Plasticité phénotypique et variabilité intraspécifique de la tolérance à la dessalure chez le loup méditerranéen Dicentrarchus labrax

Thibaut L'Honoré

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PLASTICITÉ PHÉNOTYPIQUE ET VARIABILITÉ INTRASPÉCIFIQUE DE LA TOLÉRANCE À LA DESSALURE CHEZ LE LOUP MEDITERRANÉEN *DICENTRARCHUS LABRAX*

Présentée par Thibaut L'HONORÉ

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Sous la direction de Catherine LORIN-NEBEL et Emilie FARCY

Devant le jury composé de

François LALLIER, Professeur, Sorbonne Université	Rapporteur
Jean-Patrice ROBIN, Directeur de Recherche, CNRS	Rapporteur
David MCKENZIE, Directeur de Recherche, CNRS	Examinateur, Président du jury
Guillaume MITTA, Professeur, Université de Perpignan	Examinateur
Laurence BESSEAU, Maître de Conférences, Sorbonne Université	Invitée
Catherine LORIN-NEBEL, Maître de Conférences, Université de Montpellier	Directrice
Emilie FARCY, Maître de Conférences, Université de Montpellier	Co-encadrante

UNIVERSITÉ DE MONTPELLIER

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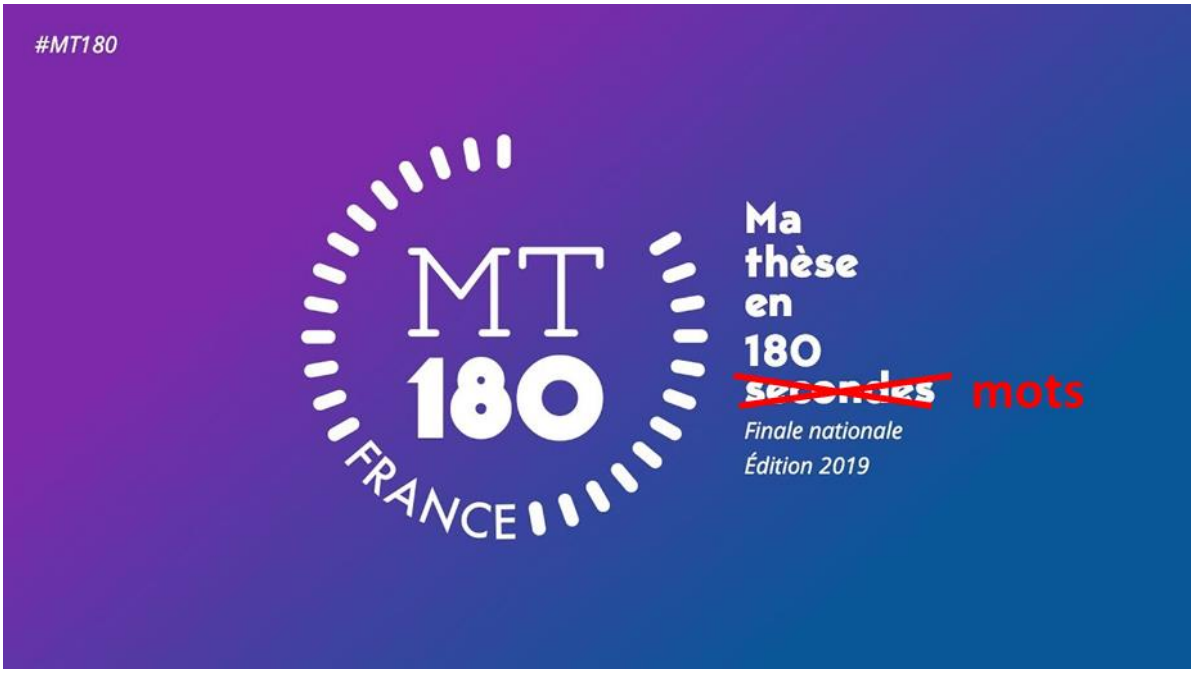
Ma thèse en 180 mots

Face à un changement dans leur environnement, les organismes doivent s'**acclimater** afin de survivre. L'acclimatation consiste en des modifications de certaines de leurs caractéristiques biologiques, ou **phénotype**. On parle de **plasticité phénotypique**. Le loup méditerranéen *Dicentrarchus labrax* est un poisson marin qui entreprend des migrations dès le stade juvénile dans les lagunes voire en rivière. En laboratoire, 30% de mortalité ont été mis en évidence en eau douce. Pendant ma thèse j'ai pu montrer que la dessalure augmentait leur tolérance à l'hypoxie, mais seulement pour de courtes durées. Les individus incapables de tolérer l'eau douce présentaient des traits comportementaux et biochimiques différents de ceux des tolérants. L'étude de l'expression des gènes a révélé que **l'intolérance à l'eau douce** serait due en partie à une **incapacité au niveau rénal** à **réabsorber les ions**. Cette étude répétée à des âges différents démontre que l'intolérance à l'eau douce est un **phénomène labile**. Cela pourrait être dû à des mécanismes de régulation épigénétiques comme la méthylation de l'ADN. Des premières réponses semblent confirmer que le passage en eau douce influe la méthylation de l'ADN.



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Liste des abréviations

SW : Seawater : eau de mer

FW : Fresh water : eau douce

FW_t : Freshwater tolerant : phénotype tolérant à l'eau douce

FW_i : Freshwater intolerant : phénotype intolérant à l'eau douce

HR : High Responsiveness : phenotype proactif

LR : Low Responsiveness : phenotype réactif

HT : Hypoxia Tolerant : phénotype tolérant à l'hypoxie

HS : Hypoxia Sensitive : phénotype sensible à l'hypoxie

NKA : Na^+-K^+-ATPase

VHA : V-type H^+-ATPase

NCC : Co-transporteur Na^+/2 Cl^-

NKCC : Co-transporteur Na^+-K^+/2 Cl^-

NHE : Échangeur Na^+/H^+

MRCs : Mitochondrion-rich cells : ionocytes

SMR : Standard Metabolic Rate : Taux métabolique standard

O_{2crit} : Saturation critique en oxygène

$O_{2deficit}$: Déficit en oxygène

ILOS : Incipient Letal Oxygen Saturation : saturation létale en oxygène

AOD : Accumulative Oxygen Deficit : déficit cumulé en oxygène

Introduction

Environment proposes, natural selection disposes

1. La Méditerranée et les changements environnementaux

La Méditerranée est un réservoir de biodiversité marine (Fig. 1) avec un fort taux d'endémisme où cohabitent des organismes tempérés et subtropicaux (Tortonese, 1985; Coll *et al.*, 2010).

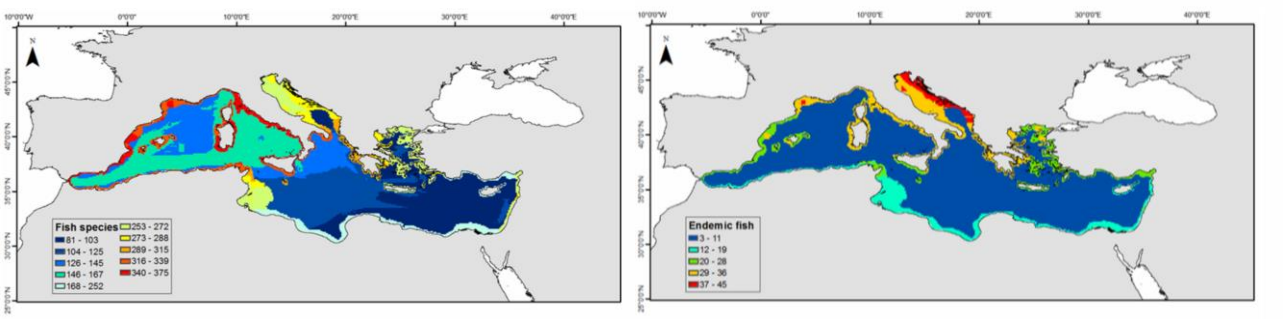


Figure 1 : Modélisation spatiale de la richesse en espèces de poissons en Méditerranée, les couleurs indiquant l'occurrence des espèces (du bleu : pas ou peu d'occurrence, au rouge : forte occurrence), d'après Coll et al., 2010

Elle est considérée comme « hot spot » du réchauffement climatique de par sa sensibilité aux perturbations d'origine environnementale ou anthropique (Giorgi, 2006; Nicholls *et al.*, 2007; Pörtner *et al.*, 2014). D'après Coll *et al.*, (2010), ces perturbations sont d'origines multiples : pollution, eutrophisation, espèces invasives, réchauffement climatique, et sont amenées à croître dans les décennies à venir (Fig. 2). Elles affectent non seulement la physiologie des organismes, mais aussi leurs interactions biotiques (compétitions, prédation), leur distribution ainsi que leur survie (Bianchi & Morri, 2000; Walther *et al.*, 2002; Verdura *et al.*, 2019). Les migrations vers le nord d'espèces subtropicales peuvent représenter un stress supplémentaires pour les populations locales et amener à de nouvelles stratégies pour les espèces locales comme la fuite ou la migration (Mannino *et al.*, 2017).

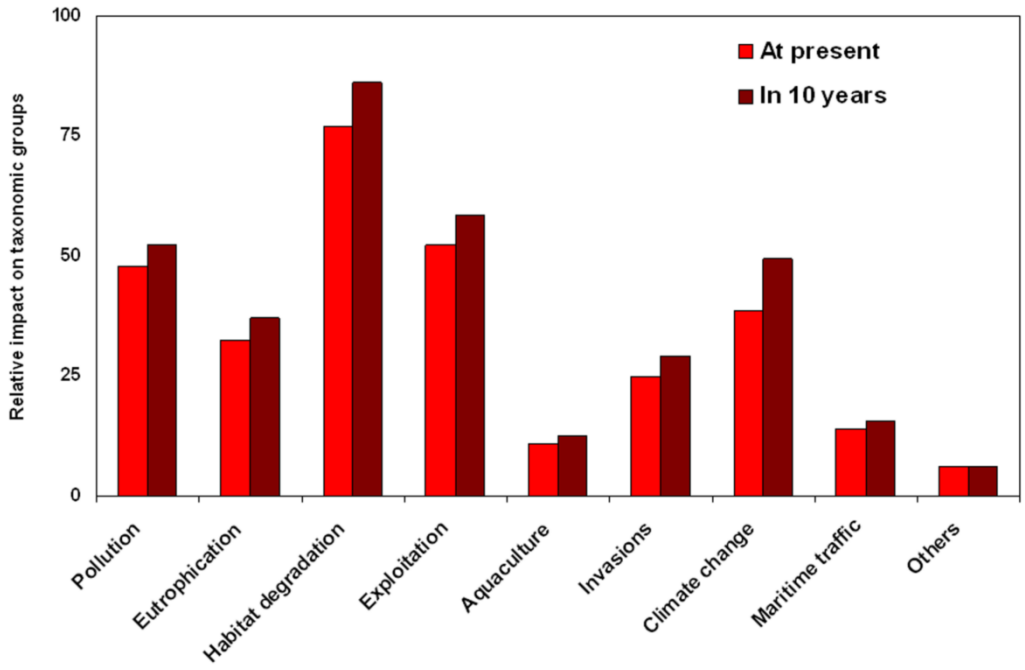


Figure 2 : Menaces actuelles et futures pour la biodiversité en Méditerranée, d'après Coll et al., 2010

L'ouest du bassin Méditerranéen est riche en **environnements** dits **de transition** tels que les lagunes et les estuaires (Pérez-Ruzafa *et al.*, 2011). Ces environnements jouent un rôle important pour les écosystèmes, servant de zone de nourrissage et de nurseries pour de nombreuses espèces de poissons. Les conditions abiotiques (salinité, température, oxygène dissous) y fluctuent énormément selon les apports à la fois pluviométriques, marins et/ou continentaux ainsi que selon la saison (Fig. 3) (Dalrymple *et al.*, 1992).

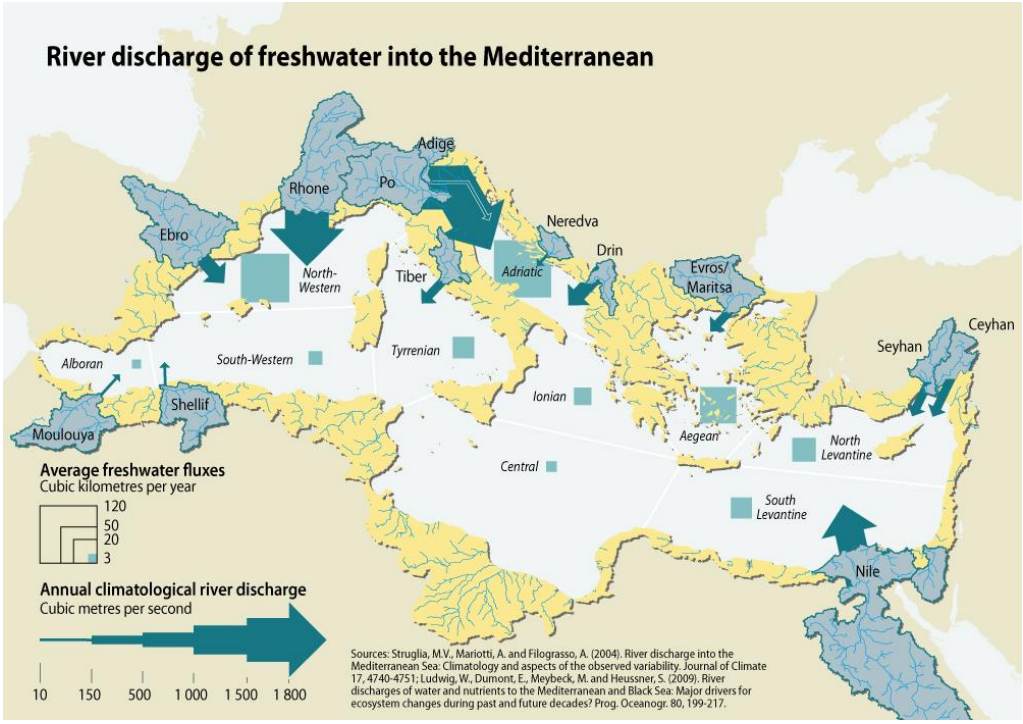


Figure 3 : Afflux d'eau douce des rivières vers la Méditerranée, d'après GRID-Arendal (http://www.grida.no/resources/5897)

D'importantes fluctuations de salinité sont retrouvées dans différentes lagunes en Méditerranée Ouest (Tableau 1). Par exemple dans l'étang d'Arnel (lagune palavasienne, Hérault, France), où de fortes densités de loups ont été relevées en 2005 et 2006 (Dufour *et al.*, 2009) (Fig. 4), la salinité a varié entre 7.8‰ et 44‰ entre les mois de mars et le mois d'août en 2013.

Tableau 1 : Salinité de lagunes et d'étangs méditerranéen où Dicentrarchus labrax est retrouvé. D'après Ifremer (2014) Réseau de Suivi Lagunaire du Languedoc-Roussillon : Bilan des résultats 2013. Rapport RSL-14/2014, p. 219

Lagunes	Salinité (‰)	
	Min	Max
Etang de Canet Saint-Nazaire	4	38
Lagune de Salses-Leucate	17	40
Etang de La Palme	10	76
Etang de Bages Sigean	29.5	40
Etangs de Campignol-Ayrolle et Gruissan	0.5	39
Etang de Vendres	0.1	33.4
Etang du Grand Bagnas	0	18
Etang de Thau	34	40
Etangs palavasiens – exemple étang d'Arnel	7.8	44
Etangs de la Camargue gardoise	2.3	29

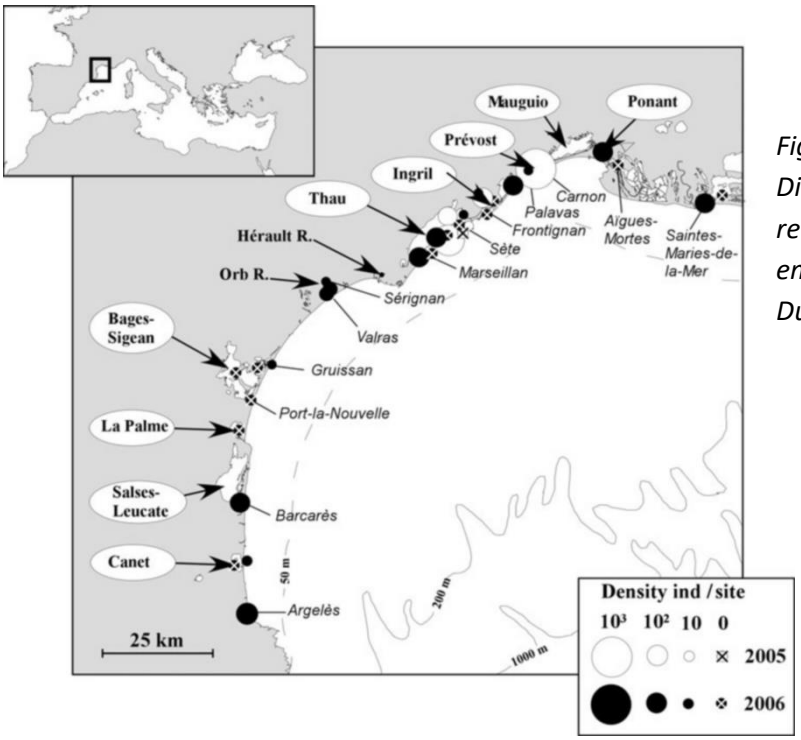


Figure 4 : Densité de Dicentrarchus labrax retrouvés par site d'étude en 2005 et 2006, d'après Dufour et al., 2009

La salinité agit comme un facteur clef dans la survie et la distribution des espèces (Wong *et al.*, 1999; Pierce *et al.*, 2012). En effet, certains poissons comme la daurade *Sparus aurata*, le mullet cabot *Mugil cephalus* ou encore le loup (ou bar) européen *Dicentrarchus labrax* migrent vers ces habitats de transition au cours de leur cycle de vie (Kelley, 1988; Waldman, 1995; Cardona, 2006; Dufour *et al.*, 2009; Vasconcelos *et al.*, 2010; Mercier *et al.*, 2012). La sensibilité de *D. labrax* aux changements environnementaux en lien avec le réchauffement climatique a déjà pu être mise en évidence, notamment aux stades juvéniles (Bento *et al.*, 2016). Cette dernière étude démontre un lien étroit entre des variables environnementales telles que la salinité et la température de l'eau et l'abondance de *D. labrax*, et ce à l'échelle européenne. D'autres travaux se sont intéressés à la vulnérabilité accentuée de *D. labrax* face aux changements environnementaux (Shrivastava *et al.*, 2019). Ces auteurs montrent pour la première fois que la vulnérabilité à l'acidification ainsi qu'à l'ammonium chez *D. labrax* est fortement augmentée aux faibles salinités. Bien que ces habitats de transition soit eux aussi vulnérables au changement global (Anthony *et al.*, 2009), on peut émettre l'hypothèse qu'ils peuvent constituer une niche potentielle pour les espèces les plus tolérantes à la fluctuation des facteurs environnementaux en limitant les futures compétitions avec les espèces invasives.

2. Acclimatation et plasticité phénotypique

Face à des conditions environnementales changeantes et selon la durée d'exposition, les organismes répondent par deux grands processus : l'adaptation et l'acclimatation. Le processus d'**adaptation** est un processus plutôt **lent**, qui se met en place au cours de **plusieurs générations.** Il répond à la **sélection naturelle,** repose sur des bases génétiques et épigénétiques et est souvent **irréversible** (Garland & Carter, 1994; Bennett, 2011). A l'inverse, le processus d'**acclimatation** est un processus beaucoup plus **rapide** qui peut apparaître **plusieurs fois au cours de la vie** d'un individu. Il peut être **réversible** et résulte de l'interaction de l'individu avec un changement de son environnement (Coles & Brown, 2003). Il repose sur des bases essentiellement **génétiques** et se traduit par des modifications internes à différentes échelles : de l'organe (modification du rythme cardiaque en réponse à un appauvrissement en oxygène), du tissu (modification de l'épithélium branchial avec la salinité et l'oxygène disponibles chez les espèces euryhalines : compromis osmo-respiratoire,

Sardella & Brauner, 2007), aux protéines (modification d'activité enzymatique en lien avec la température) jusqu'à l'expression de certains gènes (Kelly *et al.*, 2012). Ces modifications conduisent à l'apparition d'un nouveau **phénotype**, qui correspond à l'ensemble des traits biologiques d'un organisme résultant de l'interaction entre son génotype et son environnement (Johannsen, 1911).

La capacité des organismes à produire à partir de leur seul génotype, plusieurs phénotypes en réponse à des changements environnementaux est appelée **plasticité phénotypique** (Pfennig *et al.*, 2010). Les tentatives de définition du concept de plasticité phénotypique ne sont pas récentes. Déjà en 1965, Bradshaw la définit lorsque « l'expression d'un génotype peut être influencée par l'environnement ». Aujourd'hui encore de nombreuses définitions coexistent, comme celle de Callahan *et al.* (1997) « la capacité d'un organisme à altérer sa physiologie, sa morphologie ou son développement en réponse à des changements de son environnement », ou encore celle d'Eshel et Matessi (1998) comme étant « la capacité de modifier certains traits spécifiques afin de préserver la qualité des activités vitales à l'organisme ». La plasticité phénotypique est elle-même souvent divisée en deux grandes catégories selon que le phénomène est réversible ou non : la **plasticité développementale** (qui prend en compte l'ontogénèse de l'individu) non réversible, et la **flexibilité phénotypique**, réversible (Piersma & Drent, 2003). Un exemple classique de plasticité développementale chez les poissons est le cas de l'influence de la vitesse du courant sur la morphologie des salmonidés comme la truite arc-en-ciel *Oncorhynchus mykiss*, avec une corrélation presque toujours positive entre la vitesse du courant et la taille des individus (Fischer-Rousseau *et al.*, 2010). Concernant la flexibilité phénotypique, récemment Blanchard *et al.*, (2019) ont pu mettre en évidence une augmentation des capacités respiratoires aériennes chez un poisson amphibien, le killi des mangroves *Kryptolebias marmoratus* avec le temps passé hors de l'eau.

La plasticité phénotypique et la sélection ne mettent pas nécessairement en jeu les mêmes gènes, comme cela fut démontré chez deux populations de choquemort *Fundulus heteroclitus* localement adaptées à la température (Dayan *et al.*, 2015; Healy & Schulte, 2015). De telles observations ont aussi été réalisées chez les épinoches *Gasterosteus aculeatus* localement adaptées à l'eau de mer ou à l'eau douce, avec des différences de plasticité dans la réponse à des variations de salinité en termes d'expression de gènes

(McCairns & Bernatchez, 2010). Cependant il est parfois difficile de différencier l'adaptation locale de la plasticité phénotypique car celles-ci sont parfois étroitement liées et la plasticité peut devenir adaptative. En effet chez des daphnies *Daphnia magna* venant de plusieurs latitudes, il a été montré que l'acclimatation (en laboratoire) à des températures chaudes tout comme la température du site d'origine avaient le même effet positif sur la concentration en hémoglobine, en augmentant leur tolérance à des températures élevées (Yampolsky *et al.*, 2013). On parle alors de **plasticité adaptative** (Price *et al.*, 2003; Fusco & Minelli, 2010; Grenier *et al.*, 2017). Celle-ci correspond aux cas où la plasticité phénotypique permet aux organismes de répondre à un changement environnemental tout en augmentant leur *fitness* (Rago *et al.*, 2019). Cependant d'un point de vue évolutif, le coût (adaptatif) et le bénéfice de la plasticité restent débattus. En effet, nous pouvons nous interroger sur le coût énergétique (quantité d'énergie investie dans un processus biologique) et sur le coût adaptatif (impact sur la *fitness*) de la plasticité phénotypique. Il apparaît évident que dans un environnement dont les paramètres varient régulièrement, la plasticité peut s'avérer avantageuse, mais au détriment de certains traits biologiques comme la taille des organismes : on parle alors de *trade-off* (compromis) entre plasticité et coût adaptatif (DeWitt *et al.*, 1998). En revanche dans un environnement stable, la plasticité peut être très faible. Un exemple classique est le cas des poissons polaires de l'ordre des Notothénoïdes comme *Chaenocephalus aceratus*, qui ne possèdent plus d'hémoglobine, l'oxygène dissous étant présent à des taux plus important dans les eaux froides de leur habitat que dans des milieux tempérés (Holeton, 1970). En revanche, avec le réchauffement climatique diminuant les niveaux d'oxygène dans l'eau, cette perte de plasticité dans le transport d'oxygène tout comme la perte de plasticité cellulaire dans la réponse à des stress thermiques auront très probablement un impact sur leur survie (Bilyk *et al.*, 2018). Néanmoins, il nous faut nuancer la notion de coût quand on parle de plasticité phénotypique, car son étude nécessite la mesure de plusieurs traits biologiques, parfois sur plusieurs générations (Relyea, 2002).

Chez *D. labrax*, on pourrait donc se demander si la plasticité phénotypique dans la tolérance à des stress environnementaux comme la dessalure, induit un coût adaptatif. Pour cela, nous pouvons émettre l'hypothèse que chez les phénotypes les plus plastiques, l'acclimatation à la dessalure aurait un coût énergétique plus faible (lié à un métabolisme

respiratoire plus bas), traduisant un coût adaptatif plus faible de la plasticité phénotypique chez ces individus.

3. Régulation du milieu interne

Face à des variations des paramètres environnementaux, les organismes répondent selon l'une de ces trois stratégies : la **fuite**, la **conformation**, ou la **régulation** (Fig. 5). La fuite consiste pour ces organismes à chercher un environnement plus propice à leur survie, par des processus de migration ou de réaction d'enfouissement par exemple. Lorsque les organismes décident de rester dans cet environnement changeant, il faut alors distinguer deux grands types d'organismes : les **conformeurs** et les **régulateurs**.

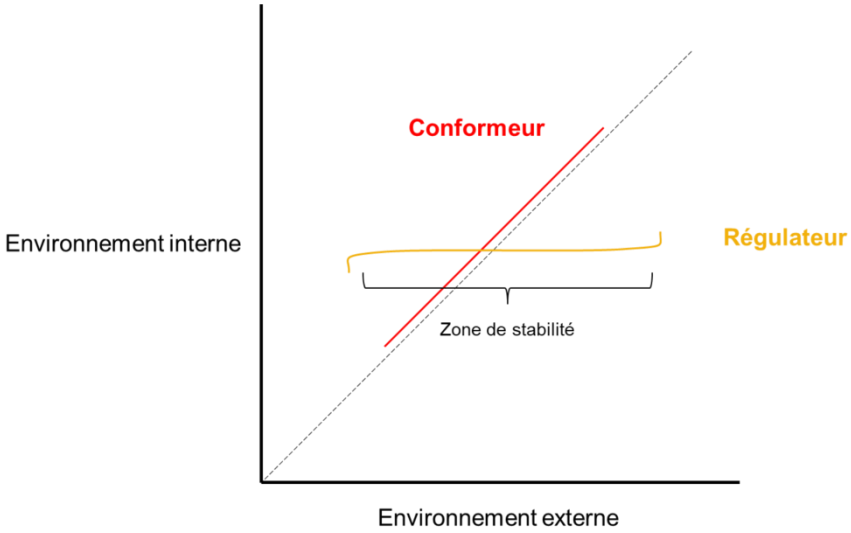


Figure 5 : Organismes conformeurs vs organismes régulateurs, d'après Wilmer et al., 2000

Les conformeurs, comme leur nom l'indique, présentent un environnement interne équivalent à l'environnement extérieur dans lequel ils vivent. Les régulateurs quant à eux possèdent une « norme » interne qui ne dépend pas du milieu dans lequel ils se trouvent. Il leur faut alors ajuster ou maintenir leur environnement interne, processus appelé **homéostasie** (Ramsay & Woods, 2014; Schulkin, 2015). Il correspond à l'ensemble des mécanismes de régulation morphologiques, métaboliques, biochimiques et moléculaires nécessaires au maintien des paramètres internes de l'organisme. On peut ainsi définir des grandes catégories d'organismes selon les stratégies qu'ils arborent face à des variations de paramètres environnementaux : on parle d'oxy-conformeurs et d'oxy-régulateurs en réponse à des variations de disponibilité en oxygène, de thermo-conformeurs et de thermo-régulateurs en réponse à des variations de température, ou encore **d'osmo-conformeurs** et **d'osmo-régulateurs** en réponse à des variations de **salinité**. Une autre stratégie possible

pour les organismes face à de nouvelles conditions environnementales peut être l'ajustement de leur physiologie et de leur comportement, processus appelé **allostasie** (Ramsay & Woods, 2014; Schulkin, 2015)**.** Par exemple, la diminution de la consommation d'oxygène en milieu hypoxique associée à la diminution du métabolisme basal chez le loup est un exemple de régulation allostatique (Zhang *et al.*, 2017).

En revanche, il existe pour chaque organisme une **gamme de tolérance** avec des points critiques à partir desquels ils ne sont plus capables de résister et de survivre. On parle aussi de **surcharge allostatique**, lorsque les organismes ne sont plus à même d'ajuster leur physiologie et/ou comportement (McEwen, 2016). Au-delà de ces limites, une forte **variabilité inter-individuelle** ou **intraspécifique** peut être détectée au sein même d'une espèce.

4. La variabilité intraspécifique

La variabilité inter-individuelle ou intraspécifique correspond à des différences dans les traits biologiques des organismes, au sein d'une même espèce, par rapport à une « norme » hypothétique appelée **norme de réaction**. Elle est définie comme un ensemble de « fonctions linéaires ou non qui caractérisent la façon dont laquelle la valeur phénotypique d'un trait pour un environnement donné, change avec l'environnement » (Hutchings, 2011). Ces normes permettent non seulement de décrire les phénotypes, mais aussi d'estimer la possible variabilité intraspécifique et la plasticité phénotypique par la variabilité observée dans leurs normes de réactions (Oomen & Hutchings, 2015). Ces normes peuvent être linéaires avec l'environnement lorsque le trait est une variable continue (comme la taille) et présenter de la variabilité inter-individuelle (en rouge, Fig. 6), ou encore discontinues avec la présence d'un seuil lorsque le trait est discret (comme la présence ou l'absence d'une caractéristique morphologique), ou bien plus complexes. Cette variabilité peut s'observer à différentes échelles : celle de la famille, de la population ou encore de l'espèce ; et peut concerner une grande diversité de traits biologiques comme la morphologie, le métabolisme aérobie, ou encore la croissance (Oomen & Hutchings, 2015). Afin d'étudier la variabilité phénotypique en réponse à un seul facteur, il faut limiter la variabilité des influences environnementales et donc essayer au maximum de travailler dans un environnement stable dont on contrôle la majorité des paramètres relatifs à l'étude (lumière, température,

nourrissage), communs entre les organismes testés, et dont on ne fait varier que le(s) paramètre(s) souhaité(s), ou autrement dit en ***common garden*** (Conover & Baumann, 2009).

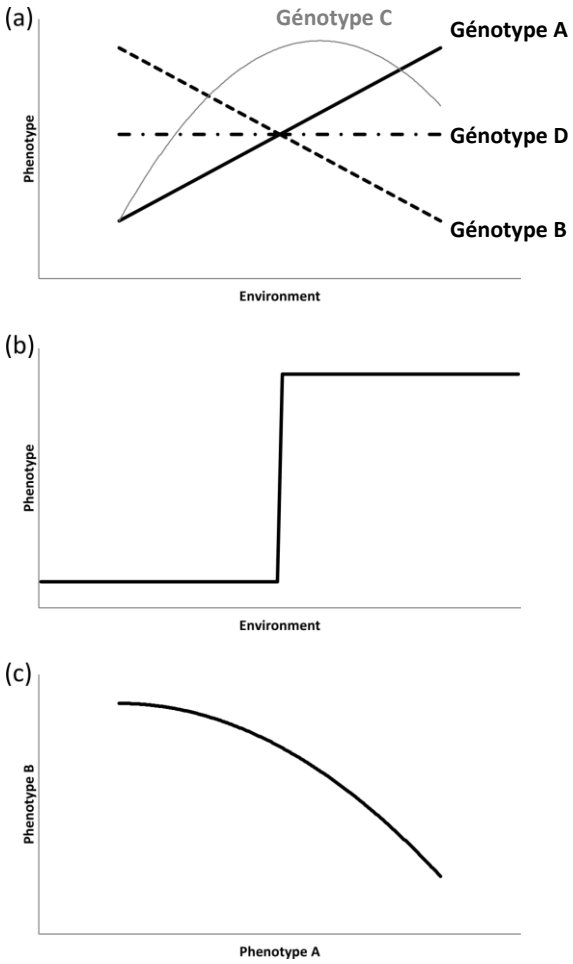


Figure 6 : Modèles théoriques de normes de réactions. (a) Normes de réaction linéaires continues pour les génotypes A et B (ligne noire pleine ou pointillée), curviligne pour le génotype C illustrant toutes les trois une réponse plastique. En revanche, le génotype D (ligne horizontale discontinue) ne montre pas de plasticité. (b) Seuil de norme de réaction discontinu et (c) norme de réaction bivariante. D'après Oomen et al., 2015

Un des gros défis de la recherche en écologie est de parvenir à comprendre et prédire les effets des changements climatiques. Pour cela, il est important de pouvoir étudier et caractériser la variabilité phénotypique car dans un contexte d'augmentation des températures, de diminution de l'oxygène disponible, et d'augmentation des perturbations climatiques (et notamment les épisodes extrêmes comme les canicules ou les fortes précipitations), la survie et la distribution des organismes ectothermes aquatiques dépendent fortement de leurs capacités d'acclimatation rapide à de telles variations environnementales ainsi que du niveau de variabilité intraspécifique (Somero, 2010; Forsman, 2015). De plus en plus d'études tendent à montrer l'importance d'étudier cette dernière quand on cherche à connaître les effets des changements climatiques (Fig. 7). Parmi elles, l'étude du coût métabolique du comportement de fuite chez un élasmobranche

Leucoraja erinacea (Di Santo, 2016) provenant de latitudes (et donc de températures) différentes puis soumis à une augmentation de température, révèle que selon la latitude d'origine, le coût de la fuite diffère. Un autre exemple est celui de la tolérance au stress oxydatif chez un cnidaire *Nematostella vectensis*, qui diffère lui aussi au sein des populations provenant de latitudes différentes (Friedman *et al.*, 2018). Ces exemples pondèrent les effets des changements climatiques et justifient une fois de plus la nécessité d'étudier la plasticité phénotypique et plus généralement la variabilité intraspécifique.

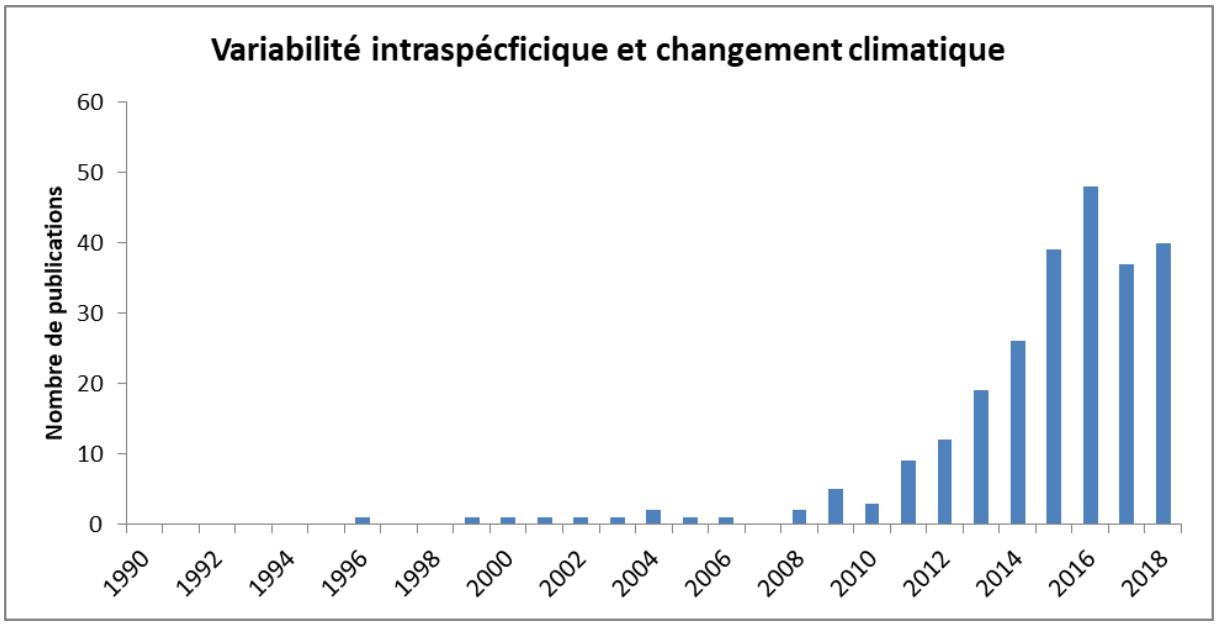


Figure 7 : Nombre de publications par an concernant la variabilité intraspécifique et le changement climatique, d'après www.ncbi/pubmed.com

Chez le loup, une forte variabilité intraspécifique a été mise en évidence concernant plusieurs traits physiologiques comme la réponse au stress, à l'hypoxie ou encore à la température. Concernant la réponse au stress, deux phénotypes émergent : les HR (*High Responsiveness*, présentant des niveaux de cortisol circulant élevés) et les LR (*Low Responsiveness*, avec de faibles niveaux de cortisol circulant) (Samaras *et al.*, 2016). Récemment il a été montré que l'existence de ces phénotypes pouvaient être due à des capacités de biosynthèse de cortisol différentes, avec notamment une surexpression de certains gènes clefs impliqués dans la biosynthèse de l'hormone comme *mc2r* et *cyp11b1* (Samaras & Pavlidis, 2018). Lorsque ces organismes rencontrent des environnements pauvres en oxygène (hypoxie environnementale, reproduite en laboratoire), deux phénotypes se distinguent : les **tolérants à l'hypoxie** et **les sensibles à l'hypoxie** (Claireaux

et al., 2013). Il a été montré que ces phénotypes particuliers dépendaient fortement des capacités cardiaques des organismes avec une meilleure contraction et relâchement cardiaque chez les phénotypes tolérants (Joyce *et al.*, 2016). Dans cet exemple, la plasticité phénotypique est directement reliée à la variabilité intraspécifique dans les performances cardiaques. Enfin, concernant la tolérance à la température, une variabilité inter-individuelle est encore observée avec des loups décrits comme **tolérants** et d'autres décrits comme **sensibles aux hautes températures**. L'existence de ces deux phénotypes a été reliée à des caractéristiques anatomiques (cœur plus gros), physiologiques mais aussi à des performances différentes entre les deux phénotypes : meilleure récupération post-exercice, consommation d'oxygène au repos plus faible chez le phénotype tolérants (Ozolina *et al.*, 2016). Concernant la salinité, une forte plasticité phénotypique est observée en eau douce en laboratoire, tout comme dans leur milieu naturel où la salinité est extrêmement variable (Cataudella *et al.*, 1991; Venturini *et al.*, 1992; Varsamos *et al.*, 2002; Lorin-Nebel *et al.*, 2006; Kokou *et al.*, 2019). Néanmoins, certaines études tendent à montrer que là aussi, il existe une forte variabilité dans la tolérance à l'acclimatation à l'eau douce (Dendrinos & Thorpe, 1985; Allegrucci *et al.*, 1994; Jensen *et al.*, 1998; Nebel *et al.*, 2005; Giffard-Mena *et al.*, 2008). Ces dernières études relèvent une mortalité d'environ 30% en eau douce caractérisant au moins deux phénotypes distincts : les individus dits **tolérants à l'eau douce** car survivants et capable de vivre longtemps (au moins 2 semaines) dans cette condition, et les individus dits **intolérants à l'eau douce** car incapables de survivre au moins deux semaines dans de tels environnements hypo-osmotiques.

5. L'osmorégulation chez les poissons

La capacité à réguler la balance hydrominérale interne des organismes est appelée **osmorégulation**. Elle réunit un ensemble de mécanismes anatomiques, morphologiques, physiologiques, biochimiques et moléculaires, et apparaît dès l'ontogénèse (Varsamos *et al.*, 2001; Kultz, 2015). Les espèces pouvant supporter de grandes variations de salinité sont appelées **euryhalines** comme c'est le cas pour le loup, le tilapia, le mulet cabot (Stickney, 1986; Nordlie & Haney, 1998; Sucré *et al.*, 2013). A l'inverse, les espèces ayant une faible gamme de salinités acceptables sont appelées **sténohalines**, comme c'est le cas chez le poisson rouge *Carassius auratus* et le poisson zèbre *Danio rerio* (Wurts, 1998) (Tableau 2).

Des différences en termes de capacités osmorégulatrices peuvent être à l'origine de la distribution différentielle des espèces comme cela a été montré chez deux espèces de gobies (Rigal *et al.*, 2008).

Tableau 2 : Salinités tolérées par différentes espèces de poissons, d'après Nordie & Haney, 1998

Espèce	Gamme de salinités tolérées (ppt)
Dicentrarchus labrax	0 à 90
Poecilia latipinna	0 à 70-80
Gambusia holbrooki	0 à 25
Cyprinodon variegatus	0 à 142.4
Floridichthys carpio	0.5 à 90
Jordanella floridae	0 à 85
Fundulus similis	0 à 100
Adinia xenica	0.5 à 95
Mugil cephalus	0 à 54

Différents organes sont impliqués dans l'osmorégulation, parmi lesquels figurent les **branchies**, le **rein postérieur** et l'**intestin** (Marshall & Grosell, 2006; Evans & Clairborne, 2009). Les branchies sont considérées comme l'interface privilégiée des échanges avec le milieu de par l'importance de leur surface d'échange, leur sensibilité aux facteurs environnementaux ainsi que par la diversité des fonctions physiologiques qui s'y opèrent : régulation acido-basique, ionique, excrétion azotée et respiration (Evans *et al.*, 2005; Sollid & Nilsson, 2006; Nilsson, 2007). Le rein quant à lui est impliqué dans la réabsorption et régulation d'ions et d'eau excrétés dans l'urine, et l'intestin dans la récupération d'ions et d'eau. Comme les branchies, le rein et l'intestin possèdent tous les deux un rôle important dans la régulation acido-basique (Claiborne *et al.*, 1994; Larsen *et al.*, 2014). Dans un environnement non iso-osmotique, afin de maintenir leur homéostasie ionique et donc leur osmolalité sanguine autour de 300-380 $mOsm.kg^{-1}$, les poissons ont recours à l'une des deux stratégies suivantes : l'hyper-osmorégulation en milieu hypo-osmotique comme l'eau douce, et l'hypo-osmorégulation en milieu hyper-osmotique comme l'eau de mer (Fig. 8) (Evans, 2010; Kultz, 2015).

5.1 En eau de mer

Pour une eau de mer à salinité « standard » de 34 ppt, l'osmolalité équivaut à 1000 mOsm kg^{-1} soit environ 3 fois plus que celle du milieu interne des poissons. Cela entraîne donc une sortie passive d'eau au niveau des interfaces comme les branchies, la peau, l'intestin, mais aussi une entrée passive d'ions (Willmer *et al.*, 2000). Afin d'éviter la déshydratation et une augmentation mortelle d'ions tels que les chlorures Cl^- ou le sodium Na^+, les poissons boivent beaucoup d'eau. Associé à cela, ils excrètent ces ions au niveau de cellules particulières appelées ionocytes ou MRC (mitochondrion-rich cells) ou encore cellules à chlorures (chloride secretory cells) situées au niveau des branchies (Marshall & Bellamy, 2010; Hwang *et al.*, 2011) et produisent peu d'urine iso-osmotique au milieu.

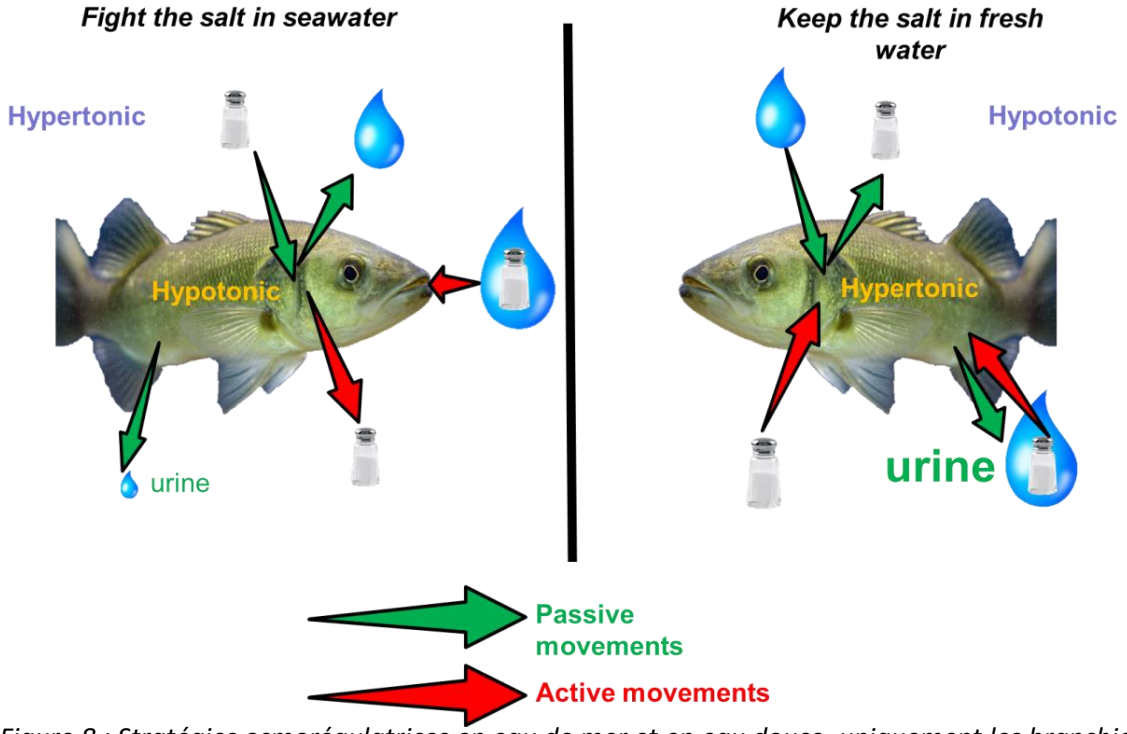


Figure 8 : Stratégies osmorégulatrices en eau de mer et en eau douce, uniquement les branchies et le rein sont représentés.

5.2 En eau douce

En milieu hypo-osmotique, ces processus prennent plus ou moins une direction opposée. En effet, l'eau entre cette fois-ci passivement et la concentration en ions diminue par fuite ainsi que par dilution. Afin d'éviter cet effet de dilution, les poissons ne boivent plus et

récupèrent les ions au niveau de ces mêmes organes. Tout d'abord leurs branchies absorbent activement des ions au niveau d'ionocytes qui sont très différents de ceux en eau de mer car ils transportent des ions vers le sang. L'intestin joue un rôle dans l'absorption d'ions *via* l'alimentation, et le rein postérieur assure la réabsorption d'ions mais devient imperméable à l'eau afin de produire une forte quantité d'urine hypo-osmotique au milieu interne (Willmer *et al.*, 2000). Le coût métabolique de l'osmorégulation en eau douce est généralement considéré comme faible chez les téléostéens marins allant de quelques pourcents jusqu'à un tiers de leur métabolisme basal, et semble être espèce-spécifique (Ern *et al.*, 2014). Chez le loup, aucune différence n'a pu être mise en évidence en termes de consommation d'oxygène après 18h passées en eau douce (Chatelier *et al.*, 2005).

5.3 Mécanismes moléculaires de l'osmorégulation

L'absorption ou la sécrétion active de certains ions sont réalisées grâce à des transporteurs ioniques basolatéraux tels que la pompe Na^+-K^+-ATPase (NKA) ou le co-transporteur Na^+-K^+-$2Cl^-$ 1 (NKCC1), et apicaux tels que le Na^+/$2Cl^-$-2 (NCC2) et NKCC2 (Evans *et al.*, 2005; Hiroi *et al.*, 2008; Inokuchi *et al.*, 2008). La NKA est un transporteur clef de l'osmorégulation chez les téléostéens que cela soit en eau douce ou en eau de mer (Wilson *et al.*, 2000; Marshall & Grosell, 2006; McCormick *et al.*, 2009). Elle est constituée de deux sous unités α et β, la première étant la sous-unité catalytique contenant les sites de liaison de l'ATP ainsi que des deux ions Na^+ et 3 ions K^+ (Evans & Clairborne, 2009). L'activité de cette pompe crée donc un gradient électrique (en créant un déséquilibre des charges de part et d'autre de la membrane) et chimique (du fait qu'elle échange du Na^+ contre du K^+). Grâce à ce gradient, le transport des ions est facilité par une multitude d'autres transporteurs membranaires. Chez le loup, l'isoforme α1 de la NKA est abondante dans les organes impliqués dans l'osmorégulation, et son expression dépend de la salinité (Blondeau-Bidet *et al.*, 2016) tout comme son activité (Jensen *et al.*, 1998; Nebel *et al.*, 2005). Au niveau des branchies, plusieurs types d'ionocytes existent et diffèrent selon les espèces, notamment par l'expression et la localisation de certains de ces transporteurs, leur morphologie et leur localisation dans l'épithélium branchial (Hwang, 2009; Hwang *et al.*, 2011). Une seconde pompe ionique essentielle pour les processus osmorégulateurs est la pompe V-H^+-ATPase (VHA). Elle permet tout comme la NKA de faciliter les échanges ioniques (en créant un

déséquilibre des charges de part et d'autre de la membrane) et acido-basique (en faisant sortir un acide) en consommant de l'ATP. L'énergie issue de l'hydrolyse de l'ATP et le gradient créée par la sortie du proton pourra alors être utilisée pour de nombreux processus dont l'osmorégulation *via* le transport actif de certains osmolytes. L'osmorégulation et la régulation acido-basique sont deux processus extrêmement liés, le transport de Na^+ et de Cl^- est souvent couplé à des transporteurs d'acides (Na^+/H^+) ou de bases (Cl^-/HCO_3^-). Chez le loup, plusieurs études décrivent les processus osmorégulateurs au niveau des branchies permettant de proposer un modèle hypothétique (Fig. 9) (Lorin-Nebel *et al.*, 2006; Giffard-Mena *et al.*, 2008; Bossus *et al.*, 2013; Blondeau-Bidet *et al.*, 2016, 2019). Au niveau du rein, la plupart des transporteurs présents dans les branchies sont aussi exprimés au niveau des tubules distaux et collecteurs. En revanche, des différences de niveaux d'expression et de localisation sont retrouvées entre ces deux organes des fois impliquant différentes isoformes ou gènes paralogues, comme c'est le cas chez le cotransporteur NKCC avec une expression plus importante de *nkcc2* apicale dans le rein et une expression plus forte de *nkcc1* basale au niveau des branchies (Cutler & Cramb, 2008; Kato *et al.*, 2010; Markadieu & Delpire, 2014; Esbaugh & Cutler, 2016).

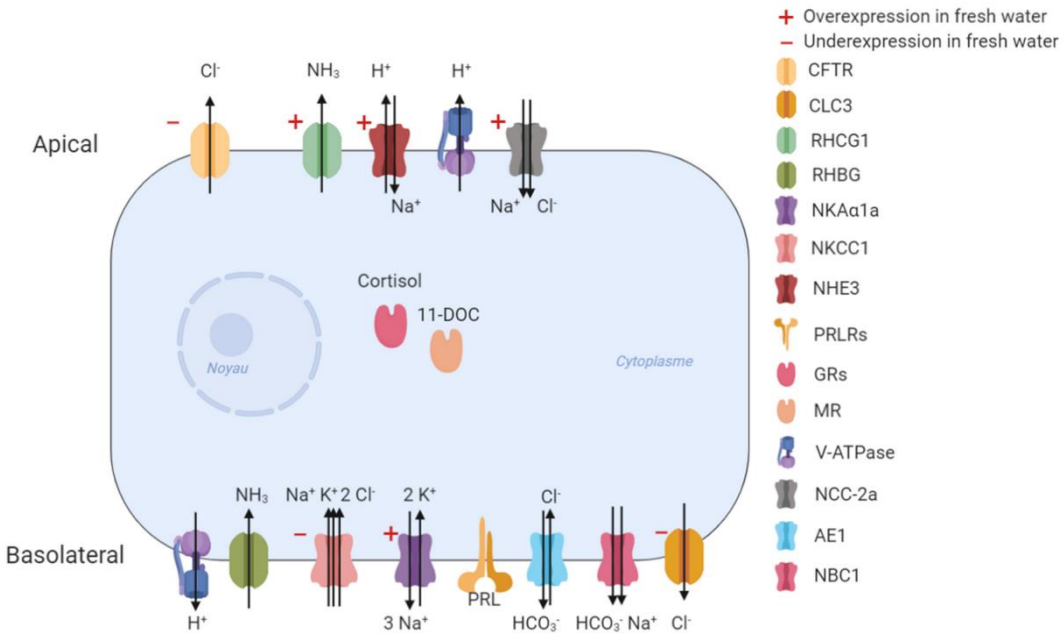


Figure 9 : Modification attendues du profil transcriptionnel des ionocytes branchiaux chez Dicentrarchus labrax après un transfert de deux semaines depuis l'eau de mer vers l'eau douce

5.4 Contrôle hormonal de l'osmorégulation

La mise en place des mécanismes d'acclimatation à des salinités différentes est sous contrôle hormonal : prolactine, hormone de croissance (GH) et cortisol notamment (McCormick, 2001). Elle répond principalement à la **prolactine** pour le passage en milieu hypo-osmotique (Dharmamba & Maetz, 1972; Manzon, 2002; Breves *et al.*, 2013, 2014a) et aux corticoïdes comme le cortisol (Terova *et al.*, 2005; Takahashi & Sakamoto, 2013). Chez le loup, l'importance de la prolactine dans les processus osmorégulateurs a déjà été démontrée (Terova *et al.*, 2005; Varsamos *et al.*, 2006; Boutet *et al.*, 2007; Ky *et al.*, 2007; Pavlidis *et al.*, 2011). Elle agit non seulement sur l'expression des récepteurs à la prolactine (Lee *et al.*, 2006; Ky *et al.*, 2007; Fiol *et al.*, 2009), mais aussi *via* l'activation des récepteurs à la prolactine, sur celle de transporteurs ioniques clefs dans l'osmorégulation (Tomy *et al.*, 2009), ainsi que sur la prolifération d'ionocytes (Cruz *et al.*, 2013) et la perméabilité cellulaire (Tipsmark *et al.*, 2010; Bossus *et al.*, 2017). Par exemple chez le tilapia *Oreochromis mossambicus*, l'hypophysectomie réduit les niveaux circulants de prolactine et affecte les capacités osmorégulatrices. En effet, le passage en eau douce de ces animaux euryhalins entraine une diminution de leur osmolalité sanguine ainsi que des niveaux d'expression plus faibles du gène codant pour le NCC au niveau des ionocytes branchiaux par rapport à des animaux non opérés (Dharmamba *et al.*, 1967; Breves *et al.*, 2014b). Ces études montrent donc un lien étroit entre le contrôle hormonal par la prolactine et le transporteur NCC qui est impliqué dans l'absorption de Cl^- et Na^+ du milieu vers le sang. L'hormone de croissance (GH) joue aussi un rôle important dans l'osmorégulation, mais plutôt en eau salée. Chez le saumon Atlantique, une augmentation de GH stimule le taux de boisson (Fuentes & Eddy, 1997). Elle possède aussi un effet stimulateur sur l'expression de NKCC1 (Pelis & McCormick, 2001) et sur l'activité de la NKA chez cette même espèce (Tipsmark & Madsen, 2009) ainsi que chez la daurade *Sparus auratus* (Sangiao-Alvarellos *et al.*, 2006). En revanche, son implication dans l'osmorégulation chez les autres téléostéens non-salmonidés reste peu élucidée (Mancera & McCormick, 1998; Breves *et al.*, 2010).

6. Les télomères : nouveaux marqueurs de stress environnementaux chez les ectothermes ?

Les télomères sont les parties distales des chromosomes. Ils sont très conservés chez les vertébrés et sont constitués de répétitions de motifs TTAGGG (Blackburn & Gall, 1978). Ils jouent un rôle clef dans la protection et la stabilité des chromosomes. Ces motifs peuvent aussi se retrouver ailleurs sur les chromosomes, reliques de fusions passées entre chromosomes, on parle alors de séquences télomériques interstitielles (ITS) (Rocco *et al.*, 2001; Ocalewicz *et al.*, 2004; Mudry *et al.*, 2007; Foote *et al.*, 2013). La longueur des télomères raccourcit de façon naturelle avec le vieillissement du fait d'une réplication incomplète (Harley *et al.*, 1990; Webb *et al.*, 2013). Ce raccourcissement peut être accéléré

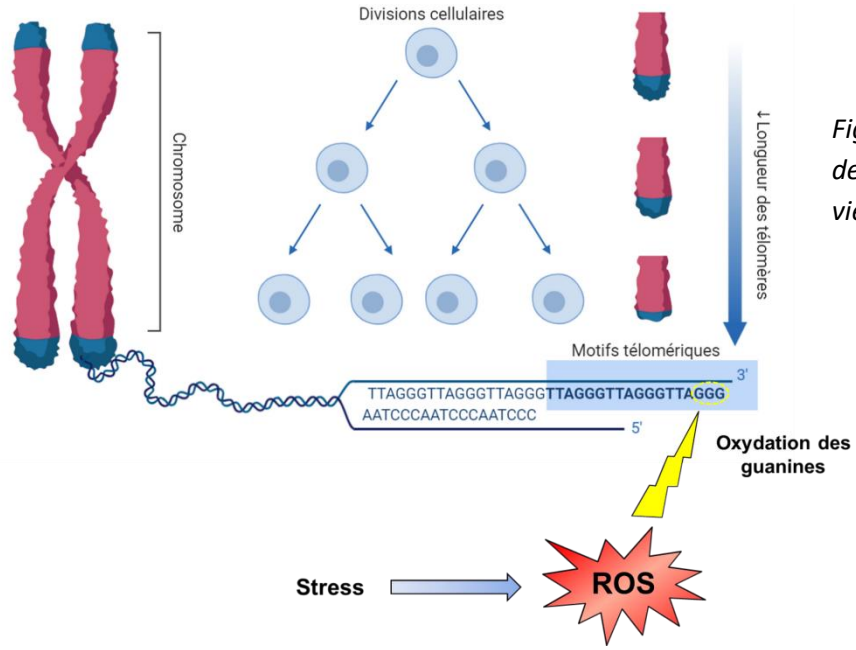


Figure 10 : Raccourcissement des télomères avec le vieillissement et le stress

sous l'effet de stress environnementaux (Epel *et al.*, 2004; Starkweather *et al.*, 2014) notamment *via* une diminution de l'activité de la télomérase, enzyme qui permet de maintenir ces séquences au cours des générations cellulaires (Blackburn, 2005). Elle est constituée d'un brin d'ARN servant de matrice pour l'unité catalytique ou *telomerase reverse transcriptase* : TERT (Blackburn, 1991). La longueur des télomères est déjà utilisée comme biomarqueur de sénescence chez vertébrés modèles notamment l'humain mais aussi le poisson zèbre (Cawthon *et al.*, 2003; Carneiro *et al.*, 2016). Elle est très utilisée en cancérologie, les cellules cancéreuses ayant une activité de la télomérase très importante car ce sont des cellules dites immortelles (Blasco, 2005; Skvortsov *et al.*, 2011). Récemment, l'étude de la dynamique des télomères (c.-à-d. longueur des télomères, activité de la

télomérase et expression de la TERT) en écophysiologie est de plus en plus utilisée comme biomarqueur de stress environnementaux chez les ectothermes (Anchelin *et al.*, 2013; Henriques *et al.*, 2013; Rollings *et al.*, 2014; Naslund *et al.*, 2015). Chez l'esturgeon sibérien *Acipenser baerii* et la truite *Salmo trutta,* une augmentation de température diminue la longueur des télomères (Debes *et al.*, 2016; Simide *et al.*, 2016). De plus, l'activité de la télomérase diminue avec des stress de jeûne, hypoxiques ou même une exposition au benzopyrène chez différents poissons (Peterson *et al.*, 2015). L'expression de la TERT dépend aussi de l'influence environnementale. En effet chez le medaka *Oryzias latipes* par exemple, un stress hypoxique entraine une surexpression de *tert* notamment dans le foie et les gonades (Yu *et al.*, 2006). Ces stress provoquent, en effet, une augmentation du nombre d'espèces réactives à l'oxygène (ROS) qui peuvent oxyder les guanines (Fig. 10) très présentes dans les motifs télomériques (Boonekamp *et al.*, 2017). De plus, le lien entre les niveaux de glucocorticoïdes comme le cortisol et le stress oxydatif mis en évidence par Kurz *et al.* (2004) suggère que différents profils de cortisol (HR et LR par exemple chez le loup) pourraient être à l'origine de différents profils de dynamique de télomères. *Dicentrarchus labrax*, par son incroyable plasticité phénotypique et par sa grande variabilité intraspécifique face aux challenges environnementaux apparaît comme un modèle d'intérêt afin d'étudier la dynamique des télomères.

7. Intérêt du loup méditerranéen *D. labrax* comme modèle biologique

Le loup ou bar européen *Dicentrarchus labrax* (Linné, 1758) est un téléostéen euryhalin et eurytherme dont l'aire de répartition s'étend du nord-est de l'océan Atlantique jusqu'à la mer Noire en passant par la Méditerranée et les côtes nord-africaines. Plusieurs lignées ont été décrites récemment notamment les lignées Atlantique et Méditerranée Ouest et Est (Duranton *et al.*, 2018). Dans cette thèse, nous étudierons des loups méditerranéens provenant de lignées Méditerranée Ouest. Ce sont des poissons essentiellement marins qui se reproduisent sur les côtes (Jennings & Pawson, 1992; Waldman, 1995). Dès la métamorphose au stade juvénile, ils peuvent migrer vers les habitats de transition comme les lagunes, les estuaires, voire même en eau douce comme les rivières (Fig. 11) (Pawson *et al.*, 1987; Barnabé, 1989; Dufour *et al.*, 2009; De Pontual *et al.*, 2019). Il s'agit d'une espèce euryhaline (0 – 90 ppt, Barnabé, 1989) très importante d'un point de vue économique.

L'aquaculture représente 97% de la production mondiale de bar en 2016 selon la FAO (191 003 tonnes produites en aquaculture *vs* 5752 tonnes par pêche).

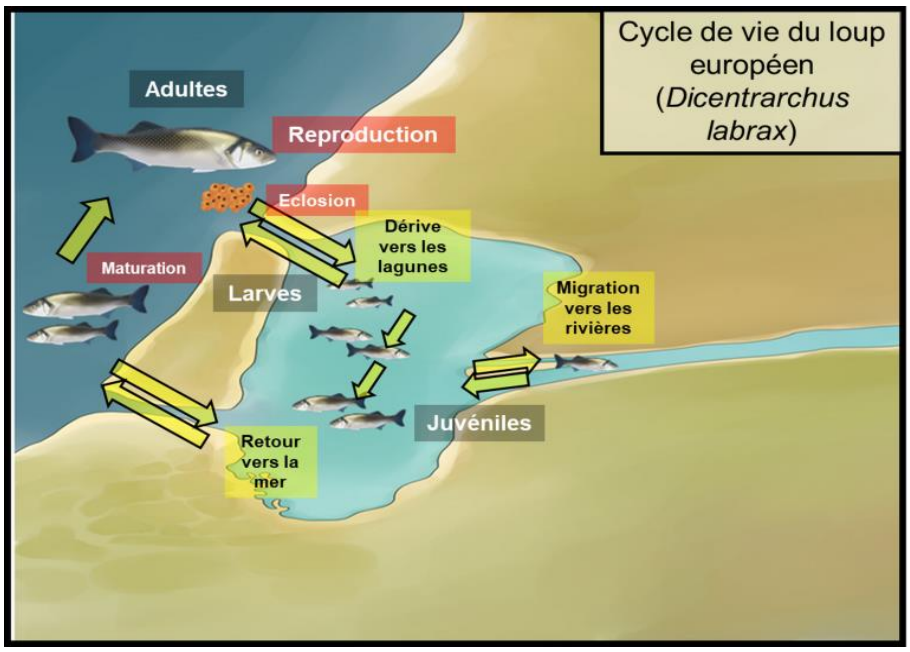


Figure 11 Cycle de vie de Dicentrarchus labrax

L'espèce *D. labrax* constitue aussi un modèle marin de plus en plus étudié en laboratoire, avec depuis la dernière décennie l'acquisition de données physiologiques, comportementales, génomiques, génétiques et épigénétiques (Fig. 12, Vandeputte *et al.*, 2019).

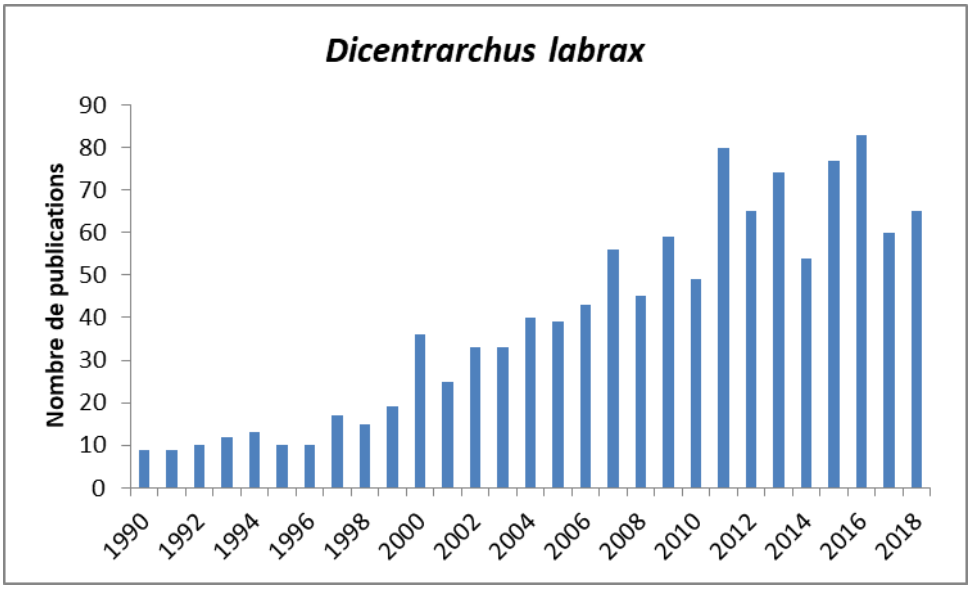


Figure 12 : Nombre de publications par an concernant Dicentrarchus labrax, d'après www.ncbi/pubmed.com

Certaines études suggèrent que les différences de tolérance à la dessalure observées chez *D. labrax* pourraient expliquer des différences d'occupation d'habitats liées à leurs capacités osmorégulatrices. Cependant ces études ne révèlent pas de lien clair entre génotype et phénotype (Lemaire *et al.*, 2000; Guinand *et al.*, 2014, 2015; Tine *et al.*, 2014). Jusqu'à présent peu d'études ont tenté de décrire les différents traits biologiques et phénotypiques caractéristiques et/ou responsables de cette variabilité dans la tolérance à l'eau douce chez le loup (Nebel *et al.*, 2005; Giffard-Mena *et al.*, 2008). De plus, des marqueurs épigénétiques, tels que le méthylome par exemple pourraient être analysés dans un contexte de dessalure, et pourraient permettre de mieux appréhender l'acclimatation en milieu hypo-osmotique ainsi que les causes de la variabilité phénotypique.

8. Objectifs de la thèse et organisation du manuscrit

Les travaux déjà réalisés sur l'acclimatation à l'eau douce chez le loup soulèvent la question de la variabilité intraspécifique observée dans la tolérance à la dessalure. Depuis un peu plus de 20 ans, le nombre de publications liant plasticité phénotypique et changements climatiques ne cesse d'augmenter, tout comme celles liant plasticité phénotypique et variabilité intraspécifique ou changements climatiques et variabilité intraspécifique (Fig. 13). L'étude de la variabilité intraspécifique en lien avec la plasticité phénotypique est essentielle pour la compréhension de la survie ainsi que de la répartition des organismes dans un contexte de changement global.

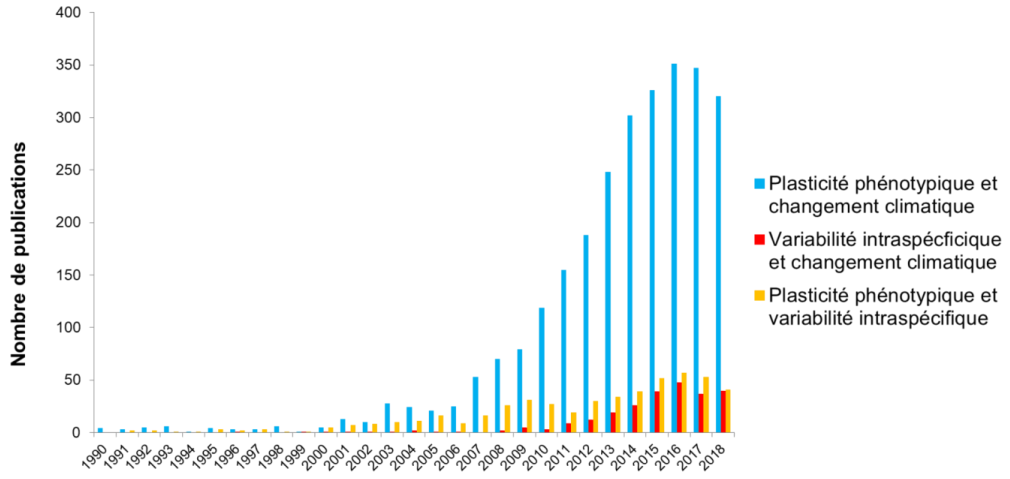


Figure 13 : Nombre de publications par an concernant les liens entre plasticité phénotypique, variabilité intraspécifique et changement climatique, d'après www.ncbi/pubmed.com

Les objectifs de cette thèse sont de mieux appréhender l'acclimatation à l'eau douce chez ces organismes migrateurs décrits comme très euryhalins, et de mieux caractériser la variabilité phénotypique dans la tolérance à l'eau douce chez ces organismes. Pour cela des approches **comportementales**, **métaboliques**, **biochimiques** mais aussi **moléculaires**. Un travail sur les régulations **épigénétiques** a également été démarré dans le cadre de cette thèse mais il ne figure pas sous forme de chapitre dans ce manuscrit. Les premiers résultats seront abordés en ouverture dans la discussion.

Les différents chapitres s'articulent autour de ces objectifs et tentent d'apporter de nouvelles connaissances sur l'acclimatation en eau dessalée ainsi que sur la variabilité intraspécifique rencontrée chez les loups méditerranéens. Ainsi, plusieurs questions ont été posées :

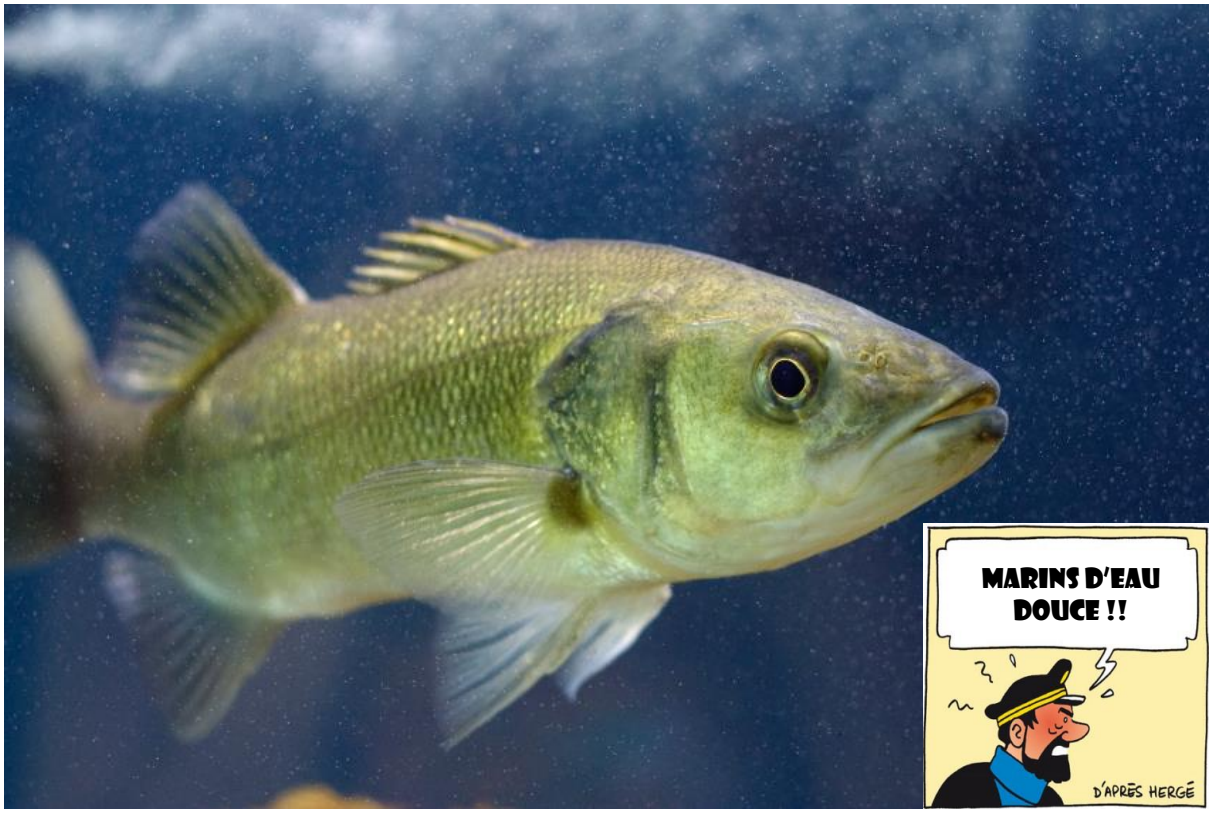
1. Quelles sont les conséquences d'un transfert en **eau douce** sur le **métabolisme respiratoire** des loups en normoxie et en hypoxie ? Dans un contexte de changements climatiques, l'eau douce constitue-t-elle un refuge **viable** pour les loups européens et quels sont les mécanismes impliqués dans l'acclimatation à long terme ?
2. Comment **identifier** et **caractériser** la **variabilité intraspécifique** dans la **tolérance à l'eau douce** chez le loup méditerranéen ? Quelles sont les caractéristiques **comportementales**, **biochimiques** et **moléculaires** de l'intolérance à l'eau douce chez le loup méditerranéen ? (Chapitre 2)
3. Quelles sont les **bases transcriptionnelles** de la **variabilité inter-individuelle** observée dans l'**acclimatation à l'eau douce** ? Quel(s) **organe(s)** présente(nt) des réponses différentielles entre les phénotypes dans l'intolérance à l'eau douce ? (Chapitre 3)
4. La dynamique des télomères est-elle affectée par des stress hypo-osmotiques ? Les individus répondent-ils différemment selon leur phénotype de tolérance à l'eau douce ? (Chapitre 4)

Les différents chapitres seront introduits par un résumé du contexte de l'étude ainsi que par les questionnements auxquels nous tenterons d'apporter une réponse dans le chapitre. Les chapitres sont tous écrits en anglais, car ils correspondent à des articles publiés

(Chapitre 2, publié dans *Marine Biology*), soumis (Chapitre 3 dans *Gene*) ou en préparation (Chapitre 1 bientôt soumis dans *Conservative Physiology Part B* et Chapitre 4 dans *Fish Biology*).

Chapitre I :

Comment le loup méditerranéen *Dicentrarchus labrax* s'acclimate-t-il à de longues expositions en eau douce ?



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Prologue du Chapitre 1

Dans ce premier chapitre, nous avons étudié la dynamique temporelle de l'acclimatation des loups à l'eau douce ainsi que le coût métabolique d'une telle acclimatation.

Ce chapitre discute des données provenant d'un suivi temporel de loups exposés en eau douce pendant deux mois, et regroupe des données histologiques (structure des branchies), biochimiques (pressions osmotiques, dosages sanguins de Na^+ et de Cl^-), moléculaires (mesure d'expression de certains transporteurs de Na^+ et de Cl^-) et physiologiques (respirométrie pour étudier le métabolisme aérobie et anaérobie). Ce travail a été effectué avec l'aide de deux stagiaires : Jeanne Naudet (étudiante en médecine vétérinaire) pour la partie suivi temporel et Marie Gimenez (étudiante en Master 1 Ecosystèmes) pour l'histologie et une partie du traitement des données issues de l'expérimentation en hypoxie. L'acquisition des données de respirométrie a été réalisée en collaboration avec David McKenzie (chercheur CNRS) à la station expérimentale de Palavas-les-Flots.

Ce travail (osmorégulation) a été valorisé sous forme de présentation orale au Congrès d'Ecophysiologie Animale III (CEPA III, Strasbourg, du 6 au 8 novembre 2017) : « Phenotypic plasticity in freshwater acclimation in sea bass *Dicentrarchus labrax* ».

Ce chapitre est écrit en anglais fera l'objet d'un article soumis au journal *Comparative Physiology Part B* sous la forme : « How do European sea bass *Dicentrarchus labrax* cope with long-term freshwater exposure? New insights into successful acclimation » L'Honoré Thibaut, Lorin-Nebel Catherine, McKenzie David, Gimenez Marie, Naudet Jeanne, Farcy Emilie.

How do European sea bass *Dicentrarchus labrax* cope with long-term freshwater exposure? New insights into successful acclimation

L'Honoré Thibaut*, Lorin-Nebel Catherine, McKenzie David, Gimenez Marie, Naudet Jeanne, Farcy Emilie

Univ Montpellier, CNRS, IFREMER, IRD, MARBEC, Montpellier, France

***Corresponding author:**

Thibaut L'Honoré

Univ Montpellier, CNRS, IFREMER, IRD, MARBEC

Place E. Bataillon,

34095 Montpellier cedex 05, France

Tel: +33 4 67 14 93 94

thibautlhonore@gmail.com

Key words: phenotypic plasticity; freshwater acclimation; osmoregulation; respirometry; gill plasticity

Abstract

Confronted with new biotic and abiotic changes, euryhaline teleosts, able to cope with extreme salinities, could shelter in more stable habitats such as lagoons and fresh water (FW) in rivers. The European sea bass *Dicentrarchus labrax* undertakes such migrations during its life cycle, but upon climate change, the importance of hypo-osmotic environments could potentially increase. Facing long-term exposure to fresh water following its migration, sea bass physiological capacities are pushed to their limits regarding metabolism and osmoregulation. In this study we aimed at describing how sea bass will cope with a long stay in FW, focusing first on blood parameters (osmolality, Na^+, Cl^-) variations after FW exposure from 1 h to 2 months. These parameters exhibited a significant drop after few hours spent in FW but blood osmolality recovered between 2 weeks and 2 months. Branchial *ncc-2a*, renal *nka α1a* and *ncc1* exhibited a differential expression according to time spent at low salinity, supporting the hypothesis of the presence of different phases for FW-acclimation. Higher standard metabolic rate (SMR) was higher in FW acclimated sea-bass indicating a cost of osmoregulation following long-term acclimation. Osmo-respiratory compromise was investigated by measuring hypoxia tolerance and gills plasticity. Lower O_{2crit} and higher incipient lethal oxygen saturation (ILOS) were measured in FW than in SW, associated with shorter lamella and wider filaments in FW. $O_{2deficit}$ was also lower in FW, suggesting lower anaerobic capacities in FW than in SW. Long-term FW acclimation and related hypoxia tolerance of sea bass is discussed in this study.

Introduction

According to IPCC and climate change scenarios, the Mediterranean Sea has been defined as a "hot-spot" for biodiversity sensitivity (Giorgi, 2006; Nicholls *et al.*, 2007; H. O. Pörtner *et al.*, 2014). Mean temperature and salinity are going to increase steadily, while extreme events of hypoxia and local precipitations will increase in frequency, with consequences for species distribution and survival. Southern species migration towards northern parts of Mediterranean Sea as well as Red Sea alien species migration through the Suez Canal (Lessepsian migration), may represent a supplementary stress on local ecosystem, and thus could drive to novel strategies in Mediterranean species (Mannino *et al.*, 2017). One possible strategy for seawater fish like *D. labrax* to avoid non-optimal temperature and salinities encountered in the Southern parts of the Mediterranean Sea might be more and more incursions in transitional habitats like lagoons or even fresh water (FW) streams where only resilient species could live, acting as protected areas from new interspecific interactions. Cases of freshwater

colonisations from marine organisms have been reported associated with the increase of coastal human activities (Ricciardi & MacIsaac, 2000; Wolff, 2000). *Eurytemora affinis* for example, a brackish water copepod, invaded freshwater habitats multiple times leading to the establishment of efficient hyper-osmoregulatory strategies in populations facing FW habitats (Lee *et al.*, 2012; Lee, 2016).

The European sea bass *D. labrax* is a euryhaline and eurythermic species commonly found in the Mediterranean Sea. It breeds offshore but larvae are drifted inshore and then swim to transitional habitats (estuaries, lagoons) serving as nurseries. In these habitats, salinity fluctuates widely, from hypersaline to brackish and even FW (Kelley, 1988; Barnabé, 1989; Waldman, 1995; Dufour et *al*., 2009; Vasconcelos et *al*., 2010). The osmoregulatory capacities of sea bass are well-described. European sea bass can acclimate successfully to a wide range of salinities (Cataudella et *al*., 1991; Venturini et *al*., 1992; Varsamos et *al*., 2002; Lorin-Nebel et *al*., 2006; Kokou et *al*., 2019) however mortalities are recorded in full freshwater probably linked to its marine origin (Nebel *et al.*, 2005; Guinand *et al.*, 2014; L'Honoré *et al.*, 2019). Coping with wide salinity variations constitutes a physiological challenge for fishes, even euryhaline species. In FW, the major osmoregulatory organs are the gills and the posterior kidney (Evans & Clairborne, 2009). Following FW transfer, it has been already demonstrated that sea bass blood parameters such as blood osmolality, Na^+ and Cl^- blood levels decrease until 10 days (Jensen *et al.*, 1998). To better understand both short- and long-term FW acclimation, the dynamic of these parameters remains to be characterised during a time-course study for longer acclimation time. Maintaining hydro-mineral balance in FW for long periods requires powerfull hyper-osmoregulation strategies, *i.e* teleosts must absorb ions (mainly Na^+ and Cl^-) through their gills and kidney and excrete excessive water through the urine (Marshall and Grosell, 2006; Evans and Clairborne, 2009). Ion's dynamic is performed by specific transporters and channels localised in particular cells known as ionocytes (Hiroi & McCormick, 2012). Ionocytes express a wide diversity of Na^+ and or Cl^- transporters as among them the Na^+/K^+-ATPase (NKA), the Na^+/Cl^- cotransporters NCC (NCC1, NCC-2A) and the Na^+/H^+ echanger-3 (NHE3) (Blondeau-Bidet *et al.*, 2016, 2019). In these latter studies following FW exposure, sea bass exhibited a higher branchial expression of *nka α1a*, *ncc-2a* and *nhe3* after 2 weeks, indicating their importance in FW acclimation mechanisms.

Changing salinity may not only affect osmoregulatory processes but also, potentially, gas exchange as a consequence of the differential availability of O_2 in the surrounding media. Modification of the structure of the gill epithelium as well as changes in the distribution and diversity of different cell types at the gill surface have been reported previously including in European sea bass (Randall & Malyusz, 1972; Sardella & Brauner, 2007; Masroor *et al.*, 2018). The trade-off between osmoregulation and respiration is known as the osmorespiratory

compromise (Nilsson & Sundin, 1998; Matey *et al.*, 2011). The gill is in fact a multi-operatory organ performing gas and ion exchange between the blood and the external media. To cope with rapid salinity change, a high plasticity in gill morphology is required for osmoregulation and gas exchange (Kelly & Woo, 1999; Masroor *et al.*, 2018). The osmo-respiratory compromise has been investigated in sea bass, with shorter lamellae and wider filament in FW than in SW at 18°C (Masroor *et al.*, 2018). The metabolic cost of osmoregulation is generally considered low in fish although it can range from few percent to one-third of fish basal metabolism, and seems species-specific (Bœuf & Payan, 2001; Chatelier *et al.*, 2005; Ern *et al.*, 2014; Ern & Esbaugh, 2018). Basal metabolism can be approximated by the resting oxygen consumption rate at a given temperature generally called the standard metabolic rate (SMR) (Fry, 1971; Clark *et al.*, 2013; Chabot *et al.*, 2016). Salinity can induce changes in SMR since hypersaline exposure in Mozambique tilapia *Oreochromis mossambicus* triggered a "tissue-level metabolic suppression" (Sardella & Brauner, 2008). However, it has to be considered that changes in SMR can also be influenced by changes in other physiological functions such as cardiovascular functions in response to salinity changes (Sollid, 2005; Olson & Hoagland, 2008). In some cases, fish encounter situations requiring anaerobic production of ATP or/and a decrease of their metabolism (predation, escaping, low oxygen water, etc). In hypoxic conditions, physiology can be pushed at its limits and survival will rely on individual's anaerobic capacities (Nilsson & Östlund-Nilsson, 2008). As a first response to hypoxic environment, fishes compensate the decline of partial pressure in oxygen across the gill by increasing oxygen uptake through hyperventilation or adjust their cardiovascular response (Farrell & Richards, 2009). The direct consequence of hyperventilation is the increase of passive water and ion movements across the gill epithelium which, in FW, generally reduces blood osmolality and requires increased active ion transports to maintain hydromineral balance and thus the cost of osmoregulation. Hypoxia tolerance is usually estimated by measuring the O_{2crit} corresponding to the critical oxygen partial pressure at which oxygen demand no longer meets oxygen supply (Pörtner & Grieshaber, 1993). Individuals with low O_{2crit} are supposed to take up and deliver oxygen efficiently to cope with metabolic depression (Claireaux & Chabot, 2016). Hypoxia tolerance can differ regarding environmental parameters such as temperature, as it was highlighted in Atlantic killifish *Fundulus heteroclitus* which exhibited a reduced hypoxia tolerance after a thermal stress (McBryan *et al.*, 2016). In sea bass juveniles exposed to hypoxia, several metabolic changes have been measured: a lower growth rate associated with an increase in both glycogen stores and efficiency in digestive protein functions (Zambonino-Infante *et al.*, 2017; Cadiz *et al.*, 2018). In this study, the minimum saturation of oxygen at which fish lose their equilibrium (LOE) was monitored as well as the incipient lethal oxygen saturation (ILOS) as a proxy for hypoxia tolerance. ILOS

was already measured in European sea bass facing acute oil exposure in SW, highlighting two phenotypes according to hypoxia tolerance with a disctinction at around 6 % saturation (Zhang *et al.*, 2017). Anaerobic capacities were also indirectly estimated by calculating the cumulative ambient oxygen deficit ($O_{2deficit}$), as it is described in Nelson & Lipkey (2015) and Claireaux & Chabot (2016). $O_{2deficit}$ stands for anaerobic glycolytic capacities that compensate for the energy supply below O_{2crit} (Maccormack *et al.*, 2006). The last indices used to characterise hypoxia tolerance was the accumulated oxygen deficit (AOD) because of its sensitivity to environmental factors. In sea bass exposed to oil, it was demonstrated that AOD is one of the most sensitive indexes (Zhang *et al.*, 2017). Influence of salinity on hypoxia tolerance is still discussed in the literature and thus needs to be clarified in short but also in long-term FW acclimation in European sea bass. Furthermore, the influence of oxygen availability on sea bass behavior has already been highlighted using risk-taking behavior experiments, with sea bass having relatively high SMR becoming more active in low-oxygen conditions (Killen *et al.*, 2012).

In this study, we aimed at describing experimentally how sea bass juveniles would cope with long-term FW exposure in order to determine the metabolic costs linked to long-term stays in fresh water areas, and to determine sea bass osmoregulatory strategies in these areas. Physiological and metabolic statuses are important to determine in the case of long-term (beyond 1 month) exploitation of freshwater habitats in this species. In this study we thus investigated the effect of freshwater exposure on (i) the dynamic of blood parameters, (ii) the expression levels of main osmoregulatory genes, (iii) the metabolic status *via* SMR and also (iv) the hypoxia tolerance *via* O_{2crit}, ILOS, LOE and $O_{2deficit}$.

Material and Methods

1. Experimental design

Two hundred European sea bass *Dicentrarchus labrax* were reared at Ifremer Station at Palavas-les-flots (Hérault, France) in recirculating 20°C SW under natural photoperiod since hatching. In both experiment, when fish were transferred to FW, they were previously transferred to brackish water (BW, 15 ppt) for 24 h to avoid acute osmotic stress. In experiment 1 (Fig. 1a), 20 sea bass (8-month-old, 13.59 cm ± 0.12, 32.19 g ± 2.62) were transferred and maintained in SW during the whole experiment (2 months) and 160 sea bass (N=20 fish/tank) were randomly transferred from SW to BW for 24h (BW, T_0) then to FW for 1 h, 3 h, 24 h, 48 h, 1 week, 2

weeks or 2 months. Finally, 16 fish per condition were analysed. SW and 2-weeks FW exposed fish were the same as in L'Honoré *et al.* (2019). In experiment 2 (Fig. 1b), juveniles were maintained in SW (6-month-old, 11.90 cm ± 0.17, 21.54 g ± 0.94) and either transferred to FW following the same procedure as for experiment 1 or transferred to SW for 4 weeks. Finally, 7 fish were analysed from the SW condition and 21 fish were analysed following FW challenge.

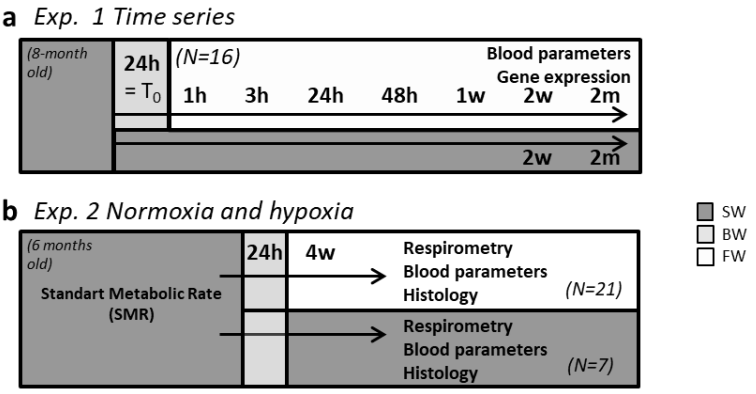


Fig. 1 Design of the 2 experiments performed in seawater (SW), brackish water (BW) or in fresh water (FW). h: hours; w: weeks; m: months; N stands for the number of fish analysed

2. Blood osmolality and ion composition

At the end of each experiment, blood was sampled in anesthetised fish (benzocaine 50 ppm) using a 1-mL syringe coated with heparin (Li-Heparin, Sigma-Aldrich, France), by puncturing blood directly in the caudal vessels. Blood osmolality was measured on 20 µL of blood using a micro-osmometer (Advanced 3300). Chloride levels were measured in duplicates immediately following blood puncture using 10 µL of blood and a chloride titrator (AMINCO, Maryland, USA). Sodium levels were quantified in duplicates by flame photometry (Sherwood, Cambridge, UK) using 1 µL of blood diluted at 1/1000 in milliQ water. The experimental procedures are further described in Masroor *et al.* (2018). Gills and posterior kidney of each fish from experiment 1 were sampled and directly stored at -80°C for molecular analysis.

3.1 RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from the gills and the posterior kidney using Nucleospin® RNA (Macherey-Nagel, Germany). Both quantity and purity of extracted total RNA was verified by measuring the A260/A280 ratio using the NanoDrop™ One/OneC Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was generated from 1 µg of RNA using the qScript™ cDNA SuperMix (Quanta Biosciences™)

providing all necessary components for first-strand synthesis: buffer, oligo(dT) primers, random primers and qScript reverse transcriptase.

3.2 Quantification of gene expression levels

Quantitative PCR (qPCR) analysis of each sample was performed in triplicate by a LightCycler®480 Real-Time PCR System (Roche, Mannheim, Baden-Württemberg, Germany) using 384-wells plates filled with an Echo®525 liquid handling system (Labcyte Inc., San Jose, CA, USA). Each well contained 0.75 µL of LightCycler-FastStart DNA Master SYBR-Green I™ Mix (Roche, Manheim, Germany), 0.037 µL of each primer (forward and reverse primers at 0.2 µM final concentration), 0.21 µL of ultrapure water and 0.5 µL of cDNA. The qPCR conditions were the same as in (L'Honoré *et al.*, 2019). The reference gene (elongation factor 1α, ef1α) was chosen according to previous studies performed on sea bass challenged to FW (Nebel *et al.*, 2005; Blondeau-Bidet *et al.*, 2016, 2019; L'Honoré *et al.*, 2019). For each organ and gene, efficiency of each primer pair was checked and indicated for each organ in Table 1. Relative expression of the target gene was performed using the comparative Ct method (threshold cycle number) according to (Blondeau-Bidet *et al.*, 2019; L'Honoré *et al.*, 2019). Ultra-pure water was used as a negative control template.

Table 1 Primer sequences used for gene expression analysis

Target gene	Primer name	GenBank accession numbers or Sequences ID	Sequence (from 5' to 3')	Efficiency	Reference
nka α1a	NKA-α1a_F	KP400258.1	CCTCAGATGGCAAGGAGAAG	2.0 (gills)	Blondeau-Bidet et al., 2016
	NKA-α1a_R		CCCTGCTGAGATCGGTTCC	2.0 (kidney)	
ncc-2a	NCC2A_F	DLAgn_00038210	ATGATGAGCCTCTTCGAGCC	2.1 (gills)	Blondeau-Bidet et al., 2019
	NCC2A_R		ACAGAAGGTGATGAGAGCAGC		
ncc1	NCC1_F	DLAgn_00172790	TGACGTACTTGATCGCTGCC	2.0 (kidney)	L'Honoré et al., 2019 (submitted)
	NCC1_R		AGTTGGTGATGGAGGCATGG		
nhe3	NHE3-F	DLAgn_00204050	GGATACCTCGCCTACCTGAC	1.9 (gills)	Blondeau-Bidet et al., 2019
	NHE3-R		AAGAGGAGGGTGAGGAGGAT	1.9 (kidney)	
ef1α	EF1α_F	AJ866727.1	GGCTGGTATCTCTAAGAACG	1.9 (gills)	Nebel et al., 2005
	EF1α_R		CCTCCAGCATGTTGTCTCC	1.9 (kidney)	

4. Oxygen consumption in normoxia and hypoxia in seawater and in fresh water

Twenty-eight 6-months old fishes were put into seawater metabolic chambers in order to measure their standard metabolic rate, SMR (mmol O_2 kg^{-1} h^{-1}) in SW. Part of the fish (N = 18) were then transferred to FW. After 4

weeks in fresh water, SMR was measured again in fresh water. Controls were measured in seawater-maintained fish at the same time (N = 7). Measurement of O_2 consumption (MO_2) were performed in a shaded quiet room for 72 h by an automated intermittent flow-through respirometry system described by McKenzie *et al.* (1995). Metabolic chambers were submerged in a thermoregulated (set at 21°C) and aerated water reservoirs. Each respirometer was connected to the software (Aquaresp, Danmark) through computer-controlled flush pumps (EHEIM, Germany). MO_2 measurements were performed by intermittent cycles of oxygen depletion and measurement (10 min), stabilisation time (1 min) and a flushing time (5 min). Oxygen consumption is calculated as indicated below (1). It takes into account the variation of the water oxygen concentration (mmol O_2 L^{-1}), the duration of the measurement (t = 10 sec), the volume of water in the respirometer (V_{resp} = 1.1 L) and the individual fish mass (M). Water O_2 saturation is measured by an Orbisphere clarke-type polarographic 856 oxygen electrode and associated meter (Orbisphere Laboratory, Geneva, Switzerland) (McKenzie *et al.*, 2003, 2007). Oxygen consumption values were minored by individual bacterial MO_2 (background respiration) measured for 1 h after the fish was removed from the respirometer. Values of MO_2 were conserved only when the linear regression of oxygen decreasing versus time yielded $r^2 > 0.90$. For each fish, SMR corresponds to the mean of the lowest 12^{th} percentile of oxygen consumption measurements (Clark et *al.*, 2013).

$$MO_2 = \Delta CO_{2,w} \times \Delta t^{-1} \times V_{resp} \times M^{-1} \quad (1)$$

After normoxic measurements in seawater and fresh water, progressive hypoxia was performed by stopping water renewal, and MO_2 was measured every 10 seconds. When fish started to lose their equilibrium, the experiment was stopped and fish were euthanised with 100 ppm of benzocaine. O_{2crit} can be defined as the critical oxygen saturation (in %) when aerobic metabolism needs to be completed with anaerobic metabolism (Rogers *et al.*, 2016). It corresponds to the oxygen saturation (%sat) when fish MO_2 starts decreasing with %sat (Fig. 5). Time spent between the last water renewal and fish loss of equilibrium (LOE) and incipient lethal oxygen saturation (ILOS) for each fish were recorded as proxies for hypoxia tolerance. Difference between O_{2crit} and ILOS called $O_{2deficit}$ was calculated by subtracting ILOS to O_{2crit}.

5. Morphometric analyses

Immediately following fish euthanasia after hypoxic measurements, first gill arch of each animal was sampled and fixed for 24h in Bouin's fixative. They were rinsed in 70% ethanol and dehydrated in increasing ethanol concentrations before being embedded in Paraplast (Sigma). Sections of 4 µm were effected on a microtome (Leitz Wetzlar) and collected on silanised slides and stained using the Masson's Trichrome staining protocol.

Gill sections were photographed under a Leica Diaplan microscope at a magnification of x 400. Filament thickness and lamella length were then measured as in Masroor *et al.* (2018) using Image J software (Image J 1.51, http://rsbweb.nih.gov/ij/). Two regions were randomly selected, photographed, and three measures were performed per region on five to six fish per condition.

6. Statistical analysis

When the data fit with normality and homoscedasticity, one-way analysis of variance was performed followed by a Tukey's post-hoc test. Conversely, if they did not, Kruskal-Wallis test was performed followed by a Dunn's test. All experimental values are reported as means ± s.e.m. All statistical differences were accepted at $P < 0.05$ and analyses were performed using GraphPad Prism (version 6, GraphPad Software Incorporated, La Jolla, CA 268, USA).

Results

1. Time series analysis of blood parameters after freshwater transfer

Blood osmolality decreased significantly after BW transfer (indicated as 0 h in Fig. 2, Dunn's test, $P < 0.05$). A gradual decrease was recorded after FW transfer until 1 week and a significant drop was recorded at 2 weeks, before returning to SW-like values at 2 months. Blood osmolality, Na^+ and Cl^- blood levels became significantly higher after 2 months in FW than after 2 weeks (Unpaired t-test, $P < 0.0001$, $P = 0.0023$ and $P = 0.0003$ respectively, Figur 2). Sodium and chloride blood levels did not change after BW transfer but exhibited a progressive decrease following FW challenge, becoming significant at 24 h and 3 h respectively (Dunn's test, $P < 0.0001$ and $P = 0.0148$ respectively, Fig 2). No significant difference was observed for blood Na^+/Cl^- ratio during the whole experiment (data not shown).

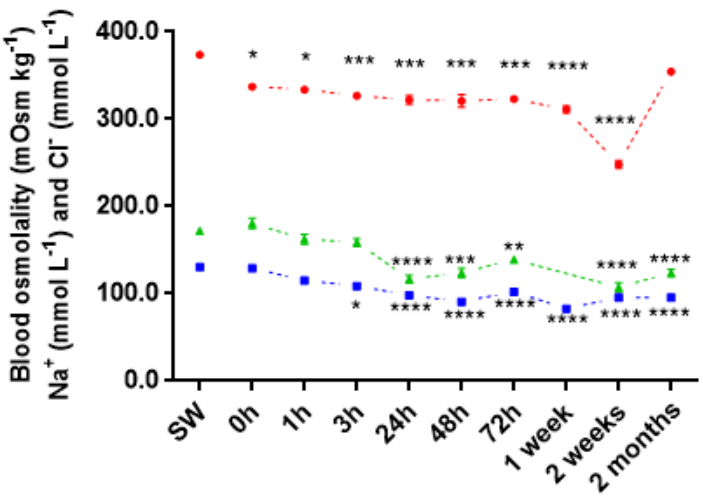


Fig. 2 Blood osmolality (●, mOsm kg^{-1}), Na^+ (▲, mmol L^{-1}) and Cl^- (■, mmol L^{-1}) measured in sea bass maintained in seawater (SW), after transfer in brackish water (BW) (corresponding to 0 h), and after freshwater (FW) transfer (from 1 h to 2 months) (means ± s.e.m). Data were obtained from experiment 1. Asterisks denote significant differences with seawater: ****: $P < 0.0001$; ***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$ (Kruskal-Wallis followed by Dunn's test, N = 10-16

2. Gene expression

Transcript levels of several genes involved in osmoregulation were measured in the gills and in the posterior kidney. Three groups were considered: fish kept in SW, FW tolerant fish that were maintained 2 weeks in FW (FW_t) and fish that were maintained 2 months in FW (long-term : FW_{LT}). In this experiment about one third of fish was not able to cope with FW and died. They were characterised as freshwater intolerant fish in L'Honoré *et* al. (2019).

At the gill level, *nka α1a* exhibited a nearly higher but not significant different relative expression in FW_t than in SW (Tukey's test, $P = 0.0519$, Fig. 3a). Moreover, no significant difference was measured in FW_{LT} compared to both SW and FW_t (Tukey's test, $P = 0.6261$ and $P = 0.2651$). Expression levels of *ncc-2a* were significantly higher in FW_t compared to SW but no difference was observed between FW_t and FW_{LT} (Dunn's test, $P = 0.0027$ and $P = 0.2994$ respectively, Fig. 3c). FW_{LT} exhibited no significant difference in *ncc-2a* expression compared to SW (Dunn's test, $P = 0.1252$). *Nhe3* expression levels were not different between the three groups (Kruskal-Wallis test, $P = 0.0999$, Fig. 3e). However, a nearly higher but not significant different relative expression was measured in FW_t compared to SW (Dunn's test, $P = 0.0960$).

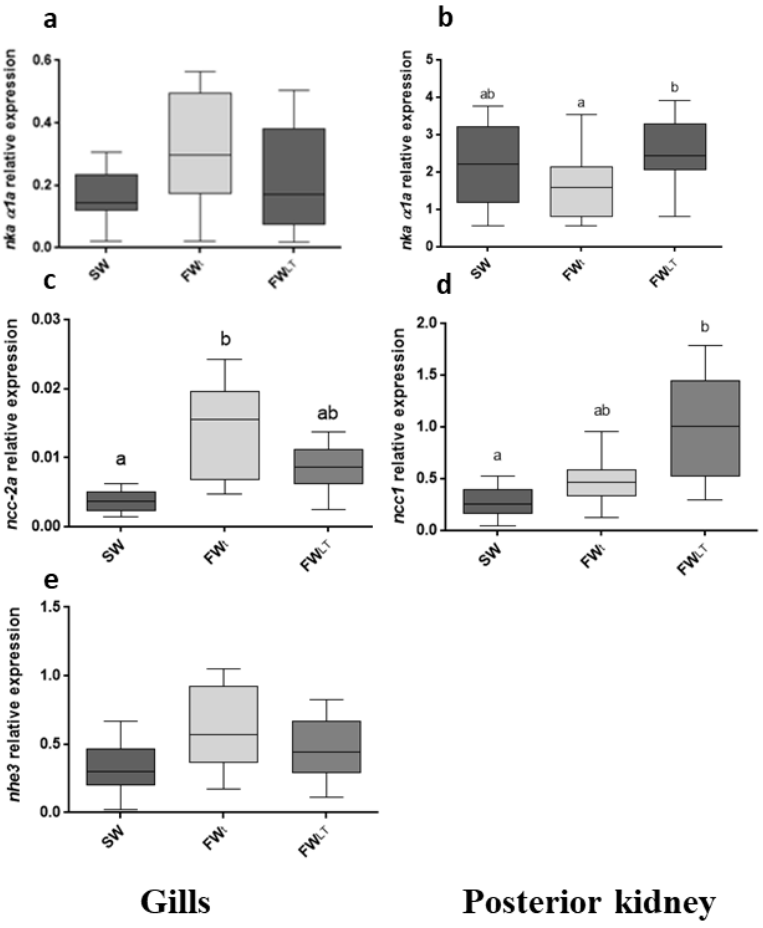


Fig. 3 Relative expression of **(a-b)** *nka α1a*, **(c)** *ncc-2a*, **(d)** *ncc1*, and **(e)** *nhe3* in the gills and in the posterior kidney of sea bass maintained in seawater (SW), after two weeks (FW_t) and 2 months (FW_{LT}) in fresh water. The expression has been normalized according to the expression of the elongation factor *ef1α*. Data were obtained from experiment 1. Different letters denote significant differences between groups (Kruskal-Wallis followed by Dunn's test, $P < 0.05$, means ± s.e.m, N = 6-16)

In the posterior kidney, *nka α1a* expression levels were relatively lower in FW_t than in SW and significantly lower in FW_t than in FW_{LT} (Tukey's test, $P = 0.06443$ and $P = 0.0227$ respectively, Fig. 3b). *Ncc1* relative expression was not significantly different between SW and FW_t but it was significantly higher in FW_{LT} than in SW (Dunn's test, $P = 0.1219$ and $P < 0.0001$ respectively, Fig. 3c). FW_{LT} exhibited a higher but not significant expression of *ncc1* than FW_t (Dunn's test, $P = 0.0957$).

3. Respirometry analysis in normoxia and in hypoxia

During the experiment in fresh water, a mortality of about 30% was observed in the raceway or in the metabolic chambers. Controls that have spent 4 weeks in seawater did not exhibit any significant difference in standard metabolic rate (SMR) between the two measurement periods (Paired t-test, $P = 0.6434$, Fig. 4a). In normoxic conditions, significantly higher SMR was measured in fresh water than in seawater (Paired t-test, $P = 0.0043$, Fig. 4b). After progressive hypoxia, blood osmolality in FW was significantly lower compared to SW (Unpaired t-test, $P < 0.0001$, Fig. 1S).

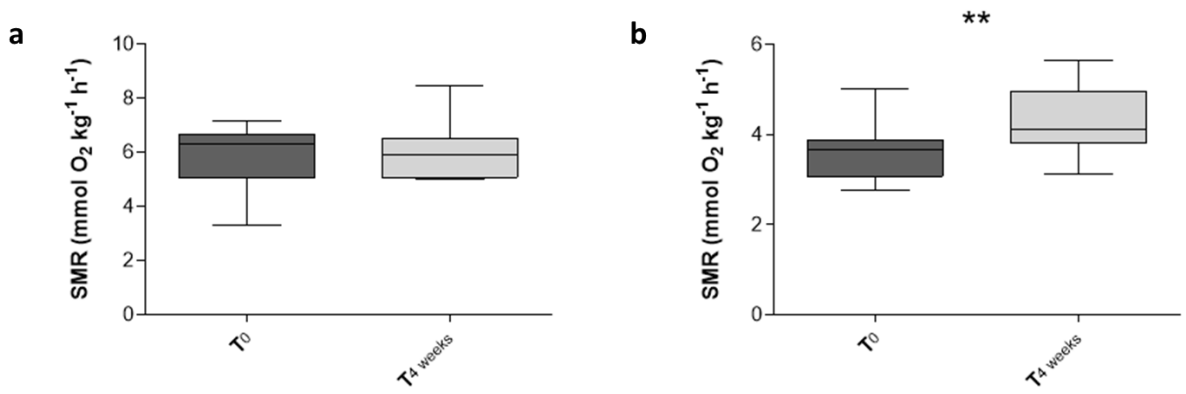


Fig. 4 Standard metabolic rate (mmol O_2 kg^{-1} h^{-1}) measured in fish maintained in seawater (SW, T_0) and then transfered for 4 weeks ($T_{4\ weeks}$) to SW **(a)** or fresh water (FW) **(b)** with a 24 h acclimation at 15 ppt before FW transfer (means ± s.e.m). Data were obtained from experiment 2. Asterisks denote significant differences between salinities (Paired t-test, $P = 0.6434$ and $P = 0.0043$, N = 7 and N = 17)

Following oxygen decrease in both salinities, fish maintained their respiration unchanged until hypoxia became severe enough to trigger a linear decline in MO2 as %sat dropped (Fig. 5). Fish challenged to fresh water exhibited a significant 21% lower O_{2crit} than fish maintained in SW (Unpaired t-test, $P = 0.0046$, Fig. 6a). Conversely, ILOS was significantly more than 2 times higher in freshwater fish than in seawater fish (Unpaired t-test, $P < 0.0001$, Fig. 6b). Accumulated oxygen demand (AOD) was lower in freshwater fish than in SW fish (Mann-Whitney test, $P = 0.0030$, Fig. 7a). Finally, $O_{2deficit}$ was higher in SW than in FW (Unpaired t-test, $P < 0.0001$, Fig. 7b).

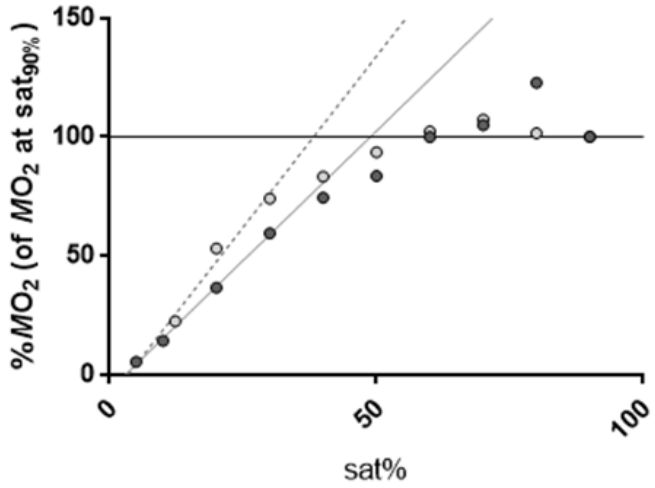


Fig. 5 Effects of progressive hypoxia upon percentage of oxygen uptake (%MO_2) compared to MO_2 at 90% of oxygen saturation ($sat_{90\%}$) in 6-month-old European sea bass maintained for 4 weeks in seawater (SW, dark grey dots) or in fresh water (FW, light grey dots). Linear regressions through the percentage of MO_2 at sat_{90} are plotted in SW (solid lines) and in FW (scattered lines) when MO_2 decreased with %sat. In SW the curve is fitted by $y = 2.1818x - 6.5591$, ($r^2 = 0.9985$) and in FW the curve is fitted by $y = 2.8802x - 10.008$, ($r^2 = 0.9668$). The horizontal line shows normoxic $MO_{2,Smax}$, set at 100%. The intersection of the two lines indicates the O_{2crit} below which the sea bass could no longer regulate aerobic metabolism. Data were obtained from experiment 2 (means ± s.e.m)

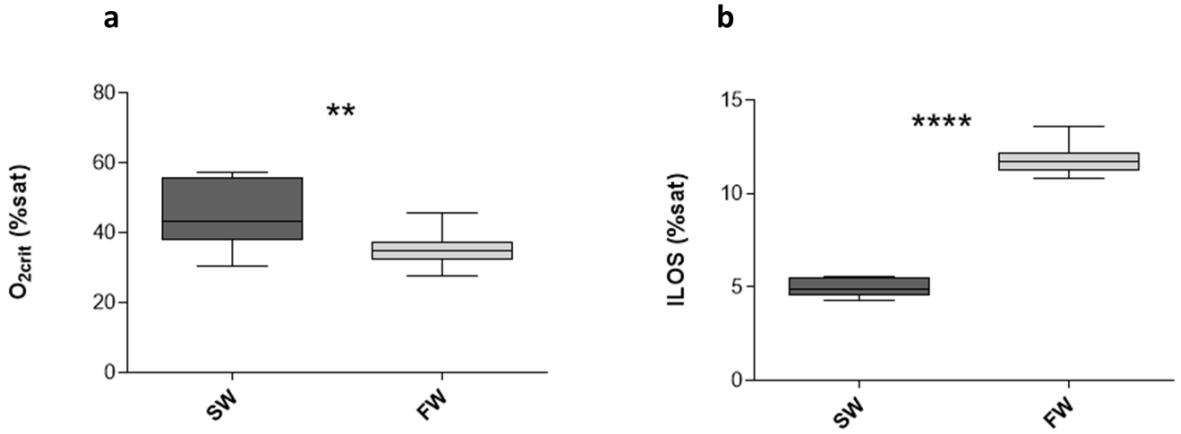


Fig. 6 Effect of salinity on **(a)** critical oxygen saturation (O_{2crit}, %sat) and **(b)** incipient lethal oxygen saturation (ILOS, %sat) measured in sea bass following a 4-week transfer to either seawater (SW) or fresh water (FW) (means ± s.e.m). Data were obtained from experiment 2. Asterisks denote significant differences between salinities ****: $P < 0.0001$; **: $P < 0.01$ (Unpaired t-test, $P = 0.0046$ and $P < 0.0001$, N = 7 in SW and N = 18 in FW)

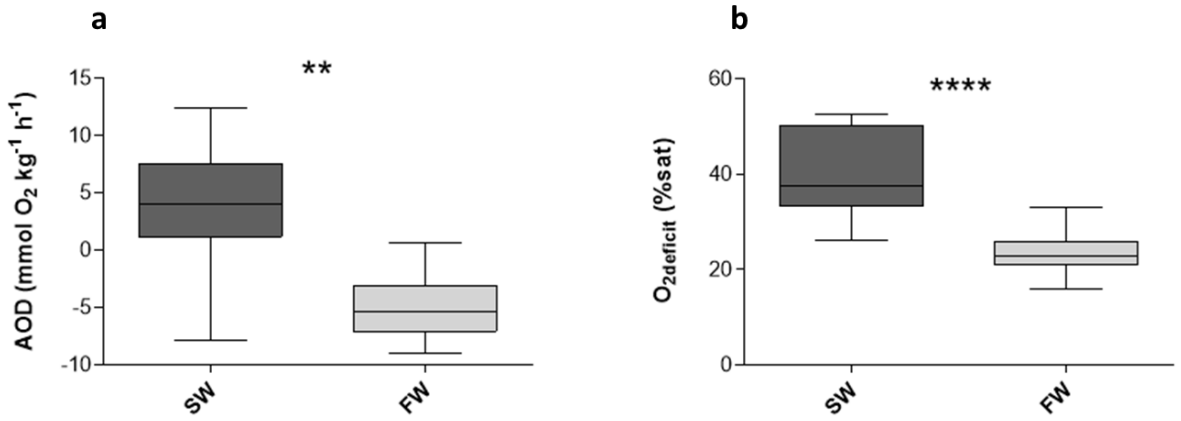


Fig. 7 Effect of salinity on **(a)** accumulated oxygen deficit and **(b)** cumulative ambient oxygen deficit ($O_{2deficit}$, %sat) in 6-month-old European sea bass maintained 4 weeks in seawater (SW) or in fresh water (FW). Data were obtained from experiment 2 (means ± s.e.m), Asterisks denote significant differences between salinities (Unpaired t-test and Mann-Whitney test, $P < 0.0001$, N = 7 in SW and N = 18 in FW)

4. Morphometric analyses

Following hypoxic and FW exposure, sea bass exhibited a significant shorter lamella length and a wider filament thickness than in SW fish exposed to hypoxia (Mann-Whitney test, $P < 0.0001$ for both, N = 5-6, Fig. 8).

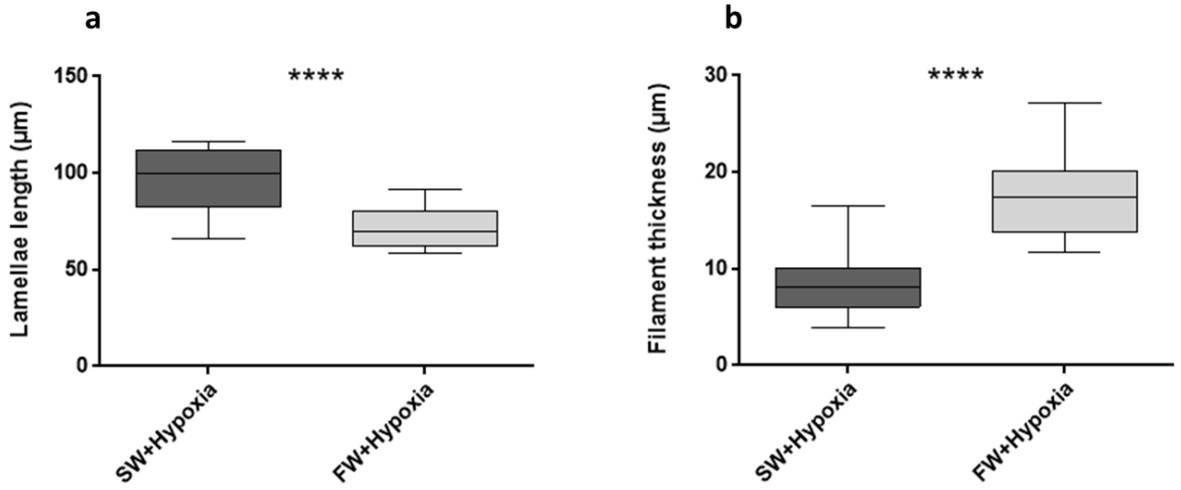


Fig. 8 Effect of salinity and hypoxia on (a) lamella length and (b) filament thickness in 6-month-old European sea bass maintained 4 weeks in seawater (SW) or in fresh water (FW) and challenged to hypoxia. Data were obtained from experiment 2 (means ± s.e.m), Asterisks denote significant differences between salinities (Mann-Whitney test, $P < 0.0001$ for both, N = 5-6)

Discussion

The aim of this study was to investigate long-term acclimation of sea bass to FW regarding osmoregulation and respiratory metabolism. European sea bass exhibited a contrasted response regarding FW acclimation: 30% mortality was observed as in previous studies (Nebel *et al.*, 2005; Giffard-Mena *et al.*, 2008; L'Honoré *et al.*,

2019), but those which survived were able to live at least 2 months in FW, switching from the SW to the FW phenotype with a long-term phase of FW-acclimation enabling blood parameters and gene expression levels to reach levels close to SW conditions. Their metabolic capacities seemed to differ between salinities after a long-term FW acclimation, since they had a higher SMR in FW and a better resilience to hypoxic challenge as shown by a lower O_{2crit} than in SW fish, but lower anaerobic capacities as shown by a lower $O_{2deficit}$ and AOD. One possible explanation could be the reduced lamella length and increased filament thickness in FW, illustrating the osmo-respiratory compromise.

1. Blood parameters dynamic following FW exposure

The significant drop in blood osmolality as well as in ion levels after two weeks in FW are consistent with a previous study in 8-month-old juvenile sea bass (Bossus *et al.*, 2011), who reported a significant decrease in blood osmolality 24 h after FW transfer until 30 days. However, we highlight for the first time a recovery between 2 weeks and 2 months. FW hydromineral balance regulation is generally characterised by first a rapid response to limit ion leakages (which might occur immediately following FW transfer to avoid excessive drop of ion osmolality) and then, the progressive establishment of relatively long- term mechanisms to recover and maintain hydro-mineral balance. A two-phase response was reported in other fish species (milkfish, pufferfish, stripped sea bass) following exposure to FW, with an acute or adjustive response occurring the day after transfer, followed by a regulatory phase (Lin *et al.*, 2004, 2006; Madsen *et al.*, 2007). In European sea bass, it seems that at two weeks, the regulatory phase is not complete as mineral balance is not fully restablished, indicating a long-term phase in the FW-acclimation process occurring between 2 weeks and 2 months following transfer. Although Na^+ and Cl^- blood levels were higher after 2 months than after 2 weeks in FW, they were still lower than SW values whereas blood osmolality recovered. These results suggest that coping with FW for 2 months involved the synthesis and the accumulation of other osmolytes to adjust blood osmolality such as organic osmolytes (Burg & Ferraris, 2008).

If we compare blood osmolalities obtained during time series after 2 months in FW (exp. 1) to blood osmolality following hypoxia (exp. 2), it appeared that blood osmolality is slightly lower (by 4 %) than for fish that have not been exposed to hypoxia. These results show that hypoxia combined to low salinity might affect blood osmolality in fish, and strengthen the hypothesis of a blood osmolality recovery between 2 weeks and 2 months. The significantly higher blood osmolality in SW following hypoxia than SW control is concordant with hyperventilation triggered by hypoxia, increasing amount of salts diffusing in the blood (Farrell & Richards,

2009). In the red drum (*Scianops ocellatus*) such differences were not measured even after 24 h of hypoxia, but their hypoxia conditions were moderated (50% air saturation, Ern & Esbaugh, 2018).

2. Short and long-term FW acclimation gene expression strategies

Regarding ion transporters in the gills, *nka α1a* expression levels were almost but not significantly higher after 2 weeks in FW compared to SW as it was demonstrated previously (Blondeau-Bidet *et al.*, 2016; L'Honoré *et al.*, 2019). Moreover, a concordant higher activity of the Na^+/K^+ ATPase pump has been reported contributing to the maintenance of the hydromineral balance (Nebel *et al.*, 2005). After 2 months of FW acclimation, *nka α1a* expression seems to decrease to levels close to SW conditions. *Nhe3* expression levels displayed the same pattern as *nka α1a* with a relatively higher expression after 2 weeks in FW followed by a decrease after 2 months. NHE3 has been previously localised apically in NHE3-type ionocytes of European sea bass, notably detected in lamella (Blondeau-Bidet *et al.*, 2019). This cell type is considered to be involved in Na^+ uptake and acid secretion (Kumai & Perry, 2012). Considering the *ncc-2a* co-transporter, its higher expression after 2 weeks in FW than in SW has been already highlighted in L'Honoré *et al.* (2019) as well as in other species (Breves *et al.*, 2011; Inokuchi *et al.*, 2017). The apical NCC2 (or NCC-like) expressed in gill ionocytes is supposed to trigger Na^+ and Cl^- uptake (Hiroi *et al.*, 2008; Inokuchi *et al.*, 2008; Bollinger *et al.*, 2016; Blondeau-Bidet *et al.*, 2019). Interestingly, after two months in FW, it seemed that *ncc-2a* as well as *nhe3* expressions returned to intermediate values between SW and 2 weeks in FW which is concordant with blood parameters. It may indicate that other ion uptake mechanisms are activated in long-term acclimated fish that remain to be identified.

At the posterior kidney level, gene expression levels of *nka α1a* exhibited a slight decrease after 2 weeks in FW followed by a recovery to SW-type expression levels after 2 months in FW. In the literature, no difference in *nka α1a* expression has been reported in sea bass exposed for 2 weeks in FW (Nebel *et al.*, 2005; Blondeau-Bidet *et al.*, 2016; L'Honoré *et al.*, 2019). We then analysed a main transporter involved in Na^+ and Cl^- uptake at the kidney level of sea bass (L'Honoré *et al.*, submitted), identified as NCC1 and supposedly expressed in apical membranes of collecting ducts (Kato *et al.*, 2010; Inokuchi *et al.*, 2017). *Ncc1* expression levels did not change in FW_t compared to SW fish but they were significantly higher after 2 months in FW (FW_{LT}). This high expression might be part of the long-term strategy in sea bass to take up ions. The data obtained at the kidney and gill level also state an over-expression of two main transporters involved in ion uptake at the kidney but not the gill level which enhances the importance of the kidney in hyper-osmoregulatory processes, notably following a long-term challenge.

3. Standard metabolic rate and hypoxia tolerance

The higher SMR following salinity challenge is not concordant with the results obtained by Chatelier *et al.* (2005), although in this latter study only 3 sea bass were exposed to FW in metabolic chambers for 18 h instead of 4 weeks and 18 animals in our experiment. Resting metabolic rate (RMR) in *F. heteroclitus* exhibits the same pattern than the sea bass in this study with higher RMR following 3 weeks of acclimation at low salinities (Brennan *et al.*, 2016). In the literature, there is no clear trend of standard metabolic rate change with salinity, and seems to depend on the species and the considered salinity (Ern *et al.*, 2014; Christensen *et al.*, 2017; Ern & Esbaugh, 2018). Nevertheless, we observed a high inter-individual variability in SMR as described in the literature certainly corresponding to different metabolic phenotypes (Claireaux & Lagardère, 1999; McKenzie et *al.*, 2014; Zhang *et al.*, 2017). More individuals should be analysed in future investigations to identify different phenotypes regarding SMR in FW. In previous studies, different phenotypes have been described in FW regarding FW tolerance but the metabolic cost has not been identified and might also differ between tolerant and intolerant fish to FW (L'Honoré *et al.*, 2019). At the same temperature, the partial pressure of O_2 is about 20% higher at 20°C at 100% O_2 sat in fresh water than in seawater (Saroglia *et al.*, 2009). Having this in mind, oxygen consumption could be increased in SW to compensate decreased oxygen availability, but this seems not to be the case in sea bass. At low salinity, proteolysis is enhanced and the osmotic regulation is disturbed, modifying the energetic demands (Wu *et al.*, 2017). These latter authors revealed that reduced salinities can also increase the basal metabolism through an increase in anaerobic metabolism indicated by elevated lactate measured in different tissues in juvenile flounder *Paralichthys olivaceus*. In sea bass, the higher SMR following 4 weeks in FW may reflect a long-term cost of osmoregulation and not the metabolic cost of the acute phase response.

Regarding hypoxia resistance, we observed that sea bass have a two times higher O_{2crit} in SW compared to previous studies with the same species in SW where O_{2crit} was around 20 % sat (Zhang *et al.*, 2017). In SW, the higher O_{2crit} may indicate that fishes are less efficient to adjust their physiology to regulate their metabolic rate. One explanation could be a lower cardiorespiratory capacity in SW (Seibel, 2011). The high SMR could also be linked to fish feeding only 2 weeks following the FW challenge as reported in L'Honoré *et al.* (2019) adding another potential source of stress or physiological impairment. Nutritional status has an impact on osmoregulation capacities and anaerobic metabolism as in FW, fish can also absorb some ions through food (Nilsson & Östlund-Nilsson, 2008; Sinha *et al.*, 2015). In fresh water, the 20% lower O_{2crit} indicates that fish are efficient in adjusting their physiology to maintain their basal metabolism with oxygen decrease. Joyce *et al.*

(2016) highlighted that a hypoxia-tolerant phenotype can be distinguished from hypoxia-sensitive phenotype not only by differences in O_{2crit}, but also by a more powerful cardiac contraction and relaxation. Intraspecific variability in cardiac performance could contribute to variability in O_{2crit} and should be investigated in future studies. In contrast, FW-acclimated sea bass exhibited a lower AOD and $O_{2deficit}$, indicating a possible lower depletion of glycogen stores and accumulation of lactate according to (Zambonino-Infante *et al.*, 2017; Cadiz *et al.*, 2018) but this needs to be confirmed by glycogen and lactate measurements. This is possibly due to their 1.5 times lower weight for a relative same length compared to fish maintained in SW. ILOS was higher in FW than in SW but we did not observe more variability between individuals than between treatments as it was previously demonstrated (Claireaux *et al.*, 2013; Joyce *et al.*, 2016; Mauduit *et al.*, 2016). ILOS reflects the extreme limit of hypoxia tolerance in the different tissues, such as the depletion of ATP reserves or glycogen stores (Vornanen *et al.*, 2009; Speers-Roesch *et al.*, 2013). Since we measured shorter lamellae in FW than in SW (because the water has more dissolved oxygen), it may be affect gas exchange capacity in FW exposed to severe hypoxia. Long-term hypoxic events are susceptible to occur more often with climate change, notably in transitory habitats as lagoons and estuaries. If sea bass meet long-term hypoxic events as well as strong anaerobic demands in FW, they might have more difficulties to withstand in FW environments as they would do in SW and this could be critical for their survival (Claireaux & Chabot, 2016).

Conclusion

The different approaches presented in this study demonstrate how fresh water affects sea bass osmoregulatory capacities, basal respiratory metabolism and hypoxia tolerance. Sea bass exhibited several phases as a response to long-term freshwater challenge. Yet, the long-term regulatory phase that allows sea bass to recover from hydromineral imbalance deserves further investigations. We highlighted for the first time that the metabolic cost of such long-term FW acclimation is higher than in SW conditions suggesting a cost of osmoregulation in FW even after long-term. It has to be noted that only a part of sea bass survived long-term FW challenge, which could indicate that some phenotypes are better equipped to face long-term FW exposure than others. Our data provide evidence that even after long-term FW transfer, sea bass extreme hypoxia tolerance and anaerobic capacities differed from SW fish possibly linked to gill remodeling as a result of osmo-respiratory compromise. However, moderated hypoxia tolerance estimated by O_{2crit}, being lower in FW indicates that sea bass are well equipped to tolerate facing short-term hypoxic events. Sea bass are in fact known to migrate to hypo-osmotic environments, notably lagoons and estuaries which are also characterised by low O_2 levels, notably in the summer months due to phytoplanctonic blooms. As a response, lower hypoxia resilience and anaerobic

capacities could become critical if fish have to stay in these habitats for longer durations. Given that multiple stressors are going to affect coastal organisms like sea bass, understanding how they can cope with these stressors could help us predicting their future migration patterns and distributions.

Acknowledgments

We would like to thank Beatrice Chatain, Stephane Lallement, Marie-Odile Blanc and Alain Vergnet of Ifremer Palavas-les-flots for helping us in the maintenance of the fish. The project used instruments that were financially supported by the 2015-2020 CPER CELIMER (funded by the French Ministry of Higher Education, Research and Innovation, the Occitanie Region, Montpellier Méditerranée Metropolis, Sète Agglopole Méditerranée, Ifremer, IRD).

Compliance with ethical standards

Conflict of interest: The authors have no conflict of interest to declare.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The experimental design has been approved by the French legal requirement concerning welfare of experimental animals (APAFIS permit no. 9045-201701068219555). Informed consent: Informed consent was obtained from all individual participants included in the study.

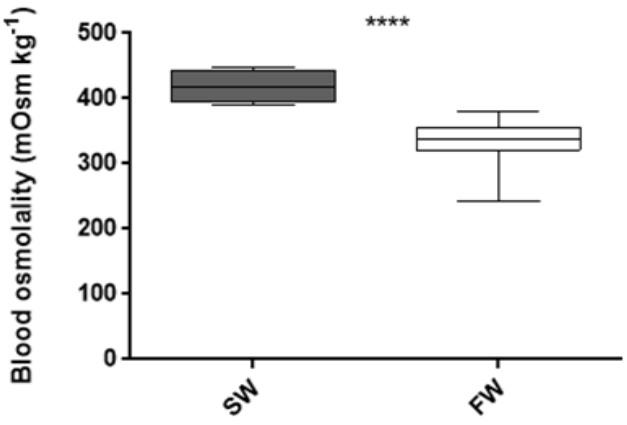


Fig. 1S Effects of progressive hypoxia and salinity upon blood osmolality in 6-month-old European sea bass maintained 4 weeks in seawater (SW) or in fresh water (FW). Data were obtained from experiment 2 (means ± s.e.m), Asterisks denote significant differences between salinities (Unpaired t-test, $P < 0.0001$, N = 7 in SW and N = 21 in FW)

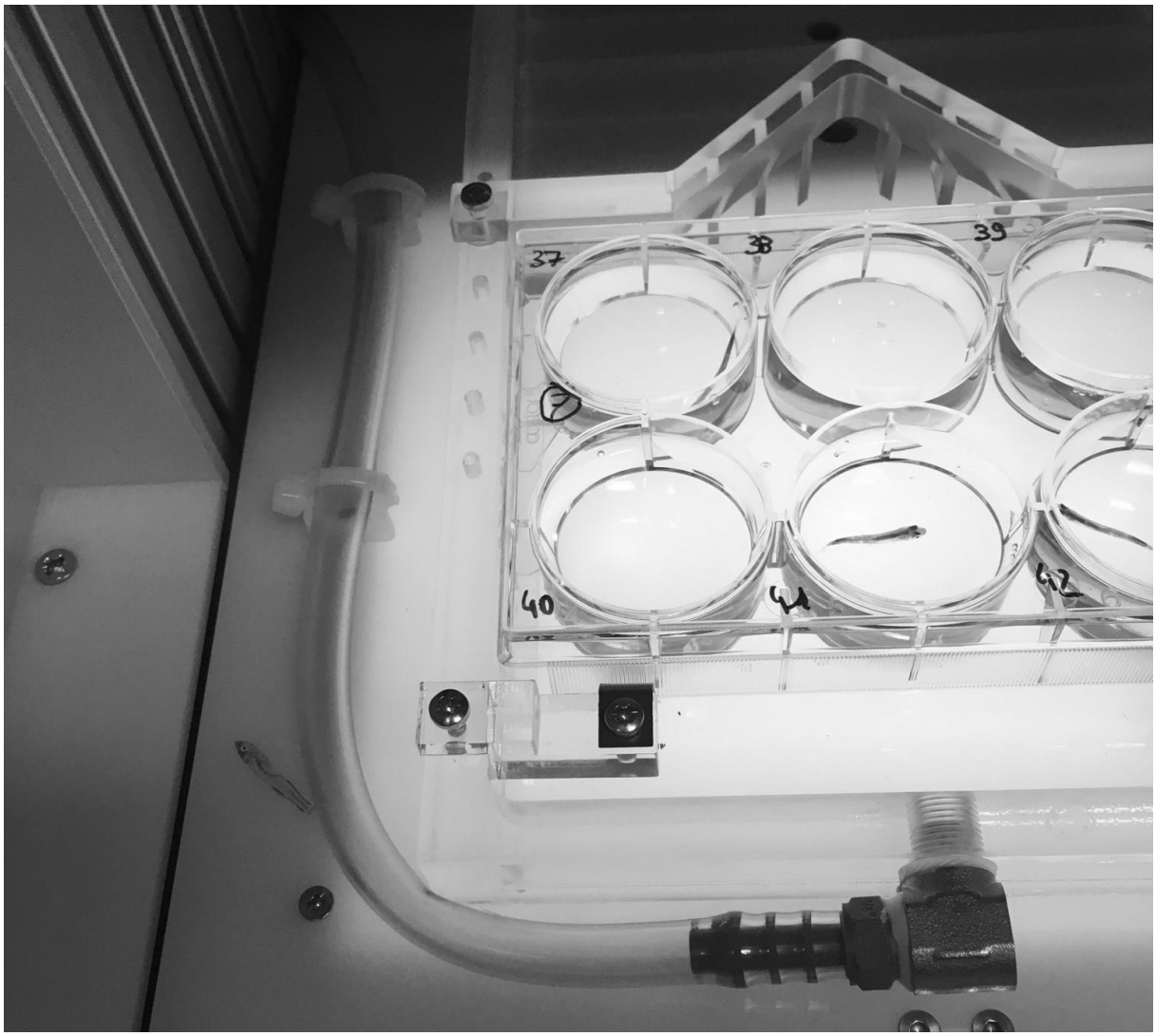
Résumé du Chapitre 1

Avec les changements climatiques, l'augmentation de la température de l'eau, l'augmentation des épisodes extrêmes de précipitations et la migration vers le Nord d'espèces plus tropicales, les poissons euryhalins capables de tolérer de grandes variations de salinités pourraient trouver refuge face à ces nouvelles contraintes biotiques et abiotiques dans les environnements à salinité changeante comme les lagunes voire l'eau douce des rivières. Le loup européen *Dicentrarchus labrax* entreprend de telles migrations au cours de son cycle de vie. Cependant avec les changements climatiques, le temps passés dans ces environnements dessalés peut être amené à changer. Chez les poissons d'origine marine, les faibles salinités rencontrées dans ces milieux affectent fortement les capacités physiologiques des organismes, que ce soit la réponse au stress en général, le métabolisme ou encore l'osmorégulation. Dans cette étude, nous avons caractérisé comment les loups s'acclimatent en eau douce à court (quelques heures), moyen (2 semaines) et long terme (2 mois), notamment en s'intéressant aux variations des paramètres biochimiques du sang (osmolalité, Na^+, Cl^-). L'ensemble des paramètres sanguins mesurés diminue significativement seulement après quelques heures passées en eau douce mais ils retournent à des valeurs proches de celles en eau de mer entre deux semaines et deux mois. Une analyse des niveaux d'expression de plusieurs transporteurs clefs dans les échanges de Na^+ et de Cl^- en eau dessalée au niveau des branchies et du rein postérieur a été réalisée. Les gènes *ncc-2a* au niveau des branchies et le gène *ncc1* au niveau du rein, tous deux impliqués dans l'absorption ou la réabsorption d'ions Na^+ et Cl^- présentent une expression différentielle en fonction du temps passé en eau dessalée, soutenant l'hypothèse que les mécanismes impliqués dans la phase tardive dans l'acclimatation à l'eau douce diffèrent de ceux mis en jeu à court et à moyen termes. Un taux métabolique standard (SMR) plus élevé après 1 mois en eau douce par rapport à l'eau de mer indique un coût métabolique de l'osmorégulation supérieur en eau douce même après une acclimatation à long terme. L'étude du compromis osmo-respiratoire a été réalisée par la mesure de la tolérance à l'hypoxie et l'étude histologique de la plasticité morphologique des branchies. Nous avons pu mesurer en eau douce une saturation létale en oxygène (ILOS) plus faible et une saturation critique (O_{2crit}) plus élevé en eau douce qu'en eau de mer, associées à des lamelles plus courtes et des filaments plus épais. Le déficit en oxygène $O_{2deficit}$ (O_{2crit} – ILOS) est également plus faible en eau douce, ce qui suggère des capacités métaboliques en conditions anaérobies plus limitées en eau douce qu'en eau de mer.

L’acclimatation à long terme à l’eau douce ainsi que la tolérance à l’hypoxie du loup sont discutées dans cette étude.

Chapitre II :

Variabilité intraspécifique dans la tolérance à l'eau douce chez le loup méditerranéen *Dicentrarchus labrax*



Crédits : Thibaut L'Honoré

« *If everything seems to be going well, you have overlooked something* » inspired from Murphy's laws

Prologue du Chapitre 2

Lors des expositions en eau douce de juvéniles de loups âgés de 8 mois, environ 30% de mortalité ont été observés dès 24 h et ce jusqu'à 2 semaines. De telles observations avaient déjà été réalisées précédemment (Nebel *et al.*, 2005; Giffard-Mena *et al.*, 2008). Dans cette étude, nous nous sommes attachés à caractériser ce phénotype particulier des individus intolérants à l'eau douce.

Pour cela, un dispositif d'étude comportemental a été mis en place afin de caractériser plus précisément les traits comportementaux observés visuellement jusqu'alors. Cette partie a été réalisée avec l'aide d'Alizée Bourges (stagiaire Master 1) sur des loups âgés de 8 mois. Le stress lié au choc hypo-osmotique ainsi que le lien existant entre celui-ci et l'osmorégulation nous ont ensuite conduits à mesurer l'expression des récepteurs aux glucocorticoïdes et minéralocorticoïdes (GRs et MR) et de la pompe Na^+/K^+-ATPase au niveau de deux organes osmorégulateurs : branchies et rein postérieur. Cette expérimentation a ensuite été répétée sur des loups âgés de 5 mois. En revanche, cette fois-ci les individus caractérisés comme étant intolérants à l'eau douce ont été marqués à l'aide de puces RFID avant d'être transférés en eau de mer en dispositif *common garden*, avec des individus caractérisés comme tolérants à l'eau douce ainsi qu'avec des témoins maintenus en eau de mer. Après une période de récupération de 5 mois en eau de mer, l'exposition en eau douce a été réitérée sur ces mêmes individus phénotypés à 5 mois, afin de déterminer si les capacités osmorégulatrices sont stables ou labiles dans le temps.

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ORIGINAL PAPER

Are European sea bass as euryhaline as expected? Intraspecific variation in freshwater tolerance

L'Honoré Thibaut[1] · Farcy Emilie[1] · Chatain Béatrice[2,3] · Gros Romain[1] · Ruelle François[3] · Hermet Sophie[1] · Blondeau-Bidet Eva[1] · Naudet Jeanne[1] · Lorin-Nebel Catherine[1]

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Abstract

In teleosts, the regulation of hydromineral balance has a direct impact on several physiological functions, biochemical processes, and can influence behaviour, distribution and survival. As European sea bass *Dicentrarchus labrax* undertake seasonal migrations from seawater (SW) to brackish, estuarine and fresh water (FW) in their habitat, this study investigates their capacity to tolerate fresh water and explores intraspecific variations in physiological responses. Juvenile *D. labrax* were transferred from SW to FW at various ages. Freshwater-tolerant and non-tolerant phenotypes were discriminated according to behavioural and morphological characteristics. About 30% of the fish exposed to FW were identified as freshwater intolerant following FW challenges performed at different ages. Interestingly, intolerant fish exhibited the same phenotypic traits: erratic swimming, lower speed, isolation from the shoal and darker colour. Freshwater-intolerant fish were also characterised by a significant lower blood osmolality compared to tolerant fish, and significantly lower Na^+/K^+-ATPase α1a expression in the posterior kidney. An imbalance in ion regulatory mechanisms was further confirmed by a blood Na^+/Cl^- ratio imbalance observed in some freshwater-intolerant fish. The analysis of glucocorticoid and mineralocorticoid receptor expression levels in gills and kidney revealed significant differences between freshwater-intolerant and -tolerant fish in both organs, suggesting differential stress-related responses. This study clearly shows an intraspecific difference in the responses following FW transfer with a decreased renal ion uptake capacity as a major cause for freshwater intolerance.

Introduction

Phenotypic plasticity, defined as the ability of a single genotype to produce multiple phenotypes in response to the environment, can be a strategy for organisms to cope with rapid environmental changes (Debat and David 2001; Pfennig et al. 2010; Parsons et al. 2011). Organisms living in lagoons and estuaries have to cope with fluctuating environmental parameters such as temperature, salinity and oxygen levels. In estuaries and lagoons, salinity plays a critical role in species distribution and influences migration patterns of many marine organisms at different stages of their life cycle (Wong et al. 1999; Pierce et al. 2012). Some fish undertake seasonal migrations towards these habitats such as the gilthead seabream *Sparus aurata* and the flathead grey mullet *Mugil cephalus* (Cardona 2006; Mercier et al. 2012). Differences in environmental parameters such as salinity can drive differential habitat distribution between species or even within a species. In gobies *Pomatoschistus sp.* or in grey mullet species, variable osmoregulatory capacities are responsible for the differential distribution observed (Lasserre and Gallis 1975; Rigal et al. 2008).

European sea bass *D. labrax* are considered a euryhaline species using transitional habitats like lagoons and estuaries as nurseries and feeding grounds (Kelley 1988; Barnabé 1989; Waldman 1995; Dufour et al. 2009; Vasconcelos et al.

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✉ Lorin-Nebel Catherine
catherine.lorin@umontpellier.fr

[1] Univ Montpellier, CNRS, IFREMER, IRD, UM, MARBEC, Montpellier, France

[2] Univ Montpellier, CNRS, IFREMER, IRD, UM, MARBEC, Palavas-les-Flots, France

[3] Univ Montpellier, CNRS, IFREMER, IRD, UM, LSEA, Palavas-les-Flots, France

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2010). Differential distribution of sea bass linked to different salinity regimes between habitats has been suggested, with some animals entering low-salinity habitats whereas others stay in the sea (Lemaire et al. 2000; Tine et al. 2014; Guinand et al. 2015). A successful acclimation to fresh water (FW) has been shown in several experimental studies, following direct or progressive FW transfer (Cataudella et al. 1991; Venturini et al. 1992; Varsamos et al. 2002; Lorin-Nebel et al. 2006; Kokou et al. 2019). Differential mortality has also been recorded in juveniles facing freshwater environments, suggesting differential physiological capacities to cope with FW within a same species (Dendrinos and Thorpe 1985; Allegrucci et al. 1994; Jensen et al. 1998; Nebel et al. 2005; Giffard-Mena et al. 2008). Intraspecific differences in habitat distribution linked to patterns of genetic differentiation were investigated in sea bass sampled in the field using genetic analyses (mini- and microsatellite markers) and transcript level expression. These studies did not reveal a direct genotype–phenotype relationship regarding the correlation between gene expression levels and habitat (e.g., lagoons or SW) (Lemaire et al. 2000; Guinand et al. 2014, 2015), but further investigations using next-generation sequencing technologies should be used to address this question (Dufresne et al. 2014).

In previous studies, European sea bass showed a wide range of blood osmolality (from 214 to 316 mOsm kg^{-1} in FW) with respect to the osmotic stress endured (Nebel et al. 2005; Giffard-Mena et al. 2008). The differential mortalities recorded in fish facing FW makes *D. labrax* a good candidate for investigating phenotype plasticity and intraspecific variability regarding freshwater tolerance. In a recent study analysing intraspecific divergence in stickleback *Gasterosteus aculeatus*, the plasticity in salinity tolerance has been directly linked to environmental salinity using reaction norms for survival and the expression of the Na^+/K^+-ATPase (NKA) (McCairns and Bernatchez 2010). Intraspecific variability linked to salinity tolerance has also been described in two populations of mummichog, *Fundulus heteroclitus*, with different mortalities observed during a FW challenge, probably linked to different habitats of ancestral fishes (Scott et al. 2004b). The authors concluded that this divergence in salinity tolerance could result in different capacities to absorb Na^+ and Cl^- at the gill level, leading to blood osmolality changes and causing death in intolerant fish (Scott et al. 2004a, 2005b).

The NKA is a membrane protein that provides an electrochemical gradient that is essential for the activation of major membrane channels and transporters (Marshall and Grosell 2006). In FW gills and posterior kidney, NKA plays a major role in ion uptake (Kiilerich et al. 2007a; McCormick et al. 2008). Gill and renal NKA activity have been already analysed in FW-intolerant sea bass (Nebel et al. 2005). A lower renal NKA activity was measured in intolerant fish compared to FW-tolerant fish, whereas the opposite pattern was recorded for gill NKA activity. Among two *nka α1* paralogs, NKA α1a is the main paralog expressed in osmoregulatory tissues of sea bass notably following FW transfer (Blondeau-Bidet et al. 2016). It still remains to be investigated if intraspecific variation regarding FW tolerance involves differences in *nka α1a* expression in osmoregulatory organs as it was the case for NKA activity.

Cortisol is a glucocorticoid hormone that is involved in the metabolic regulation and is an essential component of the stress response (Mommsen et al. 1999). Cortisol is also involved in the endocrine control of osmoregulation, by regulating different hormones and osmotic effectors responsible for gill remodelling, ionocyte differentiation and gill permeability (Franklin et al. 1992; McCormick 2001; Ágústsson et al. 2003; Boutet et al. 2007; Tomy et al. 2009; Kelly and Chasiotis 2011; Liew et al. 2015; Bossus et al. 2017). The response of fish to stress is associated with elevated plasma cortisol levels (Vijayan and Moon 1994; Wendelaar Bonga 1997; Barton 2002; Caruso et al. 2005) and can thus interfere with other physiological functions but also trigger changes in behaviour and swimming patterns (Øverli et al. 2007; Martins et al. 2012). Inter-individual differences in the stress response associated with differential cortisol levels among individuals have been reported in several species (Øverli et al. 2007; Williams 2008; Houslay et al. 2018) including sea bass according to their hierarchical position (i.e., dominant vs subordinate fish) (Samaras and Pavlidis 2018; Carbonara et al. 2019). Cortisol has the potential to be a physiological ligand of both glucocorticoid receptors (GRs): GR1 and GR2, but also mineralocorticoid receptors (MR) (Bury et al. 2003; Sturm et al. 2005). Corticoid levels and their receptors are, according to the considered species, supposed to be modified in response to salinity exposure (Sakamoto and McCormick 2006; Kiilerich et al. 2007b, 2011; McCormick et al. 2008). Teleosts do not produce aldosterone but another mineralocorticoid, 11-deoxycorticosterone (DOC) (Jiang et al. 1998), that interacts with fish mineralocorticoid receptor (MR) and might be involved in osmoregulatory processes in some species (McCormick et al. 2008; Takahashi and Sakamoto 2013). Both GRs and MR are found in osmoregulatory organs (Greenwood et al. 2003; Takahashi and Sakamoto 2013) and their role in osmoregulation has been demonstrated several times in fish species (Sloman et al. 2001; Scott et al. 2005a; Kiilerich et al. 2011). Cortisol as well as GR1 and GR2 are involved in ionocyte differentiation of medaka *Oryzias latipes* embryos (Trayer et al. 2013).

GR1, GR2 and MR mRNA levels have been measured in sea bass larvae during ontogeny (Pavlidis et al. 2011; Tsalafouta et al. 2014) and in the brain of adults (Sadoul et al. 2018). The expression patterns remain to be characterised in osmoregulatory sites of sea bass to determine if

the expression of these receptors varies following freshwater exposure.

The main objective of this study was to investigate the intraspecific differences in freshwater tolerance in juvenile sea bass. We studied inter-individual differences through the investigation of (i) morphological traits by identifying fish with a darker colour; (ii) behavioural traits by comparing swimming speed and distance; (iii) physiological parameters by analysing blood parameters (osmolality, Na^+ and Cl^- concentrations) as well as (iv) transcript levels of selected genes involved in osmoregulation and the stress response. The capacity to tolerate FW was compared following single or repeated FW exposures to determine if successive freshwater challenges differently affect sea bass.

Materials and methods

Experimental design

The experiments were conducted according to the guidelines of the European Union (directive 86/609) and of the French law (decree 87/848) regulating animal experimentation. The experimental design has been approved by the French legal requirement concerning welfare of experimental animals (APAFIS permit no. 9045-201701068219555).

European sea bass *Dicentrarchus labrax* (Linnaeus 1758) juveniles were obtained by crossing factorially unrelated wild native West Mediterranean breeders (40 males and 23 females) to obtain a large genetic diversity. They were reared at Ifremer Station at Palavas-les-flots (Hérault, France) in recirculating 20 °C SW under a natural photoperiod. FW acclimation procedure was the same for the two experiments performed in this study (see below). Juvenile sea bass were previously maintained in SW (osmolality: 1208 mOsm kg^{-1}), in which dechlorinated tap FW (osmolality: 8 mOsm kg^{-1}) was added to get 15 ppt brackish water (BW; osmolality: 475 mOsm kg^{-1}). Following 24 h acclimation at 15 ppt, fish were slightly anesthetised (benzocaine 25 ppm), and transferred to dechlorinated tap FW (Fig. 1). The container size and type were adapted to the size/density of fish and were different according to the experiment as indicated below. Pellet food (Le Gouessant, France) was given to the fish daily, but the fish maintained in FW did not feed during the 2 weeks of FW challenge, whereas SW fish did.

Experiment 1: characterisation of freshwater-intolerant and -tolerant fish

Eight-month-old juvenile sea bass ($N = 300$, 13.59 ± 0.12 cm, 32.19 ± 2.62 g) maintained in SW were transferred in FW as described above. They were divided into three 250 L raceways containing FW. Fish were observed to detect atypical behaviours (i.e., erratic swimming, isolation from the shoal associated with low reflexes and a low velocity) and dark colour/stronger pigmentation as previously described following a freshwater challenge (Nebel et al. 2005). Fish exhibiting these traits were collected and identified as the freshwater-intolerant phenotype (FW_i). Intolerant fish were identified from 1 to 14 days in FW with a clear peak between 24 and 48 h post-transfer. Sampling was done only at day 14. Fish that did not show atypical behaviours as described above were identified as freshwater tolerant (FW_t). To minimise the risk of identifying FW_i as FW_t, both phenotypes were sampled after 2 weeks in FW. Fish maintained in SW

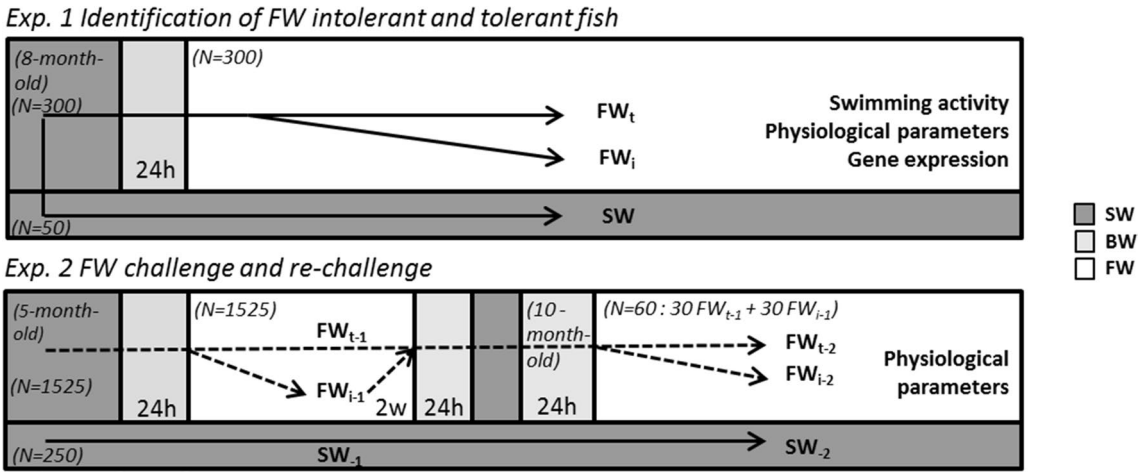

Fig. 1 Experimental design of the two experiments performed in fresh water (FW). In experiments 1 and 2, two phenotypes were distinguished in FW using behavioural traits: freshwater-intolerant fish (FW_i, FW_{i-1} and FW_{i-2}) and freshwater-tolerant fish (FW_t, FW_{t-1} and FW_{t-2}). Fish maintained in seawater (SW) were considered as controls (SW, SW_{-1} and SW_{-2}). *BW* brackish water, *h* hours, *w* weeks

($N = 50$) were used as controls and some of them ($N = 15$) were randomly sampled at the same time. Blood was sampled in anaesthetised fish (benzocaine 50 ppm) using a 1-mL syringe coated with heparin (Li-Heparin, Sigma-Aldrich, France) by puncturing blood directly in the caudal vessels for further analyses and fish were killed by a lethal dose of benzocaine (100 ppm). The first left gill arch and the posterior kidney (one-third of the kidney length, sampled in the most posterior part of the kidney) were immediately excised and immerged into RNA*later* (Qiagen, Valencia, CA). All samples were stored at − 80 °C until analysis.

Experiment 2: successive FW challenge and re-challenge

1525 juvenile sea bass (5 months old; 5.16 ± 0.03 cm, 1.37 ± 0.03 g) were transferred from SW to BW (24 h) and then to FW following the procedure described above. Fish behaviour was observed twice a day to identify tolerant and intolerant fish to FW using the behavioural features described above. Two weeks after FW transfer, fish were anaesthetised (benzocaine 25 ppm) and blood ($N = 25$/condition) was sampled by puncturing blood directly in the heart in FW-intolerant (FW_{i-1}), FW-tolerant (FW_{t-1}) and SW control fish (SW_{-1}). Fish were killed after using a lethal dose of anaesthetic (benzocaine 100 ppm).

The remaining FW_{i-1} fish (identified and then separated from the other fish between 1 and 14 days of FW acclimation) and FW_{t-1} were transferred to BW for 24 h, tagged using RFID tags (Dorset ID, Netherlands) and transferred to SW for recovery during a period of 5 months. FW_{t-1} and FW_{i-1} were kept in a 1500-L tank in a common garden with 250 SW control tagged fish (SW_{-1}) that have not been challenged to FW.

Following a 5-month recovery period, 30 animals of each phenotype, FW_{i-1} (10 months old; 15.2 ± 0.3 cm, 44.1 ± 3.3 g) and FW_{t-1} (15.2 ± 0.3 cm, 43.6 ± 2.3 g), were re-challenged to FW. Fish that showed FW intolerance during the second challenge were called FW_{i-2}, and FW-tolerant fish were called FW_{t-2}. Thirty fish from the same batch (10 months old) maintained in SW that never experienced FW were called SW_{-2} (Fig. 1). Two weeks after FW transfer, fish were anaesthetised (benzocaine 50 ppm) and blood ($N = 25$/condition) was sampled in the three groups as for the first FW challenge using the same procedure as for Experiment 1.

Swimming activity

Swimming activity was monitored in FW_i, FW_t and SW fish from experiment 1 after 14 days to confirm the visual clues observed in the shoal. Individual fish ($N = 9$–15) were maintained for 1 h in a 5 L black tank containing either FW (for FW_i and FW_t) or SW. Then, they were transferred carefully to a 30-L green tank filled with water at the same salinity, to measure their movement and their relative speed. A camera (Olympus) was placed above the tank and fish movement was recorded for 10 min. Then, two periods of 2 min were selected and analysed using Icy Software (icy.bioimageanalysis.org): from 30 s to 2.5 min and from 5.5 to 7.5 min. The movement and the relative speed of each fish were measured using the plugins Mice Profiler Tracker and Mice Profiler Video Label Maker (De Chaumont et al. 2012). Fish were killed after using a lethal dose of anaesthetic (benzocaine 100 ppm).

Blood osmolality and ion composition

Blood osmolality was measured on 20 µL of blood with a micro-osmometer (Advanced 3300). Chloride levels were measured in duplicates immediately following blood puncture using 10 µL of blood and a chloride titrator (AMINCO, Maryland, USA). Sodium levels were quantified in duplicates (or triplicates when enough blood was available) by flame photometry (Sherwood, Cambridge, UK) using 1 µL of blood. These protocols have already been used previously in Masroor et al. (2018).

Gene expression

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from gills and posterior kidney using Nucleospin® RNA (Macherey–Nagel, Germany). Quantity and purity of extracted total RNA were verified by measuring the A260/A280 ratio using the NanoDrop™ One/One^C Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was generated from 1 µg of RNA using the qScript™ cDNA SuperMix (Quanta Biosciences™) providing all necessary components for first-strand synthesis: buffer, oligo(dT) primers, random primers and qScript reverse transcriptase. RNA extraction and reverse transcription were carried out according to the manufacturer's instructions.

Quantification of gene expression levels

Primers used in this study are indicated in Table 1. Quantitative real-time (qRT) PCR analysis was performed by a LightCycler®480 Real-Time PCR System (Roche, Mannheim, Baden-Württemberg, Germany) using 384-well plates filled with an Echo®525 liquid handling system (Labcyte Inc., San Jose, CA, USA). Each well contained 0.75 µL of LightCycler-FastStart DNA Master SYBR-Green I™ Mix (Roche, Manheim, Germany), 0.037 µL of each primer (forward and reverse primers at 0.2 µM final concentration), 0.21 µL of ultrapure water and 0.5 µL of diluted cDNA.

Table 1 Primer sequences and efficiencies used for gene expression analysis

Target gene	Primer name	GenBank accession numbers	Sequence (from 5′ to 3′)	Efficiency	References
nka α1a	NKA-α1a_F	KP400258.1	CCTCAGATGGCAAGGAGAAG	2 (gills)	Blondeau-Bidet et al. (2016)
	NKA-α1a_R		CCCTGCTGAGATCGGTTCC	2 (posterior kidney)	
mr	MR_F	JF824641.1	GTTCCACAAAGAGCCCCAAG	2 (gills)	Tsalafouta et al. (2014)
	MR_R		AGGAGGACTGGTGGTTGATG	2 (posterior kidney)	
gr1	GR1_F	AY549305.1	GAGATTTGGCAAGACCTTGACC	1.7 (gills)	Pavlidis et al. (2011)
	GR1_R		ACCACACCAGGCGTACTGA	1.6 (posterior kidney)	
gr2	GR2_F	AY619996.1	GACGCAGACCTCCACTACATTC	1.8 (gills)	Pavlidis et al. (2011)
	GR2_R		GCCGTTCATACTCTCAACCAC	2.1 (posterior kidney)	
ef1α	EF1α_F	AJ866727.1	GGCTGGTATCTCTAAGAACG	1.9 (gills)	Nebel et al. (2005)
	EF1α_R		CCTCCAGCATGTTGTCTCC	1.9 (posterior kidney)	

The dilution of the samples was determined according to the standard curves generated for each primer pair. The qRT-PCR conditions were the same as in Blondeau-Bidet et al. (2019). The reference gene *ef1α* was chosen according to previous studies performed on sea bass challenged to FW (Nebel et al. 2005; Mitter et al. 2009; Blondeau-Bidet et al. 2016). For each organ and gene, efficiency was determined and ranged from 1.6 to 2. Relative expression of the target gene was performed using the comparative Ct method (threshold cycle number) according to Pfaffl (2001). Each sample was analysed in triplicate and ultrapure water was used as a negative control template (NTC).

Statistical analysis

Normality and homoscedasticity were verified using the D'Agostino-Pearson and Bartlett tests, respectively. If the data fit with these conditions, one-way analysis of variance was performed followed by a Tukey's post hoc test. Conversely, if the normality and homogeneity of variances were not verified, Kruskal–Wallis test was performed followed by a Dunn's test. All experimental values are reported as means ± s.e.m. All statistical differences were accepted at $P < 0.05$ and analyses were performed using GraphPad Prism (version 6, GraphPad Software Incorporated, La Jolla, CA 268, USA).

Results

Swimming activity

Intolerant fish exhibited erratic swimming, whereas tolerant fish were swimming in the shoal. Mean speed (cm s^{-1}) and distance (cm) travelled were highly correlated (Spearman test, $P < 0.0001$, Fig. 1S). Both parameters exhibited the same tendency in animals that shared the same phenotype. A significantly lower value was measured in FW_t and in FW_i compared to SW regarding mean speed (Dunn's test, $P = 0.0375$ and $P = 0.0205$, Fig. 2). Mean distance travelled was significantly lower in FW_t and in FW_i compared to SW (Dunn's test, $P = 0.0087$ and $P = 0.0226$). Interestingly, FW_t fish showed a tendency to an increased swimming activity compared to FW_i but this was not statistically significant, due to a higher inter-individual variability in FW_t.

Blood parameters

Experiment 1: single FW challenge

Blood osmolality was significantly higher in SW (373.7 ± 3.6 mOsm kg^{-1}) compared to both FW groups (FW_t: 268 ± 3.6 mOsm kg^{-1} and FW_i: 213.6 ± 6.6 mOsm kg^{-1}; Dunn's test, $P = 0.0027$ and $P < 0.0001$) (Fig. 3a). Blood osmolality was also significantly different between FW_t

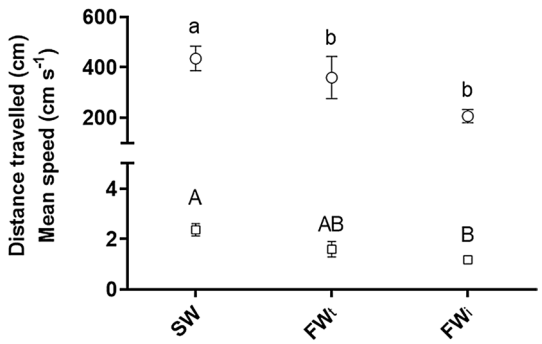

Fig. 2 Mean distance travelled (○, cm) and mean speed (□, cm s^{-1}) measured in 8-month-old sea bass acclimated to seawater (SW) and following freshwater (FW) challenge (means ± s.e.m). Data were obtained from experiment 1. FW_i: intolerant fish to FW, FW_t: tolerant fish to FW. Different letters denote significant differences between phenotypes (Kruskal–Wallis followed by Dunn's test, $P < 0.05$, $N = 9–14$)

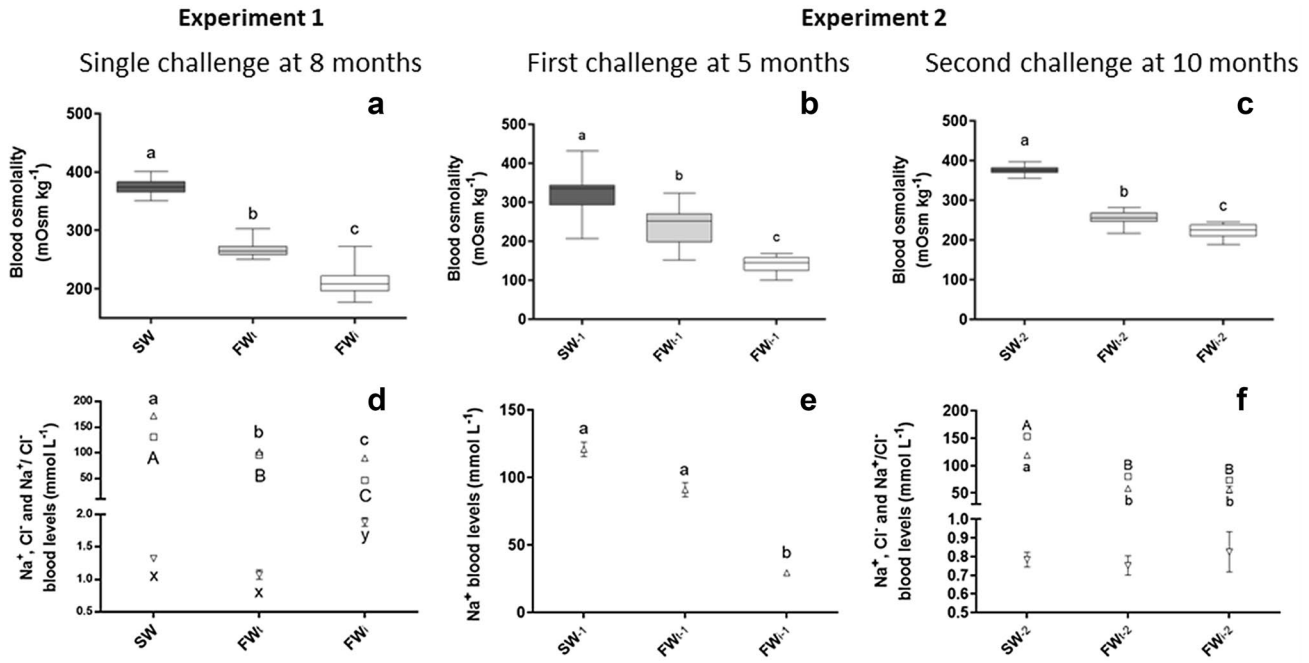

Fig. 3 Blood parameters related to each experiment. **a**, **b**, **c** Blood osmolality (mOsm kg^{-1}); **d**, **e**, **f** blood sodium levels (△, mmol L^{-1}), blood chloride levels (□, mmol L^{-1}) and sodium–chloride ratio (▽). $SW_{-1,-2}$: control fish in SW, $FW_{i,i-1,i-2}$: intolerant fish to FW, $FW_{t,t-1,t-2}$: tolerant fish to FW. Different letters denote significant differences between groups. $N = 9–32$

and FW_i ($P = 0.0205$), with FW_i exhibiting significantly lower blood osmolality compared to FW_t and a higher inter-individual variability. Blood sodium levels significantly decreased in FW compared to SW, respectively, by 41% in FW_t and by 48% in FW_i (Dunn's test, $P = 0.0018$ and $P < 0.0001$; Fig. 3d). Blood chloride levels were significantly different between each group (Kruskal–Wallis test, $P < 0.0001$; Fig. 3d) and decreased by 27% for FW_t and by 65% for FW_i compared to SW. The sodium–chloride ratio (Fig. 3d) did not differ between SW and FW_t but was significantly increased in FW_i compared to SW and FW_t (Dunn's test, $P < 0.01$ and $P < 0.0001$, respectively).

Experiment 2: successive FW challenges

Blood osmolality was significantly different between each group following both successive FW challenges (Kruskal–Wallis test, $P < 0.0001$, Fig. 3b, c). Regarding sodium levels, a significant difference was observed between FW-intolerant fish (FW_{i-1}, FW_{i-2}) and SW fish (SW_{-1}, SW_{-2}, respectively) (Tukey's test, $P < 0.0001$, Fig. 3e, f). A significant difference between freshwater phenotypes regarding blood sodium levels was observed only in fish that faced FW for the first time (Fig. 3e) (Tukey's test, FW_{i-1} vs FW_{t-1}, $P < 0.0001$ and FW_{i-2} vs FW_{t-2}, $P = 0.9495$). Following the second FW challenge, chloride levels were significantly lower in FW (FW_{i-2} and FW_{t-2}) than in SW fish (SW_{-2}) (Tukey's test, $P < 0.0001$, Fig. 3f), but no significant difference was observed between FW phenotypes ($P = 0.4395$). High inter-individual variability was observed in FW_{i-2} regarding blood Na^+/Cl^- ratio, but no significant difference was found between the three groups (Fig. 3f). Chloride levels could not be measured following the first FW challenge (in FW_{i-1}, FW_{t-1}), because insufficient blood volume was collected due to the small size of the fish.

Relative gene expression levels in gills and posterior kidney

In gills, significantly higher *nka α1a* expression levels were measured in FW (FW_t and FW_i) compared to SW (Tukey test, $P = 0.0437$ and $P = 0.0142$; Fig. 4a). No significant differences were measured between FW_t and FW_i. In the posterior kidney, a significantly lower expression of *nka α1a* was measured in FW_i compared to SW and FW_t (Dunn test, $P = 0.0021$ and $P = 0.0484$; Fig. 4b).

Concerning corticoid receptor expression levels, *gr1* was the most expressed gene compared to *mr* in the gills and compared to *gr2* and *mr* in the posterior kidney, with a higher expression of *mr* compared to *gr2* in this latter organ (Fig. 5). In gills, a significantly lower expression of glucocorticoid receptor 1 (*gr1*) (Fig. 5a) was measured in FW_i compared to SW (Dunn test, $P = 0.0480$), without any significant differences between FW phenotypes. The transcript levels of glucocorticoid receptor 2 (*gr2*) was below the limit of reliable quantification. A significantly higher

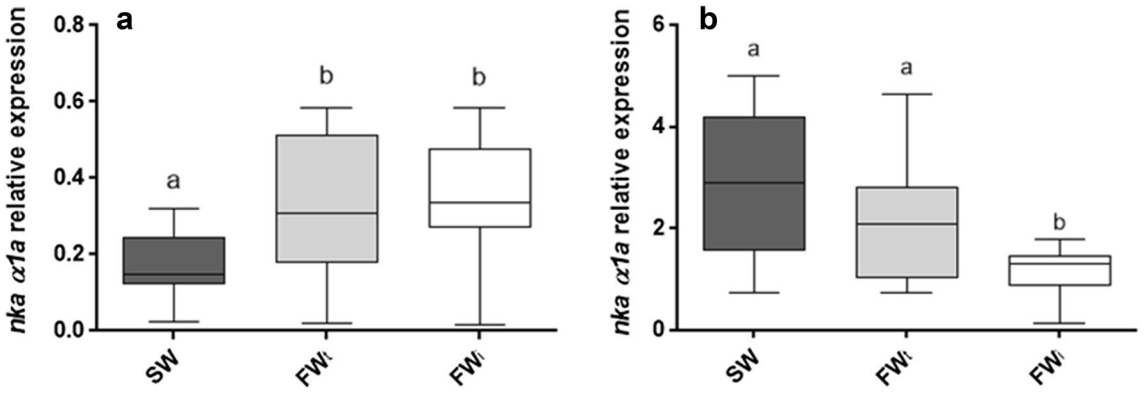

Fig. 4 Relative expression of *nka α1a* in gills (**a**) and posterior kidney (**b**), in seawater (SW) or after 2 weeks in fresh water (FW). The expression has been normalized according to the expression of the elongation factor *ef1α*. Data were obtained from experiment 1. Different letters denote significant differences between groups (one-way Anova followed by Tukey's test, $P<0.05$, means ± s.e.m, $N=6$–16). SW control fish, FW_i intolerant fish to FW, FW_t tolerant fish to FW

expression of branchial mineralocorticoid receptor (*mr)* (Fig. 5d) was measured in FW_i compared to FW_t (Tukey test, $P=0.0303$). In the posterior kidney, both *gr1* and *gr2* expression levels were significantly lower in FW_i compared to SW (Dunn and Tukey tests, $P=0.0067$ and $P=0.0037$, Fig. 5b, c), with a significant difference measured between FW_t and FW_i for *gr1* (Dunn test, $P=0.0010$). *Mr* expression was significantly lower in FW_i compared to FW_t (Tukey test, $P=0.0334$, Fig. 5e).

Phenotype occurrence following repeated FW challenges

Following the first FW challenge in experiment 2, about 28% of the 5-month-old sea bass were identified as freshwater-intolerant fish (FW_{i-1}) after 2 weeks (Fig. 6a). As the same fish were re-challenged to FW after 5-month recovery in SW, both successive FW transfers could be compared for each individual (Fig. 6a, b). The percentage of FW-intolerant fish following the second FW challenge (FW_{i-2}) was about 31%. Within these 31%, only 18% were previously identified as intolerant fish ($FW_{i-2|\,i-1}$). FW_{i-2} fish also include 13% fish that were previously identified as tolerant to FW ($FW_{i-2|\,t-1}$).

Discussion

In the wild, sea bass undertake migrations towards transitional waters from the age of 2–3 months (Dufour et al. 2009; Pérez-Ruzafa et al. 2011). To better understand intraspecific variation in freshwater tolerance, we performed single or repeated FW challenges in juvenile sea bass. In particular, two successive FW challenges interrupted by a 5-month recovery in SW have been performed with the aim to better understand if FW tolerance is maintained during development or can be modified following a second challenge. The osmotic challenges performed in this study were gradual using a 24 h acclimation at intermediate salinity as recommended in this species to prevent long-term stress effects (Kokou et al. 2019). Although sea bass is considered as a euryhaline species (Cataudella et al. 1991; Varsamos et al. 2002), the FW challenge caused differential mortalities as previously shown (Dendrinos and Thorpe 1985; Allegrucci et al. 1994; Jensen et al. 1998; Nebel et al. 2005; Giffard-Mena et al. 2008). This highlights differential individual abilities to cope with low salinities in sea bass, which requires further understanding.

These and other studies suggested that this differential acclimation capacity might be a driver for differential habitat distribution (Guinand et al. 2015). Our study highlighted for the first time that the freshwater tolerance capacity (or incapacity) can change in the same individual over time in response to repeated freshwater challenges. Indeed, the lability of the freshwater tolerance suggests that sea bass might enter unstable habitats like lagoons or estuaries in spring and display differential mortalities upon low-salinity exposure. This may also suggest that, as shown in amphihaline species like salmons (McCormick 2013), there may be an optimal physiological window (metabolic reserves, stress level, circulating hormones levels) that would allow sea bass to handle strong salinity variations.

Identification of freshwater-intolerant fish

FW-intolerant fish were characterised using a suite of sublethal traits, such as darker colour, erratic and slow swimming, as well as isolation from the shoal. These characteristics were previously described in sea bass (Nebel et al. 2005; Giffard-Mena et al. 2008) and may be the consequence of several specific metabolic and physiological disorders.

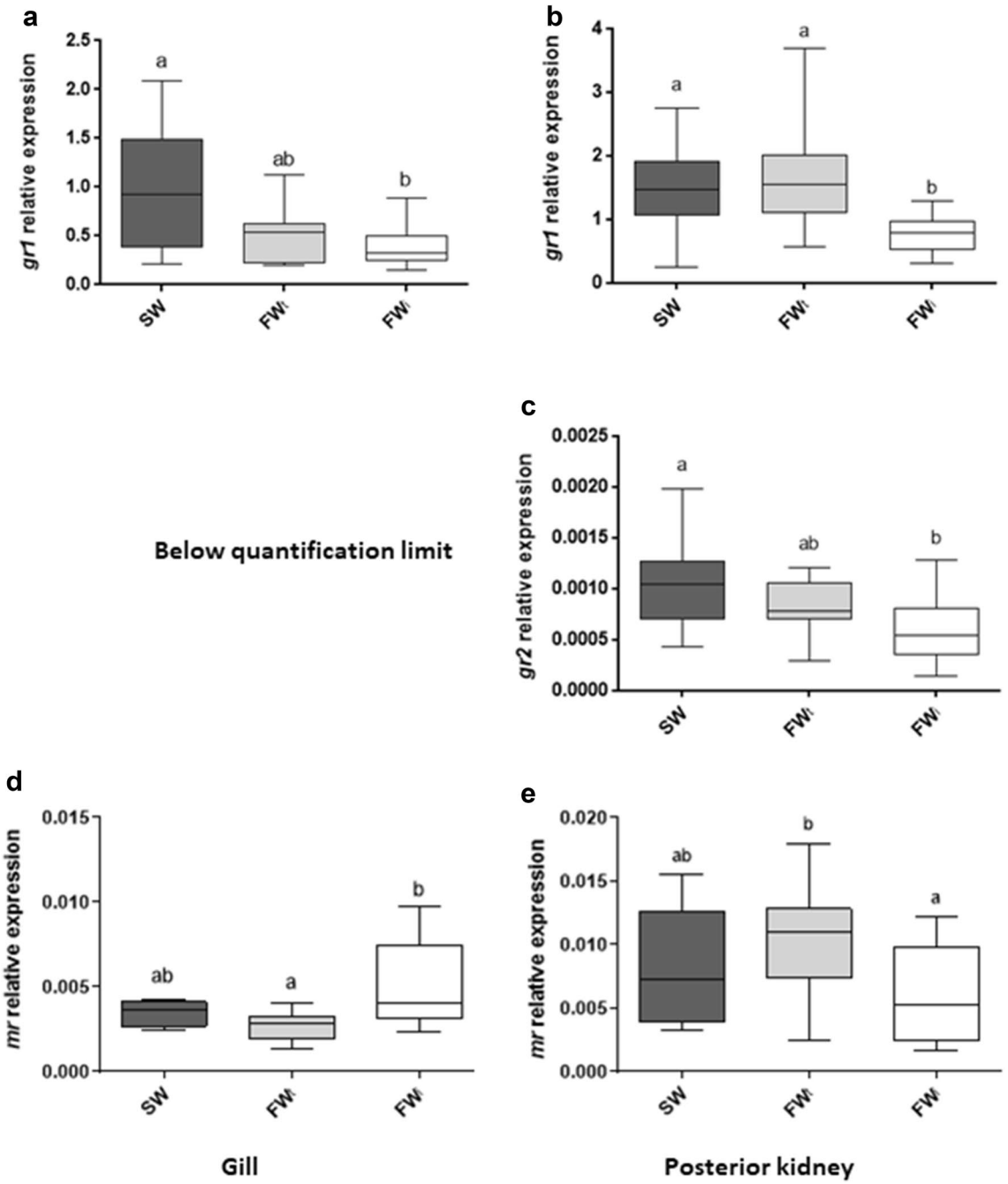

Fig. 5 Relative expression of *gr1* (**a**, **b**), *gr2* (**c**) and *mr* (**d**, **e**) in gills and posterior kidney in seawater (SW) or after 2 weeks in fresh water (FW). The expression has been normalized according to the expression of the elongation factor *ef1α*. Data were obtained from experiment 1. Different letters denote significant differences between groups (Kruskal–Wallis followed by Dunn's test, $P < 0.05$, means ± s.e.m, $N = 11–16$). SW control fish in SW, FW_i intolerant fish to FW, FW_t tolerant fish to FW

The sea bass skin colour can vary from light grey to dark grey depending on the colour of the surrounding environment and the stress condition (Kesbiç et al. 2016; Costa et al. 2017). According to several authors (Kittilsen et al. 2009; Backström et al. 2014; Castanheira et al. 2016), skin pigmentation pattern could be informative of the stress level. It is known that skin pigmentation results from interactions between pigment genes (i.e., melanocortin receptor 1 and 2: MC1R and MC2R) and the hypothalamic–pituitary–interrenal axis through corticoids such as cortisol (Mommsen et al. 1999; Barton 2002). This may explain how pigmentation profiles are correlated with cortisol stress response. According to the literature (Pottinger and Carrick 2001; Khan et al. 2016), low-responsive (LR) rainbow trouts are

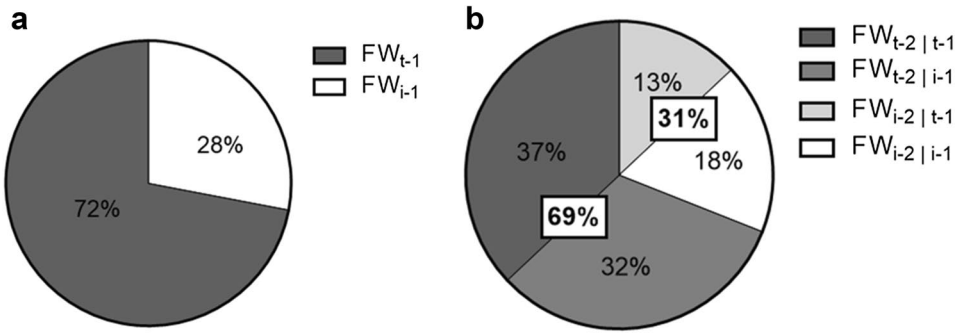

Fig. 6 Percentage of occurrence of each phenotype observed following the first FW challenge (**a**, $N = 1525$) and in re-challenged fish (**b**, $N = 60$, 30 per phenotype selected from the first challenge (**a**)). $FW_{y-2|x-1}$ correspond to the phenotype y observed in the second challenge considering the phenotype x observed at the first FW challenge

less responsive to adrenocorticotropic hormone released under stress conditions, produced less cortisol, triggering less expression of agouti signalling protein (ASIP), an antagonist of MC1R, causing more MC1R signalling and therefore more skin pigmentation. Such differing cortisol profiles (i.e., fish displaying a high (HR) or low (LR) cortisol response to stress) have been evidenced in sea bass (Samaras et al. 2016; Samaras and Pavlidis 2018) and should be further explored following FW exposure as a stress factor. Plasma cortisol measurement would undoubtedly be very informative to further understand the link between FW tolerance, skin pigmentation and stress. Compared to other teleost species, the European sea bass is characterised by extremely high resting and post-stress cortisol levels (Fanouraki et al. 2011). Therefore, cortisol analysis requires specific blood sampling procedures to avoid cortisol peaks due to sampling stress, which could not be achieved in this study. The analysis of mRNA levels of corticoid receptors (GRs, MR) provided complementary information related to cortisol regulation pathways that will be further developed in the discussion below. In this study, the darker colour was an efficient external/morphological indicator to detect the intolerant phenotype, but it has to be outlined that some intolerant fish did not exhibit this pattern. It was not possible to determine the proportion of fish showing accentuated pigmentation, because about half of intolerant fish could not be observed before they died.

Measurements of swimming velocity in individual fish have shown that intolerant fish tend to be slower than tolerant fish. Unlike tolerant fish, intolerant fish exhibit erratic swimming and did not swim in the shoal. Intolerant fish were probably isolated from the shoal as a consequence of their reduced speed (Fig. 2), but also as a consequence of the apparent delay they had to follow the shoal during a change of direction. These general observations might be linked to neuronal disturbances as shown by Nilsson et al. (2012). In this study, blood chloride levels were decreased in FW-intolerant compared to FW-tolerant fish, but this observation was not made consistently over the study (shown in experiment 1 performed on 8-month-old fishes, Fig. 3d, FW_t vs FW_i). A possible chloride loss could be the cause of decreased chloride levels and may indicate leakiness of the gill epithelium, e.g., less tight junction-related proteins might be expressed (Chasiotis et al. 2012; Bui and Kelly 2014; Bossus et al. 2015). This could also be linked to decreased chloride uptake at the gill and/or renal levels. As Cl^- uptake is tightly linked to HCO_3^- excretion, we can hypothesise that decreased blood Cl^- levels also affect HCO_3^- levels (Brauner and Baker 2009). Since the most common inhibitory neurotransmitter in vertebrates, the $GABA_A$, is sensitive to Cl^-/HCO_3^- gradients, fish behaviour may be directly affected by the Cl^- imbalance we have measured (Nilsson et al. 2012; Lai et al. 2016). More investigations are needed in sea bass to show a potential link between FW intolerance, alteration of swimming capacity and potential neural disturbances. It has to be noted that decreased swimming activity might also be linked to altered metabolism and differential energy allocation that should be further investigated in intolerant vs tolerant fish to FW.

Differential osmoregulatory traits in FW

Two groups were distinguished using the above-mentioned sublethal traits: freshwater-intolerant (FW_i, FW_{i-1}, FW_{i-2}) and freshwater-tolerant (FW_t, FW_{t-1}, FW_{t-2}) fish. After a period of 2 weeks in FW, the blood osmolality decreased to around 270 mOsm kg^{-1} in FW_t, 240 mOsm kg^{-1} in FW_{t-1} and 250 mOsm kg^{-1} in FW_{t-2}, which represents a decrease of 28, 26 and 33% compared to respective SW controls (Fig. 3a–c). 12% lower blood osmolalities in FW-tolerant compared to SW sea bass have previously been described by Nebel et al. (2005), probably due to a more gradual transfer over a period of 3 weeks instead of 24 h as chosen in this study. Interestingly, FW-intolerant fish of this latter study exhibited a 32% lower blood osmolality than FW-tolerant fish. This is consistent with the results of this study where FW-intolerant fish consistently exhibit a lower blood osmolality than FW-tolerant fish with a range going from 10% to 40% depending on the experiment. The strongest blood osmolality difference between FW phenotypes was recorded in younger fish analysed (5 months old, 40% lower in FW_{i-1}), suggesting that the capacity to regulate blood

osmolality after abrupt FW exposure is variable according to age and more efficient in older juveniles. However, the same proportion of FW-intolerant fish was found (around 28–30%) whatever the age. Also, we observed that the decrease in blood osmolality and Na^+ level were clearly reduced after FW re-challenge, suggesting more efficient acclimation mechanisms after re-challenge. But again, the proportion of intolerant fish remained around 31% after re-challenge. Different acclimation responses might be linked to developmental plasticity with fish having more efficient physiological capacities at specific developmental stages (Crozier and Hutchings 2014). Differential phenotypic flexibility, i.e., the ability within individuals to exhibit reversible changes in physiology, metabolism, behaviour, could also explain different responses observed in sea bass juveniles following FW challenges (Forsman 2015). Beside age differences, 10-month-old fish in our study have been challenged to FW twice which is not the case for 8-month-old fish. Salinity-induced phenotypic plasticity should be analysed more deeply using other traits (epigenetic markers for example, Trautner et al. 2017) to have a better understanding of the differential acclimation response in sea bass. Differential methylation patterns have been reported among individuals within a species and might be a valuable trait to be compared in sea bass phenotypes (Metzger and Schulte 2017). Concerning ions profiles, it appeared that differences in blood Na^+, Cl^- and/or Na^+/Cl^- ratio were detected between FW-intolerant and -tolerant fish. A slight increase in the Na^+/Cl^- ratio after FW transfer has been shown in previous studies on *D. labrax* (Jensen et al. 1998; Masroor et al. 2018). In our study, a significant higher Na^+/Cl^- ratio was observed in FW_i (Experiment 1, Fig. 3d), and a slightly higher and highly variable ratio in FW_{i-2} (Experiment 2, Fig. 3f) compared to tolerant fish. A possible explanation of altered Na^+/Cl^- ratio can be a more important efflux of chloride in intolerant fish to FW as reported in *F. heteroclitus* exposed to FW (Scott et al. 2004a, b). These authors also suggest that intraspecific variation in freshwater tolerance can be a result of abnormal balance between sodium and chloride that could lead to a compensatory increase in blood HCO_3^- resulting in blood alkalosis. As for the strong ion difference (SID) (Goss et al. 1992), the Na^+/Cl^- ratio gives an idea of the acid–base status of fish. Since this ratio is higher in FW_i than in FW_t (or SID of FW_t is lower than SID of FW_i), we can hypothesise that FW_i are in a more alkaline state than FW_t. Plasma pH measurements could help to confirm this hypothesis. Similar results were obtained in horned sculpin *Myoxocephalus octodecimspinosus* where chloride loss was detected in intolerant fish following long-term freshwater exposure (Claiborne et al. 1994). Some marine fish able to cope with low-salinity environments balance their chloride blood levels by minimising Cl^- efflux and by meeting chloride demands through the diet (Wood and Laurent 2003). This seems not to be the case in sea bass during our experiment, as fish did not feed throughout the 2 weeks of FW challenge, certainly because of the salinity/or operatory stress. However, even if sea bass tolerate fasting for long periods (Caruso et al. 2011; McKenzie et al. 2014), starvation might be a factor to take into account, since food intake can contribute to the maintenance of the hydromineral balance. Fasting can thus be considered as an additional challenge that might exacerbate the physiological limits of each individual in this study, and we have not measured any significant differences in size and weight between FW-tolerant and FW-intolerant fish in all experiments suggesting that there is no correlation between fish size and ion profiles (Table 1S). The lower blood osmolality and differing Na^+ or Cl^- levels observed in FW-intolerant fish might also be partially due to a failure in the renal ion reabsorption as suggested previously by Nebel et al. (2005)In this previous study, renal Na^+/K^+-ATPase activity was shown to be significantly lower in FW-intolerant compared to FW-tolerant fish. Our results clearly demonstrate a significant lower *nka α1a* expression in FW_i compared to SW and to FW_t (Fig. 4b), which would be consistent with a lower renal NKA activity. Nebel et al. (2005) also reported a lower renal tubule density in FW_i compared to both SW and FW_t. These results suggest that active ion reabsorption in urinary tubules might be altered in FW_i due to a lack in NKA activation at the transcription and potentially protein levels. In FW_t, an increased branchial *nka α1a* expression and NKA activity (Nebel et al. 2005) seem to be essential for maintaining ion balance (Scott et al. 2004a, b; Lin et al. 2004; Marshall and Grosell 2006; Nilsson 2007; Evans and Clairborne 2009; Tipsmark et al. 2011). No significant difference was found in gill *nka α1a* expression between FW_t and FW_i, suggesting that ion uptake mechanisms at gill level might not be affected in FW_i. This is not consistent with previous study that show that branchial NKA activity was significantly higher in FW intolerant compared to FW tolerant and SW controls (Nebel et al. 2005). These discrepancies may be explained either by a differential timing between transcript levels and NKA activity responses or other post-transcriptional regulations (Zhao et al.2016). Differences in NKA activity could also be due to changes in the transcription level of other *nka* paralogs (Blondeau-Bidet et al. 2016).

Osmoregulation and corticoid receptors

Glucocorticoid receptors GR1 and GR2 are implicated in ionocyte differentiation at the gill level (Cruz et al. 2013; Trayer et al. 2013), and they are also necessary with MR to trigger modifications in *nka* expression through the cortisol signalling pathway to promote successful salinity acclimations (Kiilerich et al. 2007a; McCormick et al. 2008).

In this study, we demonstrated that the differential tolerance to FW is correlated to differential corticoid receptors

transcriptional regulation. FW_i have a significant lower expression of *gr1* compared to SW in gill and posterior kidney. *Gr1* expression was significantly lower in FW_i compared to FW_t in the posterior kidney (Fig. 5a, b). Moreover in the posterior kidney, *gr2* expression levels were significantly lower in FW_i compared to SW. *Gr2* expression was slightly but not significantly decreased in FW_i compared to FW_t (Fig. 5c). These results are concordant with the literature in FW-acclimated steelhead trout (Yada et al. 2008), in which a lower expression of branchial *gr1* and *gr2* was measured compared to SW-acclimated fish. Interestingly, several studies highlighted a positive control of GRs on *nka α1a* expression. GR inhibitors inhibit *nka α1a* expression levels in Atlantic salmon *Salmo salar* (McCormick et al. 2008; Kiilerich et al. 2011). In the posterior kidney, our results are concordant with this hypothesis, since a lower expression of *gr1, gr2* was found in FW_i compared to SW and FW_t (Fig. 5b, c) correlated to lower expression levels of *nka α1a*. FW-intolerant sea bass identified in this study exhibit a significantly higher mRNA expression of branchial *mr* than FW-tolerant fish (Fig. 5d), but the opposite pattern was recorded in the posterior kidney (Fig. 5e). A significant increase of *mr* has already been demonstrated following FW exposure in rainbow trout gills and kidney (Kiilerich et al. 2007b, 2011). Sloman et al. (2001) suggested a putative role of MR in gill ionocyte proliferation following a hypo-osmotic challenge. Therefore, a higher *mr* mRNA expression may indicate an increased ionocyte differentiation in FW_i compared to FW_t. This is consistent with the observation made by Nebel et al. (2005) where ionocyte density was significantly higher in intolerant fish (by around 70%) supposedly as a compensatory response to low blood osmolality. In contrast, the lower expression of *mr* in the posterior kidney could be linked to the decrease in tubule density observed in FW-intolerant fish observed by Nebel et al. (2005). The localisation of MR transcripts or proteins in the posterior kidney would allow determine if and which renal epithelia are targeted.

FW tolerance following repeated FW challenges

Our results show that about 28% of fish were intolerant to FW at the age of 5 months, and that a relatively similar percentage is found at the age of 10 months following a second freshwater challenge (31%, Fig. 6a, b). These percentages are in agreement with previous studies performed in sea bass aiming at identifying freshwater-intolerant fish at various ages (2, 3 and 6 months) (Nebel et al. 2005; Giffard-Mena et al. 2008; Guinand et al. 2014). Although intolerant fish exhibit similar morphological and behavioural traits, the same fish does not necessarily remain FW intolerant throughout its life (Fig. 6b). Indeed, we found that 64% of the fish detected as FW_i after the first challenge were identified as tolerant after the re-challenge. Moreover, smaller differences between freshwater phenotypes were observed comparing their blood osmolality in the second challenge. This suggests a positive effect of the first FW challenge on the freshwater tolerance in those fish, maybe due to an increase in osmoprotective genes controlled by "osmolality/salinity-responsive enhancers" (Wang and Kültz 2017). Conversely, 26% of the FW_{t-1} lost their tolerance following a second FW challenge. Blood osmolality was about 10% lower in FW_{i-2} than in FW_{t-2} whereas after a first FW challenge we measured a 40% lower value (Fig. 3b, c). Concerning Cl^- and Na^+/Cl^- ratio, it appeared that there is no significant difference between intolerant and tolerant fish after a second FW challenge, whereas we found significant differences for fish after the first FW challenge (in animals where Cl^- levels could be determined) (Fig. 3d, f). This highlights that FW tolerance shows a certain lability over time and that there are different still unknown causes for FW intolerance. The same type of lability was observed in stress experiments. In gilthead sea bream exposed to a net-restraining stress test (Castanheira et al. 2016), coping style was measured both over time and at several life history stages and the authors showed different responses between successive experiments performed on the same individuals. According to these authors, different behavioural responses might be linked to physiological modifications during lifetime (Castanheira et al. 2016). However, not all "stress on stress" experiments show a positive effect of the early stress, such as chasing stress in the Senegalese sole *Solea senegalensis* (Conde-Sieira et al. 2018). Furthermore, in *Salmo salar,* the effect of rearing temperature, by modifying the diel thermal minima, does not increase acute thermal tolerance (Corey et al. 2017). In these examples, conditioning or repeated stress show habituation or a hypersensitive stress profile, respectively (Korte et al. 2007). In our study, the possible change of FW tolerance during repeated FW exposure might be at least in part linked to an intraspecific variability in the stress response profile, due to the key roles of corticoid receptors and cortisol in the maintenance of homeostasis. Finally, 45% of fish exhibit a different phenotype after re-challenge. The phenotype "tolerance to FW" seems to be a plastic phenotypic trait, not only related to the genotype and to age or ontogenesis. It rather seems linked to other unidentified factors that could possibly involve a combination of genetic and epigenetic factors that need to be analysed in further studies (Angers et al. 2010; Massicotte et al. 2011). In particular, the implication of epigenetic modifications (i.e., DNA methylation) has been shown following temperature acclimation in sea bass (Anastasiadi et al. 2017), and could be analysed here regarding the phenotypic variation observed following a salinity challenge, as mentioned above (Turner 2009; Hawes et al. 2018; Jeremias et al. 2018).

Conclusion

This study is the first to demonstrate an intraspecific variation in hypo-osmotic tolerance following single or repeated FW challenges in sea bass. We demonstrated that freshwater intolerance occurs at different ages in juvenile sea bass, in similar proportions, and with comparable disorders of hydromineral balance. Differences in the expression levels of corticoid receptors and osmoregulatory genes between freshwater phenotypes reinforce the hypothesis of insufficient ion reabsorption at the kidney level. Overall, this study suggests that the freshwater tolerance is a plastic trait, labile over time that involves phenotypic plasticity and/or epigenetic regulation.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. The experimental design has been approved by the French legal requirement concerning welfare of experimental animals (APAFIS Permit No. 9045-201701068219555).

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Résumé du Chapitre 2

Chez les téléostéens, la régulation de l'équilibre hydrominéral a un impact direct sur une multitude de fonctions physiologiques, de processus biochimiques, et peut influencer leur comportement, leur distribution ainsi que leur survie. Comme le loup européen *Dicentrarchus labrax* entreprend des migrations saisonnières de l'eau de mer vers les eaux saumâtres des lagunes et estuaires voire même en eau douce, cette étude porte sur leur capacité à tolérer l'eau douce. Elle vise à explorer davantage la variabilité intraspécifique dans les réponses physiologiques de l'acclimatation à l'eau douce. Pour cela, des juvéniles de *D. labrax* ont été transférés de l'eau de mer en eau douce à différents âges. Les phénotypes tolérants et intolérants à l'eau douce ont été discriminés en fonction de leurs caractéristiques comportementales et morphologiques : une nage erratique, une vitesse plus lente, une isolation par rapport au banc ainsi qu'une couleur parfois plus sombre. Environ 30% des loups exposés en eau douce ont été identifiés comme intolérants à l'eau douce aux différents âges. Les intolérants à l'eau douce sont toujours caractérisés par une osmolalité sanguine et une expression de la Na^+/K^+-ATPase α1a significativement plus faibles que celles des tolérants à l'eau douce dans le rein postérieur. Les régulations différentielles observées chez certains loups intolérants à l'eau douce dans les mécanismes de régulation des ions ont été ensuite confirmées par un déséquilibre dans le ratio sanguin Na^+/Cl^- indiquant une alcalose métabolique. Le lien entre stress et osmorégulation a été étudié via l'analyse de l'expression des récepteurs aux glucocorticoïdes et minéralocorticoïdes au niveau des branchies et du rein. Elle révèle des différences significatives entre les phénotypes en comparant les deux organes, suggérant des réponses différentielles dans les mécanismes de régulation hormonale de l'osmorégulation et une possible interaction avec l'axe hypothalamo-hypophysaire de réponse au stress. Cette étude démontre clairement une différence intraspécifique dans les réponses physiologiques des loups suivant une exposition à l'eau douce, notamment une capacité plus faible de réabsorption ionique au niveau du rein comme possible cause de l'intolérance à l'eau douce. En exposant une deuxième fois les loups à l'eau douce après 5 mois de récupération en eau de mer, nous mettons en évidence que le trait « intolérance à l'eau douce » n'est pas stable au cours du temps. En effet, le phénotype des individus n'est pas nécessairement le même que lors du premier challenge en eau douce. Pourtant, la proportion de phénotype intolérant à l'eau douce est la même : 30% d'intolérants. Cela suggère des mécanismes de régulations aléatoires ou épigénétiques.

Chapitre III :

Caractérisation transcriptionnelle de la variabilité intraspécifique dans la tolérance à l'eau douce chez le loup méditerranéen *Dicentrarchus labrax*

Crédits : Thibaut L'Honoré

Prologue du Chapitre 3

Après avoir mieux caractérisé les phénotypes extrêmes observés en eau douce chez le loup, nous avons voulu explorer plus en détails la réponse hypo-osmotique de ces individus au niveau de l'expression de certains gènes impliqués dans les processus de régulation ionique. Pour cela nous avons mesuré l'expression de transporteurs ioniques impliqués non seulement dans l'osmorégulation mais aussi dans la réponse acido-basique, ainsi que l'expression des récepteurs à la prolactine, principale hormone de l'acclimatation en milieu hypo-osmotique.

Ce chapitre correspond à un article soumis dans Gene « Inter-individual variability in freshwater tolerance is related to transcript level differences in gill and posterior kidney of European sea bass» L'Honoré Thibaut, Farcy Emilie, Blondeau-Bidet Eva, Lorin-Nebel Catherine. Il a également fait l'objet d'une présentation orale que j'ai effectuée en juillet 2019 à Séville au congrès de la SEB (Society of Experimental Biology).

Inter-individual variability in freshwater tolerance is related to transcript level differences in gill and posterior kidney of European sea bass

L'Honoré Thibaut, Farcy Emilie, Blondeau-Bidet Eva, Lorin-Nebel Catherine*

Univ Montpellier, CNRS, IFREMER, IRD, UM, MARBEC, Montpellier, France

***Corresponding author:**

Catherine Lorin-Nebel

Univ Montpellier, CNRS, IFREMER, IRD, UM, MARBEC

Place E. Bataillon,

34095 Montpellier cedex 05, France

Tel: +33 4 67 14 93 91

catherine.lorin@umontpellier.fr

Key words

Intraspecific variation, osmoregulation, freshwater acclimation, ion transporters, SLC26A6, NKCC, PRLR

Abstract

Acclimation to low salinities is a vital physiological challenge for euryhaline fish as the European sea bass *Dicentrarchus labrax*. This species undertakes seasonal migrations towards lagoons and estuaries where a wide range of salinity variations occur along the year. We have previously reported intraspecific differences in freshwater tolerance, with an average 30% mortality rate. In this study, we bring new evidence of mechanisms underlying freshwater tolerance in sea bass at gill and kidney levels. In fresh water (FW), intraspecific differences in mRNA expression levels of several ion transporters and prolactin receptors were measured. We showed that the branchial Cl^-/HCO_3^- anion transporter (*slc26a6c*) was over-expressed in freshwater intolerant fish, probably as a compensatory response to low blood chloride levels and potential metabolic alkalosis. Moreover, prolactin receptor a (*prlra*) and Na^+/Cl^- cotransporter (*ncc1)* but not *ncc-2a* expression seemed to be slightly increased and highly variable between individuals in freshwater intolerant fish. In the posterior kidney, freshwater intolerant fish exhibited differential expression levels of *slc26* anion transporters and $Na^+/K^+/2Cl^-$ cotransporter 1b (*nkcc1b*). Lower expression levels of prolactin receptors (*prlra, prlrb*) were measured in posterior kidney which probably contributes to the failure in ion reuptake at the kidney level. Freshwater intolerance seems to be a consequence of renal failure of ion reabsorption, which is not sufficiently compensated at the branchial level.

Introduction

In transitional habitat such as lagoons, fish have to deal with a wide range of changing environmental parameters and therefore a high phenotypic plasticity is beneficial to cope with fluctuating environments. Salinity can rapidly drop in these habitats through freshwater (FW) supplies by rainfalls or rivers. Low-salinity environments can lead to differential distributions of marine species according to their acclimation capacity (Wong *et al.*, 1999; Pierce *et al.*, 2012). Differential habitat distribution has also been observed between individuals within the same species as in stickleback *Gasterosteus aculateus* and in mummichog, *Fundulus heteroclitus*, showing a differential capacity to regulate ions in fresh water (Scott *et al.*, 2004; McCairns & Bernatchez, 2009). To maintain hydromineral balance in FW environments, fish have to minimise diffusive ion losses and compensate them by actively (re)absorbing ions at the gill and posterior kidney levels (Hickman & Trump, 1969; Dantzler, 1996). The European sea bass, *Dicentrarchus labrax*, is known to undertake seasonal migrations in transitional habitats where salinity fluctuates (Kelley, 1988; Barnabé, 1989; Waldman, 1995). Previous studies highlighted intraspecific differences in FW tolerance in this species at different ages (Nebel *et al.*, 2005; Giffard-Mena *et al.*,

2008; L'Honoré *et al.*, 2019) with about 30% mortality following FW exposure. In these latter studies as for *F. heteroclitus* in Scott *et al.* (2004), authors concluded that the divergence in osmoregulatory capacities may result in different capacities to absorb and reabsorb Na^+ and Cl^- at gill and/or kidney levels. In Nebel *et al.* (2005), it has been suggested that the kidney might be responsible for the osmoregulatory failure detected in FW intolerant fish, linked to a low renal NKA activity and a lower kidney tubular density. In L'Honoré *et al.*, (2019), authors highlighted that intraspecific variation in FW tolerance of sea bass is supported by strong differences in *nka α1a* expression in the posterior kidney while no difference was measured at the gill level. Authors also showed differences in corticosteroid receptors mRNA levels (*gr1, gr2* and *mr*) with lower expression levels in FW intolerant fish compared to FW tolerant. This suggests an impaired hormonal and stress regulation between both freshwater-tolerance phenotypes. Pituitary prolactin (PRL) is a key hormone involved in FW acclimation by promoting the maintenance of the hydromineral balance (Hirano 1986; Manzon, 2002; Sakamoto & McCormick, 2006; Breves *et al.*, 2014; Bossus *et al.*, 2017). PRL interacts with PRLR to regulate Na^+ efflux, water permeability and the differentiation of ionocytes expressing NCC as shown in tilapia and zebrafish (Dharmamba *et al.*, 1967; Dharmamba & Maetz, 1972; Jason P. Breves *et al.*, 2010; Breves *et al.*, 2013). Prolactin receptors are cell surface receptors known to be expressed in osmoregulatory organs of many teleost species (Manzon, 2002). As for many fish species like *T. rubripes*, two paralogs of *prlr* were identified in the sea bass genome called *prlra* and *prlrb* (Lee *et al.*, 2006; Tine *et al.*, 2014). It remains to be determined if both *prlr* paralogs display the same expression pattern according to salinity and if mRNA levels differ between freshwater-tolerance phenotypes in European sea bass.

In fish, blood pH levels are salinity-dependent with lower blood pH at low salinity than in SW as it was demonstrated in European sea bass by Shrivastava *et al.* (2019). In 8 month-old sea bass, freshwater intolerance was characterised by a lower blood osmolality associated with an elevated Na^+/Cl^- ratio, indicating a metabolic alkalosis (L'Honoré *et al.*, 2019). To regulate a high blood pH, fish have to excrete bases as HCO_3^-, mainly associated with Cl^- uptake (Maetz & García Romeu, 1964; Goss & Wood, 1990, 1991; Tresguerres *et al.*, 2006). The apical anion transporters SLC26A6, involved in Cl^-/HCO_3^- and Cl^-/oxalate exchanges, are known to be widespread among species in osmoregulatory organs including the posterior kidney (Mount & Romero, 2004; Sardella & Brauner, 2007; Xie *et al.*, 2013; Knauf *et al.*, 2018) and gills (Perry *et al.*, 2009; Boyle *et al.*, 2015) and could be potential entry routes for HCO_3^- in intolerant sea bass to FW. In the gulf toadfish for example, *slc26a6* was highly expressed in kidney (Grosell *et al.*, 2009) to reabsorb Cl^- from the lumen to the blood. Among three different SLC26 anion transporters in zebrafish (SLC26A6, SLC26A3 and SLC26A4, called *za6*,

za3 and *za4*), *za6* seemed to be the most expressed in gills and was overexpressed when fish were transferred to water with low Cl^- or $NaHCO_3$ (Perry *et al.*, 2009). Moreover, gene knockdown of *slc26a6c* resulted in a reduction in Cl^- uptake in zebrafish larvae confirming the major role of this transporter in Cl^- uptake (Bayaa *et al.*, 2009; Perry *et al.*, 2009). Guh *et al.* (2015) localised SLC26 genes apically in gill ionocytes, called SLC26 cells, but to our knowledge nothing is known about its localisation in the fish kidney. In sea bass, the only record about SLC26A6 (most probably SLC26A6c) is very recent and shows a high mRNA expression in the anterior intestine to promote Cl^- transepithelial transport to the blood and HCO_3^- excretion (Alves *et al.*, 2019).

The basolateral Na^+/K^+-ATPase (NKA) (Hwang *et al.*, 2011; Kumai & Perry, 2012) is a key player in Na^+ and Cl^- uptake and its importance in FW acclimation has been demonstrated in many fish species (Hiroi *et al.*, 2008; Inokuchi *et al.*, 2008; Watanabe *et al.*, 2008; Hsu *et al.*, 2014; Bollinger *et al.*, 2016). *Nka α1a* transcriptional expression in the posterior kidney was previously shown to be related to FW tolerance in sea bass (L'Honoré *et al.*, 2019). One other key cotransporter known for Na^+ and Cl^- uptake in fish is the Na^+/Cl^- cotransporter NCC-2A or NCC-like (SLC12A10 or SLC12A10.2), that plays a crucial role in freshwater acclimation in many fish species including European sea bass (Inokuchi *et al.*, 2008; Wang *et al.*, 2009; Hwang *et al.*, 2011; Blondeau-Bidet *et al.*, 2019). SLC12A10 is localised apically in FW-type branchial ionocytes (*i.e.* NCC-type cells) in several species (Hiroi *et al.*, 2008; Inokuchi *et al.*, 2008; Guh *et al.*, 2015; Blondeau-Bidet *et al.*, 2019). It is highly expressed in FW compared to SW fish gills (Hiroi *et al.*, 2008; Inokuchi *et al.*, 2008; Wang *et al.*, 2009; Blondeau-Bidet *et al.*, 2019). In posterior kidney, *slc12a10.1* paralog has been shown to be highly expressed in zebrafish and in Mozambique tilapia gills compared to other organs (Hiroi *et al.*, 2008; Wang *et al.*, 2009). The Na^+/H^+ exchanger-3 (NHE3) is also localised in the apical membrane of another ionocyte subtype at the gill level (Inokuchi *et al.*, 2008; Watanabe *et al.*, 2008; Hwang *et al.*, 2011; Blondeau-Bidet *et al.*, 2019) and functionally coupled to several other ion transporters facilitating Na^+ uptake (Dymowska *et al.*, 2015). Anion exchanger 1 (AE1) is mainly known to play a role in bicarbonate transport to the blood and Cl^- secretion at the gill and kidney (mammal medullary collecting duct cells) levels (Barone *et al.*, 2004, Lee *et al.*, 2011). AE1 is thought to be functionally linked to apical V-H^+-ATPase (VHA) in order to complete acid secretion. At the gill level, AE1 is localised basolaterally in zebrafish HR cells (Lee *et al.*, 2011). Its role was investigated in medaka *Oryzias latipes* and in zebrafish gills (Lee *et al.*, 2011; Hsu *et al.*, 2014; Liu *et al.*, 2016). In both species, the two analysed paralogs (*ae1a* and *ae1b*) were expressed in gills with *ae1b* being over-expressed in a low-Na^+ environment. It has also been suggested that AE1 is involved in Cl^- absorption and HCO_3^- secretion (Evans *et al.*, 2005; Hwang & Lee, 2007; Hwang & Perry, 2010) but its localisation in basolateral membranes of ionocytes in

pufferfish *Tetraodon nigroviridis* and in milkfish *Chanos chanos* seems not in favour with this hypothesis (Tang & Lee, 2007; Tang *et al.*, 2011). In sea bass, no data is available on VHA and AE1 localization. VHA mRNA expression and protein activity measurements have shown the presence of VHA in sea bass gills in freshwater media (Sinha *et al.*, 2015; Blondeau-Bidet *et al*., 2019).

Regarding $Na^+/K^+/2Cl^-$ cotransporters (NKCC), three paralogs have been described in teleosts: basolateral NKCC1a and NKCC1b mainly expressed in the gills and dedicated to NaCl secretion, and the apical NKCC2 mainly expressed in the kidney and intestine and attributed to NaCl reabsorption (Haas & Forbush III, 2000). NKCC1 paralogs are expressed in SW-type ionocytes in numerous teleost species including the European sea bass (Lorin-Nebel *et al.*, 2006; Inokuchi *et al.*, 2008; Buhariwalla *et al.*, 2012; Breves *et al.*, 2014). NKCC2 was detected in tilapia and in *D. labrax* intestine (Hiroi *et al.*, 2008; Alves *et al.*, 2019), but there is no data available on its localisation in the gills and in the posterior kidney of *D. labrax*.

Sea bass exhibiting a freshwater intolerant phenotype face a severe hydromineral imbalance and we will analyse if this failure originates from a failure in ion uptake at the kidney and/or gill levels. The involvement of the gill in differential ion uptake capacity of sea bass will be analysed by comparing the expression profile of the main ion transporters in tolerant and intolerant fish to freshwater. Little information is available about ion transporter expression at the kidney level. This is a first tentative to identify key renal ion transporters in freshwater kidney and to detect intraspecific differences in the transcriptional profile between tolerant and intolerant fish to fresh water.

Materials and methods

1. Tissue sampling

European sea bass juveniles (N=350) were reared at Ifremer Station at Palavas-les-flots (Hérault, France) in recirculating SW (osmolality: 1208 mOsm kg^{-1}, Na^+: 515 mmol L^{-1}, Cl^-: 737 mmol L^{-1}) under a 12/12 hours light/dark photoperiod at 20°C. At the age of 8 months (13.59 ± 0.12 cm, 32.19 ± 2.62 g), 300 fish were then transferred to brackish water (BW; osmolality: 475 mOsm kg^{-1}) for 24h and then transferred to dechlorinated tap FW (osmolality: 8 mOsm kg^{-1}, Na^+: 2 mmol L^{-1}, Cl^-: 3.5 mmol L^{-1}) for two weeks. The 50 remaining fish were transferred from SW to SW as controls. Pellet food (Le Gouessant, France) was proposed to fish daily, but the fish did not feed during the two weeks of FW challenges.

Fish were sampled two weeks following FW challenge. FW intolerant fish were identified according to their behavior *e.g.* an atypical swimming (darker color sometimes associated with a lower velocity, isolation from the shoal) as described previously (Nebel *et al.*, 2005; L'Honoré *et al.*, 2019). Fish that did not show any of these traits were considered as FW tolerant fish. In this study we compared three groups: FW tolerant fish (FW_t, N=15), FW intolerant fish (FW_i, N=15), and SW control fish (SW, N=13). After the 2-week FW challenge, fish were killed by a lethal dose of benzocaine (at 100 ppm) and the first left gill arch and the posterior kidney (last posterior third of the excretory kidney) were excised and immerged into RNAlater (Qiagen, Mississauga, ON, Canada) for 24h at 4°C and then stored at -80°C for further analyses.

The experiments were conducted according to the guidelines of the European Union (directive 86/609) and of the French law (decree 87/848) regulating animal experimentation. The experimental design has been approved by the French legal requirement concerning welfare of experimental animals (APAFIS permit no. 9045-201701068219555).

2. Gene expression

2.1 RNA extraction and complementary DNA (cDNA) synthesis

Tissues were thawed one ice in lysis buffer using the total RNA extraction kit (Nucleospin® RNA, Macherey-Nagel, Germany) before performing the extraction. Quantity and purity (A260/280 ratio) of extracted RNA were verified using a spectrophotometer (NanoDrop™ One/OneC Spectrophotometer, Thermo Scientific, Waltham, MA, USA). One microgram of RNA was used to generate the complementary DNA (cDNA) using the qScript™ cDNA SuperMix (Quanta Biosciences™) providing all necessary components for first-strand synthesis: buffer, oligo(dT) primers, random primers and qScript reverse transcriptase.

2.2 Quantification of gene expression levels

384-wells plates were filled with an Echo®525 liquid handling system (Labcyte Inc., San Jose, CA, USA). Each well contained a mix composed by 0.75 µL of LightCycler-FastStart DNA Master SYBR-Green I™ Mix (Roche, Manheim, Germany), 0.037 µL of each primer (forward and reverse primers at 0.2 µM final concentration), 0.21 µL of ultrapure water and 0.5 µL of cDNA. The dilution of the samples was determined according to the standard curves generated for each primer pair. Quantitative real-time PCR (qRT-PCR) was performed using a

LightCycler®480 Real-Time PCR System (Roche, Mannheim, Baden-Württemberg, Germany), with the conditions described in Blondeau-Bidet *et al.*, 2019. All the primers used are listed in Table 1. For each organ and gene, efficiency was determined and ranged from 1.8 to 2.1 (Table 1).

Elongation factor 1 α (ef1α) was chosen as a reference gene according to previous studies performed on sea bass challenged to FW (Nebel *et al.*, 2005; Mitter *et al.*, 2009; Blondeau-Bidet *et al.*, 2016, L'Honoré *et al.*, 2019). Relative expression of the target gene was performed using the comparative Ct method (threshold cycle number) according to L'Honoré *et al.* (2019). This method enabled to compare the expression pattern between genes within the same organ. Ultra-pure water was used as a negative control template, and each sample was analysed in duplicate.

Table 1 Primer sequences used for the gene expression analysis

Target gene	Primer name	Sequences ID	Sequence (from 5' to 3')	Efficiency	Reference
ef1α	EF1α F	AJ866727.1	GGCTGGTATCTCTAAGAACG	1.9 (gills)	Nebel et al., 2005
	EF1α R		CCTCCAGCATGTTGTCTCC	1.9 (posterior kidney)	
nkcc1a	NKCC1A F	DLAgn_00123120	AGTTGGCAGTAAGGAGGTGG	2.1 (gills)	Blondeau-Bidet et al., 2019
	NKCC1A R		TCAGACTCAGAGGAGACTTGG	1.9 (posterior kidney)	
nkcc1b	NKCC1B F	DLAgn_00080120	TCAGCTCACAGTTCAAGGCC		This study
	NKCC1B R		TTGTGGAGTCCATAGCGGC	2.1 (posterior kidney)	
nkcc2	NKCC2 F	DLA_LG5_005810	GACACTGTGGAGGACGATGG		This study
	NKCC2 R		AGCATGCATCTCACCAGGAC	2.0 (posterior kidney)	
nhe3	NHE3 F	DLAgn_00204050	GGATACCTCGCCTACCTGAC	1.9 (gills)	Blondeau-Bidet et al., 2019
	NHE3 R		AAGAGGAGGGTGAGGAGGAT	1.9 (posterior kidney)	
ncc1	NCC1 F	DLAgn_00172790	TGACGTACTTGATCGCTGCC	1.9 (gills)	This study
	NCC1 R		AGTTGGTGATGGAGGCATGG	2,0 (posterior kidney)	
ncc2-a	NCC2-A F	DLAgn_00038210	ATGATGAGCCTCTTCGAGCC	2.1 (gills)	Blondeau-Bidet et al., 2019
	NCC2-A R		ACAGAAGGTGATGAGAGCAGC	2.0 (posterior kidney)	
slc26a6a	26A6-A F	DLAgn_00129890	TCATGTGTGTGTCTCCCAGC		This study
	26A6-A R		GAGAGTGCTACCAGGCTGAC	2.0 (posterior kidney)	
slc26a6b	26A6-B F	DLAgn_00133440	ATACCTGTGGAGCTGCTTGG		This study
	26A6-B R		TCAGGGCAAAGATTCGTCCC	1.9 (posterior kidney)	
slc26a6c	26A6-C F	DLAgn_00221070	GAAACGGACACAGAGGAGGG	1.9 (gills)	This study
	26A6-C R		GTCTGGTGTTCGAGGAGTGG	2.0 (posterior kidney)	
ae1a	AE1-A F	DLAgn_00101870	TCTGAAGGAATCGGTGGTGC	1.9 (gills)	This study
	AE1-A R		CTCGTTCTGGATCTCGGTGG		
ae1b	AE1-B F	DLAgn_00193420	TGAACAAGGGTGAGATCCGC		This study
	AE1-B R		ACAAAGCGAATAGGGACGGG	2.0 (posterior kidney)	
prlra	PRLR-A F	DLA_LG19_005350	GGGACAGAGGCAGAAGACAT	2.0 (gills)	This study
	PRLR-A R		GAGGTAGGAGGATGTGGAGC	2.0 (posterior kidney)	
prlrb	PRLR-B F	DLA_LG20_006210	GAATGACAATGGGCCTCTGC	2.0 (gills)	This study
	PRLR-B R		GTTCCGTTGCTTCTGGGTTT	2.0 (posterior kidney)	

3. Phylogenetic analysis and protein comparisons

Phylogenetic analyses of *slc26a6* and *nkcc (slc12a1 and slc12a2)* have been performed (Figs 1, 2). Nucleotide sequences from different species were obtained at Ensembl or NCBI (Tables 2, 3). Sea bass sequences were

obtained from the sea bass genome project (Tine *et al.*, 2014). Multiple nucleotide alignments were performed with MUSCLE V3.8.31 (Edgar, 2004) and ambiguous regions were removed with Gblocks V0.91b (Talavera & Castresana, 2007). The phylogenetic trees were inferred using the maximum likelihood (ML) method implemented in the PhyML program (v3.1/3.0 aLRT) (Guindon & Gascuel, 2003). Best model of evolution was selected using Modelgenerator V.85 (Keane *et al.*, 2006) following the corrected Akaike Information Criterion (with four discrete gamma categories) and used to construct a phylogenetic tree. Bayesian posterior probabilities were computed with MrBayes 3.2.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). Two different runs with four incrementally heated simultaneous Monte Carlo Markov chains were conducted over one million generations, applying respective substitution models determined for each partition in PartitionFinder2 (Lanfear *et al.*, 2017). Trees were sampled every 100 generations to produce 10 000 trees. In order to estimate posterior probabilities, 25% of the trees were discarded as a burn-in stage, observing when average standard deviation of split frequency (ASDSF) values dropped below 0.01. Tree were generated and robustness of the phylogeny assumption was evaluated by bootstrapping procedure from 1000 data set replicates and with posterior probabilities (PP). The phylogenetic tree of *slc26a6* was rooted with the *slc26a6* sequences of two reptilians, the Chinese soft-shelled turtle (*Pelodiscus sinensis*) and the painted turtle (*Chrysemys picta bellii*). The phylogenetic tree *of nkcc (slc12a1 and slc12a2)* was rooted with the *ncc3* sequences of the tropical frog (*Xenopus tropicalis*) and the lizard (*Anolis carolinensis*).

Amino acid identities (in %) were performed with MUSCLE V3.8.31 (Edgar, 2004) implemented in Geneious® 9.1.8 software (Biomatters).

4. Statistical analysis

Analyses were performed using GraphPad Prism (version 6, GraphPad Software Incorporated, La Jolla, CA 268, USA). When parametric conditions were verified, one-way analyses of variances were performed followed by a Tukey's post-hoc test. In the case of non-parametric conditions, a Kruskal-Wallis test was performed followed by a Dunn's post-hoc test. Data are represented as box and whisker plots showing median, minimum and maximum values. Statistical differences were accepted from $P < 0.05$.

Tables 2 (left) Cl^-/HCO_3^- exchanger (*slc26a6*) **& Table 3 (right)** NKCC 1, 2 (*slc12a1/slc12a2*) nucleotide sequences used in the phylogenetic analysis

Nucleotide sequence ID	Fish species	Gene
XM_024104973.1	*Chrysemys picta bellii*	*slc26a6*
DLAgn_00129890	*Dicentrarchus labrax*	*slc26a6a*
DLAgn_00133440	*Dicentrarchus labrax*	*slc26a6b*
DLAgn_00221070	*Dicentrarchus labrax*	*slc26a6c*
XM_680900.6	*Danio rerio*	*slc26a6*
XM_001344207.7	*Danio rerio*	*slc26a6b*
FJ170818.1	*Danio rerio*	*slc26a6c*
XM_012876467.2	*Fundulus heteroclitus*	*slc26a6a*
XM_021318616.1	*Fundulus heteroclitus*	*slc26a6b*
XM_012872750.2	*Fundulus heteroclitus*	*slc26a6c*
ENSGACP00000002987	*Gasterosteus aculeatus*	*slc26a6*
ENSGACT00000007648.1	*Gasterosteus aculeatus*	*slc26a6b*
ENSGACT00000001978.1	*Gasterosteus aculeatus*	*slc26a6c*
ENSORLP00000011507	*Oryzia latipes*	*slc26a6a*
XM_011476835.3	*Oryzia latipes*	*slc26a6b*
ENSORLP00000002390	*Oryzia latipes*	*slc26a6c*
ENSONIT00000015705.1	*Oreochromis niloticus*	*slc26a6a*
ENSONIT00000002151	*Oreochromis niloticus*	*slc26a6b*
ENSONIP00000018142	*Oreochromis niloticus*	*slc26a6c*
XM_014577432.2	*Pelodiscus sinensis*	*slc26a6*
CAF95115.1	*Tetraodon nigroviridis*	*slc26a6a*
CAG06912.1	*Tetraodon nigroviridis*	*slc26a6c*
AB200328.1	*Takifugu rubripes*	*slc26a6a*
AB200329.1	*Takifugu rubripes*	*slc26a6b*
AB200330.1	*Takifugu rubripes*	*slc26a6c*

Nucleotide sequence ID	Fish species	Gene
JN180944.1	*Anabas testudineus*	*nkcc1a*
AJ486858.1	*Anguilla anguilla*	*nkcc1a*
AJ486859.1	*Anguilla anguilla*	*nkcc1b*
AJ564602.1	*Anguilla anguilla*	*nkcc2a*
AJ564603.1	*Anguilla anguilla*	*nkcc2b*
XM_003223867.1	*Anolis carolinensis*	*ncc3*
DLAgn_00080120	*Dicentrarchus labrax*	*nkcc1a*
DLAgn_00123120	*Dicentrarchus labrax*	*nkcc1b*
DLA_LG5_005810	*Dicentrarchus labrax*	*nkcc2*
ENSGMOT00000004750.1	*Gadus morhua*	*nkcc1a*
ENSGMOT00000001772.1	*Gadus morhua*	*nkcc1b*
ENSGMOT00000009346.1	*Gadus morhua*	*nkcc2b*
ENSGACT00000024304.1	*Gasterosteus aculeatus*	*nkcc1a*
ENSGACT00000019494.1	*Gasterosteus aculeatus*	*nkcc1b*
ENSGACT00000022179.1	*Gasterosteus aculeatus*	*nkcc2*
AY513737	*Oreochromis mossambicus*	*nkcc1a*
AY513738	*Oreochromis mossambicus*	*nkcc1b*
AY513739.1	*Oreochromis mossambicus*	*nkcc2*
ENSORLT00000024013.2	*Oryzias latipes*	*nkcc1a*
ENSORLT00000021359.2	*Oryzias latipes*	*nkcc1b*
ENSORLT00000038800.1	*Oryzias latipes*	*nkcc2*
GU066877.1	*Sarotherodon melanotheron*	*nkcc1*
ENSTRUP00000004453	*Takifugu rubripes*	*nkcc1a*
ENSTRUP00000027260	*Takifugu rubripes*	*nkcc1b*
ENSTRUT00000043584.2	*Takifugu rubripes*	*nkcc2*
CAG09669.1	*Tetraodon nigroviridis*	*nkcc1b*
CAF99849.1	*Tetraodon nigroviridis*	*nkcc2*
XM_002934280.4	*Xenopus tropicalis*	*ncc3*

Results

1. Phylogenetic analysis of sea bass *slc26a6* and *nkcc* paralogous genes

Three paralogs of *slc26a6* have been identified in the sea bass genome (Tine *et al.*, 2014). The three paralogs are called in this study *slc26a6 a*, *slc26a6 b* and *slc26a6 c* according to their phylogenetic position (Fig. 1) computed by maximum likehood analysis against sequences of selected teleost fish (Table 2). Comparatively, three paralogs were identified in *O. niloticus*, *O. latipes*, *G. aculeatus*, *F. heteroclitus* and in *D. rerio* whereas two paralogs were identified in *T. nigroviridis* (a and c clades). The sea bass *slc26a6* paralogous genes obtained in this study strongly grouped with other teleosts *slc26a6* paralogs. Sea bass *slc26a6c* is grouped with *slc26a6c* of *O. niloticus*, *O. latipes*, *T. nigroviridis* and shared 86%, 80% and 56% identity in amino acid sequences respectively. Sea bass SLC26A6c only shares 49% and 54% amino acid identity with sea bass SLC26A6b and

SLC26A6a. *Slc26a6b* of sea bass is grouped with *O. niloticus slc26a6b*, sharing 80% identity in amino acid sequences but only 53% with sea bass SLC26A6a. Sea bass *slc26a6b* is strongly grouped with *O. latipes* and *T. nigroviridis*, sharing 71% and 67% identity in amino acid sequences respectively.

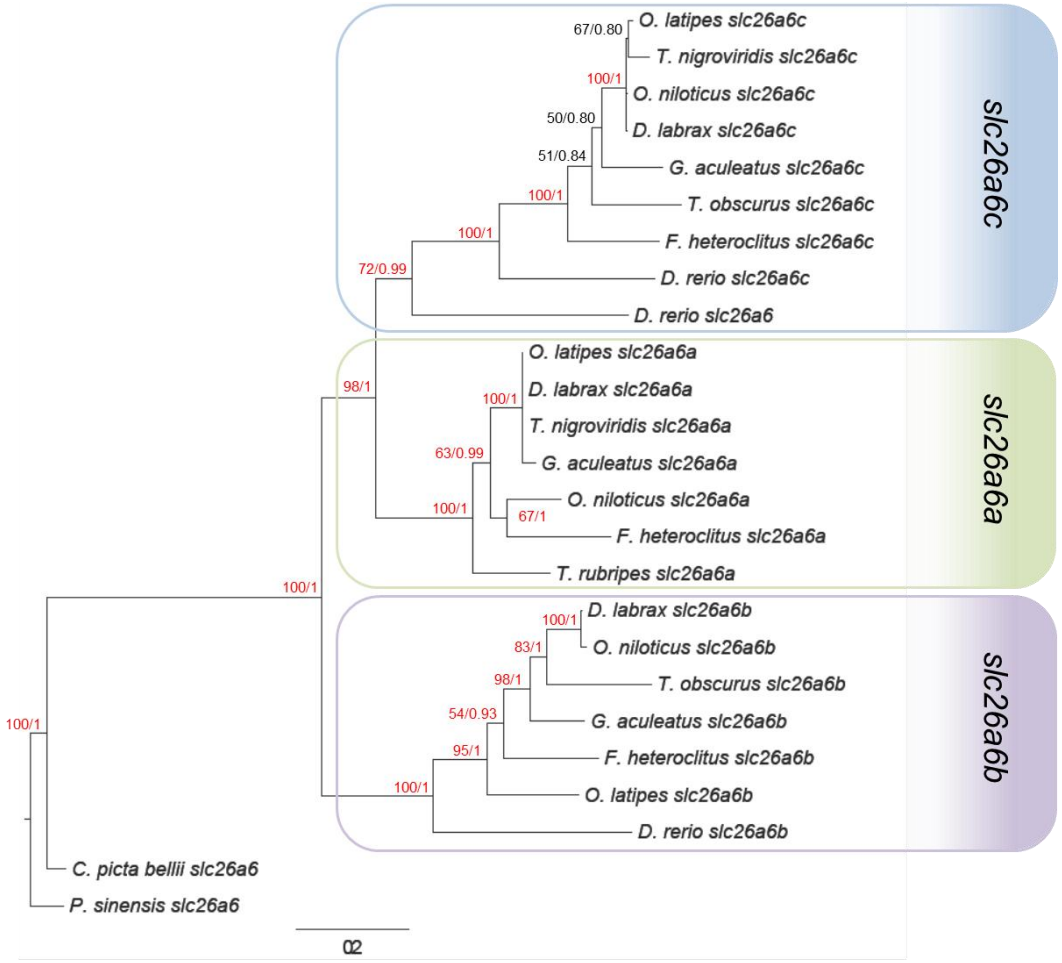

Fig. 1 Bayesian tree of *slc26a6*. A ML tree had identical topology for all nodes. Bootstrap values (in %) from the ML are shown before the Bayesian PP, confident nodes are indicated in red. Branch lengths represent the degree of divergence, with the scale bar indicating the distance representing 0.2 substitutions per position. The blue frame mainly comprises sequences of teleost *slc26a6c*. The green frame mainly comprises sequences of teleost *slc26a6a* and purple frame comprises sequences of teleost *slc26a6b*.

Two *slc12a1* (*slc12a1a* and *slc12a1b*) and one *slc12a2* were identified in the sea bass genome that correspond to NKCC1a, NLCC1b and NKCC2 according to their phylogenetical position (Fig. 2), computed by maximum likehood analysis against sequences of selected teleost fish (Table 3). Three *nkcc* genes were also found in *O. mossambicus*, *O. latipes*, *T. rubripes*, *G. aculeatus* and *G. morhua* while two were found in *T. nigroviridis* (1b and 2 clades). Sea bass *nkcc* paralogs strongly grouped with teleost *nkcc* sequences. Sea bass *nkcc1a* and *nkcc1b* are grouped with *O. latipes nkcc1a* and *nkcc1b* and share 90.2% and 91.9% identity in amino acid sequences respectively. Sea bass NKCC1a and NKCC1b only share 77.8% identity between each other. Sea bass *nkcc2* is

strongly grouped with *T. nigroviridis* and shares 82.5% amino acid identity whereas it only shares 57.1 and 55.6% identity with NKCC1a and NKCC1b of sea bass amino acid sequences.

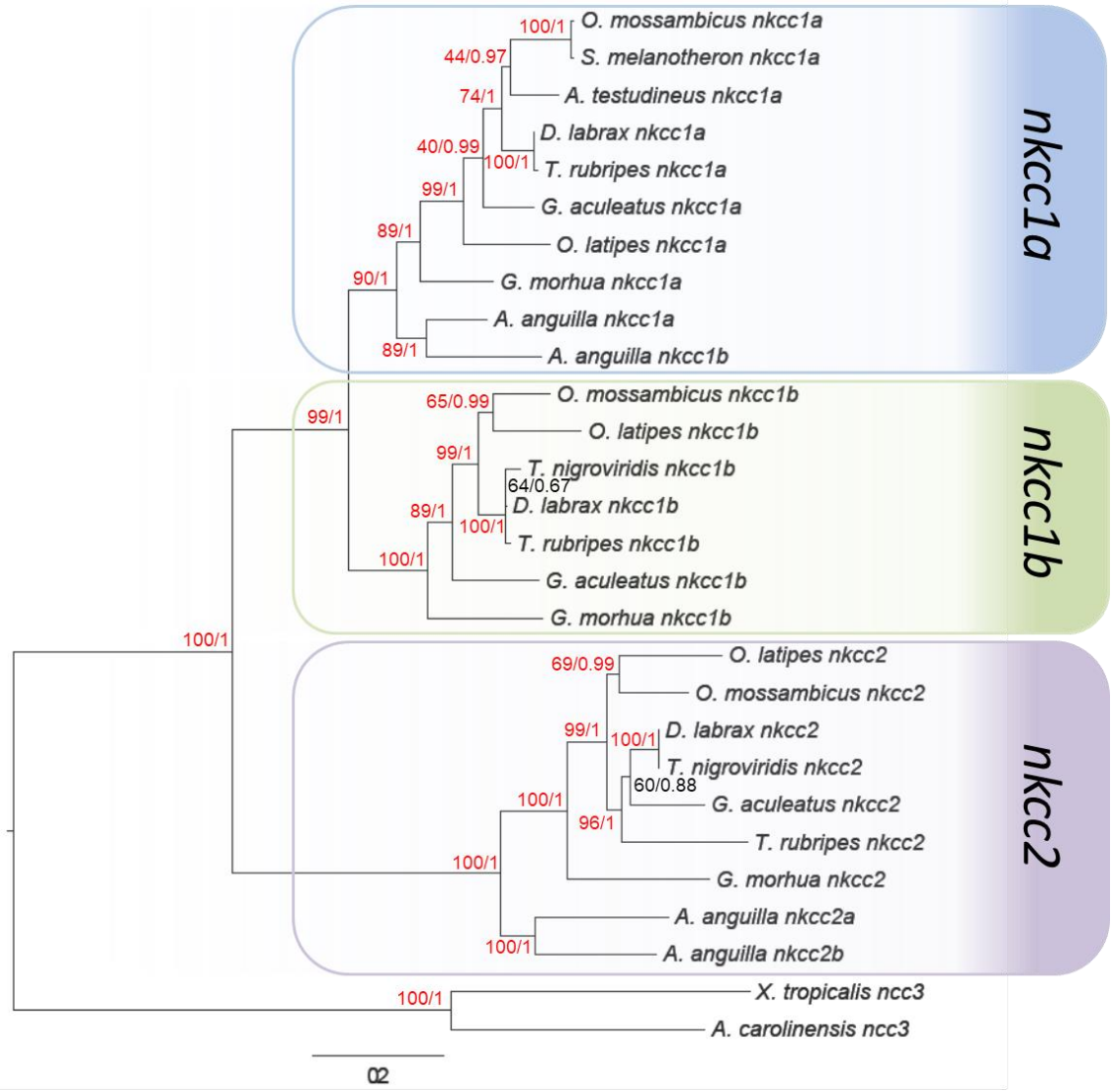

Fig. 2 Bayesian tree of *nkcc1a*, *nkcc1b* and *nkcc2* (*slc12a1a*, *slc12a1b* and *slc12a2*). A ML tree had identical topology for all nodes. Bootstrap values (in %) from the ML are shown before the Bayesian PP, confident nodes are indicated in red. Branch lengths represent the degree of divergence, with the scale bar indicating the distance representing 0.2 substitutions per position. The blue frame mainly comprises sequences of teleost *nkcc1a*. The green frame mainly comprises sequences of teleost *nkcc1b* and purple frame comprises sequences of teleost *nkcc2*.

2. Relative gene expression in osmoregulatory tissues

Expression of selected genes was quantified in the gills and in the posterior kidney (Figs 3, 4, 5). Analysed genes were: *ae1a* (*slc4a1-1*), *ae1b* (*slc4a1-2*), *nhe3* (*slc9a3*), *nkcc1a* (*slc12a2*), *nkcc1b (slc12a2-like)*, *nkcc2 (slc12a1)*, *vha-b (atp6v1b)*, *ncc1 (slc12a3)*, *ncc-2a (slc12a3-like)*, *slc26a6a*, *slc26a6b*, *slc26a6c*, *prlra* and *prlrb*. Among them, *ae1b*, *slc26a6a*, *slc26a6b* and *nkcc2* expression levels have not been quantified in the gills whereas *ae1a*

and *nhe3* expression levels have not been quantified in the posterior kidney because their expression was below the quantification limit.

2.1 Gill gene expression

Ae1a was not differentially expressed between SW and both FW groups (FW_t and FW_i, Kruskal-Wallis test, P = 0.1534, Fig. 3A). *Nkcc1a* expression was significantly lower in both FW phenotypes (FW_t and FW_i) compared to SW (Dunn's test, $P < 0.0001$ and $P = 0.0095$ respectively, Fig. 3B), but no significant differences were measured between FW phenotypes.

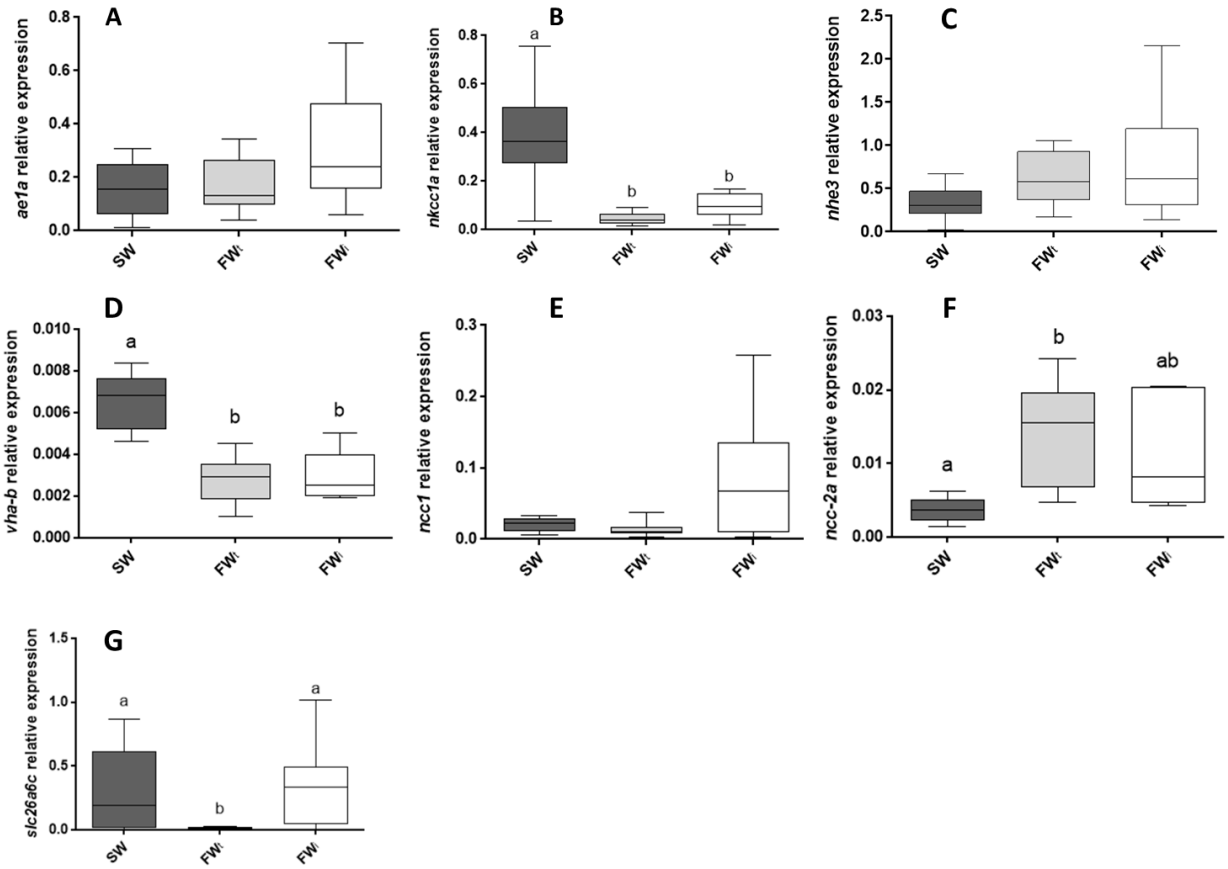

Fig. 3 Relative expression in gills of (A) *ae1a / slc4a1-1*, (B) *nkcc1a / slc12a2-1* , (C) *nhe3*, (D) *vha-b*, (E) *ncc1*, (F) *ncc-2a* and (G) *slc26a6c* in SW and after two weeks in FW. The expression has been normalised according to the expression of the elongation factor *ef1α*. Different letters denote significant differences between groups (one-way Anova followed by Tukey's test or Kruskal-Wallis followed by Dunn's test, $P < 0.05$, means ± s.e.m, N=6-16). SW: control fish in SW, FW_i: intolerant fish to FW, FW_t: tolerant fish to FW

Nhe3 relative expression showed no significant differences between SW, FW_t and FW_i, (Kruskal-Wallis test, P = 0.0648, Fig. 3C) but a higher variability was observed in freshwater conditions. *Vha-b* expression levels were significantly higher in SW than in both FW phenotypes (FW_t and FW_i), but no differences were measured between FW_t and FW_i (Dunn's test, P = 0.0083, P = 0.0264 and $P > 0.9999$ respectively, Fig. 3D). No

significant differences have been measured for *ncc1* expression levels between the three groups (Kruskal-Wallis test, $P = 0.0999$, Fig. 3E), but there is more variability in FW_i compared to SW and FW_t, with an almost significant difference between FW_t and FW_i (Dunn's test, $P = 0.0955$). *Ncc-2a* expression was significantly higher in FW_t than in SW (Dunn's test, $P = 0.0072$, Fig. 3F), but no differences were measured between SW and FW_i as between FW_t and FW_i (Dunn's test, $P = 0.0632$ and $P > 0.9999$ respectively). Among *slc26a6* paralogs, only *slc26a6c* expression has been detected in the gills. FW_t exhibited a significantly lower expression of *slc26a6c* compared to both SW and FW_i (Dunn's test, $P = 0.0311$ and $P = 0.0369$, Fig. 3G) whereas no difference was observed between SW and FW_i (Dunn's test, $P > 0.9999$).

Prolactin receptor *prlra* relative expression was significantly higher in FW_i compared to SW fish (Dunn's test $P < 0.0001$, Fig. 5A). No difference in *prlra* expression was recorded between SW and FW_t or between FW_i and FW_t (Dunn's test, $P = 0.0778$ and $P = 0.0761$ respectively) but a high variability is observed in FW_i. *Prlrb* expression was lower in FW groups compared to SW, but only significant between FW_t and SW fish (Dunn's test, $P = 0.0064$, Fig. 5B). No difference was observed between FW_i and FW_t. Overall, *prlra* mRNA levels were higher than *prlrb* levels in freshwater tolerant group (Unpaired t-test, $P = 0.0048$).

2.2 Posterior kidney gene expression

No significant differences were measured in *ae1b* expression levels between SW and FW_t. However a significantly lower expression was measured in FW_i compared to SW but not to FW_t (Dunn's test, $P = 0.0255$ and $P > 0.9999$, Fig. 4A). No significant differences were measured in *ncc1* and *nkcc1a* expression levels between the three groups (Kruskal-Wallis test, $P = 0.0967$ and $P = 0.2192$ respectively, Fig. 4B, C). Concerning *nkcc1b*, no differences were measured between SW and FW_t (Dunn's test, $P > 0.9999$, Fig. 4D) but FW_i expression levels were significantly lower than in FW_t and SW (Dunn's test, $P = 0.0319$ and $P = 0.0053$ respectively).

Nkcc2 expression was not significantly different between SW and FW_t or between FW_t and FW_i, but it was significantly lower in FW_i than in SW (Dunn's test, $P = 0.1663$, $P = 0.8540$ and $P = 0.0068$ respectively, Fig. 4E). In FW_t, among *slc12a* cotransporters analysed, *ncc1* is the most expressed paralog in the posterior kidney followed by the 5-fold less expressed *nkcc2* and the 25- and 50-fold less expressed *nkcc1a* and *nkcc1b*.

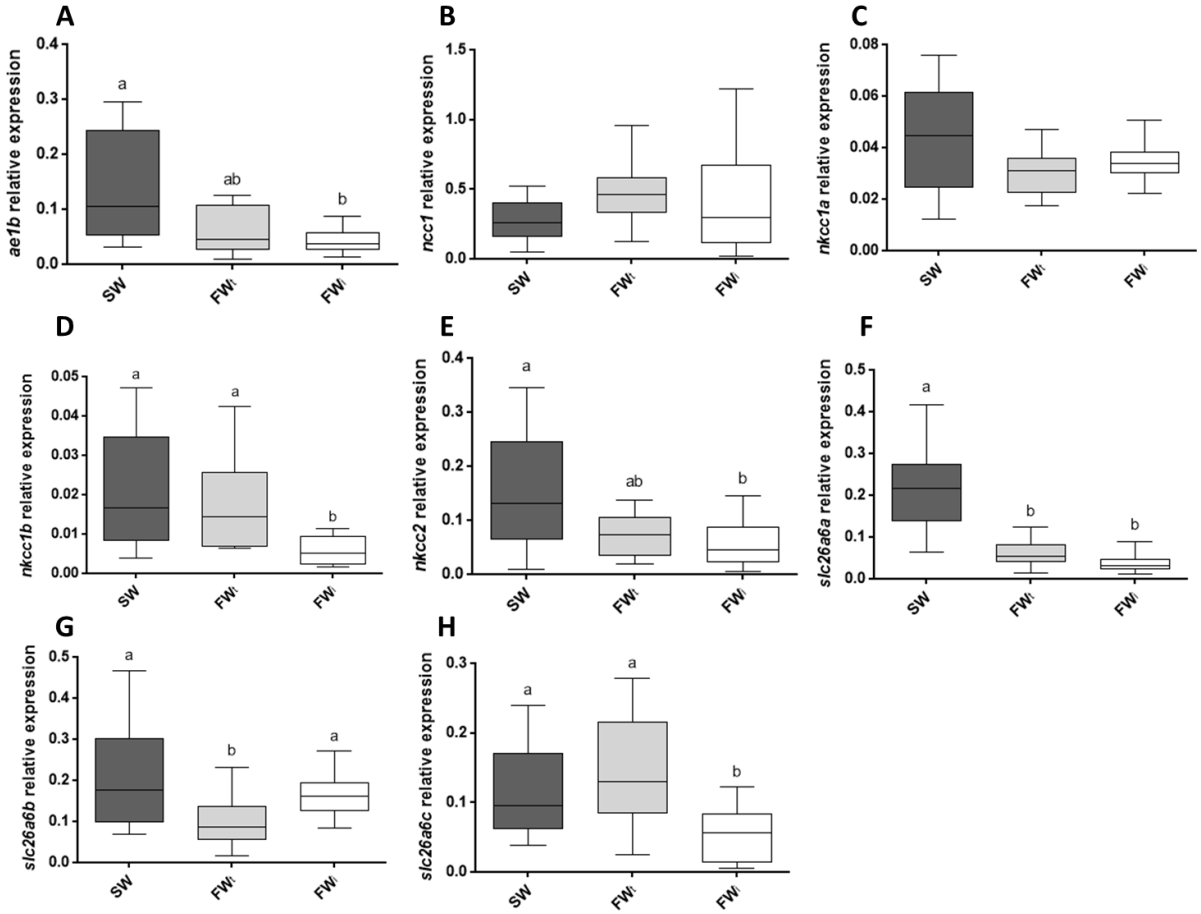

Fig. 4 Relative expression in posterior kidney of (A) *ae1 b / slc4a1-2,* (B) *ncc1 (slc12a3),* (C) *nkcc1a / slc12a2,* (D) *nkcc1b / slc12a2-like,* (E) *nkcc2 (slc12a1),* (F) *slc26a6a,* (G) *slc26a6b* and (H) *slc26a6c* in SW and after two weeks in FW. The expression has been normalised according to the expression of the elongation factor *ef1α.* Different letters denote significant differences between groups (one-way Anova followed by Tukey's test of Kruskal-Wallis followed by Dunn's test, $P < 0.05$, means ± s.e.m, N=6-16). SW: control fish in SW, FW_i: intolerant fish to FW, FW_t: tolerant fish to FW

Concerning *slc26a6* paralogs, *slc26a6a* and *slc26a6b* exhibited a significant lower relative expression in FW_t compared to SW (Dunn's test, $P = 0.0037$ and $P = 0.0109$ respectively, Fig. 4F, G). In FW_i, *slc26a6a* expression was not significantly different from FW_t, whereas *slc26a6b* was significant higher than in FW_t (Dunn's test, $P = 0.0014$ and $P = 0.0207$, Fig. 4F, G) but not significantly different from SW (Dunn's test, $P > 0.9999$). *Slc26a6c* expression levels were not significantly different between SW and FW_t (Tukey's test, $P = 0.4731$, Fig. 4H), but they were significantly lower in FW_i than in SW and FW_t (Tukey's test, $P = 0.0420$ and $P = 0.0014$ respectively). Overall, in FW_t the three paralogs were similarly expressed. *Prlra* expression was significantly higher in FW_t than in SW (Tukey test, $P < 0.0001$, Fig. 5C) whereas *prlrb* expression levels did not differ between SW and FW_t (Tukey test, $P = 0.4901$, Fig. 5D). A significantly lower expression of both *prlr* paralogs (*prlra*, *prlrb*) was measured in FW_i compared to FW_t (Tukey test, $P = 0.0175$ and $P = 0.0429$ respectively, Fig. 5C, D). As for the gills, kidney *prlra* mRNA levels were higher than *prlrb* levels in freshwater tolerant group (Unpaired t-test, $P < 0.0001$).

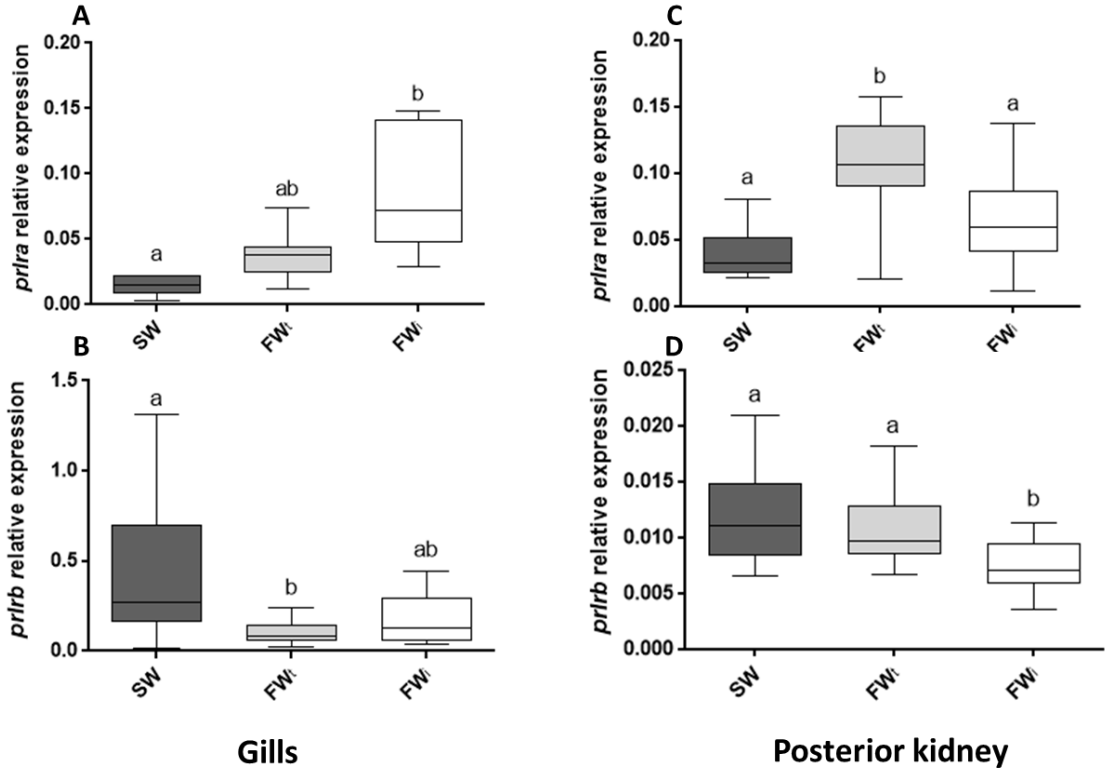

Fig. 5 Relative expression in the gills and in the posterior kidney of *prlra* (A-C) and *prlrb* (B-D) in SW and after two weeks in FW. The expression has been normalised according to the expression of the elongation factor *ef1α*. Different letters denote significant differences between groups (one-way Anova followed by Tukey's test or Kruskal-Wallis test followed by Dunn's test, $P < 0.05$, means ± s.e.m, N=6-16). SW: control fish in SW, FW_i: intolerant fish to FW, FW_t: tolerant fish to FW

Discussion

The comparative analysis of key genes and proteins involved in the maintenance of hydromineral balance in fish exhibiting different capacities to tolerate freshwater is a powerful tool to investigate intraspecific variation in FW tolerance in euryhaline species. In FW, fish have to minimise ion loss and compensate through active ion uptake occurring at interfaces with their surroundings. Most studies investigating freshwater osmoregulation focused at the gill level in adults or at integument level in larvae (mostly zebrafish), showing that freshwater tolerance relies on the expression of key genes involved in hyper-osmoregulatory mechanisms (Evans & Clairborne, 2009). Less data are available on the involvement and importance of the kidney in ion re-uptake. In this study, we highlight the importance of taking into account not only the gill but also the posterior kidney in order to fully understand the mechanisms of ion and acid-base regulation.

1. Ion uptake capacities are not altered in gills of FW intolerant sea bass

Regarding gill ion uptake, slightly higher *nka α1a* expression levels and NKA protein activity were previously measured in FW_i compared to FW_t in sea bass (Nebel *et al.*, 2005; L'Honoré *et al.*, 2019). This could indicate a

compensatory response to the blood ion imbalance. Regarding other transporters, we measured no difference in *ae1a* (*slc4a1a*) relative expression between SW and FW phenotypes in gills but a higher variability in FW_i. AE1b has been demonstrated as the dominant paralog in medaka gills and it was more expressed in FW media than in saline waters from 10 to 30 ‰ (Liu *et al.*, 2016). This seems not to be the case in sea bass, as we could only quantify *ae1a*, which was not differently expressed between salinities. In medaka gills, an opposite response was measured regarding the two AE1 paralogs, AE1a and AE1b, when comparing SW and FW-acclimated fish suggesting different functions according to the salinity. Using inhibitors and the scanning ion-selective electrode technique (SIET), the role of AE1 seemed to be attributed to acid and Cl^- secretion in seawater-acclimated medaka larvae (Liu *et al.*, 2016). In sea bass, the physiological role of AE1 paralogs still needs to be investigated. Moreover, a different expression pattern is observed between *ae1a* and *vha-b*, which is not consistent with a functional link between apical VHA and basolateral AE1a as shown in medaka. The slightly higher *ae1a* expression in FW_i compared to FW_t is therefore probably not linked to an increased acid secretion. A 3-fold lower expression of *nkcc1a* was measured in FW compared to SW acclimated fish gills, as it has previously been shown in this species (Lorin-Nebel *et al.*, 2006; Blondeau-Bidet *et al.*, 2019) and many other teleosts (Hiroi *et al.*, 2008; Inokuchi *et al.*, 2008 Bollinger *et al.*, 2016). This is consistent with the role played by basolateral NKCC1 in ion secretion. According to Inokuchi *et al.* (2017) and Blondeau-Bidet *et al.* (2019), freshwater transfer in Japanese and European sea bass might be followed by a migration towards gill lamellae of seawater-type ionocytes differentiating into freshwater-type ionocytes (NHE3-type cells) involved in Na^+ uptake. The observed decrease of *nkcc1a* expression in FW_i suggests a successful functional shift from SW to FW-type ionocytes. *Nhe3* seems also slightly more expressed in both freshwater phenotypes compared to SW but unlike previous studies (Blondeau-Bidet *et al.*, 2019), there is no significant difference between salinities. Interestingly, the variability of expression levels for *nhe3* is higher in FW_i compared to the other conditions, which could indicate differential responses within this group.

We measured a higher expression of *ncc-2a* in FW acclimated sea bass than in SW as it was already demonstrated in this species (Blondeau-Bidet *et al.*, 2019). This reinforces the hypothesis of the differentiation of *ncc-2a* expressing ionocytes in FW, as it was observed in many other fish species such as medaka and tilapia, exhibiting an higher relative expression of *ncc* (homologous to sea bass *ncc-2a*) in FW than in SW conditions (Hiroi *et al.*, 2008; Inokuchi *et al.*, 2008 Bollinger *et al.*, 2016). FW_i fish also increase slightly but not significantly their *ncc-2a* expression suggesting that at least some individuals successfully differentiate NCC-type cells in gill filaments as shown previously in this species (Blondeau-Bidet *et al.*, 2019). Interestingly,

expression levels of *ncc1* were slightly higher and extremely variable in FW_i compared to the other groups, which was surprising as *ncc1* expression in the gills is generally low (Inokuchi *et al.*, 2017). A compensatory role of *ncc1* in Na^+, Cl^- uptake in intolerant fish gills could be hypothesised as fish gills are known to have multiple ion uptake pathways, that compensate each other in case of stressful and ion-poor environments (Hwang & Lee, 2007; Hwang *et al.*, 2011).

Slc26a6c expression is significantly higher in FW_i compared to FW_t, indicating that FW_i may compensate their low blood Cl^- levels through an increased Cl^- absorption and also activate the secretion of HCO_3^- to restore blood pH levels (L'Honoré *et al.*, 2019). In zebrafish, a higher expression level of *za6c* was observed in the gills in hypo-osmotic conditions which is consistent with an involvement of this ion exchanger in chloride uptake in apical membranes (Bayaa *et al.*, 2009; Perry *et al.*, 2009). By displaying enhanced expression levels of Cl^- uptake genes such as *ncc1* and *slc26a6c*, FW_i sea bass seemed to activate Cl^- absorption at the branchial level to compensate low Cl^- circulating levels measured previously (L'Honoré *et al.*, 2019).

2. FW intolerant sea bass differentially regulate chloride reabsorption by the kidney

In FW_i posterior kidney, lower expression of *nka α1a* were previously measured compared to FW_t, associated with lower Cl^- blood levels possibly due to a renal ion leakage (L'Honoré *et al.*, 2019). In this study, we have measured a lower expression of two other transporters involved in acid-base and hydromineral regulations (*ae1b* and *slc26a6c*) in FW_i compared to FW_t suggesting that FW_i are actually less efficient to compensate ion loss at the kidney level. Contrary to zebrafish in which *ae1a* is the predominant paralog of AE1 in the kidney, in sea bass only *ae1b* could be detected (Lee *et al.*, 2011). Expression of *ae1b* was low in FW_i compared to the other conditions. Its basolateral localisation has been demonstrated in goldfish (Fehsenfeld & Wood, 2018), suggesting a role in acid-base regulation. FW_i may decrease the expression of this transporter in order to limit excessive HCO_3^- levels in the blood as these fish already experience blood alkalosis (L'Honoré *et al.*, 2019). In sea bass kidney, expression levels of *ncc1* (*slc12a3*) are higher than other *slc12a* cotransporters, which suggest that NCC1 is predominant in sea bass kidney for ion uptake. It is slightly more expressed in FW_t compared to SW fish and is highly variable in FW_i. *Ncc1* (*slc12a3*) has been demonstrated to be also highly expressed in FW in Japanese sea bass kidney compared to SW (Inokuchi *et al.*, 2017). In mefugu *Takifugu obscurus*, *ncc* (homologous to sea bass *ncc1*) is localised apically and highly expressed in collecting duct of FW-acclimated fish, with a putative role in Na^+, Cl^- reabsorption (Kato *et al.*, 2010). In killifish *F. heteroclitus*, rainbow trout *Oncorhynchus mykiss* and torafugu *Takifugu rubripes*, NKCC2 was localised apically and a role in Na^+, Cl^-

reabsorption was suggested (Katoh *et al.*, 2008; Kato *et al.*, 2010). Compared to *ncc1*, *nkcc2* was about 6.5 times less expressed in sea bass kidney in FW_t and it rather seems downregulated in FW media *vs* SW. Its role in ion uptake seems therefore secondary. FW_i sea bass exhibited the lowest expression levels of *nkcc2* and are thus certainly not using this ion uptake pathway to compensate ion loss. The basolateral *nkcc1a* and *nkcc1b* are respectively 15.8 and 27 times less expressed than *ncc1* in FW_t which is consistent with the role of NKCC1 paralogs in ion excretion rather than ion absorption. Whereas *nkcc1a* expression did not change between the different treatments and phenotypes, *nkcc1b* showed significantly lower expression in FW_i compared to the other conditions. The physiological significance of this transcriptional down-regulation needs to be further explored.

Among the three SLC26A6 paralogous genes investigated in the posterior kidney of sea bass, two exhibited significant lower expression in FW_t compared to SW: *slc26a6a* and *slc26a6b*. *Slc26a6c* expression is not significantly modified between SW and FW_t, but it was lower in FW_i. A higher *slc26a6c* expression in low-Cl^- FW compared to normal FW has already been demonstrated in zebrafish gills and kidney with a suggested role in HCO_3^- excretion associated with a Cl^- (re)absorption (Bayaa *et al.*, 2009). SLC26A6 family can exchange various anions with Cl^- such as sulfate (SO_4^{2-}), oxalate ($[COO^-]_2$) and bicarbonate (HCO_3^-) (Mount & Romero, 2004). The role of SLC26A6a in SO_4^{2-}/Cl^- exchange in mefugu proximal tubules has been already highlighted. According to Kato *et al.* (2009), SLC26A6a is acting as the main apical SO_4^{2-}/Cl^- exchanger and its expression is enhanced under SW conditions. Our results are concordant with this study since both FW sea bass phenotypes (FW_t and FW_i) exhibited lower *slc26a6a* expression levels than in SW. Less is known about the role of SLC26A6b in the kidney. SLC26A6a and SLC26A6b have been shown to be both implicated in HCO_3^- secretion by the intestine (Xie *et al.*, 2002) but their role in SO_4^{2-} or/and HCO_3^- secretion needs to be further investigated in the kidney of sea bass and other teleost species.

3. Prolactin receptor transcript levels differ according to FW tolerance capacities

Contrary to mammals where only one *prlr* gene exists, teleost can have two distinct paralogs due to genome duplication events (Ocampo Daza & Larhammar, 2018). The relationship between PRLR and gene expression patterns of osmoregulatory-related genes has not yet been described in sea bass and needs more attention. Gill *prlr* paralog expression levels seem to depend on circulating PRL levels as shown in Seale *et al.* (2012) for *prlra* and on extracellular osmolality as shown for *prlrb* in Fiol *et al.* (2009). Pituitary PRL acts as one of the major endocrine signals for FW acclimation in euryhaline teleosts (Manzon, 2002). Lower *prlra* expression levels in SW compared to FW have been already shown in Mozambique tilapia (Breves *et al.*, 2010; Breves *et al.*, 2011),

whereas no differences where observed concerning branchial *prlrb* relative expression between salinities. In our study, *prlr* paralogs expression patterns are not concordant with those described in tilapia gills. Even if *prlra* seems to be slightly more expressed in FW_t than in SW, *prlrb* is significantly overexpressed in SW compared to FW_t. We have previously shown that FW_i exhibited decreased blood osmolality levels (L'Honoré *et al.*, 2019), which could be one of the trigger to activate branchial *prlr* expression. In fact, we measured slightly but not significantly higher mRNA levels of both *prlr* paralogs in FW_i compared to FW_t. In zebrafish and Nile tilapia, PRL and PRLR positively regulate the expression of *ncc* in gill filament cultures *in vitro* (Breves *et al.*, 2013, 2014), which suggests that NCC-type cells might express PRLR. In our study, we also highlighted higher branchial *ncc1* relative expression in FW_i compared to FW_t, suggesting a similar regulation of *ncc1* expression levels by *prlr*. Moreover, slightly higher *nka α1a* expression levels and increased gill NKA activity were previously measured in FW_i compared to FW_t (Nebel *et al.*, 2005; L'Honoré *et al.*, 2019), potentially linked to an increased number of ionocytes to compensate low blood osmolality and low circulating chloride levels.

In the posterior kidney, *prlra* is the predominantly expressed paralog (10-fold higher expressed than *prlrb*) whereas in the gills, *prlrb* was 10-fold more expressed than *prlra*. No differences in *prlra* relative expression have been reported following salinity change in Seale *et al.* (2012) but a lower relative expression of *prlrb* was shown in FW compared to SW (Fiol *et al.*, 2009; Breves *et al.*, 2011). It seems different in sea bass posterior kidney where significantly higher expression levels of *prlra* are measured in FW_t compared to SW. Moreover, no differences were observed for *prlrb* between SW and FW_t. Interestingly, both *prlra* and *prlrb* were less expressed in FW_i compared to FW_t, highlighting that FW_i and FW_t do not exhibit the same regulation mechanisms in response to the FW challenge. We showed a differential expression of prolactin receptors in FW between FW_t and FW_i, with a higher, though not significant, over-expression of branchial prolactin receptors in FW_i and a significantly lower expression of both renal prolactin receptors. These results suggest that freshwater intolerance in sea bass might be due to an altered PRL-associated endocrine response in FW_i.

Conclusion

This study is the first to highlight that freshwater intolerance in sea bass is linked with lower mRNA expression of *slc26a6* genes involved in Cl^- uptake in the posterior kidney. Regarding prolactin receptors, we showed a differential endocrine control in FW between the tolerant and intolerant sea bass associated to the incapacity to maintain blood hydromineral balance in FW_i sea bass. Facing strong rainfalls during their migration to transitional habitats like lagoons, sea bass may undergo strong salinity decreases triggering mortality in the

freshwater intolerant phenotype. Thus, freshwater intolerance could affect some sea bass in their migrations by preventing them to access transitional environments. It remains to be characterised how freshwater intolerance in *D. labrax* affects seasonal migratory behaviour in sea bass.

Acknowledgments

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

Highlights

- Freshwater intolerance in not due to an altered ion uptake at the gills
- Cl^- uptake-related genes are less expressed in the kidney of freshwater (FW) intolerant fish *vs* FW tolerant fish
- Branchial and renal prolactin receptors are differentially expressed between freshwater tolerance phenotypes

Résumé du Chapitre 3

L'acclimatation à de faibles salinités constitue un véritable challenge physiologique pour les organismes marins, mêmes pour les plus euryhalins comme le loup européen *Dicentrarchus labrax*. Ce poisson entreprend des migrations saisonnières vers les environnements de transitions comme les lagunes et les estuaires, caractérisés en partie par de grandes variations de salinités au cours de l'année. Nous avons pu mettre en évidence une variabilité intraspécifique dans la tolérance à l'eau douce chez cette espèce, avec en moyenne 30% d'individus incapables de supporter la dessalure. Dans cette étude, nous apportons de nouvelles informations quant aux mécanismes moléculaires à l'origine de l'intolérance à l'eau douce chez le loup aux niveaux branchial et rénal. En eau douce, des différences intraspécifiques dans les niveaux d'expression d'ARNm de plusieurs transporteurs ioniques et récepteurs à la prolactine ont pu être mesurées.

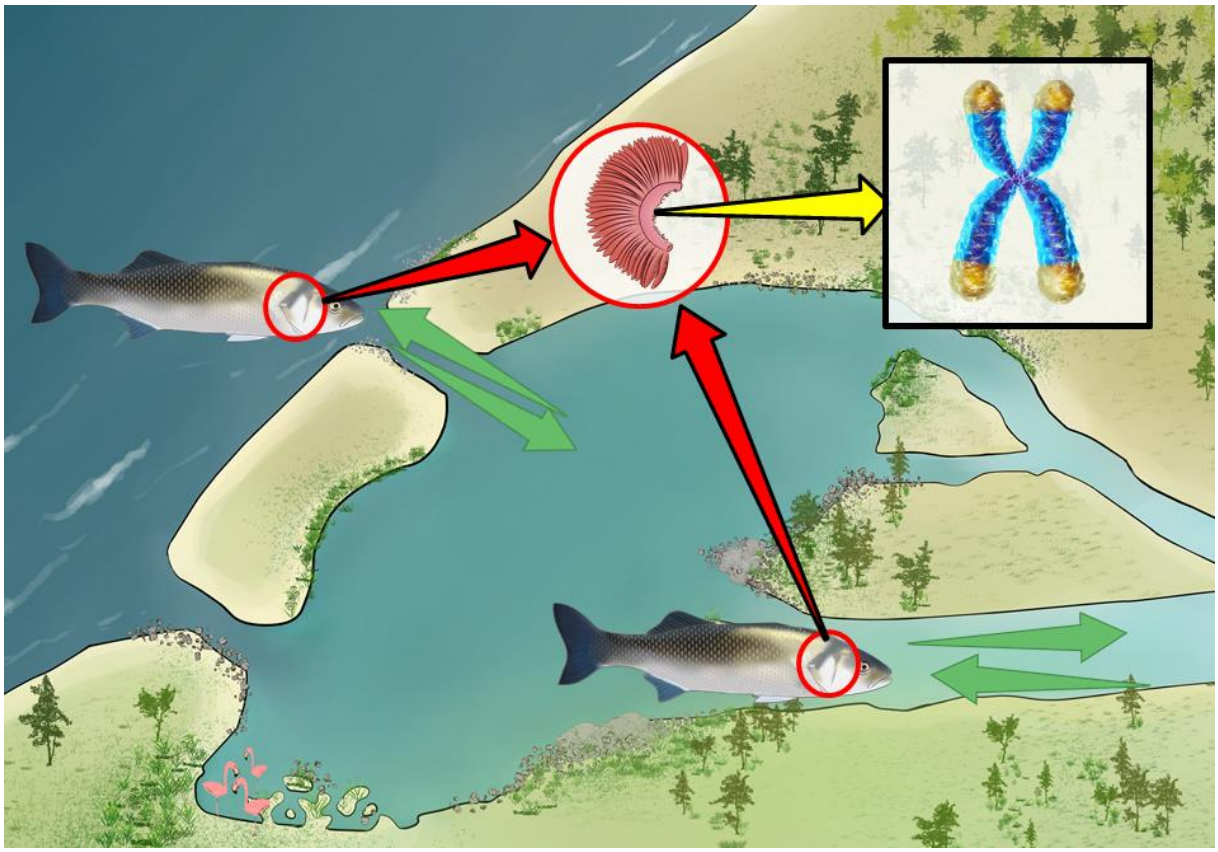
Nous avons montré que le transporteur anionique Cl^-/HCO_3^- *slc26a6c* était surexprimé au niveau branchial chez les individus intolérants. Il est localisé apicalement dans les ionocytes d'autres espèces et est impliqué dans l'absorption de Cl^- et la régulation acido-basique. Chez les intolérants, il a probablement un rôle dans la compensation de la faible osmolalité sanguine mesurée précédemment et la potentielle alcalose sanguine. De plus, le récepteur à la prolactine *prlr a* et le cotransporteur Na^+/Cl^- *ncc1* mais pas *ncc-2a* semblent tous deux légèrement surexprimés chez les intolérants et très variables entre les individus.

Dans le rein postérieur, les intolérants à l'eau douce montrent une surexpression du transporteur Cl^-/HCO_3^- *slc26a6* ainsi qu'une sous-expression du cotransporteur apical $Na^+/K^+/2Cl^-$ (*nkcc1b*) suggérant une moins bonne réabsorption d'ions au niveau rénal. De plus faibles niveaux d'expression des deux récepteurs à la prolactine (*prlr a* et *prlr b*) ont aussi été mesurés, ce qui illustre une dérégulation du contrôle hormonal par la prolactine, qui pourrait expliquer au moins en partie, la défaillance du rein à réabsorber les ions.

Ainsi d'après les résultats obtenus au niveau des transcrits, l'intolérance à l'eau douce semble être une conséquence d'une incapacité au niveau rénal à réabsorber les ions, qui n'est pas suffisamment compensée au niveau branchial.

Chapitre IV :

La dynamique des télomères est-elle un bon marqueur moléculaire de stress hypo-salins chez le loup *Dicentrarchus labrax* ?



Crédits : Thibaut L'Honoré

Prologue du Chapitre 4

Jusqu'à présent, nous avons cherché à mieux caractériser l'effet des stress hypo-osmotiques sur la physiologie des loups ainsi que sur la plasticité phénotypique associée. Dans ce dernier chapitre, nous avons cherché à mettre au point une méthodologie permettant de mettre en évidence des stress osmotiques passés, afin de mieux appréhender les effets à plus long terme mais aussi d'entrevoir des applications futures *in situ*.

Nous avons dans un premier temps localisé les motifs télomériques sur des caryotypes de loups par hybridations *in situ* réalisées sur la plate-forme de cytogénomique du CeMEB. Nous avons ensuite testé une méthodologie existante (Cawthon, 2002 & 2009, Appleby, 2016) de mesure de longueur de télomères par qPCR en prenant soin d'évaluer la variabilité opérationnelle entre deux réplicas techniques. Plusieurs méthodes de quantification de la longueur relative des télomères ont été comparées avec un focus sur l'utilisation d'un gène de référence correspondant soit à un gène présent en simple copie, soit un gène présent en plusieurs copies. La mise au point de la mesure des niveaux d'expression du gène *tert* codant pour la sous-unité catalytique de la télomérase chez le loup a été effectuée. Une fois ces deux mises au point réalisées, nous avons cherché à mesurer l'impact d'un stress hypo-osmotiques sur la dynamique des télomères chez des loups à deux stades différents : le stade larvaire (59 jours post-éclosion) et le stade juvénile (5 mois).

Ce dernier chapitre a été possible *via* l'obtention de crédits du CeMEB (Projet ERJ TELOMEL, Labex, Montpellier, 2019). Les résultats seront présentés aux 4ème Congrès d'EcoPhysiologie Animale (Octobre 2019, Rennes).

Are telomere dynamics good molecular markers of hyposaline stress in European sea bass *Dicentrarchus labrax*? A methodological approach

L'Honoré Thibaut[1,*], Lorin-Nebel Catherine[1], Blondeau-Bidet Eva[1], Perez Julie[2], Veyrunes Frédéric[2], Farcy Emilie[2]

[1]Univ Montpellier, CNRS, IFREMER, IRD, MARBEC, Montpellier, France

[2]Univ Montpellier, CNRS, IRD, EPHE, ISEM, Montpellier, France

Key words: telomere length, *tert* expression, freshwater acclimation, phenotypic plasticity, intraspecific variability

***Corresponding author:**

Thibaut L'Honoré

Univ Montpellier, CNRS, IFREMER, IRD, MARBEC

Place E. Bataillon,

34095 Montpellier cedex 05, France

Tel: +33 4 67 14 93 94

thibautlhonore@gmail.com

Abstract

Stressful events can alter organism physiology at several levels triggering allostatic responses. Telomeres are well-conserved repetititive DNA sequences mainly localised at chromosome's ends. They play a crucial role in DNA stability. Telomere length (TL) and telomerase replication transcriptase (*tert*) expression levels are new tools to assess environmental stress consequences in organisms like fish. The effect on hypo-saline stress naturally encountered during sea bass seasonal migrations is investigated through *tert* expression and TL in two developmental stages: larvae and juvenile. The methods available for TL measurements are numerous. They differ in sensitivity and cost. There are debates due to operatory variability and bias due to possible interstitial telomeric sequences. In this study, we highlighted the presence of interstitial telomeric sequences in sea bass karyotypes with fluorescent *in situ* hybridisation (FISH) at proximal and distal part of each chromosome. Then, we developed a quantitative method to measure TL and *tert* expression in the European sea bass *Dicentrarchus labrax* by quantitative real-time PCR. For the calculation of the relative telomere length through T/R ratio, two reference genes were tested: one single copy gene *mc2r* and one multicopy gene *18S*. Two calculation methods were compared: one using DNA quantity or the other using ΔΔCt method. Both methods exhibited the same tendency but calculating TL with *mc2r* as a reference seemed more sensitive. An increase in TL was detectable in 70-dph larvae's head exposed during 11 days at 0.4 ng µL of 17-β-oestradiol (E2) in sea water. Interestingly, this increase in TL was correlated to a strong 7-fold increase in *tert* expression in larvae's head after 11 days E2 exposure. Conversely, the exposure to a moderate 5-ppt hyposaline stress of 11 days performed in 2 month-old sea bass or an acute 2 weeks freshwater stress in 5 month-old fish did not trigger any significant TL or *tert* expression change. The use of these methods as potential molecular proxies to study the effect of environmental stress in sea bass still requires some development but it could be a promising tool.

Introduction

Telomeres are eukaryotic well conserved terminal regions of chromosomes, composed of repetitive sequences of TTAGGG in vertebrates whose length is maintained by the telomerase (Blackburn and Gall, 1978). Telomerase is constituted of telomerase RNA providing the template for the catalytic subunit telomerase reverse transcriptase (TERT) (Blackburn, 1991). It plays a crucial role in chromosome stability over replications (Blackburn, 2005). Telomere length (TL) undergoes natural shortening with ageing due to incomplete replication (Harley *et al.*, 1990; Webb *et al.*, 2013). Telomere length and telomerase activity are often used to study ageing in human. In human, chronic oxidative stress and life stressors can accelerate telomere attrition by decreasing telomerase activity or *tert* expression levels (Epel *et al.*, 2004; Houben *et al.*, 2008; Starkweather *et al.*, 2014). A

link between the decrease in telomere length and increase in diseases, cancer and mortality was demonstrated in humans (Cawthon *et al.*, 2003; Blasco, 2005) but also in zebrafish (Carneiro *et al.*, 2016). Environmental stressors can trigger oxidative stress through an increase in reactive oxygen species (ROS) production. ROS oxidise DNA and particularly telomeres which are rich in guanine, that succumb to oxidation more than other DNA bases (Oikawa and Kawanishi, 1999; von Zglinicki, 2000, 2002; Kawanishi and Oikawa, 2004). Oxidative stress can also reduce telomerase activity as shown *in vitro* (Kurz *et al.*, 2004; Boonekamp *et al.*, 2017). In human T lymphocytes, elevated cortisol levels trigger a decrease in telomerase activity (Choi *et al.*, 2008). However, as highlighted by Reichert & Stier (2017), these relationship have still to be clarified *in vivo*. More recently, some studies have examined the potential use of telomere length as a stress marker in fish (Anchelin *et al.*, 2013; Henriques *et al.*, 2013; Naslund *et al.*, 2015; Debes *et al.*, 2016). The link between glucocorticoid levels and oxidative stress highlighted by Kurz et al. (2004) suggests that chronic stress and high levels in glucocorticoids may be linked with TL shortening. However, in brown trout *Salmo trutta*, exogenous cortisol triggers a 2 week increase in the antioxidant glutathione levels but the measurement of the oxidation of thiols in glutathione as a sensor of oxidative stress levels did not reveal an imbalance between pro-oxidants and antioxidants (Birnie-Gauvin *et al.*, 2017). Stress response mediated by cortisol strongly interact with osmoregulation in teleosts, since cortisol is also known to play a key role in the maintenance of hydromineral balance in freshwater and seawater conditions (Franklin *et al.*, 1992; Mommsen *et al.*, 1999; Kelly and Chasiotis, 2011; Liew *et al.*, 2015). If salinity changes affect cortisol levels, we can suggest that salinity changes may indirectly affect TL, through corticosteroids modulation. In sea bass, we have recently shown differential responses to freshwater. Intolerance to freshwater, was correlated to higher expression of mineralocorticoids receptors in the gills and lower expression of glucocorticoids receptors in the posterior kidney (L'Honoré *et al.*, 2019). In fish, gills are an interesting organ to consider for TL dynamics analysis since gills are a multifunctional organ directly in contact with the surrounding water. Gills are involved in immunity, nitrogen excretion, acid-base regulation, gas exchange and osmoregulation. In particular, the presence of mitochondrion-rich cells (or ionocytes), that play a crucial role in osmoregulation (Evans *et al.*, 2005), suggests that the response to salinity change may increase ROS production, as a by-product of cellular respiration. Indeed, mitochondria are known to be the main sources of ROS production within cells (Lambert and Brand, 2009). However, in marine teleosts, there is no evidence that hyposaline stress triggers oxidative stress. Conversely, Sinha et al. (2015) have shown in *D. labrax* that fish acclimated to brackish or hypo-saline water (20, 10, 2.5 ppt), oxidative stress indices (i.e. xanthine oxidase activity, H_2O_2 and malondialdehyde) remained unaffected in hepatic tissues of fed fish. In

another study in sea bass (Lorin-Nebel *et al.*, in prep), a 2-week FW exposure resulted in a decrease of hepatic antioxidant defences (catalase and superoxide dismutase) but no clear oxidative damage was observed. Branchial epithelium is characterised by a rapid cell turnover and remodelling in response to fluctuations of environmental factors like oxygen availability, temperature and salinity (Sollid & Nilsson, 2006; Nilsson *et al.*, 2012; Masroor *et al.*, 2018). In this study, we consider the gills as a potential organ of interest to study the relationships between hypo-osmotic stress and TL in sea bass.

The European sea bass *Dicentrarchus labrax* is a euryhaline and eurythermic species that breeds offshore but larvae sometimes drift inshore. At juvenile stages, *D. labrax* undertake seasonal migration in transitional habitats, where salinity may fluctuate widely from hypersaline to even fresh water (FW) (Pawson *et al.*, 1987; Kelley, 1988; Waldman, 1995; Dufour *et al.*, 2009). These habitats may exhibit strong temperature and salinity variations throughout the year, making these environments stressful for marine organisms. Stress exposure in early life stages can have long-term effects on physiology and fitness (Burton and Metcalfe, 2014). The relationship between temperature change and telomere attrition has been the most studied in fish. In mosquitofish *Gambusia holbrooki*, a decrease in temperature for 24 h was associated with a decrease in telomere length (Rollings *et al.*, 2014), whereas an increase in temperature triggered telomere attrition in the Siberian sturgeon *Acipenser baerii* and in brown trout (Debes *et al.*, 2016; Simide *et al.*, 2016). In medaka *Oryzias melastigma*, hypoxia up-regulated *tert* expression levels (Yu *et al.*, 2006). In three fish species (medaka, red snapper *Lutjanus argentimaculatus* and orange-spotted grouper *Epinephelus coioides*), it has been demonstrated that telomerase activity was repressed with environmental stressors such as exposure to benzopyrene (Peterson *et al.*, 2015). The gene *tert* encoding the catalytic unit telomerase reverse transcriptase was found expressed in all tissues analysed in European hake *Merluccius merluccius* and Atlantic cod *Gadus morhua*, with a higher expression whentissue renewal was elevated, suggesting a role of *tert* in maintaining long-term cell proliferation capacities (López de Abechuco *et al.*, 2014). We hypothesised that species undertaking migrations to fluctuating environments, such as sea bass, are potentially more subjected to TL reduction due to telomerase activity or *tert* expression changes associated to chronic stress in fluctuating environments such as lagoons or estuaries. To our knowledge, the effect of salinity fluctuation on telomere dynamics has never been studied in marine teleosts. The wide inter-individual variability found in the European sea bass either in glucocorticoid profiles (Samaras *et al.*, 2016), freshwater acclimation (Nebel *et al.*, 2005; L'Honoré *et al.*, 2019) or hypoxia tolerance (Claireaux *et al.*, 2013; Joyce *et al.*, 2016) when comparing extreme tolerance phenotypes makes sea bass an interesting candidate species to study telomere dynamics as a molecular biomarker of environmental stress.

The presence of interstitial telomeric sequences (ITS) is a key issue to investigate TL in a novel species. In fact, using quantitative real-time PCR (q-PCR) method, ITS measurement yields to over-estimate TL (Foote *et al.*, 2013). The presence and the quantity of ITS are shown to be species-specific (Ocalewicz *et al.*, 2004; Mudry *et al.*, 2007; Ocalewicz, 2013) and perhaps individual-specific, as it was highlighted in three bird species (Foote *et al.*, 2013). Prior to study TL in sea bass, ITS presence and localisation have to be elucidated in *D. labrax* genome. Karyotype analysis of *Dicentrarchus labrax* revealed 24 pairs of chromosomes gradually decreasing in size (Sola *et al.*, 1993). Previous studies in sea bass have shown no relationship between ageing and blood TL using the telomeric restriction fragment (TRF) method which does not include ITS because restriction enzyme do not have access to ITS since DNA is not denatured (Haussmann & Vleck, 2002; Horn *et al.*, 2008). But the wide diversity and sensitivity of TL measurement methods may explain this negative result (Dagnall *et al.*, 2017; Lai *et al.*, 2018). The q-PCR–based method developed by Cawthon (2002) consisting in the relative quantification of telomeric hexamer repeats, is probably the most widely used technique to assess TL and was never tested in sea bass. This technique is easy to conduct, requires small amount of starting DNA but exhibits large inter-laboratory variations and only provides a relative TL (Lai *et al.*, 2018). Nevertheless, this technique was successfully used in many fish species (Hatakeyama *et al.*, 2008; Hartmann *et al.*, 2009; Ocalewicz, 2013; Gao and Munch, 2015; Simide *et al.*, 2016). Measuring *tert* expression by q-PCR could provide another molecular marker, not influenced by ITS, which could be regulated more rapidly than TL.

The first aim of this study was to determine the occurrence and the localisation of telomere sites in *D. labrax* genome using FISH technique in order to test if interstitial telomeric sites are detected. Then, we have used the q-PCR method developed by Cawthon (2002, 2009) to test whether (i) q-PCR is a reliable technique to measure TL in sea bass and what normalisation gene is the most appropriate (single or multicopy gene) and (ii) to characterise if hyposaline stress affects telomere dynamics in *D. labrax*. For this, relative TL and *tert* mRNA expression were measured (Cawthon, 2002, 2009) in sea bass exposed to hyposaline stress. A moderate 5-ppt hyposaline stress of 11 days was performed in 2 month-old sea bass when cell turnover is important, with a focus on head and body telomere length and *tert* mRNA expression. The natural estrogen 17-β-oestradiol (E2) was used as positive control as it is known to activate *tert* expression and telomerase activity (Misiti *et al.*, 2000; Bermudez *et al.*, 2006; Zhou *et al.*, 2013). The effect of an acute 2 weeks freshwater stress was investigated in 5 month-old sea bass, with a focus on TL in gills in relation with freshwater tolerance phenotypes, previously described in L'Honoré et al. (2019, submitted).

Materials and methods

1. Experimental design

Fish were issued from in vitro fertilisation of unrelated wild native West Mediterranean breeders (40 males and 23 females) in order to obtain a large genetic diversity. They were grown at the Ifremer Station at Palavas-les-flots (Hérault, France) under a 16/8 hours light/dark photoperiod in SW at 20°C.

For the experiment assessed on larvae, , 120 sea bass larvae of 59-dph (1.47±0.13 cm) were randomly transferred either in SW, in 5 ppt water, in SW containing 0,00008% ethanol (Fluka) or in SW containing 0.4 $ng.L^{-1}$ E2 (98% purity, Sigma) in a recirculated and aerated system made up of two McDonald jars of 8 litres each and a 5-litre beaker, representing a total volume of 22 L. E2 and ethanol were continuously delivered to the exposure system using a peristaltic pump (IPC-N, Ismatec) at a flow rate of 2 mL/h, with a renewal rate of 12 hours. For each condition, 4 litres were renewed every 48h. Larvae were fed daily with artemia nauplii from AF INVE®. Temperature, salinity, dissolved oxygen concentration and ammonia were checked every day. At 70 dph (following an exposure time of 11 days), 30 larvae of each condition were euthanised in 100 ppm benzocaine and divided in two samples (15 larvae for RNA extraction and 15 larvae for gDNA extraction). The head was separated from the body by cutting under a binocular magnifier (X40, Realux, France) between the opercula and the pectoral fins and then stored at -80°C for further analysis.

For the experiment assessed on juveniles, 5 month-old juveniles were obtained according to L'Honoré *et al.* (2019). Briefly, fish were pre-acclimated for 24h in 15 ppt water before being transfered in freshwater (FW) for 2 weeks. Tolerant and intolerant phenotypes were sorted according to fish behaviour as described in L'Honoré et al. (2019). Fish exhibiting erratic swimming, isolation from the shoal associated with low reflexes and stronger pigmentation were identified as freshwater-intolerant phenotype (FW_i). These animals were characterised by an osmoregulatory deficiency in FW (L'Honoré et al., 2019, submitted). The three groups analysed were: seawater control, freshwater tolerant fish (FW_t) and FW_i. At the end of the exposure, gills were sampled on 100 ppm benzocaine euthanised fish and stored at -80°C.

The experiments were conducted according to the guidelines of the European Union (directive 86/609) and of the French law (decree 87/848) regulating animal experimentation. The experimental design has been approved by the French legal requirement concerning welfare of experimental animals (APAFIS permit no. 9045-201701068219555).

2. Fluorescent *in situ* hybridisation on telomere sequence DNA and microscope analysis

Karyotype analysis and fluorescent *in situ* hybridisation were realised at the Cytogenetic platform (ISEM-CBGP, CeMEB, Montpellier, France). The head kidney was sampled in 2 males and 2 females at the age of 10 months and maintained in SW. Oligonucleotide telomeric probe $(TTAGGG)_7$ already labelled with Cy3 at its 5' end was purchased (biomers.net, Ulm, Germany). Fluorescent *in situ* hybridisation (FISH) was performed following the same procedure as described in Ozouf-Costaz *et al.* (2015), counterstaining the chromosomes with DAPI (4',6-diamidino-2-phenylindole)-antifade solution (Vectashield, Vector Laboratories, Peterborough, UK). Preparations were then analysed using a Zeiss Axioplan 2 Imaging epifluorescence microscope fitted with CCD camera and Cytovision 7.4 software (Applied Imaging, San Jose, CA).

3. gDNA extraction

Genomic DNA (gDNA) extraction was performed on 70-dph sea bass larvae separated in two parts, head and body and gills of 5 month-old sea bass juveniles using the Maxwell® 16 Buccal Swab LEV DNA Purification Kit (Promega, Charbonières, France). Samples were eluted in 50 µL of ultrapure water. Quantity was verified using a Qubit dsDNA BR Assay Kit (ThermoFisher Scientific), concentrations ranging from 60 to 200 µg mL^{-1}. Purity was also verified using the NanoDrop™ One/One^C Spectrophotometer (Thermo Scientific, Waltham, MA, USA) through A260/A280 and A260/A230 ratios.

4. RNA extraction and reverse transcription

RNA extraction was performed using the total RNA extraction kit (Nucleospin® RNA, Macherey-Nagel, Germany). Quantity and purity of extraction products were verified using a UV spectrophotometer (NanoDrop™ One/OneC Spectrophotometer, Thermo Scientific, Waltham, MA, USA). Reverse transcription was performed using one microgram of RNA using the qScript™ cDNA SuperMix (Quanta Biosciences™) providing all necessary components for first-strand synthesis: buffer, oligo(dT) primers, random primers and qScript reverse transcriptase.

5. Quantitative real-time polymerase chain reaction

To measure relative gene expression, we used 384-wells plates filled with an Echo®525 liquid handling system (Labcyte Inc., San Jose, CA, USA). Each well contained a mix composed by 1.5 µL of LightCycler-FastStart DNA Master SYBR-Green I™ Mix (Roche, Manheim, Germany), 0.27 µL of each primer (forward and reverse

primers at 0.9 µM final concentration), 0.23 µL of ultrapure water and 1 µL of cDNA or gDNA. Efficiency of each primer pairs reported on Table 1 were obtained by standard curves performed on all-samples pools of cDNA (*pcna*, *tert*, *casp8*, *casp9* and *l13*) or gDNA (*tel*, *mc2r* and *18S*). *Pcna* was used as a cell proliferation marker (Sadoul *et al.*, 2018) whereas *casp 8* and *casp 9* were used as extrinsic or intrinsic cell apoptosis markers respectively (Olsson and Zhivotovsky, 2011). For *tert*, *pcna*, *casp 8* and *casp 9* mRNA expression analyses, the q-PCR conditions were as follows: 2 min denaturation at 95 °C followed by 35 cycles (95 °C for 30 s, 61 °C for 45 s and 72 °C for 1 min) followed by a final elongation step at 72 °C for 4 min. The reference gene *L13* was chosen according to previous studies performed on sea bass (Mitter *et al.*, 2009). Relative expression of *tert*, *pcna*, *casp 8* and *casp 9* were determined using the comparative ΔΔCt method (threshold cycle number) with SW 59-dph larvae as a reference, as described in Pfaffl (2001).

Table 1 Primer sequences used for relative telomere length and gene expression analysis

Target gene	Primer name	Sequences ID	Sequence (from 5' to 3')	Efficiency	Reference
Pcna	PCNA F	DLAgn_00120330	CAGAGCGGCTGGTTGCA	1.7 (head + body)	Sadoul et al., 2018
	PCNA R		CACCAAAGTGGAGCGAACAA		
Tert	TERT F	DLAgn_00199170	GGGTCAGGGGCTTCTTGTAC	2.1 (head+body)	This study
	TERT R		AGAAACAGGCTCGAACCAGG		
Casp8	CASP8 F	FJ225665	TGTCAGGGAAGCCTCTACCA	2.1 (head+body)	Paiola et al., 2018
	CASP8 R		CATCCCCAGCAGGAAGTCAG		
Casp9	CASP9 F	DQ345775	CGAATGCAACCGAGCACAAA	1.9 (head+body)	Paiola et al., 2018
	CASP9 R		ACTAACGACCGCCAATGAGG		
Tel	TEL G		ACACTAAGGTTTGGGTTTGGGTTTG GGTTTGGGTTAGTGT	2 (gills)	Cawthon et al., 2009
	TEL C		TGTTAGGTATCCCTATCCCTATCCC TATCCCTATCCCTAACA	2 (head+body)	
L13	L13 F	DT044539	TCTGGAGGACTGTCAGGGGCATGC	2 (head+body)	Mitter et al., 2009
	L13 R		AGACGCACAATCTTGAGAGCAG		
Mc2r	MC2R F	FR870225	CATCTACGCCTTCCGCATTG	2 (gills)	Samaras & Pavlidis, 2018
	MC2R R		ATGAGCACCGCCTCCATT	2.0 (head+body)	
18S	18S F	KU820862	AGGAATTGACGGAAGGGCAC	2 (gills)	Masroor et al., 2018
	18S R		TAAGAACGGCCATGCACCAC	1.8 (head+body)	This study

For relative telomere length analysis, the single copy gene *mc2r* and the multicopy gene *18S* were tested according to previous studies (Wang *et al.*, 2013). Conditions used in the PCR were adapted from (Cawthon, 2009) with some modifications as follows: 15 min denaturation at 95 °C followed by 2 cycles (94° C for 15 s,

49° C for 15 s) followed by 35 cycles (95 °C for 15 s, 62 °C for 10 s and 74 °C for 15 sec). Relative TL was performed using the ratio between telomere and reference gene signals known as T/R ratio, calculated by two distinct methods: the ΔΔCt method as described in Cawthon (2002) using these formulae:

$$T/R = 2^{-\Delta\Delta Ct}$$

$$\Delta\Delta Ct = \Delta Ct \text{ (70-dph condition)} - \Delta Ct \text{ (59dph-condition)}$$

$$\text{with } \Delta Ct = Ct \text{ (telomere primers)} - Ct \text{ (reference gene primers)}$$

The DNA quantity method as described in Cawthon (2009). Briefly, the T/R ratio for an experimental DNA sample is T, the number of nanograms of the standard DNA that matches the experimental sample for copy number of the telomere template, divided by R, the number of nanograms of the standard DNA that matches the experimental sample for copy number of the reference gene.

6. Statistics

Statistical analyses were performed on GraphPad Prism (version 6, GraphPad Software Incorporated, La Jolla, CA 268, USA). Since q-PCR analyses fitted normality test (D'Agostino-Pearson test) but not homoscedasticity test (Bartlett test), non-parametric tests were performed (Kruskal-Wallis test followed by Dunn's test). Since data for correlation analysis fitted with normality assumption, Pearson correlation tests were used. Experimental values are reported as means ± s.e.m. and *p*-value threshold was set at 5%. Coefficients of variation within the plate and between the two-plates have been calculated to estimate inter-assay variability.

Results

1. Localisation of DNA telomere sequences in *D. labrax* karyotypes

Karyotype analyses confirmed the presence of 2n=48 chromosomes as expected in sea bass (Figure 1, Sola et al., 1993). FISH revealed that telomere sequences were localised distally but ITS localised proximally were also found. We did not notice any difference in signal intensity or localisation of telomere sequences between males and females using FISH.

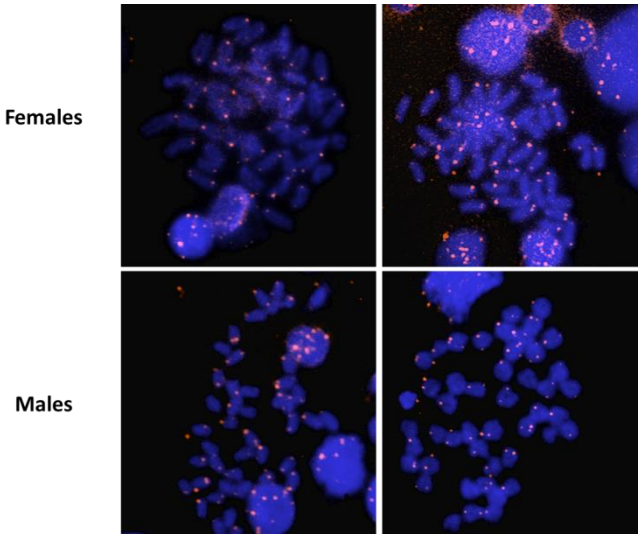


Figure 1 Fluorescent in situ hybridisation (FISH) in 10-month-old European sea bass using telomeric probe $(TTAGGG)_7$ labelled with Cy3 at its 5' end, indicated by red colour. 2 males and 2 females were analysed with N = 3 slides per fish

2. Relative telomere length measurements

2.1 Method validation

Primer efficiency of the single copy gene *mc2r* and the multicopy gene *18S* were validated between 1.8 and 2.0 (Figure 2). The primers' specificity was checked using the melting point (T_m) of the product for each primer pair and displayed a unique pike at the expected temperature (Figure 3). An inter-plate assay was performed to investigate the potential variability between two different q-PCR runs. The inter-plate correlation r^2 were respectively above 0.98 and 0.99 for the relative telomeric hexamer repeat quantification with*mc2r* and *18S* as reference genes (Pearson test, $P < 0.0001$ for each gene, Figure 4).

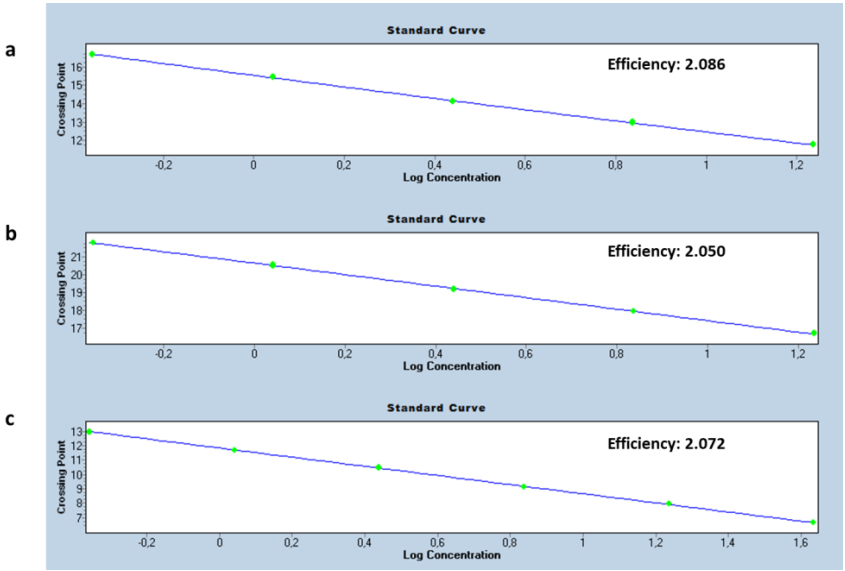


Figure 2 Standard curves for **(a)** *tel*, **(b)** *mc2r* and **(c)** *18S* primers, used to determine T/R ratios. Five to six concentrations of a pool of 16 genomic DNA samples of gills in 5-month-old sea bass were prepared by 1.5-fold serial dilution (100 ng, 40 ng, 16 ng, 6.4 ng, 2.56 ng, 1.024 ng)

Coefficients of variation (CVs) did not exhibited values > 3% for both intra-assay CV and inter-assay CV as resumed in Table 2 (see at the end). Obtained results using the two methods of T/R calculation were highly

correlated either with DNA from 5-month old sea bass gills and either with DNA from sea bass larvae's head and body (Pearson's test, $P < 0.0001$, Figure 5).

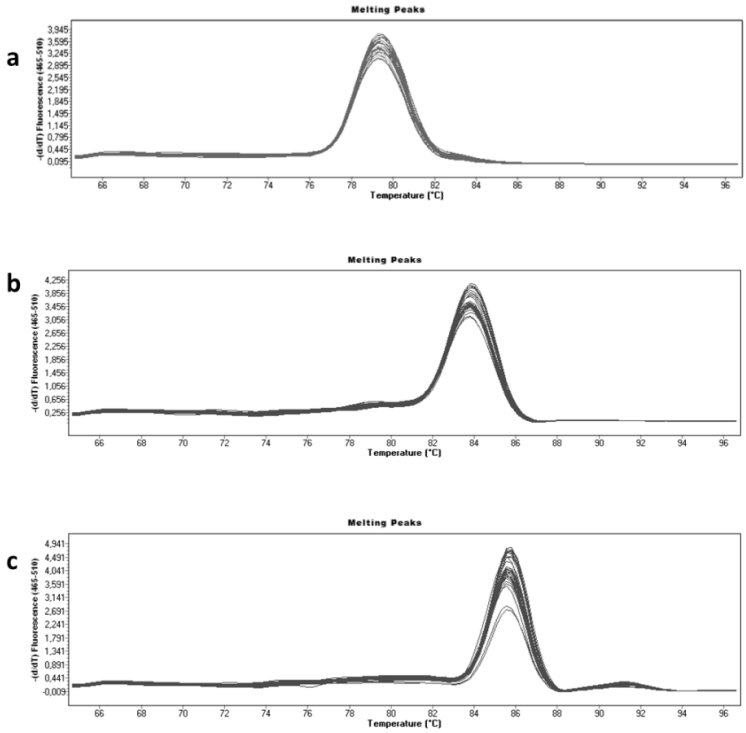


Figure 3 Melting curves performed in gills for (a) tel, (b) mc2r and (c) 18S primers, performed on a pool of 16 genomic DNA samples of 5-month old sea bass illustrating the specificity of the primers

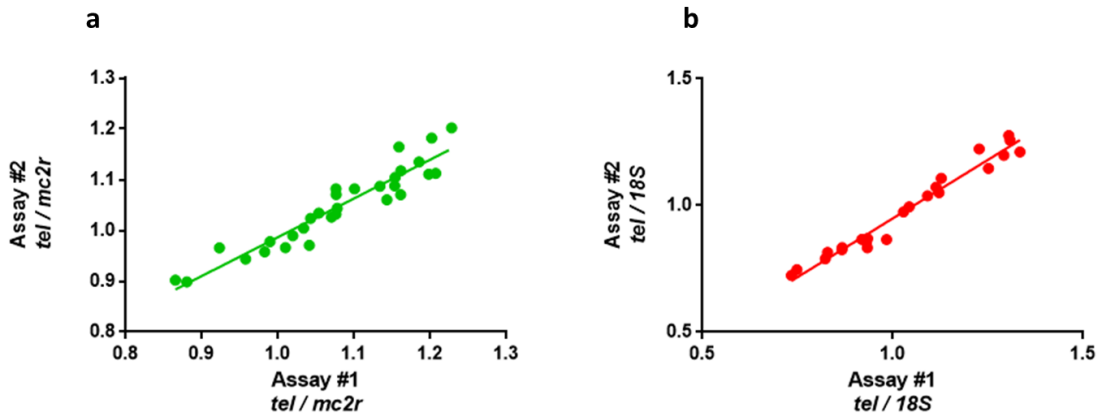


Figure 4 Inter-assay variation of T/R ratio using DNA quantity calculation. The two assays were performed separately using 16 genomic DNA samples measured in duplicates. The average of the duplicate values was used for T/R ratio calculation. Assay #1 was plotted against Assay #2 in order to study the linear correlation depending on the the reference gene used **(a)** *tel / mc2r* ($r^2 = 0.9866$, Pearson test, $P < 0.0001$) and **(b)** *tel / 18S* ($r^2 = 0.9659$, Pearson test, $P < 0.0001$)

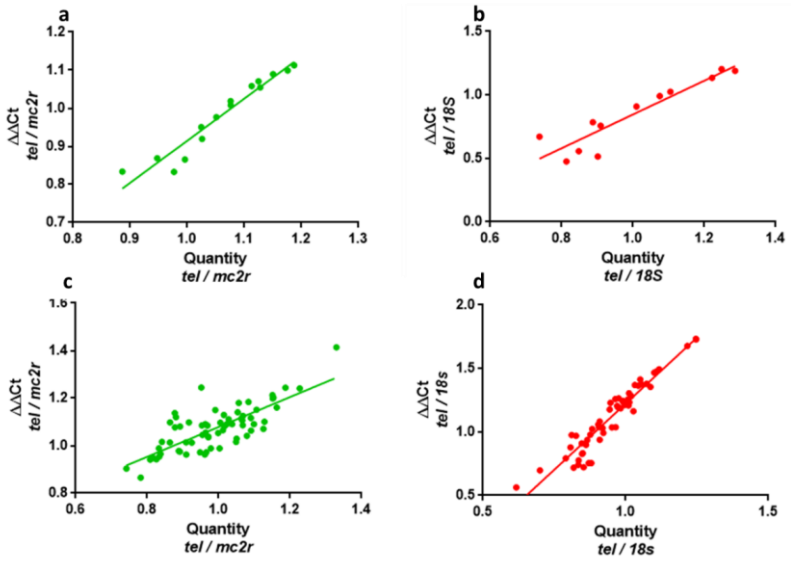


Figure 5 Comparaison of T/R ratio calculation methods of **(a-b)** 16 genomic DNA samples of gills in 5 month-old sea bass measured in duplicates and **(c-d)** 68 genomic DNA samples of 59-dph sea bass heads. The T/R ratio was calculated using **(a-c)** the single copy gene *mc2r* and **(b-d)** the multiple copy gene *18S* as reference. The T/R ratio calculated by the DNA quantity method was plotted against the T/R ratio calculated by the ΔΔCt method (**a**: r^2 = 0.9324, **b**: r^2 = 0.8560, **c**: r^2 = 0.6068 and **d**: r^2 = 0.9079, Pearson test, $P < 0.0001$)

2.2 Telomere length and freshwater exposure

In gills of 5month-old sea bass, since CVs were very low, the mean of T/R of assay #1 and assay #2 was used for each sample and reference gene (R) (Figure 6). We measured no significant difference in relative TL of 5 month-old sea bass between SW, FW_t and FW_i whatever the reference gene (*mc2r* or *18S*) or the method used (DNA

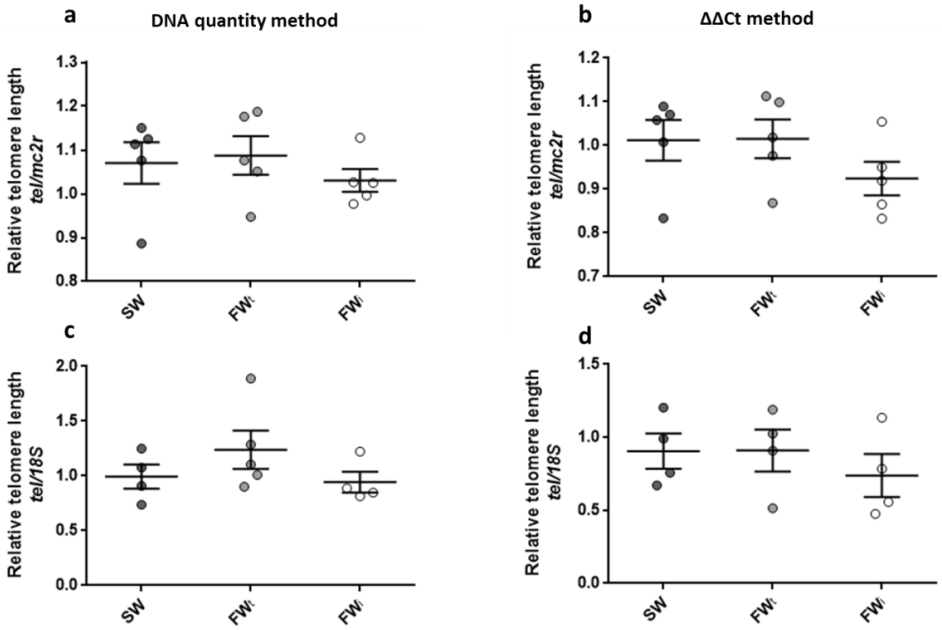


Figure 6 Relative telomere length expressed as T/R ratio with mc2r (a-b) or 18S (c-d) as reference genes. The T/R ratio was calculated using (a-c) DNA quantity or (b-d) ΔΔCt methods in gills of 5 month-old sea bass in seawater and after 2 weeks in fresh water (Kruskal-Wallis test, P > 0.05, means ± s.e.m, N=3-5). SW: control fish in seawater, FWi: intolerant fish to fresh water, FWt: tolerant fish to fresh water

quantity method: Kruskal-Wallis test, $P = 0.4969$ and $P = 0.2361$ respectively or ΔΔCt method: Kruskal-Wallis test, $P = 0.1905$ and $P = 0.6478$ respectively).

In the body of 2 month-old sea bass larvae, we did not measure any significant difference in relative TL between the five conditions using *mc2r* or *18S* as reference gene and whatever the method used (Kruskal-Wallis test, $P = 0.0999$, $P = 0.0320$, $P = 0.2465$, and $P = 0.3303$ respectively, Figure 1S). However in the head, relative TL were generally higher in 70-dph larvae compared to 59-dph larvae. This increase was statistically significant for 5ppt, SW + E2 0.4 and in SW + solvent conditions (Dunn's test, $P < 0.0001$, $P < 0.0001$ and $P = 0.0090$, Figure 7a) but not SW condition ($P = 0.0609$) using *mc2r* as reference gene and the DNA quantity method. Furthermore, relative TL was also significantly higher after 11 days in SW + E2 0.4 than in SW (Dunn's test, $P = 0.0388$). Using the ΔΔCt method and *mc2r* for calculating T/R, SW + E2 0.4 and SW + solvent conditions displayed significant higher relative TL compared to the 59 dph condition (Dunn's test, $P = 0.0011$ and $P = 0.0139$ respectively, Figure 7b). With *18S*, a significantly higher relative TL was measured between SW + E2 0.4 and 59 dph $_{SW}$ condition using the ΔΔCt method (Dunn's test, $P = 0.0121$, Figure 7c) whereas no difference could have been measured between the five conditions with the DNA quantity method (Dunn's test, $P > 0.05$, Figure 7d).

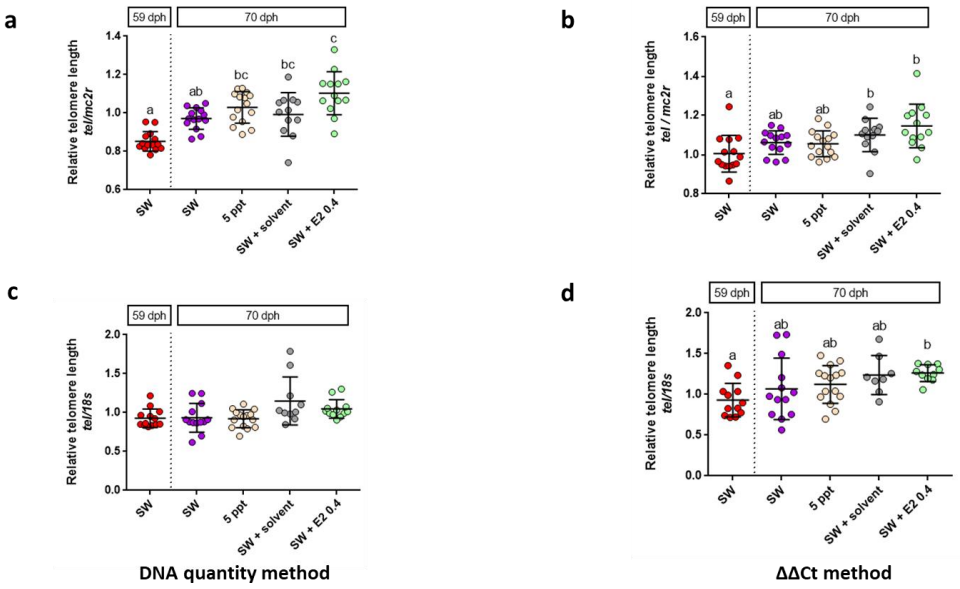


Figure 7 Relative telomere length using the DNA quantity method **(a,c)** or ΔΔCt method **(b,d)** in the head of 59-dph sea bass larvae maintained in seawater (59 dph $_{SW}$), then transfered for 11 days in: SW (70 dph $_{SW}$), 5 ppt water (70 dph $_{5ppt}$), 17-β-oestradiol at 0.4 ng.L^{-1} (70 dph $_{SW + E2\ 0,4}$) or in ethanol at 0.00008 % (70 dph $_{SW + solvent}$). The expression has been normalised according to the expression of *mc2r* or *18S*. Different letters denote significant differences between groups (Kruskal-Wallis followed by Dunn's test, $P < 0.05$, means ± s.e.m, N=13-15)

Overall, the results are consistent whatever the method used but the signal/noise ratio is stronger using the single copy *mc2r* as a reference gene, and the ΔΔCt method for relative TL calculation, so that statistically significant differences between conditions could be highlighted.

3. *Tert, pcna, casp8, casp9* expression in sea bass larvae following salinity decrease and 17-β-oestradiol exposure

In larvae's head, a significantly higher expression of *tert, pcna, casp 8* and *casp 9* was measured after 11 days in SW + E2 0.4 compared to 59-dph fish (Dunn's test, P = 0.0006, P = 0.0028, P = 0.0010 and P = 0.0043 respectively, Figure 8a-b-c-d). *Pcna* expression was significantly down-regulated after 11 days in hyposaline condition compared to SW, SW + E2 0.4 and SW + solvent conditions (Dunn's test, P = 0.0176, P < 0.0001 and P = 0.0012, Figure 8b). Additionally, after 11 days *pcna* expression was significantly higher in SW + solvent than 59-dph $_{SW}$ condition (Dunn's test, P = 0.0492). *Caspase 8* expression was significantly higher after 11 days in hyposaline and SW + E2 0.4 conditions compared to 59-dph fish (Dunn's test, P = 0.0009 and P = 0.0002 respectively, Figure 8c). Caspase 8 was significantly up-regulated at 70-dph in SW + E2 0.4 than in SW + solvent (Dunn's test, P = 0.0091). Concerning *caspase 9*, hyposaline and E2 conditions also displayed significantly higher expression compared to both 59-dph and solvent control conditions (Dunn's test, P = 0.007 and P = 0.0035 for 5 ppt condition, P = 0.0043 and P = 0.0119 for E2 condition, Figure 8d).

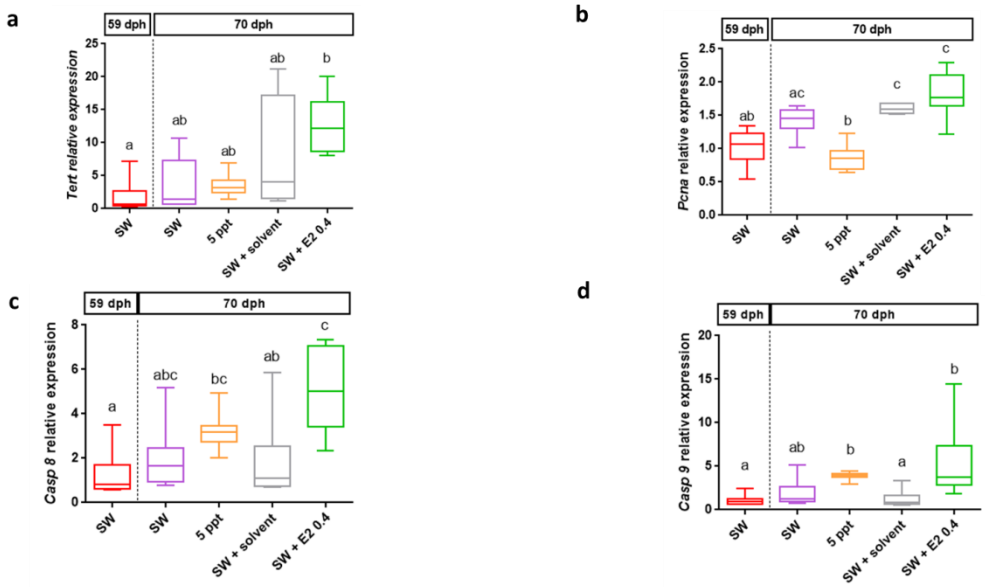


Figure 8 Relative expression of **(a)** *tert*, **(b)** *pcna*, **(c)** *casp 8* and **(d)** *casp 9* in the head of 59-dph sea bass maintained in seawater (59 dph $_{SW}$), then transfered for 11 days in SW (70 dph $_{SW}$), 5 ppt water (70 dph $_{5ppt}$), in ethanol at 0.00008% (70 dph $_{SW\ +\ solvent}$) or 17-β-oestradiol at 0.4 ng.L^{-1} (70 dph $_{SW\ +\ E2\ 0,4}$). The expression has been normalised to the ribosomal protein *L13*. Different letters denote significant differences between groups (Kruskal-Wallis followed by Dunn's test, P < 0.05, means ± s.e.m, N=4-10)

Discussion

1. Method validation

As reviewed in Lai *et al.* (2018), one potential bias for estimation of relative TL using the q-PCR method is the large variations among users and assays and within the same sample. Using the "two plate" control as described in Appleby (2016), we show that the operational variability was limited, especially by the use of the automatic pipettor Echo®525 liquid handling system. However, despite the low coefficients of variation obtained within plate and between plates, we were not able to detect variation in TL between experimental conditions in sea bass gills. This is probably due to the low number of individuals analysed in this study (n=3-5). Since a strong intraspecific variability in TL is expected, as previously observed in sea bass blood by Horn et al. (2008), more individuals should be tested. In the literature, the number of individuals per condition generally reached at least 50 individuals per condition, suggesting that increasing the replicate number may be necessary to detect differences in relative TL regarding hyposaline stress. Interestingly, in the head of 2-month-old larvae, using only 10 to 14 individuals per condition, significant changes in relative TL have been measured in 70-dph larvae compared to 59-dph larvae. In addition, this study highlights that TL and *tert* expression could reflect a general change in telomere dynamic associated with intense cell division occurring at this development stage, as suggested by the relative higher *pcna* expression measured in 70-dph larvae compared to 59-dph larvae in SW. In medaka, a higher telomerase activity was measured at the age of 2 months than before hatching (Peterson *et al.*, 2015). In the 2-month-old larvae, we have decided to separate the head and the body because organs were too small to be analysed separately at this stage. It is interesting to note that TL changes were more important in head, despite the homogenisation of the different tissues from the head (e.g. cartilage, bone, muscle, brain, eyes, and gills) and the body (e.g. kidneys, intestine, muscle). Actually, a tissue-centred approach could allow a more accurate detection of TL changes because cell proliferation and rate of telomere attrition may differ between different tissue types (Nussey *et al.*, 2014). Interestingly, though not significant, an increase of relative TL was measured after waterborne exposure to 0.4 ng L^{-1} of E2 compared the solvent condition. E2 is known inducer of *tert* expression and telomerase activity in higher vertebrates (Misiti *et al.*, 2000; Zhou *et al.*, 2013). This result should be confirmed using more individuals. However, this method did not allow highlighting any significant TL change after hyposaline stress. We cannot rule out that the q-PCR detection method is not robust enough to detect small variations in TL in the gills because of possible rapid telomere maintenance with hyposaline stress reduction. Indeed, we can hypothesise that TL attrition due to hyposaline exposure is rapidly repaired by an increase in telomerase activity or *tert* expression. Thus, we could refine the method by measuring the longest

telomere which is more susceptible to be damaged by ROS and could be less repaired than shorter telomeres (Bauch *et al.*, 2014). We also have to mention that the presence of ITS in sea bass could reduce the sensitivity of the method especially the detection of small TL changes. In a future work, it would be interesting to further explore the proportion of ITS *vs* terminal telomeric sequences in sea bass using quantitative-FISH known as QFISH. This could allow investigating whether the inter-individual variability of ITS sequences is important or if their length can be modified in response to ageing or stress. In the future, one solution to prevent potential ITS signal background using q-PCR method or to prevent intraspecific differences could be to perform longitudinal studies (i.e. repeated sampling in the same individual) using non-invasive samples. For field application using longitudinal approaches, leucocytes TL measurements could be tested as it was successfully done in humans (Scarabino *et al.*, 2019a, 2019b). In *Menidia menidia*, different studies highlighted a possible use of mucus from body or buccal cavity for DNA studies (Le Vin *et al.*, 2011; Taslima *et al.*, 2016). Mucus sampling could be proposed as a less stressful alternative to blood sampling, and applicable even in early life stages. The responsiveness of these biological matrix remains to be investigated.

The choice of the T/R reference gene was also crucial for the significance of the response obtained. While most studies used single copy genes as reference for relative TL calculation (Lai *et al.*, 2018), Wang et al. (2013) suggested that a multicopy gene like *18S* was more stable and appropriate for this calculation. In this study, the results were overall consistent whatever the method used but the signal-to-noise ratio was stronger using the single copy *mc2r* as a reference gene, and the ΔΔCt method for relative TL calculation. In the future, *tert* expression and relative TL could be completed by telomerase activity measurements to get a better view of telomere dynamics. This latter parameter has not been measured in this study because of several drawbacks notably the price, the low sensitivity and specificity of available methods, as reviewed by Skvortsov et al. (2011).

2. Hyposaline stress does not affect relative telomere dynamics in sea bass

According to our results, hyposaline stress did not trigger any significant difference in *tert* expression in 2 month-old sea bass. Interestingly, *caspase 8* and *caspase 9* expression tended to be up-regulated in hyposaline condition, which is concordant with branchial epithelium remodelling during hypo-osmotic acclimation (Nilsson, 2007; Ouattara *et al.*, 2009). However, *pcna* expression was significantly down-regulated in hyposaline condition compared to SW condition in 70-dph larvae's head. This suggests that hyposaline exposure decelerated cell proliferation. *Tert* expression tended to be higher and more variable in 70-dph fish than in 59-dph fish

possibly linked with a stronger demand in telomere maintaining. In European hake *Merluccius merluccius* and in Atlantic cod *Gadus morhua*, *tert* expression was higher in early developmental stages suggesting a higher telomerase demand possibly linked with elevated tissue renewal and long-term cell proliferation capacity maintenance (López de Abechuco *et al.*, 2014). In the head of fish exposed to E2 at 0.4 ng L^{-1}, relative TL, *tert*, *pcna*, *caspase 8* and *caspase 9* expression levels tended to be upregulated. This may indicate an activation of telomere dynamics and tissue renewal in response to estrogenic signal. The sensitivity of these genes to estrogen was already demonstrated in human (Bourdeau *et al.*, 2004). It is due to the presence of estrogen response elements (ERE) in the regulatory sequence of these genes (Bourdeau *et al.*, 2004). We can hypothesise that these ERE regulatory sequence are well conserved in sea bass but this would require further sequence analysis. The observed effect of E2 in sea bass is also concordant with the role played by estrogens in telomere dynamics in human. Estrogen exposure was shown to increase *tert* expression and telomerase activity (Misiti *et al.*, 2000; Calado *et al.*, 2006, 2009; Cen *et al.*, 2015). Based on the findings in the ovariectomised female rat model, exogenous estrogen can significantly up-regulate telomerase activity and TERT mRNA expression to exert the effects of anti-aging (Cen et al., 2006). Since telomere dynamics may differ according to gender and circulating estrogen levels (Gardner *et al.*, 2014), it could be interesting to clarify such possible differences in TL and *tert* between males and females in *D. labrax*.

In gills of 5 month-old sea bass, a high inter-individual variability in TL was observed, as expected in vertebrates (Dugdale and Richardson, 2018; Toupance *et al.*, 2019). Interestingly, FW intolerant fish exhibited a higher variability than other groups. Angelier et al. (2018) highlighted the importance of exploring the possible interactions between coping styles, glucocorticoid stress response and telomere dynamics, suggesting a potential trade-off between immediate survival and telomere protection. According to L'Honoré et al. (2019), FW intolerance in sea bass is characterised by an higher expression of mineralocorticoid receptor in the gills and a lower expression of glucocorticoid receptors in kidney and thus could reflect a different glucocorticoid response (L'Honoré *et al.*, 2019). To go further, it would be interesting to measure circulating cortisol levels and telomerase activity in order to clarify if telomerase activity, cortisol and *tert* expression are correlated in sea bass. In the future, other organs such as the posterior kidney could be investigated, because this organ is playing a crucial role in freshwater tolerance (Nebel *et al.*, 2005; Larsen *et al.*, 2014; L'Honoré *et al.*, 2019).

Following low salinity challenge, a physiological trade-off between osmoregulation and telomere maintenance seems reached, since telomere length seemed not attrited by hyposaline stress. In the wild, a stay in transitional habitats requiring active ionic regulation would potentially not represent a stress involving telomere shortening.

But in transitional waters, other environmental parameters are at stake. In particular, temperature and hypoxia have been shown to influence TL (Yu *et al.*, 2006; Debes *et al.*, 2016). The method developed in this study may be used in the field to determine whether the fluctuations of environmental parameters encountered during seasonal migration affect TL in sea bass.

Conclusion

The q-PCR method developed in this study was efficient to detect relative telomere length changes in sea bass exposed to E2 and using the T/R ratio with the single copy gene *mc2r* as a reference gene. As expected, the calculation methods of relative TL using DNA quantity or ΔΔCt methods were highly correlated. Exposure to hyposaline water triggered lower cell proliferation according to *pcna* but had no effect on *tert* expression and relative TL at the two studied developmental stages.

Acknowledgements

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Contributions

E. Blondeau-Bidet was responsible for RNA and gDNA extraction, q-PCR runs and data analysis. C. Lorin-Nebel helped in 5-month old sea bass experimental design, sampling and manuscript correction. E. Farcy was responsible for experimentation using larvae, RNA extraction and reverse transcription, ideas and manuscript corrections. J. Perez and F. Veyrunes were responsible for karyotypes and FISH analyses. T. L'Honoré was responsible for experimentation using the 5-months juveniles and was in charge of q-PCR runs, data analysis and manuscript preparation.

Significance Statement (75 words max)

Telomere dynamics is increasingly described as a potential marker of stress in metazoans. Yet, the reliability of the q-PCR method is still discussed as well as its effectiveness in detecting small TL differences. We have developed a reliable method for studying telomere dynamics in *Dicentrarchus labrax* using q-PCR and E2 as a positive control. However, telomere length and *tert* expression were not significantly affected by hyposaline stress in sea bass.

Table 2 Reproducibility of the "two-plate" telomere length assay using T/R ratio of 15 genomic DNA samples and one reference control in duplicates using DNA quantity method

	Sample	Reference	SW 1	SW 2	SW 3	SW 4	SW 5	FWt 1	FWt 2	FWt 3	FWt 4	FWt 5	FWi 1	FWi 2	FWi 3	FWi 4	FWi 5
	Assay # 1																
	Rep 1	1,14	1,16	1,08	0,88	1,15	1,16	1,07	1,21	1,08	0,96	1,19	1,03	0,98	1,04	1,14	1,01
	Rep 2	1,07	1,16	1,08	0,87	1,15	1,13	1,08	1,23	1,10	0,92	1,20	1,04	0,99	1,05	1,20	1,02
	Mean	1,10	1,16	1,08	0,87	1,15	1,15	1,07	1,22	1,09	0,94	1,19	1,04	0,99	1,05	1,17	1,01
	StDev	0,05	0,00	0,00	0,01	0,00	0,02	0,00	0,01	0,02	0,02	0,01	0,01	0,00	0,01	0,04	0,01
	CV %	4,19%	0,15%	0,02%	1,22%	0,03%	1,67%	0,37%	1,21%	1,49%	2,60%	0,99%	0,63%	0,49%	0,85%	3,31%	0,67%
																	Intra-assay CV = 1,24%
Reference gene: *mc2r*	**Assay # 2**																
	Rep 1	1,10	1,12	1,08	0,90	1,10	1,07	1,03	1,11	1,04	0,94	1,13	1,00	0,96	0,97	1,06	0,97
	Rep 2	1,09	1,16	1,07	0,90	1,09	1,09	1,03	1,20	1,08	0,97	1,18	1,02	0,98	1,03	1,11	0,99
	Mean	1,09	1,14	1,08	0,90	1,10	1,08	1,03	1,16	1,06	0,95	1,16	1,01	0,97	1,00	1,09	0,98
	StDev	0,00	0,03	0,01	0,00	0,01	0,01	0,00	0,06	0,03	0,02	0,03	0,01	0,01	0,04	0,04	0,02
	CV %	0,44%	2,93%	0,73%	0,26%	1,09%	1,09%	0,39%	5,47%	2,54%	1,66%	2,89%	1,34%	1,48%	4,48%	3,28%	1,67%
																	Intra-assay CV = 1,98%
																	Inter-assay CV = 1,61% (± 1,39%)
	Assay # 1																
	Rep 1	1,13	1,09		0,73	1,23	0,93	1,03	1,94	0,93	1,31	1,11	0,82		1,25	0,93	0,87
	Rep 2	1,09	1,12		0,75	1,33	0,98	1,04	1,98		1,30	1,13	0,83		1,29	0,92	0,87
	Mean	1,11	1,11		0,74	1,28	0,96	1,04	1,96	0,93	1,31	1,12	0,83		1,27	0,93	0,87
	StDev	0,02	0,02		0,01	0,08	0,04	0,01	0,03	0,00	0,00	0,01	0,00		0,03	0,01	0,00
	CV %	2,24%	1,92%		1,39%	5,93%	3,90%	1,00%	1,70%	0,00%	0,17%	0,91%	0,55%		2,25%	1,10%	0,05%
																	Intra-assay CV = 1,65%
Reference gene: *18S*	**Assay # 2**																
	Rep 1	1,05	1,04		0,72	1,22	0,86	0,97	1,76	0,87	1,26	1,07	0,79		1,15	0,83	0,83
	Rep 2	1,12	1,05		0,75	1,21	0,87	0,99	1,88		1,28	1,11	0,81		1,20	0,87	0,83
	Mean	1,09	1,04		0,74	1,22	0,86	0,98	1,82	0,87	1,27	1,09	0,80		1,17	0,85	0,83
	StDev	0,05	0,01		0,02	0,01	0,01	0,01	0,09	0,00	0,01	0,03	0,02		0,04	0,02	0,01
	CV %	4,52%	0,81%		2,10%	0,68%	0,65%	1,46%	4,70%	0,00%	1,07%	2,30%	2,21%		3,13%	2,81%	0,76%
																	Intra-assay CV = 1,94%
																	Inter-assay CV = 1,80% (± 1,52%)

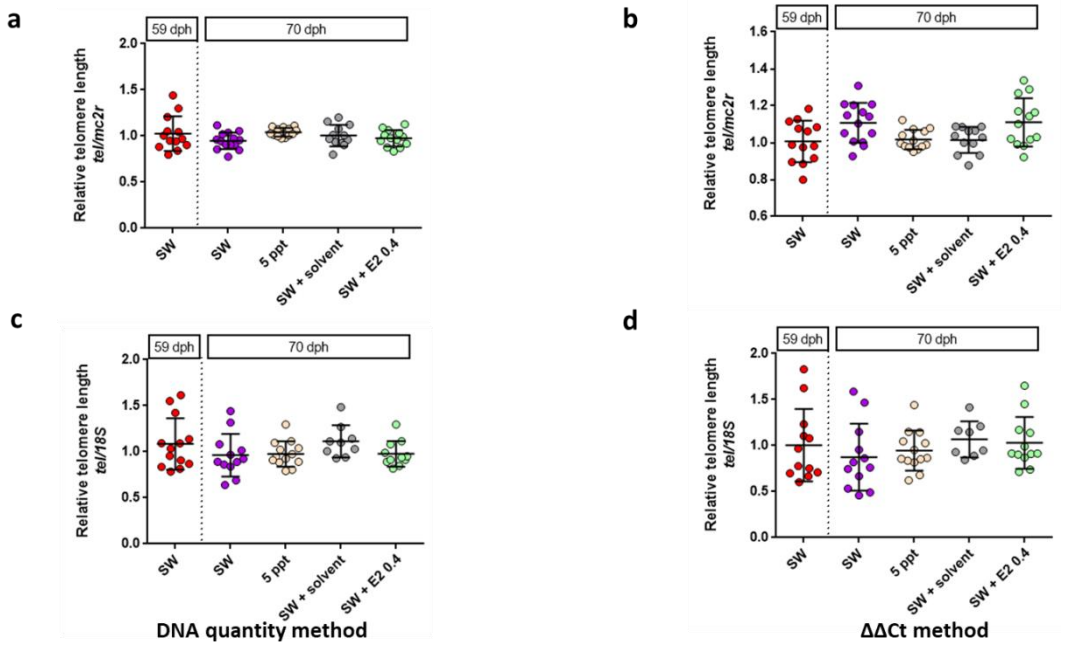


Figure 1S Relative telomere length using DNA quantity method **(a,c)** or ΔΔCt method **(b,d)** in the body of 59-dph sea bass larvea maintained in seawater (59 dph $_{SW}$), then transfered for 11 days in: SW (70 dph $_{SW}$), 5 ppt water (70 dph $_{5ppt}$), 17-β-oestradiol at 0.4 ng.L^{-1} (70 dph $_{SW + E2\ 0,4}$) or in ethanol at 0.00008 % (70 dph $_{SW + solvent}$). The expression has been normalised according to the expression of *mc2r* or *18S*. Different letters denote significant differences between groups (Kruskal-Wallis followed by Dunn's test, $P < 0.05$, means ± s.e.m, N=13-15)

Résumé du Chapitre 4

Les événements stressants peuvent modifier la physiologie de l'organisme à plusieurs niveaux, et déclencher des réponses allostatiques. Les télomères sont des séquences d'ADN répétitives très conservées dans le vivant, principalement localisées aux extrémités des chromosomes. Ils jouent un rôle crucial dans la stabilité de l'ADN. L'étude de la longueur des télomères (LT) ainsi que des niveaux d'expression de la sous-unité catalytique de la télomérase (TERT), constituent de nouveaux outils pour évaluer les conséquences des stress environnementaux chez les organismes comme les poissons. L'effet du stress hypo-salin rencontré naturellement lors des migrations saisonnières du bar européen *Dicentrarchus labrax*, est ici étudié via l'expression du gène *tert* et de la LT à deux stades de développement : le stade larvaire et le stade juvénile.

D'après la littérature, les méthodes disponibles pour estimer la LT sont nombreuses. Elles diffèrent par leur sensibilité et leur coût. Il existe des débats en raison de la variabilité opérationnelle et du biais dû à d'éventuelles séquences télomériques interstitielles. Dans cette étude, nous avons mis en évidence la présence de séquences télomériques interstitielles dans les caryotypes de *D. labrax* par hybridation fluorescente in situ (FISH), notamment sur les parties proximales et distales de chaque chromosome. Ensuite, nous avons mis au point une méthode de quantification relative pour mesurer la LT et les niveaux d'expression de *tert* chez le loup par PCR quantitative en temps réel. Pour le calcul de la longueur relative des télomères par rapport au gène de référence (ratio T/R), deux gènes de référence ont été testés. Le premier est un gène à copie unique : le récepteur à la mélanocortine (mc2r), et le second un gène de référence à copies multiples : l'ADN ribosomal (18S). Deux méthodes de calcul ont été comparées pour déterminer le ratio T/R : l'une utilisant la quantité d'ADN déterminée à partir de la courbe standard et l'autre correspondant à une méthode utilisant les ΔΔCt. Les résultats obtenus avec les deux méthodes présentaient la même tendance, mais le calcul de la TL avec mc2r comme gène de référence semble plus sensible.

Bien que non significative, une augmentation de la LT a été décelée dans la tête de larves exposées pendant 11 jours à 0,4 ng μL^{-1} de 17-β-oestradiol (E2) dans l'eau de mer. Il est intéressant de noter que cette augmentation de la LT est corrélée à une multiplication par 7 de l'expression de *tert* dans la tête des larves après 11 jours d'exposition à l'E2. Ces résultats mériteraient d'être confirmés en utilisant d'avantage d'individus mais il est intéressant de noter qu'ils corroborent les données de la littérature, confirmant le rôle des œstrogènes dans la dynamique des télomères. A l'inverse, l'exposition à un stress hypo-salin modéré de 5 ppt pendant 11 jours chez des larves de 2 mois ou à un stress aigu de 2 semaines en eau douce chez des individus de 5 mois n'a pas déclenché de changement significatif dans la LT ni dans l'expression de la *tert*. Toutefois, chez les individus intolérants à l'eau douce, les télomères semblent être plus courts que les individus tolérants à l'eau douce. Néanmoins, ce résultat nécessiterait d'être validé avec davantage d'individus. L'utilisation de ces méthodes comme potentiels proxies moléculaires pour étudier l'effet des stress environnementaux chez *D. labrax* nécessite encore certains développements, mais pourrait devenir un outil prometteur.

Discussion

&

Perspectives

« Il faut être plastique » J-H.L.

Discussion & Perspectives

La première partie de cette discussion portera sur la plasticité phénotypique des loups suite à une dessalure, notamment *via* l'étude des conséquences d'un transfert en eau douce sur le comportement et les mécanismes osmorégulateurs à moyen et à long terme. Le métabolisme respiratoire en normoxie et en hypoxie suite à la dessalure sera discuté tout comme la question de la variabilité intraspécifique dans la réponse métabolique.

Dans une seconde partie, nous discuterons plus en détail la variabilité intraspécifique dans la tolérance à la dessalure à différentes échelles : **biochimique** et **transcriptionnelle.** Nous aborderons notamment la question de la méthode de la sélection des phénotypes au sein de cette étude, et nous discuterons des phénotypes intermédiaires. Enfin, à partir des résultats de respirométrie, nous étudierons le compromis entre plasticité phénotypique et paramètres métaboliques.

Dans une dernière partie, nous verrons comment la longueur des télomères et l'expression de la télomérase pourraient être utilisées comme potentiels biomarqueurs moléculaires de stress environnementaux chez le loup, notamment les stress hypo-osmotiques. Nous verrons si ces biomarqueurs sont suffisamment sensibles afin d'aborder des questions de variabilité intraspécifique. Enfin, nous ouvrirons sur une perspective qui s'intéressera à l'implication à un marqueur épigénétique, à savoir la méthylation de l'ADN, sa modulation suite à une dessalure et son implication potentielle dans la variabilité intraspécifique chez *D. labrax*.

1. Réponses comportementales et physiologiques à la dessalure chez le loup méditerranéen

1.1 Étude du compromis osmo-respiratoire chez le loup

La branchie est un organe clef dans les processus osmorégulateurs mais aussi respiratoires, immunitaires et acido-basique. Elle possède une grande plasticité morphologique (Sollid & Nilsson, 2006). La capacité de moduler rapidement la morphologie de la branchie afin d'optimiser la régulation ionique et les échanges gazeux en fonction de l'environnement a déjà été relevée chez de nombreux téléostéens (Sollid & Nilsson, 2006).

On parle de compromis osmo-respiratoire (Sardella & Brauner, 2007). Il a déjà été montré chez des loups maintenus à 18 °C une diminution de la longueur des lamelles et une augmentation de l'épaisseur des filaments suite à un transfert de deux semaines en eau douce (Masroor *et al.*, 2018), mais aucune mesure n'avait été réalisée à plus long terme. Dans cette thèse, nous avons pu mettre en évidence chez des loups une diminution de 25% de la longueur des lamelles branchiales après 2 mois passés en eau douce à 19 °C alors que la diminution observée par Masroor *et al.* (2018) était de 11% sans doute liée à un temps d'acclimatation plus court. La comparaison est effectuée entre des poissons de lots différents mais présentant des longueurs de lamelles similaires en eau de mer (95 µm en moyenne) à des températures comparables (18 °C pour Masroor *et al.* (2018) *vs* 19 °C pour cette étude) (Fig. 1A).

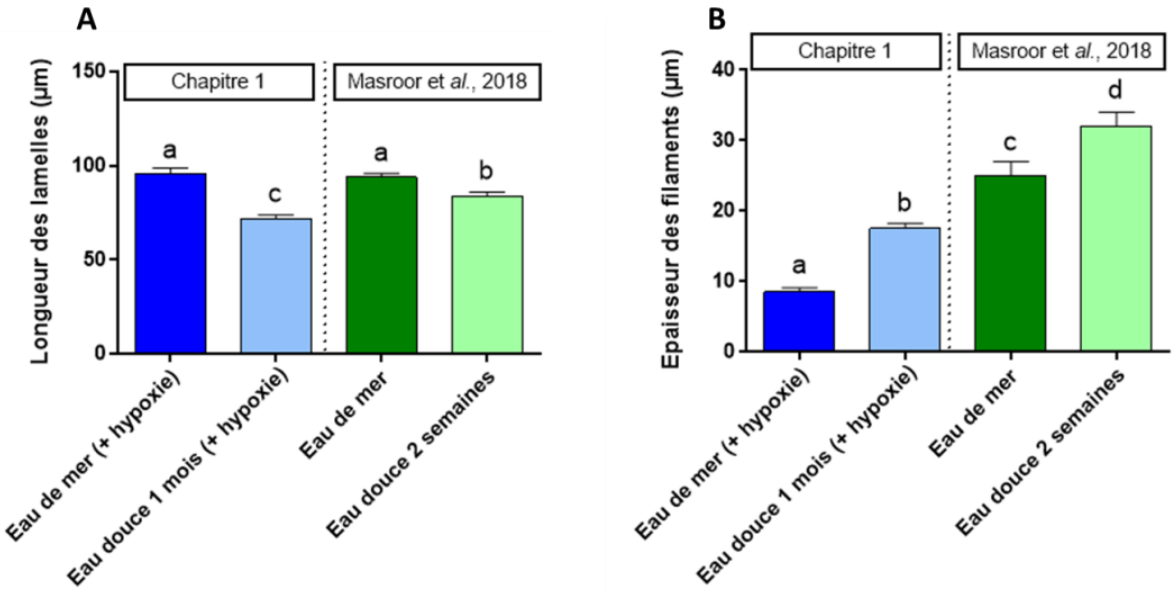


Figure 1 : ***(A)*** *Longueur des lamelles et* ***(B)*** *épaisseur des filaments de loups en eau de mer, après deux semaines ou deux mois en eau douce (moyenne ± écart-type). Les données sont tirées du Chapitre 1 de Masroor et al. (2018)*

La diminution de la longueur des lamelles est souvent reportée quand l'oxygène est plus disponible comme c'est le cas en eau douce (Saroglia *et al.*, 2002; Nilsson, 2007). Si l'on compare l'épaisseur des filaments entre cette étude et celle de Masroor *et al.* (2018), celle-ci n'est pas comparable en eau de mer mais dans les deux cas, une augmentation de l'épaisseur est relevée après deux semaines en eau douce comme après 2 mois en eau douce, respectivement de 22% (Masroor *et al.*, 2018) et 52% (cette étude, Fig. 1B).

L'augmentation de l'épaisseur des filaments peut être expliquée par le recrutement d'ionocytes pour optimiser l'absorption d'ions (Inokuchi *et al.*, 2017). Pour aller plus loin dans l'étude du compromis osmo-respiratoire chez le loup, il apparaissait intéressant d'étudier les conséquences de ces modifications morphologiques branchiales sur la physiologie respiratoire.

1.2 Métabolisme respiratoire en eau douce en normoxie et en hypoxie

Chez les organismes euryhalins, il est souvent difficile d'évaluer l'effet isolé d'un changement de salinité sur les capacités respiratoires aérobies et ainsi d'estimer le coût énergétique de l'osmorégulation dû à une forte variabilité interspécifique (Ern *et al.*, 2014; Christensen *et al.*, 2017). Ce coût énergétique est très souvent considéré faible, allant de quelques pourcents jusqu'à 30% du métabolisme basal des poissons (Bœuf & Payan, 2001; Chatelier *et al.*, 2005; Ern & Esbaugh, 2018). Chez le loup, il a été montré qu'une exposition de 18h en eau douce n'altère pas le taux métabolique standard (SMR) (Claireaux & Lagardère, 1999; Chatelier *et al.*, 2005). Après 4 semaines en eau douce, nous avons mesuré un SMR plus important chez les individus maintenus en eau douce par rapport aux individus maintenus en eau de mer (Chapitre 1). De plus, nous avons observé une forte variabilité intraspécifique des SMR renforçant encore plus l'intérêt d'avoir recours à des mesures individuelles. Les causes de cette variabilité restent à éclaircir, mais différents auteurs suggèrent des implications physiologiques pouvant avoir des répercussions sur la *fitness*. En effet, le taux métabolique est directement lié à la croissance et à la survie (Burton *et al.*, 2011). De plus, maintenir un taux métabolique haut entraîne une production plus importante d'espèces réactives de l'oxygène ou ROS, et donc pourrait impacter négativement la survie des organismes (Harman, 1956). Ainsi, le lien entre ROS, taux métabolique et *fitness* reste à étudier notamment chez les téléostéens soumis à des stress récurrents dans les environnements de transition.

Le taux métabolique rassemble la consommation d'énergie par les processus fondamentaux tels que le renouvellement protéique, la néoglucogenèse, l'activité enzymatique incluant les transports protéiques et ioniques membranaires (Rolfe & Brown, 1997). Cependant la contribution de chacun de ces facteurs pourrait différer entre les individus, notamment dû à des différences génétiques ou épigénétiques ou

comportementales. En effet, chez la souris plus de 50% de la variation du taux métabolique entre les individus ont pu être reliées à des différences de taille d'organes (foie, rein, cœur, intestins) (Konarzewski & Diamond, 1995). Du point de vue comportemental, les différences de personnalité ou *coping-style* (LR – individus proactifs : peu de cortisol circulant, réponse au stress basée sur l'adrénaline ou HR – individus réactifs : beaucoup de cortisol circulant, réponse au stress basée sur le cortisol) pourraient influencer le taux métabolique. En effet, les individus proactifs présentent généralement un taux métabolique plus haut que les phénotypes réactifs (Korte *et al.*, 2005). Biro & Stamps (2010) soulignent aussi le lien entre taux métabolique, traits comportementaux et physiologiques (un taux métabolique haut étant lié à un comportement agressif et une croissance plus importante). Ainsi, les différences en termes de taux métaboliques pourraient alors être à l'origine des différences comportementales que nous avons observées en eau douce entre les tolérants et les intolérants (Chapitre 2). Pour aller plus loin, il serait intéressant de compléter nos approches en étudiant le taux métabolique et le comportement de manière longitudinale chez le loup, afin de voir si cette relation se vérifie comme chez d'autres téléostéens (Martins *et al.*, 2011), et si elle pourrait être reliée à la tolérance à la dessalure. Pour cela, nous pourrions entreprendre un phénotypage des personnalités des loups *via* des mesures de cortisol circulant et/ou des tests comportementaux de prise de risque (ou *risk-taking)*, puis réaliser des mesures de taux métabolique suite à une dessalure afin de caractériser le lien entre *coping-style*, taux métabolique et capacité d'acclimatation à l'eau douce chez le loup.

1.3 Réponses comportementales en eau douce chez le loup méditerranéen

Le transfert en eau douce chez les téléostéens marins entraîne une cascade de réponses comportementales et physiologiques intégrant plusieurs échelles. Tout d'abord à l'échelle de l'organisme, nous avons pu mettre en évidence des différences comportementales (Chapitre 2). Les individus transférés en eau douce présentaient des vitesses de nage et une distance parcourue plus faibles que celles des individus maintenus en eau de mer. Chatelier *et al.* (2005) ont montré que 18h après un transfert en eau douce la vitesse maximale de nage des loups ne change pas, supposant que cela est lié en partie aux capacités osmorégulatrices exceptionnelles de ce modèle. Or en étudiant les paramètres sanguins (pression osmotique, niveaux en Na^+ et Cl^-), nous avons pu mettre en évidence que ceux-ci diminuaient

immédiatement après la dessalure, indiquant une nécessité d'induire rapidement des mécanismes de régulation physiologique. Nous avons pu montrer que le transfert en eau douce affectait le comportement de nage des individus, du moins à court terme. Il serait intéressant d'évaluer la vitesse maximale de nage chez des loups acclimatés 2 semaines et 2 mois en eau douce lorsque les paramètres sanguins sont très différents, afin de voir si celle-ci diffère. Dans le milieu naturel, des capacités de nage plus faibles en eau douce pourraient avoir des conséquences sur la *fitness* des loups, notamment en réduisant leurs capacités de prédation ou de fuite. On pourrait alors supposer qu'il existe un compromis (ou *trade-off*) dans ces environnements de transitions entre le coût énergétique de l'acclimatation et les bénéfices (refuge, nourrissage).

La question de la préférence de salinité a déjà été abordée chez d'autres téléostéens. Chez l'esturgeon vert *Acipenser medirostris* acclimaté à l'eau douce et/ou à l'eau de mer, Poletto *et al.* (2013) ont démontré qu'il est non seulement capable de détecter l'eau de mer mais aussi qu'il possédait une préférence vis-à-vis de celle-ci. Cela a aussi été démontré chez le choquemort *Fundulus heteroclitus*, un poisson euryhalin comme le loup, préférant l'eau de mer, qu'il soit pré-acclimaté ou non à l'eau douce (Marshall *et al.*, 2016). En revanche, chez la perche *Perca flavescens,* un téléostéen d'eau douce, Christensen & Grosell (2018) ont montré qu'elle possédait une préférence pour des salinités iso-osmotiques, limitant peut-être le coût énergétique de l'osmorégulation. Chez le loup, de telles expériences de préférences de salinité n'ont pas encore été réalisées et il serait intéressant de les mener afin de déterminer ses futures niches potentielles en lien avec le réchauffement climatique, prévoyant des modifications de salinités dans le futur, plus accentuées dans les milieux de transition comme les lagunes méditerranéennes (Pörtner *et al.*, 2014).

1.4 Mise en place des mécanismes hyper-osmorégulateurs à court terme et à long terme

Suite à une dessalure, il a déjà été montré chez la daurade et chez de nombreux téléostéens une diminution de l'osmolalité sanguine (Laiz-Carrión *et al.*, 2005). Chez le loup, une diminution de l'osmolalité sanguine a aussi été reportée après 24h en eau douce, et ce jusqu'à 30 jours (Bossus *et al.*, 2011). Dans cette thèse, nous montrons pour la première fois

un retour à des valeurs d'osmolalité proches de celles des individus en eau de mer après 2 mois passés en eau douce. L'acclimatation en deux phases à l'eau douce a déjà été suggérée chez de nombreux téléostéens marins (Lin *et al.*, 2004; Madsen *et al.*, 2007), avec une première réponse d'ajustement qui a lieu dès le transfert en eau dessalée, suivie par une phase régulatrice qui a lieu généralement quelques jours après. Chez le loup, une troisième phase d'acclimatation tardive pourrait se situer entre deux semaines et deux mois. Cette hypothèse repose non seulement sur le retour vers un équilibre osmotique après 2 mois mais aussi sur l'expression de certains gènes dans les branchies et au niveau du rein postérieur. L'augmentation de l'expression de la *nka α1a* et du *ncc-2a* branchiaux après deux semaines est, par exemple, suivie d'un retour à des valeurs proches de celles observées en eau de mer à 2 mois. De même, une diminution de l'expression de la *nka α1a* rénale après deux semaines est suivie d'un retour aux valeurs mesurées en eau de mer, suite à une exposition de 2 mois en eau douce. Ainsi, selon le temps d'acclimatation, les transporteurs ioniques mis en jeu dans les processus hyper-osmorégulateurs pourraient différer à moyen (2 semaines) et long terme (2 mois). Cela pourrait s'expliquer par la redondance des transporteurs ioniques qui assurent le même rôle, afin de permettre aux individus de sélectionner la meilleure stratégie afin d'osmoréguler selon les paramètres biochimiques de l'eau comme le pH, tel que cela a été démontré chez le poisson zèbre (Chang *et al.*, 2013). D'un point de vue moléculaire, l'étude des transcrits nous a permis de mieux caractériser l'intolérance à l'eau douce chez le loup à moyen terme (2 semaines), notamment en montrant dans le second chapitre l'importance du rein postérieur dans la réabsorption des ions chez les loups tolérants en eau douce (Chapitres 2 et 3). Nous avons aussi pu montrer une réponse hormonale différente entre les phénotypes *via* l'étude des transcrits des récepteurs aux corticoïdes et à la prolactine. Des analyses transcriptomiques des différents phénotypes identifiés dans cette thèse, comme celles réalisées par Boutet *et al.* (2006) sur le loup, pourraient permettre d'établir une représentation de la plasticité transcriptionnelle en lien avec la tolérance à l'eau douce, tout comme celle proposée par Healy & Schulte (2015) sur 2272 gènes chez le killifish. De plus, l'étude de la cinétique de mise en place des réponses transcriptionnelles apporterait d'autant plus d'informations quant au rôle des différents hormones/organes/transporteurs étudiés.

2. Plasticité phénotypique des loups méditerranéens face à la dessalure

A chaque expérimentation en eau douce menée dans cette étude, des mortalités ont pu être mises en évidence, traduisant une variabilité intraspécifique dans les capacités d'acclimatation à l'eau douce chez le loup. Bien que cette variabilité ait déjà été reportée et décrite en partie (Nebel *et al.*, 2005; Giffard-Mena *et al.*, 2008), les traits comportementaux et physiologiques restaient à approfondir. Dans le second chapitre de ce manuscrit, nous avons tout d'abord confirmé les résultats des études précédentes montrant des paramètres sanguins (osmolalité et ions Na^+, Cl^-) plus faibles chez les intolérants à l'eau douce par rapport aux individus tolérants. Nous avons aussi pu quantifier individuellement des comportements de nage plus lents chez les intolérants, simplement relevés visuellement dans le banc dans les études précédentes. De plus, de nouveaux indices possiblement à l'origine de l'intolérance ont pu être décelés, notamment au niveau rénal avec une sous-expression du gène *nka α1a* ainsi que des gènes codant pour des récepteurs aux glucocorticoïdes (*gr)* et minéralocorticoïdes (*mr)*, qui jouent un rôle clef dans la réponse aux stress. Nous avons aussi pu montrer que l'existence de deux phénotypes extrêmes (quant à leur tolérance à la salinité) était répétable et dans les mêmes proportions (30% d'intolérants) au cours du temps. Une variabilité phénotypique de la tolérance à l'hypoxie mise en évidence par Joyce *et al.* (2016) a déjà été discutée dans ce manuscrit. D'autres études réalisées chez le loup ont révélé une forte variabilité inter-individuelle dans la réponse au stress (Samaras *et al.*, 2016) et la résistance au stress thermique (Ozolina *et al.*, 2016). Chez le poisson zèbre, il a été montré que le trait résistance au stress thermique était répétable dans le temps chez un même individu (Morgan *et al.*, 2018). En revanche, nous avons mis en évidence dans cette thèse que les phénotypes tolérants et intolérants à l'eau douce ne sont pas forcément conservés au cours du temps.

2.1 Sélection des phénotypes liés à la tolérance à la dessalure

La principale difficulté dans l'étude de la variabilité phénotypique (quel que soit le trait considéré), est la détection et l'identification des différents phénotypes. C'est pour cela que les études portent généralement sur les phénotypes les plus extrêmes. Dans ce travail de thèse, nous nous sommes focalisés sur les phénotypes extrêmes tolérants et intolérants à

l'eau douce. Ces derniers ont été détectés ***a priori*** par des traits comportementaux non invasifs puis caractérisés et validés dans un second temps, par des mesures physiologiques plus invasives. L'inconvénient de ce type d'approche est l'occurrence probable d'individus faux-positifs ou faux-négatifs. Une analyse multivariée du jeu de données permet d'apporter un nouvel éclairage intéressant. Si l'on projette les paramètres sanguins (pression osmotique, Na^+ et Cl^-) sur une analyse en composante principale (ACP), les trois groupes que nous avons formés (eau de mer, eau douce tolérant et eau douce intolérant) se distinguent très bien (Fig. 2A). Les deux premiers axes expliquent 98.1% de l'inertie (95.1% et 5%), avec une contribution plutôt égale des trois facteurs dans la détermination du premier axe (pression osmotique : 34.4%, Na^+ : 33% et Cl^- : 32%). La seconde dimension en revanche est bien plus expliquée par les ions (pression osmotique : 0.8%, Na^+ : 42.6% et Cl^- : 56.5%). Cependant, il est possible de détecter *via* ces analyses des individus extrêmes en eau douce au sein des deux groupes (ou intermédiaires entre deux groupes), qui se situeraient au sein de deux ellipses de confiance, mettant en doute leur appartenance au phénotype que nous leur avons attribué. C'est le cas pour les individus intolérants FWi_01 et FWi_04 par exemple, qui semblent mieux se regrouper avec les individus tolérants car ils partagent des traits biochimiques sanguins proches (Fig. 2B). Ce retraitement de données obtenues en analyses multivariées permet toutefois de confirmer que l'approche avec *a priori* basée sur une sélection de critères comportementaux est satisfaisante, car les phénotypes identifiés sont bien corrélés entre eux au niveau de leurs paramètres sanguins (plus bas chez les intolérants) et le nombre de faux positifs ou faux négatifs reste minime.

Si l'on se focalise sur l'eau douce en prenant uniquement en compte les paramètres sanguins pour construire l'ACP, nous retrouvons 95.9% de l'inertie expliquée par les deux axes (Fig. 3A, B). En revanche, si l'on choisit de prendre l'expression des gènes mesurés dans le rein postérieur en eau douce (*nka* α1a, *prlra*, *prlrb*, *nkcc1a*, *nkcc1b*, *nkcc2*, *slc26a6a*, *slc26a6b*, *slc26a6c*, *gr1*, *gr2* et *mr*) pour construire l'ACP (étant donné qu'il s'agit de l'organe dans lequel nous avons mesuré le plus de variabilité dans les transcrits), les ellipses de confiance sont beaucoup plus proches et les deux premiers axes expliquent 59,5% de l'inertie totale au lieu de 95,9% lorsque les paramètres sanguins sont utilisés pour l'ACP (Fig. 3C, D). Cela illustre que les paramètres sanguins sont de très bons descripteurs de la tolérance à l'hypo-salinité chez le loup alors que la variabilité de l'expression des gènes

considérés est influencée (pour environ 40%) par d'autres facteurs. Ces gènes sont en effet en partie impliqués dans d'autres fonctions que l'osmorégulation (régulation du pH sanguin par exemple) et les profils d'expression observés seraient expliqués en partie par des *trade-off* fonctionnels. Pour étudier la variabilité dans la tolérance à la dessalure sans ***a priori***, une stratégie pourrait être d'échantillonner un lot d'individus à plusieurs pas de temps et de leur attribuer un phénotype ***a posteriori*** sur les critères biochimiques. Il apparaît désormais important de décrire la variabilité dans la réponse aux stress non seulement vis-à-vis des phénotypes les plus extrêmes, mais aussi vis-à-vis des phénotypes intermédiaires, car ce sont peut-être les plus réversibles.

Si l'on s'intéresse à la cinétique d'apparition des caractéristiques propres aux intolérants à l'eau douce (couleur sombre, nage lente, ou mort), l'apparition de phénotypes intolérants s'étale tout au long du challenge (Fig. 4), avec un pic d'occurrence observé 24 h après le passage en eau douce. Le nombre d'individus intolérants détectés diminue rapidement et semble se stabiliser après 4 jours, avec des individus intolérants détectés tous les jours et ce jusqu'à la fin du challenge. Les individus montrant des signes d'intolérance lors des 4 premiers jours du challenge correspondent peut-être aux individus les plus sensibles à la dessalure mais on ne peut pas exclure que ce pic soit aussi dû au stress opératoire. En faisant le choix d'échantillonner tous les individus 2 semaines après le transfert en eau douce, nous focalisons notre étude sur la tolérance à l'eau douce à moyen terme, et non sur la tolérance au stress opératoire couplé à la tolérance à l'eau douce à court terme qui ne fait pas nécessairement intervenir les mêmes mécanismes. En faisant ce choix, nous n'avons pas d'information sur les plus intolérants à la dessalure puisque ceux-ci n'ont pas été analysés. Il aurait été intéressant de caractériser ce phénotype ultra-sensible afin de déterminer la variabilité dans les réponses suite à un stress (osmotique) aigu.

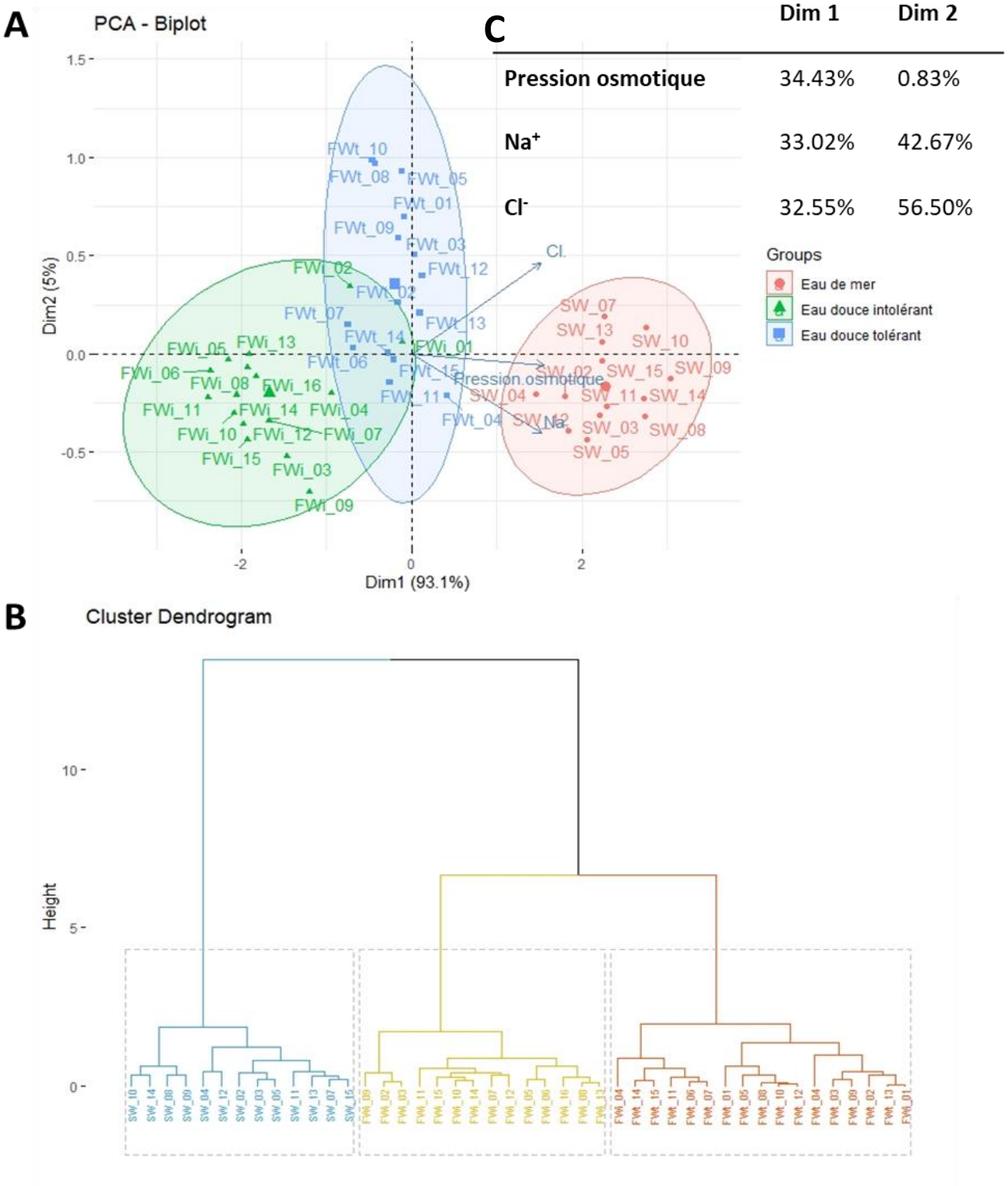
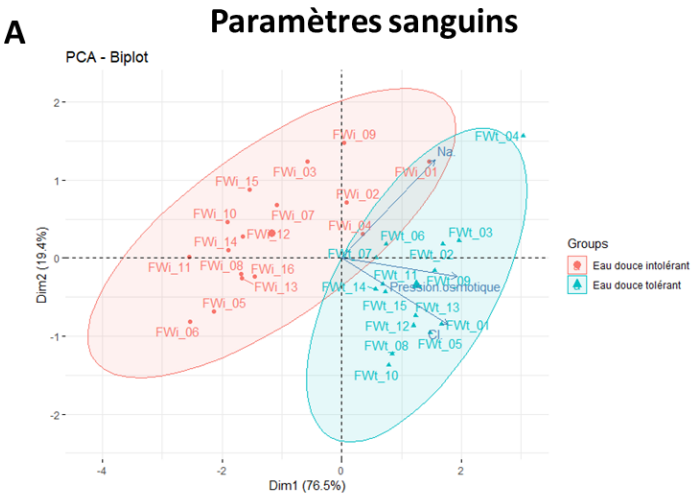


Figure 2 : ***(A)*** *Résultats de l'analyse en composante principale (ACP) construite à partir des paramètres sanguins des loups âgés de 8 mois étudiés dans le Chapitre 2,* ***(C)*** *contribution de chacun des facteurs dans la construction des axes de l'ACP et* ***(B)*** *dendrogramme réalisé à partir des résultats de l'ACP. Les individus intolérants à l'eau douce (en jaune) FWi_01 et FWi_04 entourés en noir se regroupent avec les individus tolérants à l'eau douce (FWt, en orange), tandis que les loups maintenus en eau de mer (SW, en bleu) sont tous groupés ensemble*

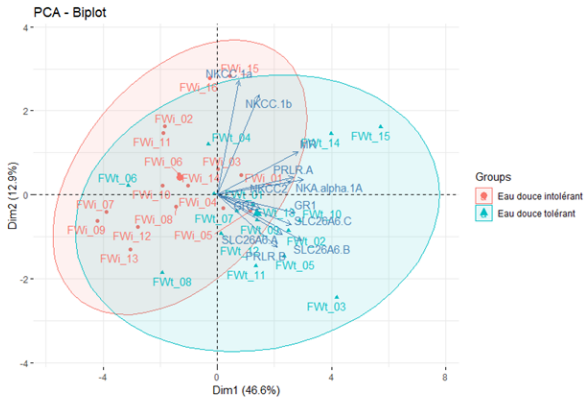
A



B

	Dim.1	Dim.2
Pression osmotique	39.9%	2.4%
Na^+	26.2%	67.1%
Cl^-	33.9%	35.5%

C



D

	Dim.1	Dim.2
Nka α1a	13.3%	0.7%
Prlra	10.5%	0.9%
Prlrb	6.5%	8.2%
Nkcc1a	0.8%	39%
Nkcc1b	3.1%	30%
Nkcc2	9.9%	0.5%
Gr1	10.8%	0.9%
Gr2	3.3%	0.5%
Mr	11.8%	5.5%
Slc26a6a	7.5%	4.7%
Slc26a6b	12.7%	6.3%
Slc26a6c	9.9%	2.6%

Figure 3 : Résultats de l'analyse en composante principale (ACP) construite à partir ***(A)*** *des paramètres sanguins ou* ***(C)*** *de l'expression des gènes dans le rein postérieur des loups âgés de 8 mois transférés en eau douce et étudiés dans le Chapitre 2, et* ***(B-D)*** *contribution de chacun des facteurs dans la construction des axes des ACPs*

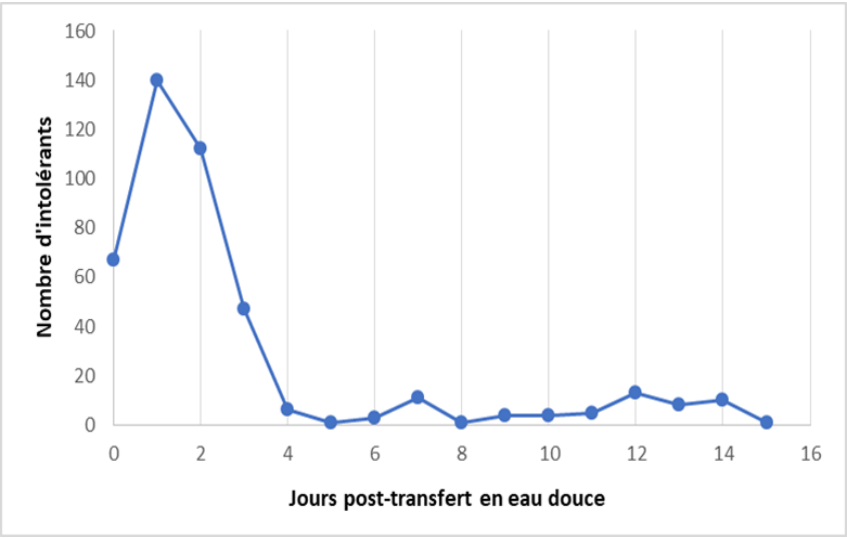


Figure 4 : Cinétique d'apparition des phénotypes intolérants à la dessalure lors de l'exposition réalisée sur 1525 loups âgés de 5 mois (Chapitre 2)

Dans leur milieu naturel, d'autres stress peuvent s'additionner au stress hyposalin : thermiques, chimiques, lumineux et biotiques (compétition, prédation), pouvant amener à minimiser les bénéfices des environnements de transitions (nourriture, refuge). Des expériences de préférence de salinité couplées à des mesures physiologiques (SMR, O_{2crit}, ILOS, pression osmotique, cortisol, prolactine) permettraient de mieux appréhender l'effet de la salinité et des autres stress environnementaux sur le métabolisme respiratoire et les capacités osmorégulatrices des loups méditerranéens. De la même façon que précédemment, nous pourrions compléter ces études par des phénotypages multiples, à savoir phénotyper les loups concernant leur limite haute thermique (C_{Tmax}), leur tolérance à l'hypoxie (HT : tolérant à l'hypoxie – HS : sensible à l'hypoxie), leur personnalité (HR – LR) tous les trois démontrés comme répétables chez le loup, puis exercer un challenge osmotique afin de réaliser un phénotypage selon leurs capacités d'acclimatation à l'eau douce. Ces perspectives d'étude apporteraient un regard nouveau sur les possibles interactions entre les différentes limites de tolérances des loups vis-à-vis des stress environnementaux multiples (thermique, hypoxique, osmotique). Cela apporterait de nouvelles connaissances sur la plasticité des loups et leurs capacités d'acclimatation et d'adaptation dans un environnement changeant. Ce type d'étude s'avère d'autant plus nécessaire que l'amplitude des variations environnementales devrait s'accentuer sous l'effet des changements globaux (Pörtner *et al.*, 2014).

2.2 Plasticité phénotypique en eau douce et compromis adaptatif

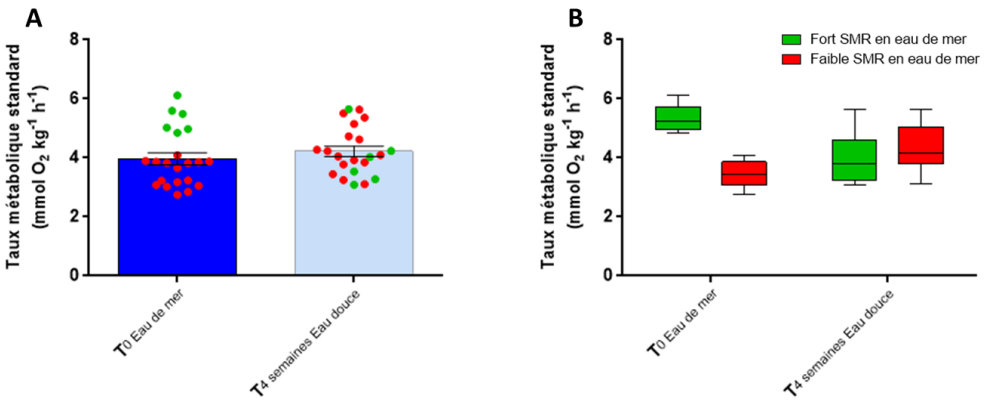
Au cours de leur vie, les loups peuvent migrer dans des environnements à salinité extrêmement variable. Ainsi, le coût de la plasticité doit être abordé sous la forme de *trade-off* entre les différentes fonctions physiologiques, tant à court terme pour l'acclimatation, qu'à long terme pour la survie et les migrations futures. Chez *Cordylophora caspia* (Hydrozoaire euryhalin), l'acclimatation à différentes salinités entraîne d'importants remaniements cellulaires et la question du coût de la plasticité phénotypique est évoquée chez des animaux exposés à des stress osmotiques, notamment afin de savoir si celle-ci est adaptative ou non (Hildebrandt *et al.*, 2018). Savoir si la plasticité est adaptative ou non reste activement débattu, d'autant plus que tous les auteurs insistent sur le manque de variables mesurées tout comme la nécessité d'approches individuelles, répétées, et

idéalement sur plusieurs générations (Via *et al.*, 1995; Hughes *et al.*, 2003; Ghalambor *et al.*, 2007). Les données acquises dans le cadre de cette thèse apportent des éléments nouveaux sur le lien entre acclimatation et coût métabolique chez le loup, ce qui pourrait alimenter les réflexions futures concernant le lien entre la plasticité de la tolérance à l'eau douce et la *fitness*.

DeWitt *et al.* (1998) suggèrent que des différences de plasticité pourraient être dues non seulement à des différences dans la mise en place des mécanismes de régulation (ontogénèse), mais aussi à des différences dans les mécanismes de détection des variations des paramètres environnementaux (osmodétection). Lorsque l'exposition en eau douce a été reproduite sur les mêmes individus après une récupération en eau de mer de 5 mois, leur phénotype (intolérant – tolérant) n'a été retrouvé que dans 30% des cas, ce qui suggère que la plasticité de la tolérance à l'eau douce chez les juvéniles relève plutôt de mécanismes d'osmodétection et/ou de capacités osmorégulatrices variables dans le temps. Relyea (2002) tout comme DeWitt *et al.* (1998) évoquent le terme de *trade-off* entre le coût énergétique de maintenance et celui de la plasticité phénotypique. Chez *D. labrax*, il a été mis en évidence que les individus qui présentaient le plus de plasticité dans la résistance à une augmentation de température étaient ceux qui possédaient un taux métabolique plus bas (Ozolina *et al.*, 2016). Ainsi, nous pouvons émettre l'hypothèse que les individus les plus plastiques seraient ceux qui possèdent (i) un coût énergétique plus faible en conditions stables, (ii) une meilleure détection des paramètres environnementaux (iii) ainsi qu'une mise en place des réponses mécanistiques plus rapide et plus efficace. Afin de répondre aux questions (ii) et (iii), il nous faudrait (ii) mesurer des osmodétecteurs comme les ORE (*Osmotic Responsive Element*, Chua *et al.*, 2016) connus pour être des sites de fixation d'un transactivateur osmo-sensible le NFAT5, et (iii) phénotyper les loups selon leur tolérance à la dessalure tout en mesurant leur taux métabolique.

A partir des résultats obtenus dans cette thèse, nous pouvons travailler sur la première hypothèse (i). Si l'on reprend les deux groupes précédemment identifiés en début de discussion (paragraphe 1.4) : un groupe avec un taux métabolique élevé en eau de mer (i.e. supérieur à la moyenne de 4 mmol O_2.kg^{-1}.h^{-1}) et un groupe avec un taux métabolique plus bas (i.e. inférieur à la moyenne de 4 mmol O_2.kg^{-1}.h^{-1}), les individus présentant un fort taux métabolique en eau de mer (en vert sur la Fig. 5A-B) semblent avoir un taux métabolique qui

diminue en eau douce, alors qu'il semble que ce ne soit pas le cas pour ceux qui possèdent un taux métabolique faible en eau de mer (en rouge, Figs 5A-B). En revanche, si l'on se focalise sur le métabolisme respiratoire et plus particulièrement sur la tolérance à l'hypoxie, il apparaît que les phénotypes les plus plastiques (*i.e.* ceux capables de mieux tolérer l'hypoxie modérée et l'hypoxie extrême) ne sont pas nécessairement ceux présentant un taux métabolique standard faible. En effet, soit le taux métabolique reste stable, soit le taux métabolique augmente en eau douce. Si l'on regarde désormais la tolérance à l'hypoxie, la majorité des indices de tolérance à l'hypoxie sont corrélés entre eux (Fig. 6C) comme chez la truite arc-en-ciel (Zhang *et al.*, 2018). En revanche contrairement à l'étude sur la truite, aucune corrélation ne semble pouvoir être faite entre le SMR en eau douce et la tolérance à l'hypoxie (ILOS, O_{2crit}, $O_{2deficit}$). En revanche, les capacités anaérobiques estimées par l'indice AOD semblent être corrélées au taux métabolique (test de Pearson, $P = 0.0045$).



C

	SMR	ILOS	O_{2crit}	AOD	$O_{2deficit}$
SMR					
ILOS	0.905				
O_{2crit}	0.382	**0.094**			
AOD	**0.004**	**0.020**	0.067		
$O_{2deficit}$	0.367	0.262	**< 0.001**	0.118	

*Figure 5 : Taux métabolique standard (SMR) de loups âgés de 8 mois en eau de mer ou après 4 semaines en eau douce avec **(A)** deux groupes formés selon leur SMR en eau de mer (haut : en vert et bas : en rouge), **(B)** visualisation sous forme de box-plot des résultats et **(C)** matrice de corrélations entre le SMR et les indices de tolérances à l'hypoxie (Test de Pearson, p-value < 0,05 en bleu, n = 18). Les données sont issues du Chapitre 1*

Si le lien entre tolérance à la température et SMR ayant déjà été démontré chez le loup (les individus ayant le SMR le plus bas présentant une meilleure tolérance aux hautes températures, Ozolina *et al.*, 2016), le lien entre tolérance à la salinité et SMR reste à démontrer. Il serait intéressant de voir s'il existe une corrélation entre le taux métabolique standard, la tolérance à l'hypoxie et la tolérance à l'eau douce. Pour cela, il faudrait mesurer le SMR avant et après un challenge osmotique, déterminer la tolérance à la dessalure par des indices comportementaux et/ou sanguins puis évaluer la tolérance à l'hypoxie (Chapitre 2).

3. Nouvelles approches moléculaires pour l'étude des stress osmotiques chez le loup

3.1 La dynamique des télomères comme potentiel biomarqueur moléculaire de stress salins

Dans le dernier chapitre de cette thèse, nous avons mis au point une méthode permettant d'étudier la dynamique des télomères chez le loup (longueur relative des télomères et expression de la télomérase). Dans un contexte de changements globaux avec l'augmentation des épisodes extrêmes de température et de précipitations (Pörtner *et al.*, 2014), il est important d'identifier de nouvelles méthodes permettant d'estimer le stress subi par un organisme au cours de sa vie. Ceci peut avoir un intérêt afin de mieux comprendre et d'anticiper les conséquences à l'échelle individuelle des changements en cours. L'ambition de cette approche serait de pouvoir utiliser la dynamique des télomères comme marqueur de ces cumuls de stress chez le loup. Des études récentes proposent d'utiliser la dynamique des télomères non seulement comme biomarqueur de cumul de stress (Bateson, 2016), mais aussi à des fins d'écologie et de biologie évolutive (Nussey *et al.*, 2014). Chez l'étourneau sansonnet *Sturnus vulgaris*, Nettle *et al.* (2015) ont démontré que les individus désavantagés aux premiers stades de vie (plus petits, moins robustes) subissaient un raccourcissement des télomères plus important que ceux ayant été avantagés, et ce tout au long de leur vie. Ainsi, l'étude de la dynamique des télomères pourraient être fortement liée aux traits d'histoire de vie des stades précoces généralement les plus sensibles, comme le souligne Monaghan (2014).

Dans notre étude, les stress hyposalins réalisés ne semblent pas affecter la longueur des télomères ou l'expression de *tert*. Néanmoins, en milieu naturel, les animaux sont exposés à un cumul de différents types de stress : hyperthermie, hypoxie, stress social, hypersalure ou dessalure. Le lien entre raccourcissement des télomères et hyperthermie ou hypoxie a déjà été démontré chez des espèces de poissons (Rollings *et al.*, 2014; Debes *et al.*, 2016; Simide *et al.*, 2016). Pour ces raisons, la méthode développée pourrait être intéressante à tester sur des individus échantillonnés en milieu naturel pour étudier si la dynamique des télomères chez le loup peut être influencée par les traits d'histoire de vie, et notamment par des migrations dans les eaux de transitions, fortement variables d'un point de vue environnemental. Pour cela, il faudrait pouvoir coupler la mesure des télomères à l'analyse des otolithes afin de comparer des classes d'âges identiques et des individus ayant subi ou non dans leur cycle de vie des dessalures importantes.

Jusqu'à maintenant, aucune étude ne démontre un lien solide entre longueur des télomères et longévité chez les poissons. Mais peu d'études ont été réalisées à ce jour et la sensibilité des méthodes utilisées reste problématique. Compte-tenu de la détection d'ITS chez le loup, il semble nécessaire de favoriser des études longitudinales afin de suivre la dynamique des télomères chez un même individu. D'après McLennan (2016), les tacons de saumons atlantiques ont d'autant plus de chances de survivre à la migration que leurs télomères sont courts, car ces individus investissent plus d'énergie dans la smoltification. De plus, Gao & Munch (2015) montrent que chez la Capucette (*Menidia menidia*), le succès reproducteur se fait au détriment de la longueur des télomères. Ainsi, chez les poissons la longueur des télomères pourrait être considérée comme un marqueur moléculaire (à court terme du moins) de compromis des traits d'histoire de vie. L'étude de la dynamique des télomères offre de nouvelles perspectives afin d'étudier la plasticité phénotypique, et pourraient permettre de comprendre et/ou de prédire les compromis réalisés par les individus tout au long de leur vie. Après avoir validé la sensibilité de la méthode mise au point pour d'autres facteurs isolés (température, hypoxie, polluant) ou combinés, il serait intéressant d'étudier cette dynamique chez des loups sauvages couplée à des analyses du comportement migratoire.

3.2 La méthylation de l'ADN : un facteur déterminant dans la plasticité phénotypique chez les loups ?

Parmi les autres mécanismes de régulation pouvant impacter l'expression des gènes et la variabilité intraspécifique, on retrouve les **mécanismes épigénétiques** (Jaenisch & Bird, 2003). Il existe plusieurs définitions de l'épigénétique et celles-ci sont encore vivement débattues (Deans & Maggert, 2015). Nous choisissons ici d'opter pour celle de Deans & Maggert (2015) : « les **mécanismes épigénétiques** régulent l'accessibilité à l'ADN pour la machinerie transcriptionnelle et peuvent donc affecter l'expression de gènes qui sont soit **stables au cours des générations cellulaires** (mitoses ou/et méiose), ou **réversibles** et qui n'entrainent **pas de modification** dans la séquence d'ADN ». Il existe différents marqueurs épigénétiques comme l'acétylation des histones, les ARN non codants et la **méthylation de l'ADN** (Ho & Burggren, 2010).

La méthylation de l'ADN est un processus décrit dans une grande majorité du vivant, des bactéries aux eucaryotes en passant par les archées (Angers *et al.*, 2010). Elle consiste en l'ajout par des enzymes appelées **méthyltransférases** (DNMTs), d'un groupe méthyl à des bases tels que les cytosines afin de former par exemple des 5-méthyl cytosine (5mC) (Fig. 6).

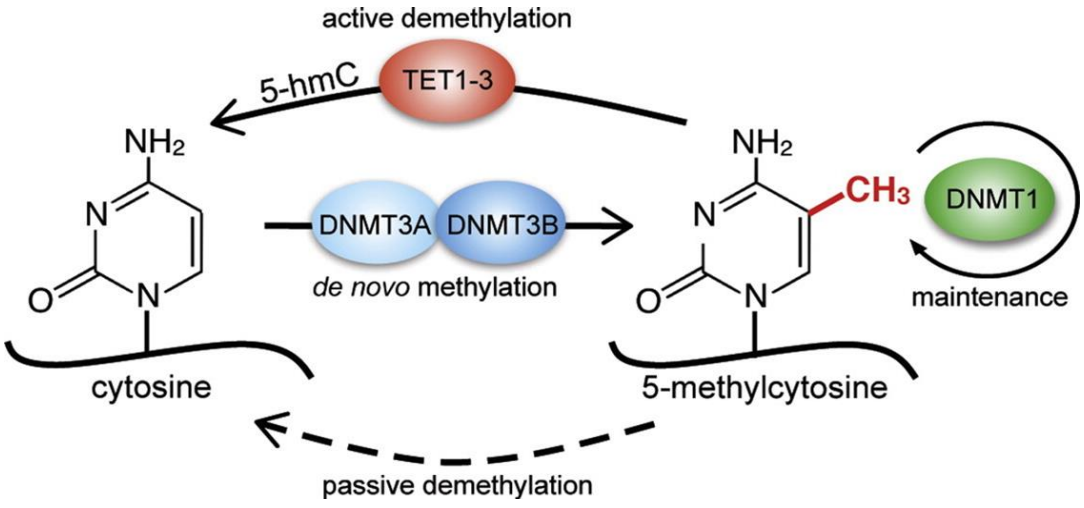


Figure 6 : Méthylation de l'ADN chez les métazoaires dont l'ADN peut être méthylé, d'après Ambrosi *et al.* (2017)

Ces enzymes sont extrêmement **conservées** au sein des *taxa* animaux et végétaux (Ponger & Li, 2005). Parmi elles, les DNMT1 assurent le maintien de ces marqueurs épigénétiques au cours des réplications cellulaires tandis que les DNMT3 assure la méthylation *de novo* (Hendrich & Bird, 2000). L'expression des DNMTs peut aussi être sous contrôle environnemental, comme cela fut démontré lors de l'exposition de larves de poisson zèbre *Danio rerio* à un stress hypothermique (Dorts *et al.*, 2016). Cela fut aussi démontré chez des larves de loup, avec une surexpression de *dnmt1* et une sous-expression de *dntm3* en conditions chaudes (Anastasiadi *et al.*, 2017). Ces groupements 5-méthyl cytosine se forment principalement au niveau de dinucléotides CpG (cytosine phosphate guanine) chez les eucaryotes (Law & Jacobsen, 2010), et sont trouvées aussi bien au niveau des gènes que des séquences inter-géniques ou au niveau des séquences répétées, tous les trois pouvant jouer un rôle dans la régulation de l'expression des gènes (Jones, 2012). En effet, si les séquences des gènes sont hyper-méthylées, alors généralement en résulte une surexpression (Yang *et al.*, 2014). A l'inverse, si ce sont les séquences promotrices, il en résulte généralement une sous-expression, car ces régions deviennent hétérochromatiques et ne sont plus accessibles aux facteurs de transcription (Chodavarapu *et al.*, 2010; Banovich *et al.*, 2014). Récemment, le lien étroit entre méthylation du premier intron et expression des gènes a été mis en évidence, illustrant la complexité du méthylome dans la régulation du transcriptome (Anastasiadi *et al.*, 2018a). La méthylation de l'ADN est fortement sous **influence environnementale** (Angers *et al.*, 2010). Elle peut permettre, par la modification de l'expression des gènes, l'émergence de nouveau phénotypes et pourrait donc expliquer, au moins en partie, la variabilité intraspécifique et la plasticité phénotypique (Massicotte *et al.*, 2011; Flores *et al.*, 2013).

De nombreux exemples illustrent l'implication de la méthylation de l'ADN sur la plasticité phénotypique et le lien existant entre la méthylation d'ADN et la physiologie des organismes. Parmi eux figure l'influence de la température sur le méthylome des épinoches (Metzger & Schulte, 2017) et des embryons de morue d'Atlantique *Gadus morhua* (Skjærven *et al.*, 2014). La salinité aussi semble influencer le méthylome des organismes marins, comme c'est le cas chez l'ascidie coloniale invasive *Didemnum vexillum* (Hawes *et al.*, 2018) et la daphnie (Jeremias *et al.*, 2018). Des différences de méthylation du génome en lien avec la salinité et l'acclimatation ont même été suggérées chez les truites communes *Salmo trutta*

(Morán *et al.*, 2013). Récemment, l'étude de la méthylation de certains gènes du loup dans un contexte d'augmentation de température ou dans un contexte d'étude du déterminisme du sexe, montre des différences d'expression de certains gènes clés du déterminisme sexuel comme celui de l'aromatase *cyp19*, ou encore le facteur de transcription *dmrt1* (Navarro-Martín *et al.*, 2011; Anastasiadi *et al.*, 2018b). Ainsi, **nous pouvons nous demander si la salinité influe le méthylome des loups, et s'il existe un lien entre la méthylation de l'ADN et les niveaux de transcrits**. De plus, la variabilité intraspécifique observée en eau douce pourrait également être analysée en utilisant des marqueurs épigénétiques tels que la méthylation des cystéines de l'ADN. D'autres formes de méthylation d'ADN existent comme la N6-adénine méthylation (6mA), aussi associée à des modifications d'expression de gènes chez *Caenorhabditis elegans* (Greer *et al.*, 2015; Luo *et al.*, 2015). Dans l'étude préliminaire que nous avons débutée à la fin de ma thèse, nous nous sommes consacrés à la 5-méthyl cytosine. Suite à une approche WGBS (Whole Genome Bisulfite Sequencing) effectués sur des tissus branchiaux de 5 individus par condition (eau de mer, ou phénotypés FW_t ou FW_i par rapport à leur tolérance en eau douce). Ces individus ont subi les mêmes conditions que celles du Chapitre 2 et 3. Nous avons pu déterminer des différences de méthylation globale de l'ADN selon la salinité (Fig. 7).

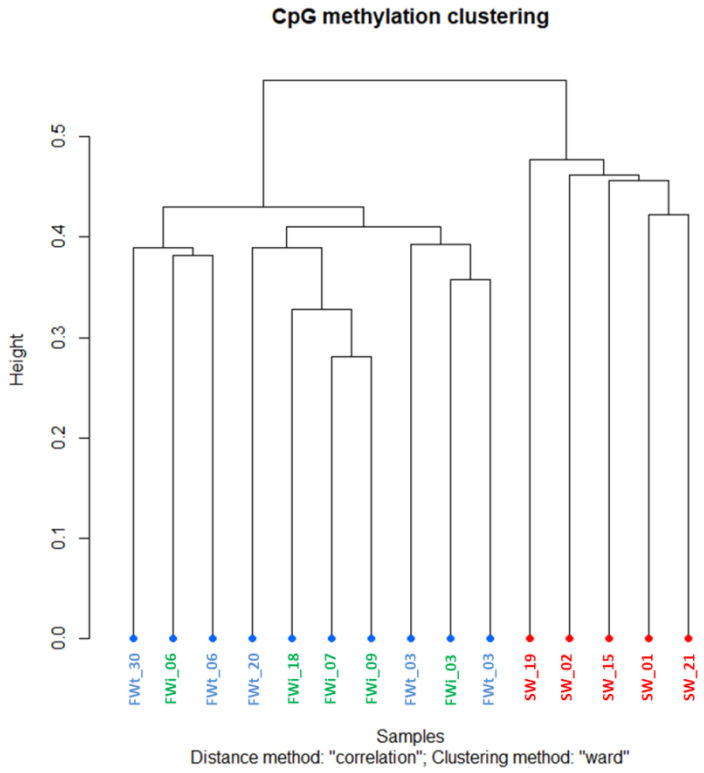


Figure 7 : Dendrogramme réalisé à partir de la méthylation de l'ADN chez des loups âgés de 5 mois maintenus en eau de mer (SW, en rouge), ou après 2 semaines en eau douce (FWt : tolérants, en bleu et FWi : intolérants, en vert ; les phénotypes étant identifiés par les indices comportementaux et validés par des mesures physiologiques du Chapitre 2)

Ainsi, le méthylome des individus maintenus en eau de mer (SW) semble différer de ceux maintenus en eau douce pendant deux semaines. En revanche, le méthylome des individus caractérisés comme tolérants à l'eau douce (FWt) ne semble pas différer fortement de celui des individus intolérants à l'eau douce (FWi). L'analyse de la méthylation des gènes jouant un rôle important dans l'acclimatation en eau douce et la réponse au stress ainsi que de leurs séquences promotrices seront étudiés en perspective de cette analyse de méthylome global. Le lien entre le méthylome, l'expression des *dnmts* et le comportement a déjà été mis en évidence chez le poisson zèbre et l'épinoche mais reste à déterminer chez le loup (McGhee & Bell, 2014; Knecht *et al.*, 2017). Il reste aussi à clarifier si les variations de plusieurs paramètres environnementaux rencontrés dans les lagunes (oxygène, température, salinité) peuvent impacter le méthylome des loups et l'influence possible sur leur comportement. De plus, l'association de ces paramètres pourrait modifier la sensibilité des loups à la dessalure et ainsi impacter la variabilité intraspécifique observée en laboratoire.

Une dernière perspective de ce travail de thèse pourrait être de s'intéresser au lien entre méthylation de l'ADN et raccourcissement des télomères. Chez les plantes, bien que les motifs télomériques soient différents de ceux des animaux, notamment par leur richesse en cytosine (TAAACCC chez les plantes *vs* TTAGGG chez les animaux), la méthylation des régions subtélomériques régule positivement l'homéostasie de la longueur des télomères chez *Arabidopsis thaliana* (Vaquero-Sedas & Vega-Palas, 2014; Vega-Vaquero *et al.*, 2016). La question de la possible régulation épigénétique de la longueur des télomères chez les animaux reste une question encore ouverte (Blasco, 2007). Des études récentes chez des cellules embryonnaires de souris démontrent que la méthylation de l'ADN limite l'élongation des télomères (Dan *et al.*, 2017). Récemment, la méthylation des télomères et des régions subtélomériques (Fig. 8) a même été utilisée comme biomarqueur de maladies liées à l'âge chez l'homme (Hu *et al.*, 2019; Lu *et al.*, 2019). Il serait intéressant d'étudier les interactions possibles entre méthylation et dynamique de la longueur des télomères chez les animaux notamment les poissons dans différentes conditions de salinité.

Au cours de cette thèse, nous nous sommes focalisés sur des populations issues de lignées sauvages de Méditerranée Ouest. Or en milieu naturel où les facteurs stressant sont multiples, des comportements d'évitement des faibles salinités ou bien de la mortalité

différentielle pourrait être observés à l'entrée des lagunes. La proportion de loups intolérants à l'eau douce n'ayant jamais été étudiée *in vivo*, elle reste à élucider. De plus, il serait intéressant de voir si ces phénomènes de tolérance différentielle à la dessalure sont partagés chez les autres souches génétiques de *D. labrax* comme les lignées de Méditerranée Est et d'Atlantique, qui possèdent potentiellement des caractéristiques écologiques et migratoires différentes de celles de la lignée Méditerranée Ouest sur laquelle nous nous sommes focalisés dans cette thèse (Duranton *et al.*, 2018; De Pontual *et al.*, 2019).

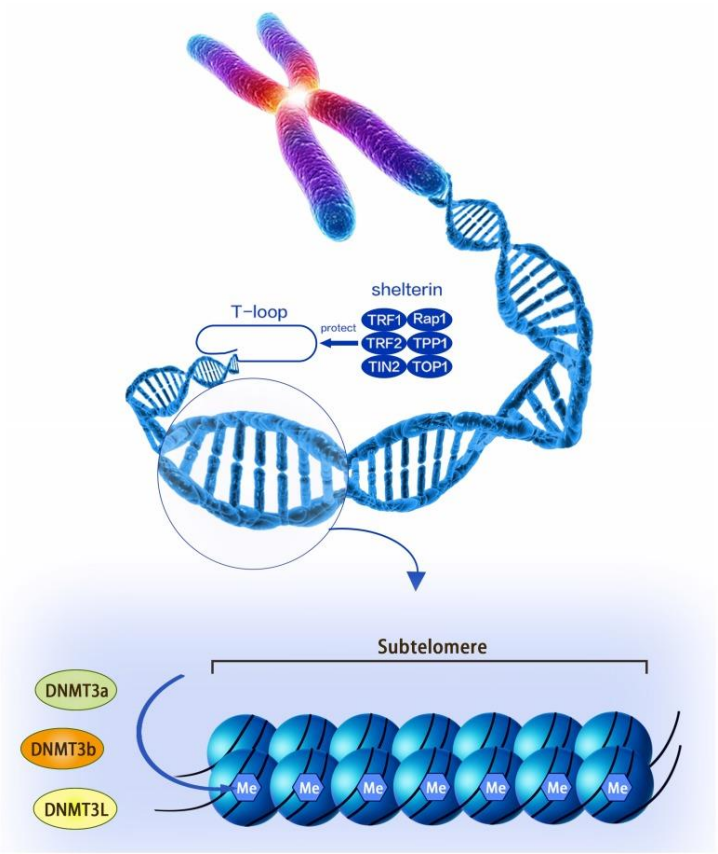


Figure 8 : Schéma structural des télomères et des subtélomères (régions entre les télomères et la chromatine). Les niveaux de méthylation de ces régions pouvant être régulés par les enzymes DNMT3L, DNMT3a et DNMT3b, d'après Hu *et al.* (2019)

Conclusion

« C'est la nature » E.F.

Conclusion

Dans cette thèse, nous nous sommes intéressés à la variabilité inter-individuelle de la tolérance à l'eau douce chez une espèce euryhaline : le loup méditerranéen *D. labrax*. En s'intéressant au métabolisme respiratoire, nous avons pu mettre en évidence une meilleure tolérance à une hypoxie modérée en eau douce qu'en eau de mer. Cependant en eau douce, les loups deviennent plus sensibles à des évènements hypoxiques intenses (Chapitre 1). Nous avons aussi identifié des traits caractéristiques de la tolérance à l'eau douce comme par exemple des paramètres biochimiques sanguins différents, indiquant une capacité différentielle à réguler les ions circulants. Celle-ci semble être due à des capacités osmorégulatrices différentes notamment au niveau du rein postérieur (Chapitre 2). Au niveau des transcrits, les individus intolérants à l'eau douce présentent une expression plus faible de certains transporteurs impliqués dans l'absorption / réabsorption de de chlorures notamment (Chapitre 3). De plus, l'équilibre acido-basique semble perturbé chez les individus intolérants à la dessalure. Il serait intéressant de compléter ces études par des mesures de pH sanguins afin de mieux caractériser la variabilité intraspécifique dans la régulation acide-base en lien avec la dessalure. En replaçant ces résultats dans un contexte naturel où les dessalures sont couplées à d'autres facteurs changeants comme l'augmentation de température et l'acidification des eaux, la variabilité inter-individuelle dans la tolérance à la dessalure pourrait influencer de manière significative les comportements des loups, notamment migratoires. Ce travail de thèse a permis de mettre au point une méthode d'étude de la dynamique des télomères chez le loup qui permet de mesurer la longueur des télomères tout comme l'expression de la télomérase (Chapitre 4). Concernant l'étude du méthylome du loup, l'objectif futur est de cibler certains gènes d'intérêt pour voir si les différences de méthylation sont corrélées à des différences d'expression génique. Ces travaux de thèse ont permis d'identifier certains gènes clefs dont l'expression diffère en fonction du phénotype. Globalement, ce travail de thèse apporte des éléments pour alimenter la réflexion sur les questions de plasticité phénotypique, variabilité intraspécifique et adaptabilité des espèces aux changements globaux.

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Annexes

Annexe 1 : Liste de publications

Manuscrit publié

- **L'Honoré, T.,** Farcy, E., Chatain, B., Gros, R., Ruelle, F., Hermet, S., Blondeau-Bidet, E., Naudet, J., Lorin-Nebel, C., 2019. Are European sea bass as euryhaline as expected? Intraspecific variation in freshwater tolerance. Marine Biology 166, 102. https://doi.org/10.1007/s00227-019-3551-z

Manuscrit soumis

- **L'Honoré, T.**, Farcy, E., Blondeau-Bidet, E., Lorin-Nebel, C., 2019. Inter-individual variability in freshwater tolerance is related to transcript level differences in gill and posterior kidney of European sea bass. *Soumis dans Gene, le 10.09.2019*

Manuscrits en préparation

- **L'Honoré, T.**, Lorin-Nebel, C., McKenzie, D., Gimenez, M., Naudet, J., Farcy, E.. How do sea bass *Dicentrarchus labrax* cope with long-term freshwater exposure? New insights into successful acclimation.
- **L'Honoré, T.**, Lorin-Nebel, C., Blondeau-Bidet, E., Perez, J., Veyrunes, F., Farcy, E.. Are telomere dynamics good molecular markers of environmental stress in European sea bass *Dicentrarchus labrax*? A focus on hyposaline stress.
- **L'Honoré, T.**, Cosseau, C., Farcy, E., Blondeau-Bidet, E., Lorin-Nebel, C.. Differential DNA methylation rate as an epigenetic determinant of the salinity stress response in the European sea bass.

Annexe 2 : Participation à des projets scientifiques

Projet TELOMEL (LabEx CeMEB, projet Equipe de Recherche Junior, 2019) : Etude de la longueur des télomères et de l'expression de la télomérase comme potentiels biomarqueurs moléculaires de stress salins chez le loup méditerranéen *Dicentrarchus labrax*. Porteur du projet : Thibaut L'Honoré

- Mise au point et réalisation des mesures moléculaires
- Encadrement d'un stagiaire de Master 1
- Présentation des résultats au Congrès d'EcoPhysiologie Animale IV (CEPA IV), Octobre 2019, Rennes, France

Projet SALSA (LabEx CeMEB, 2019-2020) : Etude de la variabilité phénotypique des loups en lien avec la dessalure au niveau du méthylome. Coordinatrices : Catherine Lorin-Nebel & Céline Cosseau

- Etude du méthylome de loups selon leur phénotype en lien avec la tolérance à la dessalure

Projet Pituitox (EMBRC, 2015) : Effet du 4-Nonylphénol sur le fonctionnement d'hypophyses en culture chez le loup européen *Dicentrarchus labrax*. Coordinatrice : Catherine Lorin-Nebel & Laurence Besseau

- Etude de l'expression des gènes sur les hypophyses en culture
- Dosage hormonal dans les milieux de culture

Annexe 3 : Communications scientifiques

Congrès internationaux :

- **SEB 2019 - Society for Experimental Biology, Séville, Espagne, Juillet 2019 :** Molecular characterisation of phenotype plasticity related to freshwater tolerance in European sea bass. **L'Honoré, T.***, Farcy, E., Blondeau-Bidet, E., Lorin-Nebel, C.
- **SEB 2018 – Society for Experimental Biology, Florence, Italie, Juillet 2018 :** Are all European sea bass as euryhaline as expected? Phenotypic plasticity in fresh water. **L'Honoré, T.**, Farcy, E., Chatain, B., Gros, R., Ruelle, F., Hermet, S., Blondeau-Bidet, E., Naudet, J., Lorin-Nebel, C*.

Congrès nationaux :

- **CEPA IV – Congrès d'EcoPhysiologie Animale IV, Rennes, France, Octobre 2019 :** Nouvelles approches moléculaires pour l'étude du stress salin chez le loup *Dicentrarchus labrax*. **L'Honoré T.***, Blondeau-Bidet E., Cabar M., Lorin-Nebel C., Cosseau C., Farcy E.
- **CEPA III - Congrès d'EcoPhysiologie Animale III, Strasbourg, France, Novembre 2017 :** Phenotypic plasticity in freshwater acclimated sea bass. **L'Honoré, T.***, Farcy, E., Chatain, B., Gros, R., Ruelle, F., Hermet, S., Blondeau-Bidet, E., Naudet, J., Lorin-Nebel, C.

Workshops :

- **Mélanger pour mieux innover, Strasbourg, France, Mars 2019 :** Multiple approaches to estimate intraspecific variability facing salinity challenges in European sea bass *Dicentrarchus labrax*. **L'Honoré, T.***, Farcy, E., Blondeau-Bidet, E., Lorin-Nebel, C.

**présentateur - présentatrice*

Annexe 4 : Autres activités scientifiques

Co-Encadrement de stagiaires :

- Mathieu Cabar, Master 1 (3 mois, 2019) : Etude du comportement des loups face à la dessalure, mise au point des mesures de longueur des télomères et d'expression de la télomérase.
- Marie Gimenez, Master 1 (2 mois, 2018) : Etude du compromis osmo-respiratoire chez le loup, histologie et mesure des paramètres morphologiques des branchies.
- Jeanne Naudet, Etudiante vétérinaire (1 mois, 2017) : Suivi des expérimentations en eau douce, échantillonnage et mesure des paramètres biochimiques.
- Alizée Bourgès, Master 1 (2 mois, 2017) : Mise au point des expérimentations en eau douce chez le loup.

Médiation scientifique

- **Bénévole au sein de l'association Pint of Science (2019) :** rencontre avec les chercheurs, mise au point d'une stratégie de vulgarisation et de communication scientifiques.
- **Ma thèse en 180 secondes (2019) :** participation jusqu'à la finale régionale, expression, vulgarisation et présence sur scène.
- **Atelier découverte de l'étang de Thau (2017) :** encadrement de collégiens le temps d'une journée sur différentes thématiques autour de la lagune de Thau et de sa biodiversité.

Enseignements

- **Licence 1 :** Biologie intégrative (TD & TP)
- **Licence 2 :** Du génotype au phénotype (TD & TP)
- **Licence 3 :** Adaptations des organismes marins aux environnements extrêmes (CM & TP)
- **Formation continue en aquaculture (SFC) :** Présentation des modèles mollusques et crustacés en biologie marine (TP)

Résumé de la thèse

Face à un changement dans leur environnement, les organismes peuvent être amenés à migrer ou à s'acclimater. L'acclimatation repose sur la capacité des organismes à pouvoir modifier leur phénotype, c'est à dire certains de leur traits biologiques (métabolisme, comportement, physiologie) afin de pouvoir survivre dans un environnement donné. On parle alors de plasticité phénotypique. Les individus d'une même espèce peuvent présenter des différences au sein de leur génotype et phénotype. On parle alors de variabilité inter-individuelle ou intraspécifique. Cette variabilité peut se retrouver dans la plasticité que possèdent les organismes à répondre à des changements rapides ou à long terme de leur environnement. Le bar ou loup Européen *Dicentrarchus labrax* est un poisson marin dont l'aire de répartition s'étend des côtes nord européennes jusqu'aux côtés africaines en Atlantique en passant par la Méditerranée et la mer Noire. Il entreprend des migrations saisonnières dès le stade juvénile dans les lagunes et les estuaires voire parfois même en rivière. C'est donc une espèce plastique en termes de physiologie osmorégulatrice, capable de supporter une grande gamme de salinité : de 0 à 90 ppt. En revanche, en laboratoire une forte variabilité dans la réponse osmorégulatrice en eau douce a pu être mise en évidence. Les objectifs de cette thèse étaient de caractériser la plasticité phénotypique et la variabilité intraspécifique du loup méditerranéen en lien avec la dessalure.

Pour cela, des loups ont été transférés en eau douce et étudiés par différentes approches. La première m'a permis de d'appréhender les effets de la dessalure sur le métabolisme respiratoire et la tolérance à l'hypoxie. En eau douce, les capacités respiratoires des loups diffèrent de celles en eau de mer avec un taux métabolique plus haut, ainsi qu'une tolérance à l'hypoxie plus accrue. De plus, l'étude des transcrits révèle des réponses différentes après deux semaines et après deux mois passés en eau douce. Les individus incapables de tolérer l'eau douce ont été caractérisés par des traits comportementaux (faible vitesse, déplacement) et biochimiques (faible pression osmotique, chlorure et sodium sanguins) qui diffèrent par rapport aux tolérants à la dessalure. L'étude des transcrits (transporteurs ioniques et récepteurs aux hormones) a révélé que l'intolérance à l'eau douce serait due en partie à une incapacité au niveau rénale à réabsorber les ions et donc à maintenir constante leur balance hydrominérale. L'étude de la variabilité phénotypique des loups méditerranéens en eau douce a été répétée à des âges différents. Elle démontre que la tolérance/l'intolérance à l'eau douce est un phénomène stable en termes de proportions (30% d'intolérants), mais labile avec le temps (ce ne sont pas forcément les mêmes individus qui sont tolérants ou intolérants à la dessalure suite à des transferts successifs). Cela suggère des mécanismes de régulation aléatoire, génétiques et/ou épigénétiques. Une étude préliminaire de la méthylation des cystéines de l'ADN a permis de déduire que le transfert en eau douce influe sur la méthylation globale de l'ADN. Le lien entre variabilité intraspécifique liée à la tolérance à l'eau douce et méthylation de l'ADN reste à déterminer.

La dernière approche menée a consisté à étudier la dynamique des télomères (longueur des télomères et expression de la télomérase) comme potentiels marqueurs de stress hypo-osmotiques chez le loup. La méthode, désormais au point n'indique aucun effet du stress hypo-osmotique sur la dynamique des télomères. Néanmoins, la réponse des loups intolérants à l'eau douce reste à confirmer en augmentant le nombre d'individus. Ces résultats préliminaires suggèrent que les transitions vers les environnements hypo-osmotiques ne provoquent pas plus de dommages oxydatifs au niveau branchial. Il serait intéressant d'aborder l'approche de la dynamique des télomères dans le cadre d'autres stress environnementaux et de creuser l'approche sur d'autres organes.

Abstract

Facing environmental change, organisms may have to migrate or to acclimate. Acclimation is based on the ability of organisms to modify their phenotype, i.e. some of their biological traits (metabolism, behaviour, physiology) in order to survive in a given environment. This is called phenotypic plasticity. However, individuals of the same species may differ within their genotype and phenotype. This is referred as inter-individual or intra-specific variability. This variability can be found in the plasticity of organisms to respond to rapid or long-term changes in their environment. The European sea bass *Dicentrarchus labrax* is a marine fish whose distribution range extends from the northern European coasts to the African coasts of the Atlantic Sea towards the Mediterranean and Black Sea. It undertakes seasonal migrations from the juvenile stage in lagoons and estuaries and sometimes even in rivers. It is therefore a plastic species in terms of osmoregulatory physiology, capable of withstanding a wide range of salinity: from 0 to 90 ppt. On the other hand, in the laboratory, a high variability in the osmoregulatory response was observed in fresh water. The objectives of this thesis were to characterise the phenotypic plasticity and intraspecific variability of the Mediterranean Sea bass in relation to desalination.

For this, fish were transferred to fresh water and studied using different approaches. The first one allowed understanding the effects of desalination on respiratory metabolism and hypoxia tolerance. In fresh water, the respiratory capacities of sea bass differ from those maintained in seawater with a higher metabolic rate, as well as a higher tolerance to hypoxia. In addition, the quantification of the transcripts reveals different responses after two weeks and two months in fresh water. Individuals unable to tolerate fresh water were characterised by differential behavioural traits (lower velocity, displacement) and biochemical traits (lower osmotic pressure, blood chloride and sodium) than those of freshwater tolerant fish. The transcript levels (ionic transporters and hormone receptors) revealed that intolerance to fresh water is partly due to an inability at the renal level to reabsorb ions and thus maintain hydromineral balance. The study of the phenotypic variability of Mediterranean Sea bass in fresh water has been repeated at different ages. It shows that tolerance/intolerance to fresh water is a stable phenomenon in terms of proportions (30% intolerant), but labile over time (it was not necessarily the same individuals being tolerant or intolerant to fresh water following successive transfers). This suggests random, genetic and/or epigenetic regulatory mechanisms. A preliminary study of DNA cysteine methylation concluded that freshwater transfer influences overall DNA methylation. The link between intraspecific variability related to freshwater tolerance and DNA methylation remains to be investigated.

Finally we studied telomere dynamics (telomere length and expression of the telomerase gene) as potential markers of hypo-osmotic stress in sea bass. The method, now optimised, does not indicate any effect of hypo-osmotic stress on telomere dynamics. Nevertheless, the response of intolerant sea bass to fresh water has yet to be confirmed by increasing the number of individuals. These preliminary results suggest that transitions to hypo-osmotic environments do not cause more oxidative damage at the branchial level. It would be interesting to use this approach in the context of other environmental stressors and to explore other organ's telomere dynamics.

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Face à un changement dans leur environnement, les organismes peuvent être amenés à migrer ou à s'acclimater. L'acclimatation repose sur la capacité des organismes à pouvoir modifier leur phénotype, c'est à dire certains de leur traits biologiques (métabolisme, comportement, physiologie) afin de pouvoir survivre dans un environnement donné. On parle alors de plasticité phénotypique. Les individus d'une même espèce peuvent présenter des différences au sein de leur génotype et phénotype. On parle alors de variabilité inter-individuelle ou intraspécifique. Cette variabilité peut se retrouver dans la plasticité que possèdent les organismes à répondre à des changements rapides ou à long terme de leur environnement. Le bar ou loup Européen *Dicentrarchus labrax* est un poisson marin dont l'aire de répartition s'étend des côtes nord européennes jusqu'aux côtés africaines en Atlantique en passant par la Méditerranée et la mer Noire. Il entreprend des migrations saisonnières dès le stade juvénile dans les lagunes et les estuaires voire parfois même en rivière. C'est donc une espèce plastique en termes de physiologie osmorégulatrice, capable de supporter une grande gamme de salinité : de 0 à 90 ppt. En revanche, en laboratoire une forte variabilité dans la réponse osmorégulatrice en eau douce a pu être mise en évidence. Les objectifs de cette thèse étaient de caractériser la plasticité phénotypique et la variabilité intraspécifique du loup méditerranéen en lien avec la dessalure.

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