Towards the development of clonal lines in the European sea bass (Dicentrarchus labrax L) : application of uniparental reproduction techniques with an insight into sea bass eggs

Julie Colleter

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Présentée par **Julie COLLETER**

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Mme Daniela BERTOTTO, Associate professor, Université de Padoue	Rapporteur
Mr Albert IMSLAND, Professor, Université de Bergen	Rapporteur
Mme Edwige QUILLET, Directrice de recherches, Inra	Examinatrice
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In the middle of difficulty lies opportunity.

Albert Einstein

La vérité vient rarement des réponses que tu reçois […] la vérité naît de l'enchaînement logique des questions que tu poses.

Daniel Pennac

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Abstract & résumé

Towards the development of clonal lines in the European sea bass (*Dicentrarchus labrax* L.): application of uniparental reproduction techniques with an insight into sea bass eggs

Clonal lines are a powerful scientific tool for improved genetic characterization of organisms used in research. Inbred fish lines can be produced in only two generations using uniparental reproduction techniques. Androgenesis, achieved with variable success in several freshwater species, has been attempted in the European sea bass (*Dicentrarchus labrax* L), a marine fish of commercial and scientific interest. The low yields of progenies inheriting only the paternal genome after UV-irradiation of eggs led to considerations on the occurrence of UV screening compounds in pelagic eggs. Mycosporine-like amino acids and gadusol were found in many marine and freshwater organisms, but their occurrence in fish eggs was not clearly related to a behavioral pattern and while gadusol appeared in higher proportions in pelagic marine eggs compared to benthic species, this statement did not apply in freshwater, and moreover the kind of compounds was related to phylogeny. Further studies on DNA photorepair could enlighten hypotheses to understand the mechanisms underlying the disparate results obtained in inducing androgenesis in different fish species. Gynogenesis was reported successful to produce clonal founders in the sea bass, but high numbers of meiotic individuals contaminating fully homozygous progenies highlighted the need for efficient DNA markers to distinguish mitotic gynogenetic individuals. Furthermore, gonad development was highly delayed in gynogenetic progenies enhancing the difficulties to produce clonal lines. A high variability between individuals in the success of uniparental reproduction brought out gamete characterization and quality as a prerequisite.

Keywords: *Dicentrarchus labrax*, clonal lines, androgenesis, gynogenesis, MAAs, gadusol, microsatellite markers, homozygosity, heterozygosity, recombination rate.

Vers le développement de lignées clonales chez le loup de mer (*Dicentrarchus labrax* L.): mise en œuvre des techniques de reproduction uniparentale et aperçu de quelques caractéristiques de l'œuf

Les lignées clonales sont un outil puissant pour une meilleure caractérisation génétique des organismes utilisés en recherche. En utilisant les techniques de reproduction uniparentale, de telles lignées peuvent être développées chez les poissons en seulement deux générations. L'androgenèse avait déjà été réalisée chez plusieurs espèces d'eau douce et a été tentée chez le loup, une espèce d'intérêt économique et scientifique. Le faible nombre d'individus contenant effectivement uniquement le patrimoine génétique du père, après irradiation des œufs aux UV, a soulevé des questions quant à la présence de composés photoprotecteurs dans les œufs pélagiques. Les acides aminés de type mycosporines et le gadusol ont été recensés dans de nombreux organismes marins et d'eau douce, mais leur présence n'a pu être reliée au comportement flottant ou coulant des œufs. Tandis que le gadusol apparaissait en plus grande quantité dans les œufs marins flottants, ce résultat était beaucoup moins clair en eau douce et le type de composé photoprotecteur semble lié à la phylogénie. D'autres études sur les mécanismes de photoréparation de l'ADN pourraient éclairer les résultats obtenus dans les expériences d'androgenèse. La gynogenèse avait déjà été reportée chez le loup mais le grand nombre d'individus méiotiques contaminant les descendances homozygotes produites a conduit à valider des marqueurs de l'ADN efficaces à distinguer les individus mitotiques. De plus, un retard de développement des gonades a été observé, augmentant les difficultés à obtenir les lignées clonales. La grande variabilité observée entre les individus quant au succès des reproductions uniparentales a montré que la caractérisation et la qualité des gamètes sont un préalable indispensable.

Mots-clé : *Dicentrarchus labrax*, lignées clonales, androgenèse, gynogenèse, MAAs , gadusol, marqueurs microsatellites, homozygotie, hétérozygotie, taux de recombinaison.

Résumé substantiel

Vers le développement de lignées clonales chez le loup de mer (*Dicentrarchus labrax* L.): mise en œuvre des techniques de reproduction uniparentale et aperçu de quelques caractéristiques de l'œuf

Le loup de mer, ou bar commun, *Dicentrarchus labrax* L., est une espèce euryhaline et eurythermique vivant le long des côtes marines européennes (Jobling et al., 2010). Le loup est une espèce gonochoristique, à différentiation sexuelle précoce et sensible à la température, qui ne montre pas de caractère sexuel secondaire. L'unique saison de ponte naturelle est hivernale, entre Janvier et Mars en Méditerranée et légèrement plus tardive en Atlantique. Les œufs sont de petite taille (1.1-1.3 mm), contiennent une ou plusieurs gouttelettes lipidiques et flottent en surface quand la salinité est supérieure à 34‰ (Barnabé et al., 1976). La croissance des embryons dépend de la température et l'éclosion intervient 5 jours après fécondation à 13°C, intervalle de temps diminué à des températures plus élevées (Jobling et al., 2010). Chez les poissons téléostéens, l'œuf est le produit final d'un long processus de développement ovocytaire, il contient toute l'information génétique et les composés nutritifs nécessaires au développement de l'embryon (Brooks et al., 1997).

L'aquaculture du loup, aidée par le développement des techniques de reproduction artificielle et le contrôle du grossissement en captivité, s'est très fortement développée en moins de 25 ans avec une évolution de la production passant de quelques milliers à 153 000 tonnes en 2012 (FAO, 2014). Malgré la domestication du loup, de nombreuses écloseries s'approvisionnent dans le milieu naturel pour entretenir leurs stocks de géniteurs et la mise en place de programmes de sélection génétique permettra d'augmenter la rentabilité économique de cette filière. Le loup, comme de nombreuses espèces de poissons, ne présente pas de chromosomes sexuels distincts, le déterminisme du sexe semble être influencé par des facteurs génétiques avec d'importantes variations induites par l'environnement. Cet aspect est le sujet de nombreuses recherches car la production de populations femelles présente un intérêt économique : croissance rapide et individus plus gros (Haffray et al., 2007; Piferrer et al., 2005).

L'objectif de cette thèse était initialement de développer des lignées clonales chez le loup, en produisant, dans un premier temps, des individus homozygotes par reproduction uniparentale qui seront les géniteurs de futures lignées clonales. La première partie de ce travail explique les principes et quelques utilisations possibles des lignées clonales en recherche scientifique. La seconde partie est, quant à elle, plus orientée sur l'androgenèse. Après une revue bibliographique des différentes expériences d'androgenèse chez les poissons, les différentes techniques d'irradiation, de diploïdisation et de vérification des descendances produites sont présentées et discutées. La troisième partie présente l'expérience d'androgenèse réalisée chez le loup durant cette thèse, ces résultats bien que négatifs rapportent, pour la première fois, l'induction de l'androgenèse chez une espèce marine et ont été publiés dans la revue open

access PlosOne. La quatrième partie analyse la présence, dans les œufs de loup et d'autres espèces, de composés permettant de limiter la pénétration et les dommages liés à l'irradiation aux UV, cette hypothèse n'explique que partiellement les résultats d'androgenèse obtenus chez le bar. La cinquième partie présente la production d'individus homozygotes par gynogenèse, avec une caractérisation du développement et l'utilisation de marqueurs microsatellites adaptés à la distinction des individus mitotiques, les seuls totalement homozygotes.

Partie 1. Le développement de ressources génétiques : les lignées clonales

Les poissons représentent le groupe le plus large et le plus primitif des vertébrés, ce groupe a évolué et s'est adapté à de multiples environnements, ce qui en fait un sujet d'études dans de nombreux domaines en relation avec l'évolution phylogénétique, la reproduction, et la génétique des vertébrés en général. L'expérimentation animale doit répondre aux 3 critères de haute qualité en matière de réplicabilité, répétabilité et reproductibilité, les lignées clonales sont ainsi un outil remarquable pour améliorer la qualité des expérimentations (Bongers et al., 1998).

Chez les poissons, la fécondation est externe et les gamètes peuvent être manipulés ce qui rend la production de lignées isogéniques clonales possible en uniquement 2 générations de reproduction uniparentale successives. La production de lignées clonales pures ou de lignées clonales recombinées peut-être effectuée par androgenèse ou gynogenèse. L'androgenèse consiste à féconder des œufs génétiquement inactivés par rayonnement UV, X ou gamma pour produire des embryons ne contenant que le patrimoine génétique du père. L'état haploïde n'étant pas viable à terme, la diploïdie des embryons est rétablie en utilisant un choc physique de température ou pression, ou en utilisant du sperme diploïde. La gynogenèse, quant à elle, produit des embryons contenant uniquement l'ADN provenant de la femelle, elle est achevée par fécondation des œufs avec du sperme inactivé génétiquement. La diploïdie peut être restaurée par un choc physique qui peut être appliqué à 2 moments distincts : un choc précoce, quelques minutes après la fécondation, entraîne la rétention du second globule polaire et produit en majorité des individus gynogénétiques méiotiques hétérozygotes, tandis qu'un choc tardif bloquant la première division cellulaire produit des individus mitotiques complètement homozygotes. Des lignées clonales ont été développées chez quelques espèces de poissons bien que, d'une manière générale, la survie des individus homozygotes soit très faible et leur fécondité très réduite, ce qui entraîne quelques difficultés à perpétuer ces lignées (Komen and Thorgaard, 2007).

Les lignées isogéniques clonales sont un outil remarquable en recherche notamment parce qu'elles permettent l'étude d'un même génotype dans différentes conditions environnementales et l'identification de QTLs. Chaque individu clonal représentant un haplotype, l'utilisation d'individus homozygotes simplifie les analyses génomiques comme le séquençage et la cartographie. Leur utilisation a, par exemple, permis d'étudier le déterminisme du sexe chez le tilapia, une espèce qui possède des chromosomes sexuels différenciés mais dont le sexe peut être modifié par des facteurs mineurs (Müller-Belecke and Hörstgen-Schwark, 1995). La variance génétique est aussi mieux caractérisée dans les lignées

clonales et le nombre d'individus utilisés pour estimer l'héritabilité de certains caractères est diminué (Bongers et al., 1997b). Dans le domaine de la cartographie du génome, la gynogenèse méiotique permet d'estimer les distances des gènes par rapport au centromère (Purdom, 1993), tandis que l'androgenèse et la gynogenèse mitotique permettent de cartographier précisément et d'analyser les recombinaisons différentielles selon le sexe des individus. L'une des utilisations les plus répandues à l'heure actuelle, où les clones présentent un intérêt majeur, est dans l'étude des QTLs. Après la comparaison de différentes lignées clonales pour un caractère d'intérêt, l'analyse de la ségrégation et la relation au phénotype dans une lignée clonale recombinée puis la mise en œuvre de techniques de séquençage, de nombreux QTLs liés à la résistance aux maladies, la croissance embryonnaire ou encore la physiologie du stress ont pu être détectés chez la truite arc-en-ciel (Komen and Thorgaard, 2007).

Partie 2. Etat de l'art, revue bibliographique sur l'androgenèse chez les poissons

L'induction de l'androgenèse a été réalisée dans diverses expériences ayant donné des résultats positifs, bien que souvent variables, chez une vingtaine d'espèces de poissons d'eau douce. La mise au point du protocole commence généralement par la caractérisation de la dose et de la durée optimales pour une irradiation des œufs permettant d'inactiver le patrimoine génétique femelle tout en conservant la viabilité de l'œuf à permettre un développement embryonnaire. La dose optimale d'irradiation est très variable selon les espèces, les doses d'UV recensées pouvant varier de 37 à 346 $mJ.cm^{-2}$, les doses de rayonnement X de 100 à 420 Gy et les doses de rayonnement gamma de 150 à 880 Gy. Il est, cependant, difficile de comparer l'efficacité de l'irradiation entre les espèces car de nombreux facteurs peuvent avoir un effet, tels que la taille et la forme des œufs, la position du pronucléus femelle à l'intérieur du cytoplasme ainsi que les propriétés physico-chimiques et optiques du chorion. Le choc de pression hydrostatique ou de température utilisé pour diploïdiser les embryons produits après fécondation est peu variable en intensité : de 7000 à 11000 psi ou de 37 à 42.5°C durant quelques minutes, mais le moment d'application du choc est, quant à lui, extrêmement variable: il peut être appliqué d'une vingtaine de minutes à plus de 7h après la fécondation en fonction de l'espèce concernée. Les haploïdes doublés, ainsi produits, montrent des taux de survie extrêmement faibles dus, entre autres, à l'expression de gènes récessifs délétères affectant le développement et la viabilité des embryons ainsi qu'à des dommages liés à l'irradiation et au choc physique appliqués aux œufs. Dans certains cas, quand l'application de l'androgenèse chez une espèce présente des difficultés, une alternative possible est d'utiliser une autre espèce pour fournir les œufs et de réaliser une fécondation uniparentale interspécifique. Bien que la compatibilité nucléo-cytoplasmique ne soit pas toujours assurée, l'androgenèse interspécifique a été réalisée avec succès dans plus d'une vingtaine d'expériences.

La première étape de l'androgenèse consiste à inactiver le matériel génétique femelle en utilisant soit des UV qui vont former des dimères cyclobutane-pyrimidine, lésions mutagènes, et la réticulation de l'ADN entraînant l'inactivation de la molécule d'ADN, soit des rayons X

ou gamma qui eux détruisent la molécule d'ADN. Le choix de la méthode d'irradiation est primairement basé sur des critères de praticité technique, les UV étant plus simples d'utilisation autant du point de vue installation que sécurité, mais doit aussi intégrer le fait que les UV ont une pénétration plus faible et peuvent aussi donner des fragments chromosomiques, que l'on pensait induits uniquement par les rayons ionisants, plus fréquents en conditions sous-optimales d'irradiation (Lin and Dabrowski, 1998). La seconde étape consistant à restaurer la diploïdie est réalisée par un choc physique. Le choix entre température et pression semble, lui, spécifique à l'espèce étudiée. Les mécanismes cytologiques induits par les manipulations chromosomiques sont encore assez peu connus mais il semble qu'un choc thermique chaud change les propriétés intracellulaires et modifie l'équilibre des fuseaux chromatiques entraînant la rétention du second globule polaire ou l'interruption de la première mitose, un choc thermique froid semble être beaucoup moins efficace qu'un choc chaud ou de pression à provoquer l'endomitose. Les fuseaux métaphasiques sont très sensibles à la pression hydrostatique et les mouvements des chromosomes sont contrôlés par des contractions des fuseaux chromatiques, c'est pourquoi, il semble qu'un choc de pression élevée agisse sur les microtubules empêchant les mouvements des chromosomes. Certains effets secondaires délétères, tels que des déstabilisations et déformations d'autres organelles, semblent aussi être provoqués suite à un choc de pression. Les effets cellulaires des différents types de choc appliqué pour rétablir la diploïdie semblent être différents et la modalité d'application semble être elle aussi importante : le moment d'application de la pression apparaît plus précis et tardif que la chaleur (Diter et al., 1993). Les difficultés à déterminer les mécanismes cellulaires mis en jeu et impactés lors des manipulations chromosomiques sont encore mal connus, et il a été de plus montré que la température ou la pression appliquées pour provoquer une endomitose bloqueraient non pas la première mais la seconde division cellulaire (Zhang and Onozato, 2004).
La dernière étape importante d'une reproduction uniparentale est la vérification de son succès, tant du point de vue de la ploïdie que de l'origine du patrimoine génétique hérité dans les embryons. Différentes techniques ont été utilisées pour déterminer la ploïdie des individus produits, telles que : le 'syndrome haploïde' caractérisé par des malformations de type nanisme, microcéphalie ou microphtalmie ; des techniques de comptage des chromosomes grâce aux caryotypes ou préparations NOR et encore, la cytométrie de flux qui permet de mesurer le contenu relatif de l'ADN nucléaire dans chaque cellule. Chaque technique présente ses avantages et inconvénients en termes de précision, temps, technicité et coût. La transmission du patrimoine génétique d'un seul parent est quant à elle vérifiée par des techniques phénotypiques à l'aide de marqueurs morphologiques récessifs, par des techniques enzymatique permettant de distinguer les allozymes, ou encore par des techniques moléculaires de plus en plus courantes, de type RFLP, RAPD ou marqueurs microsatellites.

Partie 3. Inactivation génétique d'oeufs de loup de mer (*Dicentrarchus labrax* L.) par irradiation UV: observations et perspectives

L'androgenèse, reproduction uniparentale où seul l'ADN nucléaire du père est transmis à la descendance, est une des méthodes permettant d'obtenir des lignées clonales mais est aussi

un outil scientifique dans l'étude du déterminisme du sexe et permet la préservation d'espèces en voie de disparition dont le sperme peut être cryoconservé. Cette technique a été appliquée chez plusieurs espèces de poissons d'eau douce en utilisant notamment du sperme conspécifique pour féconder des œufs irradiés aux UV, plus faciles à utiliser, mais n'est pas reportée chez les poissons marins. L'induction de l'androgenèse chez le loup de mer a donc été tentée, cette espèce étant un poisson marin d'intérêt économique en Méditerranée et Océan Atlantique dont la caractérisation génétique est encore un sujet d'étude pour l'amélioration des performances de croissance et le déterminisme du sexe. Après une recherche des conditions d'irradiation UV permettant d'inactiver le patrimoine génétique de l'œuf, l'étude s'est portée sur une caractérisation de composés photoprotecteurs de type MAAs et gadusol, déjà mis en évidence dans les œufs flottants et transparents d'autres espèces marines.

Afin d'étudier la capacité des UV à inactiver le patrimoine génétique de la mère, des œufs provenant de différentes femelles ont été exposés à différentes doses incidentes et durées d'irradiation (de 7.2 à 72 $mJ.cm^{-2}.min^{-1}$ pendant 30 s à 12 min, selon les doses). Pour homogénéiser le traitement, les œufs ont été soumis a une légère agitation durant toute la durée de l'irradiation et les lampes UV diffusaient leur rayonnement au-dessus et en-dessous de boîtes de Pétri en quartz choisies comme contenant pour faciliter la pénétration des UV. Le taux de survie pendant le développement embryonnaire était analysé à 3 stades de développement : fécondation, 50 et 74 heures après fécondation, par prélèvement d'un échantillon d'œufs provenant de chaque traitement. La ploïdie des larves produites à l'éclosion était ensuite vérifiée par cytométrie de flux après marquage de l'ADN nucléaire avec de l'iodure de propidium. Afin de contrôler que les descendances produites étaient bien issues du père uniquement, l'analyse de 9 marqueurs microsatellites a été menée séparément sur 3 descendances réalisées après fécondation d'œufs irradiés avec la seule dose ayant permis d'obtenir les individus haploïdes recherchés. Des échantillons d'œufs irradiés et non irradiés ont ensuite été analysés en spectrophotométrie puis HPLC-MS afin de déterminer la présence de composés absorbant et atténuant les rayonnements UV.

L'analyse des survies durant le développement embryonnaire a montré que la dose incidente et la durée d'irradiation ainsi que l'interaction entre ces 2 facteurs ont eu un effet sur les taux de survie observés. De manière générale, la survie diminuait avec des doses incidentes et des durées d'irradiation plus importantes. L'analyse de la ploïdie des larves produites a montré que l'irradiation UV était plutôt inefficace à inactiver le patrimoine génétique femelle, car seul le traitement 60 $mJ.cm^{-2}$ a permis d'obtenir 3 larves caractérisées comme haploïdes sur 21 larves écloses. Malgré de nombreuses malformations ressemblant aux anomalies décrites comme le syndrôme haploïde, la quasi-totalité des traitements ont conduit à la production d'individus diploïdes. L'analyse des marqueurs microsatellites a de plus confirmé que la majorité des larves produites, après une irradiation de 60 $mJ.cm^{-2}$, étaient d'origine biparentale et que seules quelques larves montraient des contributions maternelles incomplètes pour n'obtenir finalement qu'une seule larve ne montrant aucune contamination maternelle. L'analyse spectrophotométrique n'a révélé aucune différence d'absorbance entre œufs irradiés et non irradiés dans la gamme de longueurs d'onde correspondant aux UV. Enfin, la caractérisation plus précise obtenue après HPLC-MS a révélé la présence de gadusol dans les œufs de loup.

L'étude réalisée n'a permis d'obtenir que peu d'individus haploïdes héritant du patrimoine génétique du père uniquement, et malgré la large gamme de doses d'irradiation testées, une seule a pu être faiblement efficace. Parmi les différentes doses testées, plusieurs s'étaient révélées efficaces à inactiver le patrimoine génétique des œufs d'autres espèces de poissons d'eau douce. Les œufs provenant d'espèces variées semblent donc avoir des sensibilités variables à l'irradiation UV, dues à des différences dans les propriétés du chorion ainsi que dans l'organisation interne de l'œuf notamment quant à la position du nucléus. Le génotypage des individus obtenus après irradiation des œufs de 3 femelles différentes a, de plus, montré une certaine variabilité dans l'effet des UV sur différents œufs au sein d'une même ponte comme entre pontes, et le succès de l'androgenèse semble aussi dépendre de facteurs de qualité des œufs. Ce fait avait aussi été mis en évidence dans une étude menée pour l'induction de l'androgenèse chez le tilapia, bien que les mécanismes mis en jeu n'aient pas été identifiés précisément (Myers et al., 1995). L'observation microscopique des larves a montré de nombreuses malformations caractéristiques du syndrome haploïde sur certains individus à quasiment toutes les doses, mais l'analyse en cytométrie de flux n'a révélé que peu d'haploïdes et la caractérisation des génotypes a, de plus, montré des contaminations d'ADN maternel inégales et parfois même très faibles (à un seul locus). Ces individus probablement aneuploïdes ne pouvaient pas être mis en évidence par cytométrie et des analyses plus précises comme des caryotypes auraient peut être permis de mettre en évidence la présence de fragments de chromosomes maternels. L'aneuploïdie de certains individus avait été observée après induction de l'androgenèse chez d'autres espèces mais uniquement lorsque la dose d'irradiation utilisée était trop faible pour inactiver le patrimoine génétique femelle et une fois la dose optimale dépassée, seuls des individus haploïdes étaient produits. Ceci est en contradiction avec les résultats obtenus chez le loup montrant toutes sortes de ploïdies y compris à la dose permettant d'obtenir quelques individus haploïdes. Plusieurs hypothèses peuvent être proposées afin d'expliquer une partie des résultats obtenus. La première est l'expression d'allèles paternels récessifs ayant un effet délétère à l'état homozygote ainsi que la présence de fragments chromosomiques d'origine maternelle provoquant des complications lors des divisions cellulaires et des défauts de développement embryonnaire. Une autre hypothèse proposée et développée en partie dans cette étude est la présence de mécanismes de protection face aux rayons UV. Les mécanismes de photoréparation de l'ADN sont bien connus et expliquent que, notamment pour l'induction de l'androgenèse, les manipulations soient réalisées dans l'obscurité. Comme ces mécanismes de réparation de l'ADN, bien que très efficaces, ne peuvent l'être à 100%, de nombreux organismes exposés aux UV durant leur cycle de vie peuvent limiter les dommages des rayonnements par la présence de composés tels que le gadusol et les MAAs qui possèdent des proprieties d'absorption des UV. Le gadusol, en particulier, montre des capacités d'absorption des UV-C et des UV-B, en fonction du pH, et est présent dans les œufs flottants et transparents de loup et de morue (Plack et al., 1981). L'étude de la présence de ce type de composés photoprotecteurs dans les œufs de différentes espèces de poissons marines et d'eau douce, chez lesquelles l'androgenèse a pu être induite devrait donner plus d'informations sur cette hypothèse. L'utilisation des UV pour induire l'androgenèse chez le loup semble donc être inadaptée aux caractéristiques des œufs et d'autres méthodes devraient être testées, telles que les rayons ionisants (X ou gamma), un choc thermique froid tel que développé chez la

loche (Morishima et al., 2011) ou une androgenèse interspécifique utilisant des œufs pouvant être inactivés par irradiation UV, bien que le succès de ces diverses méthodes ne puisse être garanti.

Partie 4. Une analyse de la presence de composés protégeant des UV dans des oeufs de poissons de latitudes tropicale à arctique en eaux douces et marines

Le rayonnement UV peut être divisé en 3 classes énergétiques selon la longueur d'onde: les UV-C (200-280 nm) sont absorbés par la couche d'ozone et, bien qu'une partie des rayonnements UV-B (280-315 nm) le soit aussi, les radiations UV atteignant la surface terrestre sont composées d'UV-B et d'UV-A (315-400 nm), qui même s'ils sont les moins énergétiques sont connus pour leurs nombreux effets dommageables sur les organismes vivants. Bien que le degré de rayonnement UV atteignant le milieu aquatique à une profondeur donnée varie en fonction de la qualité de l'eau et de ses capacités d'absorption, les organismes vivants sont aussi soumis aux effets des rayonnements UV, il a ainsi été observé que les UV affectent la reproduction, le développement, la croissance et le comportement de nombreux organismes aquatiques (Cockell and Knowland, 1999). L'effet des UV le plus important est la modification de l'ADN entraînant des mutations et des dégénérations cellulaires, mais des dommages liés à la production de radicaux libres d'oxygène ont aussi des conséquences létales pour les cellules. Les œufs et les larves sont les stades de développement les plus sensibles aux effets des UV et les organismes aquatiques ont ainsi développé des mécanismes leur permettant de se protéger contre les effets néfastes d'une exposition aux UV. Les mécanismes de photoréparation de l'ADN, basés sur l'activité enzymatique de la photolyase, sont présents chez de nombreux organismes d'origines phylogénétiques variées (Sinha and Hader, 2002). Mais comme ces mécanismes ne peuvent être efficaces à 100%, de nombreux organismes, tels que les poissons, ont développé des processus comportementaux leur permettant d'éviter les rayons UV pendant tout ou partie de leur cycle de vie. Certaines espèces ont ainsi des comportements reproducteurs adaptés à la protection des stades précoces fragile: les pontes nocturnes ou dans des nids, les pontes à différentes profondeurs ou encore les incubations buccales sont des comportements observés chez les poissons. Des composés photoprotecteurs tels que les MAAs et le gadusol ont été trouvés dans de nombreux organismes aquatiques de toutes origines phylogénétiques, sous toutes latitudes et dans tous types d'habitat aquatique. La voie métabolique 'shikimate', par laquelle le gadusol semble être précurseur des MAAs, n'existe pas chez les animaux, et l'origine des MAAs est ainsi présumée alimentaire. Ces composés sont retrouvés accumulés majoritairement dans les œufs et les gonades femelles, mais aussi dans l'épiderme et les yeux des poissons, à l'inverse, les spermatozoïdes et gonades mâles ne contiennent pas de MAAs. Leur capacité photoprotectrice provient de leur cycle aromatique contenant un système d'électrons π qui absorbent les photons émis pas le rayonnement UV et dissipent l'énergie absorbée sous forme de chaleur (Shick and Dunlap, 2002). Cette étude a donc été menée pour 2 objectifs, le premier étant l'analyse de l'hypothèse de photoprotection expliquant la faible réussite de l'induction de l'androgenèse chez le loup en comparant les composés observés dans les œufs de loup avec ceux d'autres espèces de poissons chez lesquelles l'androgenèse a mieux

fonctionné. Le second objectif était de mieux caractériser les MAAs présents dans les œufs de poissons de différentes origines géographiques, environnementales et phylogénétiques, cette étude comparative n'ayant encore jamais été réalisée chez les poissons.
Les œufs flottants ou benthiques de 21 espèces de poissons marins et d'eau douce, vivant en zones tropicale, tempérée et arctique, ont donc été conservés dans de l'éthanol 96% et analysés en spectrophotométrie sur 2 appareils successifs, le premier donnant une idée générale du profil et de l'absorbance, le second permettant de caractériser plus finement le type de MAAs observé dans les échantillons.
Les profils d'absorbance des œufs de 6 espèces ayant pu être inactivés génétiquement par irradiation UV ont été comparés avec les œufs de loup et 4 espèces avaient effectivement un profil d'absorbance faible dans la gamme de longueurs d'onde des UV tandis que 2 espèces (la carpe et la vandoise) ont montré un profil de très haute absorbance jusqu'à 310-320 nm, similaire au spectre observé chez les œufs de loup. Après un tri séparant les espèces marines des espèces d'eau douce, les profils observés dans les œufs benthiques et flottants ont pu être comparés. Les résultats ont montré que tandis qu'en mer une dichotomie benthique/flottant a pu être observée avec une forte absorbance des œufs flottants, à l'exception de la daurade, et une faible absorbance dans les œufs coulants, les profils obtenus en eau douce ne pouvaient être comparés sur ce principe. La caractérisation plus précise du type de composé comparée en fonction de l'ordre phylogénétique a montré que le gadusol, dont le pic d'absorbance a été observé vers 280-290 nm, était plutôt omniprésent dans tous les groupes phylogénétiques et que l'ordre des Salmoniformes, principalement, semblait se distinguer par le type de composés avec des pics d'absorbance de 320 à 360 nm.
La présence de gadusol et de MAAs dans les œufs des différentes espèces de poissons analysées ne semble donc pas liée uniquement à un type de comportement flottant/coulant mais des évolutions phylogénétiques ont pu être mises en évidence. Bien qu'une caractérisation HPLC-MS soit nécessaire pour confirmer ces résultats et qu'une quantification soit indispensable à certaines conclusions, le gadusol semble être omniprésent dans la majorité des espèces considérées tandis que d'autres types de MAAs semblent plus specifiques à certains taxons. Une autre étude menée avec des yeux de poissons avait déjà montré que le type de composé et leur concentration dépendaient à la fois du groupe phylogénétique et du niveau de rayonnement UV de l'habitat écologique de l'espèce (Thorpe et al., 1993). De nombreuses questions sont encore à élucider, telles que le rôle biologique de ces composés photoprotecteurs, les voies métaboliques permettant leur conversion et/ou dégradation ainsi que leur origine et leur conservation au cours de l'évolution. Malgré tout, la présence de gadusol et/ou de MAAs ne permet pas de conclure sur la sensibilité d'une espèce au rayonnement UV et les mécanismes de photoréparation, par exemple, devraient aussi être considérés, ceci afin de comprendre les adaptations spécifiques développées par différents poissons de diverses origines géographiques, environnementales et phylogénétiques.

Partie 5. Induction de la gynogenèse mitotique chez le loup de mer (*Dicentrarchus labrax* L.): vérification génétique, survie, croissance et développement gonadique

La gynogenèse, autre reproduction uniparentale permettant d'obtenir des individus n'héritant que du patrimoine génétique femelle, est réalisée par fécondation d'œufs avec du sperme inactivé génétiquement puis restauration de la diploïdie par un choc précoce quelques minutes après fécondation induisant la rétention du second globule polaire (gynogenèse méiotique) ou un choc tardif produisant des individus homozygotes par inhibition de la première division cellulaire (gynogenèse mitotique). La gynogenèse mitotique a déjà été appliquée avec succès chez le loup afin de produire des lignées clonales et les conditions optimales d'irradiation UV du sperme et de choc pression décrites (Bertotto et al., 2005). Les difficultés rencontrées ayant aussi été expliquées, une attention toute particulière leur a été portée notamment par l'utilisation de marqueurs microsatellite adaptés afin de distinguer les individus gynogénétiques mitotiques qui sont totalement homozygotes parmi des individus gynogénétiques méiotiques hétérozygotes ou biparentaux résultant d'une irradiation incomplète du sperme eux aussi héhétrozygotes. La distance gène-centromère peut être calculée en réalisant une gynogenèse méiotique puis en observant la proportion d'hétérozygotes pour le gène considéré. Dans le cadre des expériences de gynogenèse mitotique, les meilleurs marqueurs ont une fréquence de recombinaison maximale et sont donc les plus éloignés du centromère (Purdom, 1993). Les individus homozygotes produits en gynogenèse souffrent de la forte consanguinité et des manipulations intenses et, bien souvent, survivent en très petit nombre avec une fécondité réduite. L'objectif de cette étude était donc de produire des individus homozygotes, parents fondateurs de lignées clonales, de caractériser leur survie et croissance ainsi que le développement des gonades et de vérifier, après validation des marqueurs, leur statut mitotique et donc leur complète homozygotie.

Les œufs issus de 11 femelles ont donc été fécondés séparément avec du sperme non irradié (témoin) et du sperme irradié avec des UV provenant de 11 mâles different. Après une incubation séparée pendant 4 jours, les descendants ont grandi en communauté dans 3 bassins témoins et 3 bassins traités en gynogenèse. La survie a été caractérisée par le taux de fécondation et le taux de développement embryonnaire à 72h après fécondation, puis durant l'élevage larvaire, la croissance a été comparée entre témoins et gynogénétiques à partir de mesures réalisées sur des individus photographiés. A 111 jours après l'éclosion, les individus ont été marqués individuellement et leur ADN prélevé, puis leur croissance suivie durant le grossissement jusqu'à 431 jours après éclosion où tous les individus non mitotiques ont été euthanasiés et leur sexe et gonades analysés. Les descendances témoins, gynogénétiques ainsi que 3 descendances produites par gynogenèse méiotiques ont été génotypées grâce à 12 marqueurs microsatellites afin de déterminer l'origine uniparentale, la validité des marqueurs recombinants et vérifier le statut homozygote des descendances gynogénétiques mitotiques.

Le développement embryonnaire précoce a été clairement affecté par le traitement appliqué et une variabilité entre femelles au succès de la gynogenèse en terme de survie a été démontrée. Durant l'élevage larvaire comme pendant le grossissement, l'analyse des performances de croissance n'a pas montré de différences significatives entre les témoins et les individus traités quelque soit leur statut génétique. Différents types d'individus ont, en effet, été

observés dans les descendances traitées par gynogenèse mitotique : des biparentaux provenant d'une femelle, des individus hétérozygotes méiotiques dans toutes les descendances et un faible nombre d'individus confirmés homozygotes mitotiques après validation des marqueurs microsatellites. Sur les 12 microsatellites analysés, 1 est situé en position télomérique extrême et 1 en position centromérique, les autres ont montré des taux de recombinaison variables mais ont permis de montrer que les individus gynogénétiques hétérozygotes contaminant les descendances gynogénétiques mitotiques produites étaient d'origine méiotique et provenaient d'une non disjonction des chromosomes durant la seconde division méiotique ou de la rétention tardive du second globule polaire. L'étude des gonades a de plus montré que le traitement gynogénétique a retardé le développement des gonades des individus traités en gynogenèse et ce quelque soit leur statut génétique.
Cette étude a donc montré que bien que leur nombre soit faible, la production d'individus homozygotes gynogénétiques mitotiques est possible chez le loup, leur statut génétique ayant été validé par l'usage de marqueurs microsatellite adaptés. Les marqueurs microsatellite ayant un fort taux de recombinaison et/ou étant les plus éloignés du centromère sont les plus adaptés pour la vérification de l'action de la gynogenèse sur la première mitose et donc de la validation du statut complètement homozygote des individus analysés. La contamination des descendances produites par des individus hétérozygotes d'origine méiotique a aussi été montrée, et bien que leur présence reste encore mal expliquée, elle pourrait résulter de l'utilisation d'œufs de mauvaise qualité donc la maturation aurait été retardée conduisant le choc pression à bloquer non pas la première mitose mais l'expulsion du second globule polaire ou entraînant une non-disjonction des chromosomes à la seconde division méiotique. Dans cette étude, contrairement à ce qui avait pu être observé chez d'autres espèces, les performances de croissance n'ont pas semblé être affectées par le traitement tandis que le développement des gonades a été clairement retardé. Une diminution de la fécondité chez les individus homozygotes avait déjà été démontrée dans d'autres travaux et expliquée par la forte consanguinité ou la présence de fragments chromosomiques d'origine paternelle (Komen and Thorgaard, 2007). Dans cette étude, le retard semble aussi dû à un effet délétère du choc pression car tous les individus produits par gynogenèse, quelque soit leur statut génétique, ont montré ce type de développement anormal des gonades.

Ce travail a finalement montré que la production de lignées clonales chez le loup est possible mais aussi que diverses difficultés sont à prendre en considération pour appliquer les techniques d'androgenèse et de gynogenèse chez toute espèce de poissons. Les spécificités biologiques du poisson, des œufs, du sperme doivent être considérées afin de choisir la ou les méthodes les plus adaptées pour un succès maximum, les œufs du loup sont par exemple difficiles à irradier aux UV et semblent sensibles au moment et au type de choc utilisé pour rétablir la diploïdie. La présence de composés photoprotecteurs, proposée comme hypothèse pour expliquer la difficulté à inactiver le patrimoine génétique des œufs de loup, a dû être reconsidérée car les résultats n'ont pas indiqué de relation claire entre le comportement flottant ou coulant des œufs et la présence de gadusol et MAAs, qui semblent respectivement

omniprésent ou spécifiques à certains taxons. La gynogenèse bien que fonctionnelle soulève aussi des questions quant à la présence d'un grand nombre d'individus hétérozygotes et, bien que leur origine biparentale ou méiotique ait pu être démontrée, l'évènement cytologique expliquant leur présence reste encore inconnu. Il semble, de plus, que le choc pression appliqué sur les œufs ait un effet à long terme et une nouvelle fois, les évènements cytologiques et cellulaires sous-jacents restent un mystère.

L'un des points importants soulevé tout au long de ce travail est l'importance de la qualité des œufs, un facteur variable qui peut jouer un rôle prépondérant dans le succès des manipulations chromosomiques et reproductions uniparentales. Les mesures de survie et taux d'éclosion, mesures utilisées pour caractériser la qualité d'une ponte, ne donnent pas d'information sur les facteurs qui déterminent la qualité des œufs, or celle-ci dépend des propriétés internes (génétiques, physico-chimiques, nutritives) de l'œuf mais aussi des conditions environnementales durant l'incubation (Brooks et al., 1997). De nombreux facteurs sont connus pour avoir un effet sur la qualité des pontes, outre la variabilité saisonnière et individuelle, et surtout en conditions d'élevage. Il s'agit des traitements hormonaux utilisés pour synchroniser les pontes, de l'âge des géniteurs, de facteurs plus environnementaux tels que le type d'alimentation, la photopériode, la température et le stress induit par les pratiques d'élevage, ainsi que de facteurs génétiques inhérents aux géniteurs utilisés, qui bien que difficiles à caractériser parmi les autres facteurs pouvant modifier la qualité des pontes, ont un effet majeur sur la fécondité.

Mots-clé : *Dicentrarchus labrax*, lignées clonales, androgenèse, gynogenèse, MAAs, gadusol, marqueurs microsatellites, homozygotie, hétérozygotie, taux de recombinaison.

Introduction

Introduction

1. The European sea bass

1.1. Phylogeny and geographical distribution

The scientific name of the European sea bass, *Dicentrarchus labrax* (Linnaeus, 1758), has been generally accepted in Europe since the late 1960s. Sea bass belongs to the Order Perciformes, the largest known order of fish which comprises 25% of all vertebrates, and to the suborder Percoidei. The family in which *D. labrax* should be classified is still controversial since authors disagree between the use of Serranidae or Moronidae. The genus *Dicentrarchus* represents two European species: *D. labrax* and the spotted sea bass *Dicentrarchus punctatus* (Bloch, 1972) (Fig. 1), that are found in the coastal waters of the eastern Atlantic Ocean, the Mediterranean and Black seas and may also enter freshwater. The two species are distinguished mainly on the basis of morphological features: different numbers of scales on the lateral line, differential teeth distribution and the presence or absence of black spots on the upper part of the adult body (Pickett and Pawson, 1994).

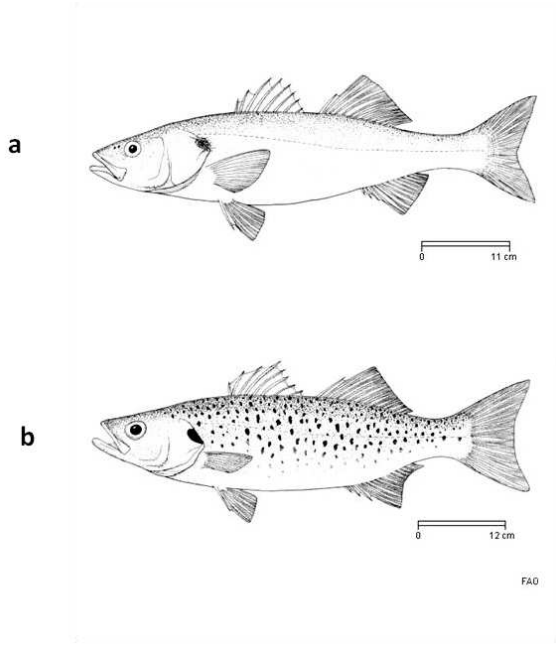

Figure 1. Drawings of a) *Dicentrarchus labrax* and b) *Dicentrarchus punctatus*. From: Bauchot (1987).

D. labrax is an euryhaline (able to adapt to 3‰ up to full strength sea water) as well as an eurythermic species that survives a wide range of temperatures ranging from 2 to 32°C (Jobling et al., 2010). The European sea bass are able to frequent coastal waters down to 100 m depth but are more commonly found in shallow water and also occur in estuaries and

brackish water. Its natural feeding strategy is based on predation with a diet mainly constituted of small fishes and invertebrates including shrimps, crabs and mollusks.

The European sea bass geographical distribution has been genetically characterized and can be divided into three main groups: Atlantic, western Mediterranean and eastern Mediterranean. Fish populations from the Atlantic and Mediterranean regions showed clear genetic differentiation. Although at smaller geographical scales the results are not so clear and questionable, some genetic differentiations are recognised. Naciri et al. (1999) reported two distinct groups among populations from the North Sea, Britanny, Portugal, Morocco, the Alboran Sea and the western Mediterranean based on a study of allele-frequency variation at six microsatellite loci: the Atlantic group including the Alboran Sea east of Gibraltar Strait and the western Mediterranean group with a transition corresponding to the Almeria-Oran oceanographic front. Furthermore, Patarnello et al. (1993) suggested that the Mediterranean populations might be structured into two geographical groups with differences between eastern and western populations and a tendency to form separated stocks. This hypothesis was confirmed later by Bahri-Sfar et al. (2000), who demonstrated that Mediterranean sea bass populations are divided into two major groups corresponding to populations living in the eastern and western basins and delimited by the Siculo-Tunisian Strait.

1.2. Fisheries and aquaculture

The European sea bass is valued for food, prized in recreational fisheries and in small-scale commercial fisheries throughout its range. In 2012, the global captures were 8990 tones with a small decrease since 2010 (FAO, 2014).

D. labrax was the first non-salmonid fish species to be commercially cultured in marine environments in Europe and is still the most important marine fish species widely cultured in the Mediterranean area. Greece, Turkey, Italy, Spain, Croatia and Egypt are the largest aquaculture producers. In less than 25 years, sea bass production grew from a few thousand tones to around 153 000 tones in 2012 (FAO, 2014). Farmed sea bass is usually marketed as portion-sized fish which corresponds to weights ranging between 250-450 g and a growth period of 14-24 months depending on water temperatures (Jobling et al., 2010).

European sea bass production was sustained by the control of spawning in captivity that allowed the development of mass production techniques to provide the required number of juveniles for commercial growing. On-growing in sea cages is the most common system of production, followed by cultures on land in tanks, raceway tanks and ponds. Sea farms are supplied with juveniles from hatcheries and fed commercial diets for marine finfish. To provide a sufficient number of high-quality eggs and larvae, hatcheries maintain their own broodstock in recirculated systems under fully controlled environmental conditions. The proper control of the entire production cycle sustained the development of farming activities but some points still need to be improved; as an example, the skewed sex ratio in favor of males commonly observed in cultured stocks affects productivity and sparks research interest,

as females have the potential to grow faster and attain ultimate larger sizes than males (Piferrer et al., 2005; Saillant et al., 2001b).

1.3. Reproductive biology

The European sea bass is a gonochoristic species without externally visible morphological secondary sex characters. Under farming conditions sexual differentiation appears quite early during development. Saillant et al. (2003) showed that most fish reached sexual differentiation at the age of 8 months. According to the same authors ovarian differentiation started at 168 days post-fertilization (dpf) and was completed at 419 dpf. In contrast, precocious males differentiated at 168 dpf while the others remained undifferentiated until the age of 250 dpf. The European sea bass exhibits ontogenetic plasticity amenable to environmental control, with sexual differentiation being especially sensitive to temperature alterations. Pavlidis et al. (2000) showed that temperature changes during the very early developmental stages are capable of affecting the process of sex differentiation.

Felip et al. (2006) showed that 2-year old cultured males are sexually mature with a proportion of precocious fish being able to mature as early as 1 year of age. For cultured females, sexual maturity is attained only by the end of the third year of life (Bruslé and Roblin, 1984). There is only one natural spawning season per year, which takes place during winter. Most of the females spawn in January-March in the Mediterranean Sea and one to a few months later in the Atlantic Ocean. The spawning season takes place during the period of low water temperatures and short and/or increasing day length. Typically the male maturity season starts 2 months before female ovulation (around November in the Mediterranean area) (Prat et al., 1990). The eggs are transparent and relatively small (1.1-1.3 mm diameter), contain one or several oily droplets, are neutrally buoyant and remain suspended in the upper layers of the water column when salinity remains above 34‰ (Barnabé et al., 1976). The rate of development depends on water temperature; at 13°C eggs hatch about 5 days post-fertilization (dpf) and the time to hatch decreases with increasing temperatures. Newly hatched larvae are 4-4.5 mm in length and rely on the yolk sac for nutrition during the first few days. First feeding occurs at around 10 days post-hatching (dph) at 16°C. At the same time, the swim bladder becomes visible as a reflective ovoid bubble, so the young fish are capable of maintaining buoyancy despite having metabolized the low-density oil droplet(s) that were present in the yolk sac (Jobling et al., 2010).

In teleost fish, the egg represents the final product of oocyte development, a long process that can take a year or more. Only water and some dissolved chemicals can pass into an ovulated egg, thus all genetic information and nutritive compounds necessary for embryonic development are incorporated during oocyte maturation within the ovary (Brooks et al., 1997). Oocyte development can be divided into 6 developmental stages: oogenesis, primary oocyte growth, cortical alveolus stage, vitellogenesis, maturation and ovulation (Tyler and Sumpter, 1996). During the early stages of oocyte development, DNA replication occurs; homologous chromosome pairs form, shorten and thicken. Chromosomes then unpair

and the oocyte enters a long period of cytoplasmic growth characterized by accumulation of yolk reserves, this stage is called vitellogenesis. A hormonal signal induces meiosis and the oocyte starts maturation. During this final phase, chromosomes arrest in second meiotic metaphase before the oocyte is released from the ovary, ready for fertilization (Bobe and Labbe, 2010; Brooks et al., 1997).

The primary signals triggering oocyte growth and maturation are environmental. In fish, the hypothalamus releases gonadotrophin-releasing hormone (GnRH) in response to external stimuli. GnRH activates the anterior pituitary gland to release gonadotrophins (GtH I and II). GtH I act on granulosa and thecal cells, stimulating the synthesis of oestradiol-17β which in turn acts on the production of precursors of yolk protein and egg shell proteins. GtH II functions later in oocyte development, acting on the follicle cells to stimulate the synthesis of progesterones. These latter hormones control the final stages of oocyte development and egg ovulation (Brooks et al., 1997).

An egg needs not only all the amino acids, lipids and carbohydrates that make up an embryo but also many other compounds such as calcium, vitamins and metals. These materials are derived from maternal sources that must be incorporated during the growth of the oocyte to sustain later the developing embryo. Vitellogenesis is the stage when most nutritive products are taken up and stored in the oocyte, and the different materials present in the oocyte must be positioned correctly within the oocyte, and protected from turnover and degradation until their requirement (Brooks et al., 1997).

1.4. Genetic improvement and development of genetic resources

Although the domestication of European sea bass was initiated in the mid 1980s, a large proportion of the commercial broodstock currently kept in captivity by hatcheries remain unselected and are maintained by recruiting from wild populations or raising juveniles acquired on the market (Haffray et al., 2007). Fish growth is a character of major interest for producers as production costs can be significantly lowered by shortening the duration of the rearing cycle (Saillant et al., 2006). Similarly to other farmed fish, one of the sexes is more beneficial. Because females grow larger than males, another potential gain in the industrial production of European sea bass would be the production of all-female stocks (Haffray et al., 2007). Under certain conditions, larval and juvenile production practices can lead to proportions of males as high as 100%, with males being 10 to 40% smaller than females (Piferrer et al., 2005).

The diploid karyotype of the European sea bass consists of 48 small chromosomes, most of which are acrocentric (Aref'yev, 1989). Nucleolar organizing regions (NORs) are located at the terminal or near-terminal sites on the short arms of the acrocentric chromosome pair 22 (Piferrer et al., 2005). Like most teleosts, the European sea bass has no morphologically distinguishable sex chromosomes (Devlin and Nagahama, 2002), therefore females and males cannot be distinguished by the karyotype. The chromosomal basis of sex

determination remains unclear and is a subject of research for aquaculture purposes as well as for fish general biology (Schartl, 2004). The fertilization rate, early developmental survival, hatching success, length of larvae and sex ratio of progenies are under parental influences (Saillant et al., 2001b; Saillant et al., 2002; Saillant et al., 2003). As epigenetic factors like temperature during early developmental stages can affect the process of gonadal sex differentiation, parental influence may extend to different sensitivities to effects of temperature (Piferrer et al., 2005). Saillant et al. (2002) suggested the possibility of selecting genotypes less sensitive to the masculinizing effects of high temperature, in order to obtain higher proportions of females in production. A polygenic hypothesis of sex determinism has been proposed by Vandeputte et al. (2007a) showing that the genetic and environmental components of sex were of comparable magnitude, and that the genetic component was essentially additive and linked to growth.

In several cultured fish species, selection for growth has proven effective with 5 to 20% gain per generation. Studies on European sea bass showed that growth rate had an important additive genetic component and that response to selection for this character was high though parameters as fish density could interfere in the heritability and genetic variation estimates (Saillant et al., 2006; Vandeputte et al., 2009b). However, Dupont-Nivet et al. (2008) obtained low genotype-by-environment interactions during the on-growing stage and suggested that selection on weight would give similar results in different farming sites except for highly divergent systems.

2. Thesis presentation

2.1. General objectives

The initial objective of this thesis was the development of isogenic or clonal lines in the European sea bass as part of a larger research effort on EU aquaculture research infrastructure (AquaExcel, EU project). The first step to attain this goal was the production of fully homozygous individuals by androgenesis, a form of uniparental reproduction. Because sea bass males become sexually mature earlier than females, androgenesis was the only way to produce inbred lines within the 3 years of this work. Our results obtained during the androgenesis investigations raised the question of the biological specificity of European sea bass eggs and led to the study of general characteristics of eggs from various species in different environments, especially in the context of solar and UV radiations. In addition, female uniparental reproduction (gynogenesis) was also induced during this thesis to obtain the clonal founders expected in the AquaExcel project. This technique was previously reported to be successful, but its application also led to considerations on the genetic inheritance in gynogenetic progenies and gonadal development. The main limiting factors during this work were related to the egg biology and quality. Thus, this thesis aims at providing some hypotheses on the biology of sea bass eggs which is relevant for artificial reproduction and chromosome manipulations in this species.

2.2. Organization of the thesis chapters

The first part of this thesis will explain the principles and uses of isogenic clonal lines in fish research. After a short introduction on this topic, this part will present the two techniques used to produce clonal founders: androgenesis and gynogenesis. Clonal lines are a useful tool for fish research studies and some applications in sex determination, genome mapping and QTL detection will be presented.

The second part is devoted to a state of knowledge on androgenesis. A literature review on this technique used for uniparental reproduction in fish will be the main objective of this chapter. After a presentation of intraspecific and interspecific androgenesis in fish, the chapter will focus on UV-irradiation and pressure shock, their impact and a comparison with alternative methods for genetic inactivation and chromosome doubling. An important step towards the development of clonal lines is the assessment of homozygosity in androgenetic progenies, and a section will deal with the different methods employed for this purpose; this part will finish with the presentation of the different steps and techniques, their limits and perspectives.

The third part of the thesis is dedicated to androgenesis in the European sea bass: the different experiments performed and the results obtained. The perspectives on UV-resistance will be presented as well as the possible alternative methods. This part is presented in the form of a paper recently published in PlosOne (Colléter et al., 2014).

The fourth part is devoted to the characterization of UV screening compounds in the eggs of several fish species and a comparison of the protection against UV exposure in fish from different habitats. The eggs from freshwater and marine species ranging from arctic to tropical latitudes were analyzed and compared in terms of UV absorbance characteristics. In addition, the presence or absence of specific UV screening compounds and their possible role in the lifestyle of these species were analyzed.

The fifth part of the thesis is devoted to mitotic gynogenesis. Based on previous works, this experiment was performed to mass-produce homozygous individuals as an alternative to androgenesis. This work led to several considerations on the yield of homozygous gynogenetic individuals, the effects on gonad development and the variability between the different female fish and their eggs.

The conclusion will be more oriented to perspectives of this work and the different points that need to be taken into account when performing uniparental reproduction and developing clonal lines in the European sea bass as well as in other fish species. Aspects related to egg quality will be addressed and the different hypotheses and outcomes of this work put into a general prospect for future research efforts on these topics.

Part 1. Development of genetic resources: clonal lines

Part 1: Development of genetic resources: clonal lines

1. Fish in scientific research

Fish represent the largest and most primitive vertebrate group and show a remarkable variety of unique adaptation responses to multitudes of aquatic environments, ecological restrictions and social interactions. This variability makes fish extremely attractive for the study of several biological and evolutionary questions and some of them have become important animal models in the areas of evolution, development, reproduction, and genomics of vertebrates in general. Around 27 977 species have already been described, a number that is expanding every year (Nelson, 2006). Understanding fish biology cannot be accomplished without experimentations using live animals.

High quality animal experiments have (Bongers et al., 1998; Dave, 1993):
- a high replicability, which corresponds to low variations between replicates in a single experiment
- a high repeatability, which means that there is a low variation between repeated tests within the same laboratory
- a high reproducibility, that allows low variations between tests using the same protocol but performed at different laboratories.

The use of outbred stocks, which is nowadays still mainly the case in experimental studies, especially decreases reproducibility. Using genetically well-defined animals is a methodological requirement to improve the quality of experiments (Bongers et al., 1998; Festing, 1992).

2. Clonal lines and uniparental reproduction

2.1. Principles

Isogenic clonal lines, also named fully inbred lines, are populations of genetically identical and completely homozygous individuals.

In conventional inbreeding, approximately 20 generations of full sib-mating are needed to obtain near completely homozygous animals (F= 0.986); these strains can be designated as inbred as stated in 1952 by the Committee of Standardized Genetic Nomenclature for mice. In mammalian research, inbred strains of mice and rats in particular have contributed to many areas of research (Bongers et al., 1998).

In fish, due to external fertilization, handling of the gametes is possible and easier than in mammals so that clonal lines can be produced in only 2 generations. Androgenesis and mitotic gynogenesis are the two techniques of artificial uniparental reproduction leading to

progenies inheriting the nuclear genetic content from only one parent which can be applied to produce inbred lines. Fully homozygous individuals can be produced using these techniques in only one generation, each offspring being unique and a clonal founder. The second generation is the replication of the first generation individuals by a second round of androgenesis or gynogenesis giving rise to many individuals all being genetically identical to a specific inbred clonal founder. Fish offer the chance to produce fully inbred lines rapidly and with the possibility of directly selecting specific genotypes. By crossing 2 inbred lines, heterozygous clones also named 'F1 hybrids' can be developed, the offspring produced are genetically identical but the genome is partially heterozygous: this kind of individuals allow the possibility of examining single major gene effects on a standardized background (Bongers et al., 1998). The F1 hybrids are more vigorous and interesting for the isolation of mutants because they are more likely to be free of recessive lethal genes (Streisinger et al., 1981). The F1 hybrids can be reproduced by androgenesis and gynogenesis to produce new homozygous individuals combining the genomes of two clonal lines also named 'recombined clonal lines'.

2.2. Androgenesis

Androgenesis is a form of uniparental development in which the nuclear genetic material is entirely of paternal origin. It is attained by artificial fertilization of genetically inactivated eggs following exposure to gamma (γ), X-ray or UV irradiation. This step leads to haploid androgenetic progenies which are inviable in fish and generally die before or soon after hatching. Viable androgenetic individuals can be produced after restoration of diploidy in the egg by suppression of mitosis-I using a pressure or thermal shock leading to doubled haploids (DH). Other techniques involve the use of diploid sperm from tetraploid fish or dispermic egg activation via fusion of sperm nuclei making the diploidization step unnecessary (Fig. 2) but these techniques do not produce homozygous DH (Komen and Thorgaard, 2007; Pandian and Kirankumar, 2003).

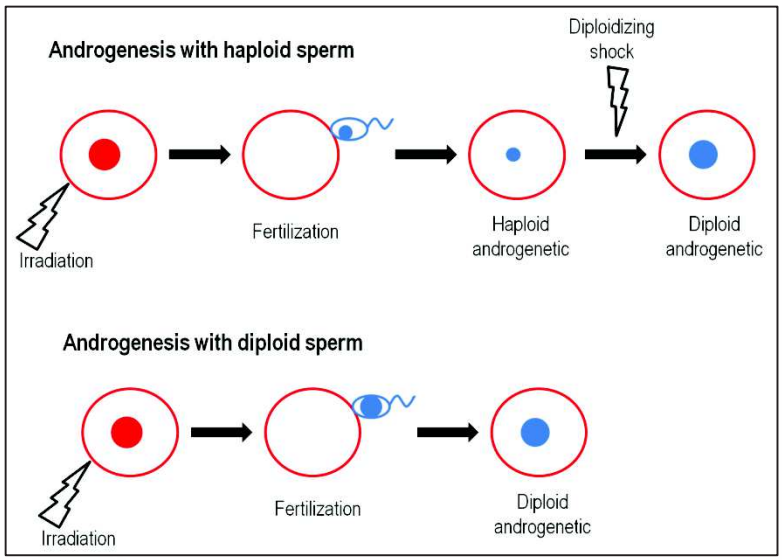

Figure 2. Schematic representation of androgenesis principle.

Androgenesis is a useful tool for the production of homozygous clones and the study of sex determination. For instance, in fish species which have a genetic sex determining system of XX/XY, androgenesis is a rapid way to generate YY individuals. This technique also allows the preservation and recovery of unique strains or endangered species from cryopreserved sperm, because as yet oocytes cannot be cryopreserved. In addition, it also permits the study of physiological effects of mitochondrial variations (Brown et al., 2006).

Fish spermatozoa are free of acrosome; they enter the eggs through the micropyle during fertilization. This characteristic allows heterospecific insemination, meaning that an egg can be activated by the spermatozoa of another fish species. Thus, attempts at inducing androgenesis in a fish species can be achieved either using homologous sperm (intraspecific androgenesis) or heterologous sperm (heterospecific androgenesis) (Pandian and Kirankumar, 2003).

2.3. Gynogenesis

Gynogenesis is a form of uniparental reproduction that leads to progenies inheriting only the maternal set of chromosomes (Fig. 3). It is attained by artificial ‘fertilization’, or more properly activation, of eggs with genetically inactivated spermatozoa. Although ‘activation’ of eggs could be used in the case of gynogenesis, ‘fertilization’ has been chosen throughout the text for easier reading. Genetic inactivation is achieved using UV or ionizing irradiation procedures as indicated previously. This procedure leads to the development of haploid gynogenetic progenies that die before to a few hours after hatching in fishes. Diploidy can be restored using a pressure or thermal shock. Unlike androgenetic progenies, diploidization can be achieved at two different times during the gynogenetic process. The

prevention of meiosis II is achieved with a shock applied shortly after fertilization and leads to the retention of the 2nd polar body producing meiotic gynogenetic progenies, with various proportions of heterozygous and homozygous individuals depending on the maternal heterozygosity and the loci analyzed, due to specific crossing over and recombination events during oogenesis. Doubled haploids (DH) are produced by the suppression of mitosis using a late shock, after the 2nd polar body extrusion and before the 1st cell division. DH are expected to be 100% homozygous mitotic gynogenetic progenies.

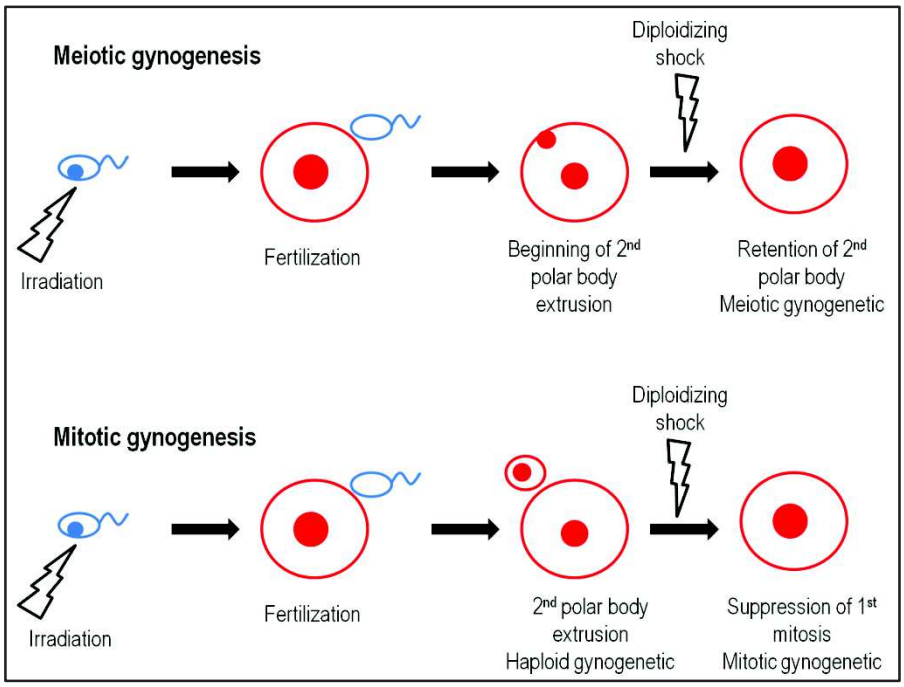

Figure 3. Schematic representation of meiotic and mitotic gynogenesis.

Gynogenesis has been achieved in several freshwater and marine species as reviewed by Komen and Thorgaard (2007). Gynogenesis is a tool for studies on sex determination, the production of all-female stocks and the production of clonal lines. In species having a sexual dimorphism in favor of females, the production of monosex female stocks is of high commercial value. When female is the homogametic sex, the use of gynogenesis in combination with hormonal sex-reversal allows the production of all-female populations by proper breeding schemes (Dunham, 2004). As meiotic gynogenesis is technically far easier to induce and leads to significantly higher survival than mitotic gynogenesis, it is more commonly used in sex determination studies to assess the homogamety or heterogamety of the female parent (Pandian and Koteeswaran, 1998). Females produced by mitotic gynogenesis have the potential to generate genetically identical progenies (clones) by a second generation of gynogenesis.

2.4. General survival and reproductive performance of doubled haploids

Gynogenetic and/or androgenetic doubled haploids, and clonal lines have been produced in both marine and freshwater species. It has to be noted that only successful productions are reported in the literature, few unsuccessful attempts are presented and the hypotheses underlying failure remain mostly not detailed (Komen and Thorgaard, 2007).

The yields of doubled haploids reported in the literature are usually low and the reduction in survival is mainly explained by inbreeding depression (expression of homozygous deleterious mutations) and damages induced by the manipulations.

A reduced fertility of doubled haploids is also usually reported in most fish species. For example in Nile tilapia (*Oreochromis niloticus* L.), only 10 out of 77 gynogenetic females produced viable eggs (Müller-Belecke and Hörstgen-Schwark, 1995). Meiotic gynogenesis performed on doubled haploids produced by a first generation of mitotic gynogenesis showed again very low survival rates and the reproductive traits among clones were highly variable (Müller-Belecke and Hörstgen-Schwark, 2000). In rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792), a reduced fertility at 2 years of age was also observed with a reduced number of females spawning for the first time. Moreover, the mitotic gynogenetic females spawned later in the season with one week delay and had a longer reproductive season due to a larger variability between females in the spawning date (Quillet, 1994). A drastically reduced fertility was also observed in mitotic gynogenetic loach females, results showed low numbers of mature fish and poor egg quality and no propagation of clonal lines could be performed in the loach (*Misgurnus anguillicaudatus* Cantor, 1842) (Arai, 2001).

3. Uses of clonal lines in fish research

Clonal lines are a unique and an extremely valuable tool in fish research. The genetic uniformity allows comparison of the same genotype under different conditions. As each clonal progeny represents a single haplotype, clones represent a unique biological tool to identify quantitative trait loci (QTLs) for many parameters such as growth rate or disease resistance and their genetic correlations or epistatic interactions. Clones are also relevant for the detection of genotype-by-environment interactions and the phenotypic plasticity. Clonal individuals bearing a doubled haploid genome are also a special tool for genomic analyses such as genetic mapping or genome sequencing since it facilitates considerably the contig or scaffold assembly.

3.1. Sex determination

Mitotic gynogenesis and androgenesis can be used to obtain information on the sex-determining mechanism in the studied fish species as well as in fish more generally. In species where female is the homogametic sex, with XX/XY sex determination, gynogenetic progenies are expected to be all-female. Androgenetic progenies should produce XX and YY individuals (viable in fish), and the YY males should theoretically give all-male progenies following crossing with an XX female. In species where female is the heterogametic sex, with ZW-ZZ sex determination, the reverse situation is expected: androgenetic progenies are expected to be all-male while gynogenesis should give equal proportions of male and female progenies and the WW females produced (which are also viable in fish) should give theoretically all-female progenies when crossed with a normal (ZZ) male. In contrast to mammals and birds, only about 10% of fish species possess morphologically differentiated sex chromosomes. Therefore, male and female karyotypes cannot be distinguished. Despite this, many fish species do have sex chromosomes although they are homomorphic (Carrasco et al., 1999; Devlin and Nagahama, 2002). Existence of a genetic sex determination has to be identified indirectly through progeny testing coupled to sex-inversion. Sex-inversion with steroid treatments is another approach which permits the creation of specific genotypes such as XX and YY males or ZW males and WW females, all interesting for sex-determining studies (Baroiller et al., 2009a). As mentioned earlier, these time-consuming procedures can be accelerated and facilitated with androgenetic and gynogenetic experiments. In an increasing number of fish species studied, the genetic sex determination (GSD) can be overridden by environmental factors and recessive mutations can affect sex determination, leading to unexpected sex-ratios in doubled haploid progenies (Baroiller et al., 2009b; Devlin and Nagahama, 2002).

In the Nile tilapia (*Oreochromis niloticus*), sex determination is primarily XX/XY but can be overruled by temperature (Baroiller et al., 1995; Cnaani et al., 2008; Devlin and Nagahama, 2002). Müller-Belecke and Hörstgen-Schwark (1995) found a high proportion of males (35.3%) among mitotic gynogenetic progenies, suggesting that one or two minor sex-determining factors could override the genetic sex determination when acting in combination or occurring in homozygous state and may account for the sex ratios observed. Sarder et al. (1999) found both males and females in one clonal line, suggesting that this line was homozygous for an allele or a combination of alleles at an autosomal locus or loci which could cause female to male sex reversal. Karayücel et al. (2004) confirmed this hypothesis on gynogenetic offspring from a heterozygous red female and found a genetic linkage between the red gene and a gene that caused female to male sex reversal. Ezaz et al. (2004a) studied departures from predicted sex ratios by androgenesis on XY male parents and mitotic gynogenesis on XY female parents (neo-females or sex-reversed genetic males) with progeny testing generally confirming the expected sex ratios based on genetic sex determination. However, they also observed some deviations from the expected sex ratios, which varied significantly between families, and concluded that some autosomal, heritable and polymorphic factors may influence sex ratio in this species. Parental factors of both maternal

and paternal origin have been shown through diallel crossings of several XX females and XY males, establishing that minor genetic factors also contributed to sex in the Nile tilapia (Baroiller and D'Cotta, 2001). In fact, sex in this species is complex with more than one locus, under the influence of sex chromosomes, temperature and minor genetic factors (Baroiller et al., 2009a; Cnaani et al., 2008).

Such deviations from expected sex ratios have also been observed in other fish species after the production of doubled haploid progenies. For example, the existence of recessive mutations in sex-determining genes has been discovered in the rainbow trout (*Oncorhyncus mykiss*) and the common carp (*Cyprinus carpio* L.) (Komen et al., 1992a; Quillet et al., 2002). A recessive autosomal mutation (*mas*) was discovered in gynogenetic common carp: the XX *mas*/*mas* individuals become masculinised and develop an intersex gonad or a testis. Crossed with an XX *mas*/+ female, the XX *mas*/*mas* male can produce both sexes: XX *mas*/*mas* male and XX *mas*/+ female.

3.2. Genetic variance of inbred lines

The use of inbred strains has statistical implications since genetic variation within inbred strains is absent. A reduction in the variance between animals in the same experiment increases statistical precision.

In any outbred population, the total variance (V_{tot}) can be partitioned in genetic variance (V_G), environmental variance (V_E) and the correlation between these components (V_{GE}). Genetic variance is also divided into an additive genetic component (V_A), a dominance component (V_D) and an interaction component (V_I) (Falconer and Mackay, 1996).

$V_{tot} = V_G + V_{GE} + V_E$ with $V_G = V_A + V_D + V_I$

In clonal lines, heterozygotes are absent and $V_D=0$. The interaction effects are thought to be negligible ($V_I=0$). In the case of fish, fully inbred individuals are obtained in only one generation, the formula for genetic variance between and within families applied therefore is:

$V_{A\text{-tot}} = V_{A\text{-between families}} + V_{A\text{- within families}}$

$= 2fV_A + (1+F-2f)V_A = (1+F)V_A$

where f is the coefficient of coancestry (Malécot relationship) among individuals of the same family (Falconer and Mackay, 1996). When offspring are fully inbred (F=1) in one generation, the Malécot relationship (f) among individuals belonging to the same family is ½. As a result, the genetic variance within these families is equal to the additive genetic variance between families and the total amount of genetic variance is doubled (Bongers et al., 1997b).

$V_G = 2V_A$

This demonstration shows that the genetic mean of the progeny reflects the additive genetic value or breeding value of the parent. In fully inbred populations, the additive genetic variance within families is zero since the parents only produce one type of gamete. As a result, between family additive genetic variance becomes $2V_A$. The production of inbred lines in only one generation is an interesting tool for fish studies and selection programs as a direct

estimation of the additive genetic variance of a certain trait and the breeding value of individual parents can be computed.

Bijma et al. (1997) developed general formulas to estimate the breeding values and the variance components using clonal lines. The authors showed that the optimum family sizes for estimating heritabilities are smaller in gynogenetic sib families than in conventional full-sib families. However, an advantage in accuracy of the estimated heritability for gynogenetic sib families was found only for heritabilities below 0.35.

The small optimal family size and the characterization of the genetic variance in fully inbred populations show that in selective breeding programs or in experiments aimed at genetic characterization, the use of inbred lines can reduce the number of fish used, improve the characterization of variability factors, and give direct information on the breeding value of the parent or heritability of a specific trait.

Using the formulas for heritability estimation and additive genetic effects, Bongers et al. (1997b) analyzed gonad development and egg quality in homozygous gynogenetic individuals of common carp. Five mitotic gynogenetic progenies were produced and revealed a high between- and within-family variance for gonado-somatic index (GSI) and egg quality. High heritabilities were obtained (0.71 for GSI at 13 months of age, 0.72 for percentage of normal larvae after fertilization at 19 months) and the authors suggested that these traits were influenced by additive genetic effects and that homozygous individuals may be selected from their families to produce early or late maturing inbred clonal lines with high egg quality. Tanck et al. (2001b) studied the heritability for the intensity of stress-related cortisol response in common carp. Using progenies produced by androgenesis, the authors observed a high heritability estimate for stress-related cortisol increase: 0.60 (0.37-0.90), and concluded that the intensity of the stress response due to a cold shock is heritable in the population used, though some environmental effects could be confounded with sire effects.

F1 hybrids are also a useful tool in this kind of studies aiming to determine the relative importance of dominance and additive genetic effects. Bongers et al. (1997c) studied testis development in common carp by crossing 4 androgenetic homozygous males with 3 gynogenetic homozygous females, all parents were selected for slow and fast gonad development. The variation for the onset of spermatogenesis between the different F1 crosses was linked to additive genetic differences between the parent inbred lines. The female parent affected the testis-somatic index more than the male parent and additive genetic variance for the onset of spermatogenesis and testis development was revealed in the F1 hybrids used, without detection of dominance and interaction effects.

3.3. Genetic mapping

Meiotic and mitotic gynogenesis as well as androgenesis are useful tools for gene-centromere mapping, ordering of linkage groups and the construction of genetic maps.

Meiotic gynogenetic diploids are produced by retention of the second polar body (or inhibition of the second meiotic division) and depending on the frequency of recombination events, the individuals produced are more or less homozygous and can be used to estimate gene-centromere distances (Purdom, 1993; Streisinger et al., 1981). Mitotic gynogenesis and androgenesis produce 100% homozygous individuals, each individual representing a unique sperm or egg haplotype. This kind of individual is an invaluable tool for genetic mapping as its uniformity can be perpetuated and used for detailed mapping and associated studies (Young et al., 1998).

A F1 hybrid of rainbow trout was produced by crossing two clonal lines and 76 doubled haploids were produced by androgenesis from this recombinant male. These individuals were analyzed for segregation of AFLP, multilocus DNA fingerprinting, microsatellite and RAPD markers. The analysis of 476 markers (475 molecular markers and sex) allowed the production of a detailed linkage map in the rainbow trout, comprising 42 linkage groups and covering more than half of the estimated genome length (Young et al., 1998). This map was related later to a microsatellite map (Sakamoto et al., 2000) and enriched with more than 900 markers (Nichols et al., 2003b) which allowed the genetic analysis of complex traits in rainbow trout clonal lines and the detection of QTLs (detailed in the coming section). A wider microsatellite linkage map was developed later, covering the whole set of chromosome arms, using doubled haploid lines of rainbow trout (Guyomard et al., 2006).

In fish and other organisms, the rate of genetic recombination events is not uniform along the whole chromosome length and may vary between sexes (Komen and Thorgaard, 2007; Singer et al., 2002). In rainbow trout, females have much lower recombination rates in telomeric regions compared to males (0.14:1), while recombination rates within regions proximal to the centromere are much higher in females (10:1) (Sakamoto et al., 2000). In the zebrafish (*Danio rerio* Hamilton, 1822), the recombination rate is significantly suppressed in male gametogenesis relative to female (2.74:1), especially near the centromere (Singer et al., 2002). In this kind of study, the use of gynogenesis or androgenesis has practical implications and knowledge of the recombination rate in males and females has applications in genetic analyses. For example, high recombination rates allow the distinction between closely linked markers and are useful for fine mapping, by enhancing the ratio of genetic distance to physical distance. Low recombination rates facilitate mutation or QTL detection and localization to a particular linkage group as well as maintaining relationships of linked alleles, useful to study epistatic interactions between mutations or QTLs (Komen and Thorgaard, 2007; Singer et al., 2002).

3.4. Use of clonal lines for QTL search

Genetic analyses of traits of interest like growth, resistance to disease, and reproductive performance are needed in breeding programs. The use of clonal lines to detect QTL markers is a fruitful approach to understand the molecular mechanisms underlying the traits of interest. The use of clonal lines and recombined clonal lines to detect QTLs is

extensive and all the below examples represent only a small proportion of the investigations carried out to date, and which have allowed considerable progress in fish genetic and genomic research.

The first step of QTL detection is the characterization of phenotypic variation between different clonal lines. The phenotypic variation among clonal lines can be analyzed by comparison of the clones or by comparison in crosses with outbred individuals. Robison et al. (1999) analyzed differences in development rate of 4 rainbow trout clonal lines by crossing eggs from outbred females with sperm from clonal lines. The authors found one clonal line exhibiting a faster development relative to the other lines. Quillet et al. (2007) studied disease resistance among 9 rainbow trout homozygous clonal lines, propagated by meiotic gynogenesis of a doubled haploid female or within-clone single pair mating. This study revealed a high variability in viral infection between the different clonal lines. Among them, 3 were fully resistant to viral hemorrhagic septicemia virus (VHSV) exhibiting a survival higher than 95% at 27 and 32 days after infection, while other were highly susceptible with survival rates under 5% and lower than the controls. These extreme phenotypes observed between clonal lines can be used for the detection of QTLs and candidate genes to study the genetic factors involved in disease resistance.

The second step of QTL detection is to analyze segregation and linking to phenotypes among recombined doubled haploids. In rainbow trout, Robison et al. (2001) found a major locus influencing embryonic development rate by analysis of 2 androgenetic families from males obtained by crossing 2 clonal lines previously differentiated for slow (OSU line) and fast development (Swanson) (Robison et al., 1999). From the cross between the 2 clonal lines, F1 hybrids were obtained that could be reproduced by androgenesis to obtain doubled haploids, which increased the power to detect QTLs compared to a traditional backcross and allowed the use of powerful analytical techniques. The analysis of 170 individuals using 222 markers allowed the detection of a QTL influencing time to hatch on 2 linkage groups. The authors also found evidence of a QTL influencing body length at swim-up on the same linkage groups but the dataset was not sufficient to determine if one gene underlies both QTLs or if the QTLs represent two separate but tightly linked genes (Robison et al., 2001). Nichols et al. (2007) produced androgenetic doubled haploids using F1 hybrid males from 2 clonal lines (OSU x Clearwater) and found the same major QTL influencing time to hatch.

In rainbow trout, behavioral differences have been found between 4 clonal lines crossed with outbred females (Lucas et al., 2004). Significant genetic effects on mean swim level, hiding, foraging, startle response (defense to sudden stimulus or threat) and aggression level were found among clonal lines, and the 2 clonal lines derived from populations reared in captivity for a long time (at least 25-50 generations) exhibited reduced predator avoidance and increased aggression level compared to the clonal lines derived from more recently domesticated populations. Further investigation on the genetic factors underlying these differences could give information on the behavioral patterns influenced by domestication and relevant to aquaculture. Genetics of stress response were analyzed using clonal lines with differing levels of domestication and two significant QTLs with opposing additive effects on cortisol levels were detected. Two QTLs were also detected for juvenile body mass, one of

which overlapped with a QTL for cortisol levels, indicating a possible link between these traits. A complex genetic control of stress physiology and relationship with growth rate were suggested (Drew et al., 2007).

Studies on resistance to pathogens and disease in rainbow trout showed major histocompatibility regions located on 4 different chromosomes. The genetic factors determining the membrane glycoproteins playing a role in pathogen elimination were mapped on 2 recombined clonal lines (Phillips et al., 2003). A single major QTL related to natural killer cell-like activity was localized to one linkage group among a recombined clonal line (Zimmerman et al., 2004). Resistance to *Ceratomyxa shasta* was analyzed in clonal lines and showed different susceptibilities but the QTL analyses revealed multiple loci associated with resistance and a polygenic hypothesis was suggested (Nichols et al., 2003a).

Other studies investigated nutrition in the rainbow trout with 3 major QTLs found associated with pyloric caeca numbers, showing a polygenic origin of this trait. This result was obtained after a comparison of 5 clonal lines and a QTL analysis on recombined doubled haploids to discern the genetic basis of observed differences (Zimmerman et al., 2005).

Studies on sex chromosome evolution and differentiation in rainbow trout also showed differences between clonal lines in Y chromosome structure. Two lines showed a common heteromorphic Y chromosome while 2 other clonal lines had a Y chromosome similar to the X (Felip et al., 2004). The genetic polymorphism of Y chromosome was analyzed by AFLP with recombinant clonal lines crossed to outbred females, and markers linked to the Y chromosome were identified (Felip et al., 2005).

Part 2. State of the art, review on androgenesis in fish

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1. Intraspecific androgenesis

The induction of androgenesis by the use of homologous sperm has been investigated in several freshwater species with variable success. Attempts at inactivating the maternal genome have been performed using different irradiation techniques and though some studies aimed at the production of haploid androgenetics only, other works tried to produce viable androgenetic individuals by restoration of diploidy using physical shocks. The different works reported in the literature focusing on haploid and diploid androgenesis are reviewed in Table 1 and Table 2, respectively.

Table 1. Review of the different haploid androgenesis experiments performed on fish species. Survival is presented as % of surviving individuals at different developmental stages and the success of androgenesis induction as yield. Hpf is used for hours post fertilization and dpf for days post fertilization.

Species	Irradiation	Dose	Survival (and yield) of haploid androgens	Reference
Zebrafish (*Danio rerio*)	UV	144 mJ/cm²	47% with abnormalities at 24 hpf	(Ungar et al., 1998)
Muskellunge (*Esox masquinongy* Mitchill, 1824)	UV	66-132 mJ/cm²	22,50%	(Lin and Dabrowski, 1998)
Tilapia (*Oreochromis niloticus*)	UV	54 mJ/cm²	18.53% at pigmentation stage	(Karayücel and Karayücel, 2003)
Stinging catfish (*Heteropneustes fossilis* Bloch, 1794)	UV	> 125 mJ/cm²	85% relative hatching	(Christopher et al., 2012)
Loach *(Misgurnus anguillicaudatus)*	UV	> 37.5 mJ/cm²	24-35% at hatching (90%)	(Arai et al., 1992)
	UV	> 150 mJ/cm²	30.3-39.5% at hatching	(Fujimoto et al., 2007)
	Cold-shock	0-3°C for 60 min	15,7% hatching (several ploidies, not 100% androgens)	(Morishima et al., 2011)
Dace (*Leuciscus leuciscus* L.)	UV	384 mJ/cm²	hatching over 15%	(Kucharczyk et al., 2008b)
Ide (*Leuciscus idus* L.)	UV	346-461 mJ/cm²	hatching over 15%	(Kucharczyk, 2001)
Masu salmon (*Oncorhynchus masou* Brevoort, 1856)	Gamma	500-600 Gy	40% at 40 dpf	(Arai et al., 1979)
Rainbow trout (*Salmo gairdneri* Richardson, 1836)	Gamma	300 Gy	46% at 20 dpf	(Parsons and Thorgaard, 1984)
Rainbow trout (*Oncorhynchus mykiss*)	Gamma	300 Gy		(Patton et al., 2007)
Plaice (*Pleuronectes platessa* L.)	Gamma	100-1000 Gy		(Purdom, 1969)
Flounder (*Platichthys flesus* L.)	Gamma	100-1000 Gy		
Russian sturgeon (*Acipenser gueldenstaedtii* Brandt & Ratzeburg, 1833)	Gamma	220 Gy	20-50 % at moving stage	(Grunina et al., 2006)
Stellate x beluga sturgeons (*Huso huso* L.)	Gamma	220 Gy	20-40% at moving stage	
Russian x Siberian sturgeons (*Acipenser baerii* Brandt, 1869)	Gamma	220 Gy	40-50% at moving stage	

Table 2. Review of the different intraspecific diploid androgenesis performed on fish species. Survival of haploid (n) and diploidized (2n) individuals is presented as % of surviving individuals at different developmental stages and the success of androgenesis induction as yield in % of androgenetic individuals. pf means post fertilization, hpf is used for hours post fertilization and dpf for days post fertilization. The conditions of diploidization procedure are detailed for shocks by temperature or pression, duration and timing pf, or by use of diploid/fused sperm when no shock was performed.

Species	Irradiation	Dose	Survival (and yield) of androgens	Diploidization (shock or 2n sperm)	Reference
Common carp (*Cyprinus carpio*)	UV	175 mJ/cm²		40°C for 2 min at 30 min pf	(Tanck et al., 2001a)
		250mJ/cm²	*n*: 53.9% at hatching *2n*: 11.6-18.3% at hatching	40°C for 2 min at 26-28-30 min pf	(Bongers et al., 1994)
		175 mJ/cm²	*2n*: 13.9% (irradiation not 100% effective)	40°C for 2 min at 30 min pf	(Tanck et al., 2001b)
		250 mJ/cm²	*n*: 13.7-59.1% at 48 hpf *2n*: 26.1-31.9% at 48 hpf, 3% at hatching	40°C for 2 min / 2-4°C for 45 min / 7500 psi for 2 min at 1.5T_0 pf	(Rothbard et al., 1999)
	X-ray	250-300 Gy		40.5-41°C for 2-3 min	(Grunina et al., 1990)
Loach (*Misgurnus anguillicaudatus*)	UV	150 mJ/cm²	42% fertilization, 14% at hatching	diploid sperm	(Yasui et al., 2010)
		75 mJ/cm²	*n*: 9.1 to 29.7% at hatching (not 100% haploids) *2n*: 0.2% feeding	11379 psi for 1 min at 35 min pf	(Masaoka et al., 1995)
		75 mJ/cm²	*n*: 5-8% at hatching (irradiation not 100% effective) *2n*: 5-10% at hatching	diploid sperm	(Arai et al., 1995)
	Cold-shock	3°C for 30 min	*2n*: 12.29% (+ other ploidies)	diploid sperm	(Hou et al., 2013)
	UV	150 mJ/cm²	*2n*: 22.23% diplo andro (+ other ploidies)	diploid sperm	
	Cold-shock	3°C for 30 min	*2n*: 4.39% (+ other ploidies)	42°C for 2 min at 65 min pf	(Hou et al., 2014)
	X-ray	250 Gy		37°C for 3 min at 2.5 T_0 pf	(Neifakh and Grunina, 1990)
Tiger barb (*Puntius tetrazona* Bleeker, 1855)	UV	88.2 mJ/cm²	*n*: 9% at hatching *2n*: 15% at hatching	41°C for 2 min at 24 min pf	(Kirankumar and Pandian, 2003)
Tilapia (*Oreochromis niloticus*)	UV	45 mJ/cm²	*n*: 1.7% at hatching *2n*: 2% at hatching, 0.07% at yolk resorption stage	42,5°C for 3.5 min at 25 min pf	(Karayücel et al., 2002)
	UV	37.8-42 mJ/cm²	29.3% survival	41,5°C for 3.5min at 20-25 min pf	(Myers et al., 1992)
	UV	72 mJ/cm²	*n*: 27% 36hpf *2n*: 5.3% 36hpf	42.5°C for 4 min at 25 min pf	(Myers et al., 1995)
African catfish (*Clarias gariepinus* Burchell, 1822)	UV	125 mJ/cm²	*n*: 81% at hatching *2n*: 10.5% at hatching	41°C for 1 min at 33 min pf	(Bongers et al., 1995)

Buenos Aires tetra (*Hemigrammus caudovittatus* Eigenmann, 1907)	UV	69.3 mJ/cm²	*n*: 10% at hatching *2n*: 10% at hatching	41°C for 2 min at 25 min pf	(David and Pandian, 2006a)
Rosy barb (*Puntius conchonius* Hamilton, 1822)	UV	75.6 mJ/cm²	*n*: 10% at hatching *2n*: 14% at hatching, 7% attained maturity	41°C for 2 min at 24 min pf	(Kirankumar and Pandian, 2004a)
Common tench (*Tinca tinca* L.)	UV	345.6 mJ/cm²	*2n*: 1.74% at 7 dpf	40°C for 2min at 30 min pf	(Nowosad et al., 2014)
Zebrafish (*Danio rerio*)	X-ray	100 Gy	*n*: 8 to 28% *2n*: 1.3%	41.4°C for 2 min at 13 min pf	(Corley-Smith et al., 1996)
Siberian sturgeon (*Acipenser baerii*)	X-ray	200 Gy	*2n*: 12.2% at hatching	37°C for 30 min at 1.6 T_0 pf	(Grunina and Neyfakh, 1991)
Stellate sturgeon (*Acipenser stellatus* Pallas, 1771)	Gamma	220 Gy	*n*: 10-30% moving embryo	37°C for 2,5min at 1.4-1.6 T_0 pf	(Grunina et al., 2006)
Rainbow trout *(Oncorhynchus mykiss)*	X-ray	350 Gy	*n*: 28.9% eyed embryos *2n*: 10.6% eyed embryos	7000 psi for 4 min at 350 min pf	(Ocalewicz et al., 2010)
	Gamma	150 Gy	*2n*: 22% at 14 dpf	fused sperm	(Araki et al., 1995)
	Gamma	400 Gy	*n*: 11.8% eyed embryos *2n*: 42.8% at 1st feeding *2n*: 0.8% at 1st feeding	diploid sperm 9000 psi for 3 min at 345 min pf	(Thorgaard et al., 1990)
	Gamma	350-500 Gy	*2n*: 20.5% at eyed stage, 4.5% at mild yolk sac resorption stage	7000 psi for 4 min at 350 min pf	(Babiak et al., 2002b)
	Gamma	360 Gy	*n*: 25-27% at eyed-egg stage *2n*: 0.7% at 1st feeding *2n*: 0.1% at 1st feeding	9000 psi for 3 min at 320 min pf 9000 psi for 3 min at 340 min pf	(Scheerer et al., 1991)
	Gamma	350 Gy	*2n*: 42.5% at hatching, 22.5% at swim-up stage, 1% attained 2 years old	7000 psi for 4 min at 350 min pf	(Babiak et al., 2002a)
Amago salmon (*Oncorhynchus rhodurus* Jordan & McGregor, 1925)	Gamma	250 Gy	*2n*: 22% at 14 dpf	fused sperm	(Araki et al., 1995)
Rainbow trout (*Salmo gairdneri*)	Gamma	360 Gy	*2n*: 32.5-38.9%	9200 psi for 1-3 min at 345 min pf	(Parsons and Thorgaard, 1985)
	Gamma	360 Gy	*2n*: 0.9% at 1st feeding	9000 psi for 3 min at 345 min pf	(Scheerer et al., 1986)
Brown trout (*Salmo trutta* L.)	Gamma	350 Gy	*2n*: 42.7% at eyed stage	7000 psi for 4 min at 450 min pf	(Babiak et al., 2002b)
Brook trout (*Salvelinus fontinalis* Mitchill, 1814)	Gamma	350 Gy	*2n*: 25.1% at eyed stage, 4.3% at hatching	7000 psi for 4 min at 350 min pf	(Babiak et al., 2002b)
	Gamma	880 Gy	*2n*: 30% at eyed stage	8500 psi for 3 min at 450 min pf	(May et al., 1988)
	X-ray	420 Gy	*n*: 19.3-21.6% at eyed-egg stage, 0-0.5% at hatching *2n*: 12.3-16.8% at eyed-egg stage, 0.2-1.6% at hatching	7500 psi for 4 min at 420 min pf	(Michalik et al., 2014)

Amago salmon (*Oncorhynchus masou ishikawae* Jordan & McGregor, 1925)	Gamma	350 Gy	*2n*: 0.22% hatching *2n*: 0.09% hatching	fused sperm 9245 psi for 6 min at 450 min pf	(Nagoya et al., 2010)
	Gamma	450 Gy	*n*: 0.3%	9245 psi for 6 min at 450 min pf	(Nagoya et al., 1996)
Masu salmon (*Oncorhynchus masou*)	Gamma	500 Gy	*2n*: 10% at 14dpf	fused sperm	(Araki et al., 1995)
Gold fish (*Carassius auratus* L.)	Gamma	25000 Gy	*n-2n*: 7-9% fertilization, 0% survival	42°C for 2min at 34-40 min pf	(Paschos et al., 2001)

The optimal irradiation dose is generally characterized by using a range of different doses and durations as well as analyzing the most efficient dose leading to haploids only, taking into account the best survival rate. Data presented in Tables 1 and 2 were converted into a common unit to allow a comparison of the range of doses employed in the different species. UV-irradiation doses presented in $\mu W.cm^{-2}$, $J.m^{-2}$, $erg.mm^{-2}$ were converted in $mJ.cm^{-2}$ and ionizing radiation doses characterized in rad or Roetgen were transformed in Gray. UV irradiation doses ranged from 37-346 $mJ.cm^{-2}$, the lowest dose corresponding to tilapia eggs and the highest being for tench eggs genome inactivation. Ionizing radiations ranged from 100-420 Gy using X-ray and 150-880 Gy with gamma irradiation. The only outstanding gamma irradiation dose reported was for gold fish (*Carassius auratus*) which required 25000 Gy for maternal genome inactivation (Paschos et al., 2001). The range of optimal doses was large with some species requiring relatively low irradiation doses and others needing up to 10-fold higher doses for achieving a complete inactivation of the maternal genome. The comparison of irradiation efficiency among the different species is difficult as differences in egg size and shape, in the relative position of the female pronucleus, in the thickness, composition and optic qualities of the chorion may affect the efficiency of the treatment and ultimately the outcome in terms of embryo survival and ploidy status (Myers et al., 1995).

Diploidy was mainly restored using pressure or heat shock. Pressure shocks ranged 7000-11000 psi with durations from 1-4 min. Heat shocks ranged 37-42.5°C for 1-6 min except for the Siberian sturgeon (*Acipenser baerii*) whose fertilized eggs were shocked for 30 min (Grunina and Neyfakh, 1991). Shock durations were usually short and the most between-species varying parameter was the time of application required to suppress mitosis I, that ranged from 20 min after fertilization to 7.5 hpf.

Diploidy restored by using a proper physical shock (temperature or pressure) leads to doubled haploids (DH) that are expected to be homozygous at all loci as they are produced by duplication of a single set of paternal chromosomes. As reviewed in Table 2, the survival rates across species were usually very low. Some hypotheses have been proposed to explain the low yields of DHs. The first is the expression of homozygous deleterious alleles that can affect the development and viability of DH embryos. This has been suggested in a mitotic gynogenetic progeny of *D. labrax* which deviated from the Hardy-Weinberg equilibrium and showed one marker allele transmitted with a significantly lower frequency thus suggesting a

linkage to a deleterious gene (Bertotto et al., 2005). The viability of DH androgens can also be further reduced by potential damage caused by irradiation and physical shock to egg mitochondrial DNA (mtDNA) and other cytoplasm constituents (Arai et al., 1979; Thorgaard et al., 1990) though some authors showed that the optimal irradiation dose did not lead to mitochondrial DNA damages (Brown and Thorgaard, 2002; May and Grewe, 1993). Irradiation, in particular gamma- and X-rays, can also lead to maternal chromosome fragments which can cause impaired development and mortalities (see section 3.2).

In fish conservation programs, androgenesis can be a potential tool for the preservation and recovery of unique strains or endangered species from cryopreserved or fresh stored sperm. In order to improve survival rates, the use of diploid sperm from a tetraploid male or the fusion of two spermatozoa to obtain diploid progenies after fertilization may be good alternatives to the physical shock. As these progenies are usually heterozygous (except if the male originates from a clonal line), they can benefit from this condition and their performance improve.

2. Interspecific androgenesis

Under certain circumstances, the eggs of certain species may be difficult to irradiate because of the egg characteristics or technical difficulties (small quantity of eggs, no efficient protocol, etc). An alternative way to obtain androgenetic progenies is to use another species as egg donor, leading to interspecific androgenesis. Although the protocol for the restoration of diploidy remains unchanged, the use of eggs and sperm originating from different species leads to other considerations described hereafter.

Table 3. Review of the different diploid interspecific androgenesis experiment performed on fish species. Sperm donor is the species in which androgenesis was induced, egg donor is the species whose eggs were inactivated and used for fertilization. Survival of haploid (n) and diploidized (2n) individuals is presented as % of surviving individuals at different developmental stages and the success of androgenesis induction as yield in % of androgenetic individuals. pf means post fertilization, hpf is used for hours post fertilization and dpf for days post fertilization. The conditions of diploidization procedure are detailed for shocks by temperature or pression, duration and timing pf, or by use of diploid/fused sperm when no shock was performed.

Sperm donor	Egg donor	Irradiation	Dose	Survival (and yield) of androgens	Diploidization (shock or 2n sperm)	Reference
Golden Buenos Aires tetra (*Hemigrammus caudovittatus*)	Black widow tetra (*Gymnocorymbus ternetzi* Boulenger, 1895)	UV	75.6 mJ/cm²	*n*: 11% at hatching *2n*: 11% at hatching	41°C for 2min at 25min pf	(David and Pandian, 2006b)
Golden Buenos Aires tetra (*Hemigrammus caudovittatus*)	Black widow tetra (*Gymnocorymbus ternetzi*)	UV	75.6 mJ/cm²	*2n*: 1.8% hatching	fused sperm	(Clifton and Pandian, 2008)
Brook trout (*Salvelinus fontinalis*)	Brown trout (*Salmo trutta*)	Gamma		inviable		(May and Grewe, 1993)
Splake trout: lake trout (*Salvelinus namaycush* Walbaum, 1792) x brook trout (*Salvelinus fontinalis*)	Brook trout (*Salvelinus fontinalis*)	Gamma		few hatching	7000 psi for 2 min at 450 min pf	
Transgenic mud loach (*Misgurnus mizolepis* Günther, 1888)	Common carp (*Cyprinus carpio*)	UV	175 mJ/cm²	*n*: 57% at hatching *2n*: 19% at hatching	40°C for 2 min at 30 min pf	(Nam et al., 2002)
Goldfish (*Carassius auratus auratus*)	Common carp (*Cyprinus carpio*)	Gamma	250 Gy	*2n*: 17.5 to 28% hatching	40°C for 2min at 40 min pf	(Bercsenyi et al., 1998)
Siberian sturgeon (*Acipenser baeri*)	Sterlet (*Acipenser ruthenus* L.)				dispermy	(Grunina et al., 1995)
Common carp (*Cyprinus carpio*) x Crucian carp (*Carassius gibelio* Bloch, 1782)	Common carp (*Cyprinus carpio*)				heat shock	
Siberian sturgeon (*Acipenser baeri*)	Sterlet (*Acipenser ruthenus*)	X-ray	220 Gy	inviable	37°C for 2.5min at 1.4-1.6 T_0 pf	(Grunina and Recoubratsky, 2005)
Sterlet (*Acipenser ruthenus*)	Siberian sturgeon (*Acipenser baeri*)			inviable		
Russian sturgeon (*Acipenser gueldenstaedtii*)	Stellate sturgeon (*Acipenser stellatus* Pallas, 1771)			inviable		
Stellate sturgeon (*Acipenser stellatus*)	Russian sturgeon (*Acipenser gueldenstaedtii*)			inviable		
Beluga sturgeon (*Huso huso* L.)	Stellate sturgeon (*Acipenser stellatus*)			*2n*: 10% at hatching, 3.3% at 1 month of age		
Stellate sturgeon (*Acipenser stellatus*)	Beluga sturgeon (*Huso huso*)			*2n*: 0.93% hatching		
Beluga sturgeon (*Huso huso*)	Stellate sturgeon (*Acipenser stellatus*)			survival similar to control	dispermic activation	

Russian sturgeon (*Acipenser gueldenstaedtii*)	Persian sturgeon (*Acipenser persicus* Borodin, 1897)			survival similar to control		
Siberian sturgeon (*Acipenser baeri*)	Russian sturgeon (*Acipenser gueldenstaedtii*)			survival similar to control		
Bastard sturgeon (*Acipenser nudiventris* Lovetsky, 1828)	Sterlet (*Acipenser ruthenus*)			survival similar to control		
Beluga sturgeon (*Huso huso*)	Sterlet (*Acipenser ruthenus*)			survival lower than controls		
Russian sturgeon (*Acipenser gueldenstaedtii*)	Paddlefish (*Polyodon spathula* Walbaum, 1792)			inviable		
Paddlefish (*Polyodon spathula*)	Russian sturgeon (*Acipenser gueldenstaedtii*)			inviable		
Paddlefish (*Polyodon spathula*)	Stellate sturgeon (*Acipenser stellatus*)			inviable		
Sterlet (*Acipenser ruthenus*)	Russian sturgeon (*Acipenser gueldenstaedtii*)			inviable		
Russian sturgeon (*Acipenser gueldenstaedtii*)	Sterlet (*Acipenser ruthenus*)			inviable		
Sterlet (*Acipenser ruthenus*)	Stellate sturgeon (*Acipenser stellatus*)			inviable		
Stellate sturgeon (*Acipenser stellatus*)	Beluga sturgeon (*Huso huso*)			few hatching but mortality before feeding		
Bastard sturgeon (*Acipenser nudiventris*)	Beluga sturgeon (*Huso huso*)			inviable		
Common tench (*Tinca tinca*)	Common carp (*Cyprinus carpio*)	UV	345.6 mJ/cm²	*n*: hatching > 2%	40°C for 2 min at 30 min pf	(Kucharczyk et al., 2014)
	Common bream (*Abramis brama* L.)			*2n*: survival 0-2%		
Brown trout (*Salmo trutta*)	Brook trout (*Salvelinus fontinalis*)	Gamma	350 Gy	inviable	7000 psi for 4 min at 350 and 450 min pf	(Babiak et al., 2002b)
Brook trout (*Salvelinus fontinalis*)	Brown trout (*Salmo trutta*)			inviable		
Brown trout (*Salmo trutta*)	Rainbow trout (*Oncorhynchus mykiss*)			inviable		
Rainbow trout (*Oncorhynchus mykiss*)	Brown trout (*Salmo trutta*)			inviable		
Brook trout (*Salvelinus fontinalis*)	Rainbow trout (*Oncorhynchus mykiss*)			inviable		
Rainbow trout (*Oncorhynchus mykiss*)	Brook trout (*Salvelinus fontinalis*)			inviable		
Arctic charr (*Salvelinus alpinus* L.)	Brook trout (*Salvelinus fontinalis*)			*2n*: 14.8% eyed stage, 0% hatching	7000 psi for 4 min at 300-600 min pf (tested every 30 min)	
Atlantic salmon (*Salmo salar* L.)	Brown trout (*Salmo trutta*)			*2n*: 12,7% eyed stage, 0% hatching		

Goldfish (*Carassius auratus auratus*)	Loach (*Misgurnus anguillicaudatus*)	UV	150 mJ/cm²	inviable		(Fujimoto et al., 2010)
Rainbow trout (*Oncorhynchus mykiss*)	Masu salmon (*Oncorhynchus masou*)	Gamma		inviable		(Fujiwara et al., 1997)
Ide (*Leuciscus idus*)	Chub (*Leuciscus cephalus* L.)	UV	345.6 mJ/cm²	*n*: 5% at hatching (irradiation almost 100% effective)		(Kucharczyk et al., 2008a)
				2n: 2% at hactching (not 100% androgens)	36°C for 5 min at 180 min pf	
Blue tilapia (*Oreochromis aureus* Steindacher, 1864)	Nile tilapia (*Oreochromis niloticus*)	UV	59.4-69.3 mJ/cm²	*2n*: 0.90%	41,6°C for 5 min at 27,5 min pf	(Marengoni and Onoue, 1998)
Goldfish (*Carassius auratus auratus*)	Crucian carp (nigorobuna) (*Carassius auratus grandoculis* L.)	X-ray	300 Gy	*2n*: 11.4% at hatching		(Fujikawa et al., 1993)
	Crucian carp (gengorobuna) (*Carassius cuvieri* Temminck & Schlegel, 1846)					
Brook charr (*Salvelinus fontinalis*)	Brook charr (*Salvelinus fontinalis*) x Arctic charr (*Salvelinus alpinus*)	X-ray	420 Gy	*n*: 21.3% at eyed-egg stage		(Michalik et al., 2014)
				2n: 30.3% at eyed-egg stage, 6.24% at hatching	7500 psi for 4 min at 420 min pf	
Brook charr (*Salvelinus fontinalis*)	Arctic charr (*Salvelinus alpinus*)			*n*: no survival		
				2n: 1 individual at eyed-egg stage, no hatching	7500 psi for 4 min at 420 min pf	
Red crucian carp (*Carassius auratus* red var) x Common carp (*Cyprinus carpio*)	Goldfish (*Carassius auratus auratus*)	UV	300 mJ/cm²	*2n*: 4% at hatching, 41.3% survival from hatching to 1st feeding	diploid sperm	(Sun et al., 2007)
Golden rosy barb (*Puntius conchonius*)	Grey tiger barb (*Puntius tetrazona*)	UV	88.2 mJ/cm²	*2n*: 1.7%	dispermic activation / 41°C for 2 min at 24 min pf	(Kirankumar and Pandian, 2004b)
Brook charr (*Salvelinus fontinalis*) x Arctic charr (*Salvelinus alpinus*)	Brook charr (*Salvelinus fontinalis*)	X-ray	420 Gy	*2n*: 4.7% at hatching	7000 psi for 4 min at 420 min pf	(Ocalewicz et al., 2013)
	Arctic charr (*Salvelinus alpinus*)			*2n*: 1.2% at hatching		
	Brook charr (*Salvelinus fontinalis*) x Arctic charr (*Salvelinus alpinus*)			*2n*: 16.8% at hatching		
Crucian carp *(Carassius auratus gibelio*)	Common carp (*Cyprinus carpio*)	X-ray	250 Gy	very low	40°C for 2-3 min at 1,7-1,9 T_0 pf	(Grunina et al., 1991)
Rosy barb (*Puntius conchonius*)	Tiger barb (*Puntius tetrazona*)	UV		*n*: 3-9% at hatching		(Kirankumar and Pandian, 2004c)
				2n: 7-14% at hatching	41°C for 2 min at 24 min pf	

Beyond the use in conservation programs which may focus on the restoration of extinct species from cryopreserved sperm, interspecific androgenesis may allow the investigation of nucleo-cytoplasmic compatibility. It has been attempted in several freshwater species as reviewed in Table 3 and resulted in varying success. Some nucleo-cytoplasmic androgenetic hybrids developed while others were unviable though viable control hybrids could be produced. Among the possible explanations, it has been suggested that interspecific androgenesis is possible only between closely related species and between species showing similar karyotypic characteristics (Babiak et al., 2002b; Grunina and Recoubratsky, 2005). To avoid the problem of nucleo-cytoplasmic incompatibility, interspecific androgenesis has been achieved using as egg donor a hybrid of the species whose sperm is used for fertilization. This sometimes led to better results than true interspecific androgenesis as observed in the brook charr (*Salvelinus fontinalis*) (Michalik et al., 2014) .

3. Genetic inactivation

3.1. DNA damage

Several types of DNA damages after UV irradiation have been identified. The two major mutagenic lesions are cyclobutane-pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs). After UV irradiation, the CPDs are the most abundant DNA damage products (Sinha and Hader, 2002). UVC irradiation (200-280nm) produce more thymine homodimers compared to dimers containing cytosine (Mitchell et al., 1991; Mitchell et al., 1992). CPDs cause problems during replication and transcription of DNA (Tornaletti and Hanawalt, 1999). Beyond these pyrimidine dimers, UV-irradiation also causes the formation of DNA-DNA or RNA-RNA cross-links (Friedberg, 1985; Komen and Thorgaard, 2007)

The most biologically significant lesion caused by ionizing irradiation (gamma or X-ray) is double-strand breaks (DSBs) that shatter DNA (Rothkamm and Lobrich, 2003).

3.2. Comparison of irradiation methods

In attempts at inducing gynogenesis and androgenesis in different fish species, either ionizing or UV irradiation were employed by different laboratories. The choice of technique is mainly related to practical considerations and safety issues. Germicidal UVC lamps are easier to handle and have several advantages compared to X-ray or gamma irradiation (Lin and Dabrowski, 1998). UV-rays have a weak penetration ability (Lin and Dabrowski, 1998; Myers et al., 1992) and are effective only on thin layers of diluted milt or eggs while gamma-rays are very penetrating but mostly unavailable near laboratories (Chourrout, 1986).

Another issue for the choice of irradiation procedure is that X-ray and gamma irradiations may result in supernumerary chromosome fragments that can interfere during early embryonic development (Chourrout and Quillet, 1982; Ocalewicz et al., 2012; Parsons and Thorgaard, 1984) as well as at later stages, e.g. during ovarian development (Krisfalusi et al., 2000). Chromosome fragments produced after X-ray irradiation of brook trout eggs were shown to be ring chromosomes or fragments with fused sister chromatids, and their presence explained by the use of a dose inefficient at completely inactivating the maternal genome (Ocalewicz et al., 2012). In general, UV irradiation was thought to be more appropriate as no chromosome fragments were observed in sperm UV-irradiated at the doses producing haploids (Chourrout, 1986). Evidence of chromosome fragments in experiments of gynogenesis and androgenesis employing UV irradiation is seldom reported (Arai et al., 1992; Lin and Dabrowski, 1998). Overall, chromosome fragments are considered to be the consequence of suboptimal UV treatment conditions and are more frequently found in androgenesis than in gynogenetic progenies (Fujimoto et al., 2007).

4. Restoration of diploidy

In fish, suppression of meiotic or mitotic events can be achieved by physical treatments like pressure and temperature (cold and heat) shocks. Both have been applied with variable success in a variety of species for the purpose of uniparental reproduction (see Tables 2 and 3 and review by Komen and Thorgaard, 2007) and polyploidization (see review by Piferrer et al., 2009). While in general their efficiency seems to be species-specific, both present pros and cons in terms of efficiency, cost and applicability. Despite a number of investigations that have been performed, the cytological events underlying chromosome set manipulations at meiosis or mitosis still remain poorly understood. In the following sections, an overview on the knowledge on thermal and pressure shocks and their underlying mechanisms at cellular level will be presented.

4.1. Thermal shock

Thermal shocks were found to be effective at inducing polyploidy in several early attempts but the mechanism(s) implicated remained largely unknown. In amphibians (salamanders), Fankhauser and Godwin (1948) showed that the most important effect of a heat shock treatment (36°C) applied to red spotted newt *Triturus viridescens* (Rafinesque, 1820) freshly fertilized eggs was not a general breakdown of the second meiotic or maturation spindle but its submergence into the interior of the egg. They observed a loss of anchorage of the spindle outer pole in the cortex, a displacement below the surface and a rotation from a perpendicular to oblique position. The consistency of the gelated egg cortex may be changed by physical agents and it could be the underlying mechanism of the second polar body retention: a change in the equilibrium of egg cortex or maturation spindle (Dasgupta, 1962).

Gaillard and Jaylet (1975) used heat shock applied to eggs of Iberian ribbed newt *Pleurodeles waltlii* (Waltl, 1839) beginning their first cleavage to produce tetraploid embryos in amphibians. This stage corresponds to a late interphase of second mitosis and they showed that the treatment induced an aberration in the spindle formation and the aster movements' inhibition. In fish, Chourrout (1982) underlined the fact that the same heat shock at the time of metaphase-anaphase of the first mitosis was unsuccessful.

Makino and Ozima (1943) observed that a refrigeration treatment applied after fertilization on common carp eggs induced retention of the second polar body. Cold-shock treatment has also been shown to affect centrosome duplication but seemed to be less effective than hydrostatic pressure shock for the prevention of mitosis (Zhu et al., 2006). Peruzzi and Chatain (2000) also observed low survivals by using cold shock for the induction of mitotic gynogenesis in the European sea bass.

4.2. Pressure shock

The metaphase spindle is very sensitive to hydrostatic pressure. Without any shock, the movements of chromosomes from metaphase to late anaphase are achieved without increase in length of the spindle but by contraction of the gelated spindle fibers. As pressure-shock changes properties of the cortex and spindle (Pease, 1941), if the shock is applied before anaphase movements, the chromosome remain in their position and sink into the egg (Dasgupta, 1962). Elevated pressure has been shown to preferentially depolymerise microtubules and actin filaments in living cells (Crenshaw et al., 1996; Rousselet et al., 2001). The destabilization of centriolar microtubules by hydrostatic pressure is a reversible phenomenon and cells recover their ability to duplicate after some time (Rousselet et al., 2001).

Ocalewicz et al. (2010) showed that high-pressure treatments decrease survivability of androgenetic rainbow trout and have deleterious effect on development. It has been suggested that aside from the destabilization of the microtubules, pressure shock may induce deformations of other cell organelles (Crenshaw et al., 1996; Ocalewicz et al., 2010) or provide mosaic individuals (Zhang and Onozato, 2004). Yamazaki and Goodier (1993) showed that hydrostatic pressure applied at the first cleavage on salmon embryos altered chromosome constitution and morphology with appearance of chromosome fragments, ring chromosomes, fused chromosomes and also occurrence of dicentric chromosomes and chromosomes with "sticky" ends resulting in fragmentation at cytokynesis. Other studies showed that high pressure shocks act on DNA-protein structure and may affect development of fish embryos when applied to zygotes (Crenshaw et al., 1996; Yamaha et al., 2002).

4.3. Comparison between induction methods and resulting cytological events

Chourrout (1986) indicated that "early" pressure and heat shocks possibly alter different cell mechanisms, even though both suppressed the second division of meiosis. Heat shocks either permit or inhibit the entire disjunction and therefore may interfere with factors involved in the migration of all chromosomes, acting for example on the centrioles. Suboptimal pressure treatments prevent only part of the meiotic disjunction, possibly by impairing processes involved in the migration of individual chromosomes, for example the spindle fiber formation.

The efficient application time of pressure shocks was found to be more acute and later than heat shock, especially in the rainbow trout (Diter et al., 1993). These differences between pressure and heat shock timing indicate a different effect on cell division. Pressure shock dissolves the spindle apparatus while heat shock induces inactivation of the centrosome leading to the prevention of aster formation (Diter et al., 1993). Moreover, Chourrout (1984; 1986) recorded a gradual transition from diploidy to triploidy through aneuploidy with pressure shocks while no aneuploids were recorded using heat shock. These results suggested that heat shock interferes with factors involved in the migration of chromosomes, like aster formation and that pressure treatment could impair processes dealing with the migration of individual chromosomes like the microtubules of the mitotic spindle (Diter et al., 1993). Work with tilapia has identified several induction "windows" where the mitotic process can be arrested, but apparently these windows are treatment-specific (Don and Avtalion, 1988; Hussain et al., 1993; Myers, 1986) and each window corresponds to a developmental period where spindle fiber formation is important (Myers et al., 1992).

As spindles are inactivated or disorganized by temperature or hydrostatic shocks, it has been supposed that inactivation or disassembly of spindles blocks the anaphase movement of chromosomes and a duplicated nucleus is formed without cell division. However Zhang and Onozato (2004) showed that hydrostatic pressure treatment around the time of metaphase of the first cell cycle of rainbow trout eggs did not suppress the first cleavage but the second one. Spindles disassembled by the shock regenerated soon after treatment, resulting in the occurrence of the first mitosis. A monopolar spindle was assembled in each blastomere in the second cell cycle, and disjunction of duplicated chromosomes and cell cleavage prevented, leading to chromosome set doubling. From the third cell cycle, normal cell division was resumed. The process of chromosome set doubling seemed to be the same for both methods but hydrostatic pressure treatments led to embryos showing tripolar or tetrapolar division in the first cell cycle, their number was 2-fold higher than with heat shocks.

5. Verification of ploidy status and uniparental transmission in experiments of androgenesis and gynogenesis

The complete inactivation of the maternal (androgenesis) or paternal (gynogenesis) genome is the prerequisite for the successful induction of uniparental reproduction. Different techniques have been used to verify the status of experimental fish such as embryo and larval morphology, nuclear DNA content, chromosomes counts, microsatellite markers or DNA fingerprinting. The different techniques used in previous androgenesis experiments (reviewed in Tables 1 to 3) are detailed hereafter. As techniques evolve very fast, the development of new DNA marker technologies for verification of parental genome inactivation, such as SNPs and genotyping by sequencing, is currently ongoing and new tools will soon be available but will not be reviewed in this section.

5.1. Verification of ploidy status

- **Haploid syndrome**

In several studies dealing with androgenesis and gynogenesis, the use of a haploid control is common to verify the complete inactivation of one parental genome. Haploids are inviable in fishes and die around hatching. Another indicator of irradiation success is the morphological appearance of haploids. The 'haploid syndrome' is characterized by several abnormalities like dwarfism, microcephaly, microphtalmy and delayed development (Araki et al., 2001; Purdom, 1969; Uwa, 1965). This method of verification should only be employed as an indication of irradiation success and completed with karyological or flow-cytometry analyses. Deformities can be observed if some mutations have occurred and the presence of chromosome fragments or aneuploidy should be verified to allow the correct and unambiguous statement of ploidy status in experimental progenies.

- **Analysis of karyotypes**

This method is used for direct chromosome counting. After injection of a live fish or treatment of newly hatched larvae with a chemical agent like colchicine, which is a mitotic inhibitor which blocks spindle fiber formation, chromosomes are blocked at cell metaphase and can be fixed and stained on microscope slides. The obtained metaphase spreads are analyzed using a standard light microscopy and chromosomes counted. This technique is the more precise as chromosome counts are unambiguous and chromosome fragments can also be detected but the main disadvantage is the time required for the preparation and analysis of a high number of cells in each individual so that karyotyping may not always easily detect mosaic ploidies (Dunham, 2004; Harrell et al., 1998).

- **NORs preparations**

Silver staining is a technique to count the number of metaphase chromosome sites of nucleolus organizer regions (NORs) in cells. After injection of a chemical agent, chromosomes are spread and slides silver-stained for microscopic examination. The disadvantages of this technique are that, like chromosome counting, NORs preparations and the examination of large number of cells is time consuming. Furthermore this procedure works best on organisms having only one NOR-bearing chromosome pair in order obtain unambiguous nucleoli counts (Gold, 1984; Harrell et al., 1998).

- **Flow-cytometry**

Flow-cytometry is a method to measure relative nuclear DNA content of individual cells and estimate distributions of cells in various stages of cell cycle. It is an optical technique that measures fluorescence of stained particles, for DNA content nuclei are stained with a DNA-specific dye (Harrell et al., 1998). Flow cytometry has been used for estimation of DNA content in experimental (e.g. Lin and Dabrowski, 1998; Peruzzi and Chatain, 2000) and wild stocks (e.g. Birstein et al., 1993; Peruzzi et al., 2005). Propidium Iodide (PI) is a fluorescent dye that intercalates between DNA base pairs with little or no preference between DNA and RNA necessitating a treatment with RNase to distinguish between the two molecules, whereas 4',6-diamidino-2-phenylindole (DAPI) binds strongly to A-T rich regions of DNA (Harrell et al., 1998). The former is used to stain dead (deep frozen of fixed) cell whereas the latter is used for live cells. The advantages of flow-cytometry are its accuracy and rapidity of analysis but the technique is relatively expensive (Dunham, 2004). Chromosome analyses are more precise and generally described as superior to flow-cytometry because it is the only irrefutable method to measure ploidy as chromosome fragments and aneuploidy can be detected unambiguously (Fujimoto et al., 2007; Harrell et al., 1998). However, flow-cytometric analyses are much less time consuming and allow analyses of thousands of nuclei per individual and large number of samples.

5.2. Verification of uniparental transmission

- **Phenotypic appearance**

Many early studies used a recessive morphological character as evidence of uniparental origin. Blond or orange color, mirror scale, telescopic eye are examples of phenotypic recessive traits that can be used in gynogenesis or androgenesis studies. This kind of marker has the advantage of being easily recognizable but cannot be used alone as inherited chromosome fragments or residual heterozygosity cannot be detected (Pandian and Kirankumar, 2003; Paschos et al., 2001; Rothbard et al., 1999).

- **Allozymes**

Allozymes reflect polymorphism as they represent the variants of proteins produced by a single gene locus. Differences in the polypeptide chains of the different allelic forms result from changes in the underlying DNA sequence, and the protein products can be differentiated by their migration properties on a starch gel electrophoresis. Allozymes markers were among the earliest markers used in aquaculture genetics but their disadvantages come from the amount and quality of tissue samples required, sometimes requiring fish to be killed. Some differences in nucleotidic sequence can also be missed, silent substitutions or synonymous substitutions do not change the encoded polypeptide or its mobility and these changes cannot be revealed on the gel (Liu and Cordes, 2004).

- **Restriction fragment length polymorphism (RFLP)**

This technique implies DNA digestion by a restriction enzyme. An endonuclease recognizes specific short palindromic base pair nucleotide sequences and cuts DNA wherever these sequences are present. Before being replaced with techniques based on polymerase chain reaction (PCR), the fragments produced after DNA digestion were separated using a Southern blot analysis (electrophoresis through an agarose gel and transfered onto a membrane) and visualized after hybridization to specific probes. The number and sizes of fragments differ between individuals, populations and species and both alleles of an individual can be observed using this technique but the main disadvantage of RFLP is the relatively low level of polymorphism. In addition, some information on the sequence and the probes are required making this technique difficult to apply or time-consuming in species lacking molecular information (Liu and Cordes, 2004).

- **Random amplified polymorphic DNA (RAPD)**

RAPD uses the PCR technique to amplify unknown regions of the genome using short primers. RAPD polymorphism is due to base substitutions at the primer binding sites or to insertions and deletions between the sites and the detection of polymorphism using RAPD is relatively high (Liu and Cordes, 2004). This technique has been used in various areas of fish biology, such as species identification, detection of interspecific hybridization at the genomic level and analysis of the genome of individuals produced by whole genome manipulation (Bercsenyi et al., 1998). RAPD is a fast, sensitive technique that tests the genome at several positions and no prior knowledge on the DNA of the species is needed (Bercsenyi et al., 1998; Liu and Cordes, 2004). To analyze parental genome inheritance, a study with a few microsatellite DNA markers has a small chance of picking up the transmission of fragments from the irradiated germ cells, especially in the case of intraspecific andro/gynogenesis, where the 2 parental genomes do not differ markedly from each other but RAPD offers the possibility to analyze several positions on the genome (Bercsenyi et al., 1998). However, the disadvantages of RAPD are the difficulty to demonstrate Mendelian inheritance of the loci,

the inability to distinguish between homozygotes and heterozygotes because of the low efficiency of band intensities scoring, and the low reproducibility of this technique (Liu and Cordes, 2004).

- **Microsatellites**

Microsatellites consist of multiple copies of simple sequence nucleotide repeats arranged in tandems distributed along the whole genome. Their small size facilitates PCR genotyping and microsatellites containing a large number of repeats are the most polymorphic. Their polymorphism results primarily from the varying numbers of repeat units contained by alleles at a given locus, this leads to a large number of alleles for each microsatellite locus. In addition to their abundance, small size and high polymorphism, they are inherited following a Mendelian law. Their disadvantage is that each microsatellite locus has to be identified and appropriate PCR-primers have to be designed and labeled usually with fluorescent dyes. However, several samples can be typed in a day and the analyses are automated using fluorescent sequencers. Of all the markers previously described, microsatellites are the most powerful DNA marker type to detect polymorphism and have been widely used in many genetic investigations such as genome mapping, parentage assignment, populations structures and also homozygosity analyses (Liu and Cordes, 2004). However, a sufficient number of microsatellites have to be used in order to detect small fragments or small differences in the samples analyzed since one microsatellite represents only one locus (Bercsenyi et al., 1998).

Part 3. Genetic inactivation of European sea bass (*Dicentrarchus labrax* L.) eggs using UV-irradiation : observations and perspectives

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Genetic Inactivation of European Sea Bass (*Dicentrarchus labrax* L.) Eggs Using UV-Irradiation: Observations and Perspectives

Julie Colléter[1,2], David J. Penman[3], Stéphane Lallement[2], Christian Fauvel[2], Tanja Hanebrekke[4], Renate D. Osvik[4], Hans C. Eilertsen[4], Helena D'Cotta[1], Béatrice Chatain[2], Stefano Peruzzi[4]*

1 Cirad, Persyst, UMR Intrepid, Campus International de Baillarguet, Montpellier, France, **2** Ifremer, Laboratoire de Recherche Piscicole en Méditerranée, Station Expérimentale d'Aquaculture, Palavas Les Flots, France, **3** University of Stirling, Institute of Aquaculture, Stirling, Scotland, **4** Department of Arctic and Marine Biology, Faculty of Biosciences, Fisheries and Economics, University of Tromsø, Tromsø, Norway

Abstract

Androgenesis is a form of uniparental reproduction leading to progenies inheriting only the paternal set of chromosomes. It has been achieved with variable success in a number of freshwater species and can be attained by artificial fertilization of genetically inactivated eggs following exposure to gamma (γ), X-ray or UV irradiation (haploid androgenesis) and by restoration of diploidy by suppression of mitosis using a pressure or thermal shock. The conditions for the genetic inactivation of the maternal genome in the European sea bass (*Dicentrarchus labrax* L.) were explored using different combinations of UV irradiation levels and durations. UV treatments significantly affected embryo survival and generated a wide range of developmental abnormalities. Despite the wide range of UV doses tested (from 7.2 to 720 $mJ.cm^{-2}$), only one dose (60 $mJ.cm^{-2}.min^{-1}$ with 1 min irradiation) resulted in a small percentage (14%) of haploid larvae at hatching in the initial trials as verified by flow cytometry. Microsatellite marker analyses of three further batches of larvae produced by using this UV treatment showed a majority of larvae with variable levels of paternal and maternal contributions and only one larva displaying pure paternal inheritance. The results are discussed also in the context of an assessment of the UV-absorbance characteristics of egg extracts in this species that revealed the presence of gadusol, a compound structurally related to mycosporine-like amino acids (MAAs) with known UV-screening properties.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files. The UV absorbance raw data are available from the Dryad Digital Repository: http://doi.org/10.5061/dryad.k7s8s.

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* Email: spe002@uit.no

Introduction

Androgenesis is a form of uniparental development in which the nuclear genetic material is entirely of paternal origin. Androgenesis can be artificially induced in fish using a variety of methods, mostly involving the inactivation of the egg genome by UV or ionizing irradiation (see reviews [1 4]). Most commonly, genetically inactivated eggs are fertilized with conspecific haploid sperm and the paternal genome is doubled by suppression of the first cleavage using thermal or pressure shocks, leading to the production of doubled haploids (DH). Alternative techniques involve the use of diploid sperm from tetraploid fish [5,6] or dispermic egg activation via fusion of sperm nuclei [7], which make the diploidization step unnecessary. Despite their lower penetrance [8], UV-rays have been most widely employed as being more manageable, less damaging to the eggs and far less prone to produce chromosome fragments than ionizing irradiations [9 12]. DH androgenetics are expected to be homozygous at all loci as they are produced by duplication of a single set of paternal chromosomes. This condition adversely affects the development and viability of DH embryos because of the expression of homozygous deleterious alleles. The viability of DH androgens can be further reduced because of potential damage caused by irradiation and physical shock to egg mitochondrial DNA (mtDNA) and other cytoplasm constituents [4,13].

Androgenesis is a useful tool for e.g. the study of sex determination [3,14,15], the production of homozygous clones for research purposes [16 19], the preservation and recovery of unique strains or endangered species from cryopreserved sperm [20] and the study of physiological effects of mitochondrial variations [21]. Androgenesis has been achieved with variable success in a number of freshwater species including Nile tilapia *Oreochromis niloticus* [22,23], zebrafish *Danio rerio* [24,25], common carp *Cyprinus carpio* [26,27] and rainbow trout

Part 3: Genetic inactivation of European sea bass (*Dicentrarchus labrax* L.) eggs using UV-irradiation: observations and perspectives

1. Introduction

Androgenesis is a form of uniparental development in which the nuclear genetic material is entirely of paternal origin. Androgenesis can be artificially induced in fish using a variety of methods, mostly involving the inactivation of the egg genome by UV or ionizing irradiation (see reviews by Arai, 2001; Devlin and Nagahama, 2002; Komen and Thorgaard, 2007; Pandian and Koteeswaran, 1998). Most commonly, genetically inactivated eggs are fertilized with conspecific haploid sperm and the paternal genome is doubled by suppression of the first cleavage using thermal or pressure shocks, leading to the production of doubled haploids (DH). Alternative techniques involve the use of diploid sperm from tetraploid fish (Arai et al., 1995; Thorgaard et al., 1990) or dispermic egg activation via fusion of sperm nuclei (Grunina et al., 2011), which make the diploidization step unnecessary. Despite their lower penetrance (Thorgaard, 1983), UV-rays have been most widely employed as being more manageable, less damaging to the eggs and far less prone to produce chromosome fragments than ionizing irradiations (Carter et al., 1991; Chourrout, 1984; Disney et al., 1987; Thorgaard et al., 1985). DH androgenetics are expected to be homozygous at all loci as they are produced by duplication of a single set of paternal chromosomes. This condition adversely affects the development and viability of DH embryos because of the expression of homozygous deleterious alleles. The viability of DH androgens can be further reduced because of potential damage caused by irradiation and physical shock to egg mitochondrial DNA (mtDNA) and other cytoplasm constituents (Komen and Thorgaard, 2007; Suwa et al., 1994).

Androgenesis is a useful tool for e.g. the study of sex determination (Devlin and Nagahama, 2002; Ezaz et al., 2004a; Gomelsky, 2003), the production of homozygous clones for research purposes (Bongers et al., 1998; Grimholt et al., 2009; Lucas et al., 2004; Müller-Belecke and Hörstgen-Schwark, 2000), the preservation and recovery of unique strains or endangered species from cryopreserved sperm (Pandian and Kirankumar, 2003) and the study of physiological effects of mitochondrial variations (Brown et al., 2006). Androgenesis has been achieved with variable success in a number of freshwater species including Nile tilapia *Oreochromis niloticus* (Karayücel et al., 2002; Myers et al., 1995), zebrafish *Danio rerio* (Corley-Smith et al., 1996; Ungar et al., 1998), common carp *Cyprinus carpio* (Bongers et al., 1994; Grunina et al., 1990) and rainbow trout *Oncorhynchus mykiss* (Babiak et al., 2002a; Scheerer et al., 1986), as reviewed by Komen and Thorgaard (2007).

The complete inactivation of the maternal genome is the prerequisite for the successful induction of androgenesis (Christopher et al., 2012; Fujimoto et al., 2007; Kucharczyk, 2001; Kucharczyk et al., 2008b; Ungar et al., 1998). Different techniques have been used to verify the androgenetic status of experimental fish such as embryo and larval morphology, nuclear

DNA content, chromosomes counts, microsatellite markers or fingerprinting (Arai et al., 1992; Bongers et al., 1994; Fujimoto et al., 2007; Myers et al., 1995; Scheerer et al., 1991; Tanck et al., 2001a).

The European sea bass is a species of considerable economic importance in the Mediterranean and North East Atlantic regions both from the aquaculture and fishery perspectives. Several studies aimed at improving its culture performance have highlighted the need for better characterization of this species (Kuhl et al., 2010; Vandeputte et al., 2009a). A range of isogenic clonal lines would allow comparison over time and under different ambient conditions, estimation of genetic correlations, detection of genotype-by-environment interactions and estimation of phenotypic plasticity for complex traits (Komen and Thorgaard, 2007). Such lines would also be advantageous for other studies such as gene mapping, genome sequencing, epigenetic effects and detection of important quantitative trait loci (QTLs) for culture and research purposes.

Clonal lines of sea bass could be more rapidly achieved by androgenesis since some precocious males mature earlier as one year of age (Felip et al., 2006). Meiotic and mitotic gynogenesis have been successfully induced in the European sea bass (Bertotto et al., 2005; Peruzzi and Chatain, 2000) but no attempt at androgenesis has been reported for this species to date and to our knowledge androgenesis has not been reported in any marine species.

In this work, we explore the conditions for the genetic inactivation of the maternal genome in the European sea bass using UV-irradiation, with the future goal of producing viable diploid androgenetics. Given that the eggs of several marine teleosts (Grant et al., 1980; Plack et al., 1981), including some Mediterranean species (Chioccara et al., 1980), contain variable levels of mycosporine-like amino acids (MAAs, notably gadusol) that provide protection against ambient UV-irradiation, particularly in small, transparent and positively buoyant fish eggs (Browman et al., 2003), a preliminary assessment of the UV-absorbance characteristics of egg extracts in this species is also described.

2. Material and methods

2.1. Experimental design

In order to investigate the efficiency of UV rays at inactivating the maternal genome and induce haploid androgenesis in sea bass we exposed pools of eggs (mixed from different females) to different incident UV-doses and durations followed by activation with normal sperm. To optimize androgenesis treatment using UV rays, irradiation was provided by two sources, from above and below the eggs (Arai et al., 1992), and mechanical stirring was also used to assure homogenous egg irradiation (Bongers et al., 1994). In order to prevent DNA photoreactivation, egg irradiation and early incubation were completed under total darkness. Observations on embryo larval morphology and survival were used as indicators of treatment

conditions and supported by nuclear DNA content estimations of surviving larvae in each experiment. For the confirmation of parental inheritance in putative androgenetic larvae, different batches of eggs were exposed, in a separate experiment, to the best performing UV-conditions from the initial experiments, fertilized with untreated sperm and the resulting larvae genotyped using a set of microsatellite markers (see section 2.6). Newly hatched larvae in the European sea bass are very small, so cytometric analyses and genotyping could not be performed on the same individuals. As a positive control, the efficiency of the purpose built UV device was verified by using the eggs of a model species, the Nile tilapia, and following published procedures for the induction of haploid androgenesis in this species (Supplementary file S1). Finally, egg extracts were analyzed through spectrophotometry and High Performance Liquid Chromatography (HPLC) to look for possible UV-screening compounds (sections 2.8 and 2.9).

2.2. Broodstock and gamete collection

The sea bass broodstock (around 120 females and 40 males) was composed of domesticated and selected fish of West-Mediterranean and Atlantic origin held at the Ifremer Experimental Aquaculture Station (Palavas-les-Flots, France). Fish were aged 4 to 6 years and weighted 1 to 5 kg, they were kept in recirculated systems (8 m^3 tanks, rate of O_2 enriched water renewal: 250 $L.h^{-1}$, constant small air flow) maintained under natural conditions of temperature and photoperiod (43° 31' 40 N, 3° 55' 37 E) and fed commercial diets (NeoRepro, Le Gouessant, France). Running males were recognized by gentle abdominal pressure and held in an easy handling tank. Female maturation stage was assessed in ovarian biopsies obtained by introducing a thin catheter (Pipelle de Cornier, Laboratoire CCD, Paris, France) in the genital orifice. Oocyte diameter and germinal vesicle migration were analyzed after addition of a clearing agent (glacial acetic acid, formaldehyde, ethanol in a ratio 1:3:6) using a profile projector (Nikon V12). Females at the correct stage of development (Fauvel and Suquet, 1988) received a single dose (10 $\mu g.kg^{-1}$) of Luteinizing Hormone Releasing Hormone analogue (LHRHa, Sigma, France) in order to induce final maturation and ovulation. The treated fishes were isolated in individual thermoregulated (13°C) tanks (1.5 m^3, 17 $L.h^{-1}$ water renewal, low air flow) and 72 h after female hormonal stimulation, ovulated oocytes were collected by abdominal pressure. Sperm was drawn from the genital papilla under abdominal pressure, using 5ml syringes, after carefully wiping off water from the genital papilla and avoiding contamination with urine and/or faeces, and held at 4°C until use. At this stage, caudal fin clips were taken from parent fish and stored in absolute ethanol for future genetic analyses. Equal volumes of suitable eggs from 3-5 females were pooled in a single 1 L beaker for further treatment in each experiment.

2.3. UV-irradiation of eggs

The UV irradiation device was composed of eight UV germicidal lamps (12 W, 254 nm, Vilber-Lourmat, Marne-la-Vallée, France) fixed above and below (four lamps each) a quartz plate mechanically stirred throughout irradiation. Small aliquots of eggs (3 ml, around 3000 eggs) were poured into 8.5 cm diameter quartz Petri dishes containing 3 ml of artificial extender SGSS (Seabass Gamete Short term Storage) made of Storefish® (IMV Technologies, France) complemented with pyruvate and glutamine at 0.6 and 3 $mg.ml^{-1}$ respectively (C. Fauvel, pers. comm.), to form a single layer of eggs: the quartz plate and Petri dishes (SARL NH Verre, Puechabon, France) were employed to maximize UV transmission during treatments. Incident UV dose rates were calculated as the addition of the measured doses from above and below. The eggs were irradiated using different incident UV dose rates (7.2, 13.2, 28.8, 42, 54, 60 or 72 $mJ.cm^{-2}.min^{-1}$) and durations (0.5-12 min) according to the following combinations: low dose rates (7.2-28.8 $mJ.cm^{-2}.min^{-1}$) for 1, 2, 4, 6, 8, 10 and 12 min, high dose rates (42-72 $mJ.cm^{-2}.min^{-1}$) for 0.5, 1, 2, 4, 6, 8 and 10 min and additional 0.75 and 1.25 min treatments for 60 $mJ.cm^{-2}.min^{-1}$. Cumulative irradiation doses were calculated by multiplying incident dose rate by duration of irradiation. The lamps were switched on at least 30 min before the onset of irradiation and UV incident dose was verified at the beginning and at the end of each experiment using a VLX-3W radiometer (Vilber-Lourmat), checking both upper and lower UV sources. Egg fertilization was performed just after irradiation by adding 80 µl sperm diluted (1:4) in SGSS and 3 ml sea water (14°C, 35‰). Each experiment was replicated three times using the same oocyte pool and each UV dose was tested twice using different pools of oocytes. Control groups consisted of fertilized eggs that were not irradiated. They were handled and fertilized as above (apart from UV irradiation). All experiments were performed under total darkness in a temperature controlled room maintained at 14°C. Shortly after fertilization, control and treated eggs were incubated separately in individual 2 L tanks in a dedicated recirculated water system (temperature: 14-14.5°C; salinity: 35-36‰) until hatching. All tanks were maintained in darkness for the first 24 h of incubation before being exposed to natural light conditions.

2.4. Estimation of embryonic and larval survival

To characterize embryo development and estimate survival, three different countings were made using sub-samples of approximately 200 eggs collected from each incubator. The first counting, realized 2-4 hours post fertilization (hpf) was used to assess fertilization rate at 4-8 cells stage. The second and third countings were performed at 50 and 74 hpf, respectively, to assess further embryonic development. All observations were made using a dissecting microscope (M3C, Wild Heerbrugg, Switzerland) and representative photomicrographs were taken using a Stemi 2000-C stereomicroscope (Carl Zeiss, Germany) equipped with a ProgResC5 camera device (Jenoptik, Germany). After inspection and development assessment, each sub-sample was returned to its incubator.

2.5. Determination of ploidy

At hatching (approx. 96 hpf) samples of control and UV treated groups were collected and prepared for flow cytometric analyses. For this purpose, individual hatched larvae were gently rinsed in distilled water and placed at the bottom of a 1.5 ml Eppendorf tube. They were then dissociated by repeated manual pipetting in 1 ml of 0.05% Propidium Iodide (PI) solution, following established procedures (Tiersch et al., 1989). After 30 min of PI staining in darkness at 4°C, 10% dimethyl sulfoxide (DMSO) was added and samples were stored at -80°C until use. Flow cytometry analyses were performed using a FACS Canto II (BD Biosciences, San Jose, CA, USA) flow cytometer and measuring the fluorescence of 5000 to 10000 nuclei/larva. The ploidy status of at least 20 hatched larvae (when available, or all surviving larvae in case of a lower number) from UV-treated groups and 10 control larvae was determined in each experiment.

2.6. Microsatellite analysis

Verification of paternal inheritance was performed on presumptive androgenetic larvae coming from three different egg batches. For this purpose, 24 ml of eggs from three dams were UV-irradiated separately for 1 minute using the best performing UV dose (60 $mJ.cm^{-2}$). After irradiation, the eggs were fertilized using the sperm of one of two sires (FAxM1; FBxM1; FCxM2) and putative androgenetic progenies incubated until hatching as previously described (see section 2.3). Individual hatched larvae were stored in absolute ethanol until genetic analyses. DNA was extracted from ethanol-preserved fin clips of the parent fish and from whole individual larvae using an E-Z 96® Tissue DNA Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's protocol. Parental inheritance was assayed at 9 microsatellite loci: *Labrax-17*, *Labrax-29*, *Labrax-3*, *Labrax-8* (Garcia de Leon et al., 1995), *Dla-22* (Ciftci et al., 2002), *Dla-3* (Tsigenopoulos et al., 2003), *Dla-16*, *Dla-105*, *Dla-119* (Chistiakov et al., 2004) found on 9 different linkage groups (LG), these being LG23, LG18, LG13, LG16, LG6, LG19, LG1, LG8, LG14 respectively (Chistiakov et al., 2005). Forward primers were labeled with fluorescent dyes (Applied Biosystems). PCR reactions were carried out in 2.5 µl total volume containing 50-100 ng DNA, 0.1-1.0 µM of each primer set, 2x Qiagen Multiplex PCR (3 mM $MgCl_2$, 6 U HotStarTaq DNA polymerase) and RNA-free water. DNA amplifications and PCR were performed on a GeneAmp PCR System 9700 (Applied Biosystems). The cycling program began with a polymerase activation step at 95°C for 15 min followed by 37 cycles of 94°C for 30 s, 59°C for 90 s and 72°C for 90 s, with a final extension at 72°C for 10 min. The PCR products were electrophoresed in a 3130x Genetic Analyzer (Applied Biosystems) and alleles scored using a GeneMapper Software v3.7 (Applied Biosystems).

2.7. Spectrophotometry analyses

Egg pools from three females were UV-irradiated (see section 2.3) using four different incident dose rates (16.8, 30, 60 and 75 $mJ.cm^{-2}.min^{-1}$) and durations according to the following combinations: lower dose rates (16.8 and 30 $mJ.cm^{-2}.min^{-1}$) for 1, 2-12 min at 2 min intervals, and high dose rates (60 and 75 $mJ.cm^{-2}.min^{-1}$) for 0.5, 1, 2-12 min at 2 min intervals. Unirradiated eggs were used as controls. Immediately after treatment, samples of approximately 1500 eggs (1.5 ml egg volume) from treated and control groups were fixed in 96% ethanol and stored refrigerated until spectrophotometric analysis. Egg extracts from ethanol-stored samples were centrifuged at 2000 rpm for 4 min with a relative centrifugal force (RCF) of 1945 and 0.5 ml of the supernatant was diluted 1:2 in 96% ethanol and scanned at wavelengths of 200 to 700 nm using a Hitachi U-2900 Double Beam Spectrophotometer (Hitachi High Technologies Corporation, Tokyo, Japan). Quartz cuvettes with 1 cm light path were used throughout the analyses. One control sample was analyzed using the same protocol but at lower pH (pH 3) obtained by addition of hydrochloric acid before wavelength scanning.

2.8. Extraction and Ultra High Performance Phase Liquid Chromatography (UHPLC) analyses

Extraction was performed on 250 mg freeze-dried sea bass eggs in a 15 ml cuvette with 5 ml of methanol/water (50/50, v/v) (analytical grade, Sigma-Aldrich, St-Quentin Fallavier, France), assisted by sonication during 10 min. The sample was then centrifuged 5 min at 10,000 rpm and the final extract diluted in Acetonitrile (ACN) (1/1, v/v) (analytical grade, Sigma-Aldrich, St-Quentin Fallavier, France).

Ultra-high performance liquid chromatography (UHPLC) analyses were performed by a Dionex UltiMate 3000 RSLC system (Thermo Fisher Scientific, Waltham, USA) equipped for separation with a Kinetex HILIC (1.7 µm, 2.1x100 mm) (Phenomenex, Le Pecq, France) maintained at 40°C. The mobile phases consisted on (A) 10 mM Ammonium acetate (HPLC grade, Sigma-Aldrich, St-Quentin Fallavier, France) and (B) ACN (HPLC grade, Sigma-Aldrich, France) at constant flow-rate of 0.4 $ml.min^{-1}$ (with gradient conditions described in Supplementary file S2).

Metabolic fingerprints were measured using a Dionex UltiMate 3000 RSLC system coupled to an AB SCIEX TripleTOF 560 quadrupole-time-of-flight mass spectrometer (AB SCIEX, Concord, ON, Canada). Mass-spectrometric analysis was performed using an electrospray ion source (ESI) in both positive and negative ion mode. In the positive ESI mode, parameters were: capillary voltage of 4500V, nebulizing gas pressure of 60 psi, drying gas pressure of 60 psi, temperature of 550°C and declustering potential of 80V. The capillary voltage in negative ESI was -4000V and the other source settings were the same as for positive ESI. Information Dependent Acquisition (IDA) method was employed to collect MS

and MS/MS accurate mass. TOF MS and TOF MS/MS were scanned with the mass range of *m/z* 80-1200.

Instrument control and data acquisition were carried out with the Analyst 1.5.1 TF software (AB Sciex, Concord, ON, Canada) and the analysis was performed using Peak View 2.0 (AB Sciex, Concord, ON, Canada) also equipped with the MasterView Formula Finder and directly linked to ChemSpider database.

2.9. Statistical analyses

At all stages, survival was estimated as a percentage of developing eggs over the total number of eggs. Survival rates were calculated relative to controls after adjustment of the latter to 100%. Survival rates were arcsin square roots transformed for comparison between groups by two-way ANOVA using Statistica (Version 7.1). Data are presented as means ± standard deviations (STD). Presence of null alleles in the PCR products was analyzed using Microchecker software version 2.2.3 (Van Oosterhout et al., 2004).

3. Results

3.1. Embryonic and larval survival

Overall, fertilization rates in the controls and the different treatments ranged from 20-80%, decreasing significantly at higher UV dose rates and longer durations ($F_{4,202}$=22.344; $F_{7,202}$=3.13; $p<0.01$), but with no interaction between the two factors (p=0.11). Only survival rates relative to controls at 50 hpf are presented as no differences between data from 50 hpf and 74 hpf was observed (p=0.47). Survival rates showed dose rate and duration effects ($F_{4,40}$=254.67; $F_{7,40}$=2695.7; $p<0.001$) and an interaction between these two factors ($F_{28,40}$=18.48; $p<0.001$). Overall, larval survival fell sharply with increasing UV intensities and durations, in particular at the highest intensities (42, 60 and 72 $mJ.cm^{-2}.min^{-1}$) where survival dropped to 20% relative to controls after 1 min irradiation only (Fig. 4). At the lowest intensities (7.2 to 29 $mJ.cm^{-2}.min^{-1}$) survival rate decreased to less than 10% when eggs were irradiated up to 6 min, before reaching 0% between 10 and 12 min.

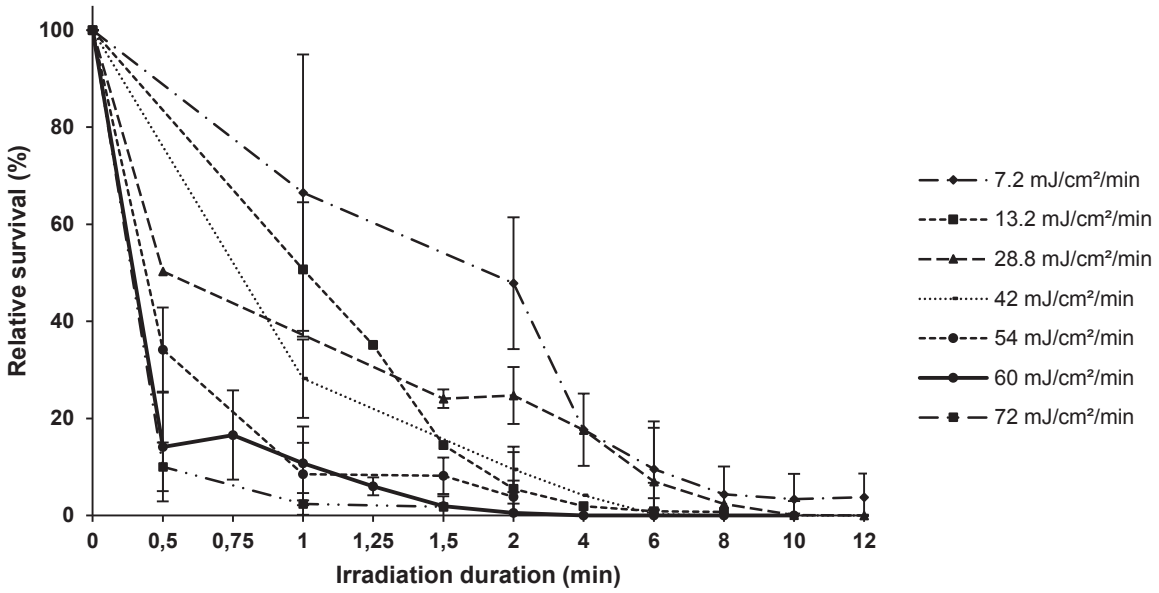


Figure 4. Percent survival relative to controls of hatched larvae issued from the different UV-irradiation treatments (7.2-72 mJ.cm^{-2}.min^{-1}) lasting 0.5-12 min. Error bars represent standard deviations of means (STD).

Cumulative UV doses (Fig. 5) showed a decrease in relative survival rates to 20% or less around 60 mJ.cm^{-2} before reaching 0% beyond 240 mJ.cm^{-2}.

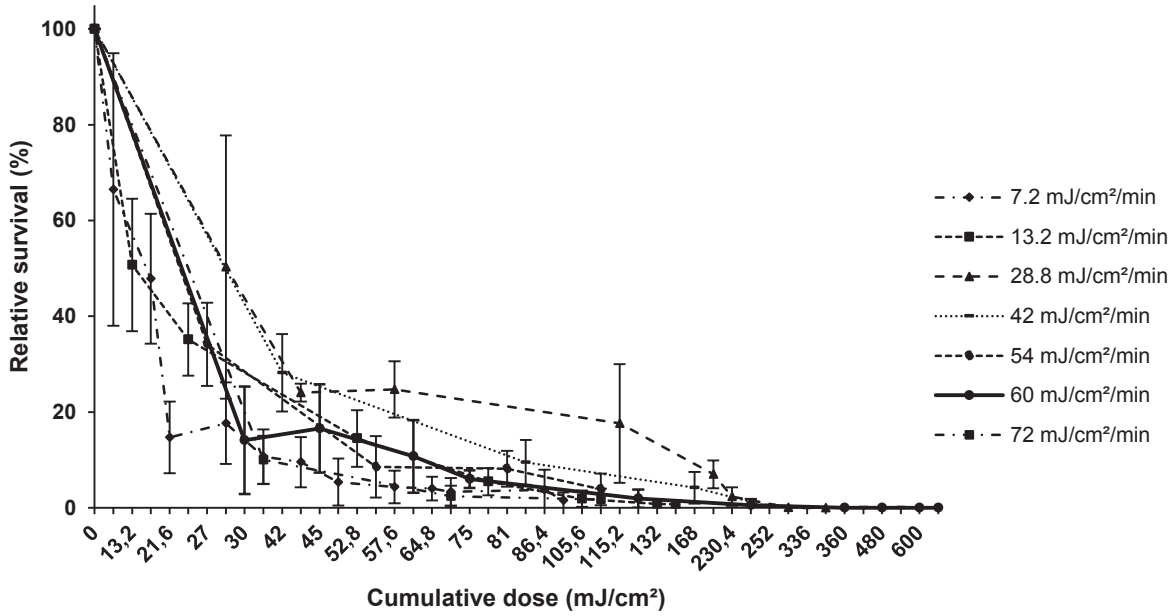


Figure 5. Percent survival relative to controls of treated groups exposed to different cumulative UV-doses in the range 7.2-720 mJ.cm^{-2}. Error bars represent standard deviations of means (STD).

3.2. Ploidy analysis

Flow-cytometry analyses showed that only one UV treatment (60 mJ.cm^{-2}.min^{-1} for 1 min) resulted in a small percentage (14%) of haploid larvae at hatching (Fig. 6). Overall, this corresponded to 3 haploids out of 21 hatched larvae and a yield of 1.4%. The analyses revealed that all other UV-treatments were ineffective at inactivating the maternal genome, yielding diploid larvae only.

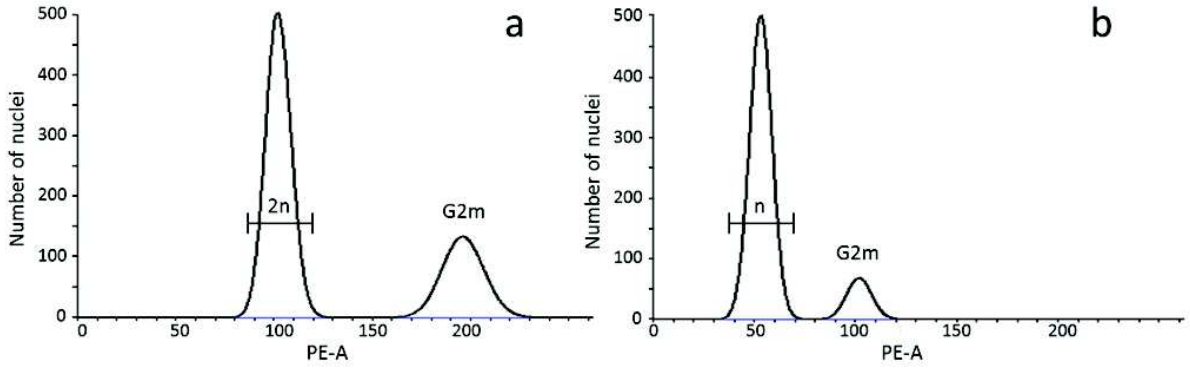


Figure 6. Representative examples of flow-cytometry histograms obtained from nuclear suspensions (5-10,000 counts) of Propidium Iodide (PI) stained larval samples. a) control diploid (2n) larva (CV: 5%); b) haploid (n) larva produced with a UV-dose of 60 mJ.cm^{-2} (CV: 10%). DNA values on the X-axis are reported in arbitrary units expressed as fluorescent channel numbers (PE-A). G2 represent mitotic peaks.

UV irradiation at nearly all doses generated a wide range of deformities, including variable proportions of abnormal embryos and larvae which were morphologically similar to haploids. Typical 'haploid syndrome' malformations included short, twisted or large bodies, curved tail, microphtalmy and microcephaly as illustrated in Fig. 7.

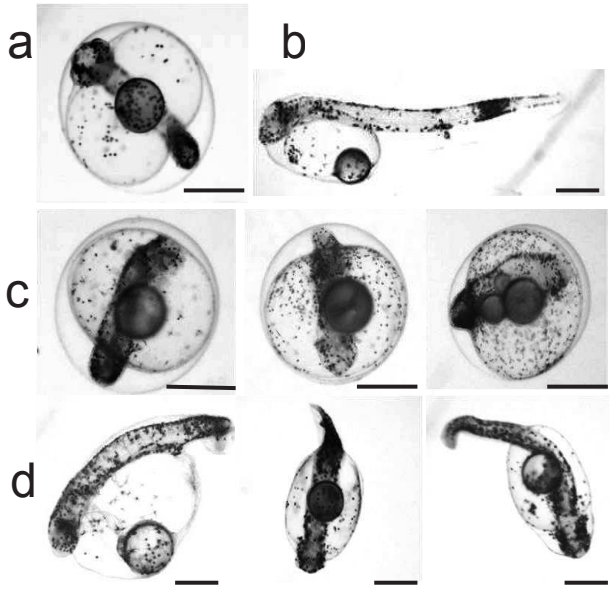


Figure 7. Morphology of control and UV-treated embryos and larvae. a) control embryo at 74h pf; b) control larva at hatching; c) UV treated embryos at 74 hpf showing microcephaly, short and large body; d) UV-treated larvae at hatching showing microphtalmy, short body and curved tail. Scale bars represent 500 µm.

3.3. Microsatellite analyses

The genetic analyses of the three progeny groups exhibited different types of microsatellite inheritance (Table 4). The progeny group A1 (FAxM1) showed 43 individuals inheriting both paternal and maternal alleles for the nine microsatellite markers analyzed and a single larva displaying exclusively the paternal allele at one locus only. Progeny group B1 (FBxM1) contained 44 larvae with both paternal and maternal inheritance, two larvae showing maternal contribution at one locus and one larva with only paternal alleles for each marker. The last of these was concluded to be an androgenetic haploid. Female C (FC) showed a null (non-amplifying) allele which was detected after analyzing the segregation profile of *Labrax-29* in its progeny, under the assumption of Hardy-Weinberg equilibrium in the transmission of alleles. Progeny group C2 (FCxM2) showed 76 larvae having inherited paternal and maternal alleles at all markers, one individual showing only paternal inheritance at one marker (*Dla-22*), one individual showing only paternal inheritance for at least five markers and one individual showed an unexpected genotype for *Labrax-29*, displaying both paternal alleles at this locus.

Table 4. Microsatellite marker loci transmission in three putative androgenetic progenies (A1, B1 and C2) produced with a UV-dose of 60 mJ.cm^{-2}. Genotypes of progenies showing only discriminant paternal alleles are presented in bold characters. For putative androgenetic progenies, homozygous or haploid alleles are only written once since genotyping cannot distinguish between the presence of one or two copies of the same allele. N represents the number of analyzed individuals in each progeny.

Fish	**N**	**Marker loci**								
		Dla-22	*Labrax-17*	*Labrax-29*	*Labrax-3*	*Dla-3*	*Dla-16*	*Dla-105*	*Dla-119*	*Labrax-8*
Female A		252/254	116/132	137/155	172/174	210/218	243/253	151/173	235/235	198/198
Male 1		230/254	116/138	133/159	136/190	216/218	237/245	157/171	225/257	198/232
Progeny A1	1	230/252	132/138	137/159	174/190	218/218	237/243	151/157	**257**	198/198
	43	bi-parental contribution at all loci								
Female B		248/252	116/134	131/155	130/176	216/228	239/241	137/145	227/257	212/212
Male 1		230/254	116/138	133/159	136/190	216/218	237/245	157/171	225/257	198/232
Progeny B1	1	**230**	116	**133**	**136**	**218**	**237**	**171**	**225**	**198**
	1	**230**	**138**	**159**	**190**	216	237/241	**157**	257	**232**
	1	**230**	116	**159**	**136**	**218**	237/241	**157**	257	**232**
	44	bi-parental contribution at all loci								
Female C		252/258	118/120	137/0	136/170	216/220	247/255	155/167	221/225	212/212
Male 2		236/254	118/138	133/159	116/136	220/226	231/265	157/157	227/235	222/232
Progeny C2	1	252/254	**138**	133/0	136	**226**	247/265	**157**	**235**	**232**
	1	254/258	118/120	**133/159**	136	216/220	231/255	155/157	225/227	212/222
	1	**236**	118/138	159/0	116/136	216/220	255/265	157/167	221/235	212/232
	76	bi-parental contribution at all loci								

3.4. Characterization of potential UV screening compounds

Spectrophotometry results showed the same wavelength scan curve for egg extracts from unirradiated controls and all combinations of UV dose rates and durations. The absorbance profiles covered the entire UV spectrum, with peaks of absorption typically around 285 nm and 269.5 nm at pH=8 and pH=3, respectively. A representative absorbance profile of egg extracts from the control group is shown in Fig. 8.

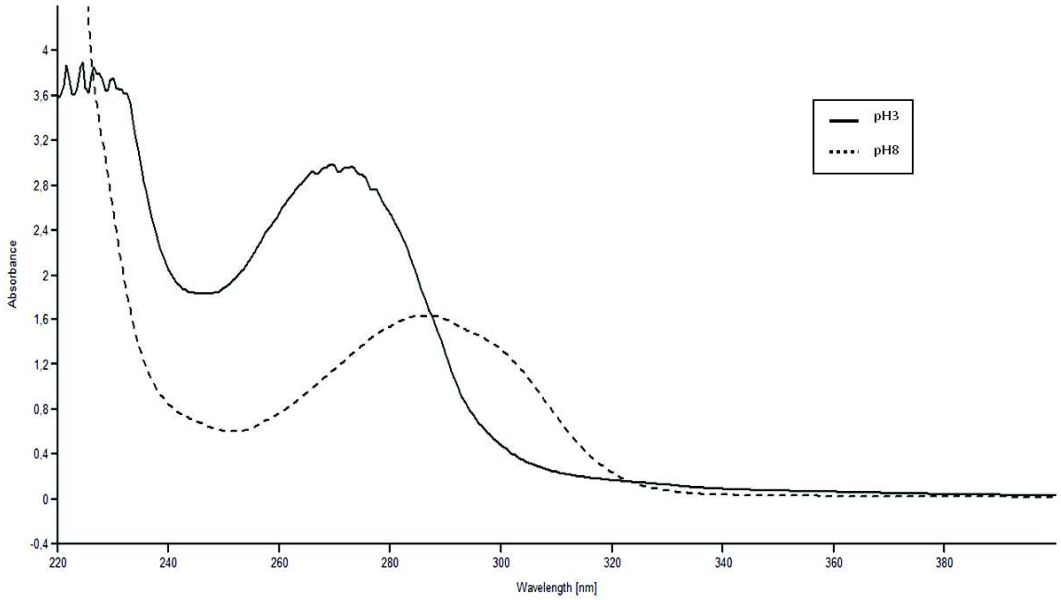


Figure 8. Absorbance spectrum of non-irradiated (control) egg extracts at pH8 (dotted line) and pH3 (solid line). Wavelengths cover almost the entire UV spectrum (UVC: 200-280 nm, UVB: 280-315 nm, and UVA: 315-400 nm). Absorption is shown as arbitrary units.

Gadusol, formula 5,6-Trihydroxy-5-(hydroxymethyl)-2-methoxy-2-cyclohexen-1-one (Supplementary file S3), was found in the sample isolated from sea bass eggs at retention time (RT) 4 min, 203.0563 Da, using mobile phase B and the negative ionization mode (Supplementary file S4).

4. Discussion

The present work indicated that UV-irradiation was largely ineffective at inactivating the maternal genome in European sea bass eggs. Despite the wide range of UV doses employed (7.2-720 mJ.cm^{-2}), only a small percentage of haploid androgenetics was produced at one of the doses tested. The different treatments covered UV dosages proven to be successful in freshwater species. For example, a UV dose of 45 mJ.cm^{-2} was effective at inactivating maternal DNA in *O. niloticus* (Karayücel et al., 2002; Myers et al., 1995). In the zebrafish, *D. rerio*, the optimal UV dose to induce haploid androgenesis was 144 mJ.cm^{-2} (Ungar et al., 1998), while in the common carp, *C. carpio*, UV-doses of 250 mJ.cm^{-2} (Bongers et al., 1994) and 175 mJ.cm^{-2} (Tanck et al., 2001a) have been successfully employed to inactivate maternal DNA. In the European sea bass, a marine species, the only dose that led to small proportions of verified haploids was 60 mJ.cm^{-2} and the androgenetic status of progenies produced at this dosage was tested using flow cytometry and DNA markers. Differential susceptibility to UV-irradiation among fish species may be attributed to

dissimilarities in the thickness, composition and optic qualities of the egg chorion. Other factors may include differences in egg size and shape, and the relative position of the female pronucleus, making it difficult to compare egg irradiation treatments across species (Myers et al., 1995). Different methods have been employed to achieve uniform UV treatment including manual or mechanical stirring of eggs kept in ovarian or synthetic fluids during irradiation from single or multiple UV-sources. In this work, we employed double UV sources (below and above) along with mechanical rotation of the eggs in order to maximize the efficiency of the treatment. The suitability of this purpose-built UV device was tested using the eggs of Nile tilapia as a positive control and haploid larvae were produced (Supplementary files S5 and S6) according to previously reported results in this species (Karayücel et al., 2002; Myers et al., 1995).

In attempts at androgenesis in other species, the use of γ-rays and X-rays led to the typical 'Hertwig effect' and such paradoxical recovery in survival rates at high irradiation doses employing UV-rays was described in the Tiger barb *Puntius tetrazona* (Kirankumar and Pandian, 2003) and the common carp (Bongers et al., 1994; Myers et al., 1995). In other species, survival rates decreased with increasing UV intensities (Arai et al., 1992; Christopher et al., 2012; David and Pandian, 2006a; Lin and Dabrowski, 1998). The genotyping results highlighted some degree of variability in the response of eggs to UV treatment and corresponding androgenetic yield, possibly ascribed to egg quality factors. Myers et al. (1995) showed evidence of female differential susceptibility to UV treatment affecting the yield of androgenetic haploids, but the mechanisms involved were not identified.

In the present study, sea bass embryos and larvae from UV-irradiated eggs possibly suffered partial denaturation of maternal genome and showed impaired development with a range of deformities similar to haploid syndrome. Similar results were observed in other species (Arai et al., 1992; Christopher et al., 2012; Fujimoto et al., 2007; Ungar et al., 1998) where larvae showed severe abnormalities like dwarfing, microcephaly, micropthalmy in most UV-treatments, even at low UV doses. In this experiment, flow cytometry and genotyping were employed to assess the ploidy status and genetically characterize hatched larvae derived from irradiated eggs. At the most efficient dose (60 $mJ.cm^{-2}$), genotyping showed a vast majority of larvae with both paternal and maternal inheritance (biparental diploids), and only one larva (progeny B) with pure paternal inheritance (androgenetic haploid) and various levels of maternal inheritance in the remaining ones. A few larvae showed only paternal inheritance for one or a few markers (progeny A1 and C2): these individuals may have been aneuploids (near diploids) lacking one or a few chromosomes or fragments. In progeny B1, conversely, two larvae showed biparental inheritance for one or a few markers: these individuals may have been aneuploids, near haploids. These kinds of individuals could not be distinguished from real haploids or diploids using flow-cytometry (which did not show any sign of aneuploidy). Chromosome analyses are generally described as superior to flow cytometric methods because chromosome fragments and single chromosomal aneuploidy can be unambiguously detected (Fujimoto et al., 2007). However, in most studies dealing with haploid androgenesis, aneuploidy was observed for low UV doses which were inefficient at inactivating the maternal nuclear DNA. Also, the frequency of

chromosome fragments and aneuploids decreased with increasing UV doses and only haploids were produced once the efficient UV dose was reached or exceeded (Arai et al., 1992; Christopher et al., 2012; Fujimoto et al., 2007).

The individual possessing both paternal alleles at one marker (*Labrax-29*) could have resulted from fertilization with an unreduced spermatozoon involving a single chromosome on which the heterozygous locus was located (LG28), the remaining microsatellite marker loci belonging to different linkage groups (Chistiakov et al., 2005). Although this remains a rare phenomenon, a small percentage (up to 1.6%) of aneuploid sperm has been previously reported for rainbow trout (Brown et al., 2008).

Several hypotheses can be put forward to explain the low success in inducing haploid androgenesis in the European sea bass. The first is the expression of recessive paternal alleles inducing high mortality at the homozygous state. Recessive mutations have been demonstrated to be one of the explanations for low survival rates of androgenetic and gynogenetic progenies in a number of species. Ungar et al. (1998) showed that UV-irradiation of the maternal genome in zebrafish eggs uncovered recessive paternal mutations at the *gol* and *oep* loci at high frequency. Bertotto et al. (2005) found one marker allele transmitted with a significantly lower frequency than the other in a mitotic gynogenetic progeny of *D. labrax*, suggesting a linkage to a deleterious gene. Another possibility for the low haploid yield in our work is impaired development and mortality due to the presence of maternal chromosome fragments. Chromosome fragments, probably of maternal origin, are considered to be a consequence of suboptimal UV treatment conditions and are more frequently reported in androgenetic than in gynogenetic progenies (Fujimoto et al., 2007). For example, interference of maternal DNA residues (participation in mitotic divisions) could be one reason for the poor viability of androgenetic muskellunge (*Esox masquinongy*) (Lin and Dabrowski, 1998) and loach (*Misgurnus anguillicaudatus*) (Arai et al., 1992). The presence of DNA fragments has been suggested as possible cause of the residual heterozygosity observed in diploid androgenetics of common carp although the maternal origin of these fragments could not be proved beyond doubt (Tanck et al., 2001a).

Another hypothesis for the low yield of haploid androgenetics based on the findings of the present study is the possible presence of some defense mechanisms against UV-irradiation in sea bass eggs. Screening compounds are known to provide a first line of defense in fish eggs (Cockell and Knowland, 1999; Sinha et al., 2007) while active DNA repair processes may be used by eggs to deal with damage caused by UV (Sinha and Hader, 2002). Photoreactivation and dark repair pathways are known processes for fixing or replacing UV-damaged DNA. In order to prevent activation of DNA-repairing mechanisms under the influence of visible light in the laboratory, the egg irradiation procedures are commonly completed under total darkness. In our case, the application of dark conditions during egg irradiation and early incubation should have prevented the possibility of light-dependent mechanisms being activated. Nevertheless, as these mechanisms can never be 100% efficient, many organisms naturally exposed to UV radiation for parts of their life-cycle can passively screen UV radiation to prevent its potential damage in the first place (Cockell and Knowland, 1999). In fish, UV-screening compounds such as gadusol and related mycosporine-like amino

acids (MAAs) are found in the eggs of Atlantic cod and other marine teleost (Arbeloa et al., 2010; Grant et al., 1980; Plack et al., 1981). In particular, gadusol shows strong absorption towards the UV-B and UV-C spectrum with pH-dependent distinctive maxima: λ_{max} (H_2O, pH<2)/nm 269 ($\varepsilon/dm^3\ mol^{-1}\ cm^{-1}$ 12400) and 296 (21800) at pH>7 (Arbeloa et al., 2011; Plack et al., 1981). Differential absorption pH-dependent was also observed in our experiment and HPLC characterization confirmed the presence of gadusol in sea bass eggs. European sea bass eggs are small, transparent and UV screening compounds like MMAs are present as observed in other marine fish producing comparable eggs (e.g. Atlantic cod). Based on our attempts at maternal genome inactivation and preliminary assessment of UV absorbance by the extracts of sea bass eggs, this last hypothesis seems plausible. Further work on the comparison of UV absorbance and chemical characterization of putative UV screening compounds like gadusol in the eggs of this and other marine species with those of freshwater species where androgenesis has been successfully reported would allow testing of this hypothesis.

If the eggs of such marine species are protected against UV, then ionizing radiation, although more difficult to work with than UV, might be more effective in successfully inducing haploid (and diploid) androgenesis in the European sea bass and other marine species. A novel method aimed at inducing androgenesis in the eggs of freshwater fish without the use of irradiation was reported by Morishima et al. (2011). These authors succeeded in producing relative high percentages of haploid androgenetic embryos among the survivors of newly fertilized cold-shocked eggs of loach (*M. anguillicaudatus*). The treatment induced the extrusion of the egg pronucleus together with the second polar body, leaving only the sperm pronucleus in the egg. Further work on this 'cold-shock technique' (Hou et al., 2014) focused on the production of androgenetic diploid loach embryos, and yielded approximately 10% diploid androgenetic larvae as well as proportions of haploid, triploid, tetraploid, pentaploid, aneuploid and mosaic larvae. Despite these constraints, the method may represent an alternative to the UV-irradiation of eggs and may be worth exploring for the induction of androgenesis in European sea bass. Another alternative method for the production of androgenetic progenies in the European sea bass could be interspecific androgenesis. The use of egg donors has been attempted in several freshwater species and resulted in varying success. The first successful attempt was the production of androgenetic goldfish (*Carassius auratus auratus*) using common carp (*Cyprinus carpio*) eggs (Bercsenyi et al., 1998). Brown and Thorgaard (2002) reported androgenetic development of rainbow trout (*O. mykiss*) with Yellowstone cutthroat trout (*Oncorhynchus clarki bouvieri* Richardson, 1836) eggs and more recently, androgenetic common tench (*Tinca tinca*) developed from common carp and common bream (*Abramis brama*) eggs (Kucharczyk et al., 2014). Other experiments of interspecific androgenesis between salmonids (Babiak et al., 2002b) and sturgeons (Grunina and Recoubratsky, 2005) led to inviable androgenetic progenies though viable hybrids could be produced. These results suggest that interspecific androgenesis is possible only between closely related species showing similar karyotypical characteristics (Babiak et al., 2002b; Grunina and Recoubratsky, 2005). To avoid nucleocytoplasmic incompatibility, interspecific androgenesis can be achieved using as egg donor a hybrid of the species whose sperm is used for fertilization. Accordingly, viable androgenetic carps were

obtained from eggs derived from the goldfish x carp hybrid females (Recoubratsky and Grunina, 2001), brook charr (*Salvelinus fontinalis*) x Arctic charr (*Salvelinus alpinus*) hybrid eggs were used to induce androgenesis in brook charr and resulted in small percentages of diploid androgenetic larvae (Michalik et al., 2014). Though the nucleocytoplasmic compatibility of European sea bass sperm with eggs from another species in which androgenesis was successful is not granted, this approach could be explored as possible alternative for the induction of androgenesis in sea bass.

Part 4. A survey of UV screening compounds in fish eggs from tropical to arctic latitudes in fresh and marine waters

Part 4: A survey of UV screening compounds in fish eggs from tropical to arctic latitudes in fresh and marine waters.

1. Introduction

The stratospheric ozone layer shields the Earth from biologically damaging solar ultraviolet radiation (UVR), even if several chemicals mainly of anthropogenic origin have depleted the ozone protective layer leading to an increase of harmful UVR in the past decades (Hardy and Gucinski, 1989). According to a recent assessment (WMO-UNEP, 2014), a reduction in the emission of ozone depleting substances by industrialized nations might allow the Earth's protective ozone layer to recover towards the middle of the current century. The distribution of total ozone over the Earth varies strongly with latitude and season, the lowest total ozone is at the equator while the highest is in polar regions with a maximum during spring (Fahey and Hegglin, 2011). Ultraviolet radiation (UVR) consists of three different energetic ranges: UV-A (315-400 nm), more energetic UV-B (280-315 nm) and the most energetic UV-C (200-280 nm). Vacuum UV (< 200 nm) and UV-C do not reach Earth surface because of atmospheric Rayleigh scattering and ozone absorption in addition to UV-B that are also strongly absorbed by ozone, mainly to 300 nm and weakly for higher wavelength (Cockell and Knowland, 1999). Until now, ozone depletion and the increase of UVR at Earth surface has stimulated concern about the effects of increased solar UVR on marine and freshwater ecosystems. UVR seems to be a natural factor varying with latitude but also according to seasons and meteorological events (Engelsen et al., 2004). In the sea, the amount of radiation reaching any given depth depends on the total amount attaining the surface, the degree of sea-surface roughness, and the scattering and absorption within the water column (Hardy and Gucinski, 1989), therefore, clear lakes and oceans in alpine and polar regions may be particularly vulnerable, because of high penetration of UV deep into the water column (Hader et al., 2007). Intertidal and epipelagic organisms are exposed to the highest levels of UVR but even planktonic and benthic organisms living deeper than 20 m sometimes experience strong UVR, depending on water properties (Booth and Morrow, 1997; Cockell and Knowland, 1999; Shick and Dunlap, 2002; Tedetti and Sempere, 2006). In clear ocean water UV-B radiation is reduced to 1% of the surface level at around 28 m depth while in productive coastal water the 1% level may occur at only 1 or 2 m depth (Hardy and Gucinski, 1989). The intensification of UVR at water surface led to concern about the exposure of living aquatic organisms to increased levels of UV light, which is known to affect aquatic ecosystems. UVR have been shown to deleteriously affect reproduction, development, growth and behavior of many aquatic organisms (Häder et al., 1998; Przeslawski et al., 2005) by affecting physiological systems especially by epithelial lesions, free radical formation and DNA damage. The most important cause of UV-induced damage is the modification of DNA that causes mutation, degeneration and cell death (Dahms and Lee, 2010). In fact, cellular damage caused by UVR occur by direct photochemical reaction like the formation of cyclobutane pyrimidine dimers (CPDs) but also via photodynamic process that produce reactive oxygen species (ROS) as singlet oxygen and superoxide radical (Tedetti and

Sempere, 2006). Fish eggs and larval stages are more vulnerable than adults, and development defects can appear in embryos when UVR causes problems in microtubules movements that occur shortly after fertilization and are necessary for axis formation in amphibians and fishes (Epel et al., 1999). Sea urchin embryos (*Paracentrotus lividus* Lamarck, 1816) show major developmental defects after exposition to UV-B, consisting in the failure of skeleton elongation and patterning, and high cellular stress (Bonaventura et al., 2005). UVR is known to cause mortality of embryos and larvae of Atlantic cod (*Gadus morhua* L.), plaice (*Pleuronectes platessa* L.), northern pike (*Esox lucius* L.), northern anchovy (*Engraulis mordax* Girard, 1854), Pacific mackerel (*Trachurus symmetricus* Ayres, 1855) and several other species (Browman et al., 2003; Hakkinen et al., 2004; Vehniäinen, 2005; Vehniainen et al., 2012; Zagarese and Williamson, 2001). Fish species can be differentially susceptible to UVR; for example the rainbow trout (*Oncorhynchus mykiss*) is more tolerant than the common carp (*Cyprinus carpio*) to immunomodulatory effects induced by UV-B exposition, but their skin sensitivity is similar in terms of mucus depletion and appearance of lesions (Markkula, 2009).

As UVR can induce developmental abnormalities, lesions and death, aquatic organisms have developed physiological, morphological and behavioral mechanisms of photoprotection, such as screening compounds, photorepairing, excision repairing and avoidance behaviors. In order to survive spontaneous and induced DNA damage that induce heritable mutations sometimes lethal, organisms have developed DNA repair mechanisms. Specialized repair proteins scan the genome continuously to avoid DNA lesions. Photoreactivation is a well-known repair mechanism based on the enzymatic activity of photolyase, an enzyme that specifically binds to CPDs and 6-4PPs (previously described as the main DNA damages induced by UVR) and reverses the lesion using the energy of light (Sutherland, 1981). Photolyases have been reported in many species in many taxa as bacteria, fungi, plants, invertebrates and many vertebrates (Sinha and Hader, 2002). Dark repair pathways are much more complex than photoreactivation and instead of reversing DNA damage use excision repair mechanisms to replace the damaged DNA with new nucleotides (Hanawalt et al., 1979). Two major categories have been described: base excision repair (BER) more common in UV resistant organisms and nucleotide excision repair (NER) observed in most organisms (Sinha and Hader, 2002). As one of the simplest ways to protect from UV damages is to avoid UVR and repair mechanisms are not 100% efficient, fish have developed behaviors to avoid UVR for their whole life cycle or during critical life stages. The reproductive behavior of some fish species is adapted to protect the sensitive larvae, and several fish species spawn in gravel or in vegetation or even during the night. The yellow perch (*Perca flavescens*), for example, spawns at different depths according to solar exposition: thus, in lakes where UV radiation can be damaging, the yellow perch spawns at greater depths (Huff et al., 2004). The kind of substrate also has its importance and a study carried out in Lake Geneva on pike (*Esox lucius*) showed that its favorite substrate was spruce branches (Gillet and Dubois, 1995). Some species like siluroid and tilapia species are mouthbreeder fish that keep the developing embryos in their oral cavity until and beyond hatching time (Kunz, 2004), protecting them from UV radiation but also from predators. Sunscreen compounds that are photoinhibitive, such as carotenoids, mycosporine-like amino

acids (MAAs), and antioxidants protect against UVR damage. MAAs are not pigments: they are transparent to visible light and have high molar absorptivity mainly for UV-A and UV-B. MAAs are predominantly superficially located in multicellular organisms and show a homogenous distribution in the cytoplasm in single cells (Shick and Dunlap, 2002). A model developed in planktonic organisms showed that cell size is a very important parameter for the efficiency of photoprotection, the limitations depending on the capacity to accumulate MAAs and the balance between concentration and osmolarity. For cells smaller than 1 µm, it has been demonstrated that sunscreens cannot be used as photoprotective mechanism and that sunscreens have efficiencies comparable to DNA repair pathways only for cells above 200 µm (Garcia-Pichel, 1994). Around 19 MAAs (Fig. 9) have been found in marine organisms and the ability to synthesize or acquire UV-absorbing MAAs is nearly ubiquitous among marine organisms under all latitudes and in almost every kind of aquatic habitat. This near-ubiquity suggests an early origin of MAAs and a functional importance that has been transmitted and retained during evolution (Shick and Dunlap, 2002).

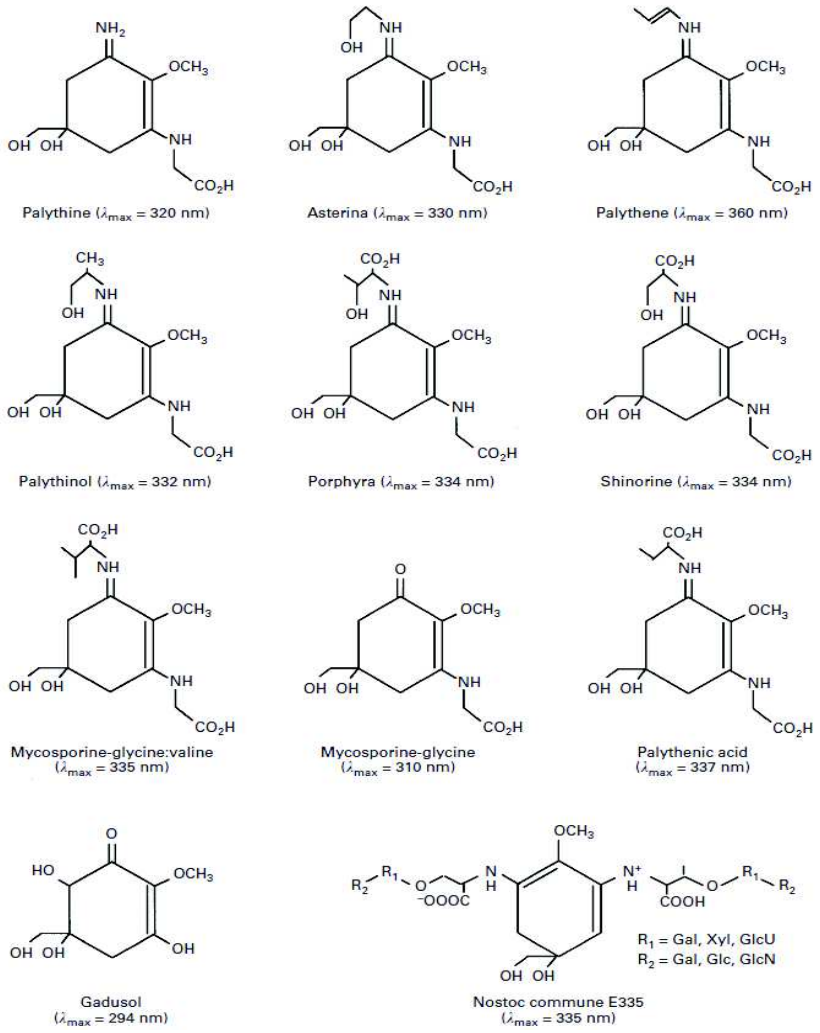


Figure 9. Structure and UV absorption maxima (λ_{max}) of common MAAs found in nature. From: Cockell and Knowland (1999).

The synthesis of MAAs by fish is unlikely as the shikimate acid pathway (the route which links metabolism of carbohydrates to the biosynthesis of aromatic amino acids) is not found in animals (Cockell and Knowland, 1999). MAAs are acquired through diet and accumulate particularly in ovaries and eggs (Chioccara et al., 1980) although MAAs have also been found in the epidermis and ocular tissues of marine fishes (Shick and Dunlap, 2002). The dietary origin of MAAs has been studied in the green sea urchin (*Strongylocentrotus droebachiensis* Müller, 1776) and results showed that higher concentrations of MAAs in the diet resulted in a better protection against UV radiation (Adams and Shick, 1996). In gametes, there is a dichotomy with ovaries and eggs containing the highest concentrations of MAAs whereas testes and sperm only have, at most, trace amounts (Shick and Dunlap, 2002). As previously stated, size may determine this difference: the observed concentrations of MAAs have a biologically relevant effectiveness in relatively large eggs (>150 µm), while in sperm, osmotic constraints in small 2-3 µm cells prevent the sufficient concentration of MAAs for an effective photoprotection (Shick and Dunlap, 2002). Freshwater fishes also contain MAAs in their lenses and skin (Shick and Dunlap, 2002). The UV absorbance properties of MAAs rely on the cyclohexenone or cyclohexenimine core structure (Cockell and Knowland, 1999). This property is based on the π-electron system, which is one of the most effective UV radiation absorbers and is the most widely represented chemical bond system that absorbs in the UV range (Cockell, 1998). Most of the MAAs containing a cyclohexanone structure absorb in the UVB range while UVA screening compounds contain a cyclohexenimine core structure (Cockell and Knowland, 1999). Another important property of a UV-screen is its capacity to dissipate the absorbed energy in order to protect sensitive biomolecules and avoid any oxidative stress. UV-screening and antioxidant properties are thus very closely related. Works on UV dissipation by MAAs showed the absence of free radical formation and a lack of fluorescence that were consistent with a high efficiency to thermally dissipate the absorbed energy (Shick and Dunlap, 2002). Gadusol (Fig. 9) is structurally related to the MAAs and 4-deoxygadusol (4-DG) is presumed to be the immediate precursor of MAAs through the shikimate acid pathway (Shick and Dunlap, 2002). Gadusols absorb strongly towards the UV-B and UV-C zones with two pH-dependent distinctive maxima: λ_{max} at pH2 is 269 nm and 296 nm at pH>7 (Grant et al., 1980). It has been found in the roes of cod and other marine fish (Chioccara et al., 1980; Grant et al., 1980; Plack et al., 1981). The concentration in cod eggs has been quantified 4 mg/g dry weight. Similar amounts have been found in the eggs of haddock (*Melanogrammus aeglefinus* L.), common dab (*Limanda limanda* L.) and flounder (*Platichthys flesus* L.) while smaller amounts have been recorded in long rough dab (*Hippoglossoides platessoides* Fabricius, 1780), plaice (*Pleuronectes platessa*) and lemon sole (*Microstomus kitt* Walbaum, 1792) (Grant et al., 1980; Plack et al., 1981). Its origin is presumably dietary but either gadusol or a precursor must be relatively common in many marine species and along the food chain since cod roes from different sea areas contained similar concentrations of gadusol and other fish species consuming different organisms also contained gadusol in their roes (Chioccara et al., 1980; Plack et al., 1981).

Previous attempts at inactivating the maternal genome of eggs using UV irradiation (UV-C) were generally successful in a number of freshwater species (see review Part 2) while largely failed in the eggs of a marine species like the European sea bass, a marine species

(Colléter et al., 2014). The presence of photoprotection mechanisms in these pelagic marine eggs was proposed as a possible explanation and gadusol was found in UV-irradiated and control eggs. The purpose of the following study is twofold. Firstly, it aims to investigate the possible occurrence of UV-screening compounds like gadusol or structurally-related MAAs in six species of fish where successful UV-inactivation of the maternal genome has been achieved in the past, comparing the results with those obtained in European sea bass eggs. Secondly, its aim is to extend the analysis of UV absorbing compounds and preliminary characterization to other freshwater and marine fish species from tropical to northern regions possessing different reproductive behaviors and egg characteristics. To our best knowledge, this is the first comparison of the UV absorbance characteristics of eggs from species across such a wide range of latitudes and under varying UVR regimes. The results are analyzed relative to the geographical distribution, phylogeny, egg characteristics and lifestyles of the different fish species.

2. Material and methods

2.1. Fish species

Eggs of 21 fish species (summarized in table 5) were sampled and analyzed through spectrophotometry. Unfertilized eggs were diluted 1:10 in 96% ethanol and stored refrigerated until analysis.

Table 5. Summary of the egg characteristics of the 21 species of fish analyzed.

Common name	Scientific name	Eggs characteristics	Ref
Order Salmoniformes			
Atlantic salmon	*Salmo salar*	Medium demersal eggs spawned in nests on gravels	(Fleming et al., 1996)
Brown trout	*Salmo trutta*	Medium demersal eggs, nests covered with sand and gravels	(Roussel and Bardonnet, 2002; Schlumberger, 2008)
European whitefish	*Coregonus lavaretus* L. (pelagic densely rakered)	Medium demersal adhesive eggs	(Amundsen et al., 2004; Luczynski et al., 1995; Skurdal et al., 1985)
	Coregonus lavaretus (littoral large sparsely rakered)	Medium demersal adhesive eggs spawned on gravels in shallow water	
Order Perciformes			
Ballan wrasse	*Labrus bergylta* (Ascanius, 1767)	Small demersal adhesive eggs deposited on benthic substrata usually in rocky reefs	(D'Arcy et al., 2012; Muncaster et al., 2010)
European sea bass	*Dicentrarchus labrax*	Small pelagic eggs	
Gilthead sea bream	*Sparus aurata* L.	Small pelagic eggs	(Ronnestad et al., 1994)

Nile tilapia	*Oreochromis niloticus*	Medium demersal eggs usually incubated in the mouth by the female	(Bhujel, 2000)
Orbicular batfish	*Platax orbicularis* (Forsskal, 1755)	Small pelagic eggs, spawning usually occurs in the night	(Gasset and Remoissenet, 2012)
Order Cypriniformes			
Bighead carp	*Hypophthalmichthys nobilis* (Richardson, 1845)	Medium bathypelagic eggs deposited among rocks of rapids, behind sandbars	(Jennings, 1988)
Common carp	*Cyprinus carpio*	Small adhesive demersal eggs deposited on vegetation	(Linhart et al., 1995)
Common dace	*Leuciscus leuciscus*	Small demersal eggs deposited on gravels	(Mann and Mills, 1985; Mills, 1981)
Ide	*Leuciscus idus*	Small demersal eggs sticking to stones or weeds in shallow water	(Winter and Fredrich, 2003)
Silver carp	*Hypophthalmichthys molitrix* (Valenciennes, 1844)	Medium bathypelagic eggs	(Esmaeili and Johal, 2005)
Tench	*Tinca tinca*	Small demersal adhesive eggs	(Linhart et al., 2000)
Zebrafish	*Danio rerio*	Small demersal eggs	(Eaton and Farley, 1974)
Order Osmeriformes			
Capelin	*Mallotus villosus* (Müller, 1776)	Small demersal adhesive eggs deposited on gravels mixed with the spawn or intertidal in sand beaches	(Nakashima and Wheeler, 2002; Sætre and Gjøsæter, 1975)
Order Gadiformes			
Atlantic cod	*Gadus morhua*	Small pelagic eggs	(MacKenzie et al., 2000; Sundnes et al., 1965)
Order Clupeiformes			
Herring	*Clupea harengus* L.	Small demersal adhesive eggs	(Holleland and Fyhn, 1986)
Order Pleuronectiformes			
Long rough dab	*Hippoglossoides platessoides*	Medium pelagic eggs	(Walsh, 1994)
Plaice	*Pleuronectes platessa*	Small pelagic eggs	(Coombs et al., 1990)
Order Scorpaeniformes			
Lumpsucker	*Cyclopterus lumpus* L.	Small demersal adhesive eggs, usually spawned in nests guarded by the male	(Goulet et al., 1986)

The different samples collected originated from wild or cultured stocks from different geographical origin:

- Brown trout and European whitefish (2 morphs): wild origin, postglacial lakes of northern Norway
- Atlantic salmon, Atlantic cod: cultured, Norway
- Ballan wrasse, capelin, herring, long-rough dab, plaice, lumpsucker: wild origin, Norwegian sea
- European sea bass, gilthead sea bream: cultured, France
- Orbicular batfish: cultured, French Polynesia
- Nile tilapia, zebrafish: cultured, United Kingdom
- Bighead carp, common carp, silver carp: cultured, Czech republic
- Common dace, ide, tench: cultured, Poland.

2.2. Spectrophotometric analyses

Egg extracts from ethanol-stored samples were centrifuged at 2000 rpm for 4 min with a relative centrifugal force (RCF) of 1945 and 1 ml of the supernatant was scanned at wavelengths of 200 to 700 nm using a Hitachi U-2900 Double Beam Spectrophotometer (Hitachi High Technologies Corporation, Tokyo, Japan). Quartz cuvettes with 1 cm light path were used throughout the analyses. Absorption spectra of blank samples (96% ethanol) were verified at all times but not plotted.

To characterize more precisely the absorbance profiles of egg extracts and the compounds involved, a second spectrophotometric analysis was performed. Egg extracts from ethanol-stored samples were centrifuged at 2500 rpm for 5 min with a relative centrifugal force (RCF) of 1945 and 150 µl of the supernatant was diluted 1:2 in 96% ethanol and scanned at wavelengths of 230 to 700 nm using a SpectraMax i3 Multi-mode Detection Platform (Molecular Devices, Sunnyvale, CA, USA), and a FilterMaxF5 Multi-mode Microplate Reader with filters at 260, 340 and 405 nm.

3. Results

3.1. Comparison of species where UV-rays were employed to inactivate the maternal genome of eggs

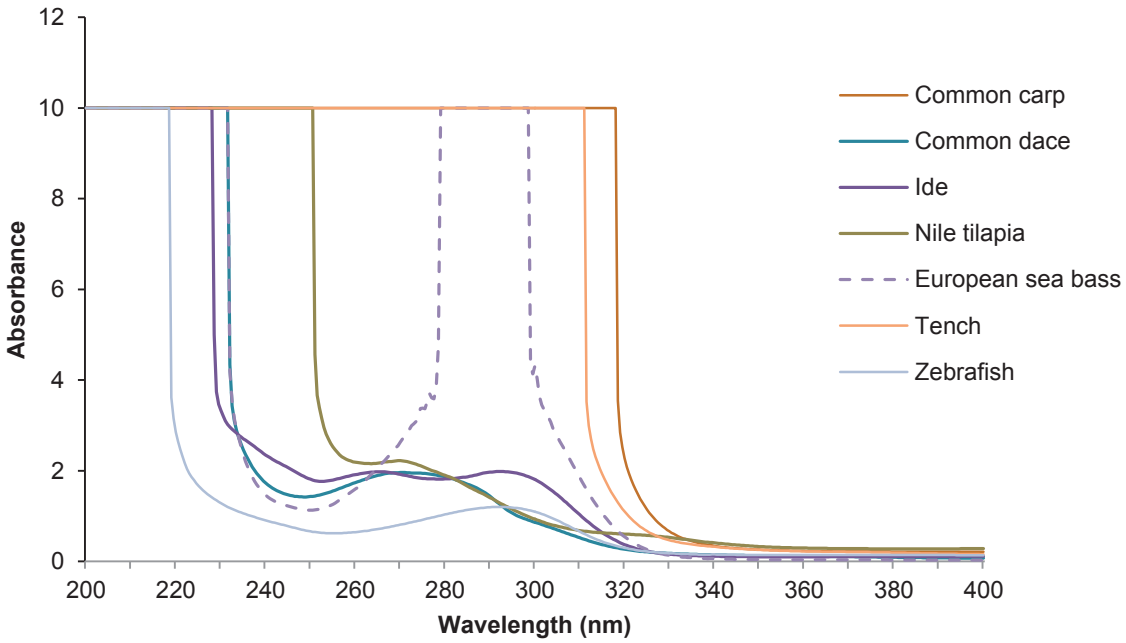


Figure 10. Absorbance profile in the UV range (200-400 nm) of egg samples from n=7 fish species where UV-rays were employed to inactivate the maternal genome. Solid lines are used for species where UV-rays successfully inactivated nuclear maternal DNA and dashed line is used for European sea bass where UV-rays were inefficient at inactivating maternal genome. Absorption is shown in arbitrary unit.

Absorbance profiles in the UV range (200-400 nm) of ethanol egg samples, presented in Fig. 10, showed low absorbance for 4 out of the 6 species analyzed, whose maternal DNA could be inactivated using UV rays. The common carp and common dace egg samples showed high absorbance, similar to the European sea bass, up to 310-320 nm. No species showed null absorbance in the range 270-300 nm, where gadusol was previously reported in the European sea bass, but the common dace, ide, Nile tilapia and zebrafish exhibited low peaks compared to the other species.

3.2. Comparison by lifestyles in marine and freshwater fish species

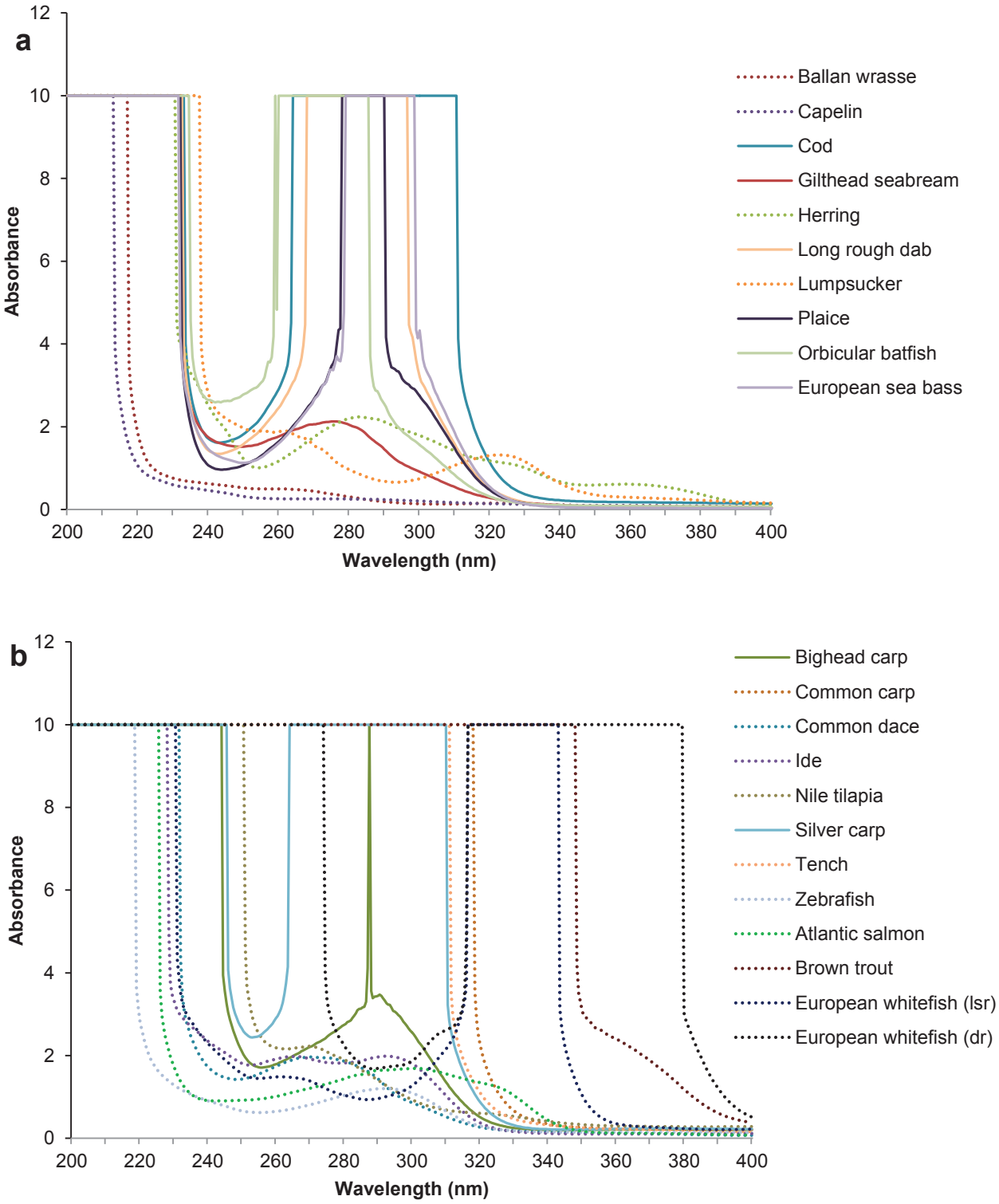
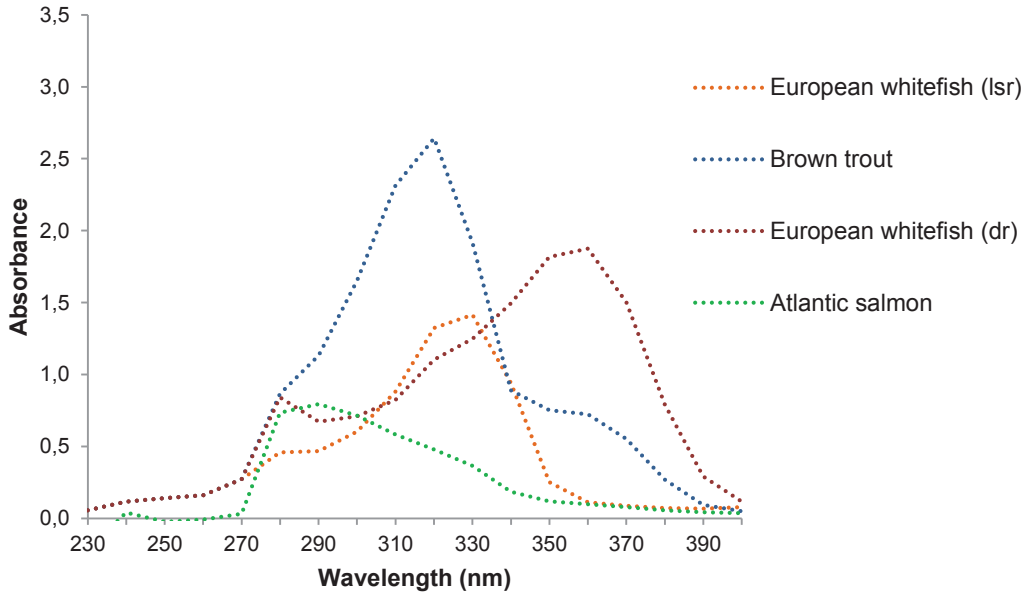


Figure 11. Absorbance profiles in the UV range (200-400 nm) of egg samples from a) n=10 marine and b) n=11 freshwater fish species. Solid lines are used for fish species with pelagic eggs and small dotted lines for species with benthic eggs. Absorption is shown in arbitrary unit.

Absorbance profiles in the UV range of ethanol egg samples of n=21 fish species classified by marine or freshwater lifestyles are presented in Fig. 11. Marine fish showed a clear dichotomy based on pelagic/benthic eggs with the exception of the gilthead sea bream. Apart for this latter egg sample, pelagic eggs showed a high absorbance in the range 260-310 nm while benthic eggs showed low absorbance profiles within the same range. The ballan wrasse and capelin showed absorbance close to 0 while the herring and the lumpsucker egg samples exhibited low absorption at around 280 nm and 330 nm, respectively. Absorbance profiles of freshwater egg samples could not be related to a pelagic/benthic lifestyle from these results. Some pelagic egg samples showed a high absorbance, especially at 285 nm (see the bighead carp), and benthic eggs showed low absorbance profiles in the range 260-310 nm, whereas the tench and the common carp benthic eggs exhibited a high absorbance profile, similar to pelagic egg species.

3.3. Spectrophometric characterization of compounds by phylogenetic classification of fish species

a



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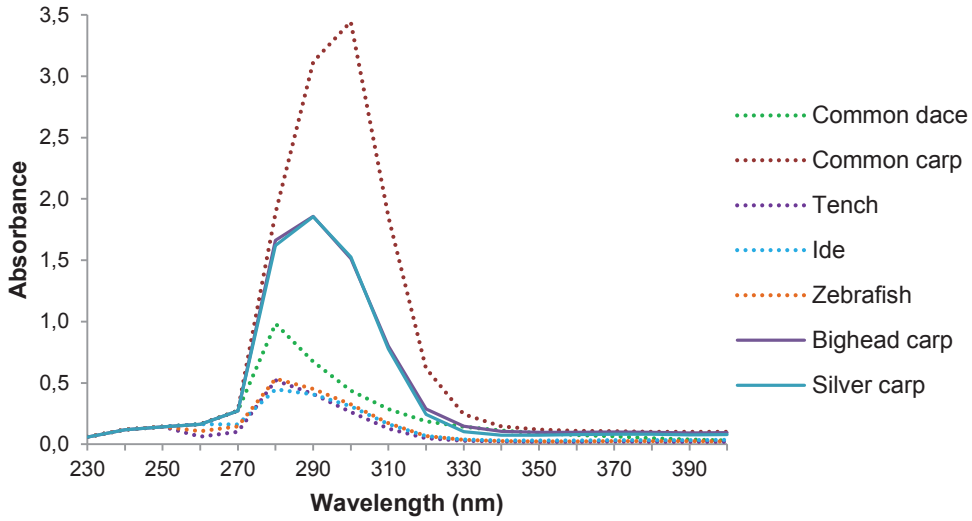
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Wavelength (nm)
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Common carp
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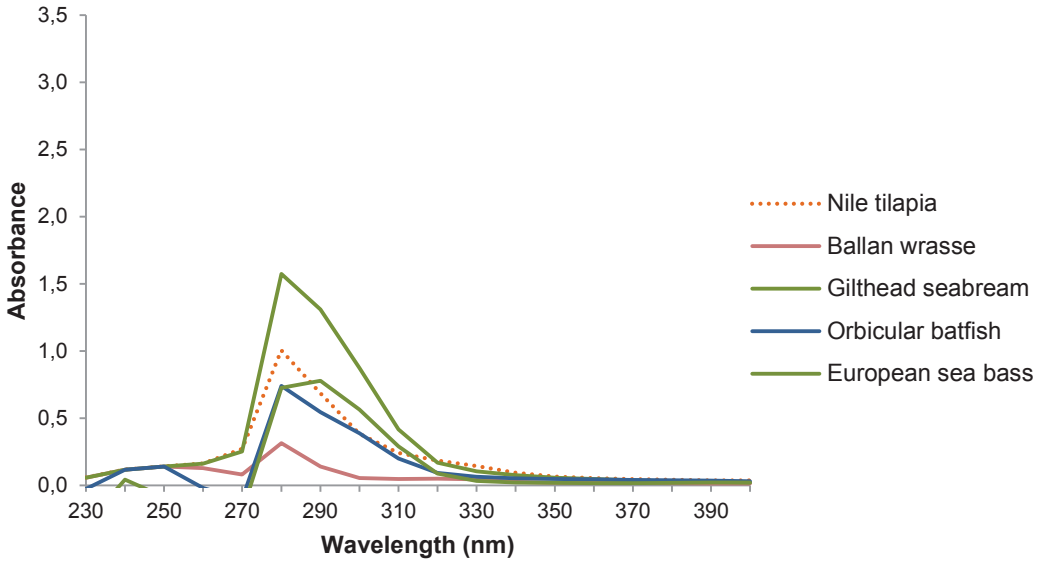
Ide
Zebrafish
Bighead carp
Silver carp

c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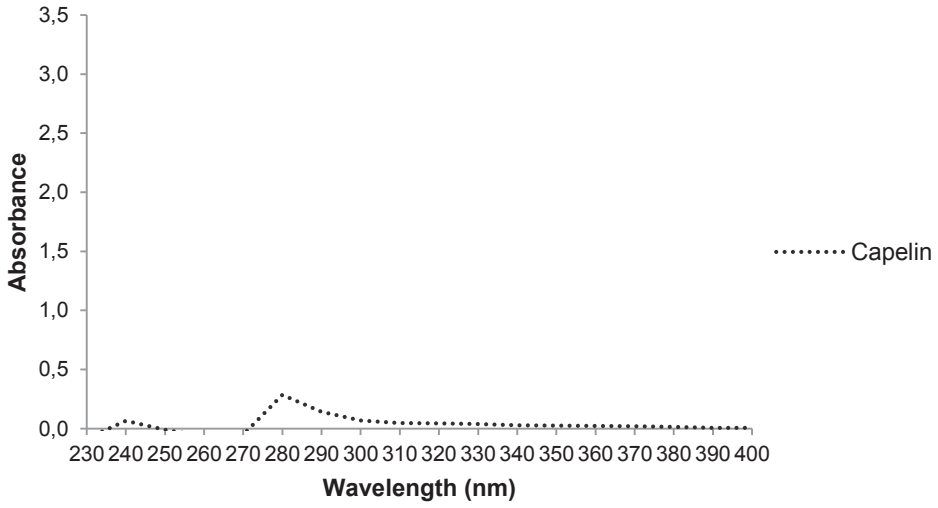
Wavelength (nm)
Nile tilapia
Ballan wrasse
Gilthead seabream
Orbicular batfish
European sea bass

d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Absorbance
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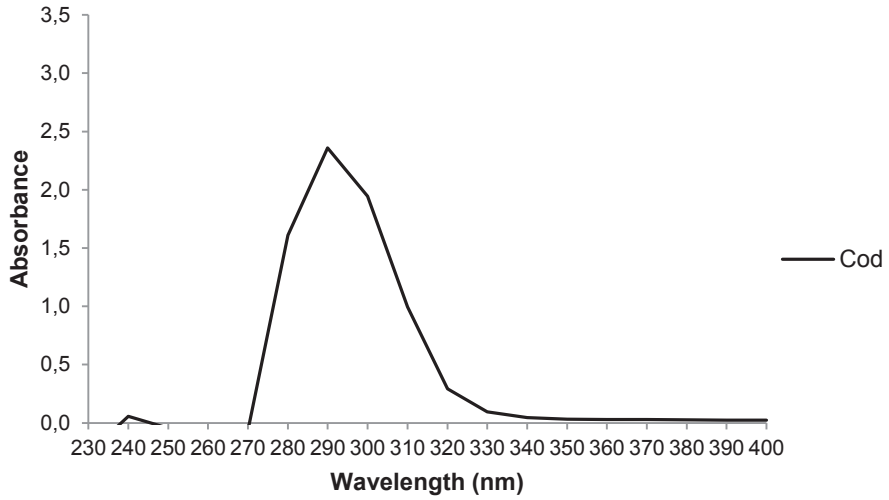
Wavelength (nm)
Capelin

e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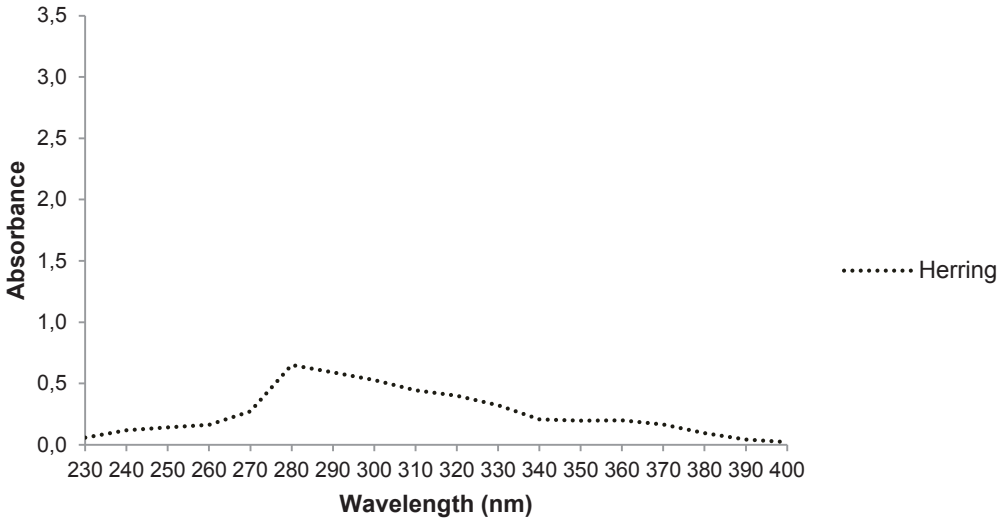
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Absorbance
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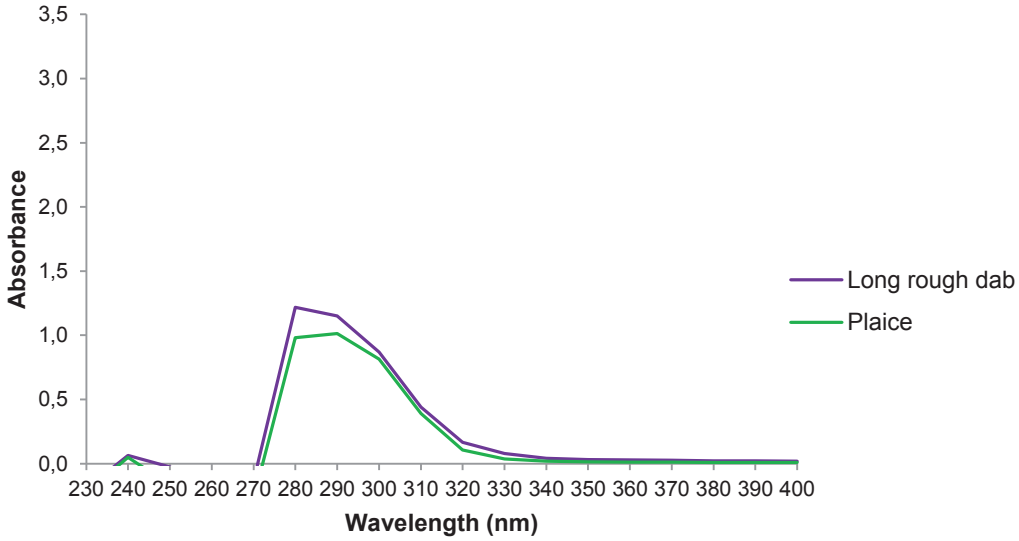
Wavelength (nm)
Herring

g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Absorbance
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Wavelength (nm)
Long rough dab
Plaice

h

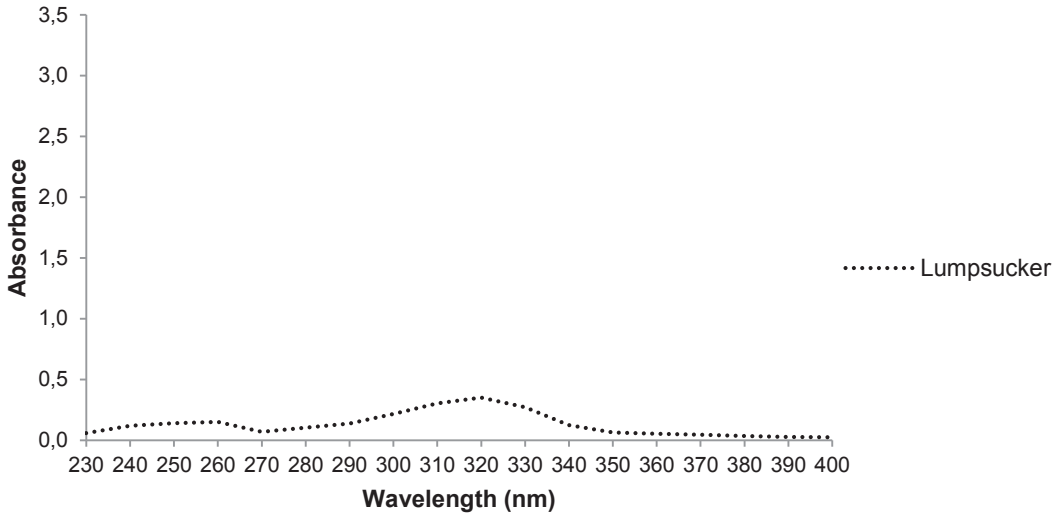


Figure 12. UV-absorbance spectra of egg samples in the range 230-400 nm. Fish species are phylogenetically classified in the Orders a) Salmoniformes, b) Cypriniformes, c) Perciformes, d) Osmeriformes, e) Gadiformes, f) Clupeiformes, g) Pleuronectiformes and h) Scorpaeniformes. Solid lines represent species with pelagic eggs and small dotted lines are for species with benthic eggs. Absorption is shown in arbitrary units.

Figure 12 (a-h) shows the different absorbance spectra obtained in the second spectrophotometric analysis after a classification by taxa and benthic or pelagic egg behavior. Most species showed a peak around 280-290 nm, which is expected to represent gadusol according to results previously obtained in Atlantic cod, European sea bass, long rough dab, and plaice (Colléter et al., 2014; Plack et al., 1981). Only the Order Salmoniformes showed differences compared to the other taxonomic groups analyzed, the main peaks being around 320, 330 and 360 nm. In the Order Scorpaeniformes, though low compared to other species, a peak at 320 nm could be observed. No species showed a null absorption and the complete absence of UV screening compounds in the eggs of some analyzed species cannot be ruled out.

4. Discussion

Gadusol is expected to have a maximal absorption around 296 nm and almost all analyzed species showed a peak in the range 280-290 nm that may be representative of the occurrence of this compound, as previously reported in the eggs and roes from Atlantic cod, European sea bass, long rough dab and plaice (Colléter et al., 2014; Plack et al., 1981). Gadusol or related compounds seemed ubiquitous among the range of species analyzed and although its occurrence resulted in different relative absorbance levels, no clear phylogenetic or behavioral pattern could be observed. Only the Salmoniformes, brown trout and European whitefish, showed some spectral differences and specificity as revealed by the

spectrophotometric analyses. The observed peaks may be representative of different MAAs reported in nature (Cockell and Knowland, 1999) which have been reported to have absorption maxima ranging 310 to 360 nm (see Fig. 10). The differences observed among Salmoniform fish may be due to differences in diet composition, with adult brown trout being largely piscivorous, and the pelagic and littoral European whitefish morphs feeding mainly on zooplankton and on benthic organisms, respectively. In the Order Scorpaeniformes, the lumpsucker eggs may also contain some MAAs as a small peak was observed at around 320 nm. Although no general correlation between egg characteristics and UV absorbance could be inferred from these analyses, some differences between marine and freshwater species could be observed. Nearly all marine species showed a trend towards stronger UV absorption in pelagic than in demersal eggs. Results were more disparate among freshwater species. Gadusol and MAAs may not be related only to a behavioral pattern and some phylogenetic patterns were detected too, with gadusol being almost ubiquitous and some phylogenetic orders showing absorbance spectra which could correspond to different MAAs. The accumulation of specific MAAs in the eggs of demersal Salmoniform fish is surprising as European whitefish eggs analyzed in this work, for example, are spawned in the autumn and do not experience any sun light due to ice-cover during most of their development. The finding of MAAs in the eggs of Salmoniform fish could reflect a remnant adaptation to UVR conditions from the past conserved along evolution. The characterization of egg extracts by high performance liquid chromatography (HPLC) and mass spectrometry (MS) is currently ongoing and should confirm these preliminary results. MAAs have not been studied as extensively in freshwater as in marine species but these compounds have been reported in freshwater cyanobacteria, microalgae, invertebrates and fish (Thorpe et al., 1993). There may be several MAAs unique to freshwater organisms but since limited data are available, the extent of MAA habitat specificity relative to geographic region or marine/freshwater environments is still unknown. The HPLC-MS characterization of our egg samples from 21 species covering a wide range of habitats might provide some clues on the MAA habitat specificity as well as the phylogenetic evolution of MAA type. The discrepancies between the relative levels of absorption between the two spectrophotometric measurements observed for some species could be ascribed to the sensitivities of the instruments employed and should be further investigated. The quantification of the compounds should thus be a major consideration and will be approached also to get information of the relative contents of MAAs and gadusol in different kind of eggs. A previous work quantified gadusol in the roes of some marine fish, and reported mean values of 4.3 $mg.g^{-1}$ dry weight in Atlantic cod, 2.9 $mg.g^{-1}$ dry weight in long rough dab and 3.47 $mg.g^{-1}$ dry weight in plaice (Plack et al., 1981). A work on pigments in fish lens showed that the types of UV-screening compounds and their concentration depend both on the phylogenetic group and the UVR regime of the habitat (Thorpe et al., 1993). A survey led on 46 marine species of molluscs, polychaetes, and one fish species showed that maturity and spawning habitat did not affect MAA composition while adult diet, phylogeny and viability affected MAA composition which varied according to the taxonomic group and occurred in relatively high concentrations in egg masses compared to adult organisms (Przeslawski et al., 2005). Fish may reduce or avoid the exposure of fertilized eggs and embryos to UVR, as these stages are the most sensitive, by adopting nocturnal spawning behaviors that allow early development in darkness, or

spawning gelatinous masses of eggs or spawning in nests hidden from UVR. Consequently, embryos of species that spawn in full sunlight are more tolerant to UVR than embryos from species that spawn in shaded habitat (Przeslawski et al., 2005) but this increased UV tolerance in connection to a reproductive behavior could not be clearly related to the occurrence of UV screening compounds, in the present study, due to specificities observed in some fish species. Häkkinen et al. (2002) showed that UV-B radiations with doses from 137-224 $mJ.cm^{-2}$ had no effect on survival and growth of European whitefish. The authors suspected the melanin pigment to be a sunscreen in this species, however they also showed that the melanin content of European whitefish embryos was half that of vendace (*Coregonus albula*). Considering the results obtained in our spectrophotometric analyses, showing compounds from the European whitefish egg sample absorbing in the UV-B spectrum from the European whitefish egg sample, the tolerance of such eggs to damaging solar radiations may be indeed related to the occurrence of MAAs.

However, the role of gadusol and MAAs in organisms is complex as the synthesis of MAAs seems induced by UVR while gadusol seems more ubiquitous, and these compounds should have biological roles, the UV screening capacity being associated with their chemical structure. A study on the role of UV-B in aquatic and terrestrial ecosystems analyzed the evolution of MAAs in plants in the different ecosystems and showed that, while visible light and long wave UV-A had only a slight effect, UV-B induced the synthesis of MAAs; but the hypothesis that increased damaging radiation could overrule increased synthesis of protective pigments was also pointed out (Rozema et al., 2002). As the shikimate metabolic pathway has not been reported in animals, the origin of MAAs in fish is likely dietary as shown both in freshwater (Mason et al., 1998) and marine (Zamzow, 2004) species. For example, the sea urchin (*Evechinus chloroticus*) gonad and egg MAA content was shown to be correlated with the algae content, itself correlated with the UVR intensities in 2 out of 4 microalgae analyzed (Lamare et al., 2004). Moreover, metabolic pathways for MAA synthesis, conversion and degradation have not yet been completely elucidated and it has been proposed that ingested MAAs could serve as precursors for conversion to gadusol and can be interconverted into different MAAs by animal metabolism (Karentz, 2001). A study on the cleaner fish, *Labroides dimidiatus,* showed that fish are able to maintain and increase MAA content of the mucus in response to UVR from stored sources though the metabolic pathways remain unknown (Zamzow et al., 2013). The role of MAAs in embryos and larvae development is still unknown. Chioccara et al. (1986) showed the occurrence of gadusol in sea urchin eggs (*Paracentrotus lividus* and *Sphaerichinus granularis*) with some concentration differences during embryonic development, its amount increasing after fertilization and decreasing after gastrula stage. Another work on *Artemia* showed that embryonic development resulted in the utilization of MAAs with the subsequent formation of gadusol and suggested that this reaction of degradation of endogenous peroxides might have the function of increasing the pool of free amino-acids available to the embryo (Grant et al., 1985). The evolutionary origin of UV screening compounds is still unknown and presumably many of them evolved for other physiological roles and later adapted to UV-screening function. As examples, the dominant MAA in cod larvae is mycosporine-glycine, a MAA known to have an antioxidant activity

(Dunlap and Yamamoto, 1995; Lesser et al., 2001) and the function of MAAs as osmotic regulators has been detected in some cyanobacteria (Singh et al., 2008).

Overall, our results on UV absorption in the eggs of marine species like European sea bass show that these small pelagic eggs may indeed be protected against damaging radiations to a greater extent than demersal eggs. Nevertheless, when the results are compared with those of freshwater species where androgenesis has been performed achieving the complete inactivation of the maternal genome, it is evident that screening compounds like gadusol may not be solely responsible for the tolerance to strong UV radiations (UV-C) used in the artificial induction of androgenesis in sea bass. Such compounds (gadusol or related MAAs) appear ubiquitous among all the studied species and may provide a first line of defense from ecologically-relevant radiations (UV-A and B) but others egg factors like highly efficient repair mechanisms might be involved and be responsible for the failure at genetic inactivation of the maternal genome in the European sea bass. Photoreparation of DNA was shown to be different according to developmental stage of zebrafish embryos, and furthermore, laboratory-raised embryos were more sensitive to UV-B than those from outdoor ponds though a competent photorepair system was confirmed in both types (Dong et al., 2007; Dong et al., 2008). UV tolerance seems species-specific and several mechanisms are implied in UVR tolerance and damage reparation. The occurrence of MAAs is not the only evidence to conclude on the sensitivity or tolerance of a species to damaging UVR and photoreparation mechanisms should also be analyzed in several fish species from different habitats to conclude on fish adaptations to different UVR regimes. Several methods have been used to detect DNA damage (see review by Sinha and Hader, 2002), but one of the most sensitive to detect UV effects in single cells is comet assay (electrophoretic technique) combined with T4 endonuclease V (an enzyme that specifically repairs UV-induced cyclobutane pyrimidine dimers in DNA). Further analyses on UV-induced damages in eggs from several fish species using this technique could provide information on the effects induced by UVR and the repair mechanisms eventually involved. Furthermore, it could help understanding how fish adapted to UVR: apart from specific reproductive behaviors, the roles of screening compounds and/or efficient DNA repair mechanisms seem crucial and have been highly conserved along evolution. MAAs and gadusol are found in many organisms in many taxa and their role in UV protection has been documented. However, some points still need to be clarified including the possible physiological roles of these compounds, their metabolic pathways in fish, and the evolutionary phylogeny specificities. The antioxidant activity of some MAAs has been reported and could be an interesting way for the development of biological sunscreens and antioxidants by the industry (Singh et al., 2008).

Part 5. Induced mitotic gynogenesis in the European sea bass (*Dicentrarchus labrax* L.) : genetic verification, survival, growth and gonadal development

Part 5: Induced mitotic gynogenesis in the European sea bass (*Dicentrarchus labrax* L.): genetic verification, survival, growth and gonadal development

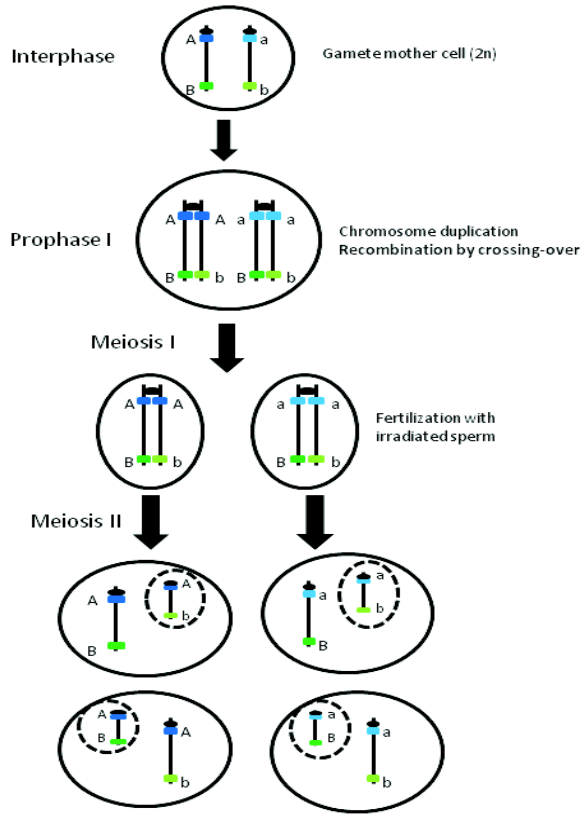
1. Introduction

The European sea bass is a species of commercial interest farmed along the Mediterranean and Atlantic coasts in Europe. As in all farmed species, selective breeding programs are now being developed for a variety of productivity traits (Vandeputte et al., 2009a). While classical breeding appears to be efficient for improving production traits like growth in the European sea bass (Vandeputte et al., 2009b), other traits like sex-ratio (Vandeputte et al., 2007b) or disease resistance appear much more challenging. For those traits with polygenic basis but potentially large QTLs, clonal lines would be a major tool to help dissect their genetic architecture. Fully inbred lines are a unique and invaluable tool in fish research especially in genetic and genomic analyses (Komen and Thorgaard, 2007). Their genetic uniformity allows comparison of the same genotype under different conditions with a better assessment of the origins of variance. They are also a unique tool for the detection of quantitative trait loci and genome mapping. Gynogenesis, the uniparental reproduction in which the nuclear genetic material is of maternal origin only, is achieved by artificial fertilization of eggs with genetically inactivated spermatozoa followed by a physical shock to restore the diploid status. The diploidization step, necessary to obtain viable individuals, can be performed at two different timings: a shock applied a few minutes after fertilization induces the retention of the second polar body and produces meiotic gynogenetic individuals, while a shock applied later suppresses the first cell division and produces mitotic gynogenetic individuals. These latter are doubled haploids (DH) and are expected to be 100% homozygous, each individual being genetically unique and the founder of a specific clonal line, while meiotic gynogenetic individuals exhibit some level of recombination, especially on the parts of the chromosomes which are located distally to the centromere (Purdom, 1993).

Gynogenesis has been achieved in the European sea bass in several previous experiments, and the optimal conditions of sperm UV irradiation and the timing for the diploidizing shock have been described. The conditions for genetic inactivation of spermatozoa while maintaining the ability of the sperm to fertilize eggs have been characterized by several authors with various results depending on the dose used for UV irradiation and the sperm dilution to avoid incomplete inactivation (Felip et al., 1999). Peruzzi and Chatain (2000) showed that the optimal UV dose to inactivate all spermatozoa with the lowest variability in survival was 320 $mJ.cm^{-2}$ with an incident dose of 40 $mJ.cm^{-2}.min^{-1}$ and sperm diluted 1:20, although strong “parental” effects in survival rates were observed. The diploidization process has been applied at different times: physical shocks applied shortly (a few min) after fertilization of eggs using irradiated sperm induced meiotic gynogenesis while late shocks (more than an hour after fertilization) produced mitotic gynogenetic progenies by endomitosis. Temperature shocks (cold and heat) were successfully used to induce the

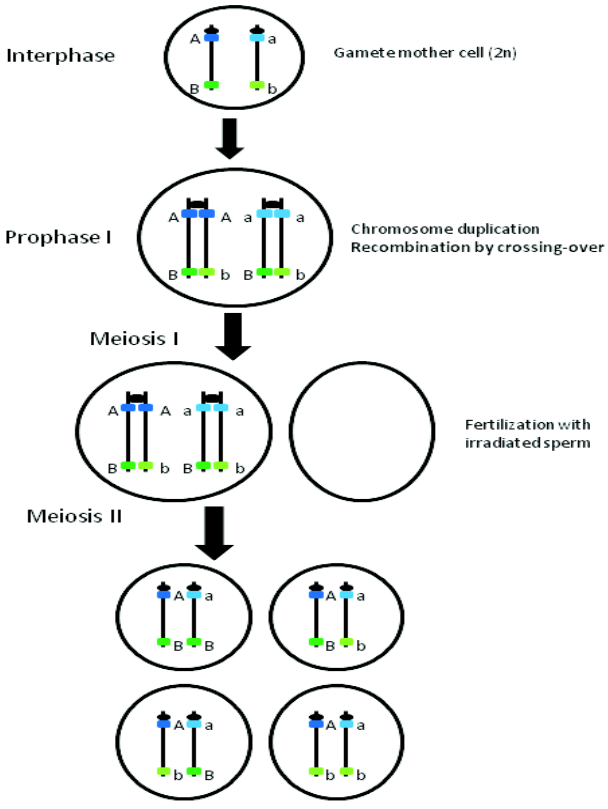
retention of the second polar body though pressure shock was shown to lead to higher survivals (Felip et al., 1998). However, in attempts at inducing mitotic gynogenesis, the success of endomitosis seemed to be more sensitive to the type of shock applied. Cold shocking was ineffective in blocking mitosis for the production of mitotic gynogenetic individuals and the use of a hydrostatic pressure shock was then chosen (Barbaro et al., 1996). Francescon et al. (2004) tried to optimize pressure shock and observed that the timing of first cleavage varied in relation to egg quality. The optimal timing for shocking eggs was around 7/10 of the first cleavage timing and a significant linear relation between shock application time (Y) and first cleavage appearance (X) was found: Y (min) = 3.013 + 0.718X (min). The success rate of DH embryo production and the survival rate at hatching significantly improved when the shock timing was monitored according to the first cleavage furrow appearance. Using this protocol, Bertotto et al. (2005) produced four progenies, of which two were fully homozygous, while two had some residual heterozygosity.

Mitotic gynogenesis is a useful tool for the production of homozygous clones but one of its prerequisite for a successful induction is the complete inactivation of the paternal genome. A contamination of mitotic gynogenetic progenies with biparental individuals leads to residual heterozygosity. These kind of progenies arise from normal eggs fertilised by sperm that escaped genome inactivation and where the pressure shock was ineffective for genome duplication (Bertotto et al., 2005). Another key point in the development of clonal founders is the necessity to distinguish true mitotic gynogenetic from meiotic gynogenetic individuals which produce residual heterozygosity as previously described in the European sea bass and other fish species including the African catfish (*Clarias gariepinus*), the rainbow trout (*Onchorhyncus mykiss*) and the common carp (*Cyprinus carpio*) (Bertotto et al., 2005; Galbusera et al., 2000; Komen et al., 1991; Quillet et al., 1991). Two different hypotheses have been put forward to explain the residual heterozygosity (see Fig. 13). Some authors proposed the inhibition of meiosis I or non-disjunction of chromosomes during this step of gamete development (Quillet et al., 1991) while others supposed a meiosis II non-disjunction origin (Galbusera et al., 2000; Komen et al., 1991) to explain the contamination of gynogenetic heterozygous diploids among mitotic doubled haploid progenies. The genotypes of the progenies produced then have to be assessed with the appropriate markers. As illustrated in Fig.12, alleles A/a of a centromeric locus and alleles B/b of a telomeric locus on a homologous pair of chromosomes will segregate differently and produce different genotypes, according to the different hypotheses: while a meiosis II non-disjunction of chromosomes or the retention of the second polar body will produce progenies with two different genotypes AABb and aaBb, a non-disjunction at meiosis I will produce four different genotypes AaBB, AaBb, AabB and Aabb.



A. Normal allele segregation with one crossing-over during prophase I.

A crossing-over happened during chromatide duplication. Only the locus B/b recombined. The resulting genotypes show heterozygosity and therefore meiotic or mitotic gynogenesis can be distinguished.
Retention of polar body will produce two different genotypes for this chromosome: AABb and aaBb.
Endomitosis will induce four different genotypes: AABB, aaBB, AAbb and aabb. All loci will then be homozygous.
When recombination happens, meiotic and mitotic gynogens will show different genotypes and will be easily detected. The frequency of recombination is proportional to the distance between the locus tested and the centromere.



B. Rare allele segregation with one crossing-over during prophase I but non-disjunction during meiosis I.

During prophase I, only the locus B/b was recombinant as described in fig. A. Non-disjunction during (or inhibition of) meiosis I was followed by a classical meiosis (resumed during meiosis II) that produced four different genotypes: AaBB, AaBb, AabB and Aabb.
This event leads to spontaneous gynogenesis that can be distinguished from mitotic gynogenesis by its heterozygosity. The distinction with meiotic gynogenesis induced by retention of the second polar body is based on the locus A/a heterozygosity. This locus is not supposed to recombine during a crossing-over because of its location close to the centromere and only a rare event like a non-disjunction during meiosis I can induce this kind of heterozygosity.

Figure 13 (previous page). The different chromosomes segregation events taking place before and after egg 'fertilization' (activation) in gynogenesis experiments. Segregation of alleles is only given as an example on one homologous pair of chromosomes. A and a represent two alleles of a locus close to the centromere with a small recombination probability (close to 0). B and b represent two alleles of a locus with a large distance to the centromere and a high recombination frequency (close to 1). Small dashed circles represent the polar body that should be released.

Crossing over (or chiasma) between a gene and its centromere can occur during prophase I of gamete meiosis, leading to some level of recombination between the alleles of homologous chromosomes present in the female parent. In a meiotic gynogenetic progeny, the frequency of heterozygous individuals (recombination rate) at a locus is a measure of crossing over frequency between the centromere and the locus: in the absence of crossing over, the meiotic gynogenetic progeny of a heterozygous female will be homozygous for one allele or the other, whereas in the case of a crossing over between the centromere and the locus in question, the meiotic gynogenetic progeny will be heterozygous. Then, if a marker is heterozygous in a female, the proportion of heterozygotes in its meiotic gynogenetic offspring will be proportional to the distance between the locus and its centromere. On the contrary, mitotic gynogenetic progenies will always be homozygous for one or the other maternal allele for all markers whatever their distance to the centromere (doubled haploids). Suitable markers to distinguish mitotic gynogenetic (mitogens) from meiotic gynogenetic individuals (meiogens) should then be located distally to the centromere to give a high microsatellite locus-centromere recombination frequency and of course have to be heterozygous in the female parent to be informative. It has to be noted that the use of the recombination rate to estimate the microsatellite locus-centromere distance relies on the hypothesis of total interference in fishes, meaning that only one chiasma occurs in any chromosome pair (Ezaz et al., 2004b; Francescon et al., 2005; Purdom, 1993).

The survival of doubled haploids reported in the literature is usually extremely low and the reduction in survival is mainly explained by inbreeding depression (expression of homozygous deleterious mutations) and damage induced by the manipulations (Komen and Thorgaard, 2007; Liu et al., 2012). To produce clonal lines, a second round of gynogenesis is required but a reduced fertility has been often reported in several fish species (Müller-Belecke and Hörstgen-Schwark, 1995; 2000; Quillet, 1994). Clonal founders were previously developed in the European sea bass (Bertotto et al., 2005) but their ability to reproduce has, to our knowledge, never been reported. As mitotic gynogenesis had already been achieved in the European sea bass with relative good yields of mitotic gynogenetic progenies, the aim of this study was to mass-produce clonal founders that could be used later for the development of clonal lines and validate the use of proper microsatellite markers for monitoring of gynogenesis experiments in European sea bass. Gynogenetic and control progenies were produced, communally reared and their genotypic status, growth and sexual characteristics analyzed. This study is, to our knowledge, the first experiment dealing with microsatellite locus-centromere-map distances in the European sea bass and the first to assess growth and gonadal development of progenies produced by mitotic gynogenesis.

2. Material & methods

2.1. Gamete collection, UV-irradiation of sperm, fertilization and incubation

This study was carried on the progenies of 11 dams and 11 males. The broodstock and gametes were handled as described in Colléter et al. (2014). Twenty-two progenies were produced by artificial fertilization of the eggs from each female with either UV-irradiated or unirradiated sperm from one male.

The UV irradiation device was composed of eight UV germicidal lamps (12 W, 254 nm, Vilber-Lourmat, Marne-la-Vallée, France) fixed above and below (four lamps each) a quartz plate gently moved by an orbital agitator throughout irradiation. After a quick motility control, 0.5 ml of sperm were diluted 1:20 in artificial extender SGSS (Seabass Gamete Short term Storage) made of Storefish (IMV Technologies, France) complemented with pyruvate and glutamine at 0.6 and 3 $mg.ml^{-1}$ respectively (C. Fauvel, pers. comm.) and poured into 8.5 cm diameter quartz Petri dishes. The quartz plate and Petri dishes (SARL NH Verre, Puechabon, France) were employed to maximize UV transmission during treatments. The lamps were switched on at least 30 min before the onset of irradiation and UV incident dose was verified at the beginning and at the end of each experiment using a VLX-3W radiometer (Vilber-Lourmat), checking both upper and lower UV sources. The total UV irradiation dose employed was 320 $mJ.cm^{-2}$ (Peruzzi and Chatain, 2000).

Eggs fertilization was performed just after irradiation by adding 5 ml of (1:20) SGSS diluted sperm to an approximate volume of 125 ml eggs and immediately after an equal volume of sea water was added (14°C, 35‰). Control groups consisted of eggs fertilized with unirradiated sperm of the same male, handled and fertilized as above (apart from UV irradiation). All experiments were performed under total darkness in a temperature controlled room maintained at 14°C.

Just after fertilization, egg batches fertilized by irradiated sperm were stored in darkness at 14°C until they were submitted to a diploidizing treatment. A pressure shock of 8500 psi for 4 min was applied at a timing calculated using the equation of Francescon et al. (2004) based on the first cleavage timing, which varied from 99 to 109 min post fertilization (pf).

Control and treated eggs were incubated separately in individual 40 L tanks in a dedicated recirculated water system (temperature: 14-14.5°C; salinity: 35-36‰). All tanks were maintained in darkness during incubation. To characterize embryo development and estimate survival, two different countings were made using sub-samples of 200 eggs collected from each incubator. The first counting, performed at 2-4 hpf was used to assess fertilization rate at 4-8 cells stage. The second counting was performed at 50 hpf to assess further embryonic development. All observations were made using a dissecting microscope (M3C, Wild Heerbrugg, Switzerland) and representative photomicrographs were taken using a Stemi 2000-C stereomicroscope (Carl Zeiss, Germany) equipped with a ProgResC5 camera device

(Jenoptik, Germany). After inspection and development assessment, each sub-sample was returned to its incubator.

2.2. A “common garden” protocol

At 74 hpf (approximately one day before hatching), samples of living embryos from 10 dams were transferred to larval rearing tanks. Six batches of eggs (3 gynogenetic treatment batches and 3 control batches) were constituted and put in separate tanks. The treated batches were constituted with 3 ml of eggs (around 3000 eggs) from each gynogenetic progeny, measured with a graduated syringe, while control batches were constituted using 2 ml (around 2000 eggs) of the corresponding control progenies. This difference in volumes provided was performed to anticipate the predicted high mortality at hatching of gynogenetically treated progenies. Each progeny was grown in triplicates except 3 treated progenies which were not sufficient to supply the triplicate and were only present in one rearing tank and the control progenies in its relative tank. As a consequence, tank 1 contained 10 different progenies while tanks 2 and 3 contained 7 progenies (Fig. 14).

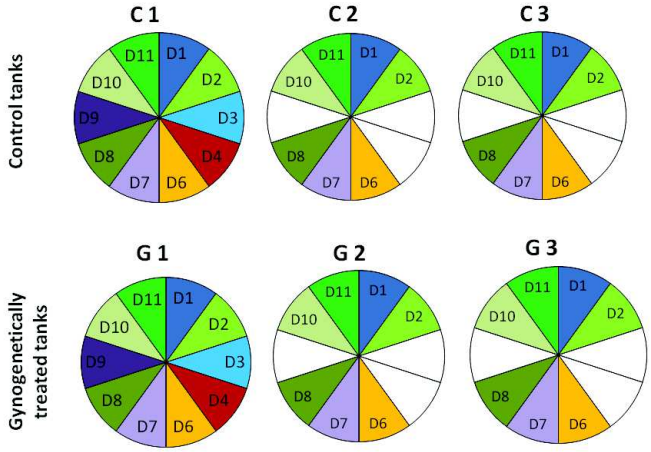


Figure 14. Schematic representation of the “common garden” protocol used for gynogenetic treatment and control progenies rearing.

2.3. Larval rearing

Larval rearing was performed in a recirculated system of 0.5 m^3 tanks with a constant salinity of 25‰, a water renewal between 10-20%.h^{-1} and an oxygenized air flow of 100-120 ml.min^{-1}. Larvae were kept in total darkness until 12 days post hatching (dph) (corresponding

to 160°C.day) when an artificial lighting of 100 lux for 12 hours a day was introduced. From 5 to 12 dph, tanks were equipped with a home-made cleaning system to remove oil and floating debris from the water surface and promote swimbladder inflation. Feeding started at 12 dph with freshly hatched nauplii of *Artemia salina*. From 28 to 84 dph according to the larvae mouth size, 1 day-old metanauplii enriched with a lipid emulsion (Selco, INVE, Ghent, Belgium) were distributed. *Artemia* prey density was adjusted to larvae consumption and maintained above 1 nauplii.ml^{-1}. From 69 days ah, the amount of preys distributed was progressively lowered and totally replaced at 84 dph by an artificial pellet (Marin Start, Le Gouessant, France) distributed during day light with a rate accorded to the approximate biomass. Temperature during larval rearing was maintained at 15°C with a progressive increase to 25°C from 80 dph. Larvae density in control tanks was roughly balanced by eye at 11, 31, 49 and 96 dph: excess larvae were randomly fished with a dip net and discarded in order to maintain similar densities between tanks.

During larval rearing, three morphometric analyses were performed at 4, 11 and 31 dph to analyse growth pattern difference between treated progenies and their controls. First feeding and swim-bladder inflation being critical stages during larval rearing were also carefully monitored. For morphometric analyses, larvae slightly anaesthetized with MS-222 (0.07 g.L^{-1}) were immobilized on the lateral side between two transparent plastic sheets and different traits measured. The analyses were performed using a dissecting microscope (M3C, Wild Heerbrugg, Switzerland) and representative photomicrographs of 30 individuals per tank at 4 dph and 50 individuals per tank at later stages were taken using a Stemi 2000-C stereomicroscope (Carl Zeiss, Germany) equipped with a ProgResC5 camera device (Jenoptik, Germany). Measures were computed using ImageJ version 1.46 calibrated with a millimeter scale to convert the image pixels in mm. The morphometric traits measured were: the total length (Lt) from the tip of the snout to the end of caudal fin, the standard length (Lst) from the tip of the snout to the end of the notochord, the head length (HL) from the tip of the snout to the end of the operculum, the head depth (HD) from the bottom of mouth cavity to the top of the head and the maximal body height (Hm). Alterations in body shape during larval rearing were identified by the study of morphometric ratios of all traits (Y) to the total length : R = Y / Lt.

2.4. Nursery and pre-growing

At 111 dph, fish were transferred to another recirculated system of 1.5 m^3 tanks supplied with a constant water renewal around 1 m^3.h^{-1} and a low air flow enriched with O_2. They were fed a commercial diet (Neo Start, Le Gouessant, France), the size of which varied according to growth, first with belt feeders and from 136 dph using on-demand feeding systems. Temperature started at 25°C and decreased to 20°C from 123 dph, then a progressive decrease (around -1°C per week) was again applied to keep the fish at 13°C from 251 dph, to allow gonad maturation. Photoperiod started at 12L:12D and decreased progressively from 206 dph to follow the natural photoperiod.

At 187 dph fishes were individually tagged with Passive Integrated Transponder glass tags and numbers per tank were equalized to 250 fish per tank. Fin clips were sampled and stored in absolute ethanol for DNA analyses. Weight was measured at 111, 187, 251, 306, 396 and 431dph. Daily Growth coefficient (DGC) was calculated as follows:

$DGC_{1\text{-}2} = (W_2^{1/3} - W_1^{1/3}) / (\text{date}_2 - \text{date}_1)$.

At 431 dph, control and non mitotic gynogenetic fish identified by genotyping were dissected in order to record their sex and gonadic weight. All manipulations on live fish were performed under anesthesia using Benzocaïne (220 ppm) to reduce stress during the measures. Dissection was performed after a lethal anesthesia of Benzocaine at 400 ppm.

After slaughtering, the gonado-somatic index (GSI) was calculated as follows:

GSI = 100*(gonads weight)/(total body weight)

2.5. DNA analyses

Three meiotic gynogenetic progenies of European sea bass previously produced following the protocol developed by Peruzzi and Chatain (2000) were analyzed using 12 microsatellite markers: *Dla-3*, *Dla-6*, *Dla-16*, *Dla-104*, *Dla-105*, *Dla-106*, *Dla-112*, *Dla-119*, *Labrax-3*, *Labrax-8*, *Labrax-17*, *Labrax-29* (Chistiakov et al., 2004; Ciftci et al., 2002; Garcia de Leon et al., 1995; Tsigenopoulos et al., 2003) in order to determine their distance to the centromere. The progeny originating from female A was composed of 28 meiotic gynogenetic individuals (meiogens), the progeny from female B contained 24 meiogens and the progeny originating from female C was composed of 44 meiogens. The different microsatellite markers belong to different linkage groups, except *Dla-104* and *Dla-106* which are on the same linkage group (LG2) (Chistiakov et al., 2005).

The 20 parents, 831 gynogenetic and 831 control fish were genetically analyzed with the same markers. Fin clip samples were sent to Labogena (Jouy-en-Josas, France) which performed DNA extraction, amplification and allele scoring. Parentage assignment was performed by exclusion using Vitassign version 8-5.1 (Vandeputte et al., 2006) by including dam genotypes both as sires and dams in order to identify gynogenetic offspring to their female parent. Only the parental pairs declared in the mating scheme were used and up to 3 mismatches were allowed.

The putative paternal contamination of gynogenetic progenies was verified in each progeny on informative markers. Microsatellite marker loci showing different alleles between the two parents were considered informative for verification of paternal contamination. Some microsatellite markers showed one allele in common between the male and female parent and could be informative about paternal contribution only in part of the offspring. Only if the discriminating paternal allele was observed or if the maternal discriminating allele was homozygous in the offspring, the microsatellite marker locus in question was considered informative for this offspring.

Mitotic gynogenetic individuals (doubled haploids) were identified by their homozygosity status for the informative microsatellite markers. Only the microsatellite markers heterozygous in the female parent were considered relevant for this purpose. If *Dla-104* and *Dla-106* were both heterozygous in the dam, only *Dla-104* was used as the two microsatellite markers do not segregate independently and give similar information. *Dla-16* was found to be positioned close to the centromere (low recombination rate in meiotic gynogenetic progenies) and was not used for meiotic/mitotic discrimination. *Dla-16* centromeric position was, however, informative to distinguish between the 2 different hypotheses of heterozygous contamination from spontaneous meiotic gynogenesis (meiosis I or meiosis II non-disjunction).

2.6. Determination of ploidy

At 400 dph, samples of blood from 42 biparental individuals in treated tanks were collected and prepared for flow cytometric analyses. For this purpose, 10 µl of pure blood collected in an heparinized syringe were diluted in 1 ml of 0.05% Propidium Iodide (PI) solution, following established procedures (Tiersch et al., 1989). After 30 min of PI staining in darkness at 4°C, 10% dimethyl sulfoxide (DMSO) was added and samples were stored at -80°C until use. Flow cytometry analyses were performed using a FACS Canto II (BD Biosciences, San Jose, CA, USA) flow cytometer and measuring the fluorescence of 5000 to 10000 nuclei/sample.

2.7. Gonad histology

Gonads from 10 controls, 14 gynogenetic and 9 biparental individuals were fixed in 10% neutral formalin for at least 48 h and subjected to standard histological procedures of dehydration and paraffin embedding. Briefly, 5 µm serial sections were stained with hematoxylin and eosin (Sigma, Norway) and observed using a DM2500 Leica (Leica Microsystems, Germany) stereoscope equipped with a DFC320 Leica camera and FireCam 3.3.1. Leica software.

2.8. Statistical analyses

All statistical analyses were performed using Statistica (Version 7.1). Genotypic and biometric data were analyzed as described in the following sections.

2.8.1. Genotypic statistical analyses

The recombination rates of the different microsatellite markers were first calculated on the meiotic progenies. The female parents were considered informative when they showed heterozygosity for the marker analyzed. The recombination rates (RR) were calculated for each marker as the proportion of heterozygous individuals in the informative progenies. Microsatellite locus-centromere distances were calculated as follows: M-C distance = (RR/2) x 100 centimorgans (cM) (Thorgaard et al., 1983).

Mendelian segregation of alleles was verified by homogeneity χ^2 tests, with p=0.001 (after a Bonferroni correction of p=0.05 for 120 individual tests) and 3 degrees of freedom, on control progenies to identify genotyping errors. The presence of null alleles was also verified using Microchecker software version 2.2.3 (Van Oosterhout et al., 2004).

The recombination rates previously found in meiotic gynogenetic progenies (RR Ggp) were used to calculate the probability of considering an offspring as mitotic when it is in fact meiotic. This probability (MMprob) was calculated as the product of (1 - RR Ggp) for each informative microsatellite marker.

To analyze the origin of heterozygous individuals in gynogenetic progenies produced using a protocol supposed to induce endomitosis, the proportions of heterozygous and homozygous individuals of each microsatellite marker found heterozygous in the female parent were calculated on the heterozygous gynogenetic offspring. These proportions were then compared to the meiotic gynogenetic offspring by χ^2 tests for each microsatellite locus with p=0.005 (calculated after Bonferroni correction of p=0.05 for 11 tests). Degrees of freedom (df) were calculated according to the number (n) of informative progenies at each locus and df = 2n-1. For the comparison of the total proportions in meiotic (Ggp) and late shocked heterozygous (Gem) gynogenetic progenies, 3 df were used to assess the significant threshold at p=0.005 (after Bonferroni correction).

2.8.2. Biometric statistical analyses

During incubation, survival was estimated as a percentage of developing embryos over the total number of eggs. Embryonic survival at 50 hpf was calculated relative to the fertilization rate. A logistic regression was used to compare fertilization success and embryonic survival between the 2 treatments, the 11 female parents and their interaction.

During larval rearing, no family or treatment result (mitotic gynogenetic, meiotic gynogenetic, biparental in the gynogenetically treated tanks) information was available. The different ratios of traits measured were arcsine square root transformed for comparison by nested ANOVA. The treatment (control or gynogenesis) was considered a fixed factor and the rearing tanks were treated as a random factor (tank effect) nested within treatment.

DGCs and GSI were arcsine square root transformed for comparison by nested ANOVA. The treatment was considered fixed factor and the rearing tanks treated as a nested random factor.

Additional analyses were performed to compare different groups of fish:

- Progenies from dam 1 were compared in 3 separate analyses:
  - A comparison between control and biparental (in gynogenetically treated tanks) individuals performed using a nested ANOVA with treatment as a fixed factor and tank as a nested random factor.
  - A comparison of control and gynogenetic individuals of dam 1 performed using a nested ANOVA with treatment as a fixed factor and tank as a random factor nested within treatment.
  - A comparison of biparental and gynogenetic individuals of dam 1 performed using a two-way ANOVA with the tanks considered a random factor crossed with the genetic status considered fixed factor.
- Gynogenetic and control progenies were compared using a two-way ANOVA with tank as a random factor nested within a fixed treatment factor. For this analysis, biparental individuals and progenies too small or not represented in all tanks were excluded.
- The parental influence and its interaction with the treatment were also compared by ANOVA, the treatment being considered fixed, the parental pair being considered as a random factor and the tank considered as a random factor.

Sex ratio between the different control and gynogenetic progenies where the number of individuals was higher than 1 were compared by a logistic regression to analyze the treatment and the parental influence on the different proportions of undetermined sex.

3. Results

3.1. Embryonic development

Fertilization rates (Table 6) in the control and the gynogenetic treatments ranged from 26.5-92.5% with no significant effect of the treatment ($p=0.66$). A significant parental effect ($\chi^2_{10}>18.31$, $p<0.001$) and a significant interaction ($\chi^2_{10}>18.31$, $p<0.001$) between female parent and treatment were found in fertilization success.

Table 6. Fertilization rates in percent measured 3-4 hpf. Results are presented per female parent and per treatment (control or gynogenesis).

	Female parent										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Control	40.5	59.0	52.0	73.5	65.5	33.5	79.0	81.0	85.5	78.0	36.0
Gynogenesis	26.5	55.0	44.5	55.0	65.0	72.0	66.0	92.5	78.5	72.0	42.0

No significant parental effect (p=0.56) was found significant (Fig. 15) while significant differences were observed for treatment (χ^2_1>18.31, p<0.001) and the interaction between dams (female parent) and treatment (χ^2_{10}>18.31, p<0.001).

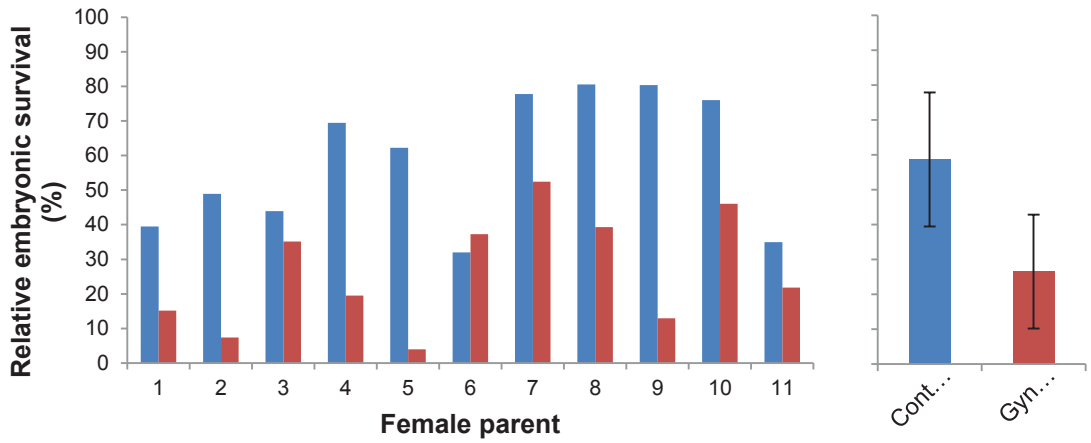


Figure 15. Embryonic survival at 50 hpf relative to fertilization rate for individual dams (left panel) and as an average (right panel) with data presented as mean ±STD. Data for control fish are shown in blue and those for gynogenetic fish in red.

These results showed that the treatment did not significantly affect fertilization, meaning that irradiated sperm did not lose its ability to fertilize eggs with the dilution used, but the treatment clearly depressed embryonic development. An interaction between the treatment and the different females was also found to be significant during embryonic development and showed the high variability between the females in gynogenesis experiments.

3.2. Larval growth

Measures performed at the different stages during larval rearing are presented in Supplementary file S7. Analysis of body shape alterations presented in Fig. 16 showed no significant treatment effect (p>0.05) at all stages for all ratios analyzed. Tank effect was found significant (p<0.05) for some traits at the 3 different stages: at 4 dph for all ratios except standard length relative to total length, at 11 dph only for head length relative to total length, and at 31 dph for all ratios except head length relative to total length.

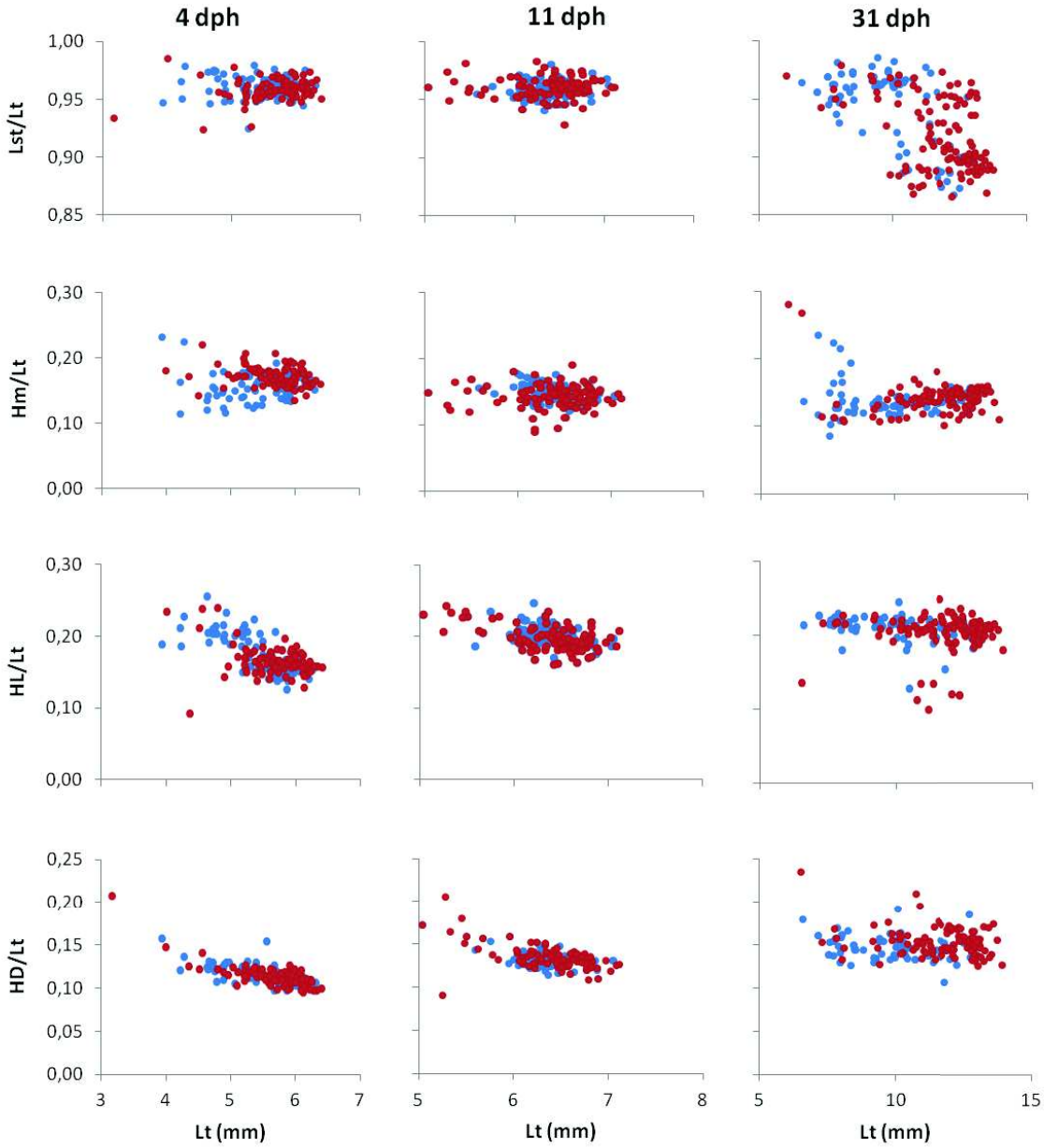


Figure 16. Development of the morphometric ratios in relation to the total length (Lt) at 4, 11 and 31 dph. In blue are the control progenies and in red the gynogenetically treated progenies. The traits measured were: standard length (Lst), maximal body height (Hm), head length (HL) and head depth (HD).

These results showed that the gynogenetic treatment did not lead to significant alterations in body shape of larvae from 4 to 31 dph.

3.3. Parentage assignment and mitotic gynogenetic status verification

3.3.1. Microsatellite markers analysis in meiotic gynogenetic progenies

Recombination rates calculated for the different markers in the three meiotic gynogenetic progenies analyzed are presented in Table 7.

Table 7. Recombination rates estimated for each microsatellite marker ranging between 0 (no recombination) and 1 (100% recombination). N shows the number of fishes genotyped in each progeny. NI represents the non-informative loci for each progeny. M-C distance shows the estimated distances between the microsatellite locus and the centromere for each marker.

Fish	N	Marker loci											
		Dla-3	*Dla-6*	*Dla-16*	*Dla-104*	*Dla-105*	*Dla-106*	*Dla-112*	*Dla-119*	*Labrax-17*	*Labrax-29*	*Labrax-3*	*Labrax 8*
Dam A	28	0.82	NI	0.00	0.79	0.71	NI	NI	0.32	NI	0.33	NI	0.81
Dam B	24	0.79	0.21	0.04	NI	0.54	0.71	0.57	0.38	1.00	0.54	0.42	0.75
Dam C	44	0.70	NI	NI	0.75	NI	0.75	0.70	0.36	0.95	0.51	0.45	0.77
Mean RR		0.77	0.21	0.02	0.77	0.63	0.73	0.63	0.35	0.98	0.46	0.44	0.78
M-C distance (cM)		38.6	10.4	1.1	38.4	31.4	36.5	31.7	17.7	48.9	23.1	21.8	39.0

Out of the 12 microsatellite markers analyzed, 7 showed a recombination frequency higher than 50% and can then be considered highly relevant for the purpose of homozygosity verification, the best one being *Labrax-17* located in a highly telomeric position. However, although some markers have a low recombination rate, the combination with other markers gives a higher power to distinguish between meiotic and mitotic gynogenetic individuals. As *Dla-104* and *Dla-106* are positioned in the same linkage group (LG2) and do not segregate independently, they give redundant information and a choice between the two of them depending on the heterozygosity of the parent should be performed before analyzing homozygosity in progenies. Ten microsatellite markers could then be considered relevant for homozygosity verification with a probability of considering a meiotic individual being mitotic of 4.95×10^{-6} if all markers are heterozygous in the female parent. Only *Dla-16* seemed inappropriate for the purpose of homozygosity verification because of its position very close to the centromere leading to very rare recombination, but could be used for other purposes as the meiotic non-disjonction analysis.

3.3.2. Parentage assignment of communally reared progenies

Out of the 1662 sampled offspring, 1653 gave good amplification and were genotyped and analyzed through Vitassign. Out of these individuals, 92.9% (1536 individuals) were assigned to a single parental pair with perfect matching, and 95.9% including 1 to 3 mismatches (1631 individuals). 0.6% of offspring (12 individuals) were not assigned or assigned to more than two parental pairs and were discarded from the analysis, and in most cases their genotype was incomplete for several microsatellite loci. Offspring with valid genotypes on less than three microsatellite markers were also discarded from further analyses. Knowing the treated or control origin of fish, the individuals assigned to multiple parental pairs or exhibiting mismatches in treated progenies were analyzed further. In control progenies, individuals showing more than two mismatches were discarded from further analyses, their genotypes were incomplete and the mismatches probably originated from errors during the genotyping process. Finally 1621 fishes were assigned to a single parental pair (see Table 8).

Table 8. Number of individuals assigned per specific progeny.

	Number of offspring assigned	
Female parent	**Control**	**Treated**
1	98	650
2	85	6
3	22	7
4	19	18
6	39	17
7	376	43
8	15	1
9	10	0
10	52	9
11	92	62

The Mendelian segregation of alleles was verified (Table 9) in each control progeny batch at every locus and all analyses showed no significant distortion in allele transmission (all $\chi^2_3 < 18.47$, $p>0.001$). The presence of null alleles was detected at *Dla-6* for dam 1, male 1 and dam 11 and at *Labrax-17* for dam 1, and taken into account in further analyses as these markers could not be informative for the verification of maternal genetic inheritance and homozygosity.

Table 9. Statistical results of allele segregation analysis. For each marker locus, knowing the genotype of the male and female parent, the proportions of alleles were analyzed in progenies and compared by a χ^2 test with 3 degrees of freedom, for which the threshold value is 18.47 for p=0.001 (after a Bonferroni correction of p=0.05 for 120 individual tests).

	Marker loci											
	Dla-3	*Dla-6*	*Dla-16*	*Dla-104*	*Dla-105*	*Dla-106*	*Dla-112*	*Dla-119*	*Labrax-17*	*Labrax-29*	*Labrax-3*	*Labrax 8*
Dam 1	215/223	119/0	241/241	262/264	155/176	245/245	108/108	222/222	130/0	111/145	120/136	210/228
Male 1	217/223	0/0	241/251	266/266	147/147	272/272	108/112	220/233	126/134	111/137	116/170	220/232
Chi-square	0.41	0.58	0.14	0.27	0.10	0.09	0.18	1.82	0.62	0.92	1.14	2.52
Dam 2	223/225	119/119	262/270	262/264	151/155	245/253	108/116	220/222	115/177	156/160	134/142	195/228
Male 2	232/236	119/119	268/268	262/266	145/145	262/272	112/112	233/259	126/126	111/139	175/175	210/220
Chi-square	2.38	0.00	0.86	1.80	0.01	3.08	0.05	2.00	3.96	0.64	0.95	7.55
Dam 3	225/225	119/119	241/262	264/264	151/172	245/245	108/112	220/262	115/115	160/160	136/145	224/228
Male 3	217/232	123/123	239/251	262/266	145/147	266/272	108/112	220/220	134/134	111/139	116/170	220/232
Chi-square	0.50	0.00	0.18	0.14	0.54	0.09	0.36	1.58	0.00	4.55	0.36	0.36
Dam 4	209/228	119/127	239/253	264/264	172/174	245/253	108/108	220/239	141/177	145/160	136/142	224/236
Male 4	217/232	123/123	239/268	266/266	147/159	272/272	108/120	220/220	126/134	137/139	116/116	210/210
Chi-square	1.38	6.23	1.38	0.00	1.96	0.08	1.28	0.46	0.77	0.77	3.77	0.08
Dam 6	223/232	123/123	251/268	266/262	145/159	266/272	108/108	257/259	126/138	137/139	116/116	210/210
Male 6	232/232	123/123	251/251	262/262	145/145	266/266	108/120	220/257	126/138	111/137	116/116	210/232
Chi-square	0.43	0.00	0.84	0.15	0.22	0.15	0.84	0.46	0.05	0.03	0.00	0.84
Dam 7	217/223	119/119	241/257	264/266	145/147	262/266	108/112	220/243	115/145	131/137	136/155	210/222
Male 7	209/225	119/127	237/262	264/266	155/174	245/245	108/108	220/227	115/115	111/156	136/142	195/197
Chi-square	6.23	1.29	1.85	2.22	1.63	3.14	0.09	1.17	0.44	8.93	2.92	4.22
Dam 8	232/238	119/123	227/243	266/266	159/167	270/270	104/112	220/255	134/141	137/163	151/177	210/210
Male 8	219/223	119/123	245/260	264/264	136/145	245/264	108/108	220/253	121/141	137/154	136/169	193/226
Chi-square	0.67	2.13	1.20	0.00	4.93	0.07	1.63	5.93	0.13	1.13	0.67	0.07
Dam 9	219/238	119/123	243/251	262/274	147/159	245/262	108/112	237/264	115/115	137/175	175/177	193/226
Male 9	217/223	119/119	251/251	266/266	147/159	262/272	112/120	220/233	134/138	111/137	116/116	210/220
Chi-square	0.40	0.27	1.07	0.40	0.20	0.80	0.30	0.40	0.00	0.30	0.80	0.40
Dam 10	227/227	119/119	223/229	262/274	145/155	262/262	108/108	220/235	115/136	154/175	159/170	195/222
Male 10	225/238	119/119	243/253	262/266	145/145	270/270	112/116	220/237	115/132	154/162	170/185	210/224
Chi-square	1.92	0.00	1.46	9.46	0.82	0.04	0.69	1.90	8.23	1.62	6.62	2.62
Dam 11	209/223	119/0	239/243	264/280	145/157	262/262	108/112	219/262	115/141	137/160	136/157	210/210
Male 11	209/217	119/119	227/266	264/266	145/170	270/274	104/112	254/262	115/138	111/137	155/173	210/228
Chi-square	7.22	0.00	1.69	1.11	1.42	0.53	0.10	2.13	1.60	2.35	0.73	1.90

3.3.3. Verification of maternal genetic inheritance in treated offspring

The putative paternal contamination of gynogenetically treated progenies was verified on each informative marker (results are presented in Supplementary file S8). For some individuals, the genotyping did not give results for some microsatellite markers. Results are presented relative to the number of informative microsatellite markers analyzed for each offspring of each progeny batch. Details of analyses are presented in Table 10.

Table 10. Analysis of paternal contamination in gynogenetic offspring. Results are presented by number of fish in each progeny showing complete to no paternal inheritance relatively to the number of informative loci. Npl represents the number of loci showing paternal inheritance and Nil the number of loci giving information of paternal allele transmission (informative loci).

Progeny 1			Progeny 2		
N fish	N pl	N il	N fish	N pl	N il
23	11	11	**1**	0	11
112	10	10	**5**	0	10
2	9	10	**Progeny 3**		
1	8	10	**5**	0	10
1	7	10	**2**	0	11
178	9	9	**Progeny 4**		
5	8	9	**8**	0	11
2	7	9	**9**	0	10
4	6	9	**1**	0	5
139	8	8	**Progeny 6**		
4	7	8	**1**	0	4
4	6	8	**6**	0	3
2	5	8	**6**	0	1
49	7	7	**4**	0	0
5	6	7	**Progeny 7**		
3	5	7	**1**	1	6
1	4	7	**2**	0	9
12	6	6	**5**	0	8
2	5	6	**12**	0	7
1	4	6	**21**	0	6
7	5	5	**2**	0	4
1	4	5	**Progeny 8**		
2	4	4	**1**	0	4
5	3	3	**Progeny 10**		
1	2	3	**2**	0	7
1	2	2	**4**	0	6
1	1	2	**2**	0	5
1	0	9	**1**	0	3
11	0	8	**Progeny 11**		
41	0	7	**3**	0	6
24	0	6	**12**	0	5
2	0	5	**24**	0	4
1	0	3	**19**	0	3
1	0	2	**3**	0	2
1	0	1	**1**	0	1

Out of the 813 analyzed offspring, 569 showed paternal contamination affecting one to all loci analyzed. This kind of biparental individuals was found mostly in one progeny (progeny 1) with 528 individuals being biparental for all microsatellite markers and 40 showing incomplete paternal contamination. In progeny 7, one individual showed incomplete paternal inheritance with contamination at only one locus. In progeny 6, the two parental genotypes were similar for several microsatellite markers and only one allele was discriminating for most of the loci considered. In this progeny, 4 individuals showed no paternal contribution but no locus was informative on the previously stated characteristics. Considering that a putative paternal contamination should have been observed if there was one, the absence of discriminating paternal alleles was considered a proof of only maternal inheritance. Given the results of this analysis, 244 individuals were considered gynogenetic and were analyzed further to assess their homozygosity status.

The ploidy status of 42 biparental offspring from dam 1 was verified by flow-cytometry and their diploid status confirmed, showing that they escaped the mitotic pressure shock, which should have made them tetraploid.

3.3.4. Verification of homozygosity in gynogenetic progenies

The mitotic status of gynogenetic offspring was verified based on the informative microsatellite markers which showed maternal heterozygosity (Supplementary file S9). Finally, only 39 putative mitotic individuals were found out of the 244 gynogenetic individuals. The others showed different levels of heterozygosity for the different markers analyzed (Table 11). The probabilities of considering a meiotic individual being mitotic according to the combinations of all informative markers are 1.30×10^{-3} in progeny 1, 6.26×10^{-6} in progeny 2, 1.10×10^{-2} in progeny 3, 1.57×10^{-5} in progeny 4, 1.37×10^{-4} in progeny 6 and 6.26×10^{-6} in progeny 7.

Table 11. Analysis of homozygosity and levels of heterozygosity in each gynogenetic progeny. Results are presented by number of fish (N fish) showing different level of heterozygosity (N recomb) relative to the number of informative loci (N markers) in each progeny.

Progeny 1		
N fish	N recomb	N markers
12	0	6
1	1	6
1	1	5
1	1	3
2	1	2
3	2	6
21	3	6
17	4	6
15	5	6
9	6	6
Progeny 2		
5	0	**9**
1	8	9
Progeny 3		
1	0	5
1	1	5
1	2	5
2	3	5
1	4	5
1	5	5
Progeny 4		
15	0	9
1	0	8
1	0	6
1	6	9
Progeny 6		
1	0	6
1	2	6
3	3	6
7	4	6
3	5	6
2	6	6

Progeny 7		
N fish	N recomb	N markers
3	0	9
1	1	4
1	2	3
1	3	9
4	4	9
8	5	9
10	6	9
5	7	9
6	8	9
3	9	9
Progeny 8		
1	4	5
Progeny 10		
1	2	6
1	3	6
2	4	6
3	5	6
1	6	6
1	2	3
Progeny 11		
1	2	8
2	3	8
11	4	8
13	5	8
17	6	8
10	7	8
1	8	8
3	4	7
1	5	7
1	2	5
2	3	5

A high variability in the proportion of homozygous individuals in the gynogenetic progenies of the different parents was observed (Fig. 17). Knowing the proportions of mitotic and meiotic individuals in the genotyped progenies and the approximate numbers of individuals from the constitution of batches (3000 eggs per dam) to 187 dph when tank were equalized and fish counted, survival from 74 hpf to 187 dph could be roughly estimated to be around 0.1% for mitotic gynogenetic progenies and to 0.6% for total gynogenetic progenies (mitotic and meiotic).

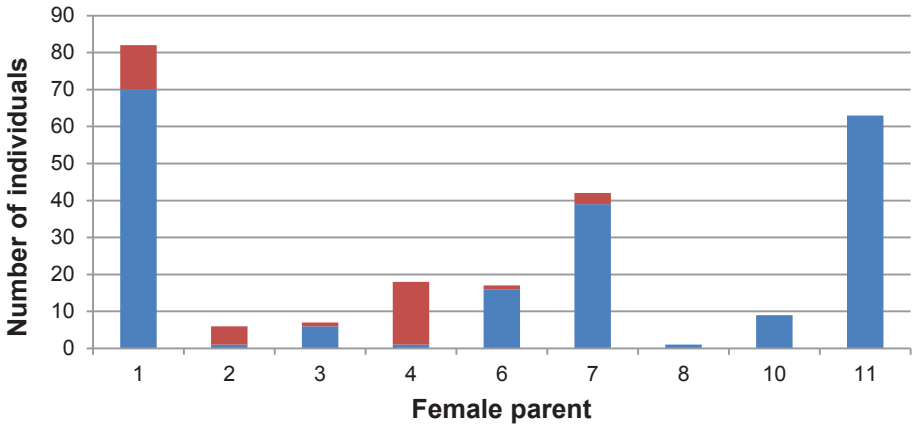


Figure 17. Proportions of meiotic and mitotic gynogenetic individuals produced from each of the 11 dams. Meiotic gynogenetics are shown in blue and mitotic gynogens in red.

3.3.5. Origin of residual heterozygosity

Table 12. Analysis of origin of heterozygous individuals in mitotic gynogenetic progenies. For each meiotic (dam A, B and C) gynogenetic progeny and heterozygous gynogens produced with a late shock applied to induce endomitosis (dam 1, 6, 7, 11, 3 and 10), results are presented by numbers of heterozygous/homozygous individuals at each microsatellite locus. The significant differences in proportions of heterozygous/homozygous individuals between the different females analyzed at each locus by a χ^2 test are indicated by a star (*). The proportions of heterozygous/homozygous individuals in the meiotic progenies (Ggp) and in the late shocked progenies (Gem) compared by a χ^2 test at each locus are significantly different when indicated by a star (*). NI represents the non-informative loci.

	Dla-3	*Dla-16*	*Dla-104*	*Dla-105*	*Dla-106*	*Dla-112*	*Dla-119*	*Labrax-17*	*Labrax-29*	*Labrax-3*	*Labrax-8*
Dam A	23/5	0/27	22/6	20/8	NI	NI	9/19	NI	9/18	NI	22/6
Dam B	19/5	1/22	NI	13/11	17/7	13/10	9/15	24/0	13/11	10/14	18/6
Dam C	31/13	NI	33/11	NI	33/11	31/13	16/28	42/2	22/22	20/24	34/10
Dam 1	40/28	NI	40/28	41/26	NI	NI	NI	NI	50/19	52/18	48/16
Dam 6	11/5	0/14	10/6	13/3	10/6	NI	7/8	16/0	9/7	NI	NI
Dam 7	10/12	0/23	17/5	20/2	18/5	17/5	9/13	20/3	14/9	14/8	16/7
Dam 11	47/16	0/59	41/18	49/14	NI	47/16	23/35	62/1	39/21	16/46	NI
Dam 3	NI	0/6	NI	4/2	NI	5/1	3/3	NI	NI	3/3	3/3
Dam 10	NI	0/9	7/2	7/2	NI	NI	2/6	8/1	7/1	5/3	4/4
Chi-square	13.16	6.04	6.59	13.16	1.36	3.63	2.02	8.93	17.91	34.14*	4.96
Ggp	73/23	1/49	55/17	33/19	50/18	44/23	34/62	66/2	44/51	30/38	74/22
Gem	108/61	0/111	115/59	134/49	28/11	69/22	44/65	106/5	119/57	90/78	71/30
Chi-square	4.16	2.24	2.52	1.87	0.04	1.95	0.53	0.27	11.67	1.73	1.17

The proportions of heterozygous and homozygous individuals for each marker in each progeny from meiotic gynogenesis and heterozygous progenies produced in mitotic gynogenesis are presented in Table 12. The statistical analysis showed no significant differences between the proportions observed in the different heterozygous offspring. These results demonstrate the retention of the polar body as a plausible explanation for the origin of meiotic individuals in gynogenetic offspring. The proportions observed on *Dla-16* particularly confirm this hypothesis, the numbers of homozygous/heterozygous individuals would be completely different (see Fig. 12) for this centromeric locus if a non-disjunction occurred during meiosis I. A significant difference ($p<0.05$) was observed between dams only for *Labrax-3* but no significant difference remained when progenies were compared by total proportions in the meiotic and late shocked progenies.

3.4. Growth

3.4.1. Control versus treated tanks

The mean growth of control and treated tanks is presented in Fig. 18 showing no significant difference in growth for the DGCs calculated between measures and for the whole period ($p=0.35$). The comparisons showed a clear tank effect (DGC total period: $F_{4,1067}=41.70$, $p<0.001$).

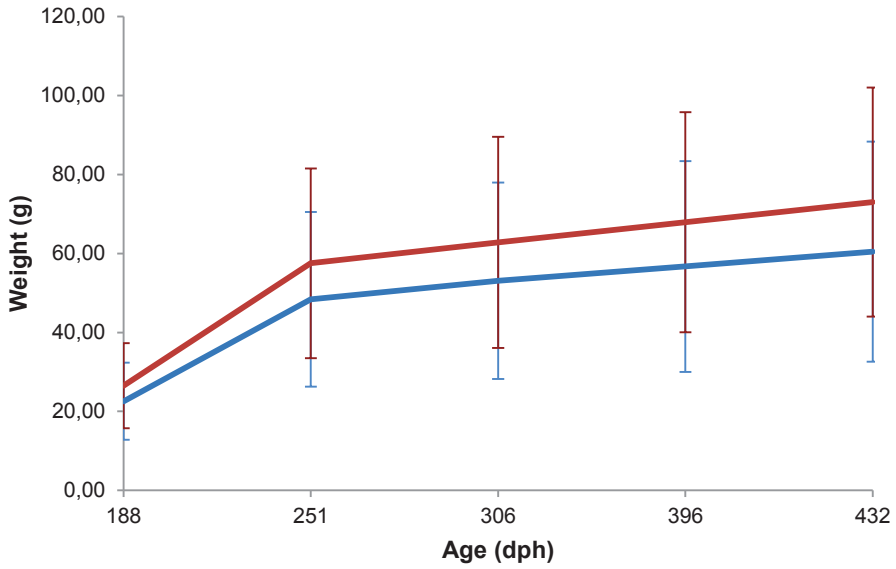


Figure 18. Growth of control and gynogenetic fish during on-growing period (188-432 dph). Data are presented by mean ± standard deviation (STD). The blue curve represents the growth of control fish and the red the one of gynogenetic fish.

However, genetic analyses showed that treated tanks contained biparental individuals (70%), meiogens (25%) and mitogens (5%), giving analyses at the tank level very little explanatory power. Additional analyses were performed to look for potential differences hidden by an improper statistical comparison.

3.4.2. Dam 1 progenies comparisons: control versus biparentals, control versus gynogens and biparentals versus gynogens

Control vs biparental offspring

Table 13. DGCs calculated from the whole analyzed period on dam 1 control and biparental treated progenies. Data are presented by mean ± SE. N shows the number of fish analyzed.

	Tank	N	DGC
Control	1	21	0.00503 ± 0.00027
	2	36	0.00472 ± 0.00014
	3	30	0.00445 ± 0.00016
	Mean	87	0.00470 ± 0.00010
Treatment	1	126	0.00541 ± 0.00007
	2	177	0.00433 ± 0.00006
	3	147	0.00513 ± 0.00008
	Mean	450	0.00489 ± 0.00005

DGC calculated during the whole analyzed period per tank and per treatment are presented in Table 13. No significant difference was found between biparental individual and control DGCs (p=0.69) but an intra-treatment (tank) effect was found significant for all periods analyzed (DGC total period: $F_{4,414}$=23.55, p<0.001).

Control vs gynogenetic offsprings

DGCs calculated for the whole analysis period per tank and per treatment are presented in Table 14. The comparison of gynogens and controls showed no significant difference (p=0.69) in growth but a tank effect for the first period analyzed (189-251 dph) and for the total period analyzed (DGC total period: $F_{4,118}$=4.558, p=0.002).

Table 14. DGCs calculated from the whole analyzed period on dam 1 control and gynogenetic progenies. Data are presented by mean ± SE. N shows the number of fish analyzed.

	Tank	N	DGC
Control	1	21	0.00503 ± 0.00027
	2	36	0.00472 ± 0.00014
	3	30	0.00445 ± 0.00016
	Mean	87	0.00470 ± 0.00010
Gynogens	1	29	0.00533 ± 0.00016
	2	20	0.00448 ± 0.00014
	3	20	0.00505 ± 0.00016
	Mean	69	0.00500 ± 0.00010

Biparental vs gynogenetic offspring

DGCs calculated for the whole period analyzed per tank and per genetic status are presented in Table 15. The comparison of biparental and gynogenetic offspring from dam 1 showed no significant difference (p=0.98) in growth but a significant tank effect at 251 dph, 432 dph and for the total period ($F_{2,513}$=55.564, p=0.02). No interaction between the tank and the genetic status were found significant at all periods analyzed.

Table 15. DGCs calculated from the whole analyzed period on dam 1 biparental and gynogenetic progenies. Data are presented by mean ± SE. N shows the number of fish analyzed.

	Tank	N	DGC
Biparental	1	126	0.00541 ± 0.00008
	2	177	0.00433 ± 0.00006
	3	147	0.00513 ± 0.00008
	Mean	450	0.00489 ± 0.00005
Gynogens	1	29	0.00533 ± 0.00016
	2	20	0.00448 ± 0.00014
	3	20	0.00505 ± 0.00016
	Mean	69	0.00500 ± 0.00010

3.4.3. Control vs gynogenetic representative progenies

Biparental individuals and progenies too small or not represented in all tanks were excluded to analyze growth between gynogens (meiotic and mitotic gynogenetic individuals) and controls in the highly contributing families. Only 4 different progenies were used in this analyze (dam 1, 6, 7 and 11). Results of DGCs calculated during the whole analyzed period are presented in Table 16. Growth remained insignificantly different ($p=0.45$) for the periods analyzed but a tank effect was found significant in all comparisons (DGC total period: $F_{4,555}=16.041$, $p<0.001$).

Table 16. DGCs calculated from the whole analyzed period on 4 control and gynogenetic representative progenies. Data are presented by mean ± SE. N shows the number of fish analyzed.

	Tank	N	DGC
Control	1	154	0.00457 ± 0.00008
	2	187	0.00411 ± 0.00008
	3	189	0.00384 ± 0.00007
	Mean	530	0.00425 ± 0.00005
Gynogens	1	65	0.00484 ± 0.00016
	2	47	0.00421 ± 0.00016
	3	53	0.00469 ± 0.00012
	Mean	165	0.00461 ± 0.00009

3.4.4. Parent-treatment interaction

Results of DGCs calculated for the whole period analyzed are presented in Fig. 19. A comparison of the treatment–parental influence was performed on the 4 representative progenies 1, 6, 7 and 11 and showed a clear interaction between the treatment and the parents ($F_{3,6}$=15.64, p=0.003).

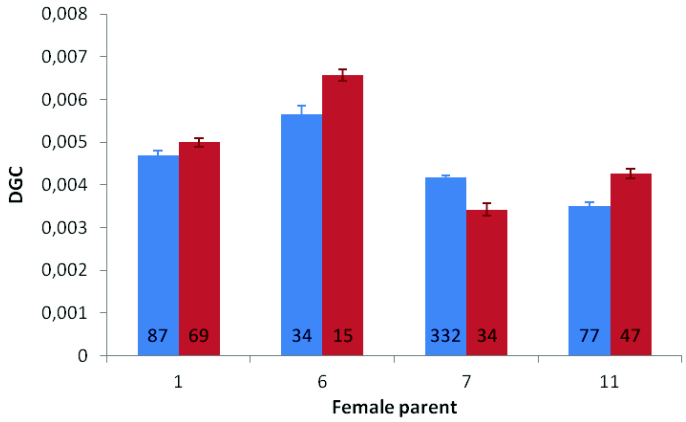


Figure 19. DGCs calculated from the whole period analyzed on 4 control (in blue) and gynogenetic (in red) representative progenies. Data are presented by mean ± SE. The numbers at the base of each column shows the amount of fish analyzed.

3.5. Gonad development and sex-ratio

3.5.1. Gonado-somatic index

Control vs treated tanks

Results of GSI measured at 432 dph are presented in Table 17.

Table 17. GSI calculated at 432 dph after slaughtering of non mitotic gynogenetic individuals. Data are presented by mean ± SE. N shows the number of fish analyzed.

	Tank	N	GSI
Control	1	168	0.065 ± 0.006
	2	159	0.057 ± 0.005
	3	149	0.027 ± 0.004
	Mean	476	0.050 ± 0.003
Treatment	1	108	0.003 ± 0.002
	2	98	0.002 ± 0.001
	3	91	0.002 ± 0.001
	Mean	297	0.002 ± 0.001

GSI was found to be significantly different between treated and control batches ($F_{1,4}$=15.20, p=0.02). A significant tank effect was also observed ($F_{4,767}$=17.06, p<0.001).

Control vs biparental individuals from dam 1

The comparison of GSI in biparental and control progenies of dam 1 (Table 18) showed a significant difference ($F_{1,4}$=12.01, p=0.02). Biparental individuals had small to inexistent gonads. A significant tank effect was also observed ($F_{4,224}$=8.31, p<0.001).

Table 18. GSI of control and biparental progenies of dam 1 calculated at 432 dph after slaughtering. Data are presented by mean ± SE. N shows the number of fish analyzed.

	Tank	N	GSI
Control	1	21	0.035 ± 0.007
	2	36	0.040 ± 0.009
	3	29	0.010 ± 0.005
	Mean	86	0.029 ± 0.005
Treatment	1	42	0.001 ± 0.001
	2	55	0.001 ± 0.001
	3	47	0.000 ± 0.000
	Mean	144	0.001 ± 0.001

Control vs gynogenetic progenies from representative dams

The comparison of ratios between gynogenetics and controls after exclusion of biparental individuals and non-represented families showed the same result: GSI is much lower for gynogens compared to the controls. However, the treatment effect was insignificant (p=0.07) contrary to the tank effect ($F_{4,347}$=6.27, p<0.001).

Table 19a. GSI of control and gynogenetic progenies of 4 representative progenies calculated at 432 dph after slaughtering. Data are presented by mean ± SE. N shows the number of fish analyzed.

	Tank	N	GSI
Control	1	67	0.059 ± 0.010
	2	85	0.041 ± 0.008
	3	80	0.014 ± 0.005
	Mean	232	0.037 ± 0.005
Gynogens	1	48	0.002 ± 0.000
	2	32	0.002 ± 0.001
	3	41	0.003 ± 0.000
	Mean	121	0.002 ± 0.001

Parent-treatment interaction

The analyze of treatment and parental effect on GSI showed a significant treatment effect ($F_{1,8}$=52.38, p<0.001) and a significant treatment-parents-tank interaction ($F_{16,329}$=3.68, p<0.001), while the parental effect, the parent-tank interaction and the interaction treatment-parent were insignificant on gonad development (p=0.22, p=0.67 and p=0.90 respectively).

Table 19b. GSI of control and gynogenetic progenies of 4 representative progenies calculated at 432 dph after slaughtering. Data are presented by mean ± SE. N shows the number of fish analyzed.

	N	GSI
Control	232	0.037 ± 0.005
Gynogens	121	0.002 ± 0.001

3.5.2. Gonadal observation

Some histological gonad analyses are presented in Fig. 20. The 10 control individual sex was clearly assessed and the gonad development at 431 dph already showed differentiated spermatogonia and primary oocytes. The 23 gonads from gynogenetic and biparental individuals remained mostly undifferentiated or in early differentiation at 431 dph.

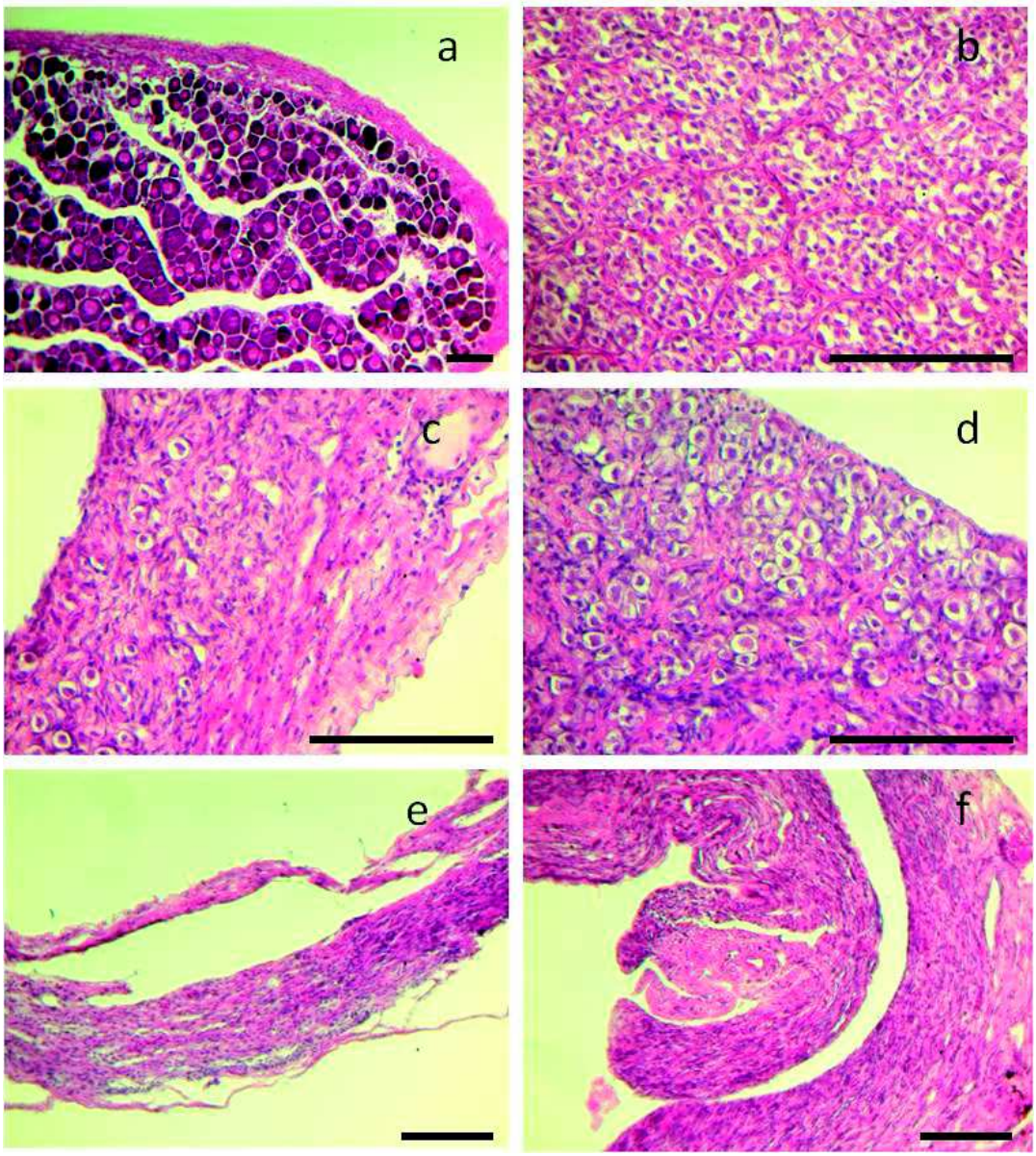


Figure 20. Pictures of histological observation of gonads at 431 dph from control (a,b), and meiotic gynogenetic and biparental progenies (c,d,e,f). a) female gonad from a control individual, b) male gonad from a control individual, c) putative female gonad from a gynogenetic individual, d) putative male gonad from a gynogenetic individual, e) undetermined gonad from a biparental individual, f) undetermined gonad from a gynogenetic individual. Scale bars represent 1 mm.

3.5.3. Sex ratio

Controls vs biparentals of dam 1

Table 20. Percentages of undetermined individuals, males and females in control and treated biparental progenies of dam 1.

	N	Undetermined	Males	Females
Control	86	10,47	58,14	31,40
Treatment	146	74,66	17,81	7,53

Sex ratio obtained in control and biparental individuals (Table 20) were clearly different showing a high number of undetermined individuals in the treated progeny. At the same age, control progeny showed a sex ratio in favor of males.

Controls vs gynogens

Percentages of females, males and undetermined fish in each family and treatment are presented in Table 21. Dam 2, dam 4 and dam 8 progenies were not included in statistical analyze as the sex ratio in gynogens was only calculated on 1 individual. The logistic regression on the proportions of undetermined individuals showed significant effects of the treatment ($\chi^2_{1,0.05}$=10.07, p<0.001), of the parental influence ($\chi^2_{5,0.05}$=32.73, p<0.001) and of the interaction between the two factors ($\chi^2_{5,0.05}$=1.05x10^{14}, p<0.001). The mean percentage of undetermined individuals was high in gynogenetic progenies (59%) compared to the control progenies (17%).

Table 21. Percentages of sex ratios in each family (dam parent) and each treatment. N represents the number of fish analyzed per condition.

		N	Undetermined	Males	Females
Dam 1	Control	86	10,47	58,14	31,40
	Gynogens	53	81,13	15,09	3,77
Dam 2	Control	73	5,48	42,47	52,05
	Gynogens	1	100,00	0,00	0,00
Dam 3	Control	15	26,67	33,33	40,00
	Gynogens	5	80,00	20,00	0,00
Dam 4	Control	16	0,00	12,50	87,50
	Gynogens	1	100,00	0,00	0,00
Dam 6	Control	34	11,76	70,59	17,65
	Gynogens	14	21,43	64,29	14,29
Dam 7	Control	125	7,20	21,60	71,20
	Gynogens	19	47,37	26,32	26,32
Dam 8	Control	13	23,08	69,23	7,69
	Gynogens	1	100,00	0,00	0,00
Dam 10	Control	38	10,53	39,47	50,00
	Gynogens	8	37,50	12,50	50,00
Dam 11	Control	74	31,08	59,46	9,46
	Gynogens	47	85,11	14,89	0,00

4. Discussion

This study showed that the production of mitotic gynogenetic individuals was possible but resulted in surprisingly low yields of homozygous individuals and a delayed gonadal development. Several hypotheses can be put forward to explain these results that stand out from previous studies carried out on the European sea bass. Bertotto et al. (2005) obtained mitotic gynogenetic progenies with comparable low overall survival (0.07%) but with higher rate of homozygous individuals (2 fully homozygous progenies and 2 progenies with 1.5-2.8% of meiotic gynogenetics). As observed in this study, the resulting mitotic gynogenetics were viable and of normal phenotypic appearance. The overall low survival of mitotic gynogens may be attributed to developmental damage caused by the pressure shock (see part 1) and the expression of genetic load.

Though previous studies in the European sea bass showed higher rates of fully homozygous progenies (Bertotto et al., 2005), large proportions of meiotic gynogenetics among progenies produced by mitotic gynogenesis were previously observed in other studies (Komen and Thorgaard, 2007; Liu et al., 2012). Galbusera et al. (2000) obtained up to 86% meiotic individuals after induction of mitotic gynogenesis in the African catfish (*Clarias gariepinus*) and explained it by a prevalent mortality of mitotic gynogenetics and the occurrence of spontaneous retention of the second polar body in surviving gynogenetic progenies which benefited from their heterozygous status and the survival of which was probably overestimated. Furthermore, Bertotto et al. (2005) did not assess the efficiency of

their microsatellite markers to distinguish mitotic from meiotic individuals. As demonstrated in this study, some microsatellite markers have high recombination frequencies due to their telomeric position on chromosomes while others have intermediate positions and variable recombination rates. *Labrax-17*, as an example of this work, has a highly telomeric position with a high locus-centromere distance (48.9 cM) which makes it a diagnostic marker for the verification of homozygosity and mitotic status. This study is to our knowledge, the first to analyze gene-centromere-map distances of 12 microsatellite loci used in studies on the European sea bass. In studying the genetic mechanisms for the occurrence of unreduced gametes and atypical reproduction, the development of sensitive microsatellite genetic markers is of high interest and a prerequisite. The preliminary analyze of microsatellite locus position on chromosomes also allowed the study of different hypotheses proposed to explain the residual heterozygosity observed in mitotic gynogenetic progenies. Biparental progenies arise from normal eggs fertilized by sperm that escaped genome inactivation and where the pressure shock was ineffective for genome duplication (Bertotto et al., 2005). However, the very low levels of paternal contamination except for the first female treated in gynogenesis proved the high efficiency of sperm UV-irradiation and the biparental progenies of dam 1 probably resulted from non- or sub-optimally irradiated sperm due to a mishandling of the mechanical stirrer during the irradiation process. A possible explanation for residual heterozygosity could be a "maturation delay effect" in the egg causing the pressure shock to block the delayed second meiotic division rather than the first mitosis. Bertotto et al. (2005) observed that the fractions of meiotic gynogenetics among mitotic gynogenetic progenies increased when stripped eggs were of poor quality with low hatching rates and concluded that the adjustment of shock timing could not completely balance the maturational variability of eggs in the same batch. Quillet et al. (1991) proposed spontaneous gynogenesis through inhibition of meiosis I, while Komen et al. (1991) suggested a meiosis II non-disjunction origin to explain the contamination of heterozygous diploids among mitotic gynogenetic progenies in rainbow trout and carp respectively. Our results suggest the retention of the second polar body or a meiosis II non-disjunction origin of the unexpected heterozygous meiotic gynogenetics. The comparisons of homozygous and heterozygous individuals for each microsatellite marker in meiotic gynogenetic progenies and the heterozygous gynogens produced with a late pressure shock confirmed this hypothesis. Furthermore, *Dla-16* which is a centromeric microsatellite locus was a diagnostic marker for analyze of the origin of heterozygosity in our gynogenetic progenies and its recombination frequency was in accordance with a non-disjunction during meiosis II. Spontaneous and induced meiogynogenesis are probably rare events but since mitotic gynogenetic individuals are also rare, the effect on the residual heterozygosity could be important (Galbusera et al., 2000; Quillet et al., 1991). The significant difference observed between dams for *Labrax-3*, a microsatellite locus having an intermediate position on chromosomes, could indicate differences in the recombinations frequency in the linkage group 13 due to variations of the chiasma position or could be the effect of random sampling of larvae.

In this study, growth seemed unaffected by the late pressure shock treatment but the gonadal development was clearly different between controls and gynogenetically treated progenies. GSI was extremely low and gonad development delayed in the progenies which

received a pressure shock regardless of their genetic status. Gonadal development has been previously reported to be repressed or impaired in gynogenesis experiments on different species including common carp (Bongers et al., 1997b; Komen et al., 1992b) and rainbow trout (Krisfalusi et al., 2000). Meiotic gynogenesis in the European sea bass was reported to have little effect on growth and gonadal development, growth performances were similar between diploid controls and meiotic gynogenetic progenies but differences were observed between progenies from the various breeders, suggesting a genetic origin (maternal effect) more than effects of gynogenesis induction (Felip et al., 2002; Francescon et al., 2005). Impaired ovarian development has been previously explained by inbreeding depression (Komen et al., 1992b) or incomplete inactivation of the paternal genome (Krisfalusi et al., 2000). In this study, the delayed gonadal development could be a consequence of the pressure shock applied to disrupt the first mitosis, i.e. a shock applied much later than for the production of meiogens.

To our knowledge, this is the first report on characterization of gynogenetic progenies after a late pressure shock applied to induce endomitosis and the work presented here led to some crucial considerations regarding attempts at producing clonal lines in the European sea bass using this approach. Possible ways to improve the technique of gynogenesis probably rely on the choice of breeders (females with high egg quality) and the handling of gametes to reduce to the minimum the variability in eggs development. Several studies already pointed out the degree of genetic and/or physiological variability between individuals involved in the methods used for gynogenesis. As sperm is UV-irradiated, the genetic contribution of males should theoretically be zero and the paternal effects restricted only to egg fertilization. Egg quality and maternal source have already been reported to affect induced gynogenesis success in several species including chinook salmon (*Oncorhynchus tshawytscha* Walbaum, 1792) (Levanduski et al., 1990), carp (Komen et al., 1991), rainbow trout (Palti et al., 1997; Quillet et al., 1991) and the European sea bass (Peruzzi and Chatain, 2000), with effect on egg ripeness and physiology. It is likely that some of the techniques used for artificial fertilization including assessment of oocyte maturation, hormone stimulation and time of stripping might not be fully mastered or adapted to the variability between females (Peruzzi and Chatain, 2000). In the sea bass, the ovulation process is slow and spreads over a wide time interval (3-4 days), varying between individuals, thus the prediction of the optimal time for stripping to obtain the eggs of highest quality can be difficult (Francescon et al., 2004). One of the critical aspects in the induction of gynogenesis is the onset time of the pressure shock. To optimize its efficacy, it was adjusted to the onset time of the first embryonic cleavage as previous studies showed that sea bass females could initiate the first cleavage furrow with significant differences up to 15 min (Bertotto et al., 2005; Francescon et al., 2004). Furthermore, the high variability in egg quality among breeders is difficult to assess and the measure of indirect parameters like the appearance of the first furrow which was inversely correlated with larval hatching in a previous work on the European sea bass (Francescon et al., 2004) could be informative on the quality of stripped eggs. Although the yields of doubled haploids were low, some homozygous individuals were produced and further work is required to assess their fertility and their ability to generate different clonal lines following a second round of gynogenesis.

Conclusion

Conclusion

1. Clonal lines of European sea bass and general considerations

1.1. Difficulties of uniparental reproduction

The different studies performed during this thesis led to several considerations, especially about the difficulties encountered when attempting to produce gynogenetic and androgenetic progenies in fish. The yields of homozygous progenies are usually very low and though clonal lines can be a very useful tool, the investment in terms of time and manipulations has to be considered in the development of such inbred lines.

As demonstrated in this thesis, eggs from different species have different characteristics that should be a priority concern when induction of androgenesis in a new species is performed. The biological specificity of different eggs has to be taken into account especially for the choice of irradiation method used to inactivate the maternal genome. UV rays are easier to handle compared to other ionizing radiations, but some eggs like the European sea bass ones seem difficult to genetically inactivate. Other methods like ionizing radiations or interspecific androgenesis could be alternatives towards the successful induction of androgenesis in this or other fish species.

The occurrence of UV screening compounds like MAAs and gadusol was one of the hypotheses proposed to explain the low success of androgenesis in the European sea bass, but the study led on 21 fish species with benthic or pelagic eggs showed some species specificities. No clear relation could be established between the occurrence of UV screening compounds, the reproductive behavior (egg characteristics) and a possible tolerance to UVR. Nevertheless, while MAAs seemed to be more phylogenetically specific, gadusol seemed ubiquitous among the different taxonomic groups. The occurrence of gadusol in eggs from species where successful androgenesis had been reported led to reconsider one the hypotheses proposed to explain the low success in the European sea bass and further studies should aim at characterizing the extent of UV damages and possible DNA repair mechanisms. DNA repair mechanisms could, indeed, be highly efficient in marine fish species with pelagic eggs and comparisons of DNA damage between UV-irradiated and non-irradiated eggs over time in different fish species, using comet assays and UV-endonuclease digestion for example, could provide information on the mechanisms underlying the high disparity of results obtained in the present androgenesis experiments.

Gynogenesis had been and has been performed successfully in the European sea bass, following established procedures. The main considerations are in the nature of the progenies obtained: few fully homozygous individuals overshadowed by the presence of high numbers of meiotic gynogenetic individuals with residual heterozygosity. This type of individuals have a higher fitness due to their heterozygous condition and their presence can lead to an overall relative low success in terms of pure homozygous mitotic gynogenetic individuals. The use of markers possessing a high discriminating power is therefore of crucial importance for the

identification of clonal founders. Moreover, putative damage induced by chromosome manipulations, as observed in gonad development, may result as a consequence of the irradiation and the diploidizing procedures employed. The extent of such damages in the mitotic gynogenetic progenies obtained during this thesis is still unknown, and the fecundity of the produced clonal founders remains to be verified as it cannot be assessed for some yearsby methods which do not require the slaughtering of fish.

Chromosome manipulations have to be performed thoroughly and as demonstrated during this thesis, high quality of gametes is a prerequisite for the success of uniparental reproductions. Egg and sperm characteristics should be evaluated before any further attempts at gynogenesis or androgenesis induction so that biological specificities could be overcome or methods adapted.

1.2. How to maintain clonal lines in order to use them in aquaculture research

The production of inbred strains of fish is performed by a gynogenetic or androgenetic reproduction of homozygous individuals. The inbred strains can be maintained by sex-reversing a proportion of the progeny in fish species having differentiated sex chromosomes. In many fish species, the phenotypic sex can be altered by the use of hormones (via treated food or by hormonal immersion) since the differentiation of gonads occurs well after hatching, tipically around metamorphosis (Pandian and Sheela, 1995). Like most fish, the European sea bass does not possess differentiated sex chromosomes or sex-linked markers, and parental and environmental (temperature) effects on sex-ratios have been demonstrated. As such, both male and female progenies can be obtained even following uniparental reproductions. Crossing two identical homozygous genotypes will allow maintenance of new stocks of the initial genotypes obtained after 2 gynogenetic or androgenetic successive reproductions provided that reproductive problems do not occur among breeders.

Heterozygous clones can also be produced by crossing two homozygous individuals from two different inbred strains. From a produced hybrid, recombinant inbred strains can be produced by androgenesis and/or gynogenesis and repeated backcrossing of a hybrid to an inbred strain. These kinds of manipulations produce congenic strains that can be used to examine single gene effects on a standardized genetic background (Bongers et al., 1998).

1.3. Uses of clonal lines for aquaculture research: pros and cons

The advantages of clonal lines in research have been described in the introduction. Briefly, clonal lines improve the quality of experiments in terms of replicability, reproducibility, and repeatability. The characterization of variability factors and the reduction of the number of fish used are direct benefits of the reduced genetic variance. Clonal lines are

used in many research areas and their use in fish research already allowed a better understanding of genetics/genomics of some 'model' species.

Some disadvantages result from the increased susceptibility of inbred strains to environment. For characters with a high heritability, which means that the phenotypic variance is mainly due to genetic variance, clonal lines are likely to be more uniform than outbred lines. For characters with a low heritability, the previous assumption may not apply anymore. The higher susceptibility of clones to environmental changes can offset the reduced genetic variance and increase the total phenotypic variance. The second generation homozygous individuals, resulting from a uniparental reproduction of homozygous breeders, can display large proportions of deformed individuals and high residual variations. Bongers et al. (1998) explained the origin of the residual variation by three components of environmental variance: first, the inter-individual variation consisting of the 'true environmental variance' to which homozygous populations are susceptible; then, the intra-individual variability also named developmental instability; to finish with the variability generated by chromosome manipulations on cytoplasmic components, also named embryonic damage. They found that the embryonic damage was the main source of variation and advise to use chromosome manipulation only for the production of homozygous breeders. They also pointed out the fact that fish are poikilothermic; this makes them more susceptible to environmental sources of variation compared to homeothermic animals.

Another disadvantage of clonal lines is the time required to produce clonal lines (2 generations with at least 3-4 years between each generation) and the small numbers of clones that will reproduce due to inbreeding depression, gonadal defects and poor egg quality.

2. A critical aspect of chromosome manipulations and artificial reproduction: the variability of egg quality

Gamete quality is one of the key factors known to influence the success of gynogenesis and androgenesis. The need for high egg quality in chromosome manipulations has been pointed by several authors (Bongers et al., 1997a; Grimholt et al., 2009; Komen et al., 1992b) and was also a crucial consideration highlighted during the different manipulations performed in this work.

2.1. Egg quality variability

The two principal measures to assess egg quality are survival and hatching rate, but these measures do not give information on the factors determining egg quality. Egg quality is difficult to define but Brooks et al. (1997) gave a definition which corresponds to the goal of this conclusion: 'The quality of an egg is determined by the intrinsic properties of the egg itself, by its genes, and by the maternal mRNA transcripts and nutrients contained within the

yolk, all of which are provided by the mother'. The environmental conditions during incubation are also encompassed in the egg quality. Studies on gamete quality are reviewed by Brooks et al. (1997), and Bobe and Labbe (2010). Briefly, wild fish display seasonal variability in egg quality and gamete quality is one of the major bottlenecks in the production of cultured stocks too. In multiple spawning fish, the quality of eggs produced in different batches over a single spawning season varies greatly, even under similar culture conditions. The physiology of breeders and the husbandry practices also have influence on the final quality of eggs.

2.2. Factors affecting quality

2.2.1. Hormonal treatments

As reviewed by Brooks et al. (1997), attempts to control the ovarian cycle with hormones in fish led to various success, some species showing no detrimental effect of hormonal stimulation while in other fish species the treatment led to bad quality eggs. In the European sea bass, Fauvel and Suquet (1998) found 4 stages of oocyte prematuration and observed that oocytes were released 72h after hormonal injection whatever the prematuration stage. But, the total volume of spawned eggs, and the fertilization and hatching rates were higher when hormonal stimulation was induced at later stages of oocyte prematuration. An overmaturation of eggs was also observed in less than 3 hours after spawning. A study on the effect of different delivery systems loaded with gonadotropin-releasing hormone agonist (GnRHa) on spawning induction, and egg and larval quality in the European sea bass showed that all treatments induced spawning 3 days later but with an egg quality relatively lower compared to spontaneously spawning fish (Fornies et al., 2001). More recently, analyses of rainbow trout egg transcriptome showed that hormonal induction of ovulation induced differences in the egg mRNA abundance of specific genes but the impact on embryonic development is unknown (Bonnet et al., 2007a). The ovarian stage at the time of spawning induction has a major impact on the developmental capacities of eggs and the differences between females in terms of oocyte development stage can lead to high variability in egg quality (Bobe and Labbe, 2010).

2.2.2. Fish age

Some studies on the effect of age on egg quality showed that quality increased with age, though no studies reported the effect of 'old age'. In rainbow trout and the European sea bass, females produced better egg quality in their second reproductive season, based on survival to eyeing in rainbow trout eggs and survival to hatching in the sea bass (Brooks et al., 1997).

2.2.3. Environmental factors

Generally, egg quality is higher in wild fish compared to captive breeders. Environmental factors are believed to be the main explanation for the decrease of egg quality in captivite broodstock as shown hereafter.

Diet

Several studies performed on the European sea bass showed that eggs from broodstock fed commercial diets had a lower survival than eggs from broodstock fed whole fish diets (Cerda et al., 1995; Navas et al., 1997). A positive relationship was observed between the fatty acid composition of the broodstock and the fatty acid composition of the egg. Other nutrients were shown to affect fecundity including vitamin E and ascorbic acid, with deficiencies in these compounds resulting in eggs with lower quality and higher mortality (Brooks et al., 1997; Izquierdo et al., 2001).

Photoperiod

Photoperiod manipulation is commonly used to advance or delay the reproductive season. The effects of photoperiod manipulations on egg quality seem difficult to characterize, as some studies on the European sea bass showed either a detrimental effect or either no effect on survival (Brooks et al., 1997; Carrillo et al., 1989). However, more recent studies on rainbow trout showed that photoperiod manipulation of spawning date can induce high defects on egg quality and that the transcriptome of unfertilized eggs can also be affected by such alteration. Overall, differences in expressed genes can be reflective of different developmental capacities (Bonnet et al., 2007a; Bonnet et al., 2007b).

Temperature

Water temperature during spawning and incubation is one of the most important factors affecting egg quality. Temperature affects metabolism, activity and structure of the developing embryo. Though spawning in the European sea bass seems to be independent of the temperature curve, the gonad maturation process needs a minimum temperature to be fully completed (Vinagre et al., 2009). Temperature has a high effect during incubation, affecting growth rate and differentiation. Three different stages of development with different thermal susceptibilities have been indicated: firstly, a period of low thermal stability during early development from fertilization to gastrulation; then, increased stability; to finish with a low stability to the end of embryonic development (Brooks et al., 1997). Temperature is an especially critical point in chromosome manipulations, the strict control of a constant temperature during all manipulations is a critical factor for the adjustment of the diploidizing

shock, corresponding to a specific timing in embryonic development (Francescon et al., 2004).

Husbandry

The main effects on egg quality due to husbandry practices are a possible induced stress of breeders and egg overripening. Disturbance and/or handling of breeders can affect the timing of reproduction or completely inhibit spawning (Schreck et al., 2001). In the brown and rainbow trouts, the confinement experienced during the final stages of maturation and periods of acute stress do not only disrupt the reproductive endocrinology but also lead to lower progeny survival rates (Campbell et al., 1994). One of the crucial point for chromosome manipulations is the time interval, between spawning (stripping) and fertilization, that affects the 'ripeness' of eggs. Overripening of eggs is perhaps the most common reason for bad egg quality (Brooks et al., 1997). A study on the effect of timing of stripping and female variability on the viability of gynogenetic and control diploid pink salmon (*Oncorhynchus gorbuscha* Walbaum, 1792) showed that the timing of stripping by itself has no influence on the viability of embryos but the female and timing of stripping-female interaction significantly affected the survival rate (Crandell et al., 1995). Moreover, a study performed on rainbow trout showed the negative effects of post-ovulatory oocyte ageing and temperature on egg quality; an increase of spontaneous triploidy was associated with increased 'ripeness' and high temperature (Aegerter and Jalabert, 2004). Any attempt at artificial fertilization, especially in chromosome manipulations, must carefully consider possible side-effects of overripening and control in consequence the timing of stripping.

2.2.4. Genetic influence

Parental genetics strongly influence fecundity and egg quality. Variability between females can for example be demonstrated by the timing of ovulation which is never exactly the same. For egg quality, the effects of parental genetics can be difficult to distinguish because of the environmental effects and their interactions. However, parental influence strongly affects fertilization, survival during incubation and hatching in the European sea bass (Saillant et al., 2001a). In common carp, a study in homozygous families showed that egg quality is genetically determined and that selection of females with high egg quality is possible (Bongers et al., 1997a).

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Supplementary files

Supplementary files

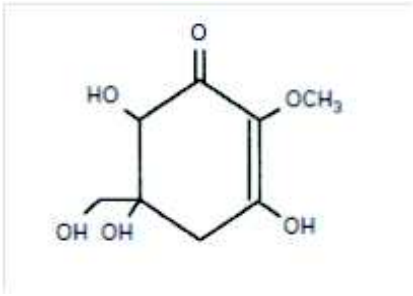
Supplementary file S1. Positive control for UV-irradiation device.

The efficiency of the UV-device (section 2.2) was tested on Nile tilapia, a species for which the successful induction of androgenesis has been reported (Karayücel et al., 2002; Myers et al., 1995). For this purpose, domesticated broodstock *O. niloticus* of Bouaké strain aged 3 years and weighting 150 to 250 g were obtained from Cirad (Montpellier, France) and transported to Ifremer Palavas-les-Flots where they were maintained and handled using standard hatchery strip-spawning techniques. A single ready to spawn female was stripped and the egg batch (around 1000 eggs) equally divided into individual Petri dishes, diluted in 10 ml freshwater and UV irradiated at 10.5 $mJ.cm^{-2}.min^{-1}$ for 0, 1, 2, 4 or 6 min. UV treated and control (unirradiated) eggs were fertilized, after removal of water, by adding 200 µl sperm diluted 1:10 in Modified Fish Ringer's solution (NaCl, KCl, $NaHCO_3$ and $CaCl_2$) (Sarder, 1998) and activated by addition of freshwater 1 min later. Treated and control eggs were reared in individual flow-through 2 L incubators at 28°C until hatching (96 hpf). All hatched larvae from UV and control treatments were rapidly separated from the yolk sac under a dissecting microscope and treated for flow cytometry as described previously (section 2.5)

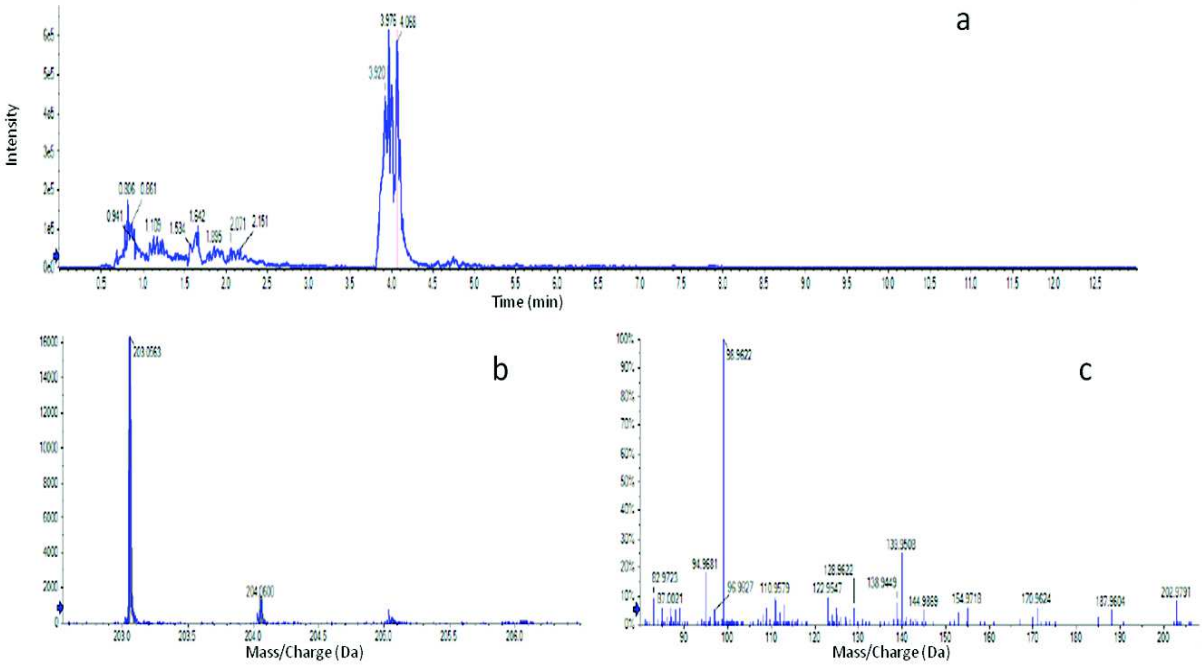
Supplementary file S2. HPLC elution gradient used for the separation of metabolic fingerprints.

Retention time (min)	Flow rate ($ml.min^{-1}$)	% A	% B
Initial	0.4	5	95
1	0.4	5	95
8	0.4	45	55
10	0.4	45	55
10.1	0.4	5	95
15	0.4	5	95

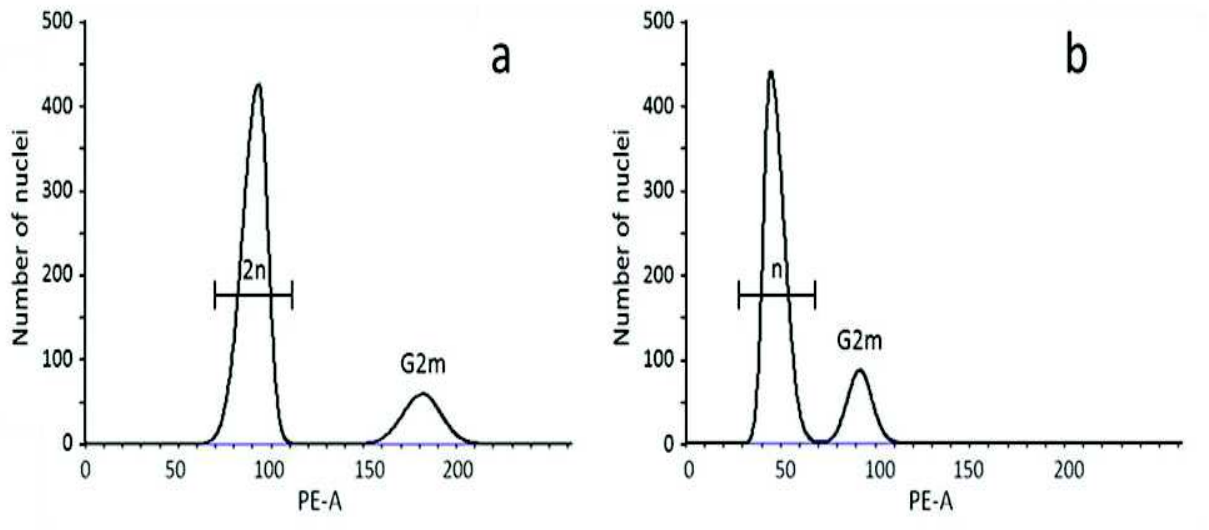
Supplementary file S3. Chemical structure of gadusol. λmax = 268 nm at pH 2.5, λmax = 294 nm at pH 7.



Supplementary file S4. Spectra obtained from HPLC analyses for the identification of gadusol. a) Extracted ion chromatogram for gadusol, m/z 203.0561 retention time 4 min. b) TOF MS spectrum from 4.154 to 4.176 min. c) TOF MS/MS spectrum from 4.068 min.



Supplementary file S5. Representative examples of flow-cytometry histograms obtained from nuclear suspensions (5-10000 counts) of Propidium Iodide (PI) stained *O. niloticus* larvae. a) control diploid (2n) larva (CV: 6.5%); b) haploid (n) larva produced with a UV-dose of 42 mJ.cm^{-2} (CV: 7%). DNA values on the X-axis are reported in arbitrary units expressed as fluorescent channel numbers (PE-A). G2 represents mitotic peaks.



Supplementary file S6. Androgenesis in Nile tilapia, *O. niloticus*.

Flow-cytometry analyses showed that 4 min irradiation with a UV dose rate of 10.5 mJ.cm^{-2}.min^{-1} was an effective treatment to induce androgenetic haploids in the Nile tilapia (Fig. S4). Of the two surviving larvae at 96 hpf out of around 150 fertilized eggs, both were haploid. All control larvae assayed (n=10) were diploid, whereas 1 and 2 min irradiation resulted in four and one diploid larvae at hatching, respectively. After 6 min irradiation, no larval survival was observed.

Supplementary file S7. Morphometric measures (in mm) performed during larval rearing. Data are presented by mean ± standard deviation (STD). The different traits measured were total length (Lt), standard length (Lst), head length (HL), head depth (HD) and maximal body height (Hm).

		Control	Gynogenesis
4 dph	Lt	5.51 ± 0.51	5.65 ± 0.53
	Lst	5.28 ± 0.52	5.41 ± 0.55
	HL	0.93 ± 0.10	0.93 ± 0.10
	HD	0.63 ± 0.05	0.64 ± 0.05
	Hm	0.88 ± 0.14	0.97 ± 0.12
11 dph	Lt	6.36 ± 0.25	6.38 ± 0.44
	Lst	6.09 ± 0.25	6.09 ± 0.48
	HL	1.28 ± 0.08	1.25 ± 0.10
	HD	0.84 ± 0.05	0.86 ± 0.07
	Hm	0.93 ± 0.08	0.90 ± 0.14
31 dph	Lt	9.93 ± 1.70	11.67 ± 1.57
	Lst	9.31 ± 1.44	10.70 ± 1.40
	HL	2.09 ± 0.34	2.35 ± 0.30
	HD	1.47 ± 0.29	1.80 ± 0.32
	Hm	1.37 ± 0.31	1.65 ± 0.30

Supplementary file S8. Verification of maternal genetic inheritance and paternal contamination in gynogenetic offspring. Discriminating alleles between the two parents for each microsatellite marker locus are in bold.

		Marker loci											
Fish	N	*Dla-3*	*Dla-6*	*Dla-16*	*Dla-104*	*Dla-105*	*Dla-106*	*Dla-112*	*Dla-119*	*Labrax-17*	*Labrax-29*	*Labrax-3*	*Labrax 8*
Dam 1		**215**/223	119/0	241/241	**262/264**	**155/176**	**245/245**	108/108	**222/222**	**130**/0	111/**145**	**120/136**	**210/228**
Male 1		**217**/223	0/0	241/**251**	**266/266**	**147/147**	**272/272**	108/**112**	**220/233**	**126/134**	111/**137**	**116/170**	**220/232**
Progeny 1	**528**	individuals showing complete paternal contamination (biparental)											
	40	individuals showing incomplete paternal contamination											
	82	individuals showing no paternal contamination											
Dam 2		**223/225**	119/119	**262/270**	262/**264**	**151/155**	**245/253**	**108/116**	**220/222**	**115/177**	**156/160**	**134/142**	**195/228**
Male 2		**232/236**	119/119	**268/268**	262/**266**	**145/145**	**266/272**	**112/112**	**233/259**	**126/126**	**111/139**	**175/175**	**210/220**
Progeny 2	**6**	individuals showing no paternal contamination											
Dam 3		**225/225**	**119/119**	**241/262**	**264/264**	**151/172**	**245/245**	108/112	220/**262**	**115/115**	**160/160**	**136/145**	**224/228**
Male 3		**217/232**	**123/123**	**239/251**	**262/266**	**145/147**	**266/272**	108/112	220/220	**134/134**	**111/139**	**116/170**	**220/232**
Progeny 3	**7**	individuals showing no paternal contamination											
Dam 4		**209/228**	**119/127**	239/**253**	**264/264**	**172/174**	**245/253**	108/108	220/**239**	**141/177**	**145/160**	**136/142**	**224/236**
Male 4		**217/232**	**123/123**	239/**268**	**266/266**	**147/159**	**272/272**	108/**120**	220/220	**126/134**	**137/139**	**116/116**	**210/210**
Progeny 4	**18**	individuals showing no paternal contamination											
Dam 6		**223**/232	123/123	251/**268**	262/**266**	145/**159**	266/**272**	108/108	257/**259**	126/138	137/**139**	116/116	210/210
Male 6		232/232	123/123	251/251	262/262	145/145	266/266	108/**120**	**220**/257	126/138	**111**/137	116/116	210/**232**
Progeny 6	**17**	individuals showing no paternal contamination											
Dam 7		**217/223**	119/119	**241/257**	264/266	**145/147**	**262/266**	108/**112**	220/**243**	115/**145**	**131/137**	136/**155**	**210/222**
Male 7		**209/225**	119/**127**	**237/262**	264/266	**155/174**	**245/245**	108/108	220/**227**	115/115	**111/156**	136/**142**	**195/197**
Progeny 7	**42**	individuals showing no paternal contamination											
	1	individual showing incomplete paternal contamination											
Dam 8		**232/238**	119/123	**227/243**	**266/266**	**159/167**	**270/270**	**104/112**	220/**255**	**134**/141	137/**163**	**151/177**	**210/210**
Male 8		**219/223**	119/123	**245/260**	**264/264**	**136/145**	**245/264**	**108/108**	220/**253**	**121**/141	137/**154**	**136/169**	**193/226**
Progeny 8	**1**	individual showing no paternal contamination											
Dam 10		**227/227**	119/119	**223/229**	262/**274**	145/**155**	**262/262**	**108/108**	220/**235**	115/**136**	154/**175**	**159**/170	**195/222**
Male 10		**225/238**	119/119	**243/253**	262/**266**	145/145	**270/270**	**112/116**	220/**237**	115/**132**	154/**162**	170/**185**	**210/224**
Progeny 10	**9**	individuals showing no paternal contamination											
Dam 11		209/**223**	119/0	**239/243**	264/**280**	145/**157**	**262/262**	**108**/112	**219**/262	115/**141**	137/**160**	**136/157**	210/210
Male 11		209/**217**	119/119	**227/266**	264/**266**	145/**170**	**270/274**	**104**/112	**254**/262	115/**138**	**111**/137	**155/173**	210/**228**
Progeny 11	**62**	individuals showing no paternal contamination											

Supplementary file S9. Verification of homozygosity status in gynogenetic progenies. Discriminating markers (heterozygous in the female parent) are in bold. MM prob represents the probability of considering a meiotic individual being mitotic. RR Ggp are the recombination rates calculated on meiotic gynogenetic progenies.

		Marker loci											
	N	*Dla-3*	*Dla-6*	*Dla-16*	*Dla-104*	*Dla-105*	*Dla-106*	*Dla-112*	*Dla-119*	*Labrax-17*	*Labrax-29*	*Labrax-3*	*Labrax 8*	
RR Ggp		0.77	0.21	0.02	0.77	0.63	0.73	0.63	0.35	0.98	0.46	0.44	0.78	MM prob
Dam 1		**215/223**	119/0	241/241	**262/264**	**155/176**	245/245	108/108	222/222	130/0	**111/145**	**120/136**	**210/228**	1.30×10^{-3}
Progeny 1	12	individuals showing homozygosity for all markers												
	70	individuals showing recombinations												
Dam 2		**223/225**	119/119	262/270	**262/264**	**151/155**	245/253	**108/116**	**220/222**	**115/177**	**156/160**	**134/142**	**195/228**	6.26×10^{-6}
Progeny 2	5	individuals showing homozygosity for all markers												
	1	individual showing recombinations												
Dam 3		225/225	119/119	241/262	264/264	**151/172**	245/245	**108/112**	**220/262**	115/115	160/160	**136/145**	**224/228**	1.10×10^{-2}
Progeny 3	1	individual showing homozygosity for all markers												
	6	individuals showing recombinations												
Dam 4		**209/228**	**119/127**	239/253	264/264	**172/174**	**245/253**	108/108	**220/239**	**141/177**	**145/160**	**136/142**	**224/236**	1.57×10^{-5}
Progeny 4	17	individuals showing homozygosity for all markers												
	1	individual showing recombinations												
Dam 6		**223/232**	123/123	251/268	**266/262**	**145/159**	266/272	108/108	**257/259**	**126/138**	**137/139**	116/116	210/210	1.37×10^{-4}
Progeny 6	1	individual showing homozygosity for all markers												
	16	individuals showing recombinations												
Dam 7		**217/223**	119/119	241/257	**264/266**	**145/147**	262/266	**108/112**	**220/243**	**115/145**	**131/137**	**136/155**	**210/222**	6.26×10^{-6}
Progeny 7	3	individuals showing homozygosity for all markers												
	39	individuals showing recombinations												
Dam 8		**232/238**	**119/123**	227/243	266/266	**159/167**	270/270	**104/112**	**220/255**	**134/141**	**137/163**	**151/177**	210/210	9.78×10^{-5}
Progeny 8	1	individual showing recombinations												
Dam 10		227/227	119/119	223/229	**262/274**	**145/155**	262/262	108/108	**220/235**	**115/136**	**154/175**	**159/170**	**195/222**	7.36×10^{-5}
Progeny 10	9	individuals showing recombinations												
Dam 11		**209/223**	119/0	239/243	**264/280**	**145/157**	262/262	**108/112**	**219/262**	**115/141**	**137/160**	**136/157**	210/210	2.85×10^{-5}
Progeny 11	62	individuals showing recombinations												