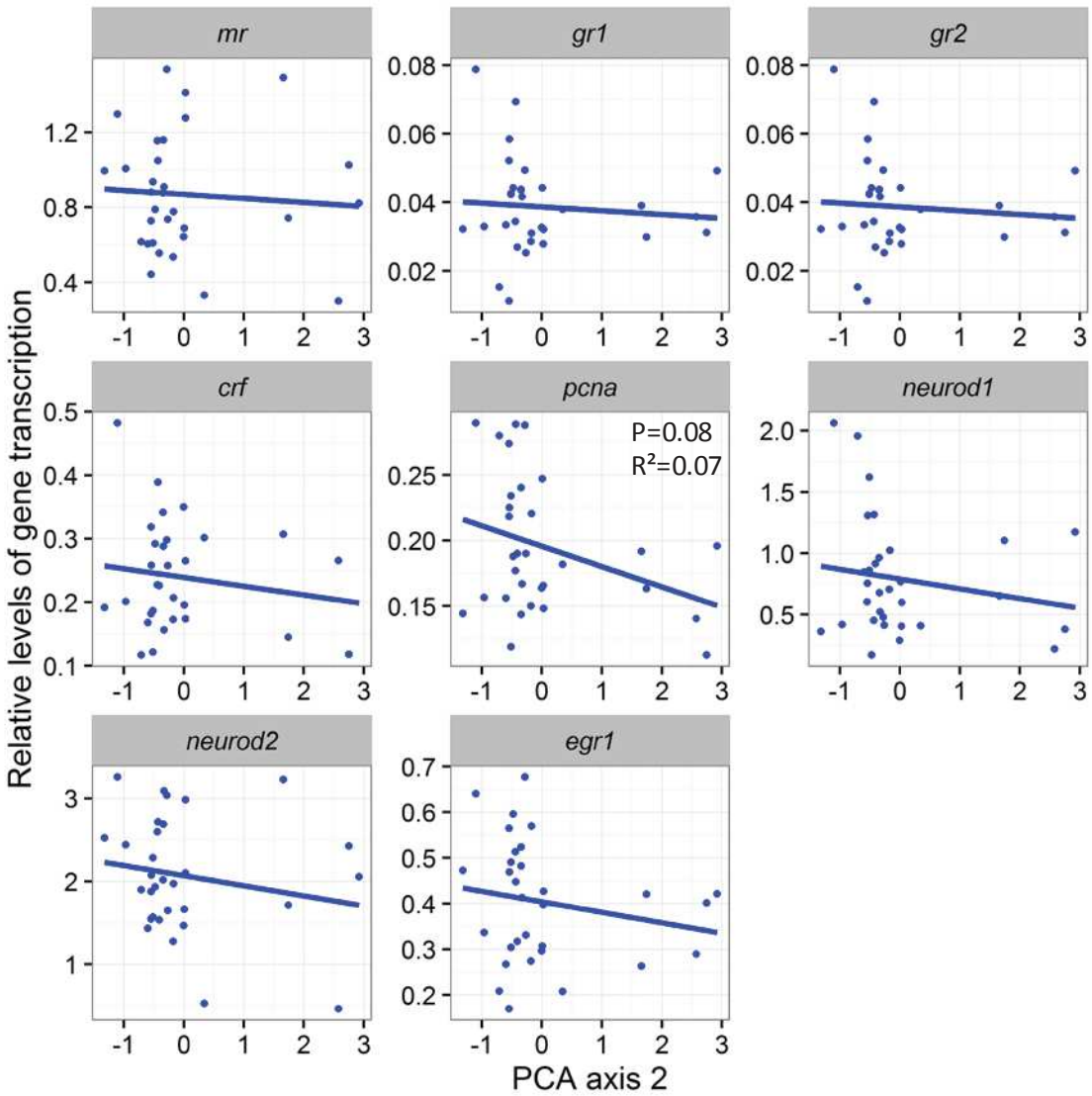


Figure S1. Relative levels of gene transcription of the *mineralocorticoid receptor* (*mr*), *glucocorticoid receptor* 1 (*gr1*), *glucocorticoid receptor2* (*gr2*), *corticotropin-releasing factor* (*crf*), *neurogenic differentiation factors* (*neurod1* and *neurod2*), *proliferating cell nuclear antigen* (*pcna*), and *early growth response* (*egr1*) relative to reference genes (*l13* and *eef1-alpha*) in the brain as a function of PCA axis 2. P-value (p) and the associated R^2 are indicated for the statistical trend of linear regression.

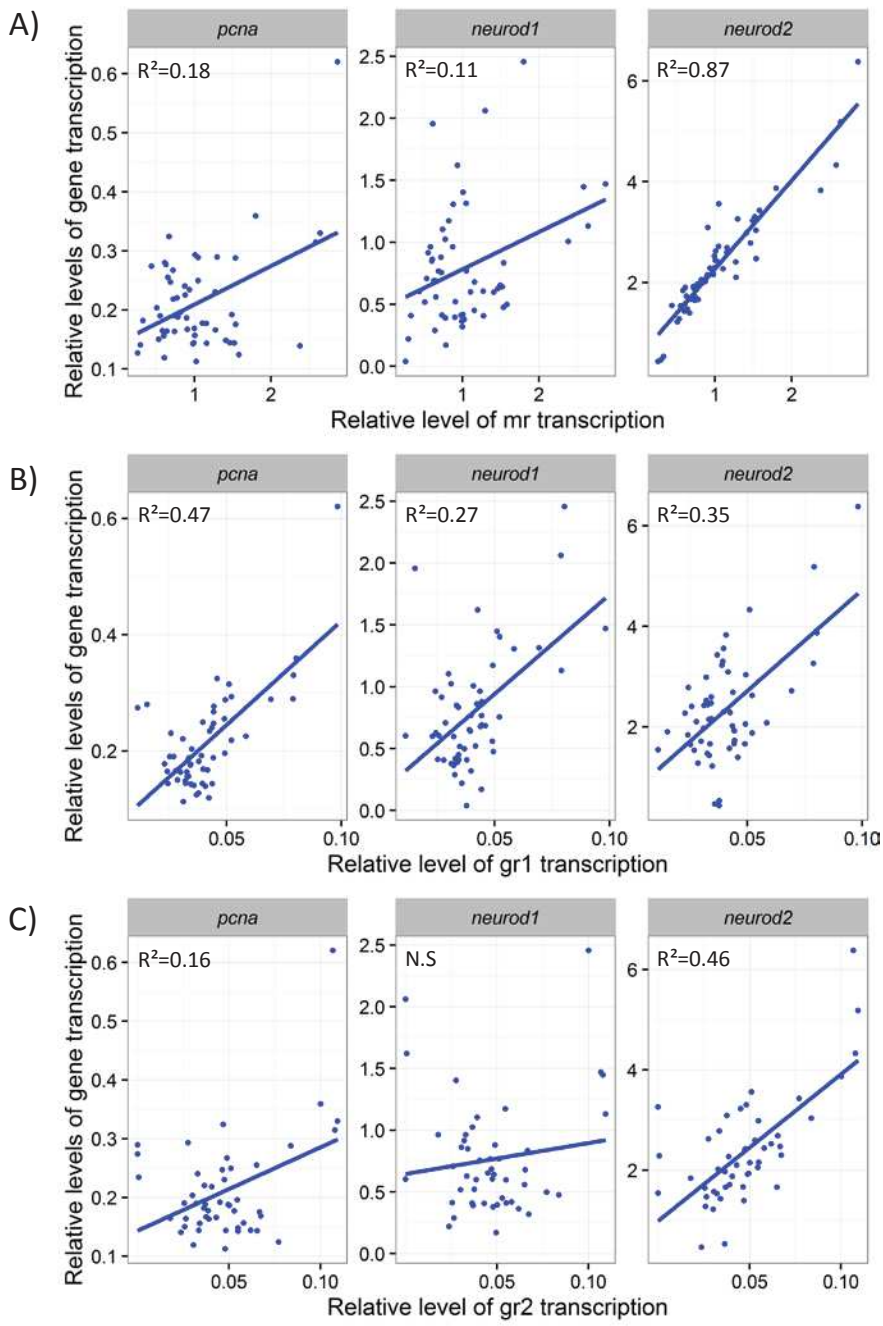


Figure S2. (A) Relative level of *mr* transcription as a function of relative level of *pcna neurod1*, and *neurod2* transcriptions; **(B)** Relative level of *gr1* transcription as a function of relative level of *pcna, neurod1*, and *neurod2* transcriptions. **(C)** Relative level of *gr2* transcription as a function of relative level of *pcna, neurod1*, and *neurod2* transcriptions. R^2 is indicated for each significant linear regression while N.S (not significant) is indicated (n=55, except for *gr2* n=49).

Partie 2

Effets d'une exposition chronique à un mélange de polluants organiques persistants sur les réponses comportementales et physiologiques des téléostéens et leur descendance

Chapitre 3

Examining multi- and transgenerational behavioural and molecular alterations resulting from parental exposure to a PCB and PBDE environmental mixture

Sébastien Alfonso
Mélanie Blanc
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Catherine Munschy
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Aquatic Toxicology

Chapitre 3 – Présentation

Contexte:

Les polychlorobiphenyles (PCB) et les polybromodiphenyles ethers (PBDE) sont des polluants organiques persistants (POP). A cause de leur usage massif lors du XXème siècle, et de leur persistance dans l'environnement, ils sont aujourd'hui présents dans de nombreux écosystèmes, notamment les écosystèmes aquatiques.

Ces polluants sont connus pour être neurotoxiques, dont certains sont considérés comme des perturbateurs endocriniens, pouvant d'affecter le comportement et les traits d'histoire de vie. Cependant, peu de choses sont connues sur les effets de ces polluants en *cocktail* et aux doses environnementales sur les générations issues de poissons exposés.

Questions:

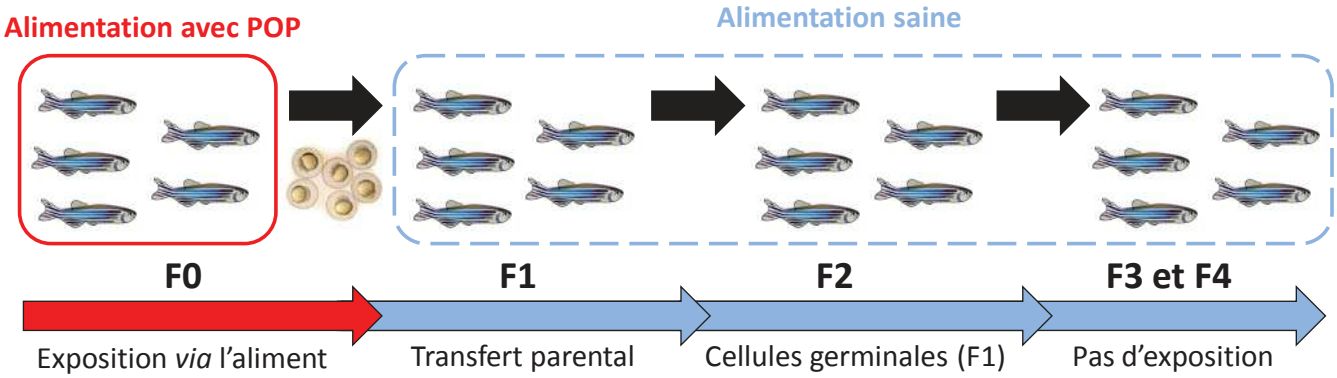
(1) Quel est l'impact d'une exposition chronique à un mélange de POP, représentatif des conditions environnementales, sur le comportement des téléostéens exposés et leur descendance ?

(2) Quels sont les mécanismes physiologiques pouvant expliquer les altérations comportementales et la transmission des effets à la descendance ?

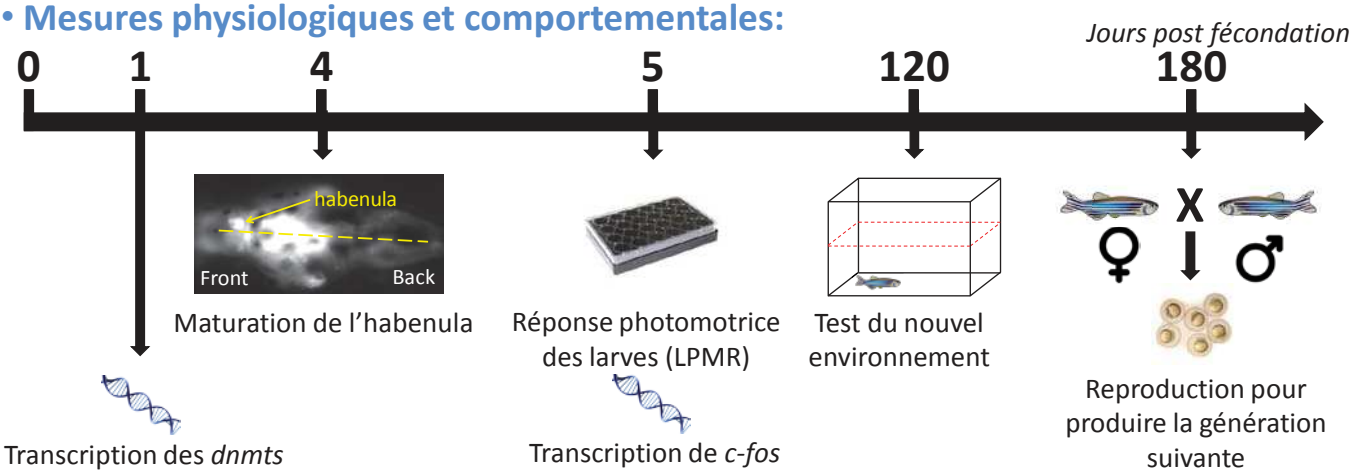
Méthode:

Les individus de la génération (F0) ont été nourris avec un mélange de PCB et PBDE représentatif de conditions environnementales (estuaire de Seine) à partir de 5 jpf jusqu'à la reproduction. Pour étudier les **réponses comportementales**, l'**anxiété** a été mesurée dans un test de nouvel environnement (F0-F3), ainsi que la **réponse photomotrice** des larves (F1-F4). Des **marqueurs liés à l'anxiété** (transcription de ***c-fos*** et la **maturation de l'habenula**, ont été analysés chez les générations descendantes. La **méthylation de l'ADN** a également été estimée à travers l'étude de la transcription des ***dnmts***.

• Exposition:



• Mesures physiologiques et comportementales:



Examining multi- and transgenerational behavioral and molecular alterations resulting from parental exposure to a PCB and PBDE environmental mixture

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Abstract

Polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) are persistent organic pollutants extensively used during the 20th century and still present in aquatic environments despite their ban. Effects of exposure to these compounds over generations are poorly documented. Therefore, our aims were to characterize behavioral responses and underlying molecular mechanisms in zebrafish exposed to an environmentally relevant mixture of PCBs and PBDEs as well as in four unexposed offspring generations. Zebrafish (F0) were chronically exposed from the first meal onward to a diet spiked with a mixture containing 22 PCB and 7 PBDE congeners in proportions and concentrations reflecting environmental situations (ΣPCBs= 1991 and ΣPBDEs= 411 ng/g). Four offspring generations (F1 to F4) were obtained from this F0 and were not further exposed. Behavior was assessed at both larval and adult stages. Mechanisms related to behavioral defects (habenula maturation and *c-fos* transcription) and methylation (*dnmts* transcription) were monitored in larvae. Exposed adult F0 as well as F1 and F3 adults displayed no behavioral change while F2 expressed anxiety-like behavior. Larval behavior was also disrupted, *i.e.* hyperactive after light to dark transition in F1 or hypoactive in F2, F3 and F4. Behavioral disruptions may be related to defect in habenula maturation (observed in F1) and change in *c-fos* transcription (observed in F1 and F2). Transcription of DNA methyltransferase (*dnmt3ba*) was also modified in all generations. Our results lead us to hypothesize that chronic dietary exposure to an environmentally relevant mixture of PCB and PBDE triggers multigenerational and transgenerational molecular and behavioral disruptions in a vertebrate model.

1. Introduction

Among chemicals massively used and emitted in the environment, persistent organic pollutants (POPs) are of greatest relevance because they are known to be hydrophobic, lipophilic, little or not metabolized by organisms, present long environmental half-life and undergo long-range atmospheric transportation (Gray, 2002; Jones and de Voogt, 1999; Luo et al., 2007). As a consequence, they persist in the environment and are significantly bioaccumulated and biomagnified in biota through trophic transfer which is considered as the most important exposure pathway (Couderc et al., 2015; Darnerud et al., 2001; Johansson et al., 2006; Muir et al., 2003; Pompa et al., 2003; Sjodin et al., 2003). Among POPs, polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) are each a family of 209 congeners resulting from the substitution of one to ten hydrogen atoms by chlorine and bromine atoms, respectively, on a common nucleus (McFarland and Clarke, 1989; Rahman et al., 2001). In both cases, their industrial production was performed as complex mixtures of numerous congeners, e.g. Aroclor, Kanechlor or Clophen in the case of PCBs (Kannan, 2000) and e.g. penta-, octa- and decaBDE according to the average number of bromines in the case of PBDEs (de Boer et al., 2000). Since awareness about their toxicity increased, regulatory rules were adopted to limit PCB use and they were progressively banned since the 70s (Breivik et al., 2007). For PBDE, the technical mixtures Penta- and octa-BDEs were voluntarily phased out in the USA in 2004 and all mixtures were banned or regulated in the EU (Kemmlein et al., 2009). Due to their persistency, they are still detected in all environmental compartments around the world and associated (eco-)toxicology issues persist (de Wit, 2002).

In wild animals, disruption of behaviors such as exploration, sociability or mating may reduce individual fitness with consequences for population sustainability. Neurodevelopmental or behavioral defects were shown in exposed vertebrates, including fish exposed to PCBs (Gonzalez et al., 2016; Lovato et al., 2016) or PBDES (Chen et al., 2012a; Chen et al., 2012c; Garcia-Reyero et al., 2014; McClain et al., 2012; Truong et al., 2014). Recently, behavioral disruptions were also observed in zebrafish offspring after parental exposure to PCBs (Péan et al., 2013) or PBDEs (Chen et al., 2012b; He et al., 2011). These behavioral disruptions in offspring could be due to maternal transfer and accumulation in eggs (Daouk et al., 2011; He et al., 2011; Nyholm et al., 2008; Yu et al., 2011) or a consequence of physiological maternal stress (Talge et al., 2007). Beyond this, epigenetics mechanisms may serve an additional explanation for changes in behavior observed in the offspring over several unexposed

generations (Skinner, 2014a, b). DNA methylation is one of the epigenetic mechanisms and is driven by DNA methyltransferases (Dnmt). It was recently shown that various chemicals such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, *i.e.* TCDD (Aluru et al., 2015) and bisphenol A (Laing et al., 2016; Liu et al., 2016) alter *dnmt* transcription in exposed zebrafish. In the case of TCDD, decrease in *dnmt3ba* transcription is associated to a decrease in methylation of the promoter of *c-fos*, (Fos proto-oncogene a transcription factor) and an increase in its transcription (Aluru et al., 2015). This is of particular interest since changes in *c-fos* transcription, which is a molecular marker for neural activity, were associated with changes in anxiety level (Chen et al., 2015; Duncan et al., 1996). Besides, another mechanism which could underlie anxiety is a defect in maturation of the habenula, a diencephalon structure. In normal situation, habenula has an asymmetrical organization revealed by differences in genes expression during development and in innervation later on (Aizawa et al., 2007; Roussigné et al., 2009). Defect in the development of such asymmetrical organization was shown to be related to modification of anxiety or fear responses in fish (Agetsuma et al., 2010; Budaev and Andrew, 2009; Lee et al., 2010).

Persistency over-generation can differ according to mechanism triggered upon exposure and behavioral defects are not necessarily observed at all stages in one generation. As examples, in F1 offspring of fish exposed to polycyclic aromatic hydrocarbons (PAHs) mixtures, there is no consistency in defects observed in larvae and adults while F2 larvae have opposite responses compared to F1 (Vignet et al., 2015). In another case, an early direct exposure to benzo[a]pyrene produced an hyperactivity in both F1 larvae and adults but not in F2 while F1 and F2 adults displayed an enhanced predator avoidance (Knecht et al., 2017). Therefore there is a need to evaluate behavioral disruptions at different ages and over several generations.

In most cases, disruptions described previously are the results of acute exposure to single PCB or PBDE congeners at high concentrations while investigations of consequences of chronic exposure to POP mixtures at environmental concentrations remain scarce. Moreover, these studies focused on exposed fish and one offspring generation. In vertebrates, germ cells which will later produce gametes are present during early developmental stages. In zebrafish embryos, primordial germ cells can be detected as early as 8 hours post fertilization (hpf) and migrated to reach their final position at the gonads at 24 hpf (Weidinger et al., 1999; Yoon et al., 1997). Therefore, in the case of POPs, which are transferred to the eggs, F1 germ cells, which will produce F2 generation, may be exposed to POPs in developing F1. Consequently, F3 generation is the first to be totally isolated from POP parental exposure and is therefore

required to distinguish between multigenerational (F1 and F2) and transgenerational (F3 and later generations) effects.

Objectives of this study were first to monitor behavioral responses in zebrafish chronically dietary exposed (F0) to an environmentally relevant mixture of PCBs and PBDEs. Second, behavioral effects were analyzed in the offspring fed with plain food, at larval and adult stages from F1 to F4 generations to examine multigenerational and transgenerational effects. Finally to identify mechanisms underlying behavioral disruptions and transmission over generations, the transcription of *dnmts* and *c-fos* were analyzed in F1 to F4 larvae, together with the lateralization of habenula (F1 to F3).

2. Material and methods

The study was conducted under the approval of the Animal Care Committee of Poitou-Charentes # 84 COMETHEA (France) under the project authorization number CE2012-23 and followed the recommendations of Directive 2010/63/EU.

Succession of exposure, generations and analyzes performed is summarized in **Figure 1**. Additional information concerning material and methods are provided in the **supplementary material** (**Text S1**).

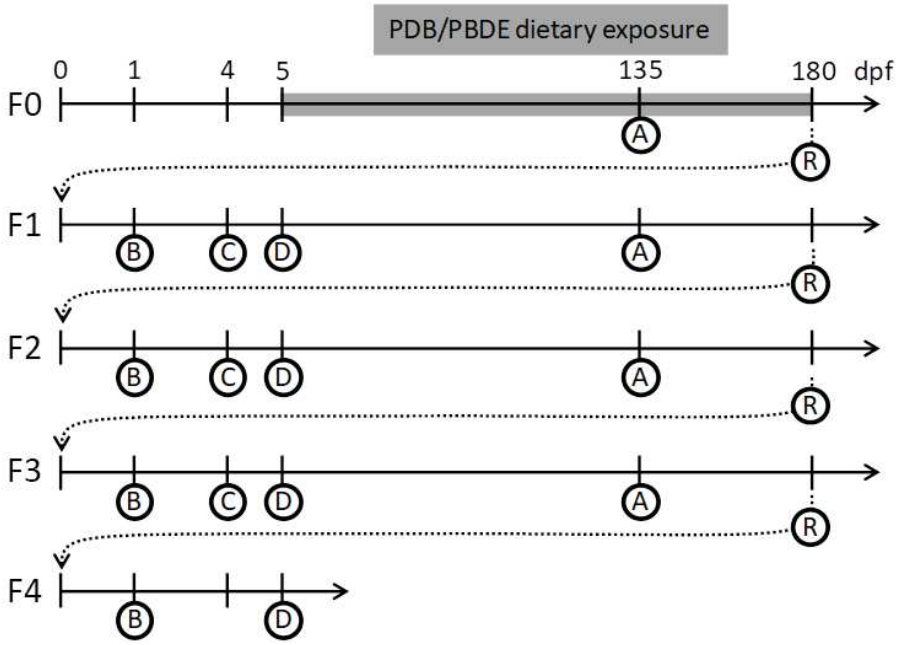

Figure 1. Experimental procedures performed in this study over time and generations. (A) Novel tank test; (B) Analyzes of *dnmt1*, *dnmt3aa*, *dnmt3ba* and *dnmt3ba* transcription; (C) Localization of habenula; (D) Analyzes of *c-fos* transcription and larval photo-motor response (LPMR); (R) Reproduction to produce next generation. dpf: days post fertilization.

2.1. Fish reproduction and rearing

The study was conducted on zebrafish using wild type strain TU (ZFIN ID: ZDB-GENO-990623-3) and a transgenic line having GFP under the control of *neurogenin1* promoter (*Tg(-8.4neurog1:gfp)*; ZFIN ID: ZDB-GENO-030904-3). Both lines were from a stock kept at the Fish Ecophysiology Platform (PEP - http://wwz.ifremer.fr/pep_eng) and originated from the European Zebrafish Resources Center (EZRC, Karlsruhe, Germany). Fish were reared at 27±1°C under a 14h/10h light/dark cycle with light onset at 9:00. Physical water conditions were maintained constant during the experiment and were within recommended ranges (Lawrence, 2007).

In all cases (treatment, generation or zebrafish line (wild-type or transgenic)), eggs were obtained by random pairwise mating using adults at around 180 dpf. For each treatment, to avoid genetic bias, spawns with fertilization rate above 80 % originating from 6 pairs , were used to produce the next generation in the following manner. Briefly, one male and one female were introduced in a spawning box (AquaSchwarz, Germany) in late afternoon, between 15 and 20 pairs were set up per treatment. Eggs were collected in the following morning and the fertilization rate was assessed within 2 h of collection. Spawns above 100 eggs and 80% fertilization rate were kept for experiments and placed in Petri dishes filled with 30 mL of the isotonic mixture E3 (1 L: 17.2 g NaCl, 0.76 g HCl, 2.9 g $CaCl_2$. 2 H_2O, 4.9 $MgSO_4$.7 H_2O) and methylene blue (250 µL/L). The day after, eggs from different spawns were evenly distributed in order to avoid spawn-effects: for each treatment, 10 eggs from each of the 6 spawns were selected to reach 60 eggs per 10 cm Petri dish. They were incubated at 28 °C during 3 days under a 14h/10h light/dark cycle. At 72 hours *post*-fertilization (hpf), after hatching, chorions were removed from the Petri dishes. At 5 dpf, larvae were transferred from Petri dishes to 1 L tanks. At 21 dpf, they were transferred from 1 L tanks to 3 L tubes located in 10 L tanks in the rearing system. At 26 dpf, tubes were gently poured in 10 L tanks. At least 5 replicates were produced per treatment. This procedure was used to produce F0 (TU and *Tg* lines) for exposure, F1 to F2 (*Tg* line) and F1 to F4 (TU) offspring generations.

2.2. MIX diet and F0 exposure and PCB and PBDE analyzes

The MIX diet was spiked with 22 PCB congeners (CB-8, -18, -28, -31, -44, -49, -52, -77, -101, -105, -110, -118, -128, -132, -138, -149, -153, -156, - 170, -180, -187 and -194) and 7 PBDE congeners (BDE-28, -47, -99, -100, -153, -183 and -209) all with purity above 98% (AccuStandard Inc., New Haven, USA). Congeners were selected according to their

abundance in environment and to span a wide range of hydrophobicity (log K_{ow}). Relative proportions of congeners were chosen to simulate environmentally relevant mixtures(Abarnou et al., 2000; Ospar, 2009, 2013). Chlorine (for PCBs) and bromine (for PBDEs) substitution numbers and detailed dietary concentrations of each congener used for diet spiking are given in **Table 1**. Briefly, MIX diets were prepared after dilution of a stock PCB and PBDE solution in isooctane solvent for incorporation as described in Daouk et al. (2011). Control diet was prepared in the same manner, *i.e.* with addition of isooctane only. F0 fish were exposed using food of appropriate size following the same schedule as described in Horri et al. (2018). Fish were fed from their first meal (5 dpf) with either a Control diet or a MIX diet (later called respectively Control or MIX treatments) until final sampling (180 dpf). F1, F2, and F3 generations were fed using commercial plain food.

PCB and PBDE concentrations were determined in Control and MIX diets, in whole F0 fish (at 180 dpf) and in F1 obtained from F0 fish after 180 days of exposure as eggs (batches of 200 eggs), larvae (5 dpf, batches of 200 larvae, 0.65±0.15 mg) and adults (whole individuals at 112 dpf, 264±12 mg). These analyzes were performed as described in Horri et al. (2018).

2.3. Behavioral experiments

For all behavioral experiments, videos were recorded and analyzed using EthoVision XT 10.1 software (Noldus, The Netherlands). The behavioral challenge room was under the same light and temperature conditions than the rearing room.

Novel tank diving test

The novel tank diving test has been validated to evaluate the anxiety level by monitoring fish vertical positioning over an observation period (Egan et al., 2009; Levin et al., 2007).

Novel tank diving tests were performed from F0 to F3 generations. In the afternoon of the day before the challenge, adult fish (135±14 dpf) were moved to the behavioral challenge room and kept isolated into 1 L tanks (AquaSchwarz, Germany) in visual contact with each other until challenge. Novel tank diving test was performed from 10:00 to 18:00 as described by Levin et al. (2007). Individual fish was bathed into a 500 mL beaker containing plain system water during 8 minutes thereafter they were directly transferred in a novel tank (trapezoid 1.5 L tank Aquatic Habitats, Apopka, FL; USA, size in cm: height 15.2 × width 7.1 × length 27.9 at the top and 22.5 at bottom) and vertical positioning recorded during 4 min.

Table 1. PCBs and PBDEs contamination of MIX and Control diet.

Congener	Br/Cl	MIX diet						Control diet			
		Targeted concentration (ng g^{-1})	Measured concentration (ng g^{-1})				Spiking efficiency (%)	Measured concentration (ng g^{-1})			Occurrence (n)
CB-8	2	28	27.20	±	1.21		97.1	0.20	±	0.05	3
CB-18	3	38	32.70	±	1.37		86.1	0.60	±	0.28	4
CB-28	3	75	69.00	±	3.35		92	0.30	±	0.07	5
CB-31	3	42	47.40	±	1.74		112.9	0.30	±	0.05	4
CB-44	4	65	66.50	±	1.83		102.3	0.40	±	0.23	4
CB-49	4	54	53.20	±	4.67		98.5	n.d.		-	n.d.
CB-52	4	65	64.10	±	2.76		98.6	0.20	±	0.04	6
CB-77	4	30	31.80	±	1.54		106	n.d.		-	n.d.
CB-101	5	150	151.90	±	6.47		101.3	0.90	±	0.48	8
CB-105	5	76	78.90	±	5.43		103.8	0.40	±	0.18	8
CB-110	5	166	170.60	±	7.03		102.8	0.60	±	0.19	8
CB-118	5	110	107.30	±	3.05		97.5	0.60	±	0.16	8
CB-128	6	37	32.60	±	1.30		88.1	0.20	±	0.07	6
CB-132	6	71	68.70	±	6.46		96.8	0.20	±	0.11	5
CB-138	6	207	199.60	±	4.94		96.4	0.60	±	0.30	8
CB-149	6	158	161.70	±	2.47		102.3	0.80	±	0.24	8
CB-153	6	280	270.40	±	14.24		96.6	0.60	±	0.42	8
CB-156	6	38	34.10	±	3.71		89.7	0.10	±	0.01	7
CB-170	7	71	69.10	±	4.76		97.3	<LOD		-	8
CB-180	7	130	129.30	±	5.55		99.4	0.30	±	0.12	8
CB-187	7	35	32.60	±	2.91		93.1	0.60	±	0.45	4
CB-194	8	35	33.60	±	3.56		96	<LOD		-	4
Sum PCBs		1991	1932.30	±	90.35		97.05	7.90		3.45	
BDE-28	3	9.95	10.78	±	0.60		108.3	0.03	±	0.01	5
BDE-47	4	49.97	49.93	±	2.91		99.9	0.35	±	0.13	16
BDE-100	5	14.94	15.95	±	1.19		106.8	0.09	±	0.04	16
BDE-99	5	30.25	30.25	±	1.63		100.0	0.11	±	0.07	16
BDE-153	6	9.74	10.18	±	0.56		104.5	0.03	±	0.004	6
BDE-183	7	100.48	130.20	±	12.65		129.6	0.14		-	1
BDE-209	10	195.73	232.53	±	31.27		118.8	0.17	±	0.11	16
Sum PBDEs		411.1	479.82	±	50.81		116.7	0.92	±	0.36	

List of PCB and PBDE congeners used in this work along with the chlorine and bromine numbers. Targeted and measured congeners concentrations (ng g-1 ww) in MIX (n = 12) diet are indicated (mean±SE), as well as the spiking efficiency. In the case of Control diet the concentration of each congener as well as the number of samples, upon 16, in which congeners were detected (Occurrence column). n.d.: not detected. LOD: limit of detection.

Novel tank diving test with prior nicotine exposure

In order to ascertain that potential difference in vertical positioning during standard novel tank diving test between Control and MIX treatments were related to anxiety, the challenge was repeated with prior exposure to nicotine. Indeed, acute exposure to nicotine is anxiolytic and induces relief from anxiety in zebrafish which is translated by a higher time spent in top area during a novel tank diving test (Levin et al. 2007). To be validated as a proper anxiety measurement, both Control and MIX treatments should display a higher time spent in top area after prior exposure to nicotine. This challenge was repeated with naïve fish to evaluate pharmacologically behavioral changes using nicotine as described by Levin et al. (2007). For this purpose, individual fish was bathed into a 500 mL beaker containing nicotine (3.33 mg/L) during 3 minutes and then transferred to a second 500 mL beaker filled with plain system water for 5 additional minutes before introduction in the novel tank and video recording as described before.

For both tests, Control and MIX fish were alternated within session to avoid time bias between treatments. For space occupancy analysis, the tank was separated into two equal volume areas (top and bottom) according to Egan et al. (2009). The time spent in the top area during challenge, inversely correlated to anxiety, was used for statistical analyzes. After these experiments, fish were euthanized, sexed, measured and weighted.

Larval photo-motor response (LPMR)

LPMR tests were performed with larvae from F1 to F4 generations. The morning before the challenge, single larvae (5 dpf) issued from Control or MIX fish were individually transferred into one well of a 24-well plate (Krystal 24, opaque wall and clear bottom microplate) for acclimatization and plates transferred to the challenge room. Equal number of larvae from each treatment was introduced in each plate to avoid potential trial effect. Plates were successively placed into the challenge box (described in Vignet et al. (2014)) for F1 and F2 or DanioVision (Noldus, The Netherlands) for F3 and F4. The LPMR tests were run in the early afternoon which is the most stable activity period for 5 dpf larvae (MacPhail et al., 2009) and they included the following 5-min steps: Light on–1 (LON1, 70 lux), Light off (LOFF, <1 lux) and Light on–2 (LON2, 70 lux) with constant infra-red light maintained during video recording. Distance travelled (cm) was recorded and summed for each 5-min step.

2.4. Localization of the habenula

Line *Tg(-8.4neurog1:gfp)* was used to monitor habenula maturation in F1, F2 and F3. Habenula maturation was not monitored in F4 generation because no differences were observed in F2 and F3. At 4 dpf, larvae were anesthetized using benzocaine (50 mg/L) and placed in microscope glass depression slides covered by 3 % methylcellulose in order to maintain them in dorsal view. Larvae were observed using an epifluorescence microscope (Olympus BX41; fluorescence source XCite series 120 Q, SCOP PRO, Itteville, France) and imaged with a DMK31AU03 monochrome camera and IC-Capture software (The Imaging Source, Germany). Each larva was ranked in one of the following categories, depending on the position of the habenula revealed by the expression of GFP: right side, left side, median. When the habenula was not detected, larvae were removed from analyzes. The monitoring of habenula localization was performed by two independent readers but not performed blind.

2.5. **dnmts** *and* **c-fos** *transcription analyzes*

F1, F2, F3 and F4 embryos were obtained as described previously. In total, between 6 and 10 biological replicates (pools of 35 embryos or larvae) were used for analyzes. Gene transcription was monitored at 1 dpf for *dnmts* (*dnmt1*, *dnmt3aa*, *dnmt3ab*, *dnmt3ba*) and at 5 dpf for *c-fos*. Embryos or larvae were mechanically disrupted using a Beadblaster (Benchmark Scientific, Dutscher, France). Total RNA extraction was done according to RNeasy Plus Universal Mini Kit protocol (Quiagen) and 2 µg were used for cDNA synthesis.

Primers (Eurofins) for targeted and reference genes were designed using the free software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) with an annealing temperature of 60°C. Primers are listed in **Table S1**. Specificity of primers was first controlled *in silico* using the Basic Local Alignment Search Tool nucleotid (BLASTn) of the ZFIN database (http://zfin.org). Experimentally, melt curve analysis as well as electrophoretic migrations of the PCR products allowed to ensure that the amplification gave rise to one specific product of the expected size for each reaction. In addition, primer and cDNA dilutions were optimized in order to avoid non-specific products such as primer dimers. Overall, qPCR experiments were conducted in 20 µl reactions with 300 to 600 nM of primers, 2X Fast SYBR Green Master Mix (Applied Biosystems) and cDNA diluted 1/5. Serial dilutions of the cDNA were made in order to perform standard curve analyses which confirmed that amplification using each primer pair was linearly proportional to the input cDNA amount. Amplicon size and associated efficiencies for each primer pair can be found in

Table S1. The amplification protocol used was as follows: initial denaturation (20 sec at 95°C) followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. Technical triplicates were run for each biological replicate. Amongst 6 investigated reference genes (*g6pd, act1b, gapdh, rpl13a, b2m, eef1),* the 3 most stable were selected using BestKeeper software (Pfaffl et al., 2004). For F1 and F2 generations, *rpl13a, gapdh* and *act1b* were used for the analyses at 24 hpf, and *act1b*, *b2m* and *rpl13a* at 5 *dpf*. For the F3 generation, *rpl13a, eef1* and *g6pd* were used for the analyses at 24 hpf, and *act1b*, *g6pd* and *rpl13a* at 5 *dpf*. Then for F4, *rpl13a, eef1* and *b2m* were used for the analyses at 24 hpf, and *act1b*, *eef1* and *rpl13a* at 5 *dpf*

The analysis software Relative Expression Software Tool (REST (Pfaffl, 2001; Pfaffl et al., 2002)) calculated fold change in transcription of a target gene (target), normalized with qPCR efficiency (E) and reference genes (ref), between Control and MIX groups according to the following formula:

$$Fold\ change = \frac{(E_{target})^{\Delta Ct_{target}\ (Control - MIX)}}{(E_{ref})^{\Delta Ct_{ref}\ (Control - MIX)}}$$

2.6. Statistical analyzes

All statistical analyzes concerning body mass and behavioral responses were performed using Statistica 9.0 software (StatSoft, USA). Normality of residuals was tested *a posteriori* using Shapiro-Wilks test and dataset were confirmed as normally distributed. Statistical analyses for habenula localization were performed in R 3.1.0 software (R Core Team, 2013). Finally, statistical analyzes of gene transcription were performed using REST software (Pfaffl, (2001); Pfaffl et al., (2002); see details below). All statistical analyzes were carried out at a 95 % level of significance and values are represented as mean ± SEM except when mentioned otherwise.

For the time spent in top area during novel tank diving test, we first performed a factorial ANOVA with two factors (generations and treatments) for water bathed fish. A second factorial ANOVA was realized in nicotine bathed fish to evaluate possible different behavioral responses after nicotine exposure with again two factors (generations and treatments). To ascertain that time spent in top area was indicative of anxiety level, a third factorial ANOVA was calculated with two factors (generations and bathing), treatment factor was removed from analysis because it was not significant.

For LPMR, repeated-measure ANOVA was applied for comparison of the distance travelled over the three time periods with the two factors generations and treatments.

All ANOVA were followed by Newman-Keuls (NK) multiple comparisons *post-hoc* tests. The analysis of the habenula position was performed using a Chi^2 test within each generation. For qPCR data, the analysis software REST automatically includes statistical analysis based on a two-sided pair-wise fixed reallocation randomization test and bootstrapping methods including 2000 iterations which allows to get a good estimate of the p-value (Pfaffl, 2001). Data are presented as the geometric average ± standard error of the fold-change relative to Control via Taylor algorithm (http://rest.gene-quantification.info).

3. Results

3.1. PCB and PBDE concentrations from F0 to F1

PCB and PBDE concentrations in MIX and Control diets were presented by Horri et al. (2018). Briefly, in the MIX diet (n=12), the total concentrations, *i.e.* summed across 22 PCB and 7 PBDE congeners, were 1932.3±90.4 ng/g (mean±SE) ww for∑PCB s and 479.8±50.8 ng/g ww for∑PBDE s. In the Control diet (n=16), the total concentrations were 245 and 522 times lower, *i.e.* 7.9±3.5 ng/g ww for∑PCB s and 0.92±0.36 ng/g ww for∑PBDE s. In addition, PCB and PBDE profiles in Control diet were completely different from the one in MIX diet (**Table 1**).

PCBs and PBDEs concentrations were monitored at 180 dpf in F0 whole individuals exposed to Control and MIX diets. Since there was no difference between sex (p>0.05; data not shown) they were analyzed together. In MIX F0 fish, the total concentrations of PCBs (n=6) and PBDEs (n=6) were 2162.5±311.0 and 103.6±12.3 ng/g ww respectively. In Control F0 fish, the total concentrations of PCBs (n=2) and PBDEs (n=6) were 26.5±0.4 and 1.8±0.9 ng/g ww.

In F1 generations∑PCBs concentration was 91.3±18.0 ng/g ww and∑PBDE s concentration was 7.2±0.5 ng/g ww in MIX eggs (n=3 batches), which was 20 and 98 fold higher than in control eggs (ΣPCBs=4.5 ng/g ww, n=2 batches and ΣPBDEs=0.074 ng/g ww, n=1 batch). At 5 dpf, total concentrations of PCBs and PBDEs were 332.5±135.8 ng/g ww and 28.0±5.8 ng/g ww respectively in MIX larvae (n=3 batches). In Control larvae, levels were respectively 90 and 20 fold lower than in MIX larvae, with ΣPCBs=3.7 ng/g ww (n=2) and ΣPBDEs below the limits of detection (0.66 ng/g ww for BDE-209 and 0.11 ng/g ww for all other congeners). Then at 112 dpf, approximately when the novel tank diving test was performed, concentrations were similar among both treatments, ΣPCBs=18.2±5.5 ng/g ww in MIX fish

(n=6) and ΣPCBs=17.2±2.5 ng/g ww in Control fish (n=3). The same applied for ΣPBDEs=0.8±0.1 ng/g ww in MIX fish (n=6) and ΣPBDEs=0.8±0.2 ng/g ww in Control fish (n=3). These values are in the range of those found in Control F0 fish.

3.2. Behavioral experiments

Novel tank diving test

Body mass of fish challenged in novel tank diving test differed between generations (Factorial ANOVA, F=3.35, df=3, p=0.02) but not between either treatments (F=0.09, df=1, p=0.76) or bathing (F=0.88, df=1, p=0.35) and there was no interaction either pairwise or combining all factors. Body mass for each factor, generations, treatments and bathing are given in **Table S2**.

The time spent in top area did not differ between generations (Factorial ANOVA, F=1.8, df=3, p=0.14) but differed between treatments (F=8.3, df=1, p=0.005) and there was a significant interaction effect between generations and treatments (F=2.9, df=3, p=0.04). No significant difference was found between treatments in F0, F1 and F3 generations (NK, respectively p=0.63, p=0.77 and p=0.48). However, in F2 generation, the offspring of MIX fish spent significantly less time in top area than Control ones (NK, p=0.005; **Figure 2**).

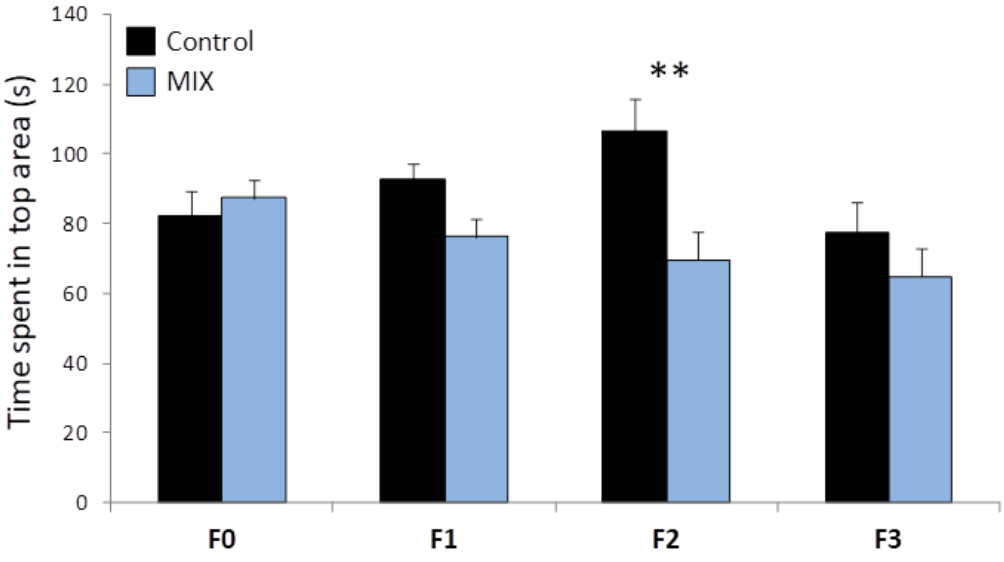

Figure 2. Time spent in top area during the novel tank diving test for each generation for water bathed Control fish (black bars, F0: n=23; F1: n=20; F2: n=19; F3: n=22) and MIX fish (blue bars, F0: n=23; F1: n=20; F2: n=19; F3: n=24). Factorial ANOVA with two factors (treatment and generation). Newman-Keuls test between treatments within generation: **: p<0.01.

Novel tank diving test with prior exposure to nicotine

In nicotine bathed fish, time spent in the top area did not differ between generations (Factorial ANOVA, F=1.9, df=3, p=0.14), treatments (F=0.1, df=1, p=0.75) and there was no interaction between generations and treatments (F=2.6, df=3, p=0.08).

When integrating all data together (water bathed and nicotine bathed fish), time spent in top area differed between generations (Factorial ANOVA, F=3.3, df=3, p=0.02). The F3 generation spent significantly less time in top area than the F2 generations (NK, p=0.003). Time spent in top area was different according to bathing factor (F=145.6, df=1, p<0.001) with nicotine bathed fish spending significantly more time in top area during the novel tank diving test than water bathed fish whatever the generation (NK, p<0.001 for all generation) but there was no significant interaction between the two factors (F=0.5, df=3, p=0.7). Time spent in top area according to generations, bathing and treatments are given in **Table S3**.

Larval photo-motor response (LPMR)

As expected for a LPMR test, distance travelled was higher during the LOFF period compared to LON1 and LON2 (repeated measures ANOVA, F=730.3, df=2, p<0.001) but also varied depending on generations (F=159.8, df=3, p<0.001) and treatments (F=13, df=1, p<0.001) and there was a significant interaction between generations and treatments (F=9, df=3, p<0.001). Post-hoc tests indicated that distance travelled during LPMR was higher in F1 generation compared to F2 generation (NK, p<0.001) which was also higher than that of F3 and F4 generations (NK, p<0.001 for both) which were similar (**Figure 3**).

Within generation, when considering the LON periods, there was no difference in LON1 between larvae issued from Control and MIX fish for F1 (NK, p=0.49) and F2 (NK, p=0.58) but there was a trend for hypoactivity both for F3 (NK, p=0.05) and F4 (NK, p=0.08). No difference was observed between Control and MIX larvae during LON2 whatever the generation (NK, p=0.32; p=0.21; p=0.22 and p=0.28 for F1 to F4 respectively). When considering the LOFF period, a difference for distance travelled between Control and MIX larvae was observed for all generations. In F1, the distance travelled was increased for MIX larvae compared to Control ones (NK, p=0.04) while for all following generations distance travelled by MIX larvae was decreased compared to the respective Control (NK, p=0.01; p=0.01 and p<0.001 from F2 to F4 generations respectively).

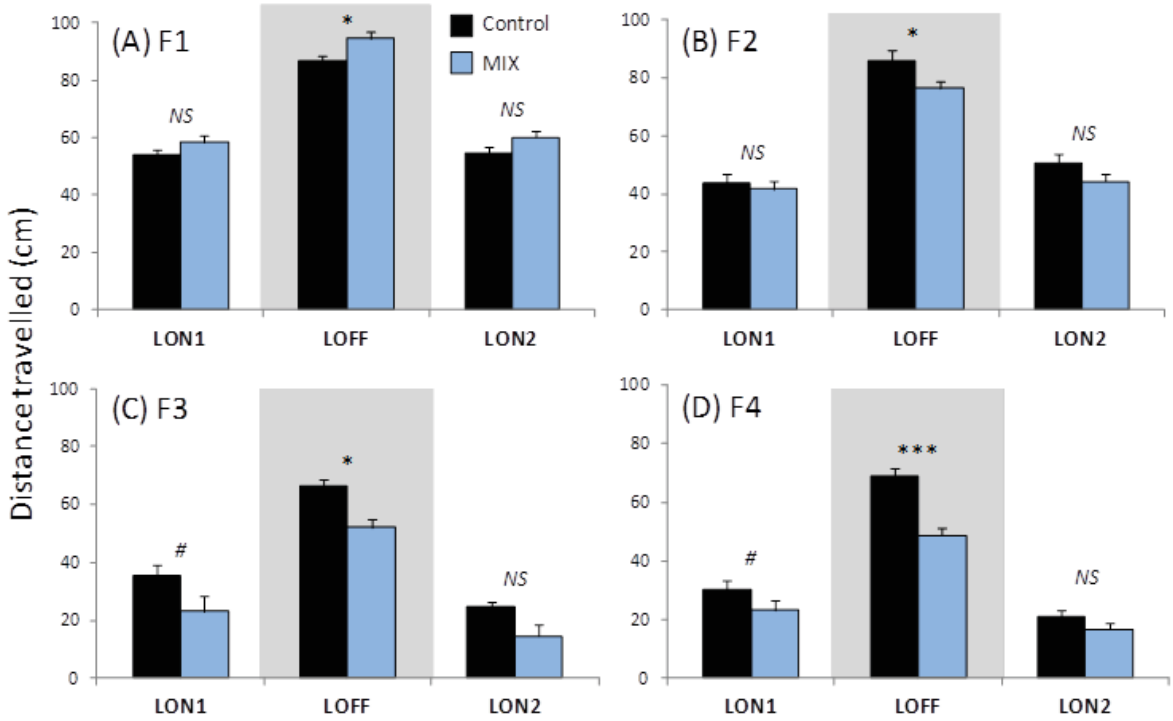

Figure 3. Distance travelled during each 5-min steps (Light on-1,LON1; light off, LOFF; Light on-2, LON2) of the larval photo-motor response (LPMR) performed at 5 dpf for **(A)** Control F1 (n=112, black bars) and MIX F1 (n=121, blue bars); **(B)** Control F2 (n=63, black bars) and MIX F2 (n=64, blue bars); **(C)** Control F3 (n=95, black bars) and MIX F3 (n=95, blue bars) and **(D)** Control F4 (n=71, black bars) and MIX F4 (n=67, blue bars). Repeated measures ANOVA on 5-min step (LON1, LOFF and LON2) with two factors (treatment and generation). Newman-Keuls test between treatments within each 5-min step: #: $p<0.10$; *: $p=0.05$; ***: $p<0.01$; *NS*: not significant.

3.3. Localization of the habenula

In 4 dpf larvae, the habenula is located at the anterior and dorsal edge of the diencephalon front and its position can clearly be monitored according to GFP signal in the *Tg(-8.4neurog1:gfp)* line (**Figure 4**). At this age, habenula position was mainly located lateral in reference to brain axis with only 40% of Control larvae having habenula in medial position. In contrast, for MIX F1 larvae habenula was medial in 80% of larvae which was significantly different compared to Control F1 ($\chi^2=20.9$, $p<0.001$). In F2 and F3 larvae, no difference was observed between Control and MIX larvae (F2: $\chi^2= 3.4$, $p=0.19$; F3: $\chi^2= 2.7$, $p=0.26$).

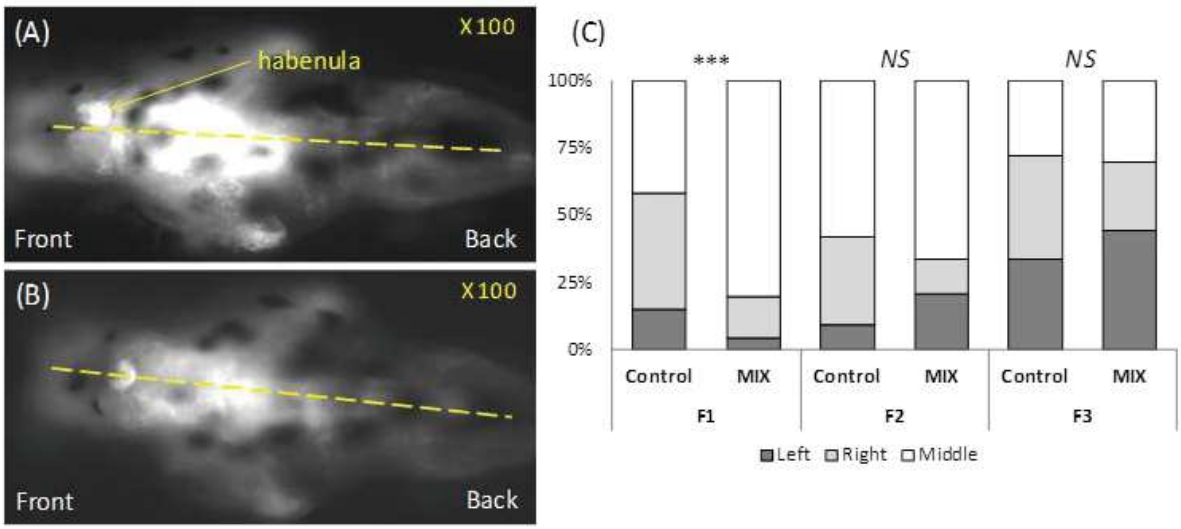

Figure 4. Dorsal view of the head of F1 *Tg(-8.4neurog1:gfp)* 4 dpf zebrafish larvae with focus on habenula in **(A)** Control F1 and **(B)** MIX F1 (in A and B, dashed line indicates the median axis); **(C)** habenula localization (left: dark grey; right: grey and middle: white) relative to the median axis in F1, F2 and F3 generations (F1: n=74 Control, n=65 MIX; F2: n=39 Control, n=74 MIX; F3: n=65 Control, n=64 MIX). Chi² test within each generation: ***: p<0.001; *NS*: not significant.

3.4. dnmts *and* c-fos *transcription analyzes*

In order to gain a better understanding of the molecular mechanisms leading to the observed behavioral disruptions, transcription of *dnmts* and *c-fos* were monitored in F1, F2, F3 and F4 embryos and larvae, respectively (**Figure 5**). In 1 dpf MIX F1 embryos, *dnmt3aa* was expressed at a similar level as in Control F1 embryos (p=0.14) while other tested *dnmts* were downregulated: *dnmt1* (fold-change relative to the Control (Fc=0.74; p=0.015), *dnmt3ab* (Fc=0.57; p=0.012) and *dnmt3ba* (Fc=0.29; p=0.003). Moreover, the transcription of *c-fos* was upregulated in 5 dpf MIX F1 larvae (Fc=2.02; p=0.009).

The transcription of *dnmt3ba* and *c-fos* remained altered in MIX F2. In contrast to F1, *dnmt3ba* was upregulated (Fc=1.86; p=0.004) and *c-fos* was downregulated (Fc=0.30; p<0.001 in MIX F2. On the contrary, *dnmt1* and *dnmt3aa* were both equally expressed in Control and MIX F2 embryos (p=0.72 and p=0.99, respectively). In F3 and F4 generations, *dnmt1*, *dnmt3aa*, *dnmt3ab* and *c-fos* were also equally expressed in Control and MIX (p=0.56; p=0.68; p=0.85; p=0.51 and p=0.19; p=0.63; p=0.67; p=0.63 respectively) while *dnmt3ba* was downregulated in MIX F3 (Fc=0.47; p<0.001) and upregulated in MIX F4 (Fc=2.00; p<0.001).

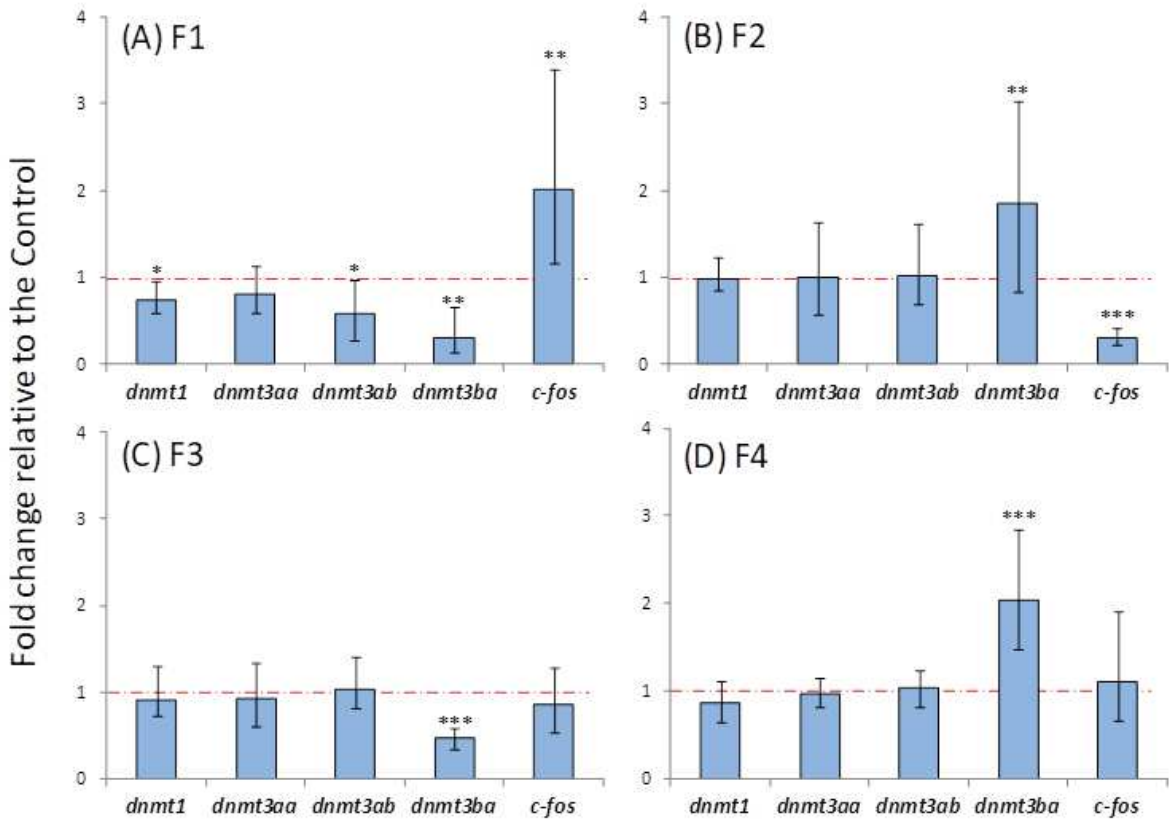

Figure 5. Comparison of *dnmt1*, *dmnt3aa*, *dnmt3ab*, *dnmt3ba* genes transcription at 1 dpf and *c-fos* at 5 dpf in MIX expressed as fold-change relative to the Control and expressed as mean±SE with n = number of biological replicates (each being a pool of 35 embryos or larvae) in **(A)** F1 (*dnmts*: n=8 Control/n=6 MIX, *c-fos*: n=7 Control/n=7 MIX); **(B)** F2 (*dnmts*: n=7 Control/n=6 MIX, *c-fos*: n=7 Control/n=5 MIX); **(C)** F3 (*dnmts*: n=7 Control/n=6 MIX, *c-fos*: n=8 Control/n=8 MIX); **(D)** F4 (*dnmts*: n=7 Control/n=9 MIX, *c-fos*: n=10 Control/n=8 MIX). Dashed red line indicating Control transcription. Pair-wise fixed reallocation randomization test: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

4. Discussion

Measurements of PCB and PBDE concentrations in Control fish showed no external or cross-contamination. This is confirmed by PCB and PBDE profiles in Control diet which were different from the mixture used in this experiment. Analyzes of the MIX diet revealed spiking efficiencies of 86 to 130% of targeted concentrations of the selected PCBs and PBDEs (Horri et al., 2018). Achieved concentrations of PCB and PBDE congeners were clearly higher in MIX F0 fish than in Control ones (83 and 59 times respectively) at 180 dpf with no difference between sexes. Environmental relevance of exposure is confirmed by the fact that, at the end of the experiment, PCB and PBDE levels in MIX F0 fish were in the range of those reported in fish from European industrialized estuaries (Law et al., 2006; Robinson et al., 2017). In F1 eggs, PCB and PBDE concentrations were respectively 30 and 108 times higher in MIX F1

than in Control F1 because of maternal transfer in oocytes (Daouk et al., 2011; Nyholm et al., 2008; Yu et al., 2011). At 5 dpf, the concentrations of both PCBs and PBDEs increased. These increased concentrations in larvae compared to eggs is likely due to the loss in weight after hatching because eggs where sampled with their chorion containing a relatively large amount of water (Foekema et al., 2012). At 112 dpf, levels of PCB and PBDE congeners were low and similar to those found in Control F1 fish. This can be explained by dilution during body growth (Sijm et al., 1992), indeed individual weight increased more than 400-fold between larval and adult stage.

The novel tank diving test was used for the determination of adverse effects of the mixture used on behavior. This test is a common protocol used to evaluate fear and anxiety in juvenile or adult zebrafish where higher anxiety state is revealed by a shorter time spent in the top area of a tank (Bailey et al., 2013; Egan et al., 2009; Levin et al., 2007). Prior exposure to nicotine, which is an anxiolityc drug, has been used in this context and induced a higher time spent in top area (Levin et al., 2007). In the present study, MIX F0, F1 and F3 adults did not show specific response; conversely, behavior of MIX F2 indicated a higher anxiety compared to Control fish. Prior exposure to nicotine confirmed the nature of the behavior we observed. Such anxiety-like behavior was already described after exposure to 6-OH-BDE-47 (Macaulay et al., 2015) and Aroclor 1254 (Gonzalez et al., 2016) in zebrafish larvae or in adults after direct embryonic exposure to CB-126 (Glazer et al., 2016). Such behavior may result from the well documented neurotoxicity of PCBs and PBDEs (Costa et al., 2014; de Cock et al., 2012; Dingemans et al., 2011; Faroon et al., 2000; Howdeshell, 2002; Ulbrich and Stahlmann, 2004). Indeed, in some cases, correlations between behavioral and neuronal disruptions have been established (Chen et al., 2012c; He et al., 2011). Overall, the novel tank diving test suggests multigenerational (F2) but no transgenerational inheritance of adult anxiety level.

LPMR is a robust widespread behavioral assay that can report locomotor defects caused by toxic compounds or drugs (Chen et al., 2012b; Péan et al., 2013; Schnorr et al., 2012; Vignet et al., 2014). Regardless of the treatment, an increase in locomotor activity was observed in response to light to dark transition in all generations (F1, F2, F3 and F4), validating the procedure for LPMR (Schnorr et al., 2012). Differences in distance travelled between generations is likely due to external factors which were better controlled for in the device used for F3 and F4 compared to the one used for F1 and F2. Because of these differences in response baseline between generations, it is not possible to directly compare behavior across generations; it is, however, possible to compare behavioral response between treatments

within generations. In MIX F1, activity during LOFF was higher compared to Control F1. Similar increase in activity during LOFF was previously observed in F1 fish larvae after parental exposure to BDE-209 (He et al., 2011) and PCBs (Péan et al., 2013) as well as in larvae after embryo-larval exposure to DE-71 (Chen et al., 2010; Chen et al., 2012a). In later generations (F2, F3 and F4), activity of MIX larvae was consistently lower during LOFF compared to Control larvae. In addition, a trend for hypoactivity during LON1 was observed in both MIX F3 and MIX F4 compared to respective Control larvae but it was not observed in MIX F1 and MIX F2. Neural circuit underlying locomotion behavior is still not completely understood, but defects in motor neurons could be involved in locomotion disruptions (Drapeau et al., 2002; Kabashi et al., 2010). Therefore, behavioral disruptions observed during LPMR from MIX F1 to F4 generations may reflect neural disruptions. For example, He et al. (2011) showed that development of secondary motor neurons could be delayed in F1 larvae issued from BDE-209 exposed fish. Moreover, Chen et al. (2012c) reported similar neurodevelopmental defects in BDE-47 exposed larvae also showing locomotion disruptions. The habenula is mainly responsible for assimilation of environmental information. At 4 dpf, the development of the habenula is completed, giving rise to a functional asymmetry between the right and the left neurons, in charge of assimilating olfactory and visual information, respectively (Bianco and Wilson, 2009; Dreosti et al., 2014). Defects in habenula maturation are related to inadequate response to environmental stimuli, such as disorientation, increased anxiety (Lee et al., 2010) or fear and lack of escape reaction (Agetsuma et al., 2010; Budaev and Andrew, 2009), making an individual more sensitive to predation (Lee et al., 2010; Okamoto et al., 2012). Alterations in habenula maturation exemplified by the incompletion of functional asymmetry in MIX F1 larvae may provide neuronal support to explain MIX F1 behavioral response in LPMR and to assimilate it to anxiety. Conversely, neither locomotor increase in larvae nor habenula maturation defects were observed in later generations.

Besides, changes in *c-fos* transcription may also provide some mechanistic understanding. For example, increased *c-fos* transcription in rodent hypothalamus is induced by challenges promoting stress or anxiety (Duncan et al., 1996). A similar relationship was demonstrated in zebrafish larvae while incubation in a drug with anxiolytic effect both relieve behavioral stress and prevent *c-fos* transcription increase (Chen et al., 2015). This provides mechanistic basis to associate increase in *c-fos* transcription in F1 and decrease in F2 larvae with an increase in activity evoking anxiety during LOFF in F1 and the opposite in F2. This mechanism cannot be proposed for later generations since the decrease in LOFF persisted

while *c-fos* transcription was unchanged between Control and MIX larvae. It is important to note than in F3 and to a lesser extent in F4, activity was also reduced in LON1. The decrease in activity during LOFF may therefore also reflect another behavioral disruption not related to anxiety in these generations. Such behavioral disruptions in larvae and/or adults over several generations may decrease individual fitness and recruitment and impact population dynamics and sustainability.

Parental exposure to POPs may trigger epigenetic modifications that can be transferred through generations and support inherited physiological disruptions, including behavior (Skinner, 2014a, b; Vandegehuchte and Janssen, 2011). Early-life stages are particularly sensitive to epigenetic disruption since *de novo* DNA methylation patterns are settled together with the overall epigenetic dynamicity to support zygotic genome activation and cell differentiation (Andersen et al., 2013). It was previously reported that direct exposure to various chemicals leads to changes in global and/or specific methylation levels of the developing zebrafish genome (Aluru et al., 2015; Corrales et al., 2014; Fang et al., 2013). In addition, methylation levels of adult gonads can be altered after chemical exposure (Laing et al., 2016; Liu et al., 2016), which may also act as a support for multigenerational inheritance of effects. In the present study, transcription of DNA methyltransferases was investigated in F1, F2, F3 and F4 larvae. *Dnmt1*, *dnmt3ab* and *dnmt3ba* were all downregulated at 1 dpf in MIX F1, suggesting an interference of the exposure with the DNA methylation pathway. Interestingly, similar modifications were obtained in F1 offspring of rats exposed *in utero* to a mixture including PCBs, organochlorine pesticides and methylmercury (Desaulniers et al., 2009). In the next generations, opposite regulations were alternatively reported in F2, F3 and F4 offspring with the up-, down- and up-regulation of *dnmt3ba*, respectively. *Dnmt3ba* is essentially expressed in the brain area and involved in neurogenesis in early-life stages (Shimoda et al., 2005). In addition, other studies suggested that changes on genome methylation level may influence animal behavior (Knecht et al., 2017; McGhee and Bell, 2014). Particularly, the present results are in agreement with previous investigations by McGhee and Bell (2014) who showed a negative correlation between transcription of *dnmt3a* and increased anxiety in F1 offspring of stickleback. Therefore, *dnmt3ba* transcription may impact behavior of F1 and F2 larvae issued from MIX fish and should be considered along with the changes in *c-fos* transcription. Indeed, this gene transcription was already shown to be induced upon exposure of zebrafish larvae to benzo[a]pyrene and TCDD in association with a decrease in its promoter methylation (Aluru et al., 2015; Corrales et al., 2014). Besides,

the maternal transfer of *dnmt3ba* mRNAs (Smith et al., 2011) may support the transgenerational inheritance of *dnmt3ba* misexpression over F3 and F4 generations. However, changes in transcription of *c-fos* and *dnmt3ba* alone are not sufficient to explain transgenerational changes in behavior since link with LPMR behavioral response is lost from the F3 generation onwards.

The differences in behavioral and molecular responses observed between generations suggest a triggering by a conjunction of several mechanisms. In F1, both early exposure to PCBs and PBDEs transferred to the eggs and epigenetic mechanisms, as changes in *dnmts* expression, may contribute to the induction of hyperactivity at larval stage evoking an anxiety-like behavior when interpreted along with molecular markers (**Table 2**). In F2, since PCBs and PBDEs concentrations in MIX F1 juveniles decreased to the level of Control F1, we can assume that there is no such transfer to the eggs. However, germ cells which will later produce gametes involved in F2 generation are present in F1 at larval stages when PCBs and PBDEs concentrations are still significantly higher in MIX F1 compared to Control F1. Therefore in F2, both early exposure of germ cells and changes in some *dnmt* expression may contribute to behavioral alterations. In F3 and F4, mostly epigenetic mechanisms (*dnmt* expression) could be mentioned while oscillation of *dnmt3ba* transcription in these generations suggests additional complexity. These results underline the importance of discriminating multigenerational and transgenerational effects upon exposure to pollutants.

In conclusion, effects of a parental exposure to POPs were highly depending on the generation that was studied. The complexity to interpret the data can be, in part, a result of the distinct ways fish from successive generations were exposed to the POPs, (direct exposure for F0 and after transfer to the eggs for F1) or to various physiological effects (F2-F3-F4). However, the distinct effects observed between the different generations emphasize new elements to take into consideration when investigating multi- and transgenerational effects. We suggest that embryonic exposure to PCBs and PBDEs is triggering hyperactivity in larvae evoking anxiety-like behavior when interpreted along with molecular markers but anxiety-like behavior is lacking in adult F0; and that the direct exposure of F2 germ cell progenitors in F1 larvae together with changes in *dnmt* expression are triggering specific effects in the F2 generation at both larval and adult stages. Further molecular analyses to identify changes in other gene transcription and in methylation profiles are ongoing with the aim to deepen our understanding of this kind of observation which has, to our knowledge, never been described.

Table 2. Effects observed between treatments on adult anxiety, larvae locomotor activity during the light off period (LOFF), *dnmt3ba* and *c-fos* transcription from F0 to F4 generations. Sign indicate a higher (+) or lower (-) response of MIX or issued from MIX exposed parents compared to control.

	Exposure to PCB / PBDE	Adult	Larvae		
		Anxiety	Locomotor activity	*Dnmt3ba*	*c-fos*
F0	Direct exposure	=			
F1	Transfer to eggs	=	+	-	+
F2	Exposure of germ cells	+	-	+	-
F3		=	-	-	=
F4			-	+	=

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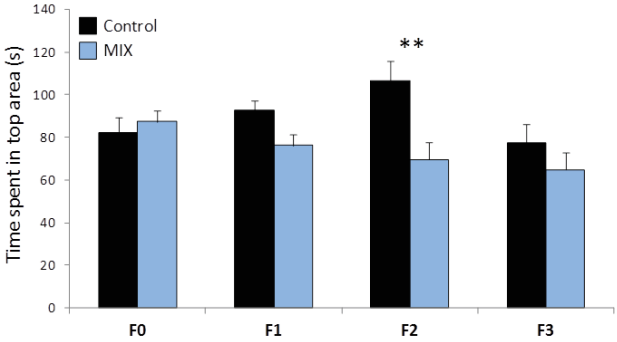
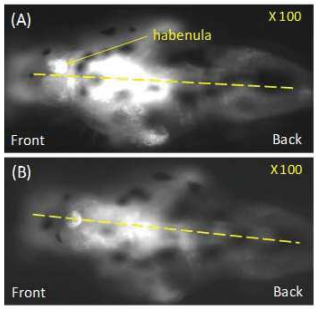
Chapitre 3 – Conclusions

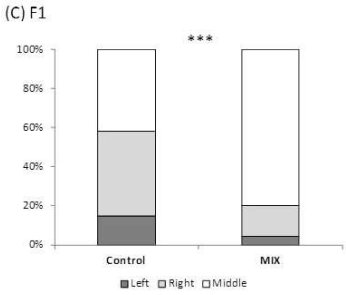
Résultats:

• Nouvel environnement:

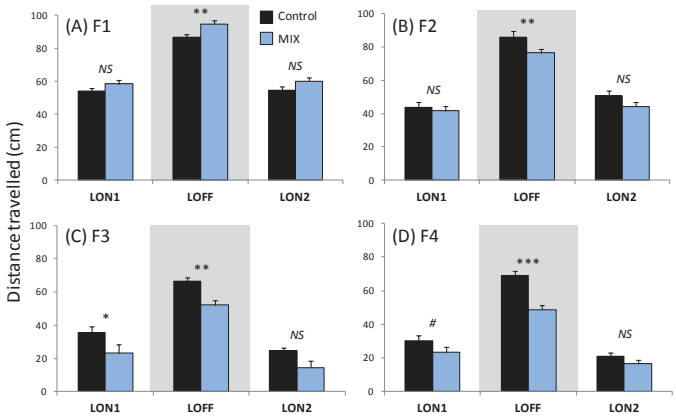


• Localisation de l'habenula:

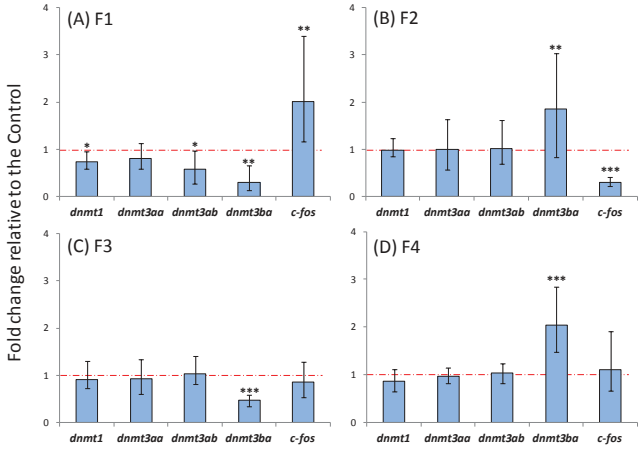




• Réponse photomotrice: larves



• Transcriptions *dnmts* et *c-fos*



*: p<0.05, **: p<0.01, ***: p<0.001

Points importants:

• Une exposition chronique par la voie alimentaire à un mélange de PCB et PBDE n'affecte pas le comportement de la génération exposée (F0) mais il affecte celui des générations suivantes (F1, F2, F3 et F4) chez les larves et/ou les juvéniles. La réponse photomotrice des larves est altérée jusqu'à la génération F4 et les juvéniles F2 MIX présentent une anxiété supérieure aux contrôles.

• La maturation de l'habenula était normale chez les générations F0 et F2 alors qu'elle a été retardée chez la génération F1. De plus, la transcription du facteur de transcription *c-fos* est altérée chez les générations F1 et F2 pouvant expliquer en partie ces altérations comportementales.

• L'altération de la transcription des *dnmts (*notamment *dnmt3ba* de F1 à F4*)*, est un mécanisme suggéré pour expliquer le transfert d'altérations comportementales jusqu'aux générations non exposées par le mélange de polluants (F3 et F4).

➔ Une exposition chronique à un mélange de PCB et PBDE représentatif de conditions environnementale est capable d'entraîner des altérations comportementales jusqu'à la génération F4.

Communications:

• **European Society of Comparative Biochemistry and Physiology Barcelone – 2017 –** *Communication orale*

• **Society of Environmental Toxicology and Chemistry – Bruxelles – 2017** *Communication orale*

Supplementary material
Examining multi- and transgenerational behavioral and molecular alterations resulting from parental exposure to a PCB and PBDE environmental mixture

Aquatic Toxicology 208, 29-38 (2019)

Text S1. Detailed material and methods

1. ***Fish rearing***

Fish were reared in heterosexual groups (n=28±5) at 27±1°C under a 14h/10h light/dark cycle. Physical water conditions were maintained constant during the experiment: conductivity 300±50 µS/cm and pH 7.5±0.5 using a mixture of reverse osmosis water and tap water, both being initially treated with sediments and activated charcoal filters. Ammonia, nitrites, and nitrates were monitored weekly and remained within recommended ranges (Lawrence, 2007). Contaminated water was collected and treated with activated charcoal.

Fish were fed three times a day, *i.e.* morning and evening with pellets and at midday with freshly hatched crustaceans *Artemia salina*. Food pellet size was adapted to fish age and mouth size. From 5 to 70 days post fertilization (dpf), fish were fed sequentially with SDS 100 µm, 200 µm, and 300 µm (Special Diet Service; Dietex international) and from 60 dpf onwards, they were fed with Inicio^{+} 500 µm (Biomar, France). Transition period between food sizes were performed with co-feeding using food of both sizes.

2. ***PCB and PBDE analyzes***

Briefly, PCBs were analyzed by pressurized liquid extraction with the SpeedExtractor E-914 (Buchi Lab., Switzerland), purified sequentially with concentrated sulfuric acid and by adsorption chromatography on Florisil column, and analyzed using gas chromatography equipped with an electron capture detector (GC-µECD). PBDEs were analyzed by pressurized

liquid extraction using an accelerated solvent extractor (Dionex Corp., USA) followed by gel permeation chromatography, silica and alumina column, and concentrated sulfuric acid treatment prior to quantification by gas chromatography coupled to mass spectrometry (GC–MS) in electron capture negative ionization mode (ECNI).

Quality assurance/Quality control for *PCB analysis*

The calibration of the system was performed within quite a range using a multi-point (6) calibration curve to define the linearity range of our detector (ECD) for all contaminants, for example from 4 to 2000 pg/µl for CB-153. The relative precision of the method was checked for this type of samples by the analysis of 6 aliquots of a homogeneous preparation of fish (laboratory control card). The results showed coefficients of variation of less than 10 % for all congeners, which indicates a satisfactory reproducibility of the method. During the analysis of the real samples, analytical blanks were systematically measured every 10 samples. The blank were about 0.2 pg/µl, 20 times less than the concentrations of the lowest standard of CB-153 (*i.e.* <0.1 ng/g), and much less for other determinants. Surrogate recoveries were 86±6% for CB-30, 97±8% for CB-198 and 102±9% for CB-209 (mean±SE calculated on n=93 analyses) and no surrogate correction was applied for the reported concentrations.

Finally, 6 replicates of a Standard Reference Material, SRM2977 (mussel, *Mytilus edulis* tissue) were analysed in order to determine the accuracy and precision of the method. PCB recoveries varied between 77 and 115%. The concentrations of the real samples were not corrected for recoveries. Moreover, the RSD values ranged from 3.8 to 19.6%, with a mean of 9.7% for all PCBs. All these results were in agreement with certified reference.

The limit of detection (LOD) was estimated taking into account a signal to noise ratio of 3, the injection volume (1 µl), the volume of the concentrated extract before injection (250 µl) and the extracted sample mass. Average value varied between 2 to 15 pg depending to congener.

Quality assurance/Quality control for *PBDE analysis*

Quality Assurance / Quality Control procedures were implemented for each batch of 8 to 10 samples, including procedural blanks, use of recovery surrogates in all samples, analysis of certified reference material and participation to inter-comparison exercises. Detailed information can be found in Munschy et al. (2011) and an update is given below.

Procedural blank samples were analysed in every batch of 8 to 10 samples using glass powder, extracted and processed in the same manner as the fish samples. Blanks were

contamination-free, with the exception of BDE-209, which was found at 0.5±0.3 pg injected (average value calculated on 14 blanks). BDE-209 concentrations were corrected from blank values in every sample. The values obtained in each blank were used to correct the values in samples analysed in the same batch.

The limit of detection (LOD) was calculated for each sample taking into account a signal to noise ratio of 3, the injection volume, the volume of the concentrated extract before injection and the extracted sample mass. Average value was 0.09±0.05 ng/g ww (n=93).

Surrogate recoveries were 88±8% for BDE-139 and 77±17% for ^{13}C BDE-209 (mean±SE calculated on n=93 analyses) and no surrogate correction was applied for the reported concentrations.

Certified reference material (WMF-01, supplied by Wellington laboratories Inc., Ontario, Canada) was routinely included in each sequence of samples and the results were within ± 2 times the standard deviations of the means of the certified values. WMF-01 consists of a freeze-dried fish sample for which certified or indicative values are given for 7 congeners (namely, BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183) at concentrations of between 0.53±0.40 ng/g dry weight (dw) for BDE-183 and 123.2±24.8 ng/g dw for BDE-47.

The laboratory regularly takes part in Quality Assurance of Information for Marine Environmental Monitoring in Europe (QUASIMEME) inter-comparison exercises for PBDEs in biota and our Z-scores are satisfactory, *i.e.* between -2 and +2 (for example, they were between -0.28 and -0.77 in 2015 and between -0.83 and +0.32 in 2016).

3. *Behavioral experiments*

For all behavioral experiments, videos were recorded with an analogue camera ICD-48E (Ikegami) and a 2.1-13.5 lens (Fujinon) linked to a computer with an acquisition card and EthoVision XT 10.1 software (Noldus, The Netherlands). Data extraction and analyzes were performed using EthoVision XT 10.1 software. Individuals with acquisition default and/or outliers values (freezing behavior) were removed before statistical analyzes (ca. 1.5 individuals per generation were removed, min=0, max=2).

Novel tank diving test

In the afternoon before the challenge, adult fish (135±14 dpf) were moved to a behavioral challenge room with similar photoperiod and temperature as in the rearing room. Fish were isolated from each other and kept into 1 L tanks (AquaSchwarz, Germany) until challenge. During the challenge, fish was recorded in side view during 4 min.

For the regular novel tank diving test, 22±2 fish were used per treatment (Control and MIX) for each generation tested (F0-F3). For the novel tank diving test including a nicotine bath n=22±2 naïve fish were used per treatment for each generation tested (F0-F3). Nicotine (Pestanal, Sigma-Aldrich Saint-Quentin Fallavier, France) solution was prepared in system water.

After novel tank diving tests, fish were euthanized by an overdose of benzocaine (500 mg/L from a stock solution at 100 g/L in ethanol; Sigma-Aldrich, Saint-Quentin Fallavier, France), sexed and measured for standard length (from tip of head to beginning of caudal fin rays, mm) and for body mass (mg).

Larval photo-motor response (LPMR)

Equal number of larvae from each treatment was introduced in each plate to avoid potential trial effect. The challenge was performed on F1 larvae (n=112 for Control and n=121 for MIX (12 spawns)), on F2 larvae (n=63 for Control and n=64 for MIX larvae (6 spawns)), on F3 larvae (n=95 for Control and n=95 for MIX larvae (8 spawns)) and on F4 larvae (n=71 for Control and n=67 for MIX larvae (6 spawns)).

4. *Localization of the habenula*

Neurogenin1 is expressed in progenitors and primary neurons of the central nervous system in early life and adult stages and it is especially expressed within the habenula (Korzh and Strahle, 2002). Larvae were maintained in dorsal view to observe the habenula located in the diencephalon, at the forebrain/midbrain boundary. At least two independent spawns were used for imaging for all generations and a total of n=74 Control and n=65 MIX (for F1); n= 39 Control and n=74 MIX (for F2) and n=65 Control and n=63 MIX (for F3).

5. dnmts *and* c-fos *transcription analyzes*

Pools of embryos (1 dpf) or larvae (5 dpf) were kept in RNALater (Sigma-Aldrich) at 4°C until further processing. Prior to RNA extraction, RNALater was removed and embryos were put in a lysis buffer (Quiagen) before being mechanically disrupted using a Beadblaster (Benchmark Scientific, Dutscher, France). Total RNA extraction was done according to RNeasy Plus Universal Mini Kit protocol (Quiagen). Quality and quantity of the extracts were checked using electrophoresis migration and spectrometric dosing. Subsequently, cDNA was synthesized from 2 µg of total RNA, using 2 µl of Superscript III Reverse Transcriptase (Invitrogen) according to its associated protocol in a final volume of 21 µl. All cDNA were diluted 5 times in Milli-Q water prior to analysis.

Table S1. Sequences of primers used for qPCR experiment with associated amplicon size and efficiency (mean ± SEM).

Gene	Forward primer (5'→ 3')	Reverse primer (5'→ 3')	Amplicon size	Efficiency %
dnmt1	GAAGAGGAGGTGTCCCAACC	GGGTCATCAGGGCTTACAGA	252	96.7 ± 1.6
dnmt3aa	GGAGCGAGACATCGAAGAAG	TTCTCTCTGCGGCTGATATG	168	98.8 ± 0.8
dnmt3ab	CCTGAATCGGAGCCCTATG	AGCCATCGTCATCGTATTGA	310	94.5 ± 1.8
dnmt3ba	GACAGAGCCAGTTCCTGTGA	GCCTCCCTCCATTTATTTCC	251	99.1 ± 0.7
c-fos	TCTCCTCTGTGGCGCCCT	GTCTGGAACCGAGCGAGCCG	602	97.3 ± 1.5
actb1	CCCAGACATCAGGGAGTGAT	CACAATACCGTGCTCAATGG	123	97.8 ± 1.0
b2m	GGAAAGTCTCCACTCCGAAAG	GTGAAGGCAACGCTCTTTGT	213	99.8 ± 0.2
eef1	CAGCTGATCGTTGGAGTCAA	TGTATGCGCTGACTTCCTTG	94	97 ± 1.1
gapdh	GTGGAGTCTACTGGTGTCTTC	GTGCAGGAGGCATTGCTTACA	174	94.2 ± 3.0
g6pd	GTCCCGAAAGGCTCCACTC	CCTCCGCTTTCCTCTC	124	97.5 ± 2.5
rpl13a	GGACTGTAAGAGGTATGCTTCCA	ACTTCCAGCCAACTTCATGG	200	99.3 ± 0.3

Table S2. Body mass (mean ± SEM; mg) for water bathed Control fish (F0: n=23; F1: n=20; F2: n=19; F3: n=22) and MIX fish (F0: n=23; F1: n=20; F2: n=19; F3: n=24) and for nicotine bathed Control fish (n=19 for all generations) and MIX fish (F0: n=20; F1: n=18; F2: n=19; F3: n=19) challenged in novel tank diving test. Related statistical analyses are given in the main text.

Bathing	Treatment	F0	F1	F2	F3
Water	Control	410±20	369±16	492±40	537±35
Water	MIX	383±16	410±27	486±22	615±39
Nicotine	Control	447±25	377±23	576±51	744±314
Nicotine	MIX	482±109	410±38	486±36	518±32

Table S3. Time spent in top area (mean ± SEM; s) during the novel tank diving test for each generation for water bathed Control fish (F0: n=23; F1: n=20; F2: n=19; F3: n=22) and MIX fish (F0: n=23; F1: n=20; F2: n=19; F3: n=24) and for nicotine bathed Control fish (n=19 for all generations) and MIX fish (F0: n=20; F1: n=18; F2: n=19; F3: n=19). Related statistical analyses are given in the main text.

Bathing	Treatment	F0	F1	F2	F3
Water	Control	82.3±7.0	92.6±4.6	106.7±9.0	77.4±9.0
Water	MIX	87.2±5.0	76.1±5.4	69.5±8.3	67.6±7.7
Nicotine	Control	139.6±10.7	167.8±13.5	155.9±10.5	144.4±19
Nicotine	MIX	162.1±10.4	130.6±20.5	179.5±12.6	122.7±16.2

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Chapitre 4

Chronic dietary exposure to an environmental mixture of Persistent Organic Pollutants (PCB, PBDE) alters boldness and anxiety syndromes over generations in zebrafish (*Danio rerio*)

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Manuscript en préparation

Chapitre 4 – Présentation

Contexte:

Les polychlorobiphenyles (PCB) et les polybromodiphenyles ethers (PBDE) sont des polluants organiques persistants (POP) retrouvés dans les écosystèmes aquatiques. Les effets des polluants sur le comportement, notamment les traits de personnalité, sont souvent négligés alors que certaines études pointent pourtant des altérations notables après exposition à divers types de polluants. Les traits de personnalités, permettant de caractériser l'individu, sont pourtant très importants et sont impliqués dans les comportements de dispersion, la recherche de nourriture ou de partenaire.
Il apparait donc important d'étudier les effets d'une exposition aux POP, à dose environnementale, sur les traits de personnalité afin de mieux appréhender les conséquences que cela peut avoir sur les populations en milieu naturel.

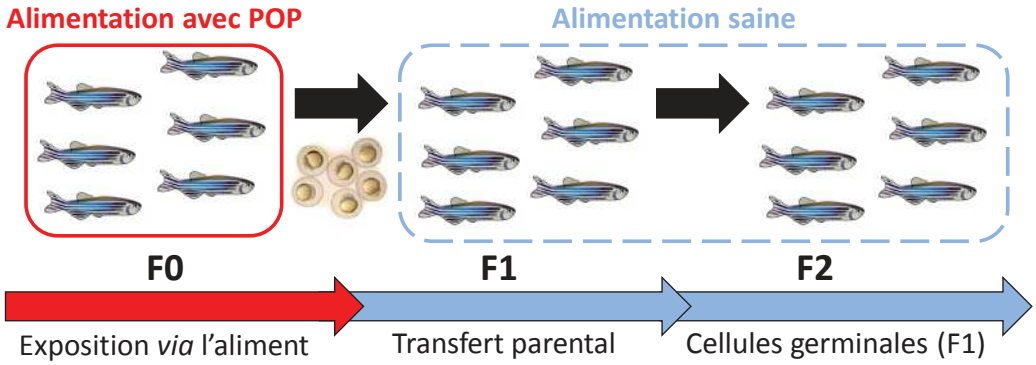
Questions:

(1) Quel est l'impact d'une exposition chronique à un mélange de POP, représentatif des conditions environnementales, sur l'expression de la personnalité et de l'anxiété ?
(2) Quels sont les mécanismes physiologiques pouvant expliquer ces possibles altérations ?

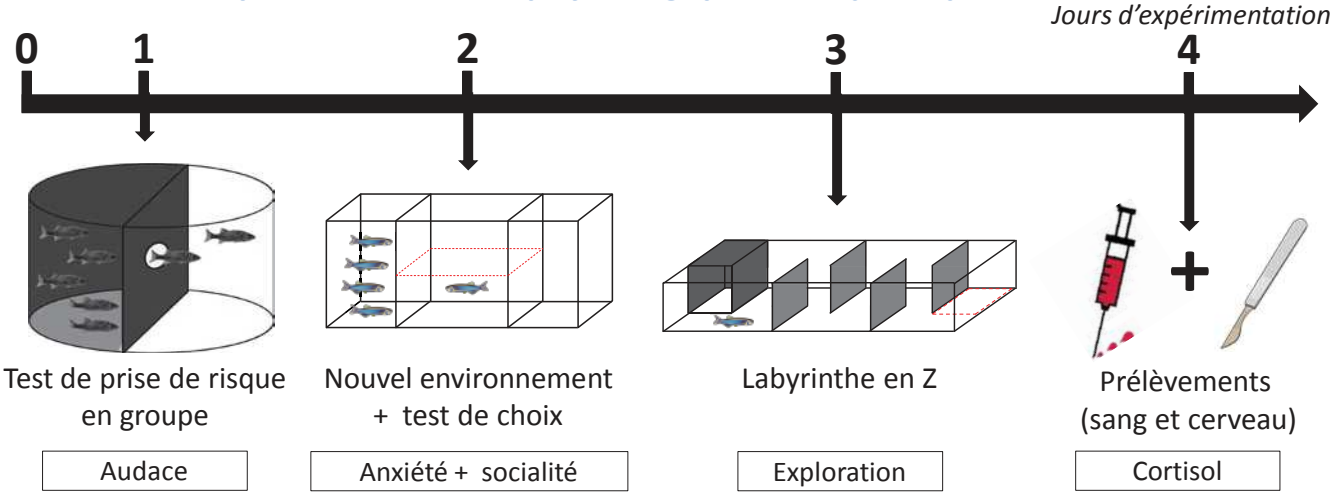
Méthode:

Le protocole d'exposition aux PCB et PBDE est le même que dans le chapitre 3. L'**audace** a été mesurée dans un test de prise de risque en groupe. Ensuite, le niveau d'**anxiété**, de **socialité** et les **capacités d'exploration** ont été mesurés respectivement dans un nouvel environnement, un test de choix et un labyrinthe en Z sur un sous échantillon de poisson. Le **lien entre les différents traits** de **personnalité** et l'**anxiété** a été évalué. Pour finir, le taux de **cortisol** sanguin a été mesuré. *Une analyse transcriptomique est actuellement en cours sur les cerveaux de ces animaux.*

- **Exposition:**



- **Mesures comportementales et physiologiques: 140 *jours* post fécondation**



Chronic dietary exposure to an environmental mixture of Persistent Organic Pollutants (PCB, PBDE) alters boldness and anxiety syndromes over generations in zebrafish (*Danio rerio*)

In preparation

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Abstract

Polychlorinated Biphenyls (PCBs) and Polybrominated Diphenyl Ethers (PBDEs) are Persistent Organic Pollutants (POPs) extensively used during the 20th century, especially as additives, stabilizers or flame retardants. Despite their total or partial ban (production and/or use), these pollutants are still present in all environmental compartments because of their chemical properties and low or no metabolization. Little is however known about the effects of an environmentally relevant mixture of PCBs and PBDEs on behavioural and physiological disruptions in teleost. Starting from 5 days post fertilization, zebrafish were chronically exposed *via* diet to an environmentally relevant mixture (MIX) containing 22 PCB congeners and 7 PBDE congeners in proportion and concentrations close to environmental situations (ΣPCBs= 1974.5 ng g^{-1} and ΣPBDEs= 488.9 ng g^{-1} wet weight in diet). MIX-exposed F0 fish produced offspring (F1 and F2 generation) which were fed using plain diet and grown into adulthood. In each generation, coping style was determined by the study of four behavioural personality axes, *i.e.,* boldness, activity, sociality and exploration; anxiety was evaluated as well as HPI axis responsiveness (cortisol). Boldness was positively correlated to activity and exploration whereas anxiety level was related to lower sociality. F0 fish did not display any behavioural syndrome disruption resulting from POP exposure whereas F1 MIX fish were bolder than F1 control, and F2 MIX displayed higher anxiety syndrome than F2 control. Cortisol response did not differ in the different treatments and generations tested. Behavioural disruptions in boldness and anxiety in offspring generation may have ecological consequences affecting fitness and hence detrimental effects on fish natural population exposed to pollutants mixtures.

1. Introduction

Several thousands of chemical products emerged in the 20th century. Their wide use resulted in the increase in their emissions in the environment. Progressively, the toxicity of these released chemicals was highlighted. Among all of them, Persistent Organic Pollutants (POPs) gather molecules that are hydrophobic, lipophilic, little or not metabolized by organisms and have a long half-life in the environment (Gray, 2002; Jones and de Voogt, 1999; Luo et al., 2007). As a consequence, they can accumulate in some environmental compartments and persist over long periods of time (Jones and de Voogt, 1999). POPs include pesticides (such as chlorinated ones as DTT) and many industrial chemicals such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs).

Polychlorinated biphenyls (PCBs) are composed of a biphenyl nucleus with chlorine at one, or all, of ten available sites (McFarland and Clarke, 1989). Two hundred and nine PCBs congeners exist depending on the number and position of chlorine atoms on the biphenyl nucleus. Because of their chemical properties, these molecules were used in several industrial domains as technical mixtures (*e.g.* Aroclor, Kanechlor or Clophen; Kannan (2000)). PCBs mixtures were used in closed systems such as hydraulic fluids, transformers and capacitors and in open systems as bactericides, insecticide additives, stabilizers or flame retardants in paints, inks and plastics (Breivik et al., 2002). As concerns about their toxicities progressively increased, regulatory rules were adopted to limit their use and they were progressively banned since the 70s.

Polybrominated diphenyl ethers (PBDEs) are composed of a diphenyl ether nucleus which can contain from 1 to 10 bromine atoms (Rahman et al., 2001). As for PCBs, 209 PBDEs congeners exist as a function of number and position of bromine atoms. Their chemicals properties, made them used as flame retardants in a large range of industrial products but also in everyday products such as electronic equipment, building materials, textile coating of vehicles or wood treatment (Darnerud et al., 2001; de Wit, 2002; Rahman et al., 2001). Technical PBDEs are manufactured as mixtures called penta-, octa- and decabromodiphenyl ethers (Penta-, Octa- and DecaBDE) according to the average number of bromines. The use of PBDEs was regulated due to high concentrations found in the environment and their related health risks. In 2003, the European Council banned the production and use of the technical mixtures PentaBDE and OctaBDE (at higher concentrations than 0.1 %; Directive 2003/11/EC). Further, some PBDEs were forbidden in 2006 in Europe. However, it is

important to note that not all countries worldwide have banned the production and use of these compounds yet.

Atmospheric fallouts related to prior incineration of these compounds as well as non-atmospheric routes such as soil leaching, and sewage discharge are the main causes of environmental contamination, including that of aquatic ecosystems (Darnerud et al., 2001; de Wit, 2002). Due to these pollutants persistence, they are still detected in all environmental compartments around the world and associated ecotoxicology issues persist (de Wit, 2002). As a consequence, many studies have been reporting PCBs and PBDEs contamination for almost 50 years in several aquatic and terrestrial organisms from polluted environment (Andersson and Blomkvist, 1981; Jensen et al., 1969; Luo et al., 2007; Robinson et al., 2017).

Presence of POPs in the environment causes contamination of organisms *via* dietary uptake which is considered as the most important exposure pathway (Darnerud et al., 2001; Pompa et al., 2003; Sjodin et al., 2003). Chronic or acute exposure to PCBs and PBDEs may have adverse effects on organisms. Indeed, these organic pollutants can act as endocrine disruptors, able to affect synthesis, transport and excretion of hormones (Legler and Brouwer, 2003; Safe et al., 2001; Yang et al., 2005) and to disrupt neuronal development (He et al., 2011; Kimura-Kuroda et al., 2007). Moreover, it was shown that PCBs and PBDEs can be transferred from one generation to another (Alfonso et al., Chapitre 3; Daouk et al., 2011; Nyholm et al., 2008; Yu et al., 2011), resulting in neural and behavioural disturbances in offspring (Chen et al., 2012; He et al., 2011; Péan et al., 2013) and even cause multigenerational and transgenerational effects (Alfonso et al., Chapitre 3). For instance, Péan et al. (2013) showed that locomotor activity of F1 larvae issued from fish exposed to a mixture of PCBs was higher during a light/dark challenge. Alfonso et al. (Chapitre 3) showed that behavioural and molecular responses were disrupted following exposure to a mixture of POPs, including PCBs and PBDEs, in F1 to F4 offspring unexposed generations. So far some studies were interested in behavioural disruptions in juveniles or adults after POP or organic pollutants exposures but most often to single compound at rather high concentration (*e.g.* Eddins et al., 2010; Klüver et al., 2015; Truong et al., 2014) and rarely using mixture at environmentally relevant concentrations. Even fewer studies were interested about how chemicals may affect teleost personality traits (Montiglio and Royauté, 2014) and their resulting coping style, an emerging research field in behavioural ecology presented hereafter.

Réale et al. (2007) suggested that animal personality could be defined through 5 *continuum* which are boldness, activity, sociality, exploration and aggressiveness. A coping style (also termed behavioural syndrome and referring to animal personality traits) is defined as a correlated set of individual behavioural and physiological characteristics that is consistent over time and across situations (Coppens et al., 2010; Réale et al., 2010). Coping style is also described as a *continuum* between two extreme phenotypes which are called Proactive and Reactive. Proactive fish are generally bolder and more aggressive (Castanheira et al., 2017; Øverli et al., 2006) and more likely to explore new environment than reactive ones (MacKenzie et al., 2009; Øverli et al., 2006). As part of the coping style theory, individual behaviour is correlated with physiological responses (Réale et al., 2010). For instance, risk taking (boldness) and activity were correlated with individual metabolic rate as was demonstrated in fish (Herrera et al., 2014; Killen et al., 2012) or in other animal groups (Careau et al., 2008). Behavioural coping style has also been linked to different hypothalamus-pituitary-interrenal (HPI) axis responses (Øverli et al., 2007; Tudorache et al., 2013, 2015) with high cortisol value most often characterizing reactive fish, different gene expression (MacKenzie et al., 2009; Rey et al., 2013) or different immune capacities (MacKenzie et al., 2009).

Even if it is poorly documented for POPs, exposures to such pollutants may trigger adverse effects on personality traits or coping-style (see **Table 1** in review Montiglio and Royauté (2014)). More recently, Royauté et al. (2015) have demonstrated that jumping spider (*Eris militaris*) showed a strong departure from their personality tendencies following an insecticide exposure. It was also demonstrated that fluoxetine exposure can reduce boldness, activity and exploration behaviour as well as behavioural consistency in female Siamese fighting fish (*Betta splendens,* Dzieweczynski et al. (2016)). Other studies have shown the oppos ite with increased boldness (escitalopram in zebrafish, Nielsen et al. (2018)) or other traits (activity, exploration, sociability) modifications (17β-trenbolone in eastern mosquitofish, Betram et al., 2018).

Behavioural responses and in particular anxiety-like behaviour (*e.g.* bottom dwelling related to predator threat) are often measured as standalone in ecotoxicological studies, and have been for a long time (Marcucella and Abramson, 1978) because they reveal various neurological or developmental defects attributed to the tested compounds (Kalueff et al., 2016; Sackerman et al., 2010). It is however rarely directly addressed in coping style studies although some authors mention that reactive fish might be more fearful and anxious (Martins

et al., 2011; Øverli et al., 2007; Thornqvist et al., 2018). One direct method to evaluate anxiety level in fish is the novel tank diving test which allow monitoring fish vertical positioning over an observation period and this test has been largely validated (Egan et al., 2009; Levin et al., 2007).

The aim of this study was to investigate how coping style and anxiety of zebrafish (*Danio rerio*, F0) was affected after being exposed to an environmentally relevant mixture of POPs (PCBs and PBDEs) and how their offspring (F1 and F2), fed unexposed diet, were affected. Fish were screened for different personality traits using first a risk-taking test as a measure of boldness. Anxiety was evaluated using a novel tank diving test followed by a shoal preference test to measure sociality, and finally fish were tested for exploration and activity using a Z-maze. In addition, HPI axis responsiveness was evaluated by measuring blood cortisol.

2. Material and methods

The study was conducted under the approval of the Animal Care Committee of Poitou-Charentes # 84 COMETHEA (France) under the project authorization number CE2012-23 and followed the recommendations of Directive 2010/63/EU.

Succession of exposure, generations and analyses performed is summarized in **Figure 1**. Here we exposed a first generation (F0) to a PCB and PBDE mixture (MIX) and reared two offspring generations (F1 and F2) for the purpose of the study. Additional information concerning material and methods are provided hereafter and in Alfonso et al. (Chapitre 3).

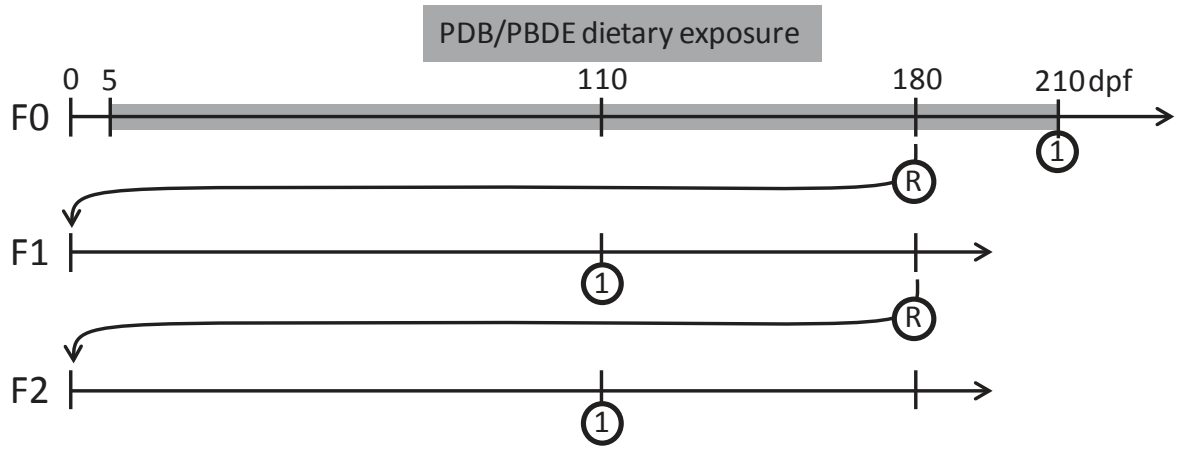

Figure 1. Experimental procedures performed in this study over time and generations. ① Personality traits measurement and novel tank diving test; ® Reproduction to produce next generation. dpf: days post fertilization.

2.1. Fish reproduction and rearing

The study was conducted on zebrafish using wild type strain TU (ZFIN ID: ZDB-GENO-990623-3) kept at the Fish Ecophysiology Platform (PEP - http://wwz.ifremer.fr/pep_eng) and originated from the European Zebrafish Resources Center (EZRC, Karlsruhe, Germany).

Fish were reared at 27±1°C under a 14h/10h light/dark cycle, with light onset at 9 am. Physical water conditions were maintained constant during the experiment and were within recommended ranges (Lawrence, 2007). In all cases (treatment or generation), eggs were obtained at around 180 dpf by random pairwise mating using adults (6 pairs, mixed spawns of 10 eggs from each spawn to reach 60 eggs per Petri dish x 5 replicates). At least 5 replicates of 30 individuals (at 30 dpf) were reared per treatment. This procedure was used to produce F0 for exposure and then F1 and F2 offspring generations.

2.2. MIX diet and F0 exposure

The MIX diet was spiked with 22 PCB congeners (CB-8, -18, -28, -31, -44, -49, -52, -77, -101, -105, -110, -118, -128, -132, -138, -149, -153, -156, - 170, -180, -187 and -194) and 7 PBDE congeners (BDE-28, -47, -99, -100, -153, -183 and -209) all with purity above 98% (AccuStandard Inc., New Haven, USA). Congeners were selected according to their abundance in environment and to span a wide range of hydrophobicity (log K_{ow}). Relative proportions of congeners were chosen to simulate environmentally relevant mixtures (Abarnou et al., 2000; Ospar, 2009, 2013). Chlorine (for PCBs) and bromine (for PBDEs) substitution numbers and detailed dietary concentrations of each congener used for diet spiking are given in **Table 1** in Alfonso et al. (Chapitre 3). Briefly, MIX diets were prepared after dilution of a stock PCB and PBDE solution in isooctane solvent for incorporation as described in Daouk et al. (2011). Control diet was prepared in the same manner, *i.e.* with addition of isooctane only. F0 fish were exposed using food of appropriate size following the same schedule as described in Horri et al. (2018). Fish were fed from their first meal (5 dpf) with either a Control diet or a MIX diet (later called respectively Control or MIX treatments) until final sampling (210 dpf). F1, and F2 generations were fed using commercial plain food.

PCB and PBDE concentrations were determined in Control and MIX diets, in whole F0 fish (at 180 dpf) and in F1 obtained from F0 fish after 180 days of exposure as eggs (batches of 200 eggs), larvae (5 dpf, batches of 200 larvae) and adults (whole individuals at 112 dpf). These analyses were performed as described in Horri et al. (2018).

2.3. Behavioural experiments and HPI responsiveness

For all behavioural experiments, videos were recorded with an analogue camera ICD-48E (Ikegami) and a 2.1-13.5 lens (Fujinon) linked to a computer with an acquisition card and EthoVision XT 10.1 software (Noldus, The Netherlands). Data extraction and analyses were performed using EthoVision XT 10.1 software.

Behavioural experiments were performed in a dedicated challenge room at 27±1°C, with a photoperiod synchronized with the rearing room. The four following screening experiments were conducted in all generations (F0, F1 and F2) at 210 dpf in generation F0 and at 110 dpf in F1 and F2 (**Figure 1**). For each generation, 40 fish per treatment were challenged in groups of 10 in a risk taking test which was performed on day 1. Thereafter, behavioural experiments including novel tank diving test, shoal preference test and Z-maze exploration test were performed on a subset of 20 fish per treatment (Control or MIX) characterized for boldness on day 1. Novel tank and shoal preference tests were performed on day 2 and Z-maze exploration test was performed on day 3. Fish were kept in isolation overnight in 1L tank in visual contact with each other. During behaviour experiments, fish were fed once a day with freshly hatched artemia in late afternoon at the end of the testing day. On day 4, fish were measured, weighted, sexed and blood and brain were sampled to measure cortisol and realize transcriptomic analyses respectively.

Day 1: boldness screening using group risk taking test (RT)

To assess boldness in our fish population we used a risk taking test in group adapted from Rey et al. (2013). In the morning before risk taking test, 4 groups of 10 fish from 4 different tanks (4 replicates) were placed into 3L tanks and transferred from the rearing room to the challenge room. In the beginning of the afternoon, each group of 10 fish was placed for a 10-min habituation in a shelter with dark wall and a sliding door (**Figure 2.A**, 20 cm in height, 20 cm in width and 14 cm in length). After this habituation period, the sliding door was gently removed allowing fish to exit the shelter during a screening period of 15-min via a 3 cm diameter hole located in the centre of an opaque partitioning plate and to explore the appended open area (**Figure 2.A**, 20 cm in height, 20 cm in width and 26 cm in length). When fish exited the shelter, they were one by one gently caught with a net and placed into a numbered 1L tank, keeping track of the rank and timing of exit (*Latency to exit shelter*). Those fish were considered as bold. Fish that still remained inside the shelter after this screening period were considered as shy. A total of 20 fish (22 for F1) per treatment for each

generation (50 % bold and 50% shy) were challenged in novel tank diving test, shoal preference test and exploration test detailed below.

Proportion of bold fish was calculated for each treatment within each generation allowing comparing boldness between treatments. *Latency to exit shelter (s)* was the variable of interest to characterize individual boldness level (continuous variable).

In order to be able to evaluate consistency of different behavioural traits across contexts between treatments (Control *vs.* MIX) within generations and independently of possible differences in boldness, we decided to select a subset of fish of similar mean boldness. For this purpose, all fish that left the shelter were kept separately in numbered 1L tanks until the end of the screening experiment. Then we selected fish to achieve a similar mean of the variable *Latency to exit* between treatment and within generation.

Day 2: anxiety (novel tank diving test) and sociality evaluation (shoal preference test)

Novel tank (NT) and shoal preference (SP) tests were run in the same multi-compartment apparatus (**Figure 2.B**, 12 cm in height, 12 cm in width and 36 cm in length). First, each individual fish was placed in the novel tank compartment (12 cm in height, 12 cm in width and 24 cm in length) and video tracked for 7 min. Then a sliding opaque door was gently opened to allow visual contact with the shoal compartment (12 cm in height, 12 cm in width and 12 cm in length) containing 3 males and 3 females of unfamiliar match-sized congeners; focal fish position was recorded during 5 min.

In this manner, the habituation period before the shoal preference test served as novel tank diving test and vertical positioning of the fish over a 6 minutes observation period was recorded. For analysis, the tank was separated into two areas according to Egan et al. (2009): top area including one half of the volume and bottom area including the other half. *Time spent in top area (s) and Distance travelled (cm)* were the variables of interest for evaluating anxiety and measuring activity level respectively.

For shoal preference test analysis, the tank was separated into three areas: near shoal area including one third of the volume near the shoal, far shoal area including one third of the volume far from the shoal and centre area including the last third of the volume. *Time spent in near shoal area (s)* and *Latency to go in near shoal area (s)* were the variables of interest used as proxy of sociality and boldness respectively.

Day3: exploration (Z-maze; ZM)

In addition, to evaluate the ability of fish to explore their environment, a Z-maze adapted from Chapman et al. (2010) was used. The Z-maze (70 cm in length, 45 cm in width and 15 cm in height) consisted of a starting area which served as a shelter during the test (3/4 of an arm) and four other arms (**Figure 2.C**). Each arm was divided virtually into four quarters of equal size, referred to as quadrants. The maze thus consisted of 17 quadrants (one adjacent to refuge zone and 16 for the 4 other arms. Fish were introduced into the starting area (equivalent to three quadrants, covered with a black slab and ending in a sliding door) and left for two minutes in the starting area; hereafter the door was gently opened. Fish activity was recorded in the 5 min following the first excursion of the fish from the refuge zone (detection in the first quadrant next to the refuge). The variables measured were the time taken to leave the starting area and thus enter in Z-maze (*Latency to enter in Z-maze*, s). We also determined the number of quadrants into which the fish ventured within the maze (17 indicating that the fish reached the end of the maze) and an exploration index was calculated as follow:
Exploration index = Farest quadrant explored / Time to reach farest quadrant starting after exit from the starting area (shelter)
Individuals with high *Exploration index* explored faster their environment than individuals with low value. *Time spent in shelter* (after first exit, s) was also recorded.

Day 4: biometry and blood sampling

On day 4, at the same time of the day for each generation, fish were euthanized by placing them during 1 minute into a 1 L container filled with 200 mL of system water with 700 µl of a benzocaine stock solution (50 $g.L^{-1}$ in 100 % ethanol; Benzocaine Sigma, Saint-Louis, USA). Individual standard length (from tip of head to beginning of caudal fin rays, mm) and body mass (mg) were measured and sex was determined. Blood was immediately sampled with EDTA coated micropipette in the cardiac area, 2 µL of blood were collected and frozen at -20°C for later cortisol analysis. Whole blood cortisol concentration was later determined by ELISA (17beta-Estradiol Saliva ELISA RE52601, IBL International, Hamburg, Germany) using a Synergy-HT (BioTek Instruments, Winooski, VT, USA). Finally, whole brain were dissected out and preserved at -80°C for further transcriptomic analyses (RNAseq data interpretation on-going at the time of writing).

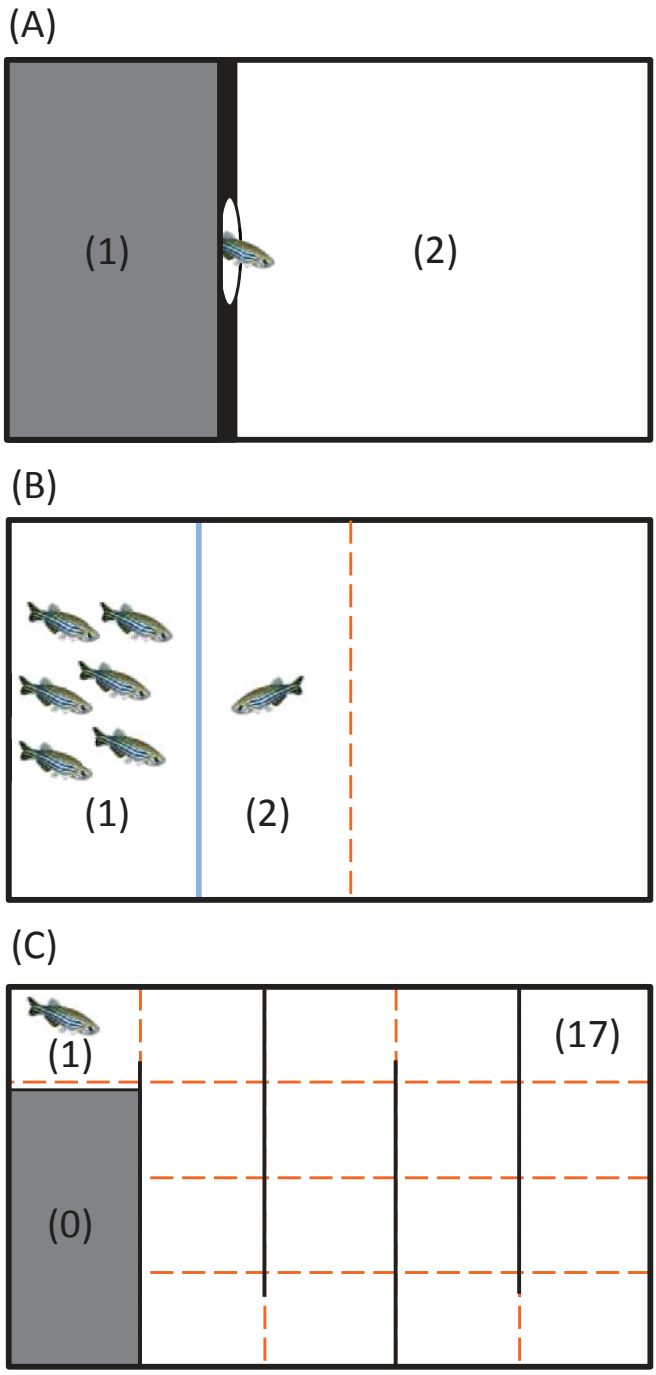

Figure 2. (A) Risk taking test apparatus composed of a shelter area (1) and an open area (2); **(B)** Novel tank and shoal preference tests multi-compartment apparatus: a closed compartment (1/3 of total volume) accommodates 6 fish (1) isolated by a transparent window and a sliding opaque door from the other 2/3 of the tank which contains the individual focal fish (2). The latter compartment (2) serves first as novel tank test to evaluate fish anxiety during a 7 min habituation period before the opaque sliding door is open to run the shoal preference test during 5 min. Area comprised between transparent window and dotted red line represents *near shoal area*; **(C)** Z-maze apparatus used to evaluate fish exploration which contains a starting area which also served as shelter (0). Quadrant (1) represents the first quadrant and quadrant (17) the last one used to calculate the exploration index. See text for apparatus dimensions.

2.4. Statistical analysis

Individuals with acquisition default and/or outliers values (freezing behaviour) were removed before statistical analyses. Around 1.5 individuals per generation were removed (min=0, max=2, 9 % of the tested fish in total). Statistical analyses were performed using R 3.1.0 software (R Core Team, 2015). All statistical analyses were carried out at 95 % level of significance. Values are represented as mean ± SEM. Normality and homoscedasticity were tested a priori using Shapiro-Wilk test and where sample sizes were too small, non-parametric statistics were used.

Fish weight and length were compared between generations and treatments with a factorial ANOVA followed by *post-hoc* Tukey HSD.

Chi-square test was performed to compare the *Proportion of bold fish* during risk taking test between treatments and within each generation. Then, still within each generation, a Wilcoxon test was done to compare between treatments (Control *vs.* MIX) the *Latency to exit shelter* (only for fish that left the shelter).

A principal component analysis (PCA) was done with individual data from all generations using ade4 package (Dray and Dufour, 2007) on 7 variables which were: *Time spent in top area* (s) and *Distance travelled* (cm; novel tank experiment); *Time spent in near shoal area* (s) and *Latency to go in near shoal area* (s; shoal preference test); *Latency to enter in Z-maze* (s), *Exploration index* and *Time spent in shelter* (s) (Z-maze test). Individual PCA scores were downloaded for the first two axes: *PCA scores axis 1* and *PCA scores axis 2;* and then a modelling approach presented below was performed on the two new variables.

Linear models were performed to explore the links between *PCA scores axis 1* or *PCA scores axis 2 (i.e.* behavioural responses during the three experiments in Novel tank, Shoal preference and Z-maze test) and *Generations* (F0, F1 and F2), *Latency to exit shelter* in Risk taking test, *Treatments* (Control *vs.* MIX), fish sex and length. ANOVA were performed to compare models one by one and discriminate the best model explaining the data (*PCA scores axis 1* and *PCA scores axis 2*). Best models for each PCA score axis are presented below:

Model 1: *PCA scores axis 1~ Generation + Latency to exit shelter (RT)*

Model 2: *PCA scores axis 2 ~ Generation + Treatment + Generation:Treatment*.

Model 1 reached best significance when *Latency to exit shelter* was included only with bold fish (*i.e.* fish that exited shelter during risk taking test), as a consequence Model 1 only concerns bold fish. Model 2 was performed with all fish (bold and shy) since *Latency to exit shelter* was not significant in the model. Homogeneity of the two model residuals was *a*

posteriori checked using Shapiro-Wilk test. For the second model *post-hoc* Tukey HSD test was performed to compare the *PCA scores axis 2* between generations and treatments.
Finally, because no effects of coping styles (bold *vs.* shy) were seen on blood cortisol concentration (Factorial ANOVA, F=0.15, p=0.69), blood cortisol concentration was compared only between generations and treatments with a factorial ANOVA.

3. Results

Fish weight and length were different between generations (Factorial ANOVA, F=172.6, df=2, p <0.001 for weight and F=104.5, df=2, p <0.001 for length) but not between treatments within generation (F=1.5, df=1, p =0.22 for weight and F=0.21, df=1, p =0.65 for length). F0 fish were heavier and longer than the two other generations (F0: 446±14 mg; F1: 233±10 mg; F2: 239±20 mg; **Supp. Figure 1**; F0: 28.0±0.3 mm; F1: 22.7±0.2 mm; F2: 22.4±0.4 mm).

3.1. PCB and PBDE concentrations from F0 to F1

PCB and PBDE concentrations in MIX and Control diets were presented by Horri et al. (2018)). Briefly, in the MIX diet (n=12), the total concentrations, *i.e.* summed across 22 PCB and 7 PBDE congeners, were 1932.3±90.4 ng/g (mean ± SE) ww for∑PCBs and 479.8±50.8 ng/g ww for∑PBDEs. In the Control diet (n=16), the total concentrations were 245 and 522 times lower, *i.e.* 7.9±3.5 ng/g ww for∑PCBs and 0.92±0.36 ng/g ww for ∑PBDEs. In addition, PCB and PBDE profiles in Control diet were completely different from the one in MIX diet (**Table 1**, in Alfonso et al., Chapitre 3).

PCBs and PBDEs concentrations were monitored at 180 dpf in F0 whole individuals exposed to Control and MIX diets. Since there was no difference between sex (p>0.05; data not shown) they were analysed together. In MIX F0 fish, the total concentrations of PCBs (n=6) and PBDEs (n=6) were 2162.5±311.0 and 103.6±12.3 ng/g ww respectively. In Control F0 fish, the total concentrations of PCBs (n=2) and PBDEs (n=6) were 26.5±0.4 and 1.8±0.9 ng/g ww.

In F1 generations∑PCBs concentration was 91.3±18.0 ng/g ww and ∑PBDEs concentration was 7.2±0.5 ng/g ww in MIX eggs (n=3 batches), which was 20 and 98 fold higher than in control eggs (ΣPCBs=4.5 ng/g ww, n=2 batches and ΣPBDEs=0.074 ng/g ww, n=1 batch). At 5 dpf, total concentrations of PCBs and PBDEs were 332.5±135.8 ng/g ww and 28.0±5.8 ng/g ww respectively in MIX larvae (n=3 batches). In Control larvae, levels were respectively 90 and 20 fold lower than in MIX larvae, with ΣPCBs=3.7 ng/g ww (n=2) and ΣPBDEs below

the limits of detection (0.66 ng/g ww for BDE-209 and 0.11 ng/g ww for all other congeners). Then at 112 dpf, approximately when the novel tank test was performed, concentrations were similar among both treatments, ΣPCBs=18.2±5.5 ng/g ww in MIX fish (n=6) and ΣPCBs=17.2±2.5 ng/g ww in Control fish (n=3). The same applied for ΣPBDEs=0.8±0.1 ng/g ww in MIX fish (n=6) and ΣPBDEs=0.8±0.2 ng/g ww in Control fish (n=3). These values are in the range of those found in Control F0 fish.

3.2. Behavioural experiments and HPI axis responsiveness

Boldness screening

During risk taking test, approximately 37 % (from 22.5 to 52.5 across generations and treatments) of tested fish exited the shelter. *Proportions of fish* that left the shelter were similar between treatments within each generation (Chi² test, F0: χ^2=0.46, p=0.50; F1: χ^2=2.51, p=0.11 and F2: χ^2=0, p=1.00; **Supp. Figure 2**).

Among fish that exited the shelter, *Latency to exit shelter* did not differ between treatments in F0 and F2 generations (Wilcoxon rank sum test; respectively F0: W=114.5, p=0.22; F2: W=61, p=0.54; Figure 3A). In contrast, MIX F1 left the shelter earlier than Control F1, *i.e.* bold MIX were bolder than control (F1: W=214, p<0.01; **Figure 3**).

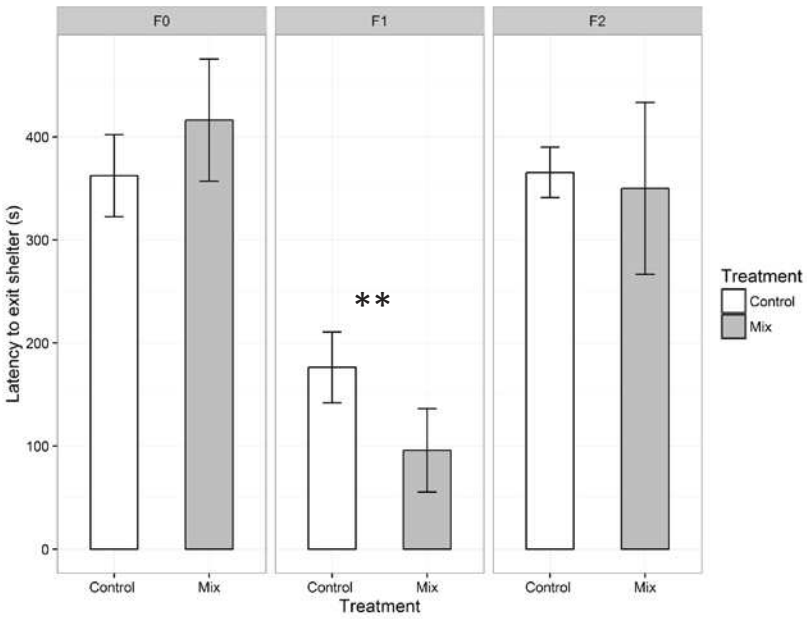

Figure 3. Mean ± SEM of *Latency to exit shelter* in risk-taking test between treatments for each generation (F0: n=19 Control/n=18 MIX; F1: n=21 Control/n=22 MIX; F2: n=17 Control/n=16 MIX); Wilcoxon rank sum test between treatments within each generation: **: p<0.01.

Behavioural syndrome

Among bold fish selected to evaluate behavioural syndrome in others experiments (*i.e.* novel tank diving test, shoal preference test and Z-maze), the *Latency to exit shelter* did not differ between treatments (Control *vs.* MIX) whatever generations (F0: W=141, p=0.34; F1: W=279.5 p=0.21 and F2: W=155.5, p=0.47; data not shown)

PCA axis 1, which explained 24 % of the variability observed, was mainly driven by *Distance travelled* (NT) – a proxy of activity, *Latency to enter in Z-maze* a proxy of boldness and *Exploration index* (ZM) which reflected individual exploration (**Table 1**). PCA axis 1 could thus be considered as individual coping style axis with *PCA scores axis 1* high values characterizing proactive individuals (high activity, high boldness and fast exploration) compared to low values which are typical of reactive individuals (low activity, shyness and slow exploration) according to Réale et al. (2010).

By contrast, PCA axis 2, which explained 21 % of the variability observed, was mainly driven by *Time spent in top area* (NT), *Time spent near shoal* area (SP) and *Time spent in shelter* (ZM). PCA axis 2 could thus be considered as reflecting the anxiety state of the individual fish. Individuals with *PCA scores axis 2* high values spent less *Time in top area*, less *Time near shoal* (SP) and spent more *Time in shelter* during Z-maze and could be considered as anxious fish compared to individuals characterized by low values for *PCA scores axis 2* (**Table 1**).

Table 1. Loadings of behavioural variables (*Time spent in top area* (NT), *Distance travelled* (NT), *Latency to go in near shoal* area (SP), *Time spent near shoal area* (SP), *Latency to enter in Z-maze* (ZM), *Time spent in shelter* (ZM) and *Exploration index* (ZM)) for the two PCA axes : values in bold are the main drivers of the related axis.

Variables	PCA axis 1	PCA axis 2
Time spent in top area (NT)	0.06	**-0.59**
Distance travelled (NT)	**0.85**	0.13
Latency to go in near shoal area (SP)	-0.12	0.08
Time spent near shoal area (SP)	0.02	**-0.76**
Latency to enter in Z-maze (ZM)	**-0.64**	0.38
Time spent in shelter (ZM)	0.17	**0.60**
Exploration index (ZM)	**0.68**	0.13

A linear model (Model 1) was performed to analyse behavioural syndrome across the three tested contexts (novel tank, shoal preference and Z-maze: *PCA scores axis 1*) as a function of *Generation* and *Latency to exit shelter* in Risk taking test (*i.e.* the model only integrate fish that left the shelter). *PCA scores axis 1* values (proxy of coping style) were explained by the factors *Generation* and *Latency to exit shelter* in risk taking test ($p<0.001$, $F=12.72$, $R^2=0.40$; **Table 2**).

Table 2. Outputs of the linear Model 1 Coping style (*PCA scores axis 1*) ~ *Generation* + *Latency to exit shelter* (in RT). Generation F1 and F2 are compared to F0. Significant parameters of the model are in bold.

Parameter	Estimate	Std Error	t value	p value
(Intercept)	2.406	0.385	6.244	**< 0.001**
Generation F1	-1.806	0.394	-4.584	**< 0.001**
Generation F2	-1.358	0.342	-3.967	**< 0.001**
Latency to exit shelter	-0.004	0.000	-5.196	**< 0.001**

Individuals that left the shelter quickly during risk taking test were also more active and explored their environment quicker than fish that left the shelter later whatever the treatment (**Figure 4**).

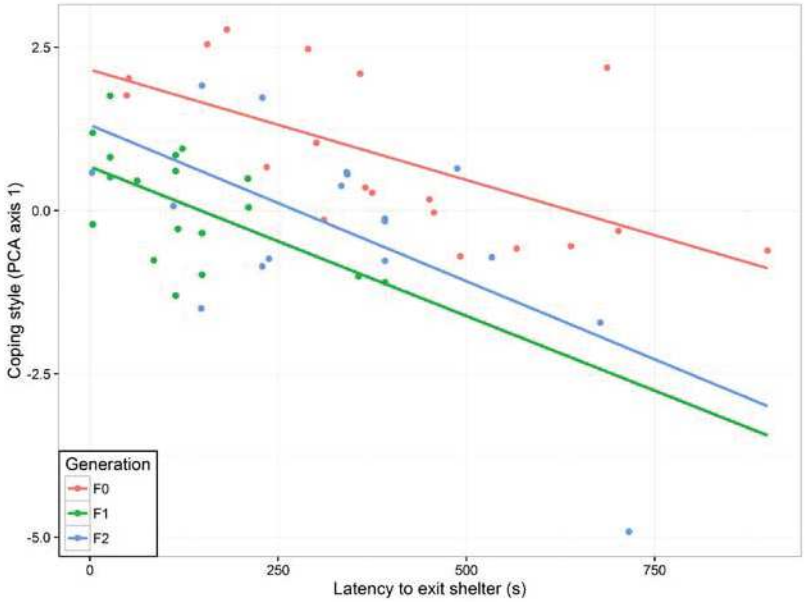

Figure 4. Linear model regression between *PCA scores axis 1*) as function of *Latency to exit shelter* in risk-taking test and generations ($R^2=0.40$).

A second linear model (Model 2) was performed to analyse behavioural syndrome across the three tested contexts (novel tank, shoal preference and Z-maze: *PCA scores axis 2*) as a function of *Generation* and *Treatment* and the interactions of the two factors. *PCA scores axis 2* values were explained by the factors *Generation* and *Treatment* and interaction between *Generation* and *Treatment* ($p<0.001$, $F=46.99$, $R^2=0.67$; **Table 3**). Post-hoc tests indicate that in F0 and F1 generations, MIX and Control fish displayed the same anxiety level ($p=0.98$ and $p=0.43$ for F0 and F1 respectively) whereas MIX F2 were more anxious than Control F2 ($p=0.002$; **Figure 5**).

Table 3. Outputs of the linear Model 2 Anxiety (*PCA scores axis 2*) ~ *Generation* + *Treatments* + *Generation:Treatment*. Generation F1 and F2 are compared to F0 and Treatment POP is compared to Control. Significant parameters of the model are in bold.

Parameter	Estimate	Std Error	t value	p value
(Intercept)	0.907	0.149	-6.089	**< 0.001**
Generation F1	-2.260	0.206	10.992	**< 0.001**
Generation F2	-0.892	0.217	4.117	**< 0.001**
Treatment MIX	-0.144	0.214	0.673	0.500
Generation F1:Treatment MIX	0.513	0.291	-1.761	0.080
Generation F2:Treatment MIX	1.075	0.314	-3.425	**< 0.001**

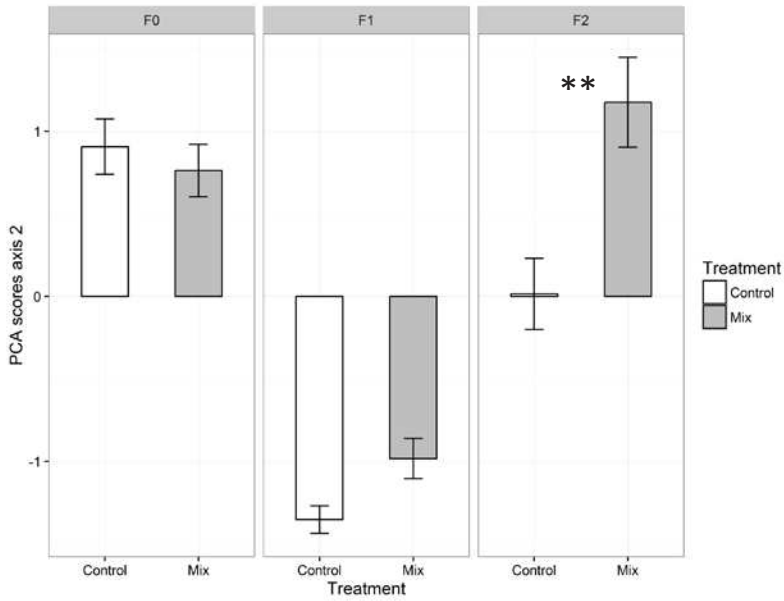

Figure 5. Mean ± SEM of PCA scores axis 2 for each followed generation (F0: n=19 Control/n=18 MIX; F1: n=21 Control/n=22 MIX; F2: n=17 Control/n=16 MIX). Comparisons between treatments within generations after *post-hoc* Tukey HSD:** : $p<0.001$.

HPI axis responsiveness

The figure showing blood cortisol concentrations according to coping styles and treatments is available in supplemental material **(Supp. Figure 3).** Blood cortisol concentration did not differ between generations (Factorial ANOVA, F=0.81, df=2, p=0.45), treatments (F=2.45, df=1, p=0.12) and there was no interaction between generations and treatments (F=0.63, df=2, p=0.53; **Figure 6**).

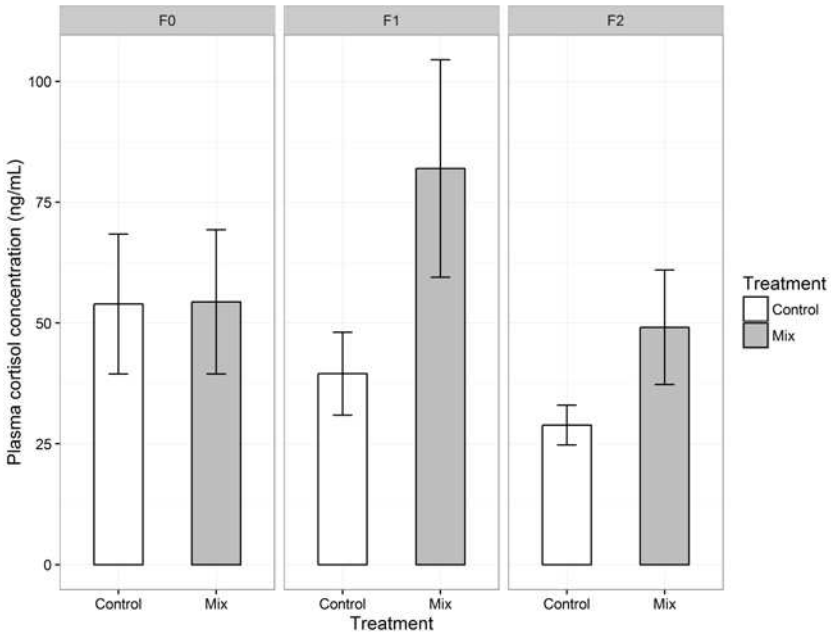

Figure 6. Mean ± SEM of blood cortisol concentration (ng/mL) in relation to generations and treatments (F0: n=19 Control/n=18 MIX; F1: n=21 Control/n=22 MIX; F2: n=17 Control/n=16 MIX).

4. Discussion

The aim of this study was to investigate how coping style and anxiety of zebrafish (*Danio rerio*, F0) were affected after exposure to an environmentally relevant mixture of POPs (PCBs and PBDEs) and how their offspring (F1 and F2), fed with unexposed diet, were affected. Thus, several challenges to characterise personality traits such as boldness, activity, sociality and exploration were performed and anxiety was also evaluated in all these generations at adult stage.

Pollutants in fish

First, as shown in Alfonso et al. (Chapitre 3), measurements of PCB and PBDE concentrations in Control fish showed no external or cross-contamination. Environmental

relevance of exposure is confirmed by the fact that, at the end of the experiment, PCB and PBDE levels in MIX F0 fish were in the range of those reported in fish from European industrialized estuaries (Law et al., 2006; Robinson et al., 2017). In F1 eggs, PCB and PBDE concentrations were respectively 30 and 108 times higher in MIX F1 than in Control F1 because of maternal transfer in oocytes (Daouk et al., 2011; Nyholm et al., 2008; Yu et al., 2011). At 5 dpf, the concentrations of both PCBs and PBDEs increased. These increased concentrations in larvae compared to eggs is likely due to the loss in weight after hatching because eggs where sampled with their chorion containing a relatively large amount of water (Foekema et al., 2012). At 112 dpf, levels of PCB and PBDE congeners in MIX F1 were low and similar to those found in Control F1 fish. This can be explained by dilution during body growth (Sijm et al., 1992). Even if there is no more PCB and PBDE in F1 MIX at 112 dpf, MIX may have exerted an effect on F2 generation. Indeed germ cells which will later produce gametes are already present in embryos. Therefore in the present case, since PCB and PBDE are detected in F1, germ cells at the root of gamete to produce F2 can be exposed.

Boldness and other personality traits: A first behavioural syndrome

The risk taking test in group is a common test to measure fish boldness level (Ferrari et al., 2015; MacKenzie et al., 2009; Rey et al., 2013). In the present experiment, proportion of bold fish was overall not different between treatments. In F1 generation however, MIX bold fish were bolder than Control bold fish whereas level of boldness was similar between treatments in F0 and F2 generations. This result echoes findings from other animal models showing that exposure to pollutants may disrupt several personality traits including boldness (Montiglio and Royauté, 2014). For instance, following an exposure to 17β-trenbolone, which is an hormonal growth promotant with androgenic endocrine disruptor activity, eastern mosquitofish displayed higher activity and faster exploration (Bertram et al., 2018). Waterborne exposure to citalopram (anxiolytic compound) increases the locomotor activity in three-spine stickleback (Kellner et al., 2016). Contrasting results were, however, observed in jumping spider where aggressive and voracious individuals from an insecticide-treated environment had lower boldness than counterparts from an insecticide-free environment (Royauté et al., 2015). Boldness was also shown to be reduced in female Siamese fighting fish exposed to fluoxetine (Dzieweczynski et al., 2016). The two last studies point to an opposite direction than ours but underlie the fact that the observed behavioural disruptions are likely linked to the pollutants properties.

Boldness is one of the most studied personality trait and is generally positively correlated with other personality traits such as activity, exploration and aggressiveness but negatively correlated to sociality according to Réale et al. (2010) which overall forms a behavioural syndrome (Sih et al., 2004). In the present study, we observed a behavioural syndrome between boldness and activity measured in novel tank diving test and exploration measured in Z-maze test. Such behavioural syndrome is consistent with literature on the topic (Biro et al., 2010; Geffroy et al., 2015; Wilson et al., 2010). Further, this behavioural syndrome was observable in all generations without any treatment effects but it was, however, different in overall levels across generations. Indeed, for the same level of boldness in risk taking test in group, F0 fish were more active and more explorative in the other challenges than F2 fish which themselves were more active and more explorative than F1 (**Table 4**). An explanation for such a difference could have been the age at which fish were screened. F0 were screened at 210 dpf whereas F1 and F2 were screened at 110 dpf. In earlier work, however, when the behavioural effects of PAH were tested, no significant differences were observed at 60 or 180 dpf (Vignet et al., 2014) leading us to set aside this explanation.

Table 4. Summary of all results obtained across generations and treatments for the different variables tested.

Variables	F0	F1	F2
Proportion of bold fish (RT)	Ctrl=MIX	Ctrl=MIX	Ctrl=MIX
*Latency to exit shelter** (RT)	Ctrl=MIX	**Bold MIX are bolder than Ctrl**	Ctrl=MIX
*PCA scores axis 1**	**Boldness syndrome: F0>F2>F1, no treatment effect**		
PCA scores axis 2	Ctrl=MIX	Ctrl = MIX	**MIX are more anxious**
	Anxiety syndrome: F0>F2>F1		
HPI axis responsiveness	Ctrl=MIX	Ctrl=MIX	Ctrl=MIX

*: for fish that left the shelter only

As explained before, POP exposure did not disrupt behavioural syndrome between boldness, activity and exploration but triggered an increase in boldness in MIX F1 generation compared to Control F1. Even if the concentration of pollutants disappeared during growth quickly in F1 fish, they were exposed to PCB and PBDE starting at embryo stage contrary to F0 which exposure started at 5 dpf. This could explain the fact that boldness was only altered in MIX F1 but not in F0 and F2. Increase in boldness, and the associated behaviour such as activity and exploration tendencies, may have ecological consequences (Conrad et al., 2011; Réale et al., 2007; Sih et al., 2004). One of the most discussed ecological consequences of boldness is

the predator-prey interactions. Indeed, bold fish tend to capture more prey being more active and explorative but this risky behaviour tends to increase the chances to be predated. This is consistent with the pace of life syndrome suggesting that bold individuals tend to have higher growth rate but shorter life than shy ones (Réale et al., 2010). Even if no behavioural disruptions were observed in MIX F0, previous study, using the same exposure protocol and environmental mixture, showed that MIX F0 had a higher growth rate but lower spawning probability than Control F0 (Horri et al., 2018). Interestingly, Debecker et al. (2016) have also shown that pesticide exposure resulted in a shorter life history according to pace of life syndrome (Réale et al., 2010) but no behavioural disruption was observed in *Ischnura* damselflies.

Anxiety: A second behavioural syndrome

We investigated the anxiety level of fish using the novel tank diving test which is a standardized test in ecotoxicological research (Bailey et al., 2013; Egan et al., 2009; Levin et al., 2007). In Alfonso et al. (Chapitre 3), we have previously shown that anxiety level measured only using the novel tank diving test was higher in MIX F2 compared to their respective controls. In the present study, we have shown that a high anxiety level of fish as measured in the novel tank diving test was also related to low sociality during shoal preference test and to a propensity to spend more time in the shelter during the Z-maze experiment. These data form another behavioural syndrome in relation to anxiety this time (**Table 4**). This anxiety behavioural syndrome was not correlated with the boldness one. In the present study, MIX F2 appeared to be more anxious than their respective control whereas MIX F0 and MIX F1 did not differ from their respective control.

An anxiety-like behaviour, was already described after exposure to POP congeners such as 6-OH-BDE-47 (Macaulay et al., 2015) or after direct embryonic exposure to CB-126 (Glazer et al., 2016). It is, however, generally with a single measure of anxiety (bottom dwelling behaviour) and not related to other behavioural traits such as exploration or sociality. In our case, sociality within the anxiety syndrome appeared to be reduced in F2 MIX compare to their respective control. A decrease of sociality behaviour was also previously observed in eastern mosquitofish after exposure to 17β-trenbolone (Bertram et al., 2018). Sociality is an important behaviour in gregarious species which allows the share of information and reduce the predation risk (Godin et al., 1988; Krause and Ruxton, 2002; Pitcher and Parrish, 1993).

Therefore lower sociality resulting from exposure to pollutants exposure can cause deleterious effects for gregarious fish populations.

By following many behavioural traits related to personality and anxiety together, we were able to be more precise concerning the characterization of behavioural disruptions resulting from POP exposure hereby increasing the understanding of potential ecological consequences.

HPI axis responsiveness

One should note that fish were sampled after 3 days of testing, in the next morning, and our measures (in average between 25 and 80 $ng.mL^{-1}$ blood cortisol) were actually close from basal level measured in control wild type zebrafish (20-200 $ng.mL^{-1}$) as reported in Ziv et al. (2013). Here we did not observe any link between blood cortisol concentration, coping style, anxiety level or treatment. This was, however, quite surprising because both coping styles and anxiety level are generally linked to HPI axis responsiveness (Backstrom et al., 2011; Egan et al., 2009; Geffroy et al., 2018; Summers et al., 2017; van den Bos et al., 2017). Nevertheless, some study report behavioural divergences without cortisol differences (Vindas et al., 2017) suggesting that the measure of cortisol alone is not sufficient to predict behavioural disruptions in the absence of an additional challenge. More information should come from genes transcription network analysis when RNAseq data analysis will be completed.

Conclusions

In conclusions, effects of a parental exposure to POPs were highly dependent on the generation that was studied as reported in Alfonso et al. (Chapitre 3). The chronic dietary exposure to POP in F0 did not trigger any effect on behavioural responses whereas F1 generation displayed higher boldness level and F2 generation displayed higher anxiety syndrome level. Basal blood cortisol concentration did not predict the observed behavioural disruptions in F1 and F2 generations and further physiological analyses are required to fully understand this. Nevertheless, the behavioural disruptions in coping style and anxiety in offspring generation may have ecological consequences and detrimental effects on fish natural population exposed to PCB and PBDE mixtures.

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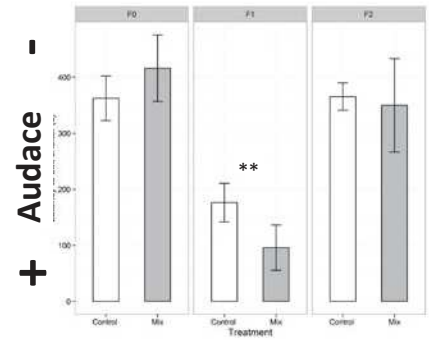
Chapitre 4 – Conclusions

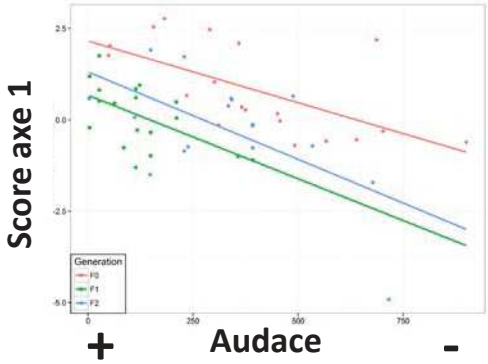
Résultats:

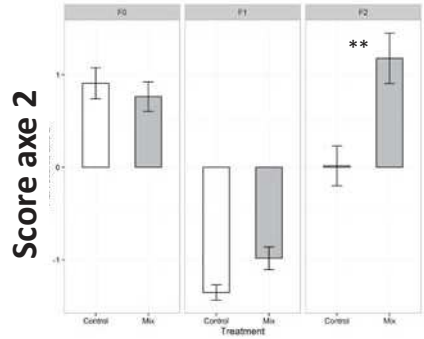
• Méthode d'analyse:

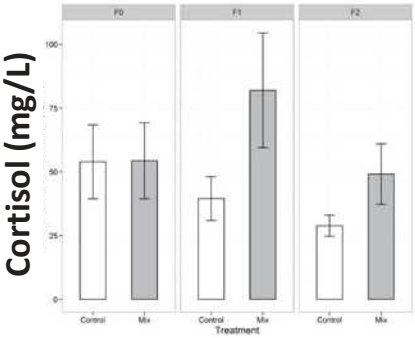
Une analyse en composante principale (ACP) a été réalisée avec les différentes variables mesurées lors des tests de nouvel environnement (**NE**), test de choix (**TC**) et le labyrinthe en Z (**ZM**). Le premier axe de l'ACP expliquait 24 % de la variabilité observée et le second axe 21 %. Les individus avec une forte valeur sur le premier axe étaient plus actifs dans le NE, plus audacieux et plus explorateurs dans le ZM. **Le premier axe représente donc un syndrome comportemental lié au *coping style*.** Les individus avec une forte valeur sur le second axe étaient, quant à eux, plus anxieux dans le NE, passaient moins de temps près du banc dans le TC (moins sociaux) et plus de temps dans le refuge lors du ZM. **Ce second axe représente un syndrome comportemental lié à l'anxiété.**

• Prise de risque:









• Légende:

- Score axe 1 +
- Activité
- Exploration
- Audace (ZM)

- Score axe 2 +
- Anxiété
+ Socialité -
- Temps passé dans le refuge (ZM) +

Traitements

Contrôle | MIX

Générations

F0 | F1 | F2

*: p<0.05, **: p<0.01

Points importants:

• Les F1 MIX sont plus audacieux que les F1 contrôles. L'audace est corrélée positivement à l'activité et la vitesse d'exploration (axe 1) formant un syndrome comportemental non affecté par le traitement.

• L'anxiété est liée à une faible socialité et un temps dans le refuge important formant un syndrome comportemental affecté par le traitement. Les F2 MIX présentaient une plus forte valeur pour ce syndrome comportemental, ils étaient donc plus anxieux et moins sociaux que les individus contrôles.

• Les concentrations en cortisol sanguin étaient identiques entre les traitements, ne permettant pas de prédire les altérations comportementales de la descendance (générations F1 et F2)

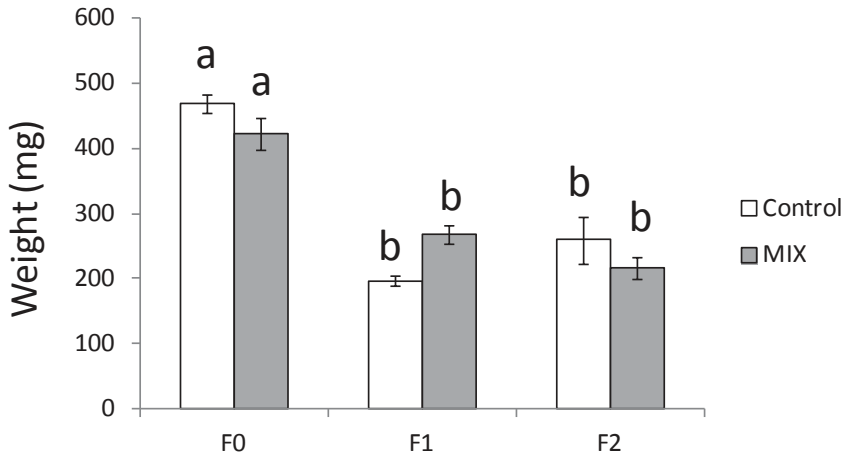
Communications:

• **Société Française d'Etude du Comportement Animal**
Caen – 2016 – *Communication orale*

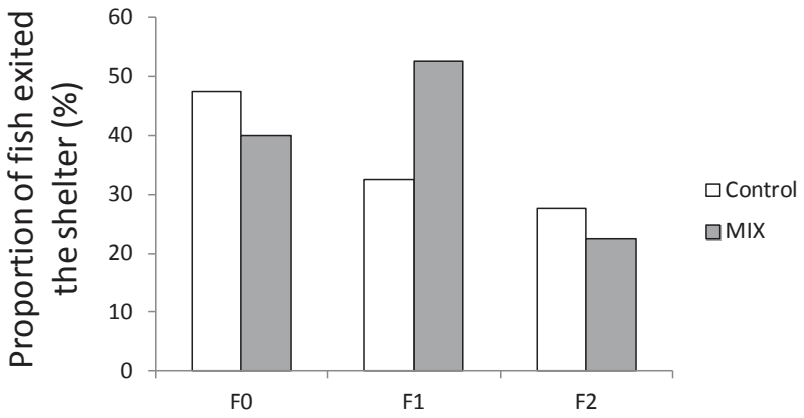
Supplementary material

Chronic dietary exposure to an environmental mixture of Persistent Organic Pollutants (PCBs, PBDEs) alters coping-style and anxiety syndromes over generations in zebrafish (*Danio rerio*)

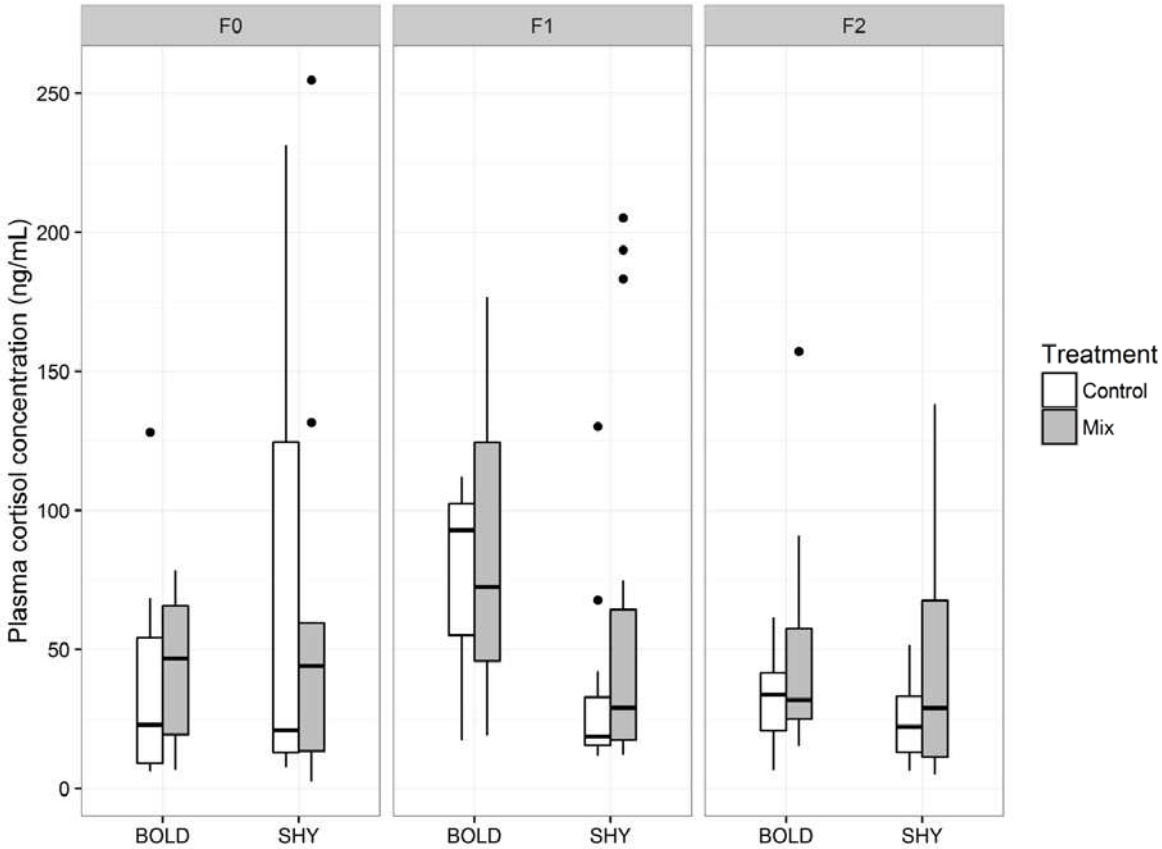
In preparation



Supp. Figure 1. Fish weight (mg) in relation to treatment for each generation (F0: n=19 Control/n=18 MIX; F1: n=21 Control/n=22 MIX; F2: n=17 Control/n=16 MIX). Different letters indicate significant differences between treatments after *post-hoc* Tukey HSD.



Supp. Figure 2. Proportion of fish exited the shelter (%) during risk taking test for each generation (F1, F2, F3).



Supp. Figure 3. Plasma cortisol concentration function of generations (F0, F1 and F2), coping styles (Bold and Shy) and treatments (Control and MIX; F0: n=19 Control/n=18 MIX; F1: n=21 Control/n=22 MIX; F2: n=17 Control/n=16 MIX). The central line of the boxplot indicates the median, while boxes lines represent quartiles and the whiskers cover 95 % of values. Expected outliers are represented by points.

Partie 3

Effets d'une exposition aigue à des facteurs de stress retrouvés en aquaculture sur les réponses comportementales et physiologiques des téléostéens

Chapitre 5

Spatial distribution and activity patterns as welfare indicators in response to water quality changes in European sea bass, *Dicentrarchus labrax*

Sébastien Alfonso
Xavier Cousin
Lucette Joassard
Béatrice Chatain
Marie-Laure Bégout

Manuscript en préparation

Chapitre 5 – Présentation

Contexte:

Le secteur de l'aquaculture connait une expansion ces dernières décennies. Les nouvelles pratiques visent souvent à limiter les apports d'eau neuve afin de minimiser les coûts de production. Cela peut conduire à de mauvaises qualités d'eau, notamment avec de faibles concentrations en O_2 et/ou de fortes concentrations en ammonium. Cela peut entraîner des conséquences sur la santé et le bien être des téléostéens. Des observations du comportement des animaux en élevage, fournissant des indicateurs prédictifs et opérationnels, pourraient permettre de qualifier rapidement l'état physiologique des individus et leur niveau de bien être afin d'ajuster les paramètres de qualité d'eau aux besoins des animaux si nécessaire.

Questions:

(1) Quelles sont les réponses comportementales des téléostéens durant/après une exposition à de mauvaises qualités d'eau (conditions avec de hautes concentrations d'ammonium (TAN), d'hyperoxie et d'hypoxie) ?

(2) La réponse physiologique de l'axe du stress est-elle corrélée aux altérations comportementales observées suite à une exposition à ces qualités d'eau dégradées?

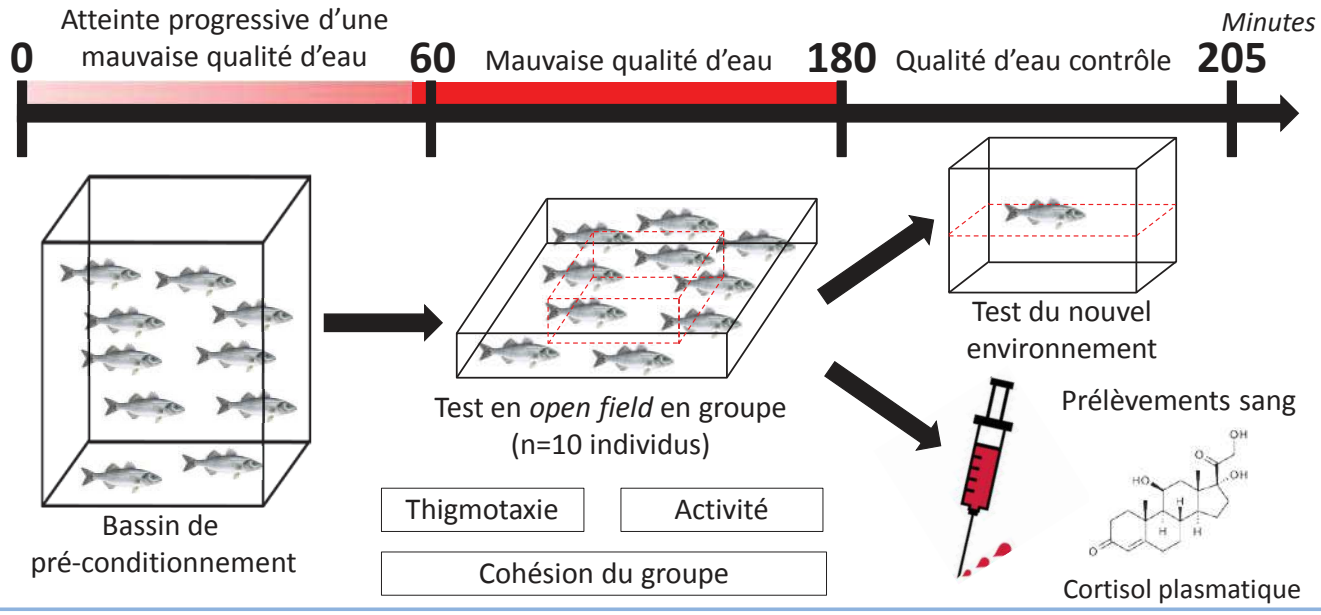
Méthode:

Les individus ont été placés dans un bassin de pré-conditionnement où la qualité d'eau a progressivement changé pour atteindre une des 3 conditions ci-dessous (**TAN, hyperoxie** ou **hypoxie**) ou une condition **contrôle**. Les individus ont été ensuite transférés dans un *open field* (OFT) où leur comportement a été suivi en groupe (**thigmotaxie** (temps passé en périphérie de l'arène, signe d'anxiété), **activité** et **cohésion de groupe**) toujours dans **les même conditions**. Ensuite un sous échantillon, a été testé individuellement dans un nouvel environnement (qualité d'eau contrôle) pour étudier **l'anxiété** *post*-stress. Le taux de **cortisol** plasmatique a été mesuré dans le second échantillon après OFT.

• Conditions:

Contrôle	Mauvaises qualités d'eau		
	TAN	Hyperoxie	Hypoxie
O_2 = 100 % TAN <2.5 mg/L	O_2 = 100 % TAN = 18 mg/L	O_2 = 100 % TAN <2.5 mg/L	O_2 = 100 % TAN <2.5 mg/L

• Protocole expérimental:



Spatial distribution and activity patterns as welfare indicators in response to water quality changes in European sea bass, *Dicentrarchus labrax*

In preparation

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Abstract

In modern aquaculture, production costs are the major driver. This has resulted in culture practices and rearing environments aimed at maximizing production capacity. Consequently, fish are exposed to unavoidable stressors, which can be detrimental to animal health and welfare. This work aims were to contribute further to the establishment of behavioural welfare indicators including the psychological dimension of stress in a model marine teleost in response to different water quality stressors observed in aquaculture condition (High Total Ammonia Nitrogen (TAN) concentration and oxygen variation). Group of ten fish (n=3) were exposed to High TAN concentration (18 $mg.L^{-1}$, equivalent to 0.8 $mg.L^{-1}$ [NH_3]), Hyperoxia (200 % O_2 saturation) or Hypoxia (20 % O_2 saturation) during 2 hours. Videos were recorded in a novel environment to assess the following behavioural indicators: group (social cohesion) and individual behaviour (distance travelled, time spent in periphery) which were compared to control condition. After this challenge, blood samples were taken in a subsample (n=10 fish/condition) to analyse plasma cortisol concentration as a physiological stress indicator. Other fish exposed to the same three conditions (n=20) were analysed individually using the novel tank diving test during 25 minutes to evaluate anxiety state *post*-stress situation. This novel tank diving test was translated from zebrafish research and validated using nicotine as an anxiolytic drug. During stress condition in a novel environment in group, fish from High TAN, Hyperoxia and Hypoxia conditions spent less time in periphery during the first minutes of the challenge, showed lower group cohesion at the end, travelled less distance and showed higher plasma cortisol concentration (except for fish from Hyperoxia condition) than fish from control condition. During novel tank diving test, a lower activity was observed in all conditions compared to control and only fish exposed to TAN showed a high vertical positioning likely related to physiological distress overruling anxiety-like behaviour. This study indicates that stressors found in aquaculture condition may quantitatively impact behavioural and physiological indicators of farmed fish stress responses and also may inhibit the normal responses observed after introduction into a novel environment.

1. Introduction

Fish production expanded importantly during the last decades, both because of the world's diminishing natural fisheries and the increase in demand for fisheries products (Conte, 2004). Fish production has increased by more than 20 million metric tons (mt) between 2004 and 2014 to reach 73.8 million mt in 2014. It represented 44.1 % of total production (including for non-food uses) from capture fisheries and aquaculture in 2014 (FAO, 2016) and as such aquaculture is now recognized as a major food production industry. Thus, as well as for terrestrial farming industry, concerns about sustainability, environmental issues and animal welfare in aquaculture are increasing (Ashley, 2007; Conte, 2004; Hixson, 2014; Martins et al., 2010; Martins et al., 2012). It is common that under aquaculture conditions, and in every fish husbandry system, variations of water quality parameters such as temperature, pH, oxygen (O_2), carbon dioxide (CO_2) or Total Ammonia Nitrogen concentrations (TAN) occur. Such variations when they reach a certain threshold and depending on the species *preferendum*, could be deleterious for fish and have economic consequences for farmers. Water quality parameters monitoring is thus important to maintain good husbandry quality and assure animal welfare and production (Conte, 2004).

Oxygen plays a vital role in the breathing processes and in the energy metabolism in aerobic organisms, thus variations in oxygen concentrations in water may have detrimental effects on life history traits (Perdersen, 1987; Pichavant et al., 2000, 2001; Rimoldi et al., 2016; Thomas et al., 2007; Wu et al., 2003). From a behavioural point of view, acute hypoxia lead fish to aquatic surface respiration by rising to the surface and ventilating the layer of water in contact with atmosphere which is rich in dissolved oxygen (Kramer and McClure, 1982). If hypoxia is prolonged, it can change locomotor activity depending on fish species and/or oxygen concentration (Chapman and Mckenzie, 2009). Moreover, in Atlantic herring (*Clupea Harengus*), hypoxic condition (20 % of O_2 saturation) lead to altered school structure by increasing school volume (Domenici et al., 2002). As well as for hypoxia, hyperoxic conditions may also disrupt behaviour in fish. For instance, it was demonstrated that hyperoxic conditions lead to an increase in ventilation rate in Giant goby (*Gobius cobitis*; Berschick et al. (1987)). Hyperoxic conditions can also affect the swimming behaviour of fish. Espmark and Baeverfjord (2009) have shown that the variance of swimming activity in Atlantic salmon (*Salmo salar*) was higher under prolonged hyperoxic conditions but without level difference compared to normoxia conditions.

Total Ammonia Nitrogen (TAN) concentration in water is another important parameter to assure fish welfare. Ammonia is known to be more toxic in its un-ionized form (NH_3); unionized ammonia nitrogen, UIA-N) and less or non-toxic in its ionic form (NH_4^+; Camargo and Alonso (2006)). NH_3 may cause negative physiological effects by causing asphyxiation by reducing the oxygen-carrying capacity, and affecting the liver, kidneys, or immune system which in turns are causing a reduction in feeding activity and could alter swimming performances, fecundity, and fish survival (Camargo and Alonso, 2006; Constable et al., 2003; Gao et al., 2017; Shingles et al., 2001). Shingles et al. (2001) have in particular shown that an increase in ammonia levels in plasma was associated with a significant reduction in individual critical swimming speed and a lower active metabolic rate in rainbow trout (*Oncorhynchus mykiss*). Other water quality factors have been shown to affect fish behaviour such as low water pH levels (Day and Butler, 1996) or carbon dioxide exposure (Sadoul et al., 2017).

Behavioural measurements have proven to be sensitive indicators of the complex existing biochemical and physiological changes that occur in response to stress (Martins et al., 2012; Scherer, 1992; Schreck, 1990, 1997). Behaviours, such as changes in food-anticipatory activity, feed intake, ventilation rate, individual and group swimming activity are commonly used as welfare indicators (Carbonara et al., 2006; Huntingford et al., 2006; Huntingford and Kadri, 2014; Martins et al., 2012). Group swimming behaviour is defined as the spatial distribution and swimming activity of groups of fish held within an aquaculture production unit and it covers shoal structure, the horizontal and vertical distribution of the group, their swimming speed and direction (Martins et al., 2012). Exposure to negative *stimuli*, such as poor water quality, is known to lead to rapid escape movements of fish group towards the bottom of the tank (Bratland et al., 2010; Stien et al., 2007) or to alter group cohesion and activity (Domenici et al., 2002; Espmark and Baeverfjord, 2009; Sadoul et al., 2014, 2017). Thus, group swimming behaviour appears to be a sensitive welfare indicator even if it is still lacking calibration effort to turn into an operational welfare indicator but some examples exist (Papandroulakis et al., 2014; Pettersen et al., 2014). Moreover, the appraisal of negative or positive stimuli and hence the psychological dimension of stress as defined for fish by Galhardo and Oliveira (2009) is seldom tackled in welfare research. There exist however a complementary measure which is the individual behavioural responses to novel environment and in particular the novel tank diving test which is worldwide used along with the measure of stereotypies such as thigmotaxis to assess anxiety in zebrafish (*Danio rerio*) in ecotoxicology

and pharmacology research (Egan et al., 2009; Levin et al., 2007; Macaulay et al., 2015; Vignet et al., 2014). Appropriateness of the novel tank diving test to evaluate anxiety was validated using drugs and acute nicotine exposure has been shown to be anxiolytic in this context (Levin et al., 2007). The novel tank diving test could thus be a helpful non-invasive tool to monitor farmed fish anxiety state *post* stress exposure hereby assessing psychological stress and contributing to the assessment of positive or negative emotions and, hence better welfare determination.

Besides behavioural measurements, cortisol is the final hormone produced through the hypothalamo-pituitary-interrenal axis (HPI) in teleost and it is acting as an activator of physiological and behavioural responses when facing a stress (Sadoul and Vijayan, 2016; Schreck and Tort, 2016; Sumpter, 1997). Among deviations of the previously cited parameters, oxygen variations and high ammonia concentrations are known to trigger the HPI axis resulting in increase in the cortisol release which is widely use an acute stress biomarker (Espmark and Baeverfjord, 2009; Knoph and Olsen, 1994; van Raaij et al., 1996).

Overall, objectives here were to contribute further to the establishment of behavioural welfare indicators including the psychological dimension of stress in a model marine teleost in response to different water quality stressors. Aims of this study were therefore first to validate the novel tank diving test as a behavioural test to evaluate anxiety-like behaviour in European sea bass *Dicentrarchus labrax*, using nicotine as an anxiolytic reference drug. The second objective was to monitor behavioural responses of fish group in a novel environment under acute stressor exposure which were high Total Ammonia Nitrogen (High TAN) concentration (18 $mg.L^{-1}$), Hyperoxia (200 % O_2 saturation) and Hypoxia (20 % O_2 saturation) along with physiological HPI axis responses. The third objective was to evaluate individual behavioural responses following stressful water quality exposure using the novel tank diving test translated from zebrafish.

2. Material and methods

Experiments were authorized by ethics committee agreement APAFIS#7098 and all procedures involving animals were in accordance with the ethical standards of the institution and followed the recommendations of Directive 2010/63/EU.

2.1. Fish rearing

Juvenile European sea bass were hatched and reared at Ifremer Palavas-les-flots research station (France, 34250) until 280 days *post* fertilization (dpf) according to sea bass rearing standard conditions (Chatain, 1994) and transferred to Ifremer L'Houmeau (France, 17137). They were then randomly separated in groups of 100 fish into 4 tanks (400 L) in a recirculating system with a flow rate of 4 m^3 per hour and water was renewed at a rate of 10 % per day. Water temperature was maintained at 21.5 ± 1°C, oxygenation at 100 % saturation, salinity was 20.5 ± 1 and pH was 8.3. The light regime was 13:11 L/D. Total Ammonia Nitrogen (TAN) concentration was < 2.5 $mg.L^{-1}$ (equivalent to 0.1 $mg.L^{-1}$ [NH_3]) The fish were fed using commercial diet Le Gouessant (France) once a day each morning at 9:00. Fish were allowed to recover from transport from Palavas-les-Flots to L'Houmeau for at least 3 months before further experiments.

2.2. Translating and validating the novel tank diving test in European sea bass

The novel tank diving test has been validated to evaluate the anxiety level of individual fish *e.g.* by monitoring zebrafish (*Danio rerio*) vertical positioning over a 6 minutes observation period (Egan et al., 2009; Levin et al., 2007). First objective of this work, was to adapt the test protocol (observation duration, tank size relative to fish size) to European sea bass, and then to validate the measure of anxiety using nicotine as a reference anxiolytic drug.

Two groups of fish (385 dpf, n=24 per group) were transferred from the home rearing tank to two 50 L tanks into the experimental room for a 1 hour acclimation period. After this acclimation period and before introducing fish into the novel tank, two different baths were applied. Control fish (n=24) were bathed one individual at a time into plain water during 8 min in a 3 L tank (24.5x15x13.5cm, AquaBox 3; Aqua Schwarz GmbH). After several trials, the following protocol was adopted for nicotine exposure: fish (n=24) were bathed one individual at a time during 5 min in a 5 $mg.L^{-1}$ nicotine solution (Pestanal®, Sigma Aldrich) followed by 3 min in plain water both in a 3 L tank. Directly after the bathing period, fish was then transferred gently alone in a novel tank (29x21x17 cm, 10 L trapezoid tank from Aquatic Habitat. Inc.), and a video was recorded in side view during 25 minutes. For space occupancy analysis, the tank was virtually separated into two areas according to Egan et al. (2009): top area including one half of the volume and bottom area including the other half. Time spent in top area (s) and latency to enter top area (s), which are both indicative of anxiety level, were measured. In addition, distance travelled (cm) and number of transitions between areas,

indicative of fish activity level were also measured for each bathing. Variables were recorded in each frame and they were summed over periods of 5 min or over the whole test duration (*i.e.* 25 min).

2.3. Exposure to stress condition and water quality characterization

In the morning before the experiment, a group of 10 fish was gently caught from the rearing tank and transferred to the behavioural room where they were maintained into a 50 L tank filled with the same system water as their home tank (**Figure 1**). Fish were kept under standard condition during 1 h for tank acclimation and then during the next hour one of the four following conditions was applied *i.e.* Control, High Total Ammonia Nitrogen (TAN) concentration, Hyperoxia and Hypoxia.

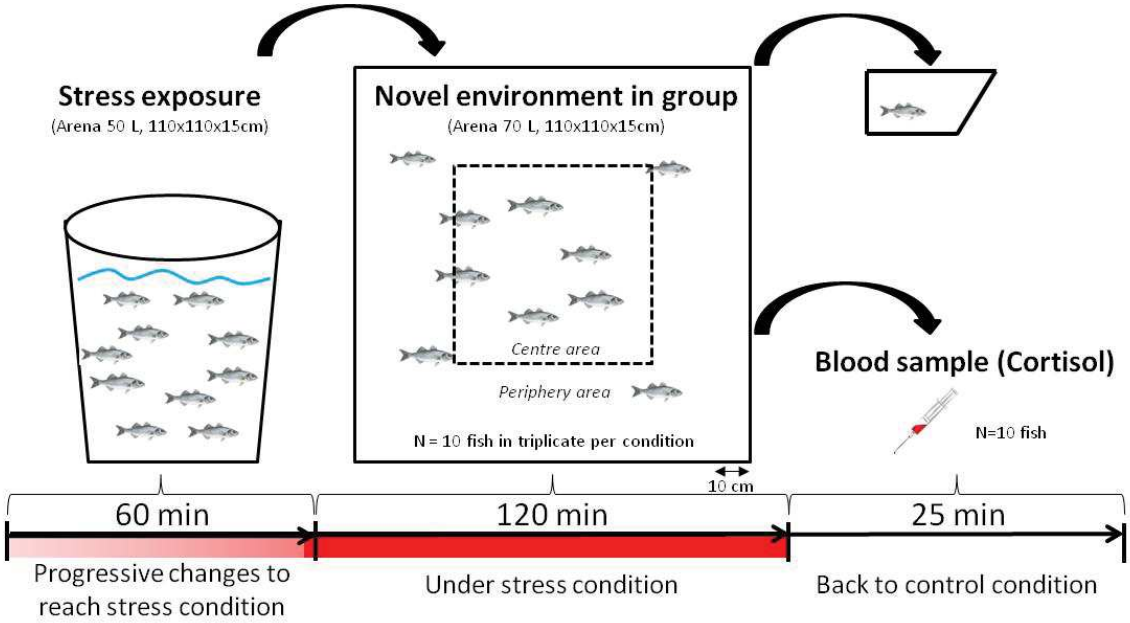

Figure 1. Experimental protocol followed for fish exposure to different water quality and behavioural and physiological measurements.

For the control condition, fish were maintained under the same standard condition than in the rearing room (*i.e.,* at 21.5 ± 1°C, 100 % O_2 saturation and TAN < 2.5 mg.L^{-1}).

For high TAN condition, 8.5 g of ammonium chloride NH_4Cl (Fluka 09711, Sigma Aldrich) dissolved in 0.5 L of system water was added twice at 30 min interval to reach a targeted TAN concentration of 18 mg.L^{-1} which correspond to 0.8 mg.L^{-1} of UIA-N (NH_3). Three water samples were taken to quantify *a posteriori* TAN concentration: (i) before adding ammonium chloride, (ii) at the beginning and (iii) at the end of the novel environment in group. Samples were stored at -22°C before further analysis. TAN concentration was

quantified using a spectrophotometer with continuous flow (Alliance Integral Futura, Frépillon, France) and analytical method is described in Petitjean (2018). Associated UIA-N concentration was determined using the equation of Johansson and Wedborg (1980) as a function of pH, temperature and salinity.

For Hypoxia condition, oxygen concentration was slowly lowered to reach 20 % of O_2 saturation using nitrogen bubbling.

For Hyperoxia condition, oxygen concentration was slowly increased to reach 200 % of O_2 saturation using oxygen bubbling. Oxygen concentration was monitored every 5 min during the all experiment using an Oxygen probe (Oxi 3310, WTW, Xylem Analytics Germany Sales GmbH & Co. KG) but only concentrations measured at 0, 30 and 60 minutes were further presented.

Experiments were performed in triplicates (n=10 fish x 3 run per condition). After the bathing period to reach stress condition, group of 10 fish were challenged into novel environment in group (see section 2.4.1). After this challenge in group, fish from two replicates were then challenged individually into novel tank diving test in plain system water (see section 2.4.2, n=20 for control, n=20 for High TAN and n=18 for Hyperoxia and Hypoxia). The last replicate was used to quantify plasma cortisol concentration following the challenge in group (see section 2.5, n=10 per condition). After blood sampling (N=9 or 10 fish per condition) or novel tank diving test, all fish were measured for weight (g, to the nearest mg) and standard length (cm, to the nearest mm) under the same anaesthesia conditions (see section 2.5).

2.4. Behavioural procedures

All behavioural experiments presented above for the translation of novel tank diving test and here below were performed in a dedicated room with same environmental parameters than the rearing room. All videos were recorded with an analogue camera ICD-48E (Ikegami) and a 2.1-13.5 lens (Fujinon) linked to a computer with an acquisition card and EthoVision XT 10.0 software (Noldus, The Netherlands) at 25 frame.s^{-1}. Data extraction and analyses were performed using EthoVision XT 13.1 software. Swaps between individuals during novel environment in group were manually corrected using the track editor module (Noldus, The Netherlands).

2.4.1. Novel environment in group

After the bathing period, the entire group of fish (n=10) was gently transferred into a novel arena (110 cm x 110 cm x 6 cm, 70 L). After 1 min, video recording started for 2 hours in top view. For each condition, water parameters were maintained similar to those obtained at the end of the bathing period for the respective condition following the same procedure explained in section 2.2.2. For space occupancy visualization heat map were produced for each 5-min period using Ethovison XT 13 (Noldus The Netherlands). For further analysis, the arena was separated into two areas: Centre area including one half of the volume and periphery area including the other half; time spent in periphery (s), indicative of thigmotaxis behaviour (Ferrari et al., 2014), was recorded. Distance travelled by each fish (cm), indicative of individual fish activity, and the interindividual distances (cm), indicative of group cohesion (Buske and Gerlai, 2011), were also recorded. Variables were recorded for each frame and they were summed (for Distance travelled) or averaged (for time spent in periphery and interindividual distances) over period of 5 min every 30 minutes (*i.e.,* four sampling periods: 0-5 min, 30-35 min, 60-65 min and 90-95 min).

2.4.2. Novel tank diving test post-*stress condition*

After novel environment challenge in group, fish were gently transferred individually into a novel tank (same as the one used for method validation with nicotine, see above) containing plain system water and fish swimming activity was monitored during 25 min to assess anxiety state and behavioural recovery following stressful conditions exposure. For space occupancy and fish swimming activity analyses, the same procedure as presented before in section 2.2 was followed.

2.5. HPI axis response

Immediately after the end of the observation period in the novel environment challenge, fish were gently caught and transferred into a 10 L tank which contains 500 $\mu l.L^{-1}$ of a benzocaine stock solution (50 $g.L^{-1}$ in 100 % ethanol; Benzocaine Sigma-Aldrich, Saint-Quentin Fallavier, France). Blood samples were obtained within 3 minutes from the venous sinus with heparinised syringes. The blood was centrifuged (5 min at 4000 g) to obtain plasma samples which were stored at -22°C until further analyses. Plasma cortisol concentration was determined by ELISA (RE52061, IBL International, Hamburg, Germany) using a Synergy-HT (BioTek Instruments, Winooski, VT, USA).

2.6. Statistical analyses

Statistical analyses were performed using Statistica 9.0 software (StatSoft, USA). All statistical analyses were carried out at a 95 % level of significance and values are represented as mean ± SEM except where otherwise mentioned. Normality and homoscedasticity were tested *a priori* using Shapiro-Wilks test and where sample sizes were too small non parametric statistics were used.

Fish weight and length were compared between tested water quality using one-way ANOVA.

For validation of the Novel tank diving test, time spent in top area and distance travelled were compared between bathing factors (Nicotine *vs.* Control) with a repeated-measures ANOVA (with 5 periods, *i.e.* 5-min, 10-min, 15-min, 20-min and 25-min) and a Tukey HSD *post-hoc* test. A Mann-Whitney U-test was also performed to compare between bathing factors, total time spent in top area, latency to enter top area, total distance travelled by fish and number of transitions between top and bottom areas.

For Novel environment challenge in group, distance travelled, distance between fish and time spent in periphery area were compared between conditions with a Factorial ANOVA (with 4 periods, *i.e.* 0-5-min, 30-35-min, 60-65min, 90-95min) followed by Tukey HSD *post-hoc* test.

For novel diving tank *post*-stress condition, time spent in top area and distance travelled were compared between conditions with a repeated-measures ANOVA (with 5 periods, *i.e.* 5-min, 10-min, 15-min, 20-min and 25-min) and a Tukey HSD *post-hoc* test. A One-way ANOVA followed by Tukey HSD *post-hoc* test were performed to compare between conditions the total time spent in top area, latency to enter top area, total distance travelled by fish and number of transitions

For plasma cortisol concentration, a Mann-Whitney U-test was performed to compare between control and each condition.

3. Results

Overall, 168 fish were used for the different experiments: 48 for the translation and validation of the novel tank diving test (50.8 ± 0.6 g, 15.4 ± 0.9 cm, n=24 per group); and for the stress condition exposures (51 ± 1 g, 15.3 ± 0.2 cm, n=30 per group) which were homogeneous between conditions (One-Way ANOVA, F=1.5, df=3, p=0.23 and F=2, df=3, p=0.13 for body weight and standard length respectively).

3.1. Validation of novel tank diving test in European sea bass

Irrespective of the bathing (Control *vs.* Nicotine) fish progressively explored the top area of the novel tank (Repeated-measures ANOVA, F=6.0, df=4, p<0.001). Nicotine bathed-fish spent significantly more time in the top area than control ones during each 5-min period of the test (Repeated-measures ANOVA, F=31.8, df=1, p<0.001; **Figure 2.A**) as well as over the total duration of the test (Mann-Whitney U-test, Z=4.8, p<0.001; **Figure 2.B**). Fish travelled also more distance progressively during the experiment whatever the bathing (Repeated-measures ANOVA, F=13.3, df=4, p<0.001) but nicotine bathed-fish travelled significantly more distance than control ones during each 5-min period of the test (Repeated-measures ANOVA, F=15.9, df=1, p<0.001; **Figure 2.C**) as well as over the total duration of the test (Mann-Whitney U-test, Z=3.5, p<0.001; **Figure 2.D**). Nicotine bathed-fish swam more between the two areas and entered quicker in the top area than control ones (Mann-Whitney U-test, Z=4.3, p<0.001 and Z=-5.0, p<0.001 respectively; **Figure 2.E,F**).

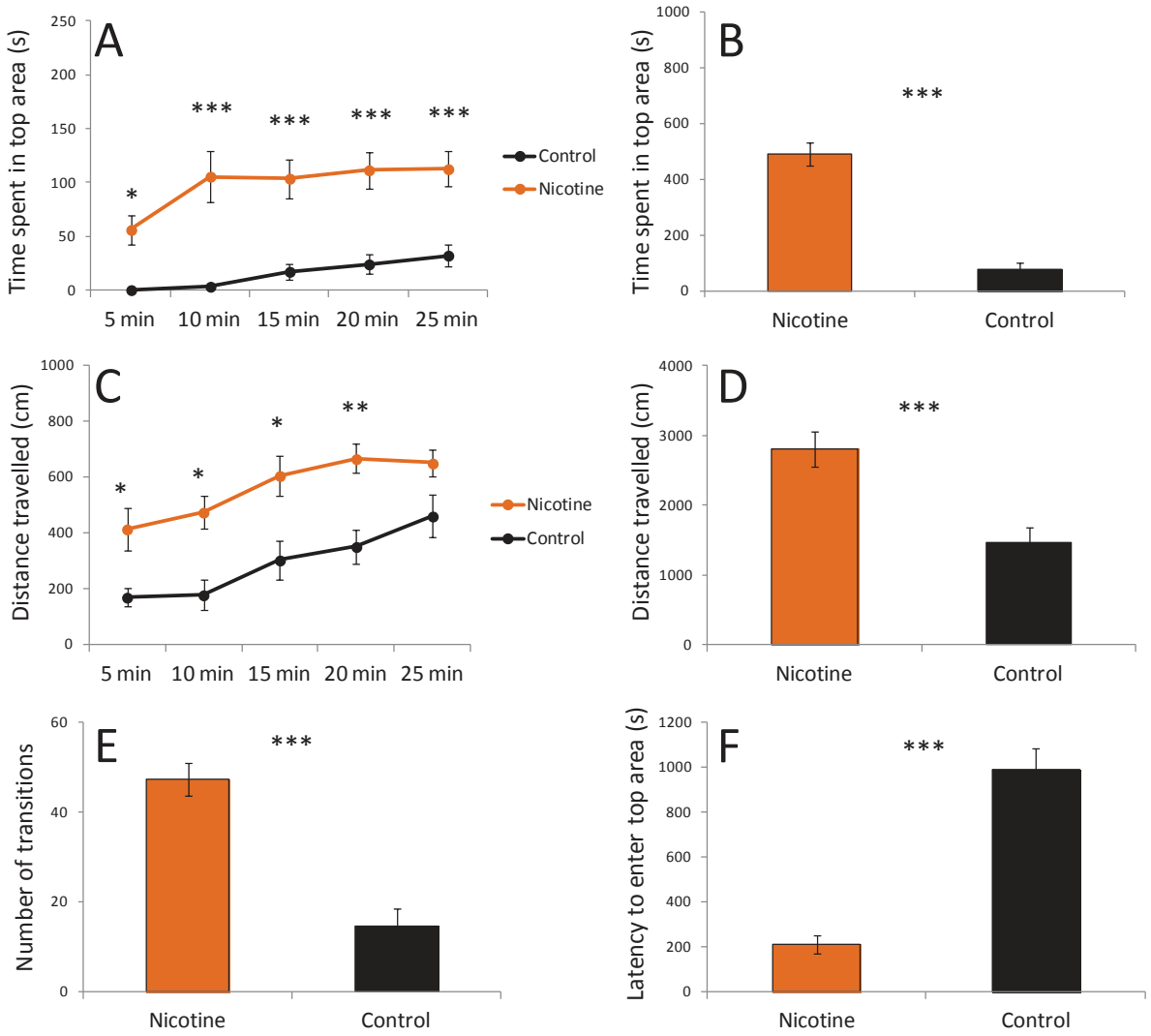

Figure 2. Swimming characteristics in the novel tank diving test. Mean ± SEM of **(A)** time spent in top area in relation to the observation period (s); **(B)** total time spent in top area (s); **(C)** distance travelled in relation to the observation period (cm); **(D)** total distance travelled (cm); **(E)** number of transitions between top and bottom areas and **(F)** latency to enter top area (s) *post*-nicotine exposure *vs.* control-condition (n=24 per condition). **(A, C)** Tukey HSD *post*-hoc: *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. **(B,D,E,F)** Mann-Whitney U-test: ***: $p<0.001$.

3.2. Water quality characterization

As mentioned before, for control condition, water oxygen concentration was maintained at 100% saturation (at 21.5 ± 1°C) and TAN concentration was < 2.5 $mg.L^{-1}$ during both bathing and novel environment in group.

For High TAN condition, oxygen concentration was 100 % and TAN concentration was 18 $mg.L^{1}$ at the end of the bathing period which corresponded to 0.8 $mg.L^{1}$ of UIA-N [NH_3]. Further, at the beginning of novel environment in group, TAN concentration was 15.3 ± 4.2

mg.L^{-1} and 18.8 ± 7.6 mg.L^{-1} at the end of the novel environment which corresponded to UIA-N concentration of 0.82 ± 0.33 mg.L^{-1}.

For both Hyperoxia and Hypoxia condition, TAN concentration was maintained < 2.5 mg.L^{-1} and oxygen concentration increased slowly during the bathing period to reach 208 ± 5.6 % of saturation Hyperoxia condition and decreased slowly during the bathing period for Hypoxia condition to reach 21.3 ± 3.8 % of saturation before starting novel environment challenge in group. All conditions were then maintained during two hours until the end of novel environment in group (**Table 1**).

Table 1. Mean ± SEM of TAN and UIA-N (mg.L^{-1}) and oxygen concentration (% O_2) for High TAN, Hyperoxia and Hypoxia conditions during bathing period (at 0, 30 and 60 min) and at the start and end of novel environment challenge in group.

Stress condition	High TAN		Hypoxia	Hyperoxia
Time (min)	TAN (mg.L^{-1})	UIA-N (mg.L^{-1})	Oxygen saturation (%)	
0	2.5 ± 0.6	0.11 ± 0.03	99.9 ± 0.5	99.4 ± 0.5
30	NA	NA	52.0 ± 2.2	158.9 ± 5.1
60	NA	NA	20.1 ± 0.1	201.1 ± 1.4
Start experiment	15.3 ± 4.2	0.67 ± 0.18	21.3 ± 2.0	208.0 ± 3.2
End experiment	18.8 ± 7.6	0.82 ± 0.33	20.3 ± 2.3	199.7 ± 10.2

3.3. Novel environment in group

Heat maps representing the mean location frequency of fish from different conditions and during different periods of the novel environment challenge in group are plotted in **Figure 3**. For control condition, fish responded to novel environment in group by staying mainly in the periphery of the arena during the first five minutes of the test. Progressively, they explored the Centre of the arena. On the contrary, the other conditions (*i.e.* High TAN, Hyperoxia and Hypoxia), were located in the Centre area from the beginning of the test. Moreover, under these conditions, fish group size enlarged during the test under exposure to stressful water quality conditions whereas fish from control condition remained aggregated.

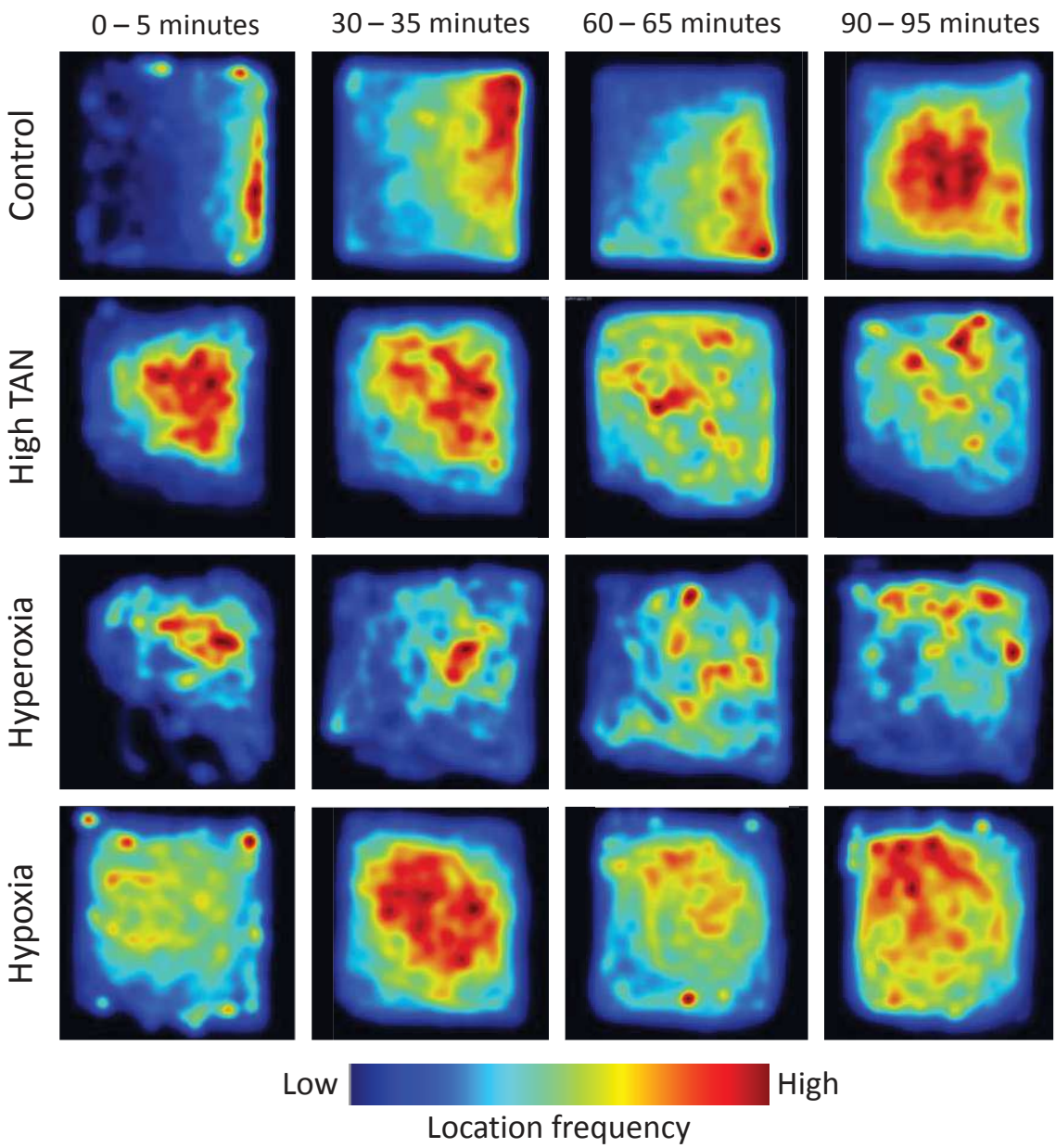

Figure 3. Spatial location of fish during the novel environment in group. Averaged heat maps of fish spatial location (n=30 per condition) into the arena of novel environment in group during the different periods. From low (blue colour) to high location frequency (red colour).

Under stress conditions, distance travelled by fish differed between conditions (Factorial ANOVA, F=355.3, df=3, p<0.001), periods (F=8.4, df=3, p<0.001) with an interaction between conditions and periods (F=9.8, df=9, p<0.001). Fish from control condition travelled progressively more distance over the challenge duration; 3178 ± 268 cm during period 0-5 min *vs.* 5148 ± 254 cm during period 90-95 min (p<0.001). Fish from High TAN, Hyperoxia and Hypoxia conditions showed a lower distance travelled compared to control fish during all periods (p<0.001 for all conditions) and it was stable over all periods (**Figure 4.A**).

Distance between fish, indicative of group cohesion, differed between conditions (Factorial ANOVA, F=18.8, df=3, p<0.001), periods (F=25.4, df=3, p<0.001) with an interaction between conditions and periods (F=30.5, df=9, p<0.001). Fish from control conditions progressively decreased their interindividual distance during the challenge; 48.3 ± 1.4 cm during period 0-5 min *vs.* 41.9 ± 1.1 cm during period 90-95 min (p<0.001). During the 0-5 min period, fish from control and Hypoxia condition showed the same distance between individuals (p=0.12), whereas fish from High TAN and Hyperoxia conditions swam closer to each other (p<0.001 for both conditions). However starting from the 60-65 min to the 90-95 min period, fish from High TAN, Hyperoxia and Hypoxia conditions showed higher dispersion than control fish (60-65 min: p<0.001 for both Hypoxia and High TAN conditions and p=0.03 for Hyperoxia condition ; 90-95 min: p<0.001 for all conditions; **Figure 4.B**).

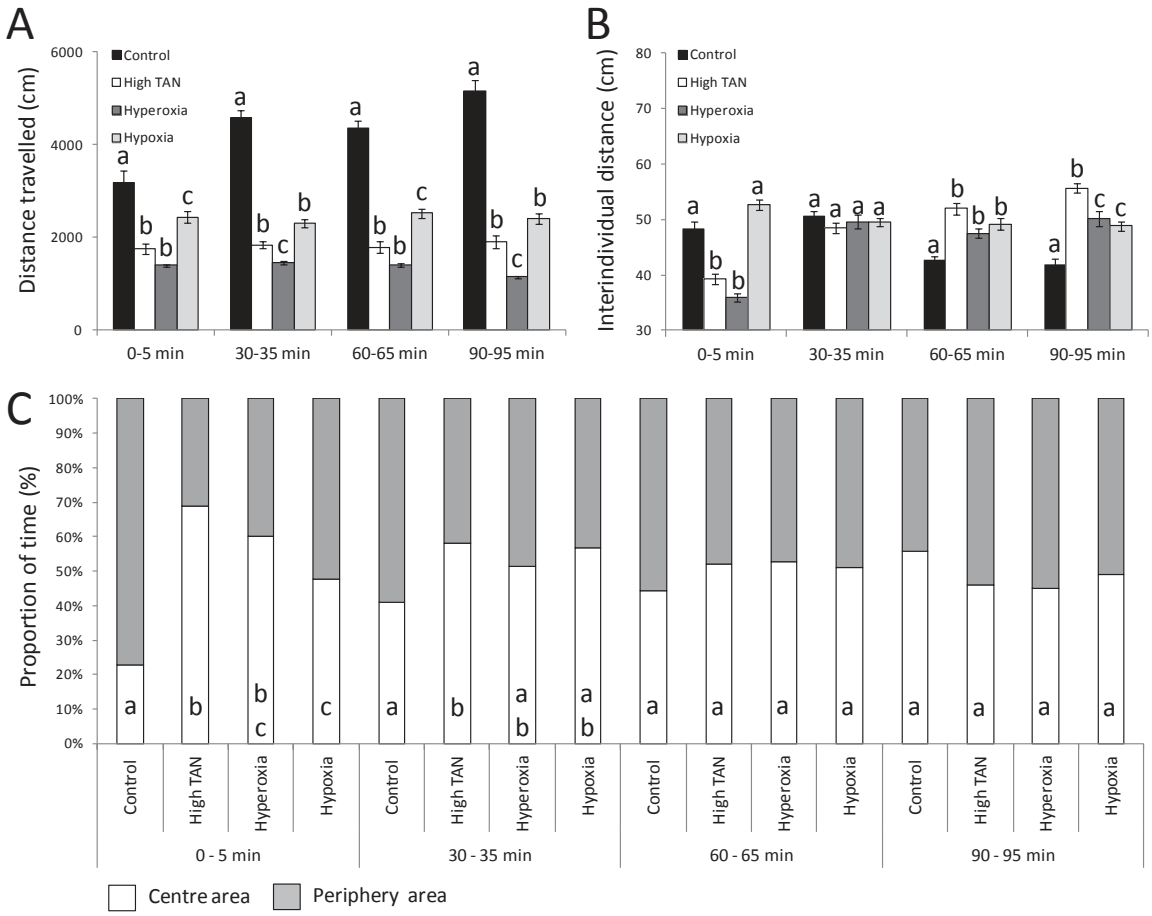

Figure 4. Swimming characteristics in the novel environment in group challenge. Mean ± SEM of **(A)** distance travelled (cm); **(B)** interindividual distance (cm); **(C)** proportion of time spent in different area (%) for each condition (n=30 control, High TAN, Hyperoxia and Hypoxia). Tukey HSD *post*-hoc: different letters indicate significant differences between conditions within observation periods.

Time spent in periphery area differed between conditions (Factorial ANOVA, F=15.3 df=3, p<0.001), not between periods (F=0.5, df=3, p=0.66) but the interaction between conditions and periods was also significant (F=10, df=9, p<0.001). Fish from control condition spent more time in Centre area starting at the second period (30-35 min) until the end (90-95 min) compared to the first 0-5 min period (p=0.003, p=0.003 and p<0.001 for 30-35 min, 60-65 min and 90-95 min respectively). During the first 0-5 min period of the test, fish from High TAN, Hyperoxia and Hypoxia conditions spent less time in periphery area than control ones (p<0.001 for both conditions) whereas they spent the same time in periphery during the rest of the test, with the exception of High TAN which still spent more time in Centre than Control during 30-35 period (**Figure 4.C)**.

3.4. Novel tank diving test *post*-stress condition

Irrespective of the condition, fish progressively explored the top area of the novel diving tank (Repeated-measures ANOVA, F=5.9, df=4, p<0.001). Condition (Repeated-measures ANOVA, F=11.8, df=3, p<0.001) and interaction between conditions and period effects were also found (Repeated-measures ANOVA, F=4.4, df=12, p<0.001; **Figure 5.A**). Total time spent in top area differed depending on conditions (One-way ANOVA, F=11.8, df=3, p<0.001; **Figure 5.B**) with High TAN bathed-fish spending significantly more time in the top area than the three other conditions (p<0.001).

Distance travelled by fish varied in relation to the duration of the test (Repeated-measures ANOVA, F=13.0, df=4, p<0.001), condition (Repeated-measures ANOVA, F=15, df=3, p<0.001) and interaction between period and condition (Repeated-measures ANOVA, F=3, df=12, p<0.001; **Figure 5.C**). Total distance travelled by fish also differed between condition (One-way ANOVA, F=15, df=3, p<0.001; **Figure 5.D**). High TAN, Hyperoxia and Hypoxia bathed-fish travelled less distance than control ones during novel tank in all cases (p<0.001 for all conditions). No difference was observed for the number of transitions between the two areas between condition (One-way ANOVA, F=2.3, df=3, p=0.8; **Figure 5.E**) as well as for the latency to enter into the top area (One-way ANOVA, F=0.5, df=3, p=0.7; **Figure 5.F**).

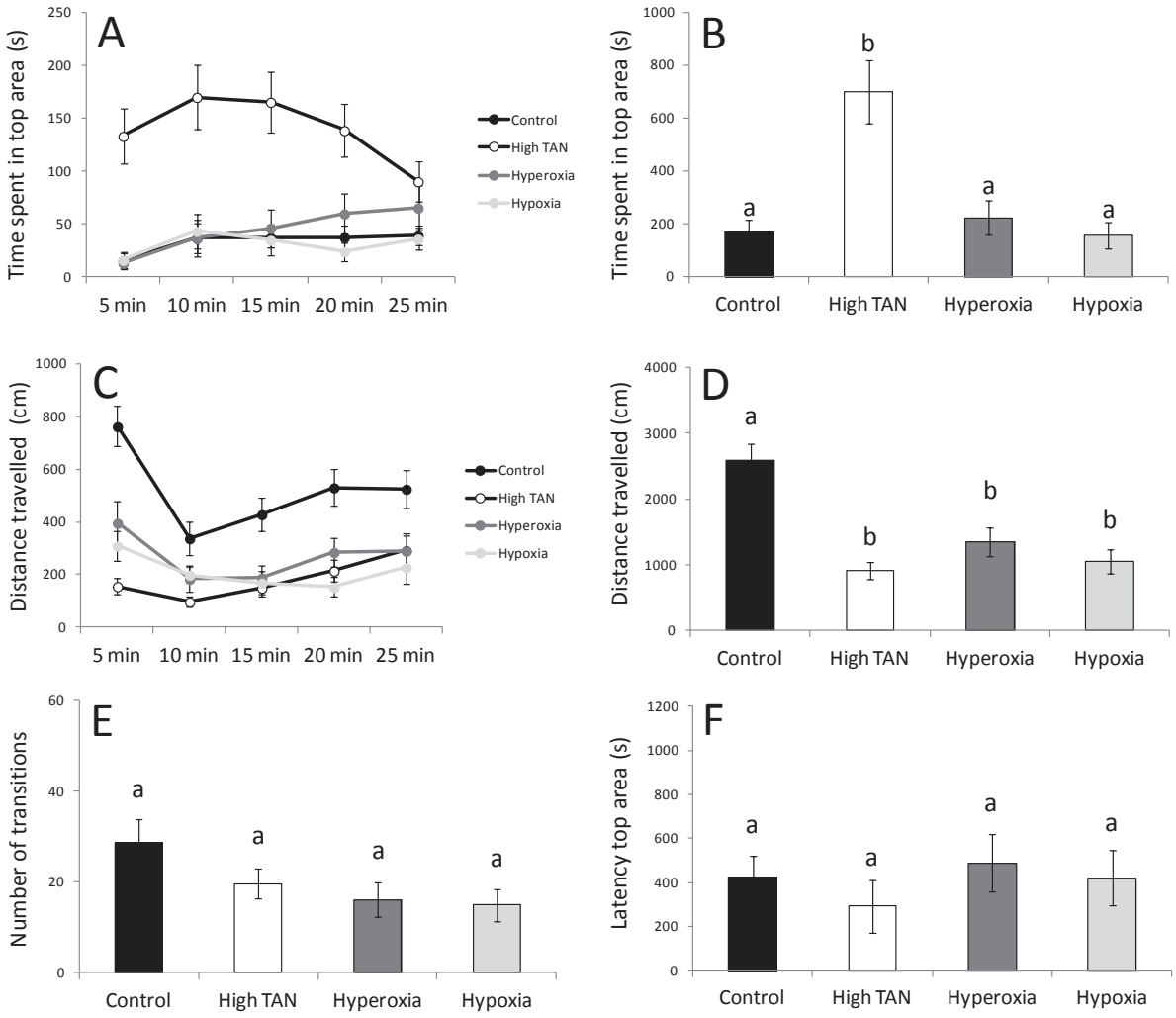

Figure 5. Swimming characteristics in the novel tank diving test. Mean ± SEM of **(A)** time spent in top area (s); **(B)** total time spent in top area (s); **(C)** distance travelled (cm); **(D)** total distance travelled (cm); **(E)** number of transitions between the two areas and (F) latency to go in top area (s) *post*-stress for fish from for each exposure-condition (n=20 control, n=20 High TAN, n=18 Hyperoxia; n=18 Hypoxia). Tukey HSD *post*-hoc: different letters indicate significant differences between conditions.

3.5. HPI axis response

Cortisol concentration in plasma for fish from Hyperoxia condition was not different from control ones (Wilcoxon sum rank test, Z=0.1, p=0.93, **Figure 6**). On the contrary, plasma cortisol concentration was higher both in fish from High TAN and Hypoxia conditions than control ones (Mann & Whitney U-test: Z=-3.5, p<0.001 and Z=-2.1, p=0.04 respectively).

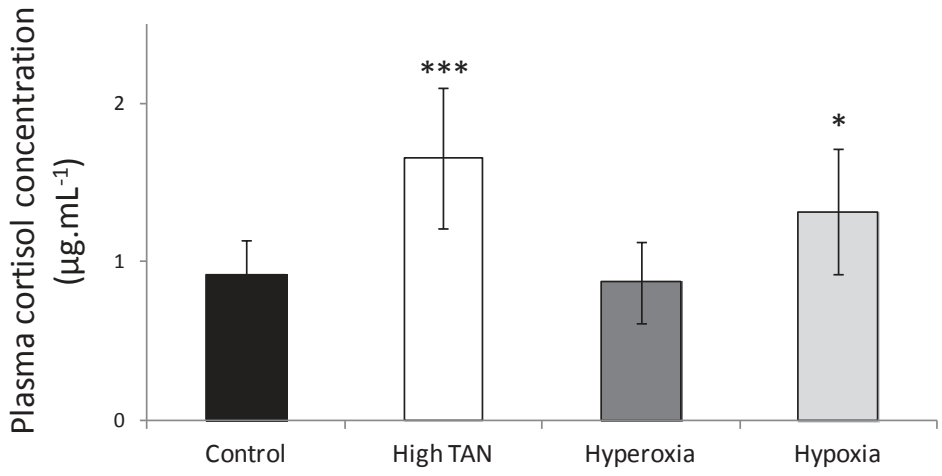

Figure 6. Plasma cortisol concentration *post*-stress in the novel environment group challenge (Mean ± SD) for each condition (n=9 control, n=9 High TAN, n=9 Hyperoxia; n=10 Hypoxia). Mann & Whitney U-test: *: $p<0.05$; ***: $p<0.001$.

4. Discussion

Overall, this study allowed the translation of the novel tank diving test to evaluate anxiety in a model marine farmed fish and this opened the possibility to evaluate psychological stress following either manipulation, biotic or abiotic factors environmental changes. Further, we have characterized responses to short term exposures to High Total Ammonia Nitrogen (TAN), Hyperoxia and Hypoxia using, along with activity, thigmotaxis, which is a stereotypical response consisting in staying in periphery instead of the centre of a novel arena following introduction (Schnorr et al., 2012; Simon et al., 1994), and group dispersion as complementary behavioural indicators measured in groups. Final objective was to evaluate individual anxiety-like behavioural responses following stressful water quality exposure using the novel tank diving test as translated from zebrafish.

Diving in an anxiogenic environment is a common behaviour in fish which has been used to define a procedure suitable to measure anxiety. Existing protocol was determined for zebrafish (Levin et al., 2007) and it has been successfully transferred to other fish species such as three-spine stickleback and fathead minnow (Margiotta-Casaluci et al., 2014; Thompson et al., 2016). Comparison of protocols used suggested the need for species-specific adaptations for *e.g.* tank size and test duration. Sea bass being a large fish characterised by a high stress level (Fanouraki et al., 2011; Levin et al., 2007) we extended test duration to 25 minutes. It is to note that whatever this extension, the proportion of time spent in the upper

part of the tank remained limited to approximately 10%. Prior acute incubation in nicotine induced the expected relief of anxiety as observed in zebrafish (Levin 2007). It is to note however that the concentration we used, 5 $mg.L^{-1}$, was much lower than the one efficient in zebrafish (100 $mg.L^{-1}$) while a concentration of 50 $mg.L^{-1}$ was not able to induce change in bottom dwelling behaviour still in zebrafish (Levin et al., 2007). Since the effect was visible already after the first 5 min of recording this is not related to a question of timing effect. In zebrafish, exposure to nicotine results in a release from bottom dwelling behaviour (swimming in the top area of the novel tank) and a lower increase in locomotor activity. In the present study, after exposure to nicotine, sea bass spent more time in the top area than control fish as found by Levin et al. (2007) but sea bass were more active (*i.e.,* travelled more distance and showed a higher transitions number between tank areas) compared to control. Changes in activity has already been shown in zebrafish after exposure to nicotine, with increase then decrease in activity over test duration or significant hyperactivity (Bencan and Levin, 2008; Levin et al., 2007). In sea bass, both increase in time spent in the top area and even more in distance travelled after exposure to nicotine was reduced over time with no more difference in distance travelled between control and nicotine-exposed fish for the last time bin. This is in agreement with the observation made in zebrafish that the increase in time spent in top area upon exposure to nicotine is reduced 20 min after exposure and abolished 40 min after (Levin et al., 2007). Altogether, this may suggest a much higher anxiolytic effects of nicotine in sea bass than in zebrafish. We can thus suggest that nicotine acts as anxiolytic drug and its effects are behaviourally observable in European sea bass as well as for zebrafish, which allow a first validation of the novel tank diving test for anxiety-like behaviour monitoring in a marine teleost, European sea bass.

Water quality measured during both bathing period and novel environment in group experiment showed that the four experimental conditions were different. Oxygen saturation was at 100 % and TAN concentration was under 2.5 $mg.L^{-1}$ in control condition whereas TAN concentration was approximately 8 fold higher in High TAN condition and oxygen saturation was 2 fold higher and 5 fold lower in Hyperoxia and Hypoxia conditions respectively. Targeted concentration of TAN, corresponding to a concentration of NH_3 of 0.8 $mg.L^{-1}$, was approximately half of the LC50 concentration for European sea bass after 96 h of exposure (Person-Le Ruyet et al., 1995). Oxygen saturation chosen both during Hyperoxia and Hypoxia conditions were known to affect fish physiology and behaviour during chronic exposure without producing any lethality (Chapman and Mckenzie, 2009; Espmark and Baeverfjord,

2009; Rimoldi et al., 2016). Both concentrations of oxygen and TAN used in the present study were quite extreme but they can occur occasionally (or even co-occur) in aquaculture conditions in case of technical failures. In the novel environment in group, control fish displayed thigmotaxis and spent most of the time in periphery area during the first 5 minutes of the test. Handling fish from bathing tank to a novel environment induced a typical thigmotaxis behavioural response. This thigmotaxis has been shown to indicate an anxiety state (Prut and Belzung, 2003; Schnorr et al., 2012). Interestingly, fish from High TAN, Hyperoxia and Hypoxia conditions spent less time in periphery than control fish, which suggest that they were not displaying anxiety-like behaviour alike control fish in response to handling. High TAN, Hyperoxia and Hypoxia conditions are factors that can directly affect fish survival (Magaud et al., 1997; Person-Le Ruyet and Boeuf, 1998; Rimoldi et al., 2016; Shimps et al., 2005) whereas handling is mostly documented for its effects on HPI axis (Barton 2002). To our knowledge, handling do not have direct effects on survival except if fish were injured which is not the case in our experiment. Therefore, our results suggest that when fish are coping with stressors affecting survival, the thigmotaxis expression in response to a psychological stressor is overruled. Further, during novel environment in group, control fish travelled progressively more distance over the test duration whereas High TAN, Hyperoxia and Hypoxia conditions expressed lower and stable distance travelled. Such lower activity under High TAN was described before in rainbow trout (Shingles et al., 2001) and under Hypoxia conditions in common sole (*Solea solea*; Dalla Via et al. (1998)) and unoperated dogfish (*Scyliorhinus canicula*; Metcalfe and Butler (1984)) whereas Hyperoxia was shown to lead to higher variability of the activity pattern in Atlantic salmon (Espmark and Baeverfjord, 2009). It is, however, important to note that behavioural responses under hypoxia were found to be clearly dependant on the intensity, duration of hypoxia and species (Chapman and Mckenzie, 2009). NH_3 is known to cause asphyxiation by reducing the blood oxygen-carrying capacity and hence alter swimming performances reported above (Camargo and Alonso, 2006; Shingles et al., 2001). Indeed, decrease in oxygen or increased in TAN concentration both lead to reduced active metabolic rate (Muusze et al., 1998; Shingles et al., 2001) and therefore explain why exposed fish are being less active than controls. It seems that fish adopted a “wait and see” strategy consisting in minimizing energy expenditure and waiting for an improvement in water quality (Clingerman et al., 2007; van Raaij et al., 1996). Finally, concerning interindividual distances, during the 0-5 min period of novel environment in group, fish from Control and Hypoxia conditions expressed the same group cohesion whereas fish from high TAN and Hyperoxia conditions were closer to each other. From 60-65

to 90-95 min periods, fish from high TAN, Hyperoxia and Hypoxia conditions, however expressed lower group cohesion than control fish. Interestingly, Domenici et al. (2002) reported the same larger dispersion in Atlantic herring under O_2 saturation of 20 % and same results were observed in Atlantic salmon under O_2 saturation of 150 % (Espmark and Baeverfjord (2009). Lower group cohesion was also observed in rainbow trout under hypercapnia by Sadoul et al. (2017). When given the possibility, it is well known that fish are able to avoid stressful environment such as hypoxic waters, with high ammonia or high CO_2 concentrations (Clingerman et al., 2007; Herbert et al., 2010; Richardson et al., 2001; Skjæraasen et al., 2008). Therefore we suggest that fish interindividual distances increase to maximize oxygen uptake (Domenici et al., 2017) and as such could be used as relevant stress indicator (Espmark and Baeverfjord, 2009; Juell, 1995). Further experiments are still needed to fully understand the observed disruptions in shoal cohesion but it overall fits well with the on growing research effort about the effects of abiotic factors on fish group behaviour (Colchen et al., 2017; Domenici et al., 2002, 2017; Espmark and Baeverfjord, 2009; Sadoul et al., 2017; Weetman et al., 1999).

Concerning HPI axis responsiveness, plasma cortisol concentrations measured were high for European sea bass and that is likely explained by the combination of handling and confinement stress they experienced (Rotllant et al., 2003; Samaras et al., 2016). Indeed, we believe that the first handling between rearing and bathing tanks and then the second between bathing tank and experimental tanks are both responsible for the high plasma cortisol concentrations. Measured plasma cortisol concentration was similar between fish exposed to Hyperoxia and control fish but was higher in fish exposed to Hypoxia and High TAN conditions. Such increase in cortisol release was previously reported in Atlantic salmon following chronic exposure to ammonia (0.15 $mg.L^{-1}$, Knoph and Olsen, 1994) and hyperoxia (150 % O_2 saturation, Espmark and Baeverfjord, 2009) or acute exposure to hypoxia (25 % of O_2 saturation) in rainbow trout (van Raaij et al., 1996). Cortisol may act as a signal to adjust behavioural responses described before to cope with Hypoxia or High TAN conditions (Barton, 2002). The lack of cortisol increase in Hyperoxia exposed fish, nevertheless displaying behavioural disruptions compared to control fish, may suggest that others physiological factors involved in stress response such as plasma catecholamines could play a key role in behavioural responses to stressors as already shown in rainbow trout (Gesto et al., 2013; van Raaij et al., 1996).

After 2 h-exposure to any of the tested conditions (High TAN, Hyperoxia and Hypoxia), fish travelled less distance in novel tank diving test than control fish under standard condition (100 % O_2 saturation and TAN < 2.5 mg.L-^{1}) during the first 5 minutes. Thereafter fish all reached the same activity level. Such behavioural recovery from hypoxia in European sea bass is rapid and similar to that reported in Nile Tilapia (*Oreochromis niloticus*, Xu et al. (2006)). To our knowledge, it is the first time that behavioural recovery from Hyperoxia and High ammonia concentration has been monitored. In addition to distance travelled, fish previously exposed to High TAN explored the top area more than control fish and decreased progressively time spent in top to reach control's level after 20 min in standard water. On the contrary, the two other conditions (*i.e.,* Hyperoxia and Hypoxia) explored progressively the top area in the same way than control fish. The fast release from bottom dwelling (or the search for access to water surface) observed in High TAN exposed fish resembled the surface swimming observed for nicotine-exposed fish. As explained before for novel environment in group, the lower anxiety-like behaviour observed in High TAN fish could be the results of fish setting priority to cope with TAN instead of expressing psychological stress effects.

In conclusions, our results showed that thigmotaxis, swimming activity, group cohesion and bottom dwelling behaviour are reliable behavioural indicators of animal health and welfare status. Moreover, this study reports the fact that it is important to distinguish between stressors that could affect survival and psychological stressors such as handling or confinement alone. Besides cortisol, others molecular factors should be sought for to fully understand individual stress responses depending on the types of stressors. Finally, the novel tank diving test seems to be a sensitive and promising challenge test for screening recovery state and thus better evaluate welfare status in farmed fish.

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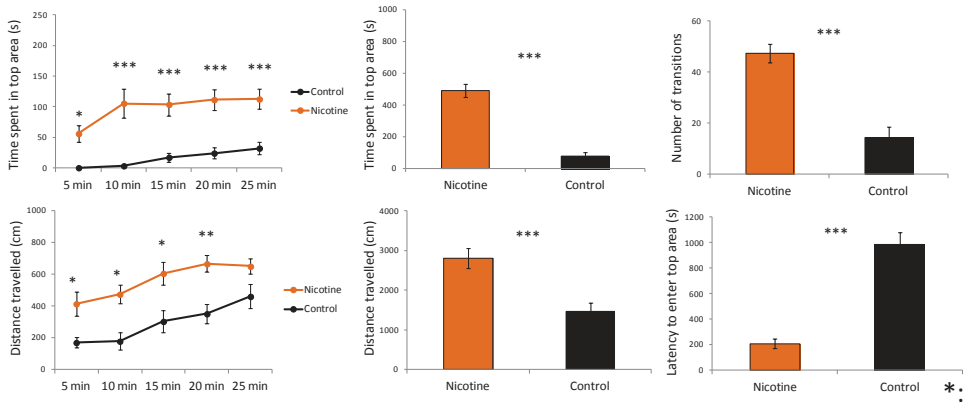
Chapitre 5 – Conclusions

Résultats:

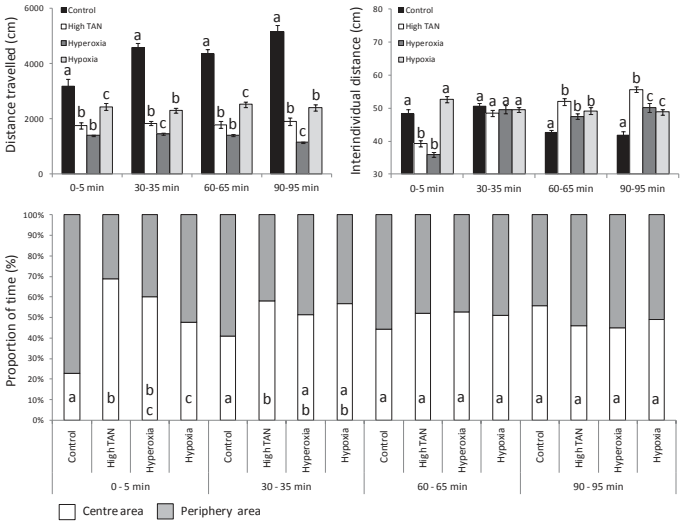
• Validation du nouvel environnement individuel: utilisation de la nicotine



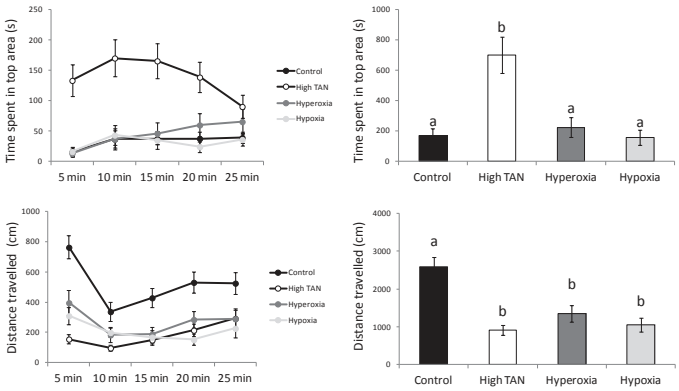
Avant utilisation du nouvel environnement comme indicateur du niveau d'anxiété, il a été validé en amont en étudiant le comportement des bars préalablement exposés à la nicotine, qui est une molécule anxiolytique.

*: p<0.05, **: p<0.01, ***: p<0.001

• *Open field* en groupe:



• Nouvel environnement individuel:



• Légende :

Contrôle	TAN
Hyperoxie	Hypoxie

Points importants:

• Suite à une exposition à une mauvaise qualité d'eau, l'activité des bars est réduite et la cohésion du groupe est plus faible. La réponse normale (thigmotaxie) suite à un test en *open field* est inhibée. Cette exposition entraîne une élévation du taux de cortisol plasmatique pour les conditions TAN et hypoxie mais pas pour la condition hyperoxie.

➔ **L'inhibition de la thigmotaxie chez les individus évoluant dans des conditions dégradées suggèrent que les bars font face en priorité aux facteurs de stress pouvant affecter leur survie *vs.* manipulation.**

• En phase de récupération dans une bonne qualité d'eau, les bars reprennent un comportement normal après 5 minutes (hyperoxie, hypoxie) ou après 20 minutes (TAN) dans le test du nouvel environnement individuel.

Communications:

• **Society for Experimental Biology – Florence – 2018**
Communication orale
• **World Aquaculture Society – Montpellier – 2018**
Communication orale

Chapitre 6

Divergent behavioural responses to hypoxia for opposed coping styles in European sea bass, *Dicentrarchus labrax*

Sébastien Alfonso
Bastien Sadoul
Béatrice Chatain
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Marie-Laure Bégout

Manuscript en préparation

Chapitre 6 – Présentation

Contexte:

Les *coping styles* sont définis comme une suite de traits comportementaux corrélés entre eux dans différents contextes et corrélés aux réponses physiologiques, stable dans le temps et le contexte. Deux réponses extrêmes sont définies: une réponse active (*proactive*) et une réponse passive (*réactive*), et sont souvent mesurées *via* le niveau d'audace des individus. Les *coping-styles* peuvent devenir importants (être une variable explicative supplémentaire des variations observées ou être sélectionnés) dans le domaine de l'aquaculture afin de maximiser les gains de production tout en améliorant les conditions de bien être des téléostéens. Des diminutions d'oxygène, conduisant à des conditions d'hypoxie, sont de plus en plus fréquentes en milieu naturel et ponctuellement en milieu d'élevage. Il apparait donc important d'étudier les capacités d'adaptation à ce phénomène en fonction des *coping styles* des téléostéens.

Questions:

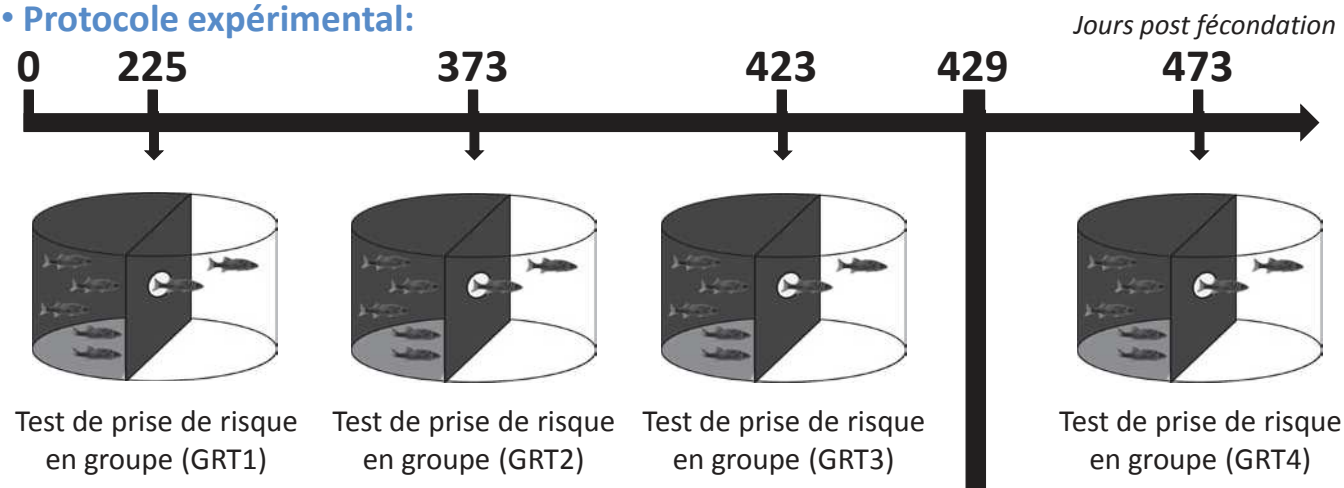
(1) Des bars catégorisés selon leur audace expriment-ils des réponses comportementales et physiologiques différentes lorsqu'ils sont soumis à une condition d'hypoxie ?

(2) L'exposition aigue à une condition d'hypoxie altère-t-elle leur niveau d'audace ?

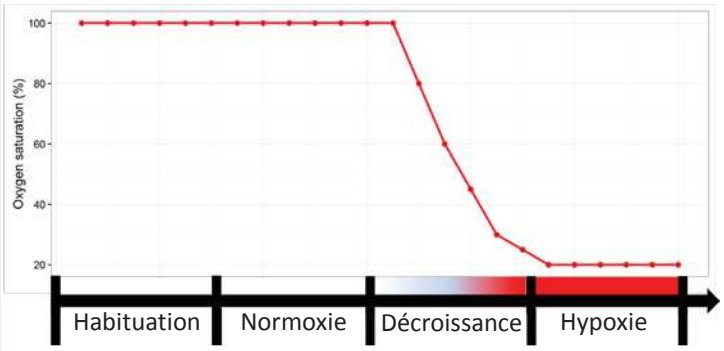
Méthode:

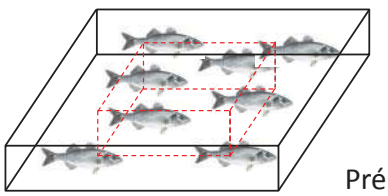
L'**audace** des bars a été mesurée **3 fois** à l'aide d'un test de prise de risque en groupe (GRT). Les individus audacieux et timides extrêmes sur l'ensemble des GRT ont été ensuite testés dans un *open field* afin d'étudier leurs **réponses comportementales** (**activité**, **thigmotaxie**, **cohésion de groupe**) face à des **conditions d'hypoxie**. Finalement, leur **audace** a été **mesurée une dernière fois** (GRT4), **34 jours après**, afin d'étudier l'impact de l'exposition aigüe à l'hypoxie sur l'audace. Le cerveau a été prélevé pour réaliser une analyse transcriptomique (*actuellement en cours*).

• Protocole expérimental:



• Challenge dans des conditions d'hypoxie:





Test en *open field* en groupe

Thigmotaxie | Activité

Cohésion du groupe

Prélèvement cerveau

Analyses transcriptomique

Divergent behavioural responses to hypoxia for opposed coping styles in European sea bass, *Dicentrarchus labrax*

In preparation

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Abstract

Coping styles are defined as a coherent set of individual physiological and behavioural differences (along five personality axes) in stress responses that are consistent across time and context. Such different behavioural and physiological responses to stressors are promising phenotypes to answer ecological and aquaculture sustainability issues including the capacity to respond to low oxygen concentration in water. The aim of the study is to analyse behavioural and physiological responses of fish to acute hypoxia exposure (20% O_2 saturation) according to their coping styles. To do so, we first characterised one axis of personality *i.e.* boldness using risk taking tests in group (GRT) three times from 225 to 423 days post fertilization (dpf) in European Sea bass. Individuals showing extreme behaviours (bold *vs.* shy) in the three tests were selected for further behavioural characterization for activity and exploration (two other axes of personality) in a novel environment. Behavioural responses in groups of 8 fish were measured in the novel environment under a hypoxia challenge at 429 dpf. At 473 dpf, fish were finally challenged in a fourth risk taking test in group to evaluate consistency in coping style characterization. Finally, brains of a sub sample of fish were preserved for further transcriptomic analysis. Shy fish during the GRT displayed higher thigmotaxis following introduction in a novel environment and also showed a higher activity both under normoxia and hypoxia conditions. Interestingly, the decrease in thigmotaxis behaviour from normoxia to hypoxia conditions was higher in shy fish than in bold ones whereas the decrease in activity and group cohesion were more pronounced in bold fish suggesting two distinct adaptive strategies in relation to individual coping styles. In addition, challenged fish showed a consistent risk taking behaviour in the last GRT, suggesting that the acute hypoxia exposure did not alter boldness level. Overall, the different responses observed when facing stressors and consistency across long time period even after stressors exposure, highlight how relevant coping styles are for further aquaculture domestication/selection processes and for a better understanding of ecological issues.

1. Introduction

Coping styles are defined as "a coherent set of individual physiological and behavioural differences in stress responses consistent across time and context" (Koolhaas et al. (1999). It has been described in various animal species, including fish, and describes a *continuum* between two extreme phenotypes, called proactive and reactive (Castanheira et al., 2017; Ferrari et al., 2015; Øverli et al., 2007; Tudorache et al., 2013). In terms of behaviour, proactive fish are bolder, more aggressive, explore their environment faster and display less flexible behaviour than reactive ones (Castanheira et al., 2017; Ferrari et al., 2015; Huntingford et al., 2010; Millot et al., 2009; Øverli et al., 2004, 2006). Moreover, correlations between these behavioural responses across time and context were established in several fish species (Castanheira et al., 2017; Geffroy et al., 2015; Øverli et al., 2007) as well as between behaviour and physiology (Ferrari et al., 2015; Johansen et al., 2012b; Killen et al., 2011; MacKenzie et al., 2009; Øverli et al., 2006; Rey et al., 2013; Silva et al., 2010) and these are essential to define coping style.

In response to adverse events, fish typical stress response involves two main axes, the catecholamines axis and the hypothalamic-pituitary-interenal (HPI) axis (Barton, 2002; Gesto et al., 2013; Sumpter, 1997). This stress response appears to be different depending on coping styles. Indeed, reactive fish respond to adverse event by displaying higher HPI activity and release of catecholamines than proactive ones (Øverli et al., 2001, 2002, 2007; Vindas et al., 2017). In fish, stress regulation is also closely related to neurogenesis (Best et al., 2017; Sadoul et al., 2018; Sorensen et al., 2011) and reactive fish as for them tend to display higher neurogenesis than proactive ones (Johansen et al., 2012a) which may explain higher behavioural plasticity in reactive fish. Moreover, coping styles also diverge in terms of metabolism, with bold fish (proactive) displaying higher metabolic rate (Herrera et al., 2014; Killen et al., 2012).

All these differences in behavioural and physiological responses to stressors are promising to tackle ecological and aquaculture fish adaptive issues in a changing world (Castanheira et al., 2017; Diaz and Breitburg, 2009). In aquatic environment, the simultaneous increase in nutrient inputs in the water from land use and the increase in temperatures from global warming both result in a decrease in oxygen concentration in the water (Diaz and Breitburg, 2009). Under aquaculture conditions, oxygen concentration can also promptly decrease due to management practices aiming at maximizing production and/or technical failures. Hypoxia

exposure may have consequences on growth (Pichavant et al., 2001; Pichavant et al., 2000), swimming capacities (Chapman and Mckenzie, 2009; Domenici et al., 2002; Lefrançois et al., 2005, 2009), coping abilities (van Raaij et al., 1996), and ultimately fitness which can be critical for fishes (Perdersen, 1987; Pichavant et al., 2000; Rimoldi et al., 2016; Wang et al., 2016; Wu et al., 2003).

The European sea bass *Dicentrarchus labrax* is a predatory species, widely distributed around Europe and West Africa (FAO, 2012), that has a key ecological role in the marine food chain (Whitehead et al., 1986) and also one of the most important farmed fish species in Europe (Tveteras and Nystoyl, 2011). Therefore, additional knowledge about behavioural and physiological responses to hypoxia in European sea bass according to their coping style may be highly relevant to improve management in a context of increase in hypoxic events frequency in natural environments (Diaz and Breitburg, 2009) and in the context of fish welfare in aquaculture facilities (Castanheira et al., 2017; Ferrari et al., 2015, 2016a).

The aim of the study was to analyse behavioural and physiological responses of fish to acute hypoxia exposure (20% O_2 saturation) in relation to their coping styles. To do so, we first characterised one axis of personality i.e. boldness using risk taking tests in group (GRT) three times at 225, 373 and 423 days *post* fertilization (dpf). Bold and shy fish were then screened in groups of 8 individuals for thigmotaxis, activity and group cohesiveness under progressive hypoxia condition to evaluate group and individual behavioural responses at 429 dpf. At 473 dpf, fish were challenged a last time in GRT to evaluate consistency in boldness trait and hence coping style characterization. Finally, brain of a sub sample of fish was preserved for further transcriptomic analyses.

2. Material and methods

Experiments were authorized by ethics committee agreement APAFIS#10745 and all procedures involving animals were in accordance with the ethical standards of the institution and followed the recommendations of Directive 2010/63/EU.

2.1. Fish rearing and pit-tagging

The reproduction, as well as larval rearing of European sea bass, was performed at Ifremer, Palavas-les-Flots (France, 34250). Eggs were incubated at 13.5±2.5°C, and larvae reared according to rearing standard following (Chatain, 1994). From 121 dpf onwards, rearing temperature was set at 21°C. At 175±4 dpf fish were anesthetized using 200 ppm of

Benzocaine (150 g/L, E1501, Sigma, Saint Louis, MO, USA), measured for weight and length (14.09±4.4 g, 103.18±9.4 mm at tagging), and 583 individuals were pit-tagged in the muscle just in front of the dorsal fin with ISO PIT tag (8 x 1.4 mm), to ensure individual identification.

Fish were kept in 3 different 1.5 m^3 tanks within a recirculating bio-filtered system under a 12/12 light/dark period until the first group risk taking test (see below). All along the rearing, fish were fed *ad libitum* by self-feeder using a standard aquaculture diet adapted to fish sizes (Neo-grower, Le Gouessant, France).

2.2. Group risk-taking test

The group risk-taking test (GRT) is a standardized test to measured boldness in fish and was adapted from Ferrari et al. (2015). GRT was performed four times at 255±4, 373±1, 423±3 and 463 dpf and thereafter called GRT1, GRT2, GRT3 and GRT4. Between GRT3 and GRT4, 14 unselected fish died, therefore a total of 574 fish were assessed in GRT4.

GRT consists in placing all the fish from a rearing tank into half of a tank (exact same 1.5 m^3 tank as the home tank), separated in two by a PVC opaque divider. Fish were gently transferred from their home tank to one half of the experimental tank under a light anaesthesia (100 ppm of Benzocaine, 150 g/L, E1501, Sigma, Saint Louis, MO, USA) between 10:00 and 11:00 am. The side containing the fish was covered with a black opaque curtain, to create a sheltered area. Both half of the tank received the same water quality, oxygenation and had the same exchange rate. After a habituation period of 2 hours, the door (a circular opening gate of 12 cm Ø in the centre or the divider) was opened and fish were left with the possibility to exit the sheltered area, therefore exhibiting a risk-prone behaviour. The door was equipped with a PIT-tag detection antenna (DORSET, The Netherlands) connected to a control device upgraded from Ferrari et al. (2016a). PIT-tag detection antenna allowed monitoring fish behaviour during the GRT, by obtaining the exact exit time (*Latency to exit (s)*) of each fish leaving the sheltered area for the first time.

As the test lasted for 20 hours, a latency of 1200 min, was assigned to fish that did not exit the sheltered area before the end of the test. Bold and shy fish were split in two groups according to the first and fourth quartiles of each individual mean Latency to exit calculated over GRT1-GRT2-GRT3 (*i.e.* fish with higher values were considered shy and fish with lower value were considered bold). Thus, among 583 fish challenged in GRT, 146 were considered

as shy and 146 are considered as bold. After GRT3, 48 fish per coping style were randomly selected among 146 screened as bold or shy for further analyses (Hypoxia challenge, see below). Those 96 fish were tested again in GRT4 with all the unselected fish.46 assessed in the three first GRTs.

2.3. Hypoxia challenge

Among fish screened during GRT1, GRT2 and GRT3, a sample of 48 individuals per coping style (*i.e* n=48 bold and n=48 shy) were challenged in a Hypoxia challenge in group of 8 fish (*i.e* n=6 trials*8 individuals per coping style).

Hypoxia challenge was adapted from Alfonso et al. (chapitre 5) and performed at 429±2 dpf (91.0±6.6 g, XX.194±4.8 cm in average). The day before the Hypoxia challenge, a group of 8 fish (either bold or shy group) was isolated from the rearing tank and kept overnight in a separated 300L tank under the same water conditions so as to avoid important handling just prior to the hypoxia challenge. The next day, fish were gently transferred to an Open field arena (75cm x 75 cm x 21.5 cm of water height, 120 L) where the Hypoxia challenge took place. A corner (15 cm x 15 cm, 225 cm^2) was isolated from the open field arena by a grid and contained a pump, oxygen and nitrogen aerators to maintain targeted oxygen concentration and an oxygen probe (Odéon, NEOTEK-PONSEL, Caudan, France) to record oxygen saturation every 5 min all along the experiment. The test lasted 2 hours and consisted in 4 periods of 30 min hereafter respectively called Habituation, Normoxia, Decrease and Hypoxia periods. Both during Habituation and Normoxia periods, oxygen saturation was maintained above 100 %. The first 30 minutes served as a Habituation period for fish and we considered after this period that the group expressed standard behaviour during Normoxia period. After one hour, oxygen saturation was slowly decreased over 30 minutes (the Decrease period) to reach the targeted saturation of 20 % which is considered as severe hypoxia. During the last 30 minutes of the experiment, the Hypoxia period, the oxygen saturation was maintained constant at 20 % of oxygen saturation.

During the Hypoxia challenge, behaviour was recorded with a DMK 31AU03 camera (The imaging source, Germany) and video were analysed using EthoVision XT 13.1 software (Noldus, The Netherlands). The arena was separated into two areas according to Schnorr et al. (2012): the centre area, representing half of the total surface and the periphery area representing the other half. *Time spent in periphery* (s), indicative of thigmotaxis behavior, *Distance travelled* by each fish (cm), indicative of activity, and *Interindividual distance*s

between fish (cm), indicative of group cohesion (Buske and Gerlai, 2011), were calculated. Variables were recorded for each frame (25 frame/s) and were either summed (*Time spent in periphery*, *Distance travelled*) or averaged (*Interindividual distances*) over 5 min or over the 30 min corresponding to each period *i.e.* Habituation, Normoxia, Decrease and Hypoxia periods, for further statistical analyses and illustrations.

2.4. Transcriptomic analyses

Fish brains (n=5 out of the 48 tested individual per coping style) were sampled after GRT4 and preserved at -80°C to perform transcriptomic analyses which are ongoing at the time of writing.

2.5. Statistical analyses

Statistical analyses were performed using Statistica 9.0 software (StatSoft, USA). All statistical analyses were carried out at a 95 % level of significance and values are represented as mean ± SEM except where otherwise mentioned.

For comparing boldness variation between bold and shy fish (sub sample of 96 individuals used in the hypoxia challenge), repeated-measures ANOVA (GRT1, GRT2, GRT3 and GRT4) and a Newman-Keuls *post-hoc* test was applied. Moreover, a factorial ANOVA was performed between coping styles (bold and shy) and challenge (Hypoxia challenge or not) to evaluate *Boldness variation* in risk taking test before and after hypoxia challenge. *Boldness variation* (%) was calculated as follow: (Latency to exit [GRT4] - Latency to exit [GRT3]) / Latency to exit [GRT3] * 100. Newman-Keuls *post-hoc* test was applied to compare between conditions.

To ensure oxygen saturation decreases were similar during hypoxia challenges between coping styles (6*2 trials), oxygen saturation (%) was compared between trials performed on bold and shy fish with repeated-measures ANOVA (with 24 periods, *i.e.* 0-5 min, 5-10 min, [...], 110-115-min and 115-120-min)..

All behavioural variables measured in Open field test (*i.e. Time spent in periphery* (%), *Distance travelled* (cm) and *Interindividual distances* (cm)) were compared between bold and shy fish with repeated-measures ANOVA (with 4 periods, Habituation, Normoxia, Decrease and Hypoxia) and a Newman-Keuls *post-hoc* test. In order to analyse the responses to Hypoxia challenge for the three variables listed above, T-test was performed between bold

and shy fish comparing *Behavioural variation* (%) between Normoxia and Hypoxia periods. This *Behavioural variation* (%) was calculated as follow: (Behaviour [Normoxia] – Behaviour [Hypoxia]) / Behaviour [Normoxia] * 100.

3. Results

3.1. Coping style screening

Fish selected for their strong divergence in latency to exit the sheltered area in GRT1, GRT2 and GRT3, also showed consistent difference in GRT4 performed after the Hypoxia challenge (Repeated-measures ANOVA, F=1437.8, df=1, p<0.001, **Figure 1.A**). Between GRT3 and GRT4, latency to exit shelter increased in bold fish (p<0.001) but was similar for shy fish (p<0.47). A similar pattern was observed for fish which were not Hypoxia challenged (p=0.33, **Figure 1.B**).

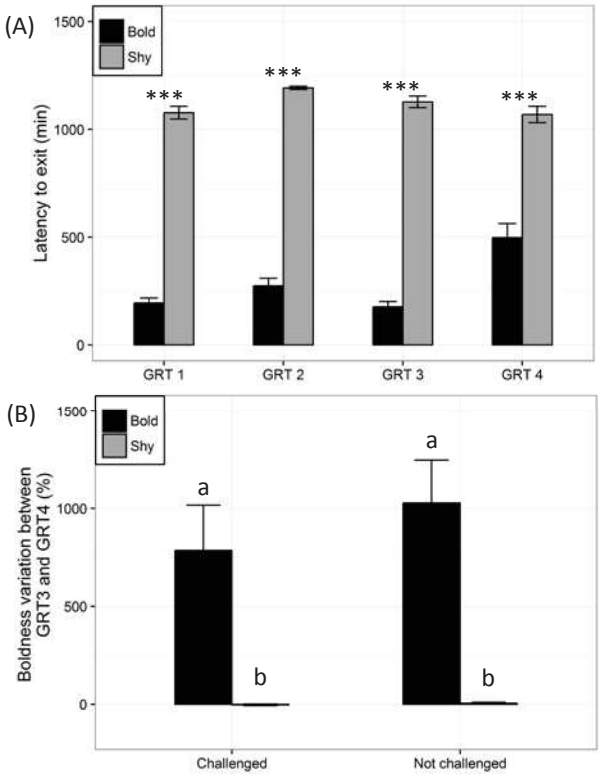

Figure 1. (A) Mean ± SEM of *Latency to exit* (min) during GRT (from GRT1 to GRT4) for bold (black bars, n=48) and shy fish (grey bars, n=48). Newman-Keuls *post-hoc* between coping styles within GRT: ***: p<0.001. **(B)** Mean ± SEM *Boldness variation* (%) between GRT3 and GRT4 for Hypoxia challenged bold (black bars, n=48) and shy fish (grey bars, n=48) and not challenged bold (black bars, n=98) and shy fish (grey bars, n=98. Different letters indicate significant differences between coping styles and challenge according to Newman-Keuls *post-hoc*.

3.2. Hypoxia challenge

Overall, 96 fish were used for Hypoxia challenge, mean body weight was 91.0 ± 4.70 g and was homogeneous between coping styles (T-test, t=0.8, p=0.43).

Oxygen saturation (%)

During the Hypoxia challenge, oxygen saturation (%) decreased significantly over the four periods as expected (Repeated-measures ANOVA, F=61.9, df=23, p<0.001) and the decrease was similar between trials involving bold or shy fish (Repeated-measures ANOVA, F=0.1, df=1, p=0.77, **Figure 2**). Moreover, there was no interaction between time course of the experiment and coping styles (Repeated-measures ANOVA, F=0.7, df=23, p=0.9). Therefore, the hypoxia challenges were similar for bold and shy fish and variables acquired during the challenge are thus fully comparable.

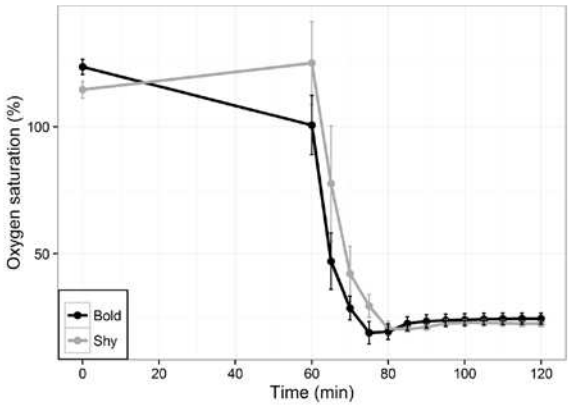

Figure 2. Mean ± SEM of oxygen saturation (%) during Hypoxia challenge for bold (black points, n=6 trials) and shy fish (grey points, n=6 trials).

Behavioural measurements

Whatever the coping styles, *Time spent in the periphery* of the arena (%), progressively decreased during the first minutes of the challenge and was stable until Hypoxia condition were reached and then *Time spent in periphery* decreased (**Figure 3.A**). *Time spent in periphery* showed a significant decrease between the periods of the experiment (Repeated-measures ANOVA, F=82.4, df=3, p<0.001), differed between the coping styles (Repeated-measures ANOVA, F=22.7, df=1, p<0.001) and the interaction between both factors was significant (Repeated-measures ANOVA, F=18.3, df=3, p<0.001 **Figure 3.B**).

In further details, for bold fish, *Time spent in periphery* was statistically similar during Habituation, Normoxia and Decrease periods but decreased during Hypoxia period compared

to previous ones (p<0.001). For shy fish, *Time spent in periphery* was higher during the Habituation periods compared to Normoxia (p<0.001), then became stable during Normoxia and Decrease periods (p=0.360) and finally decreased to reach the lowest value during Hypoxia in a similar manner than bold fish (p<0.001). *Time spent in periphery* was higher for shy fish compared to bold ones during the Habituation period (p<0.001). Bold fish displayed a lower decrease in *Time spent in periphery* between Normoxia and Hypoxia periods than shy ones (T-test, t=2.63; p=0.001, **Figure 6.A**).

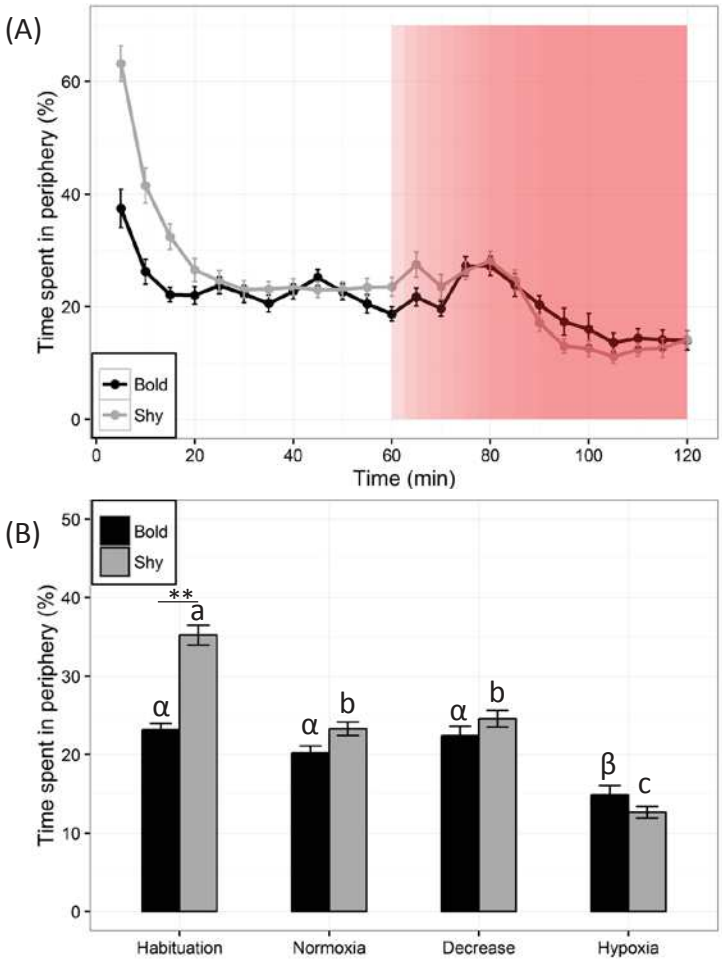

Figure 3. (A) Mean ± SEM of *Time spent in periphery* (%) during Hypoxia challenge for bold (black points, n=48) and shy fish (grey points, n=48). Progressive red background indicates Decrease period and red background indicates Hypoxia period. **(B)** Mean ± SEM of *Time spent in periphery* (%) during the different periods of the Hypoxia challenge for bold (black bars, n=48) and shy fish (grey bars, n=48). Different letters indicate significant differences between periods within coping styles (Greek letters for bold and Latine letters for shy fish) according to Newman-Keuls *post-hoc* and **: p<0.01 between coping styles inside periods.

Whatever the coping styles, *Distance travelled* by fish (cm), progressively increased during the first 30 minutes of the challenge and became stable during the following 30 minutes of the Normoxia period. Decrease in oxygen concentration induced a decrease in activity which became stable when Hypoxia period was reached (**Figure 4.A**). *Distance travelled* by fish (cm) differed between periods of the experiment (Repeated-measures ANOVA, $F=351.7$, $df=3$, $p<0.001$), differed between the coping styles (Repeated-measures ANOVA, $F=7.8$, $df=1$, $p=0.006$) and interaction of both factors was significant (Repeated-measures ANOVA, $F=9.5$, $df=3$ $p<0.001$, **Figure 4.B**).

In further details, *Distance travelled* increased between Habituation period and Normoxia for bold and shy ($p<0.001$ for both coping styles, **Figure 4.B**), and then decreased between Normoxia and Hypoxia periods ($p<0.001$ for both coping styles). Activity was similar between bold and shy fish during the Habituation period ($p=0.44$) whereas shy fish displayed higher *Distance travelled* than bold fish during Normoxia, Decrease and Hypoxia periods (respectively $p=0.03$, $p<0.001$ and $p<0.01$). However, bold fish displayed a higher *Variation in distance travelled* between Normoxia and Hypoxia conditions than shy fish ($p<0.001$, **Figure 6.B**).

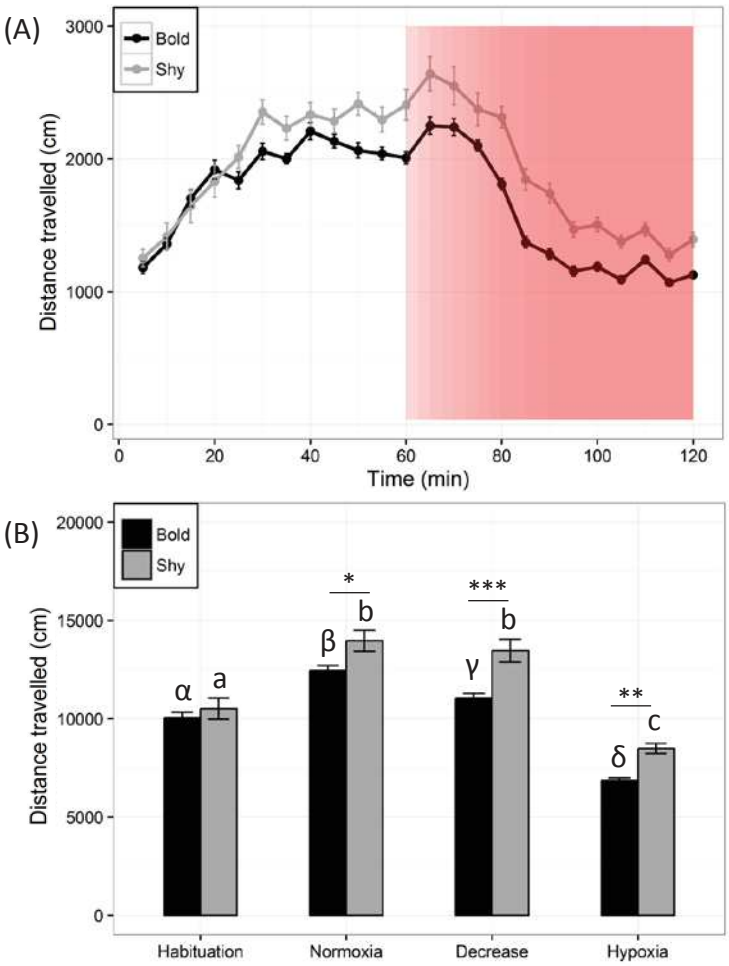

Figure 4. (A) Mean ± SEM of *Distance travelled* (cm) during the Hypoxia challenge for bold (black points, n=48) and shy fish (grey points, n=48). Progressive red background indicates Decrease period and red background indicates Hypoxia period. **(B)** Mean ± SEM of *Distance travelled* (cm) during different periods of the Hypoxia challenge for bold (black bars, n=48) and shy fish (grey bars, n=48). Different letters indicate significant differences between periods within coping styles (Greek letters for bold and Latine letters for shy fish) according to Newman-Keuls *post-hoc* and **: $p<0.01$ indicate significant difference between coping styles within periods.

Whatever the coping styles, *Interindividual distance* (cm), progressively increased following the decrease in oxygen concentration. When 20 % of oxygen saturation was reached, *Interindividual distance* increased. *Interindividual distance* differed with the periods of the experiment (Repeated-measures ANOVA, F=44.7, df=3, $p<0.001$), the coping styles (Repeated-measures ANOVA, F=6.8, df=1, p=0.01, **Figure 5.A**) and the interaction of both factors was significant (Repeated-measures ANOVA, F=3.57, df=3, p=0.01, **Figure 5.B**).

In further details, in bold fish, *Interindividual distance* decreased between Habituation and Normoxia periods (p=0.04), and between Decrease and Hypoxia periods (p=0.03) but was still higher than in Normoxia period (p<0.001, **Figure 5.B**). For shy fish, *Interindividual distance* was similar between Habituation and Normoxia periods (p=0.38), became higher in Decrease period (p<0.001), and decreased during Hypoxia (p<0.001) to reach similar level to that observed during Habituation and Normoxia periods. *Interindividual distance* was higher in shy fish during both Normoxia and Decrease periods compared to bold fish (respectively p=0.001 and p=0.03). Between Normoxia and Hypoxia periods, bold fish displayed a higher increase in *Variation in interindividual distance* than shy fish (t=2.75, p=0.007, **Figure 6.C**).

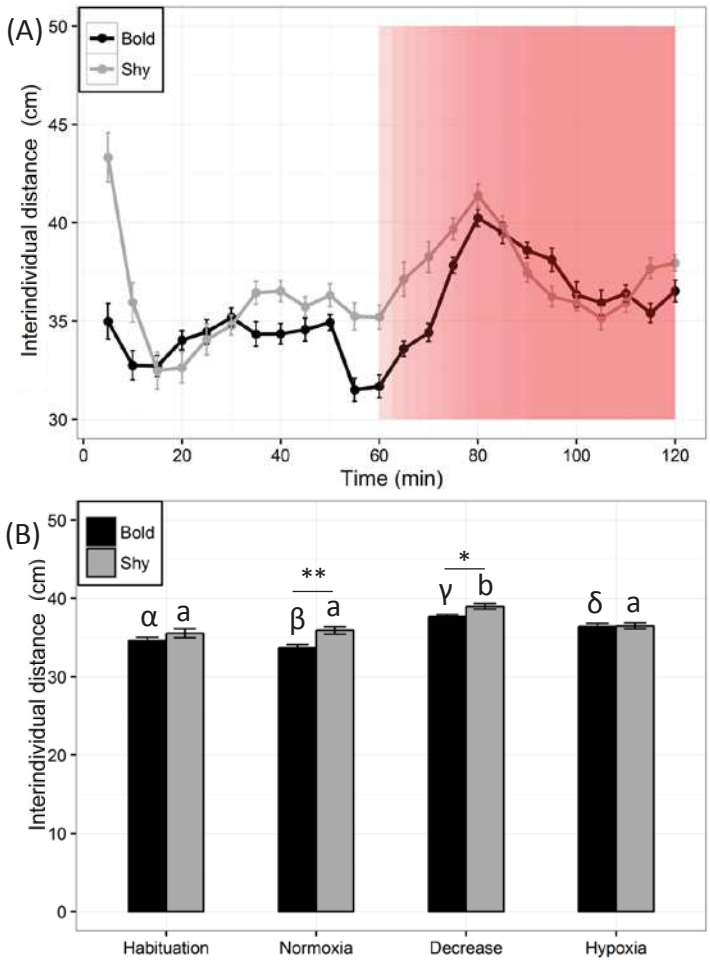

Figure 5. (A) Mean ± SEM of *Interindividual distance* (cm) during the Hypoxia challenge for bold (black points, n=48) and shy fish (grey points, n=48). Progressive red background indicates Decrease period and red background indicates Hypoxia period. **(B)** Mean ± SEM *Interindividual distance* between individuals (cm) during different periods of the Hypoxia challenge for bold (black bars, n=48) and shy fish (grey bars, n=48). Different letters indicate significant differences between periods within coping styles (Greek letters for bold and Latine letters for shy fish) according to Newman-Keuls *post-hoc* and **: p<0.01 indicate significant difference between coping styles within periods

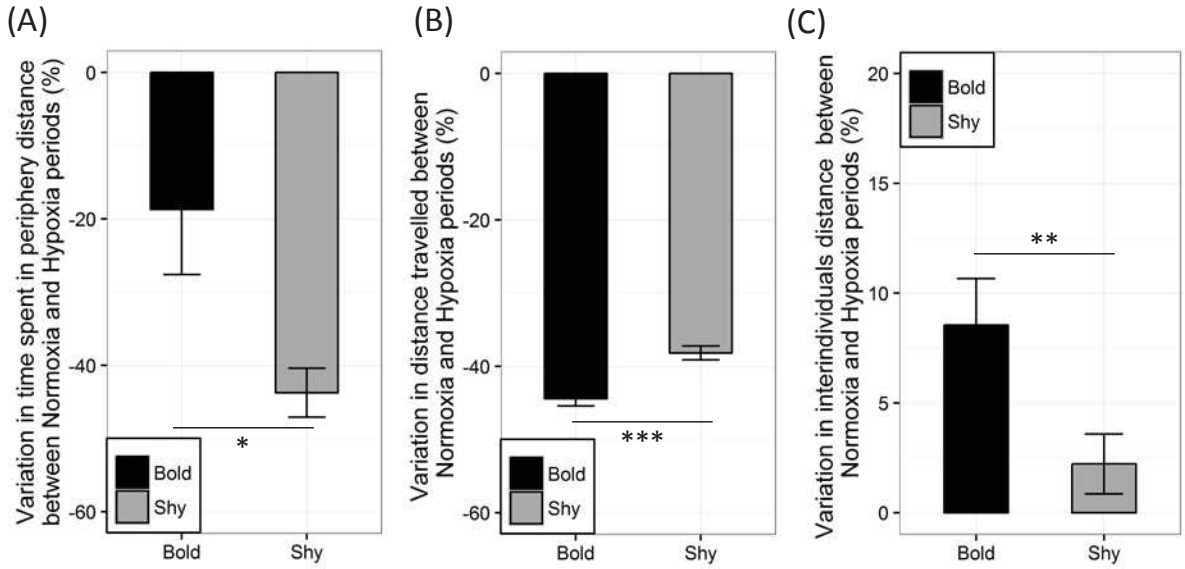

Figure 6. Mean ± SEM of **(A)** *Variation in time spent in periphery* between Normoxia and Hypoxia periods (%) **(B)** *Variation in distance travelled* between Normoxia and Hypoxia periods (%) **(C)** *Variation in interindividual distance* between Normoxia and Hypoxia periods (%) for bold (black bars n=48) and shy fish (grey bars, n=48). T-test comparisons between coping styles: *: $p<0.05$, **: $p<0.01$, **: $p<0.001$.

4. Discussion

In the present study, boldness level of European sea bass was measured three times in group risk taking test (GRT). Fish were then challenged by Hypoxia in an open field test in groups of extreme boldness (bold *vs*. shy fish), and were later challenged again in GRT a fourth and last time. European sea bass showed two divergent bold and shy traits during the three first GRTs and as expected, boldness was very consistent across GRTs (Ferrari et al., 2016a; Ferrari et al., 2015; Sadoul et al., in prep). These two distinct and consistent traits form a solid basis for distinct coping styles (Castanheira et al., 2017; Øverli et al., 2006; Øverli et al., 2007).

Introduction to a novel environment triggers thigmotaxis behaviour, which can be measured by the time spent by fish in the periphery of the arena, and is often interpreted as reflecting the anxiety level in fish (Schnorr et al., 2012; Shams et al., 2015). In the present study, fish from both coping styles displayed higher thigmotaxis during the first minutes of the challenge (0-15 minutes) compared to the following normoxia period. After 30 minutes, fish seemed to have habituated to the novel environment since all measured variables were relatively stable. Shy fish, however, displayed higher thigmotaxis than bold ones during the very first minutes of test (Habituation period) which likely reflected a higher anxiety level linked to the discovery

of the novel environment. Such higher anxiety state in shy fish in response to novelty has already been reported in literature reviews (Castanheira et al., 2017; Martins et al., 2011) and backed up by molecular markers (delta opioid and dopamine D2 receptors; Thornqvist et al., 2018). Hypoxia exposure triggered a decrease in time spent in periphery for fish from both coping styles. As reported in Alfonso et al. (Chapitre 5), stressors such as ammonia or hypoxia exposure induced an inhibition of thigmotaxis. Behavioural responses observed during the present study consolidate our hypothesis that fish first cope with stressors which directly affect survival, such as low oxygen concentration, and that overrules the expression of psychological stressors such as anxiety-like state. There were no further differences in thigmotaxis profiles between coping styles during Hypoxia exposure.

Distance travelled by sea bass increased between Habituation and Normoxia periods reflecting the usual habituation response to novel environment seen in other fish species (Levin et al., 2007). Thereafter, irrespective of coping styles, fish decreased their activity in response to Hypoxia exposure. This decrease in activity following hypoxia exposure was previously reported in several fish species including European sea bass (Alfonso et al., chapitre 5; Chapman and Mckenzie, 2009; Dalla Via et al., 1998; Metcalfe and Butler, 1984; Shingles et al., 2001). We suggest that individuals cope with this stressor by adopting a wait and see strategy consisting in minimizing energy expenditure and waiting for an improvement in water quality (Clingerman et al., 2007; van Raaij et al., 1996). Bold individuals are generally known to be more active than shy ones (Geffroy et al., 2015, 2018; Øverli et al., 2002; Réale et al., 2007) but surprisingly, in the present study, shy fish were more active than bold ones during all periods except the Habituation one. Such higher activity in shy fish was however previously reported in zebrafish larvae under standard conditions (Tudorache et al., 2015) which can be comparable to Normoxia period in our study. Differences in activity pattern between bold and shy fish persisted during both Decrease and Hypoxia periods suggesting that hypoxia exposure did not affect activity levels differentially between coping styles. Interestingly, the decreased in activity between Normoxia and Hypoxia periods was higher in bold fish than in shy ones. This result may be due to the fact that bold fish generally display higher metabolism than shy fish (Herrera et al., 2014; Killen et al., 2012) and therefore need to decrease further their activity to cope with low oxygen level.

Concerning group cohesiveness, fish from both coping style displayed higher group dispersion when experiencing hypoxia. Such group dispersion has already been described as a behavioural response to cope with hypoxia condition (Domenici et al., 2002, 2017). Indeed, as

suggested by Domenici et al. (2017) and Alfonso et al. (chapitre 5), fish move apart from each other to maximize oxygen uptake which is vital for breathing processes and aerobic energy metabolism in fish. During Hypoxia period, however, fish from both coping styles showed a higher group cohesion which could appear less adaptive. We suggest that it might only have been a short term response that changed during the last 10 minutes of the Hypoxia period when fish dispersed again.

Overall, fish from both coping styles adapted their behavioural responses to acute hypoxia by decreasing thigmotaxis and activity and increasing interindividual distances. Two different strategies were however observed with shy fish decreasing thigmotaxis more remarkably while bold fish rather decreased activity and increased interindividual distances between Normoxia and Hypoxia periods. Under basal condition (*i.e.* without any physiological challenges), shy fish are generally described as more sensitive to stressors related to novelty than bold ones (Castanheira et al., 2017; Thornqvist et al., 2018) which is also the case in the present study with shy fish showing higher thigmotaxis during the habituation period in the novel environment. Moreover, shy individuals are also generally displaying lower metabolic rate than bold ones (Careau et al., 2008; Herrera et al., 2014; Killen et al., 2012; Réale et al., 2007). Therefore, results of the present study suggest that in response to stressors, such as acute hypoxia, shy and bold fish adopt a resilient strategy for their most characteristic trait related to coping styles *i.e.* thigmotaxis for shy fish to alleviate anxiety; and lower activity and group cohesion to maximize oxygen uptake and sustain higher metabolic rate in bold fish. These results also suggest that differences between coping styles are highlighted under challenging situations (introduction to novel environment, hypoxia exposure) as reported by Killen et al. (2012) whereas under normal conditions (*i.e.* Normoxia), differences may be more subtle.

A month after the acute hypoxia exposure, fish were challenged again in GRT and differences in boldness were still consistent with the previously observed traits both during earlier GRTs and Open field test. Boldness was however affected in bold fish (*Latency to exit* from shelter increased), *i.e.* bold fish became shyer but for both hypoxia challenged and control fish and could be related to fish aging. It is known that stressors, including biotic and abiotic factors, can shape individual boldness during life (Brown et al., 2007; Ferrari et al., 2016b; Frost et al., 2007; Thomson et al., 2012). For instance, in European sea bass, fish reared under a predictable food supply grew faster and were shyer than fish reared under an unpredictable food supply (Ferrari et al, 2016b). In the present study, acute hypoxia exposure did not alter

boldness in European sea bass and this was also illustrated in an experiment using repeated random stressors performed over 3 weeks and leading to a chronic stress state which did also not affect boldness (Sadoul et al., in prep).

In conclusion, European sea bass shows divergent behaviours and boldness as evaluated in GRTs is a robust measure of coping style type (Ferrari et al. 2016a) since bold and shy traits are highly consistent across time. In novel environment, shy fish display higher thigmotaxis at the beginning of the test. Exposure to hypoxia leads to different behavioural responses in these two distinct coping styles with differential prioritized variation in thigmotaxis (in shy fish), activity and group cohesion (in bold fish). Acute hypoxia exposure does not trigger long lasting effects on fish behaviour. Overall, the different responses observed when facing stressors and consistency across long time period even after stressors exposure, highlight how relevant coping styles are for further aquaculture domestication/selection processes and for a better understanding of ecological issues.

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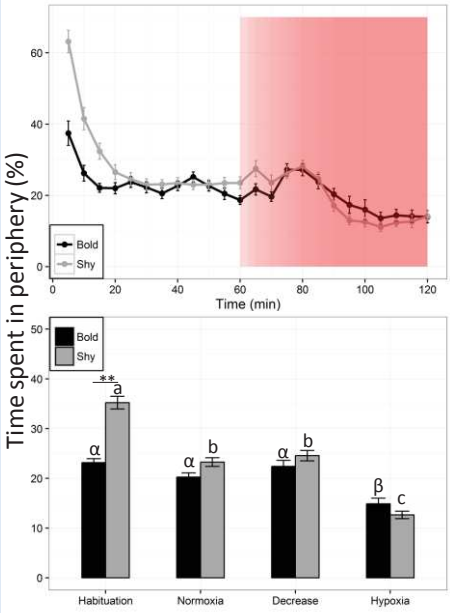
Chapitre 6 – Conclusions

Résultats :

• Thigmotaxie:



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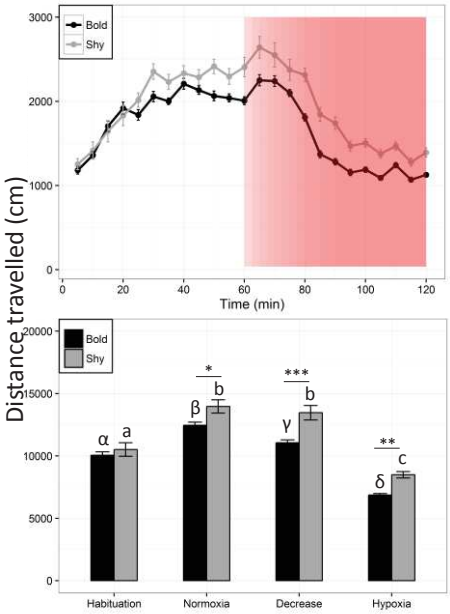
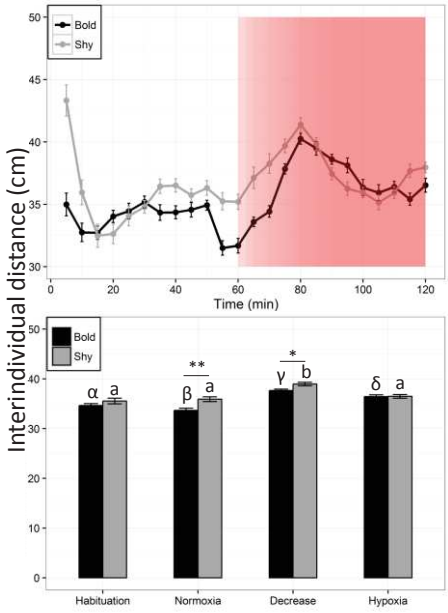


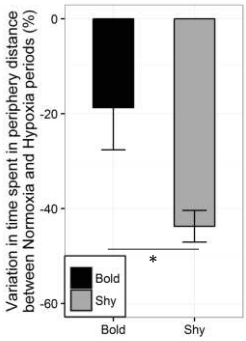
• Cohésion du groupe:



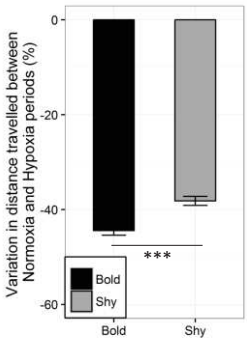
Les différences entre les périodes au sein des *coping styles* (grecques pour les audacieux et latine pour les timides). Différences entre les *coping styles* au sein d'une période: *: p<0.05, **: p<0.01, ***: p<0.001.

• Variation du comportement entre la période de normoxie et d'hypoxie:

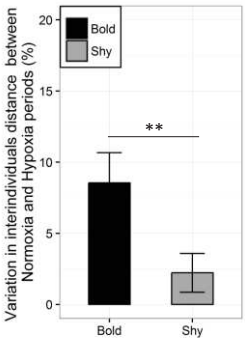
• **Thigmotaxie**



• **Activité**



• **Cohésion du groupe**



*: p<0.05, **: p<0.01, ***: p<0.001

• Variation de l'audace avant et après le challenge d'hypoxie:

• Les différences d'audace entre les individus identifiés comme timides et audacieux avant l'exposition aigue à l'hypoxie persistent toujours 1 mois après cette exposition (non illustrée).

Points importants :

• Lors de la période d'habituation, les individus identifiés comme timides passent plus de temps en périphérie que ceux identifiés comme audacieux. En condition standard (normoxie), les timides sont également plus actifs lors de l'*open field* et ont une dispersion de groupe plus importante que les audacieux.

• L'induction de l'hypoxie entraîne une amplitude plus importante de la réponse du comportement de thigmotaxie chez les individus timides et *a contrario* une amplitude plus importante sur l'activité et la cohésion du groupe chez les individus audacieux.

• Une exposition aigue à des conditions d'hypoxie n'entraîne pas d'altération de l'audace à long terme.

Discussion générale

Les objectifs de cette thèse étaient les suivants :

(1) Apporter une meilleure compréhension de la **mise en place de la personnalité**, la relation entre **les différents traits de personnalité**, leur **cohérence** à long terme et des **réponses physiologiques associées** aux différentes personnalités chez les téléostéens dans le cadre de la théorie des *coping styles*.

(2) Déterminer les **réponses physiologiques et comportementales** associées à **différents facteurs de stress chimique** (exposition chronique à un mélange de PCB et de PBDE ; variation de la qualité d'eau).

Dans une première partie, nous discuterons donc les résultats de l'objectif (1) à savoir les différents comportements caractéristiques des traits de personnalité et de leurs possibles corrélations entre eux (syndrome comportemental) pour aboutir à deux types divergents de réponses comportementales ou *coping styles*: l'un plutôt proactif (individus audacieux, actifs, explorateurs rapides) et l'autre plutôt réactif (individus timides, peu actifs, explorateurs lents). La place de l'anxiété au sein de ces types de réponse, sera discutée ainsi que la stabilité et la plasticité de ces comportements (leurs corrélations, sur de courtes ou longues périodes). Pour finir, un focus sera fait sur les mécanismes physiologiques corrélés à ces deux *coping styles*.

Dans une deuxième partie de la discussion, nous reviendrons sur les altérations comportementales et physiologiques observées suite à l'exposition aux différents facteurs de stress réalisés dans le cadre de cette thèse (stress chimique chronique : exposition à un mélange environnemental de PCB/PBDE et stress physico-chimique : manipulation /exposition aigue à des conditions d'hypoxie, d'hyperoxie et une forte valeur d'azote ammoniacal).

Nous discuterons ensuite dans une troisième partie des réponses comportementales des individus suite à un stress chimique aigu en relation avec leur *coping style* (exposition à des conditions d'hypoxie). Une mise en perspective sera ensuite faite quant à l'importance et la pertinence des connaissances approfondies sur le *coping style* en lien avec les capacités d'adaptation face au stress, et les bénéfices attendus aussi bien dans un contexte d'aquaculture que plus largement dans un contexte écologique.

1. Le *coping style* chez les téléostéens

Plusieurs traits de personnalité, caractérisant les *coping styles* ont été étudiés au cours des différents chapitres dans différents contextes et à différents stades de vie. Cette première partie de la discussion s'intéresse aux corrélations comportementales et physiologiques mises en évidence entre les différents traits de personnalité (syndrome comportemental), le possible lien avec l'anxiété, ainsi que leur stabilité (*vs*. plasticité) dans le temps.

1.1. La personnalité et les syndromes comportementaux

Dans le **chapitre 1**, nous avons mis en évidence que le **niveau d'audace d'un individu**, mesuré dans un test de prise de risque en groupe, **était corrélé positivement à son activité** dans un nouvel environnement dès le stade larvaire (8 jpf), à 21 jpf et 60 jpf chez le poisson zèbre. Chez les adultes, nous avons également observé que l'audace, toujours mesurée dans un test de prise de risque, était toujours positivement corrélée à l'activité dans un nouvel environnement mais également à d'autres comportements. Plus précisément, **un individu audacieux explorera également plus rapidement son environnement** (**Chapitre 4**). Ces deux chapitres démontrent **la présence d'un syndrome comportemental chez le poisson zèbre**. Dans le **chapitre 2**, chez le bar, nous avons également observé deux types de réponses dans un test en *open field* individuel avec une zone refuge. Une partie des individus se montrait audacieuse, quittait la zone refuge et se montrait active dans la zone ouverte alors que d'autres individus se montraient plutôt timides et restaient quasiment immobiles dans la zone refuge toute la durée du test. D'un point de vue comportemental, toutes ces observations dans les **chapitres 1, 2 et 4** sont cohérentes avec la littérature qui définit deux types de syndrome comportemental : l'un proactif avec des individus audacieux, actifs qui explorent rapidement leur environnement et sont moins sociaux, et un plutôt réactif avec des individus timides, moins actifs, qui explorent leur environnement plus lentement et sont plus sociaux (Réale et al., 2010). Le syndrome comportemental lié à l'audace observé dans le **chapitre 4**, et faisant suite à la mesure de tous ces traits de personnalité, présente des résultats très intéressants car les traits de personnalité sont rarement tous étudiés sur un même individu et leur analyse est conforme à la théorie exposée dans Réale et al. (2010).

En revanche, dans le **chapitre 6**, nous avons observé **une corrélation négative** cette fois-ci entre **le niveau d'audace,** mesuré à l'aide de trois tests de prise de risque en groupe (le même test répété à plusieurs semaines d'intervalle), et le **niveau d'activité** dans un *open field*. Cette

corrélation négative avait également été observée chez des larves de poisson zèbre (Tudorache et al., 2015). Ces résultats contradictoires à ceux des **chapitres 1** et **4**, mais parfois retrouvés dans la littérature, peuvent trouver une explication dans le métabolisme et les liens entre ce dernier et les traits de personnalité, notamment l'activité (Careau et al., 2008). En effet, selon Réale et al. (2010), les individus audacieux, exprimant une réponse plutôt proactive, sont plus actifs et présenteraient aussi un métabolisme plus élevé comme cela a déjà été démontré chez le bar en situation de challenge hypoxique (Killen et al., 2012). Le taux métabolique est le taux auquel un animal oxyde des nutriments afin de produire de l'énergie. Malgré le nombre considérable d'études menées sur l'activité et le métabolisme, la relation entre le taux métabolique de routine (TMR) et l'activité n'est pas tout à fait claire (Mathot et Dingemanse, 2015; Speakman et Selman, 2003). Elle peut être expliquée selon deux modèles différents : le modèle de performance et le modèle d'allocation qui prédisent des relations entre TMR et activités opposées (**Figure 26**).

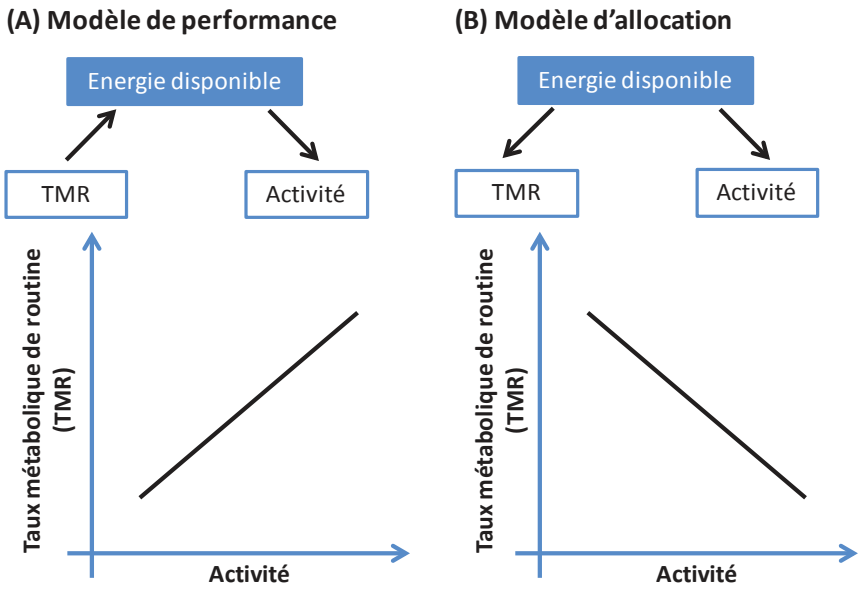


Figure 26. Relation hypothétique entre le taux métabolique de routine (TMR) et l'activité en fonction du (**A**) Modèle de performance; (**B**) Modèle d'allocation. D'après Careau et al. (2008).

Le modèle de performance prédit une relation positive entre TMR et activité, suggérant que le TMR reflète la taille des 'machines digestive et métaboliques' nécessaires pour capturer, ingérer, extraire et mobiliser l'énergie, et donc que des individus plus actifs maintiennent des niveaux élevés de rendement énergétique nécessitant des organes plus développés et/ou efficaces en soutien à leur fonction de base pour le traitement des aliments et de l'énergie et donc un coût de maintenance également supérieur (Daan et al., 1990). Le modèle d'allocation

suggère quant à lui que l'énergie dont l'individu dispose est partagée entre les coûts liés au TMR et à l'activité (Careau et al., 2008). De manière générale, l'existence des deux modèles a pu trouver des supports expérimentaux mais le modèle de performance reste majoritairement supportée chez les téléostéens (Cutts et al., 1998; Cutts et al., 1999; Mathot et Dingemanse, 2015).

En considérant que les individus audacieux sont caractérisés par un métabolisme plus élevé (Killen et al., 2012; Martins et al., 2011b), les résultats obtenus chez le poisson zèbre à différents stade de vie sont cohérents avec le modèle de performance (une relation positive entre l'activité et le TMR) alors que les résultats obtenus chez le bar semblent plus cohérents avec le modèle d'allocation. Cependant, il est également important de noter que le contexte social dans lesquels ont été faites ces mesures d'activité entre le poisson zèbre et le bar différait (*i.e.* seul chez le poisson zèbre *vs.* groupe chez le bar) et peut donc jouer un rôle dans la divergence des résultats obtenus entre les deux espèces.

- **Quid de l'anxiété ?**

Dans l'introduction, nous nous étions questionné sur l'absence de l'anxiété en tant que trait de personnalité. Nous avons mesuré de nombreuses fois l'anxiété au cours des différents chapitres et ce de manières différentes : *open field* (**Chapitres 1, 5, et 6**) et test du nouvel environnement (**Chapitres 1, 3, 4 et 5**) dont trois fois avec la mesure préalable de l'audace à l'aide *via* le test prise de risque en groupe (**Chapitres 1, 4, 6**).

Dans le **chapitre 1**, nous avons observé qu'un **plus haut niveau anxiété et une activité importante dans un nouvel environnement catégorisaient les individus plutôt audacieux** que ce soit au stade larvaire ou juvénile chez le poisson zèbre. A l'âge adulte, lorsque l'anxiété est mesurée et analysée conjointement avec les autres traits de personnalité (activité, socialité, exploration) lors d'une analyse en composante principale (ACP), il apparait que l'anxiété se projette majoritairement sur le second axe alors que les autres traits de personnalité (audace, activité et explorations) contribuent principalement au premier axe (**Chapitre 4**). Ces résultats divergents entre les **chapitres 1** et **4** suggèrent que l'anxiété ne peut pas être considérée comme un trait de personnalité. Très peu de références directes sont faites sur l'anxiété des individus en fonction de leur *coping style*. Cependant il semblerait que les individus timides soient considérés comme plus peureux (Martins et al., 2011a; Øverli et al., 2007), même si la peur est une émotion différente de l'état d'anxiété. En revanche, il a été démontré récemment que dans un test de nouvel environnement les individus audacieux

montaient rapidement dans la zone haute et y passaient plus de temps que les individus timides (Thornqvist et al., 2018) suggérant un faible niveau d'anxiété. Pour finir, dans le **chapitre 6**, nous avons observé que même en groupe, les **individus timides avaient tendance à rester beaucoup plus dans la zone périphérique que les individus audacieux** appuyant l'hypothèse selon laquelle les individus timides sont également plus anxieux que les individus audacieux.

En conclusion, même si des expériences complémentaires devront être menées, **il semblerait que l'anxiété soit généralement corrélée négativement à l'audace des individus tout comme l'audace est corrélée à d'autres traits de personnalité (activité, exploration) structurant les bases comportementales de la théorie des *coping styles***. Si l'anxiété d'un individu s'avère stable à travers le temps, du moins sur de courtes périodes, alors elle pourrait être identifiée comme un trait de personnalité et intégrée en tant que telle dans la théorie du syndrome de rythme de vie - *pace of life syndrom* - de Réale et al. (2010) sur laquelle nous reviendrons plus tard.

1.2. La plasticité *vs.* la stabilité

Comme évoqué dans la **section 1.1**, **l'audace**, mesurée lors d'un test de prise de risque en groupe, est **corrélée aux comportements mesurés suite à l'introduction dans un nouvel environnement** chez le poisson zèbre dès lors que les tests différents sont enchaînés **à court terme** quel que soit le stade de vie (*i.e.* larvaire, *post*-métamorphose et juvénile; **Chapitre 1**). De la même façon nous avons retrouvé une cohérence très forte entre le comportement d'audace, toujours mesuré dans un test de prise de risque en groupe, avec d'autres traits de personnalité comme l'exploration, l'activité et la socialité mesurées chez les adultes et à courte échéance, dans des contextes différents (**Chapitre 4**; **Figure 27.A**).

Chez le bar, l'audace mesurée grâce à un test de prise de risque en groupe, est cohérente dans le temps sur de courtes périodes (Ferrari et al., 2016a; Ferrari et al., 2015) mais également sur des périodes de temps relativement longues (plusieurs mois, Sadoul et al. (in prep), **chapitre 6**, **Figure 27.B**). Cette cohérence d'un même trait de personnalité (audace) à travers le temps avait également été démontrée chez le poisson zèbre adulte sur 10 mois (Rey et al., 2013). Les résultats de cette thèse vont dans le sens de la plupart des résultats présentés dans les publications actuelles sur le sujet et qui démontrent la présence de *coping styles* stables à travers le temps et les contextes chez les téléostéens.

En revanche, d'autres résultats viennent mesurer et apporter des précisions. Par exemple, dans le **chapitre 1**, nous avons également mis en évidence chez le poisson zèbre, que malgré la **présence des mêmes syndromes comportementaux** à partir du stade larvaire jusqu'au stade juvénile, les **individus faisaient preuve de plasticité entre ces deux stades** (**Figure 27.D**). Les individus audacieux au stade larvaire n'étaient pas forcement audacieux arrivés à l'âge adulte. De la même façon chez le bar, des individus catégorisés comme audacieux à 227 jpf dans un test de prise de risque ne l'étaient par forcément isolés dans un test de nouvel environnement à 749 jpf (**Chapitre 2**; **Figure 27.C**).

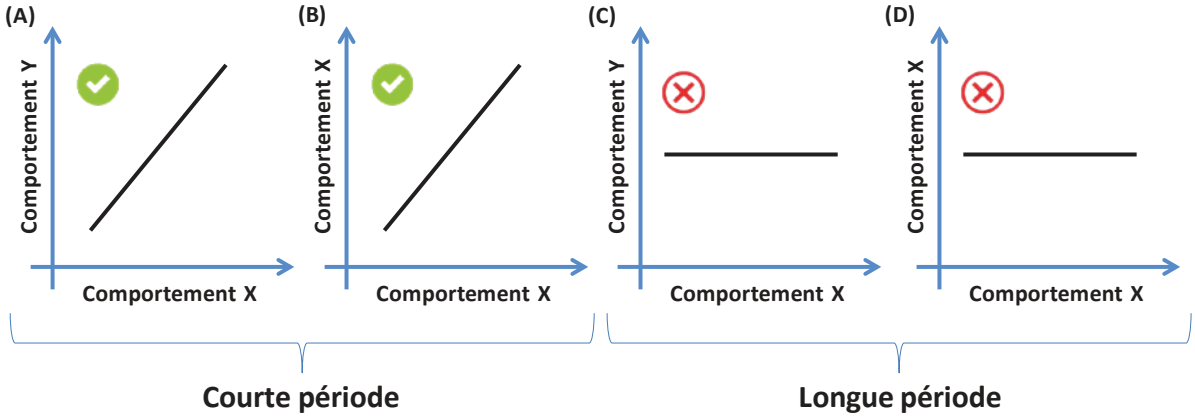


Figure 27. (**A**) Corrélations entre le comportement X et le comportement Y sur une courte période; (**B**) Corrélations du comportement X entre deux dates sur une courte période; (**C**) Corrélations entre le comportement X et le comportement Y sur une longue période; (**D**) Corrélations du comportement X entre deux dates sur une longue période.

Il est connu que des facteurs biotiques et abiotiques peuvent façonner l'expression de la personnalité chez les téléostéens (Brown et al., 2007; Ferrari et al., 2016b; Frost et al., 2007; Thomson et al., 2012). Par l'exemple, un bar nourri à heures fixes aura tendance à devenir plus timide qu'un bar nourri à heure variable (Ferrari et al., 2016b). Dans le cadre de cette thèse, les individus ont été maintenus et testés dans des conditions d'élevages identiques, en *common garden*, ces facteurs ne permettent donc pas d'expliquer cette absence de cohérence comportementale sur de longues périodes. En revanche, ces résultats suggèrent que des facteurs biotiques, comme les interactions sociales au cours de la vie sont des facteurs importants (Webster et al., 2007) qui peuvent façonner la personnalité de l'individu, de même que les changements physiologiques associés au développement peuvent modifier l'expression de la personnalité. Cette observation semble être **une caractéristique des téléostéens dont la personnalité n'est pas fixée aux stades larvaires et juvéniles** (Bell et Stamps, 2004;

Herczeg et al., 2013; Edenbrow et Croft 2013) mais semble se **stabiliser ensuite à l'âge adulte** (Polverino et al., 2016) sous réserve de l'absence de facteurs de stress externes.

Au vu de tous ces résultats, il apparait donc que si on souhaite étudier les réponses comportementales et/ou physiologiques des individus face au stress en fonction de leur *coping style*, il faut être sûr que celui-ci soit fixé du moins pour la période de l'expérimentation. Par exemple, pour une exposition à un stress aigu sur un stade de vie donné comme par exemple dans le **chapitre 6**, le *coping style* était fixé et l'expérimentation est donc possible. En revanche, si on souhaite étudier les réponses des individus en fonction de leur *coping style* face à un facteur de stress chronique sur des périodes d'expérimentation longues ou à des stades précoces, cela apparait donc plus compliqué car les traits comportementaux et physiologiques ne sont pas fixes et sont fortement susceptibles de changer au cours de la vie de l'individu. Une solution pour étudier les réponses aux facteurs de stress sur de longues périodes en fonction du *coping style* serait d'avoir des animaux sélectionnés en fonction de ces derniers car il a été démontré que le *coping style* est aussi héritable (Ferrari et al., 2016a). Une alternative serait d'étudier des lignées présentant des divergences comportementales et physiologiques proches de celles des *coping styles* comme les lignées AB et TL chez le poisson zèbre par exemple (van den Bos et al., 2017).

1.3. Les mécanismes physiologiques associés

1.3.1. La régulation du stress

D'après la théorie des *coping styles*, les individus timides sont caractérisés par une plus forte réactivité de l'axe hypothalamus-pituitaire-interrénale et des systèmes monoaminergiques liés à la production de dopamine, norépinephrine et sérotonine en réponse au stress (Castanheira et al., 2017; Øverli et al., 2001, 2007).

Dans le **chapitre 2**, nous n'avons pas observé de différences dans la concentration en cortisol plasmatique en fonction des *coping styles* à la suite de l'*open field*. Cela peut paraitre surprenant mais a déjà été observé chez d'autres espèces de téléostéens (Tudorache et al., 2013; Vindas et al., 2017b). Cela peut s'expliquer par le fait que la production de cortisol étant à son pic suite au test en *open field* individuel, cela masque potentiellement les différences entre les deux *coping styles*. Il a par ailleurs était démontré que le pic de cortisol était identique chez les deux *coping styles* suite à un stress aigu mais que des différences étaient ensuite observées dans la phase de diminution (récupération) suivant le pic (Tudorache

et al., 2013). Même si nous n'avons pas observé de différence dans la concentration en cortisol entre les individus timides et audacieux, nous avons en revanche observé des différences de transcription dans le cerveau de gènes associés à la production et la régulation du cortisol. En effet, **nous avons mis en évidence une sur-expression des gènes du facteur cortico-releasing (*crf*) et du récepteur à minéralocorticoïdes (*mr*) chez les individus timides (réactifs) comparés aux individus audacieux (proactifs)** impliqués respectivement dans la libération d'ACTH stimulant donc la libération de cortisol (Doyon et al., 2003; Wendelaar Bonga, 1997) et dans le rétrocontrôle négatif de la libération du cortisol (Alderman et al., 2012; Bernier et al., 2009; Johansen et al., 2011). Ces résultats chez le bar viennent confirmer des études précédentes menées chez d'autre espèces de téléostéens (Backstrom et al., 2011b; Geffroy et al., 2018; Summers et al., 2017; van den Bos et al., 2017) et suggèrent donc une plus forte réactivité de l'axe HPI chez les individus réactifs.

Nous avons mis en évidence que suite à un test en *open field*, les individus timides et audacieux affichaient des **concentrations en dopamine équivalentes dans le cerveau**. En revanche, **les individus timides présentaient une concentration cérébrale de norépinephrine plus élevée que les individus audacieux, ainsi qu'une métabolisation de la sérotonine plus faible (ratio 5HIAA/5HT)**. Une étude précédente menée chez des truites arc-en-ciel sélectionnées en fonction de leur réactivité de l'axe HPI suite à un stress : HR (haute réactivité, réactives) et LR (faible réactivité, proactives) est à ce jour la plus complète sur l'activité des systèmes monoaminergiques en réponse au stress chez les téléostéens (Øverli et al., 2001). Les auteurs ont mis en évidence, que dans la majeure partie des zones cérébrales, les individus réactifs présentaient une plus forte activité des systèmes monoaminergiques face au stress.

Au premier abord, leurs résultats sont donc cohérents avec les nôtres pour l'axe noradrénergique, aboutissant à la synthèse et à la libération de norépinéphrine, mais contradictoire concernant l'axe sérotonergique, où nous avons observé l'inverse, et l'axe dopaminergique où l'activité est identique entre les deux *coping styles*. Cependant, Øverli et al. (2001), ont tout de même observé un ratio 5HIAA/5HT plus important dans le télencéphale des truites LR (proactives) que chez les truites HR (réactives) en période contrôle et dans le tronc cérébral que ce soit en période contrôle ou suite à un stress de confinement. Concernant l'axe dopaminergique, son activité peut également varier entre les deux *coping styles* dans certaines régions du cerveau et pas dans d'autres (Øverli et al., 2001).

Nos résultats sont donc pour le moment globaux (les analyses du **chapitre 2** étant sur cerveaux entiers) chez le bar et nécessitent donc des études plus approfondies pour mieux comprendre les différents patrons d'activité en fonction des différentes zones cérébrales (celles à venir dans le **chapitre 6** seront sur 2 régions du cerveau et l'inter-rénale). D'autre part, il semblerait qu'il existe des interactions entre l'axe HPI et les systèmes monoaminergiques chez les téléostéens (Backstrom et al., 2011a; Carpenter et al., 2007). Outre son rôle au sein de l'axe HPI dans la libération du cortisol, de nombreuses études tendent à démontrer que *crf* est une composante majeure dans la régulation de la neurotransmission (Carpenter et al., 2009; Carpenter et al., 2007; Maier et Watkins, 2005) et *a fortiori* de la régulation du comportement de locomotion (Lowry et Moore, 2006), induisant notamment un comportement de type anxieux chez les téléostéens (Carpenter et al., 2007).

En conclusion, les mécanismes physiologiques impliqués dans la régulation du stress sont apparus différents en fonction des *coping styles* chez le bar autant sur l'axe HPI que sur les systèmes monoaminergiques. Une synthèse des différences observées au niveau physiologique et des comportements associés aux deux types de réponses, mis en évidence dans le cadre de cette thèse, est présentée sur la **figure 28**.

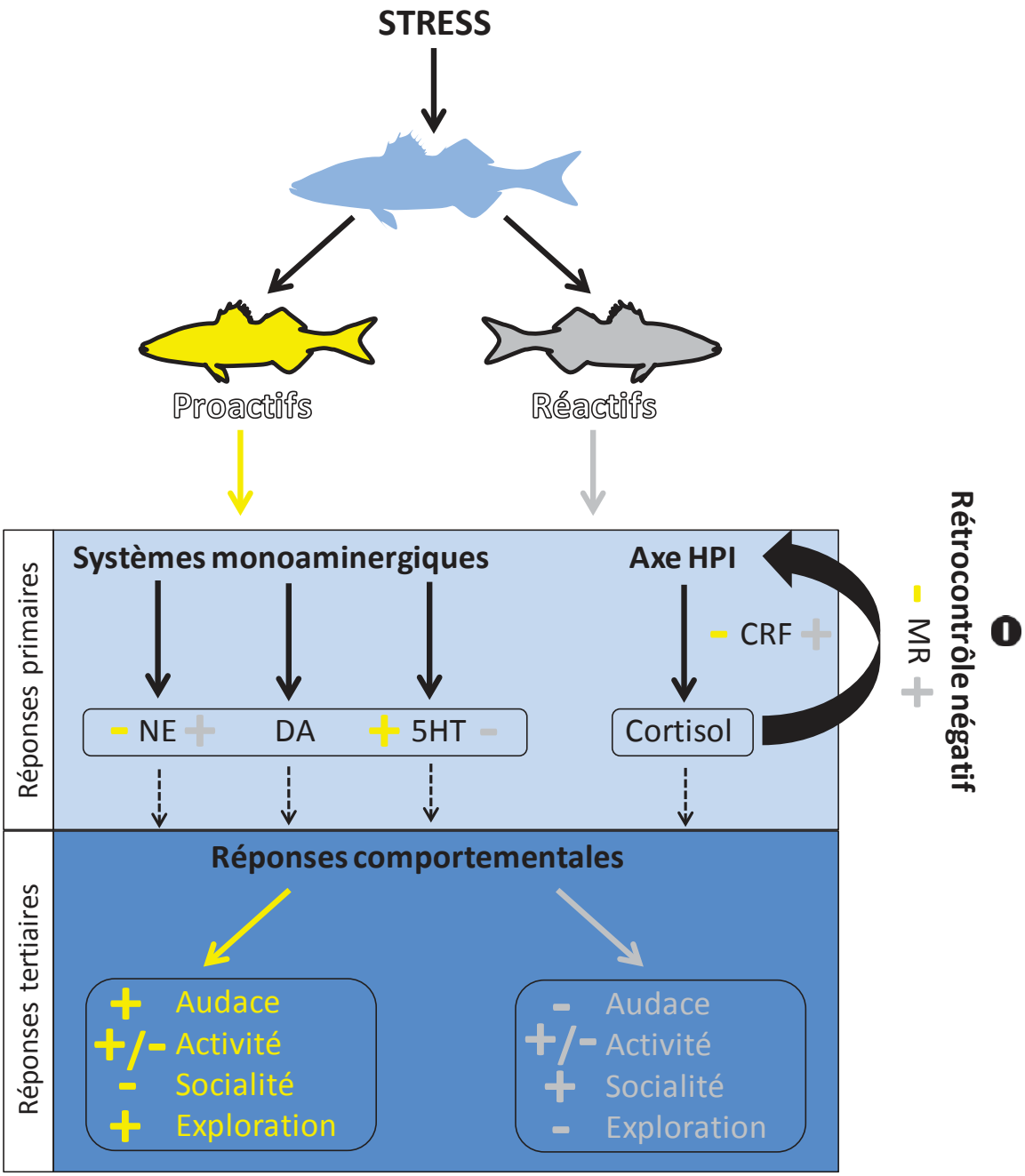


Figure 28. Synthèse des différences observées au niveau physiologique (réponses primaires) et des comportements associés (réponses tertiaires) en fonction des *coping styles* chez le bar mis en évidence dans le cadre de cette thèse. La couleur jaune fait référence à une réponse plutôt proactive et la couleur grise à une réponse plutôt réactive.

1.3.2. L'activité et la plasticité neuronale

Comme évoqué dans l'introduction générale, des études suggèrent comme cela a été montré chez les mammifères, que le stress induit des changements dans l'activité et la plasticité neuronale du système nerveux central des téléostéens (Johansen et al., 2012; Sorensen et al., 2011, 2013). Il est donc cohérent de penser que les individus éprouvant une sensibilité au stress différente (*coping styles*) présentent également des patrons d'activité et de plasticité neuronale différents (Sorensen et al., 2013). Dans le **chapitre 2**, nous avons essayé de tester cette hypothèse en mesurant l'activité et la plasticité neuronale à travers la mesure de l'expression des gènes *egr1*, qui renseigne sur l'activité neuronale (Burmeister et Fernald, 2005), le gène *pcna* codant un facteur important pour la réplication de l'ADN et qui considéré comme un marqueur de l'activité prolifératrice (Eisch et Mandyam, 2007; Mandyam et al., 2007). Enfin les facteurs de transcription *neurod1* et *neurod2* sont impliqués dans la différentiation neuronale et la survie des nouveaux neurones immatures (Korzh et al., 1998; Steiner et al., 2006).

Il est apparu que les **individus timides** (réactifs) montraient une **surexpression des gènes *egr1*** et ***neurod2*** comparés aux individus audacieux (proactifs). Ces résultats font écho aux récentes études suggérant également que des individus plutôt réactifs, catégorisés par une plus forte réactivité de l'axe HPI, sont ceux qui présentent une neurogénèse plus importante en comparaison à ce qui est observée pour les individus proactifs (Geffroy et al., 2018; Øverli et Sorensen, 2016; Sadoul et al., 2018b; Sorensen et al., 2013; van den Bos et al., 2017). Dans le cadre de cette thèse, j'ai eu d'ailleurs l'occasion de travailler avec des collègues sur cette question et nous **avons mis en évidence, que la transcription de gènes impliqués dans la régulation du stress** (*mr*, *gr1 et gr2*) **était corrélée à l'expression de gènes impliqués dans la prolifération cellulaire** (*pcna;* **Figure 29**) **et la différentiation neuronale** (*neurod1*) chez trois espèces différentes de téléostéens dans des conditions non stressantes incluant le bar (Sadoul et al., 2018b).

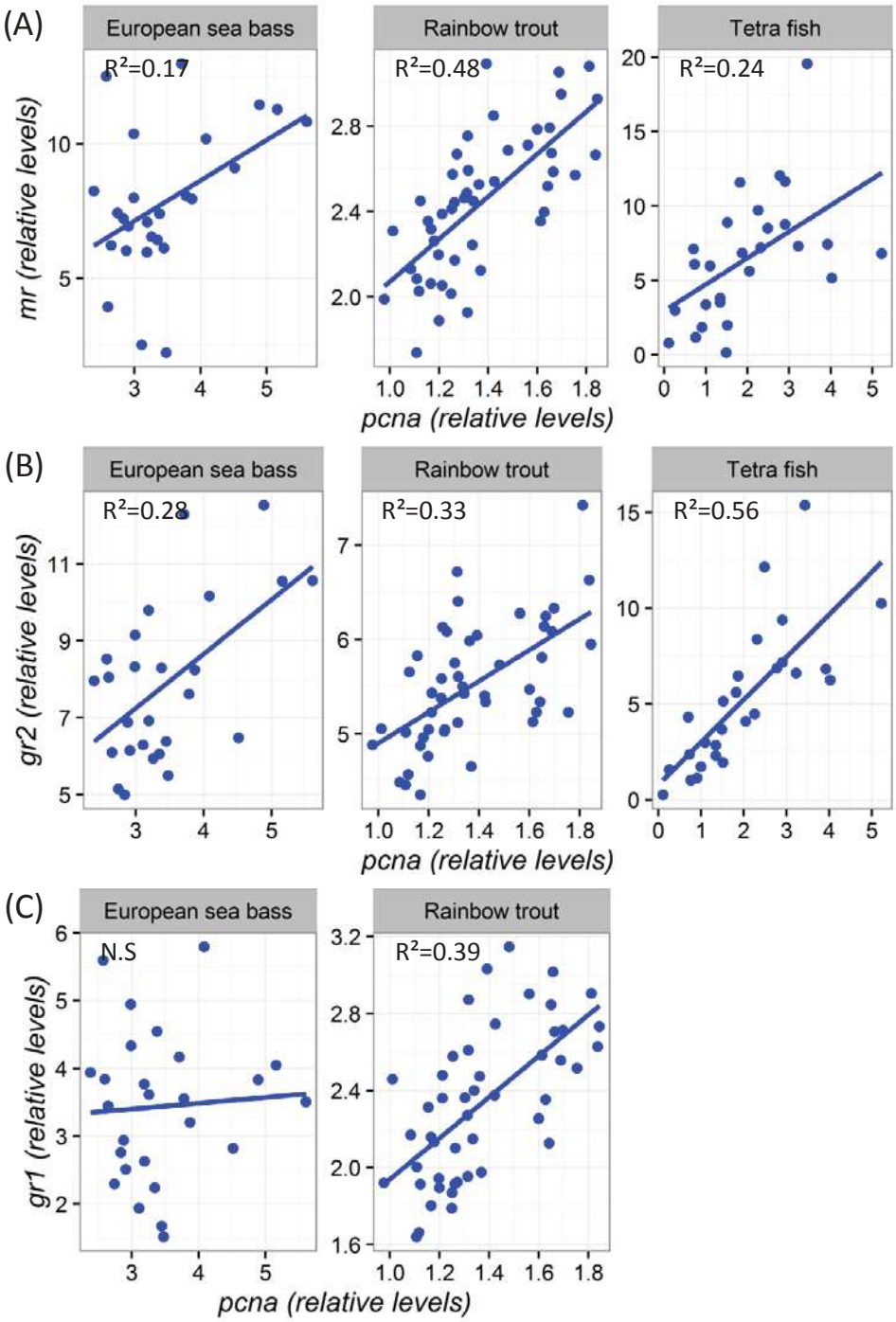


Figure 29. L'expression du gène *pcna* chez le bar, la truite arc-en-ciel et le tétra en fonction de l'expression de **(A)** *mr*, **(B)** *gr2* et **(C)** *gr*. L'expression des gènes a été normalisée avec l'expression des gènes de références *eef1-alpha* et *l13* chez le bar et la truite arc-en-ciel et *gapdh* et *18s* pour le tetra. Le coefficient de détermination (R^2) est indiqué pour chaque régression significative. Dans le cas contraire, NS (non significatif) est mentionnée. D'après Sadoul, B., Alfonso, S., Bessa, E., Bouchareb, A., Blondeau-Bidet, E., Clair, P., Chatain, B., Begout, M.L., Geffroy, B., 2018b. Enhanced brain expression of genes related to cell proliferation and neural differentiation is associated with cortisol receptor expression in fishes. Gen. Comp. Endocrinol. 267, 76-81.

Il est encore difficile à l'heure actuelle de savoir si la régulation du stress *via* l'axe HPI influence la plasticité neuronale ou inversement. Cependant, la plasticité neuronale apparait comme une composante importante permettant de caractériser les *coping styles* chez les téléostéens. Ces résultats permettraient d'expliquer pourquoi les individus audacieux ont tendance à développer des routines comportementales et se montrent moins flexibles que les individus timides (Bolhuis et al., 2004; Ruiz-Gomez et al., 2008). De la même façon, cela supporte le fait que les bars plutôt timides sont généralement aussi ceux qui explorent et actionnent les mécanismes des distributeurs d'aliment permettant ainsi l'alimentation du groupe (Benhaim et al., 2017; Ferrari et al., 2014).

Ces différences dans les réponses physiologiques et comportementales entre les individus supposent des capacités d'adaptation différentielles aux différents facteurs de stress rencontrés dans l'environnement. Cette notion sera abordée dans la troisième partie de la discussion. Pour le moment, nous allons discuter des réponses individuelles face à différents facteurs de stress sans prendre en compte *le coping* style en amont.

2. L'individu face aux facteurs de stress de l'environnement

Dans la partie 2 de cette thèse, nous nous sommes intéressés aux réponses physiologiques et comportementales des téléostéens suite à une exposition à un stress chimique chronique lié à un mélange environnemental de polluants organiques persistants (PCB/PBDE; **chapitres 3** et **4**), puis dans la partie 3 à un stress chimique aigu lié à des variations de concentration en oxygène et de forte valeur d'azote ammoniacal (**Chapitre 5**). Dans cette partie de la discussion, nous allons revenir sur les résultats obtenus dans le cadre de ces différentes expériences.

2.1. Effets d'une exposition chronique à un mélange environnemental de PCB et de PBDE sur le comportement et la physiologie

Dans les **chapitres 3** et **4**, nous avons étudié les réponses comportementales, notamment les traits de personnalité, chez le poisson zèbre exposé à un facteur de stress chimique qui était une exposition chronique à un mélange environnemental de polluants organiques persistants *via* l'alimentation (PDB et PBDE). Le comportement des générations suivantes (F1, F2, F3 et F4 dans le **chapitre 3** et seulement F1 et F2 dans le **chapitre 4**) a également été étudié tout

comme certains volets physiologiques permettant d'aborder les mécanismes possibles des altérations comportementales observées (expression de *c-fos*, niveau de cortisol) et la transmission d'effets à la descendance (expression des *dnmts*).

Concernant le protocole de contamination, les générations F1, F2, F3 et F4 n'étaient pas directement exposées aux polluants *via* l'alimentation mais des altérations peuvent être tout de même observées chez la descendance à cause notamment du stress physiologique maternel (Talge et al., 2007) et/ou par le transfert des PCB et PBDE de la génération F0 vers la génération F1 par transfert maternel *via* les œufs (Daouk et al., 2011; He et al., 2011), ce qui a également été le cas pour le mélange de polluants (**Chapitre 3**). En effet, les concentrations en PCB et PBDE chez les larves F1 à 5 jpf descendants de F0 exposés aux polluants (MIX) étaient respectivement 90 et 20 fois supérieures aux concentrations mesurées chez les individus contrôles puis diminuaient jusqu'à devenir identiques entre les deux conditions à partir de 112 jpf à cause d'un phénomène de dilution lié à la croissance (Sijm et al., 1992). Bien que la concentration en polluants chez les MIX F1 devienne négligeable avec l'âge, les cellules germinales qui produiront la génération F2 ont été exposées durant leur développement. Par la suite, les futures générations F3 et F4 seront quant à elles les premières non exposées aux polluants (directement ou indirectement).

La génération F0 ne présentait aucune altération comportementale contrairement aux générations suivantes (F1 jusqu'à F4). Chez la **génération F1**, nous avons en revanche mesuré chez **les larves MIX une hyper-activité locomotrice** lors du test de la réponse photomotrice (LPMR), ainsi **qu'une augmentation du niveau d'audace chez les adultes MIX**. Chez les **F2, nous avons observé cette fois une diminution de la réponse photomotrice des larves et une anxiété élevée à l'âge adulte** sans pour autant observer une altération de l'expression de la personnalité. Chez **les F3 et F4, nous avons observé une activité locomotrice plus faible chez les larves en réponse au changement brusque lumière/obscurité mais également dans la phase précédent ce changement mais sans toutefois de différences de niveau d'anxiété** à l'âge adulte (expérience réalisée chez les F3 seulement).

Concernant les mécanismes physiologiques potentiellement associés à ces altérations, nous n'avons **premièrement pas observé de différences en terme de concentration en cortisol plasmatique entre les traitements MIX et contrôles quelques soient les générations**. En revanche, le facteur de transcription ***c-fos* est apparu sur-exprimé chez la génération F1 et**

sous-exprimé chez la génération F2 chez les MIX par rapport aux contrôles alors qu'il est exprimé de façon identique chez les générations F3 et F4. *c-fos* est un marqueur d'activité neuronale connu pour être associé à des modifications du niveau d'anxiété (Chen et al., 2015; Duncan et al., 1996). D'ailleurs chez les F1 MIX, cette sur-expression de *c-fos* peut être associée à l'hyper-réaction lors du test LPMR, comme la sous-expression de *c-fos* observé chez les F2 MIX peut être associée à l'hypo-réaction des larves lors de ce même test. Ce mécanisme ne peut pas être proposé pour expliquer les réponses comportementales observées durant la LPMR chez les F3 et F4 car chez ces générations, *c-fos* est normalement exprimé.

Concernant les mécanismes physiologiques permettant d'aborder les transmissions d'effets à la descendance, nous avons suivi la l'expression des *dnmts* qui sont des gènes codants pour des enzymes impliquées dans la méthylation de l'ADN (Aluru, 2017). L'expression des *dnmts* a été quantifiée à 1 jpf. Parmi ces *dnmt*, ***dnmt3ba* est apparu alternativement sous et sur-exprimé chez les embryons MIX des générations F1 à F4**. Des corrélations ont été observées entre des modifications de la méthylation (niveau global de méthylation ou expression de *dnmt*) et des altérations comportementales (Knecht et al., 2017; McGhee et Bell, 2014). Cette variation d'expression de *dnmt3ba* jusqu'à la génération F4 est donc un candidat crédible pour expliquer la transmission d'effets jusqu'à ces générations non exposées aux polluants.

Comme précisé précédemment, nous n'avons pas mis en évidence d'altération comportementale chez les MIX F0 mais cela ne veut pas dire pour autant qu'il n'en existe pas. En effet, d'autres comportements liés à la reproduction, aux autres interactions sociales au sein d'un groupe, ou la compétitivité pour l'accès à la nourriture auraient pu être intéressants à étudier afin de vérifier de possibles altérations. D'ailleurs, dans le cadre de Fish'N'POPs, **nous avons mis en évidence chez les MIX F0 une altération des traits d'histoire de vie, avec notamment un taux de croissance plus important pour une plus grande longueur finale par rapport aux contrôles**. Nous avons également **observé une probabilité de ponte plus faible chez ces individus, ainsi qu'une mortalité larvaire plus importante chez la descendance (F1)** pour des larves issues des premières pontes des F0 (Horri et al., 2018). En plus, des altérations physiologiques et comportementales observées dans le cadre de ma thèse, ces altérations des traits d'histoire de vie peuvent avoir des conséquences délétères pour le devenir des populations naturelles de téléostéens exposés à ce cocktail de polluants présent dans l'environnement (Horri, 2018).

Pour conclure cette partie, une analyse transcriptomique a été réalisée et l'exploitation des données est actuellement en cours dans le cadre du **chapitre 4** tentant d'apporter de nouvelles informations sur les mécanismes corrélés aux altérations comportementales observées autant sur la personnalité que sur l'anxiété. Malheureusement, le dosage des monoamines dans le cerveau des générations F0, F1, F2 et F3, préalablement prévu dans le cadre du projet Fish'N'POPs, n'a pas été possible principalement à cause de contraintes techniques et ne pourra donc pas apporter de nouvelles informations sur l'activité des systèmes monoaminergiques.

2.2. Effets d'un stress physico-chimique en système d'élevage sur le comportement et la physiologie

Dans le **chapitre 5**, nous nous étions intéressés aux réponses comportementales d'un groupe de bars dans un *open field* dans des conditions de stress (hypoxie, hyperoxie, forte concentration d'azote ammoniacale). Les objectifs étaient dans un premier temps de **définir des indices comportementaux de bien-être** en quantifiant l'activité de nage, la cohésion du groupe et le comportement de thigmotaxie, avec comme indicateur de stress en appui, une mesure de la concentration en cortisol plasmatique. Le second objectif était d'adapter le test du nouvel environnement (*novel tank*), développé chez le poisson zèbre, chez le bar en tant que nouvel outil permettant d'évaluer l'anxiété individuelle. Cette expérience a été conduite afin apporter des solutions non-invasives (du moins, moins que des prélèvements) et pertinentes pour suivre le bien-être des téléostéens dans le cadre des systèmes d'élevage.

- **Indicateurs comportementaux face au stress chimique en système d'élevage**

Quel que soit le facteur de stress chimique auquel les bars ont été exposés dans cette expérience (*i.e.* faible ou forte concentration en oxygène (20 ou 200 % de saturation en O_2), forte concentration d'azote ammoniacal (TAN=18 mg/L), ils présentaient les mêmes altérations comportementales : à savoir une **diminution de l'activité locomotrice et une diminution de la cohésion du groupe**. Premièrement, l'activité est la variable utilisée dans la plupart des études visant à quantifier le comportement. Des études précédentes avaient déjà suggéré que des conditions d'hypoxie ou de fortes valeurs de TAN peuvent diminuer l'activité des téléostéens (Dalla Via et al., 1998; Metcalfe et Butler, 1984; Shingles et al., 2001). Même si pour l'hypoxie, il est important de noter que les variations d'activité dépendent fortement de l'espèce, de la durée et de la sévérité de l'hypoxie (Chapman et Mckenzie, 2009). En

revanche pour l'hyperoxie, c'est, à notre connaissance, la première fois que cela est démontré même si des variabilités comportementales plus fortes ont déjà été observées (Espmark et Baeverfjord, 2009). D'autres facteurs permettant de caractériser la qualité de l'eau, comme la teneur en CO_2 par exemple, peuvent être une source de stress si leur concentration dans l'eau devient trop haute (hypercapnie) et altérer l'activité locomotrice des téléostéens. Par exemple, Sadoul et al. (2017) ont démontré chez deux lignées isogéniques de truite arc-en-ciel qu'une induction d'hypercapnie dans le bassin entraînait une augmentation de l'activité locomotrice qui chutait lorsque ces conditions étaient prolongées. A l'instar d'une condition d'hypercapnie, des conditions d'hypoxie ou de fortes concentrations en ammoniac (NH_3) dans l'eau, entrainent toutes deux une diminution de l'oxygène disponible pour les téléostéens. Pour NH_3, cela est causé par une réduction de la capacité de transport d'oxygène (Camargo et Alonso, 2006; Shingles et al., 2001). Pour cette raison, **nous supposons donc que la diminution d'activité en réponse à ces facteurs de stress est une adaptation comportementale afin de minimiser la dépense énergétique associée au comportement de nage** permettant ainsi de supporter le plus longtemps possible ces mauvaises qualités d'eau en attente d'une amélioration des conditions du milieu (stratégie *wait and see*; Clingerman et al., 2007; van Raaij et al., 1996).

Dans le cadre de cette thèse, un travail réalisé, mais non présenté dans ce manuscrit car encore trop préliminaire, visait à enregistrer l'activité de nage de bars dans deux qualités d'eau différentes (n=3 individus par condition) dans de réelles conditions d'élevage (50 kg/m^3). La bonne qualité d'eau se différenciait de la mauvaise principalement par des valeurs plus faibles de CO_2 (9.2±0.8 *vs.* 13.6±1.7 mg/L), de matière en suspension (10.5±1.1 *vs.* 18.7±3.9 mg/L) et de turbidité (0.29±0.04 *vs.* 0.95±0.20 NTU). La mauvaise qualité d'eau était tout de même relativement bonne et dans la gamme des standards d'élevage en aquaculture. Les déplacements des individus étaient enregistrés à l'aide de marques électroniques, des accéléromètres de télémétrie acoustique, ce qui a permis de mesurer l'activité de nage dans leur bassin sans perturbation durant 4 semaines (~une valeur par minute), et en ayant attendu suffisamment de temps après l'implantation chirurgicale de la marque (**Figure 30**).

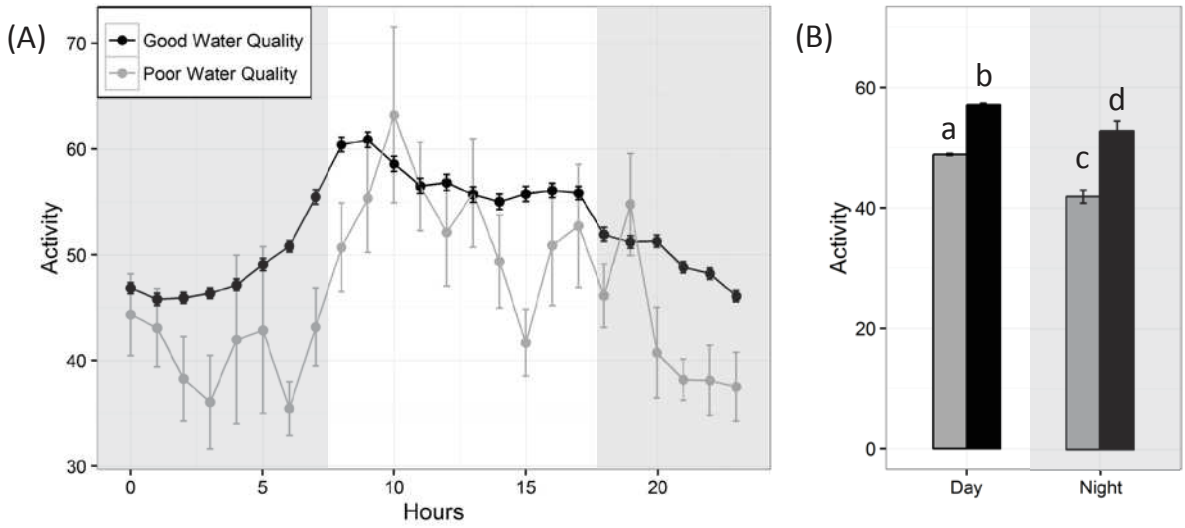


Figure 30. Activité des bars soumis à deux qualités d'eau différentes (Bonne qualité d'eau : noir et mauvaise qualité d'eau : gris) en fonction **(A)** des différentes heures de la journée et **(B)** des périodes de la journée (jour/nuit). Des lettres différentes représentent une différence significative entre les différentes conditions et périodes de la journée (Tukey HSD).

Encore une fois, on a observé **une activité plus faible des individus dans la mauvaise qualité d'eau mais également plus variable au cours de la journée**. Cette expérience ajoute un poids supplémentaire aux résultats du **chapitre 5** sur le fait qu'une diminution de l'activité des téléostéens est un indicateur pertinent et sensible en réponse à des facteurs de stress chimiques tels que des variations de la qualité de l'eau.

En plus de l'activité, **la cohésion de groupe est apparue diminuée en réponse aux différents facteurs de stress** auxquels les individus ont été exposés. Cette variable est principalement connue pour évoluer en réponse à des facteurs biotiques comme la présence de prédateurs (Seghers, 1974). Mais de récentes études soulignent le fait **que la cohésion du groupe est également un indicateur comportemental sensible à l'exposition à des facteurs de stress chimiques** (abiotiques) liés à la qualité d'eau (Colchen et al., 2017; Domenici et al., 2002, 2017; Espmark et Baeverfjord, 2009; Sadoul et al., 2017; Weetman et al., 1999). En effet, pour reprendre le même exemple que pour l'activité, les truites arc-en-ciel se dispersent en réponse à des conditions d'hypercapnie comme c'est le cas dans notre étude en réponses aux différentes qualités d'eau (Sadoul et al., 2017). D'autres expériences, menées en laboratoire, ont par ailleurs démontré que les téléostéens étaient capables d'éviter des environnements stressants comme des conditions d'hypoxie, des fortes valeurs d'ammoniac ou de CO_2 (Clingerman et al., 2007; Herbert et al., 2010; Richardson et al., 2001; Skjæraasen

et al., 2008). Les **téléostéens s'adaptent à ces facteurs de stress en s'écartant les uns des autres afin de maximiser l'absorption d'oxygène** (notamment pour des conditions d'hypoxie ou de fortes valeurs de NH_3) comme le suggère Domenici et al. (2017) en faisant référence aux conditions d'hypoxie.

La manipulation suivie de l'introduction dans un nouvel environnement conduisent à un état d'anxiété chez les téléostéens, se matérialisant par un comportement de thigmotaxie consistant à rester sur les bords de l'arène lors d'un test en *open field*, ou dans le fond du bassin lors d'un test de nouvel environnement (Egan et al., 2009; Levin et al., 2007; Schnorr et al., 2012). Dans le **chapitre 2**, nous avons observé chez les bars contrôles ce comportement de thigmotaxie dans les 5 premières minutes après introduction dans le nouvel environnement, puis une diminution dans les 30 minutes suivantes pour ensuite rester stable jusqu'à la fin du test. En revanche, chez toutes les autres conditions exposées à des facteurs de stress chimique (hypoxie, hyperoxie, forte concentration de TAN; **chapitre 5**), cette réaction normale de thigmotaxie suite à l'introduction dans un nouvel environnement a été inhibée. Les différents facteurs de stress chimique auxquels les individus ont été soumis sont connus pour affecter la survie des téléostéens lorsque l'exposition est prolongée (Magaud et al., 1997; Person-Le Ruyet et Boeuf, 1998; Rimoldi et al., 2016; Shimps et al., 2005) alors que la manipulation simple n'est pas décrite comme affectant la survie (sauf si des blessures peuvent entrainer des pathologies). D'après les résultats observés dans le chapitre 5, **nous formulons l'hypothèse selon laquelle lorsque l'individu est confronté à différents facteurs de stress, il va prioritairement s'adapter à celui qui est le plus menaçant du point de vue de son bien-être et de sa survie immédiate** (**Figure 31**). Cette hypothèse devra être testée dans le futur pour pouvoir être confirmée ou infirmée mais elle semble prometteuse pour expliquer ces résultats et suggèrent donc d'importantes capacités d'analyse de la situation chez les téléostéens (*appraisal*; Faustino et al., 2015; Galhardo et Oliveira, 2009; Galhardo et al., 2011). De plus, les mécanismes physiologiques associés à cette stratégie de *coping i.e.* répondre en priorité aux facteurs de stress les plus menaçants au détriment d'autres facteurs de stress, n'ont pas pu être totalement déterminés dans le cadre de ce chapitre.

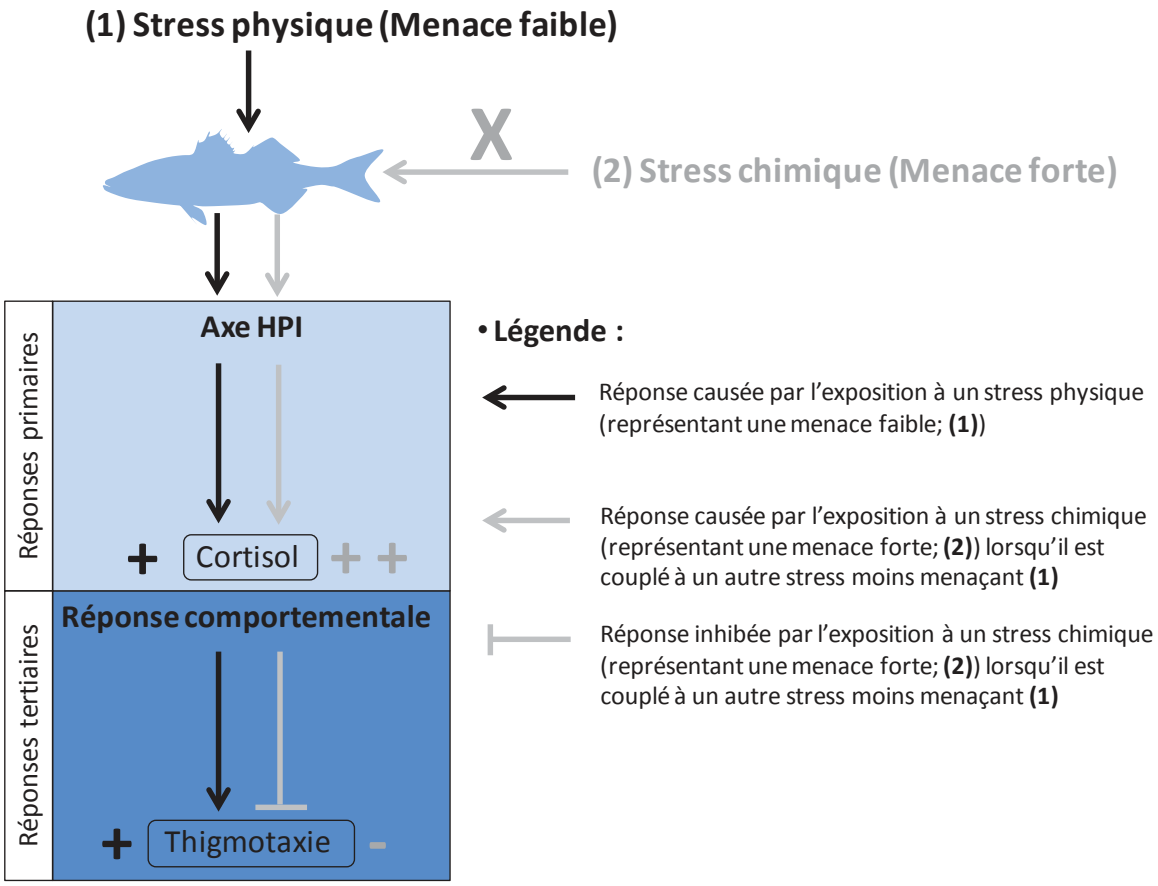


Figure 31. Réponse physiologique (primaire) et comportement associé (tertiaire) lorsque l'individu est soumis à deux facteurs de stress conjointement (physique et chimique). Les flèches noires représentent la réponse individuelle suite à un stress physique (manipulation). Les flèches grises représentent la réponse individuelle lorsqu'un stress chimique (hypoxie, forte valeur de TAN) est couplé au premier stress physique (manipulation).

La concentration en cortisol plasmatique s'est avérée plus élevée chez les individus exposés à des conditions d'hypoxie et de forte concentration en TAN par rapport aux individus contrôles. En revanche, aucune différence n'a été observée entres les individus exposés à des conditions d'hyperoxie et les individus contrôles alors que ces individus exposés présentaient les mêmes réponses comportementales que les individus exposés aux conditions d'hypoxie et de forte concentration en TAN. Des précédentes études ont rapporté des augmentations de la concentration en cortisol dans le plasma suivant une exposition chronique à de l'ammoniac (0,15 mg/L; Knoph et Olsen, 1994) et des conditions d'hyperoxie (150 % O_2 saturation; Espmark et Baeverfjord, 2009) ou des conditions aigue d'hypoxie (25 % of O_2 saturation) chez la truite arc-en-ciel (van Raaij et al., 1996). Le cortisol est connu pour agir en tant que signal permettant d'ajuster le comportement en réponse à un facteur de stress (Barton, 2002).

Cependant, de nombreuses études dans différents domaines de recherche rapportent des altérations comportementales entre différentes conditions en réponse au stress mais avec l'absence de différence dans la libération de cortisol plasmatique (Vindas et al., 2017b; **chapitre 2, 4**). Cela est principalement dû au fait que la réponse de l'axe HPI est très variable et sensible chez les téléostéens (Ellis et al., 2012) et encore plus chez le bar (Rotllant et al., 2003; Samaras et al., 2016). Nos résultats suggèrent donc que **d'autres indicateurs des mécanismes liés à la régulation du stress sont importants à mesurer pour pouvoir mieux appréhender la mise en place des réponses comportementales** chez les téléostéens comme par l'exemple la libération des monoamines dans le cerveau (Gesto et al., 2013; van Raaij et al., 1996), l'expression de gènes associés à la libération du cortisol (Vindas et al., 2017b; **Chapitre 2**) ou des monoamines cérébrales (Thornqvist et al., 2018; Vindas et al., 2017a).

Pris ensemble (**activité, cohésion du groupe et thigmotaxie**), ces **indicateurs semblent très prometteurs pour mesurer et interpréter le comportement les téléostéens en situations d'élevage** et donc réagir rapidement si des facteurs de stress liés à la qualité d'eau menace le bien-être des téléostéens et *a fortiori* leurs performances biologiques.

- **L'adaptation du nouvel environnement chez le bar (*Novel tank*)**

Dans le cadre de la recherche d'indicateurs de bien-être pertinents chez le bar, l'adaptation du test de nouvel environnement (*novel tank*) du domaine de l'écotoxicologie vers l'aquaculture est également un point du **chapitre 2** important à discuter.

A l'instar du test en *open field*, la manipulation et l'introduction dans un nouvel environnement induit un comportement d'anxiété chez le poisson zèbre se traduisant par un temps passé dans la zone haute faible en début de test qui augmente progressivement et devient ensuite stable très rapidement (Levin et al., 2007). Chez le poisson zèbre, cette réponse est observable au bout de 6 minutes dans un aquarium de 1,5 L (Hauteur x largeur x longueur; 15,2 x 7 x 28 cm) alors que ce n'est pas du tout cas chez le bar dans un aquarium de 10 L (29 x 21 x 17 cm) pour des poissons de 15 cm, qui montent très peu dans la zone haute (**Figure 32.A**). En revanche, **lorsqu'on augmente la durée d'observation à 25 minutes, on observe la même réponse comportementale chez le bar que chez le poisson zèbre sur une période de 6 minutes** (**Figure 32.B**).

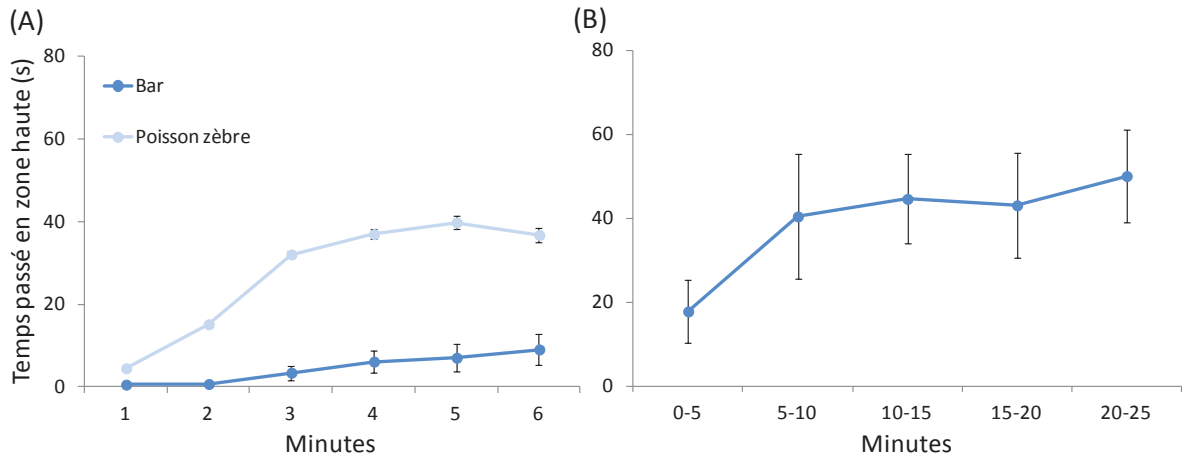


Figure 32. Temps passé dans la zone haute (s) lors d'un test de *novel tank* **(A)** chez le bar (bleu) et le poisson zèbre (bleu clair) sur une période de 6 minutes **(B)** chez le bar (bleu) sur une période de 25 minutes.

Dans le chapitre 5, nous avons étudié le positionnement vertical du bar dans le nouvel environnement après exposition à de la nicotine (molécule anxiolytique) afin de confirmer qu'un faible positionnement dans la zone haute peut être relié à un état d'anxiété comme cela a été démontré chez le poisson zèbre (Levin et al., 2007). Ces résultats ont été concluants sur une période de 25 minutes pendant laquelle le bar exprimait un temps passé dans la zone haute plus important après exposition à la nicotine et ce protocole nous a permis de **valider le *novel tank* comme un nouvel outil permettant de mesurer l'anxiété chez le bar**.

Suite à l'adaptation du *novel tank*, nous l'avons utilisé dans le **chapitre 5** pour mesurer le niveau d'anxiété dans une phase de récupération après deux heures d'exposition à différents facteurs de stress chimique (hypoxie, hyperoxie, forte valeur de TAN). Seuls les individus exposés précédemment à la condition TAN exprimaient un temps passé dans la zone haute supérieur aux individus contrôles suggérant une inhibition du comportement d'anxiété de ces individus *post*-exposition. Il est vraisemblable que les bars recherchaient en surface des conditions plus favorables en oxygène quitte à prendre des risques en se positionnant en surface. La contrainte sur l'organisme des fortes valeurs de TAN semble ainsi plus forte que des variations de la concentration en oxygène. Cependant, un retour à un comportement normal a été observé après 20 minutes *post*-exposition.

Même si nous ne pouvons pas comparer les résultats obtenus entre le test en *open field* et le *novel tank* du fait que le premier a été effectué dans de mauvaises qualités d'eau et le second avec une qualité d'eau saine, ces deux méthodes sont tout de même comparables sur d'autres aspects plus opérationnels. D'un point de vue débit d'échantillonnage, le *novel tank* réalisé en

individuel est plus robuste d'un point de vue statistique, car il permet de manipuler un grand nombre d'individus, dépendant toutefois du nombre d'individu observables en parallèle, alors que le test en *open field* en groupe nécessite une durée plus longue d'observation pour seulement un seul groupe d'individus. La mise en place de ces deux méthodes est relativement simple mais l'analyse quantitative des comportements en groupe est encore aujourd'hui plus longue et fastidieuse que l'analyse de données individuelles donnant l'avantage au *novel tank* individuel sur ce point. Il est important de noter que de nouveaux logiciels avec des interfaces conviviales sont en train de voir le jour et rendront possiblement cette analyse plus simple (Rodriguez et al., 2017, 2018). Le dernier point sur lequel ces deux méthodes diffèrent est le caractère « perturbateur » du test et cette fois-ci l'avantage est pour le test en *open field*. En effet, dans ce test les individus peuvent être testés en groupe contrairement au *novel tank* dans lequel l'isolement social est un facteur de stress supplémentaire, possiblement confondant. La célèbre citation « *good welfare = good science* » (bien-être = bonne science) peut être employé ici, suggérant ainsi que même si le débit d'échantillonnage est plus faible et l'analyse des données plus longue et compliquée pour l'*open field* en groupe, le fait qu'il soit moins invasif pour les téléostéens sociaux représente un avantage considérable sur la qualité et la robustesses des données acquises (**Tableau 6**).

Tableau 6. Comparaison de la méthode *open field* en groupe *vs. novel tank* en individuel. L'avantage à une méthode pour un critère de comparaison est symbolisé par un + et donc le désavantage de l'autre méthode pour ce critère est symbolisé par un -.

Critère de comparaison	***Open field* en groupe**	***Novel tank* individuel**
Débit d'échantillonnage	-	+
Mise en place	+	+
Analyses des données	-	+
Caractère perturbateur (isolement)	+	-
Qualité des données	+	+

3. L'importance des traits de personnalité et des *coping styles*

Dans cette partie de la discussion, nous allons revenir sur l'expérience menée dans le **chapitre 6** et discuter des capacités d'adaptation des téléostéens aux facteurs de stress en fonction de leur *coping style*, principalement en situation d'hypoxie. Ensuite, nous discuterons de ces adaptations plus largement dans le cadre du syndrome du rythme de vie (*pace of life syndrome*), et de l'importance de la stabilité de l'environnement dans un contexte aquacole ou écologique.

3.1. Des capacités d'adaptation à l'hypoxie différentes

Dans le cadre du **chapitre 6**, nous avions catégorisé le *coping style* des individus grâce à des mesures d'audace répétées avant d'étudier leurs réponses comportementales, et donc leurs capacités d'adaptation, face à des conditions d'hypoxie progressive lors d'un test en *open field*.

De manière générale, **quel que soit leur *coping style*, l'induction de l'hypoxie entraînait des altérations comportementales consistant en une diminution de l'activité locomotrice, une cohésion du banc plus faible, ainsi qu'une diminution du comportement de thigmotaxie**, variables démontrées comme des indicateurs comportementaux pertinents face à ce type de facteurs de stress dans le **chapitre 5**. Cette expérience apporte de nouvelles informations sur des points discutés précédemment avec d'une part la hiérarchisation des réponses comportementales en fonction de la dangerosité du facteur de stress sur l'individu (**section 2.2**) et d'autre part la place de l'anxiété au sein des *coping styles* (**section 1.1**).

Premièrement, quel que soit leur *coping style*, les individus diminuent leur comportement de thigmotaxie en réponse à une diminution d'oxygène. Cette réponse étaye donc l'hypothèse évoquée dans la **section 2.2** selon laquelle les individus peuvent s'adapter en priorité au stress le plus menaçant pour leur survie au détriment d'autres facteurs qui le sont moins. Deuxièmement, **la diminution du comportement de thigmotaxie suite à l'induction de l'hypoxie s'est avérée plus importante chez les individus timides que chez les individus audacieux**. Ces individus timides montraient un comportement de thigmotaxie plus élevé lors de l'introduction dans le nouvel environnement (avant toute modification de la qualité de l'eau) avant d'atteindre le même niveau que celui des individus audacieux. Ces résultats confirment le fait que les individus timides sont plus anxieux que les individus audacieux (Castanheira et al., 2017; Martins et al., 2011a; Thornqvist et al., 2018). Par ailleurs, le fait

que ces individus timides soient aussi ceux qui présentent la plus forte diminution du comportement de thigmotaxie après induction de l'hypoxie est une donnée semblant suggérer qu'en présence d'un facteur de stress (susceptible de menacer la survie de l'individu), l'individu va moduler davantage son comportement caractéristique en situation normale, comportement pouvant être de ce fait handicapant face à ce facteur de stress (*i.e.* anxiété pour les individus timides). Les individus timides montrent une plasticité plus importante dans leur comportement de thigmotaxie suite à l'induction de l'hypoxie.

Cependant, le deuxième point intéressant est que ce sont cette fois ci les individus audacieux qui **présentent une diminution de leur activité et de la cohésion du groupe plus marquée que celle des individus timides suite à l'induction de l'hypoxie**. Les individus audacieux font aussi preuve de plasticité dans leur réponse comportementale mais sur des comportements différents et fortement liés aux contraintes métaboliques (*i.e.* en maximisant plus l'oxygénation *via* l'élargissement du groupe et en minimisant leur consommation en diminuant plus leur activité). En effet, comme discuté dans la **section 1.1**, les individus proactifs possèdent un plus haut métabolisme que les individus réactifs (Killen et al., 2011) et ils ont donc besoin de sur-réagir dans leurs adaptations comportementales face à une diminution d'oxygène afin de préserver le maximum d'énergie pour pouvoir faire face à ce facteur. Pour cette raison, il me semble préférable de dire que les individus ont une plasticité différentielle selon leur *coping style* : les individus audacieux montrent une plus forte réponse dans la mise en place des adaptations comportementales visant à optimiser le métabolisme alors que les individus timides priorisent sur leur comportement anxieux.

Ce différentiel de 'cible' dans les réponses entre les deux *coping styles* suggèrent aussi que l'amplitude de la réponse est fonction du comportement 'ressenti' comme le facteur limitant l'adaptation (*i.e.* activité et cohésion de groupe chez les audacieux / anxiété chez les timides). De telles capacités d'adaptation différentes aux facteurs de stress font des *coping styles* un domaine de recherche pertinent dans le cadre de l'amélioration du bien-être des téléostéens dans un contexte d'aquaculture (Castanheira et al., 2017) et plus largement dans un contexte écologique (Conrad et al., 2011; Réale et al., 2007; Sih et al., 2012). Ces capacités d'adaptation différentielles selon les traits de personnalité ou le *coping style* sont approfondies dans la section suivante en lien avec le syndrome du rythme de vie.

3.2. Le syndrome du rythme de vie (*pace of life syndrome*)

Le syndrome du rythme de vie, *pace of life syndrome*, est une théorie récente qui inclue les traits d'histoire de vie et le concept des stratégies r et K à la théorie des *coping styles* (Réale et al., 2010). Une stratégie r est basée sur une durée de vie relativement courte avec une reproduction précoce conduisant à un grand nombre de descendants lesquels reçoivent peu de soin parentaux (MacArthur et Wilson, 1967). Au contraire, une stratégie K est basée sur une durée de vie plus longue avec une reproduction tardive produisant un faible nombre de descendants mais auxquels des soins parentaux sont donnés. Le concept de stratégies r et K a été utilisé pour définir la stratégie au niveau de l'espèce mais il peut également être discuté à l'échelle des populations, ou des individus d'une même population, par le biais des traits d'histoire de vie caractérisant les deux stratégies. **Le syndrome du rythme de vie suggère que les traits d'histoire de vie co-évoluent avec les traits de personnalité en fonction du contexte**, comme par exemple l'abondance de nourriture ou le risque de prédation pour aboutir à deux rythmes de vie différents, un rythme de vie lent (proche de la stratégie K) ou un rythme de vie rapide (proche de la stratégie r; **figure 33**).

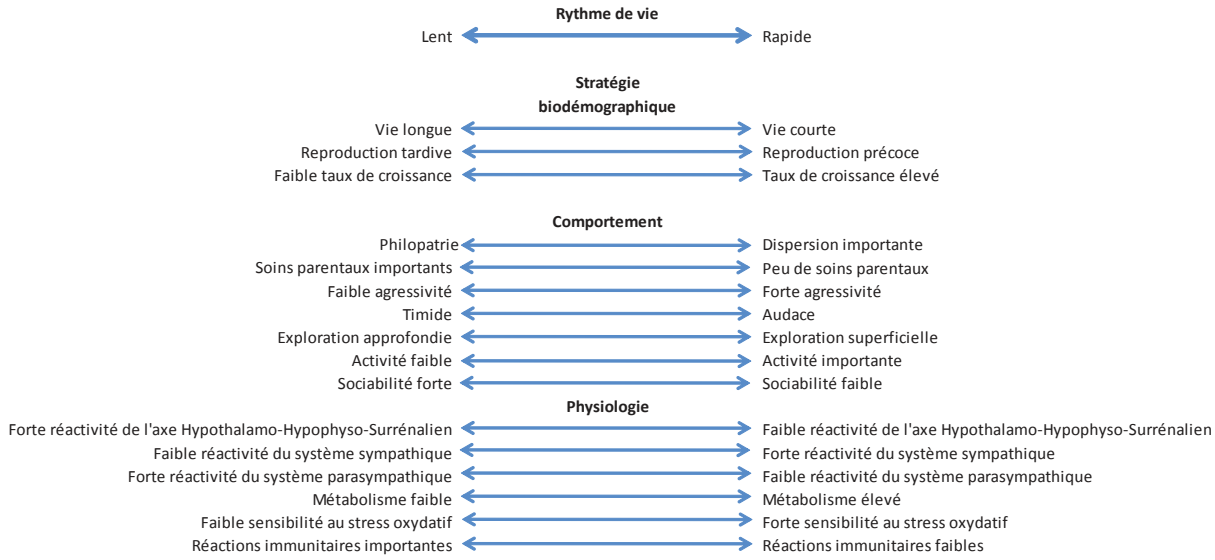


Figure 33. Variations des traits physiologiques et comportementaux en fonction des stratégies biodémographiques et des rythmes de vie d'après le syndrome des rythmes de vie. D'après Réale et al. (2010).

Par exemple, une populations de Guppy trinidadienne (*Poecillia reticulata*), exposée à un haut niveau de prédation, se montrent plus audacieuse et exprime une maturité sexuelle plus précoce et un investissement dans la reproduction important (rythme de vie rapide) contrairement à d'autres populations exposées à un plus faible niveau de prédation qui elles

présentent des traits comportementaux et d'histoire de vie opposés (rythme de vie lent; Fraser et Gilliam, 1987; Reznick et al., 1996). Un comportement plus audacieux aura tendance à conduire l'individu vers un rythme de vie rapide caractérisé notamment par un taux de croissance plus important (rythme de vie plus court). Cependant, il est important d'apporter des nuances à cette théorie dans la mesure où les individus timides sont également connus pour faire preuve de plus de plasticité dans leur réponses comportementales contrairement aux individus audacieux qui ont tendance à développer des routines comportementales (Bolhuis et al., 2004; Ruiz-Gomez et al., 2008). Il convient donc de prendre plus de précautions dans le contexte de la théorie du syndrome de rythme de vie en disant que **les individus audacieux ont un taux de croissance plus important que les individus timides lorsque les conditions du milieu sont stables** (alimentation à heure fixes, facteurs du milieu contrôlés, *etc.*) contrairement aux **individus timides qui auront tendance à avoir un meilleur taux de croissance dans un milieu plus fluctuant.** D'ailleurs, une étude récente souligne ces précautions à apporter à la théorie émise en 2010 en suggérant que cette dernière est fortement dépendante du contexte (Montiglio et al., 2018).

Dans un milieu stable, les individus audacieux sont donc théoriquement avantagés et devraient montrer de meilleures capacités d'adaptation que les individus timides et en conséquence présenter un potentiel de croissance plus important et inversement dans un milieu fluctuant. D'après la théorie de la sélection naturelle, en milieu stable, le comportement audacieux serait sélectionné au détriment du comportement timide et inversement en milieu instable (**Figure 34**). Dans le contexte de l'aquaculture, il semblerait donc judicieux de sélectionner des individus audacieux si on souhaite travailler en milieu très stable (circuit fermé en eau recirculée) ou des individus plutôt timides si on travaille en milieu instable (cages en mer, pisciculture d'étang). Cela permettrait d'avoir des individus plus adaptables à leur environnement et qui exprimeraient donc un meilleur état de bien-être. Comme évoqué en introduction générale, un état de bien-être peut conduire à de meilleures performances de croissance (Veissier et al., 2007), ce qui est une relation gagnant-gagnant pour les téléostéens et les éleveurs.

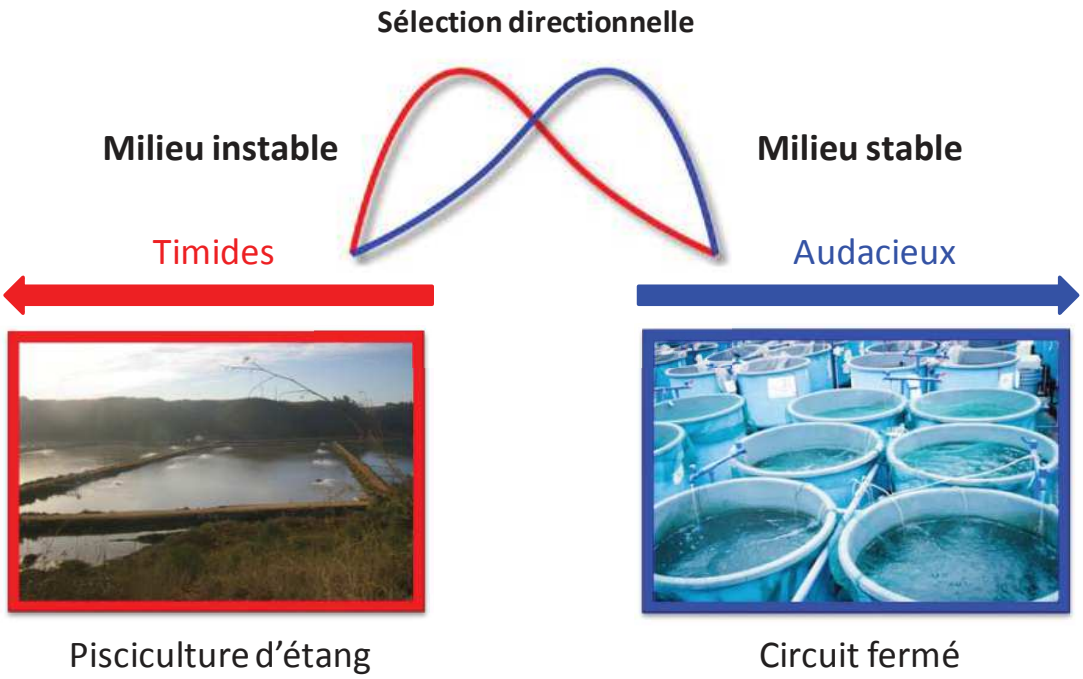


Figure 34. Sélection directionnelle au profit des individus timides dans un milieu instable (pisciculture d'étang) et audacieux dans un milieu stable (circuit fermé en eau recirculée). D'après Ferrari (2014).

Cependant, des travaux effectués lors de thèses précédentes démontrent que même dans des conditions de milieu plutôt stable, ce sont les individus timides qui présentent une meilleure croissance sur le long terme (Ferrari et al., 2016a; Millot, 2008). Les mêmes résultats ont été observés dans le cadre de cette thèse lors de différentes biométries effectuées sur des bars élevés dans un milieu contrôlé en circuit fermé (**Figure 35**). Cela suggère que les milieux dits contrôlés ne sont pas forcément aussi stables que nous le pensons pour les téléostéens. Potentiellement d'autres variables peuvent entrer en jeux auxquelles les individus timides semblent les plus à même de s'adapter et d'exprimer de meilleures performances.

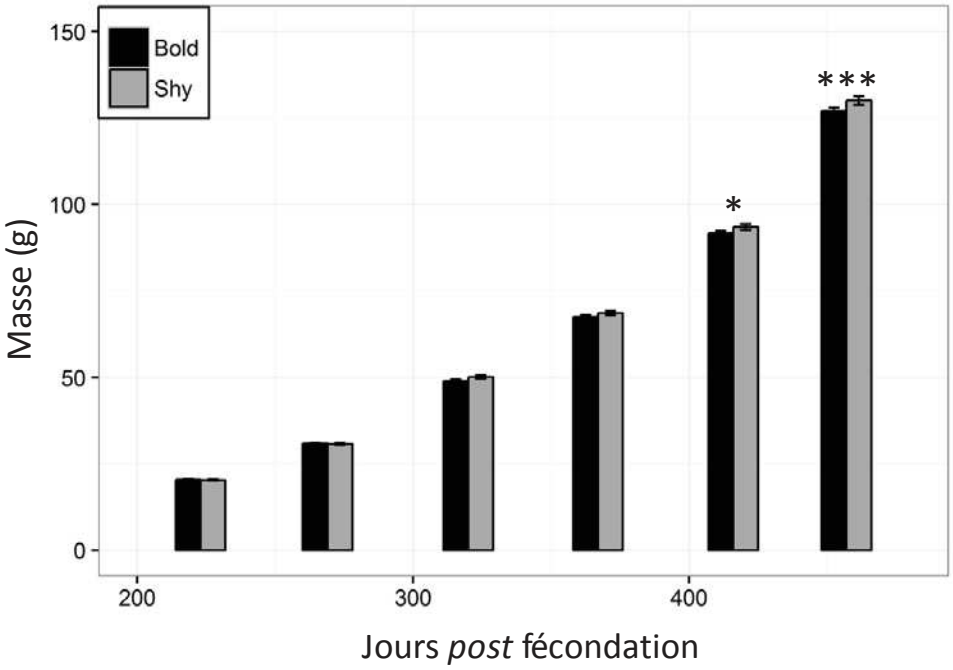


Figure 35. Masse des individus (g) sur différentes biométries réalisés à 223, 269, 320, 367, 416 et 457 jours *post* fécondation en fonction de leur trait de personnalité déterminé en test de prise de risque en groupe (Noir : audacieux, gris : timides). Les différences entre les traits de personnalité au sein de chaque biométrie sont indiquées par des astérisques : * : $p<0.05$ et *** : $p<0.001$ (Tukey HSD).

Il serait donc intéressant de rechercher par la suite si ces individus timides sont ceux qui parviennent à s'adapter et à réaliser de meilleures performances sur de longues périodes sachant que paradoxalement ils sont connus pour mettre le plus de temps à se nourrir après le transfert dans un nouvel environnement (Martins et al., 2011a, c), qu'ils sont moins compétitifs pour l'accès à la nourriture et expriment une moins bonne efficacité alimentaire que les individus audacieux (Martins et al., 2008). Serait-ce lié à leur plus forte réponse au stress *via* l'axe HPI et les systèmes monoaminergiques ? Leur plasticité neuronale plus importante leur permettrait-elle une meilleure adaptation sur le long terme ? Ces questions passionnantes autour des *coping styles* trouveront surement des réponses dans les années à venir.

4. Conclusions et perspectives

L'environnement exerce un ensemble de pressions sur les téléostéens à travers des facteurs de stress variés. Ces facteurs induisent des réactions physiologiques et comportementales chez les organismes grâce auxquelles les individus essayent de maintenir leur stabilité physiologique (homéostasie). Lorsque cette pression est trop importante, l'homéostasie ne

peut être retrouvée et cela se traduit par des altérations des mécanismes physiologiques et comportementaux à court ou long terme dépendant de la période, de la durée et de la sévérité du stress. Ces altérations peuvent alors menacer la survie, la croissance et la reproduction et donc entrainer des conséquences à l'échelle des populations expérimentales ou naturelles (**Figure 36**).

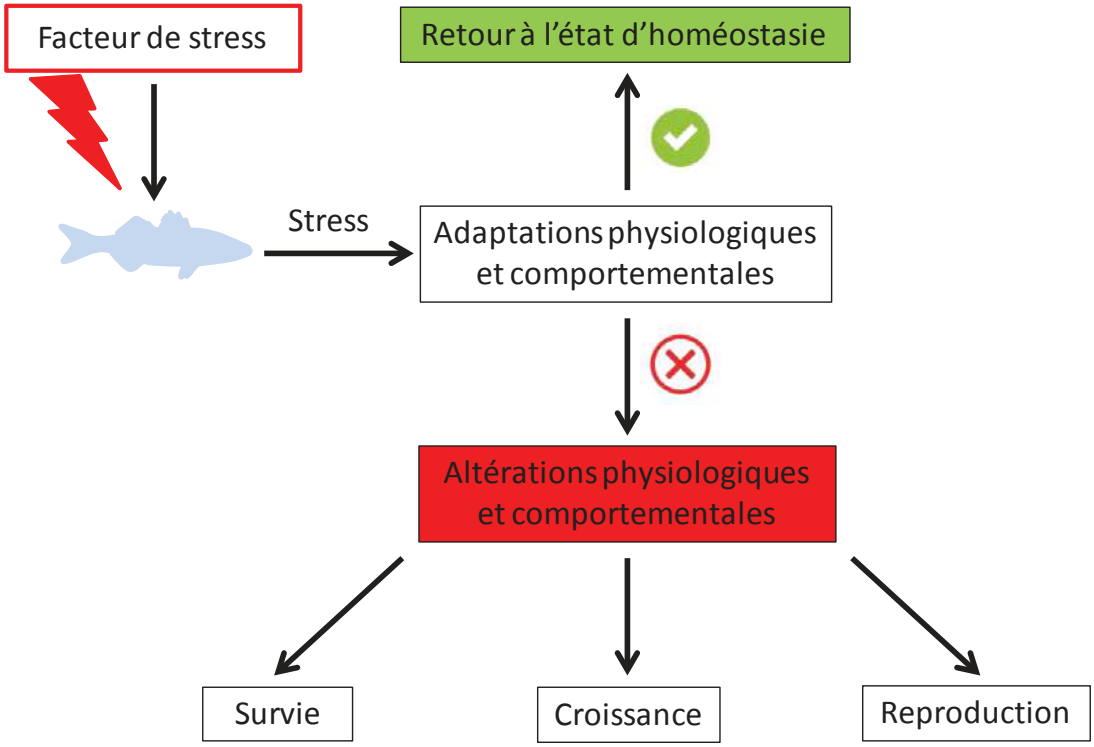


Figure 36. Réponses aux facteurs de stress et conséquences sur les traits d'histoire de vie (Survie, croissance, reproduction).

Cependant, un individu X diffère d'un individu Y de par sa physiologie, son comportement ou ses capacités d'apprentissage. Il est désormais largement démontré que ces différences interindividuelles d'un point de vue physiologique et comportemental sont interconnectées aboutissant à un *continuum* entre deux types extrêmes d'individus, les proactifs et les réactifs. Ces individus plutôt proactifs ou réactifs présentent également des différences dans leurs capacités d'adaptation face aux facteurs de stress conduisant à des états de bien-être différentiels pouvant avoir aussi des conséquences sur les performances biologiques (**Figure 37**). Ces *coping styles* peuvent donc offrir des perspectives considérables autant sur le plan du bien-être et des performances de croissance dans des conditions d'aquaculture que sur le plan écologique avec les problématiques de conservation, de pollution environnementale ou de réchauffement climatique.

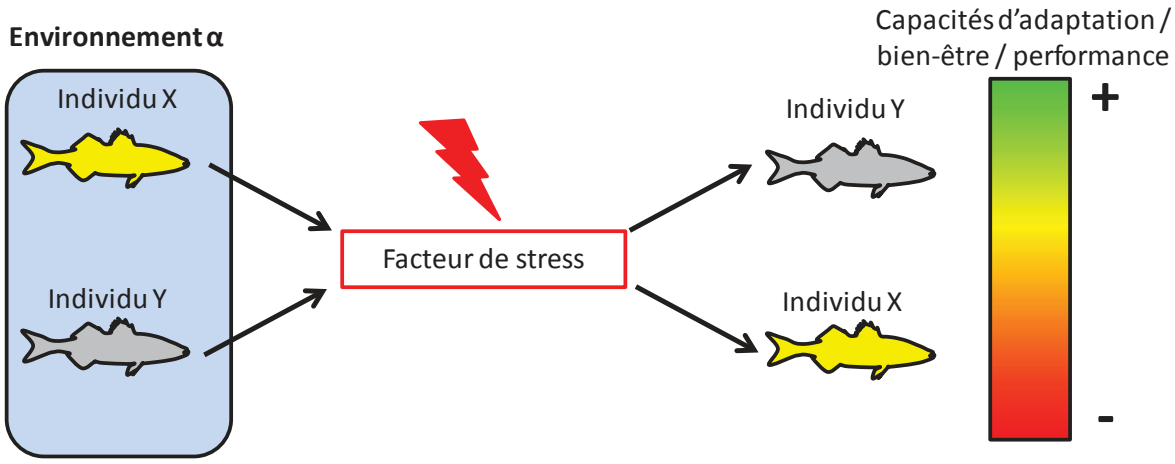


Figure 37. Réponses différentielles entre deux individus X et Y dans un environnement dans leurs capacités d'adaptation, leur état de bien-être et de performances biologiques. Des couleurs différentes représentent des individus aux personnalités différentes.

Dans le cadre de cette thèse, nous aurions souhaité étudier plus en détail les capacités d'adaptation des téléostéens en fonction de leur *coping styles*, comme par exemple suite à une exposition aux polluants ou une exposition précoce à des molécules aversives. Malheureusement la plasticité des réponses comportementales observée entre les différents stades de vie ne permet pas de caractériser un *coping style* stable et donc cohérent dans le temps dès le stade larvaire. En conséquence, les expositions très précoces aux facteurs de stress chimique ont dû être abandonnées. Que ce soit pour une exposition à des polluants ou pour d'autres facteurs de stress, je ne doute pas que des études seront menées dans le futur afin de mieux comprendre et pouvoir qualifier les capacités d'adaptation des différents *coping styles*.

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Annexes

Annexe 1

Sébastien Alfonso – French – 27 years old

PhD – Ecology and Biodiversity

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Employment history

March 2018	**Temporary teacher**, Fish behavior and applications to Aquaculture and Ecotoxicology, 17 hours of teaching at Cnam (Cherbourg, France)
2015-2018	**PhD student** at Ifremer (Palavas-les-flots, France) Working on the "Interaction between personality traits in teleost fish, environment and pollutants" under Marie-Laure Bégout's supervision
2015	**Master internship** of 6 months at Ifremer (La Rochelle, France) Working on the "Effects of chronic exposure to persistent organic pollutants on reproduction of zebrafish and behavioral abilities of offspring" under Marie-Laure Bégout's supervision
2014	**Master internship** of 3 months at University of Saint-Etienne (Saint-Etienne, France) Working on the "Influences of social status and temperament on the perception of acoustic and visual signals in cichlid males *Metriaclima zebra*" under Joël Attia's supervision

Education

2015-2019	**PhD degree** in ecology and biodiversity – University of Montpellier
2013-2015	**Master degree** in animal behavior and ecology – University of Saint-Etienne
2009-2013	**Bachelor degree** in Biological Oceanography – University of Marseille

Additional education and technical skills

2017	French qualification to design and conduct animal experiments, specialization in fish
2015	Animal personality: concepts, methodology and applications, University of Leiden

Scientific and technical skills:

Fish behavior	■■■■■	**Fish rearing**	■■■■□
Physiology	■■■■□	**Blood/brain sampling**	■■■■■
Aquaculture	■■■■□	**Behavior tracking**	■■■■□
Statistics	■■■■□	**Communication**	■■■■□
Ecology	■■■□□	**Writing**	■■■□□

Languages:

French	■■■■■
English	■■■■□
Italian	■□□□□

Software: Pack office, R, Statistica, Ethovision, Etholog, EndNote

Publications

2019

Alfonso S., Sadoul B., Gesto M., Joassard L., Chatain B., Geffroy B., Bégout M.L.. **Coping styles in European sea bass: the link between boldness, stress response and neurogenesis.** *Physiology and Behavior* (minor revisions).

Alfonso S., Blanc M., Joassard L., Keiter S., Munschy C., Loizeau V., Bégout M.L., Cousin X. (2019). **Examining multi- and transgenerational behavioral and molecular alterations resulting from parental exposure to a PCB and PBDE environmental mixture.** *Aquatic Toxicology, 208, 29-38.* https://doi.org/10.1016/j.aquatox.2018.12.021

2018

Sadoul B., Alfonso S., Bessa E., Bouchareb A., Blondeau-Bidet E., Clair P., Chatain B., Bégout M.L., Geffroy B. (2018). **Enhanced brain expression of genes related to cell proliferation and neural differentiation is associated with cortisol receptor expression in fishes**. *General and Comparative Endocrinology* , 267, 76-81. http://doi.org/10.1016/j.ygcen.2018.06.001

Horri K., Alfonso S., Cousin X., Munschy C., Loizeau V., Aroua S., Bégout M.L., Ernande B. (2018). **Fish life-history traits are affected after chronic dietary exposure to an environmentally realistic marine mixture of PCBs and PBDEs**. *Science Of The Total Environment*, 610-611, 531-545. http://doi.org/10.1016/j.scitotenv.2017.08.083

In prep.

Alfonso S., Cousin X., Sadoul B., Joassard L., Chatain B., Bégout M.L.. **Spatial distribution and activity patterns as welfare indicators in response to water quality changes in European sea bass, *Dicentrarchus labrax*.** *In preparation for Journal of Experimental Biology.*

Alfonso S., Sadoul B., Chatain B., Geffroy B., Prunet P., Bégout M.L.. **Divergent behavioral and molecular responses to hypoxia for opposed coping styles in European sea bass, *Dicentrarchus labrax*.** *In preparation for Molecular ecology.*

Alfonso S., Peyrafort M., Cousin X., Bégout M.L.. **Zebrafish behavioral syndrome is consistent at different ages from larvae to juveniles but not over life stage.** *In preparation for Journal of Experimental Biology.*

Alfonso S., Cousin X., Joassard L., Munschy C., Loizeau V., Bégout M.L.. **Chronic dietary exposure to an environmental mixture of PCBs and PBDEs alters boldness and anxiety syndromes over generations in zebrafish**. *In preparation.*

Alfonso S., Carbonara P., Zuppa W., Triplet S., Spedicato M.T., Lembo G., Bégout M.L.. **Activity of sea bass, *Dicentrarchus labrax*, in RAS system: sensitivity to water quality**. *In preparation.*

Communications

2018

Alfonso S., Leguay D., Joassard L., Jarc C., Cousin X., Chatain B., Bégout M.L.. **Behavioral and physiological responses to hypoxia, hyperoxia and high total ammonia nitrogen concentration (TAN) in European sea bass, *Dicentrarchus labrax*.** AQUA2018, World Aquaculture Society. 25-29 August 2018, Montpellier, France.

Alfonso S., Geffroy B., Sadoul B., Joassard L., Gesto M., Chatain B., Bégout M.L. **Stress coping styles in European sea bass *(Dicentrarchus labrax):* from genes to behaviour.** AQUA2018, World Aquaculture Society. 25-29 August 2018, Montpellier, France.

Alfonso S., Leguay D., Joassard L., Jarc C., Cousin X., Chatain B., Bégout M.L.. **Behavioral and physiological responses to hypoxia, hyperoxia and high total ammonia nitrogen concentration (TAN) in European sea bass, *Dicentrarchus labrax*.** Society of Experimental Biology, annual meeting. 3-6 July 2018, Florence, Italy.

Alfonso S., Geffroy B., Sadoul B., Joassard L., Gesto M., Chatain B., Bégout M.L. **Stress coping styles in European sea bass *(Dicentrarchus labrax):* from genes to behaviour.** Society of Experimental Biology, annual meeting. 3-6 July 2018, Florence, Italy.

2017

Alfonso S., Peyrafort M., Joassard L., Jarc C., Leguay D., Cousin X., Begout M.-L.. **Expression et cohérence du coping-style chez le poisson zèbre (*Danio rerio*) du stade larvaire à l'adulte.** SFECA 2017 - Colloque national de la Société Française pour l'Etude du Comportement Animal. 15-17 May 2017, Gif sur Yvette. *[Poster presentation]*

Alfonso S., Blanc M., Bringer A., Leguay D., Joassard L., Loizeau V., Munschy C., Bégout M.L., Cousin X.. **Persistent Organic Pollutants chronic dietary exposure disrupt behavioral and molecular responses over several generations in unexposed zebrafish (*Danio rerio*).** 27th SETAC Congress of The society of Environmental Toxicology and Chemistry. 7-11 May 2017, Brussels, Belgium.

2016

Alfonso S., Blanc M., Bringer A., Leguay D., Joassard L., Loizeau V., Munschy C., Bégout M.L., Cousin X.. **Deciphering behavioural disruption in zebrafish exposed to PCBs and PBDEs environmental mixtures and in unexposed offspring over several generations.** 30th ESCPB Congress of New European Society for Comparative Physiology and Biochemistry. 4-7 September 2016, Barcelona, Spain.

Alfonso S., Bringer A., Leguay D., Joassard L., Begout M.L., Cousin X.. **Does zebrafish parental trophic POPs exposure affect coping style in unexposed offspring ?** SFECA 2016 - 46ème COLLOQUE de la Société Française pour l'Etude du Comportement Animal. 22-24 March 2016, Caen, France.

Alfonso S., Blanc M., Vignet C., Joassard L., Vouillarmet A., Trenkel V., Le Menach K., Budzinski H., Bricca G., Begout M.L., Cousin X.. **1, 2, 3... different generations, different effects – multigenerational toxicity assessment of environmental mixtures.** 7th annual meeting of the EFOR Network (Réseau d'Etudes Fonctionnelles chez les ORganismes modèles). 2016, 7-8 March, Paris, France.

2015

Alfonso S., Blanc M., Vignet C., Joassard L., Vouillarmet A., Trenkel V., Le Menach K., Budzinski H., Bricca G., Begout M.L., Cousin X.. **Neurobehavioral disruption after exposure to persistent organic pollutants.** 4th International Symposium: Effect-related evaluation of anthropogenic trace substances - concepts for genotoxicity, neurotoxicity and endocrine effects. 22-23 October 2015, Aachen, Germany.

2014

Alfonso S., Attia J., Beauchaud M., Boyer N., Bertucci F., Bouchut C., Bertucci F., Mathevon N.. **Cross-sensory modulation depends on social status and temperament : an experimental investigation in a cichlid fish.** SFECA 2014 - 44ème COLLOQUE de la Société Française pour l'Etude du Comportement Animal. 8-10 July 2014, Paris, France. *[Poster presentation]*

Annexe 2

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Fish life-history traits are affected after chronic dietary exposure to an environmentally realistic marine mixture of PCBs and PBDEs

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HIGHLIGHTS

- Effects of realistic marine PCB/PBDE mixtures on fish life-history are unknown.
- Zebrafish were chronically exposed to a realistic marine PCB/PBDE mixture via diet.
- Exposed fish grew to larger sizes but their spawning probability was delayed.
- Larval survival of offspring from early spawns was decreased under starvation.
- Environmental PCB/PBDE mixture can alter fish population dynamics via life-history.

GRAPHICAL ABSTRACT

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Condition
Fertilization rate
Energy allocation
Trade-off

ABSTRACT

Polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) are persistent organic pollutants that have been shown to affect fish life-history traits such as reproductive success, growth and survival. At the individual level, their toxicity and underlying mechanisms of action have been studied through experimental exposure. However, the number of experimental studies approaching marine environmental situations is scarce, i.e., in most cases, individuals are exposed to either single congeners, or single types of molecules, or high concentrations, so that results can hardly be transposed to natural populations. In the present study, we evaluated the effect of chronic dietary exposure to an environmentally realistic marine mixture of PCB and PBDE congeners on zebrafish life-history traits from larval to adult stage. Exposure was conducted through diet from the first meal and throughout the life cycle of the fish. The mixture was composed so as to approach environmentally relevant marine conditions in terms of both congener composition and concentrations. Life-history traits of exposed fish were compared to those of control individuals using several replicate populations in each treatment. We found evidence of slower body growth, but to a larger asymptotic length, and delayed spawning probability in exposed fish. In addition, offspring issued from early spawning events of exposed fish exhibited a lower larval survival under starvation condition. Given their strong dependency on life-history traits, marine fish population

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Short communication

Enhanced brain expression of genes related to cell proliferation and neural differentiation is associated with cortisol receptor expression in fishes

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ABSTRACT

Stress enhances or inhibits neurogenesis in mammals and some fish species. The link between the two processes is still unclear. Most studies have been performed in very specific stressful or altered environments. Despite the known inter-individual divergence in coping abilities within populations, the relationship between the stress axis and neurogenesis has never been addressed in unstressed individuals. Here we correlate brain expression of the *pcna* (proliferating cell nuclear antigen) and *neurodI* (neurogenic differentiation factor 1) genes, two markers of neurogenesis, with transcripts of cortisol receptors in three fish species living in very distinct environments. Within the three species, individuals with the highest expression of neurogenesis genes were also those that expressed the high levels of cortisol receptors. Based on these correlations and the hypothesis that mRNA levels are proxies of protein levels, we hypothesize that within unstressed animals, individuals sensitive to cortisol perceive a similar environment to be more stimulating, leading to increased neurogenesis. Although it is difficult to determine whether it is sensitivity to cortisol that affects neurogenesis capacities or the opposite, the proposed pathway is a potentially fruitful avenue that warrants further mechanistic experiments.

1. Introduction

Coping with the environment is a major component of fitness traits, especially in a world with increased environmental variability and frequent extreme events (Bonier et al., 2009; Németh et al., 2013). The hypothalamo-pituitary-interrenal axis plays a central role in coping abilities of fishes, with the final product, cortisol, acting as the endocrine activator of most physiological responses to perturbations (Schreck and Tort, 2016). Most importantly, it allocates energy initially directed towards the production of structures in the liver and muscles to fuel coping behavior, osmoregulatory mechanisms, and immune responses (Sadoul and Vijayan, 2016). Cortisol is also known to operate on the brain, mostly by regulating its own production (Fryer and Peter, 1977) and its transformation into the inactive metabolite, cortisone (Alderman and Vijayan, 2012), through a negative feedback loop. These effects occur primarily by the binding of cortisol to its receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). These two receptors are found in many fish tissues, including the brain (Faught et al., 2016).

Recent studies in fish have demonstrated that cortisol has a role in brain physiology other than simply its own regulation. Cortisol treatment during development was found to increase neurogenesis, affecting brain development, in treated zebrafish, *Danio rerio* (Best et al., 2017). In contrast, a study in adult rainbow trout, *Oncorhynchus mykiss*, demonstrated that elevated cortisol levels lead to reduced cell proliferation in the brain (Sørensen et al., 2011). In addition, inhibition of GR, reducing the actions of cortisol, was found to increase the expression of genes related to neurogenesis during development (Nesan and Vijayan, 2013). These studies highlight a link between the stress axis and neurogenesis in fishes. However, the results are still inconsistent and the mechanisms involved mostly unknown. It is still unclear whether such opposing results are the consequence of differences between species or experimental set up.

Here, we investigated the relationship between the expression of genes related to neurogenesis and those involved in cortisol signaling by exploring the natural variability between individuals of three fish species from very distinct environments: European sea bass, *Dicentrarchus labrax*, from temperate marine waters, tetra fish,

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Résumé / Abstract

Dans le cadre de cette thèse, nous avons étudié la mise en place de la personnalité, ainsi que sa cohérence en fonction du contexte et des différents stades vie de l'individu et les mécanismes physiologiques associés. La personnalité est apparue cohérente entre les contextes sur de courtes périodes mais ne l'est plus entre les différents stades de vie. Les mécanismes physiologiques de régulation du stress (cortisol et monoamines), ainsi que l'activité et la plasticité neuronale divergent en fonction de l'audace des individus. Ensuite, nous avons étudié les réponses physiologiques et comportementales des téléostéens exposés chroniquement à un mélange de POP (PCB et PBDE), représentatif des conditions environnementales, ainsi que celles de leur descendance non exposée. Des altérations comportementales différentes de la réponse photomotrice larvaire, de l'audace et d'un syndrome comportemental lié à l'anxiété en fonction des générations ont été observées jusqu'à la génération F2 ou F4. Des altérations du niveau d'expression des enzymes responsables de la méthylation de l'ADN ont été également observées suggérant que des mécanismes épigénétiques sont responsables de la transmission de ces altérations chez la descendance. Ensuite, le comportement a été étudié dans des qualités d'eau dégradées afin de mettre en place des indicateurs comportementaux de bien-être pertinents. Les résultats ont montré que suite à la variation de l'oxygénation ou de la teneur en NH_3, les individus s'adaptent en diminuant leur activité de nage et la cohésion du groupe. De plus, nous avons observé que le comportement normal de thigmotaxie est inhibé lors de l'exposition à une qualité d'eau dégradée. Cela suggère une hiérarchisation des adaptations comportementales en fonction de la sévérité du stress. Enfin, nous avons étudié les réponses comportementales à une diminution du niveau d'oxygène dans l'eau en fonction de la personnalité : les individus audacieux diminuaient leur activité et leur cohésion de nage avec une plus grande amplitude que les individus timides qui eux diminuaient, avec une plus grande amplitude, le comportement de thigmotaxie. Ces capacités d'adaptation différentes face au stress, en fonction de la personnalité, apportent de nouvelles perspectives pour faire face aux enjeux dans des contextes écologique et aquacole.

In the frame of this thesis, we first studied the establishment of personality in teleost, the consistency across context and different life stages as well as the associated physiological mechanisms. Personality appeared consistent across contexts over a short time period but it was no longer the case over a long period of time, especially between different life stages. Physiological mechanisms of stress regulation, as well as activity and neural plasticity, differed according to individual boldness. Then, we studied the physiological and behavioural responses of teleost fish chronically exposed to an environmentally relevant mixture of POP (PCBs and PBDEs), as well as those of their unexposed offspring. Different behavioural alterations in larval photomotor response, boldness and a behavioural syndrome linked to anxiety have been observed between generations up to the F2 or F4 generations. Alterations in the expression of enzymes responsible of DNA methylation were also observed suggesting that epigenetic mechanisms are involved in the transmission of these behavioural alterations in unexposed offspring. Behaviour was also studied under stressful water quality conditions in order to set behavioural welfare indicators. Results showed that individuals cope with these alterations in oxygen levels or NH_3 concentration by decreasing their swimming activity and group cohesion. In addition, we observed that the normal thigmotaxis behaviour was inhibited during exposure to degraded water quality. It suggests a hierarchy of behavioural adaptations depending on stress severity. Finally, we studied the behavioural responses to a decrease in oxygen level according to personality: bold individuals decreased their activity and swimming cohesion with greater amplitude than shy individuals which decreased with higher amplitude thigmotaxis behaviour. These abilities to cope with stress, depending on the personality of teleost fish, bring new perspectives to face both ecological and aquaculture issues.