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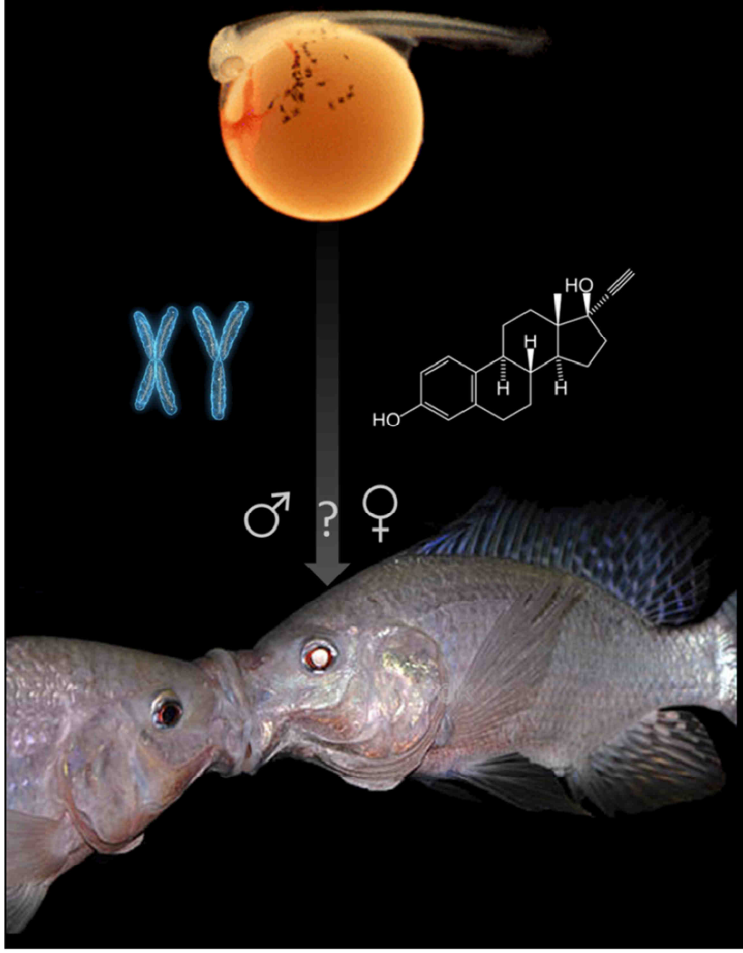
Biologie de la reproduction et analyse du déterminisme et de la différenciation du sexe à des stades précoces chez le tilapia du Nil, *Oreochromis niloticus*



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Résumé

La reproduction des poissons téléostéens se caractérise par une diversité des types de sexualité (gonochorisme vs hermaphrodisme) et des systèmes de déterminisme du sexe (génétique et/ou environnemental), ainsi que par une plasticité du contrôle génétique et endocrinien de la différenciation sexuelle incomparables parmi les autres vertébrés. Le tilapia du Nil (*Oreochromis niloticus*) est un cichlidé gonochorique possédant un système chromosomique de déterminisme sexuel à homogamétie femelle XX/XY, complété par un déterminisme environnemental, les hautes températures pouvant modifier le développement phénotypique du sexe. L'intérêt porté à cette espèce en aquaculture à conduit au développement de différentes techniques de contrôle du sexe (par traitement hormonal) permettant la production de poissons présentant différentes combinaisons de phénotype/génotype sexuels (mâles XX, XY, YY, femelles XX, XY et YY). Ces derniers constituent des outils majeurs de l'étude du déterminisme et de la différenciation du sexe et soulèvent de nombreuses questions concernant les interactions génotype/phénotype sexuels chez cette espèce. Au regard de ces considérations, nous nous sommes fixés 2 objectifs principaux :

1. Déterminer l'influence du génotype sexuel sur certains aspects liés à la biologie reproductive chez le tilapia du Nil tels que la qualité du sperme, les taux de stéroïdes sexuels (testostérone, T ; 17β-œstradiol, E2 ; 11-cétotestostérone, 11KT) et les comportements agressifs. Le génotype sexuel ne semble pas avoir d'influence sur la qualité gamétique des mâles, puisqu'aucune différence n'est observée entre les individus XX, XY et YY, alors que paradoxalement, les taux de 11KT sont plus élevés chez les mâles XX. De la même manière, les femelles présentent des taux croissants d'E2 chez les individus XX, XY et YY. Ces différences de taux de stéroïdes sexuels pourraient être liées aux taux d'agressivité (évalué par la quantification de huit comportements agonistiques dans des confrontations mâle-femelle) plus élevés des mâles XX et des femelles XY et YY et engendrer des perturbations des comportements reproducteurs. Des recherches complémentaires sont nécessaires d'une part, pour évaluer l'effet du génotype sur la qualité des gamètes des femelles et d'autre part, pour déterminer si les modifications phénotypiques observées sont liées à l'expression du génotype sexuel ou à des perturbations engendrées par les traitements hormonaux d'inversion sexuelle administrés durant la période de différenciation des gonades.

2. Explorer les mécanismes du déterminisme et de la différenciation à des stades précoces du développement. Grâce à la mise au point d'une technique d'inversion sexuelle ciblant les périodes embryonnaire et larvaire (avant 10 jpf) par des expositions courtes (4h) d'embryons âgés d'un jpf à des androgènes (11KT, 17α-méthyltestostérone), un inhibiteur de l'aromatase (Fadrozole) ou un œstrogène (17α-éthynylœstradiol), nous avons confirmé l'existence d'une période sensible de la différenciation sexuelle avant le développement des gonades. Les voies de différenciation phénotypique semblent être différentes en fonction du génotype, puisque la masculinisation des embryons XX est moins efficace (max 10%) que la féminisation d'embryons XY (max 91%). De la même manière, un probable effet chromosomique rend inefficace la féminisation d'embryons YY. La féminisation des individus XY est concomitante avec une augmentation des taux de T et d'E2 et d'expression de l'aromatase cérébrale à 4 jpf qui pourrait indiquer un rôle précoce du cerveau dans la différenciation sexuelle.

Dans l'ensemble, nos résultats nous permettent de suggérer, d'une part, que les premiers événements moléculaires de la différenciation sexuelle du tilapia sont initiés avant la formation des gonades, et d'autre part, que chez les individus sexuellement inversés, des différences sexuelles peuvent apparaître dans le cerveau avant ou en même temps que le développement des gonades et induire des modifications comportementales à l'âge adulte. La question du rôle possible du cerveau dans la différenciation des gonades reste ouverte.

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Partie 1

Introduction générale

1. Contexte général

La reproduction sexuée est le mode de reproduction le plus répandu chez les animaux, et en particulier chez les vertébrés (Otto & Lenormand, 2002). Au-delà de la production de gamètes par méiose et de leur union pour former un nouvel individu à partir du zygote, les processus évolutifs ayant abouti à la reproduction sexuée et à un dimorphisme gamétique ont engendré l'apparition de différences sexuelles non seulement dans les gonades, mais également dans les tissus somatiques jusqu'au comportement (Mank & Avise, 2009; Arbeitman et al., 2014). Que ce soit dans le choix des partenaires, les comportements reproducteurs, le mode de reproduction et de développement, jusqu'aux mécanismes cellulaires, génétiques et moléculaires du déterminisme et de la différentiation sexuels qui aboutiront au développement d'une nouvelle progéniture capable elle-même de se reproduire, les différents aspects développementaux, physiologiques et comportementaux de la reproduction façonneront la vie des individus sexués, et définiront un équilibre adaptatif des sexes à l'échelle d'une population.

Les mécanismes du déterminisme et de la différenciation sexuels sont étudiés dans des modèles appartenant aux principaux groupes de vertébrés (Schartl, 2004; Herpin et al., 2010) mais un intérêt grandissant s'est développé autour des poissons téléostéens en raison de l'extrême diversité de leurs modes de reproduction et de leurs systèmes de détermination du sexe, qu'ils soient génétiques et/ou environnementaux (Devlin & Nagahama, 2002). Il existe environ 27000 espèces de téléostéens représentant le groupe le plus riche et le plus diversifié de tous les vertébrés (Nelson, 2006). L'étude de la biologie reproductive et du déterminisme sexuel revêt un intérêt fondamental particulier pour comprendre et élucider les mécanismes biochimiques et évolutifs sous-jacents, et trouve également toute son importance dans la conservation de nos ressources écologiques et alimentaires. La dégradation physique et chimique des habitats aquatiques et la surexploitation halieutique menacent de nombreuses populations sauvages dont la compréhension du cycle de vie est indispensable pour mettre en place des mesures efficaces de protection et de gestion. Par ailleurs, la stagnation de la production halieutique et la demande alimentaire croissante ont amené un développement important de l'aquaculture au cours des dernières décennies. La maîtrise de la reproduction et le contrôle du sexe sont des éléments cruciaux pour la production aquacole en systèmes artificiels car ils permettent une meilleure gestion des stocks et une optimisation de la

production. Ces préoccupations d'ordre économique ont conduit, chez de nombreuses espèces, au développement de techniques exploitant la plasticité de la différenciation sexuelle et permettant d'inverser le sexe phénotypique, assurant la production de populations monosexes. Les techniques d'inversion sexuelle constituent par ailleurs un des outils les plus puissants dans l'étude des mécanismes du déterminisme et de la différenciation sexuelle, largement utilisées dans ce travail.

Les recherches présentées dans ce travail apportent une contribution aux connaissances de ce vaste domaine qu'est la biologie reproductive, en explorant certains aspects des processus développementaux du déterminisme et de la différentiation sexuels, et leur influence sur la biologie des géniteurs chez un téléostéen, le tilapia du Nil (*Oreochromis niloticus*), modèle remarquable non seulement du point de vue de la compréhension des aspects fondamentaux, mais également pour son intérêt économique dans la production aquacole.

2. Le déterminisme et la différenciation du sexe chez les poissons

2.1. Diversité des systèmes de détermination sexuelle

Selon la terminologie de Hayes (1998), le déterminisme du sexe désigne les mécanismes (génétiques et environnementaux) qui orientent la différenciation du sexe, tandis que la différenciation sexuelle représente le développement des testicules ou des ovaires à partir des gonades indifférenciées.

Chez les mammifères et les oiseaux, le sexe est génétiquement déterminé lors de la fécondation par la combinaison des chromosomes sexuels. Les mammifères ont un système hétérogamétique mâle, les individus XX se développent en femelles et les XY en mâles. A l'opposé, chez les oiseaux, le sexe hétérogamétique (ZW) est femelle et le sexe homogamétique (ZZ) mâle. Ce système apparemment stable n'est cependant pas la règle dans les autres groupes de vertébrés (Figure 1). De nombreux reptiles ont un déterminisme sexuel environnemental. Chez tous les crocodiliens, ainsi que chez de nombreuses espèces de tortues et certains lézards, la température d'incubation des œufs est l'unique facteur contrôlant la

différenciation gonadique. D'autres lézards ont un système XY ou ZW alors que tous les serpents ont un déterminisme strictement génétique de type ZW (Schartl, 2004). Les anoures et les urodèles présentent un déterminisme génétique XY ou ZW et chez certaines espèces, des températures extrêmes peuvent expérimentalement affecter le déterminisme génétique, mais rien n'indique que ce facteur soit un déterminant sexuel dans la nature (Herpin et al., 2010; Schartl, 2004).

Parmi les vertébrés, les poissons présentent la plus grande diversité dans les systèmes de déterminisme du sexe. Celui-ci peut être strictement génétique, environnemental ou une combinaison des deux. Le déterminisme génétique peut-être de type XY ou ZW, avec ou sans l'influence de gènes autosomaux, ou plus compliqué comme des systèmes polygéniques sans chromosomes sexuels, présentant un seul (XO, ZO) ou au contraire de multiples chromosomes sexuels (X_1X_2Y, XY_1Y_2, XWY) (Devlin & Nagahama, 2002; Schartl, 2004). A la différence des mammifères qui semblent avoir hérité leur déterminisme génétique d'un ancêtre commun, les chromosomes sexuels sont apparus de nombreuses fois chez les poissons et évoluent rapidement. On retrouve ainsi des espèces très proches avec des systèmes différents comme *O. niloticus* et *O. aureus* qui possèdent respectivement des chromosomes XY et ZW ou comme le platy *Xiphophorus maculatus* chez qui ces 2 systèmes génétiques ont été observés dans des populations différentes de la même espèce (Mank & Avise, 2009).

Chez de nombreuses espèces, le déterminisme environnemental est lié à la température mais d'autres facteurs comme le taux d'oxygène, le pH ou la structure sociale peuvent également orienter la différenciation sexuelle (Baroiller et al., 1999, 2009b; Devlin & Nagahama, 2002). Chez la plupart des espèces thermosensibles, les hautes températures induisent un développement mâle et les basses températures un développement femelle. Il existe une période sensible au cours du développement durant laquelle la température peut agir pour orienter la différenciation sexuelle (Baroiller & D'Cotta, 2001; Ospina-Álvarez & Piferrer, 2008).

La diversité des systèmes de détermination du sexe s'exprime aussi dans les différents types de sexualité chez les poissons. Tandis que le gonochorisme est la règle chez les autres vertébrés, il existe des espèces de poissons gonochoriques et hermaphrodites (protandres, protogynes ou simultanés). Un cas d'autofécondation a même été rapporté chez le

cyprinodonte *Rivulus marmoratus* (Schartl, 2004) et certaines espèces peuvent pratiquer la parthénogenèse (Devlin & Nagahama, 2002).

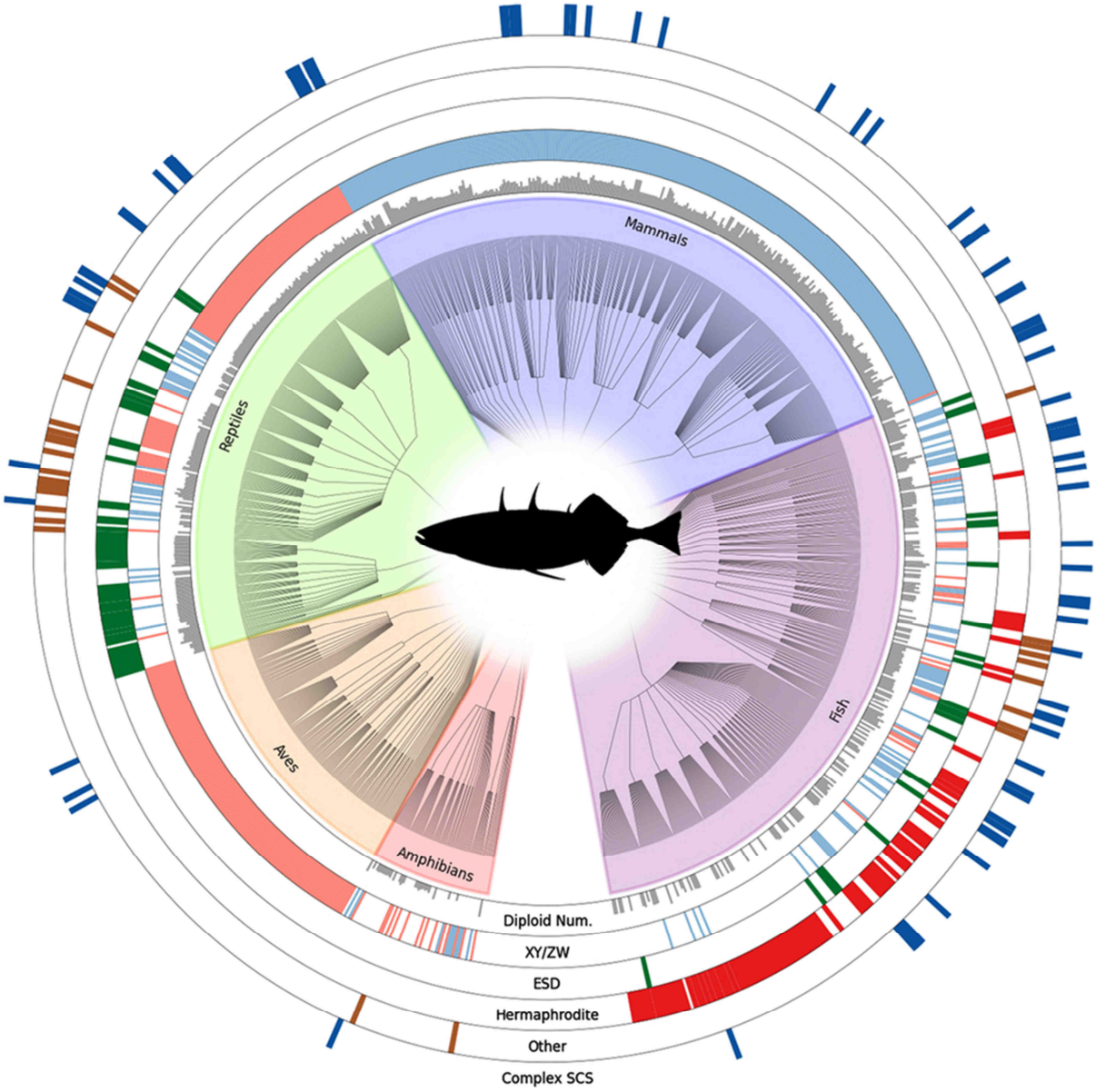

Figure 1. Distribution des systèmes de déterminisme du sexe chez les vertébrés basée sur l'analyse de 2145 espèces. La hauteur des barres grises dans le cercle intérieur représente le nombre de chromosomes à l'état diploïde. Les autres cercles représentent la présence ou l'absence de l'attribut indiqué à la base du cercle. Le cercle XY/ZW est coloré en bleu pour les systèmes XY et en rose pour les ZW. ESD : déterminisme environnemental du sexe. Le cercle 'Other' regroupe la parthénogenèse, la gynogenèse et l'hybridogenèse. Complex SCS : systèmes chromosomiques complexes (p. ex. X_1X_2Y) (The Tree of Sex Consortium, 2014).

2.2. Contrôle génétique de la différenciation sexuelle

2.2.1. Déterminants majeurs du sexe

Chez les mammifères euthériens, le gène *sry*[1] lié au chromosome Y initie une cascade d'expression génique mâle dans les gonades des embryons XY aboutissant à la différenciation testiculaire (Sinclair et al., 1990). Ce rôle de déterminant sexuel majeur semble être joué par le gène *dmrt1*[2] chez le poulet (Smith et al., 2009) et, chez le xénope *Xenopus laevis*, c'est un déterminant femelle présent sur le chromosome W (*dm-W*[3]), homologue de *dmrt1*, qui déclenche la différenciation ovarienne (Yoshimoto et al., 2008). Un autre homologue de *dmrt1*, *dmY*, est le déterminant majeur (mâle) chez le médaka (*Oryzias latipes*) (Matsuda et al., 2002). Bien que *dmrt1* soit impliqué dans la différenciation testiculaire chez les mammifères, les oiseaux et les poissons (Cutting et al., 2013), et que deux gènes homologues aient été identifiés comme déterminant majeurs, il n'existe pas de déterminant universel chez les vertébrés non-mammaliens et tous les facteurs génétiques hérités de façon différentielle entre les deux sexes peuvent potentiellement devenir des déterminants sexuels (Arnold, 2012). Les autres déterminants majeurs du sexe connus jusqu'à présent chez les vertébrés ont été découverts chez différentes espèces de poissons et ont des origines différentes : *gsdf*[4] chez *Oryzias luzonensis* (Myosho et al., 2012), *amhy*[5] chez *Odontesthes hatcheri* (Atherinopsidae) (Hattori et al., 2012), *amhr2*[6] chez le fugu *Takifugu rubripes* (Kamiya et al., 2012) et *sdY*[7] chez la truite arc-en-ciel *Oncorhynchus mykiss* (Yano et al., 2012). Cette diversité et la présence de déterminants majeurs différents chez des espèces du même genre (*Oryzias*) reflète le caractère peu conservé des déterminants majeurs du sexe et l'évolution rapide des chromosomes sexuels chez les poissons (Kikuchi & Hamaguchi, 2013).

Chez *O. niloticus*, le déterminant majeur du sexe n'est pas connu mais a été localisé sur le groupe de liaison LG1. Il semble que d'autres gènes appartenant aux groupes LG3 et LG23 soient aussi probablement impliqués dans le déterminisme sexuel chez cette espèce (Cnaani et al., 2008).

[1] sex-determining region of Y chromosome
[2] doublesex and mab-3-related transcription factor 1
[3] W-likned doublesex and mab-3 DNA-binding motif
[4] gonadal soma derived growth factor
[5] Y-linked anti-Müllerian hormone
[6] anti-Müllerian hormone receptor type II
[7] sexually dimorphic on the Y chromosome (related to interferon regulatory factor 9)

2.2.2. Gènes impliqués dans la différenciation sexuelle

Contrairement aux déterminants majeurs du sexe, les acteurs génétiques situés en aval dans la cascade de la différenciation sexuelle semblent avoir été relativement conservés au cours de l'évolution. *Foxl2*[8] et *cyp19a1*[9] sont impliqués dans la différenciation ovarienne et *dmrt1*, *sox9*[10], et *amh*[11] dans la différenciation testiculaire chez les poissons, les oiseaux et les mammifères. Ce caractère conservé n'est cependant qu'apparent puisque ces gènes peuvent avoir des fonctions et des positions différentes dans le processus de différenciation selon les groupes. Par exemple, chez les mammifères, *sox9* active le gène de l'*amh* dans les cellules de Sertoli des testicules en développement conduisant à la régression des canaux de Müller. Chez les oiseaux, l'expression de l'*amh* précède celle de *sox9* qui est exprimé chez les mâles et les femelles (Cutting et al., 2013). Les poissons ne possèdent pas de canaux de Müller et le rôle de l'*amh* n'est pas clair. Chez *Odontesthes hatchery*, le gène de l'*amh* a évolué en déterminant majeur sur le chromosome Y.

Un bref résumé des fonctions et profils d'expression connus pour ces gènes, et d'autres impliqués dans la régulation de la différenciation sexuelle chez les poissons est présenté ci-dessous (détails de leur expression au cours de la différenciation sexuelle chez le tilapia du Nil à la Figure 2).

Sox9

Chez les mammifères, *sox9* est la cible directe de *sry* et son expression est nécessaire pour la différenciation testiculaire (Siegfried, 2010). Deux copies paralogues[12] de ce gène, *sox9a* et *sox9b* ont été identifiées chez plusieurs espèces de téléostéens (Chiang et al., 2001; Johnsen et al., 2013; Nakamoto et al., 2005). Chez le médaka, *sox9a* est exprimé dans les ovocytes des ovaires adultes. *Sox9b* est exprimé dès la formation d'une ébauche de gonade mais un dimorphisme sexuel apparaît après les premiers signes de différenciation gonadique. Bien qu'une augmentation de son expression soit observée durant la morphogenèse testiculaire (Nakamoto et al., 2005), ce gène ne semble pas impliqué dans la différenciation testiculaire mais remplirait un rôle dans le maintien de la lignée germinale (Nakamura et al., 2012). Par

[8] forkhead transcriptional factor L2
[9] cytochrome P450, family 19, subfamily A (aromatase)
[10] Sry-related HMG-box protein 9 gene
[11] anti-Müllerian hormone
[12] Copies issues d'un même gène ancestral par un événement de duplication.

contre, chez la truite arc-en-ciel (*Oncorhynchus mykiss*), *sox9a* est exprimé très tôt dans les gonades en développement avec un niveau d'expression plus élevé chez les mâles apparaissant avant les premiers signes de différenciation (Vizziano et al., 2007).

Chez le tilapia, le niveau d'expression de *sox9a* est similaire dans les gonades XX et XY de 9 à 29 jours post-fécondation (jpf), durant la différenciation sexuelle, suivi d'une augmentation spécifiquement dans les gonades XY à partir de 39 jpf, après la différenciation histologique des gonades (apparition des canaux efférents et de la cavité ovarienne) (Ijiri et al., 2008; Kobayashi et al., 2008). Cependant, D'Cotta et al. (2007) ont observé une expression plus élevée de *sox9a* et *sox9b* dans les gonades XY entre 20 et 25 jpf (Figure 2). Bien que plus précoce, ce dimorphisme apparaît après le début d'une prolifération différentielle des cellules germinales primordiales entre mâle et femelle (Kobayashi et al., 2008). Le rôle des gènes *sox9* doit encore être précisé chez le tilapia mais il ne semble pas intervenir dans les premières phases de la différenciation testiculaire.

Amh

Chez les mammifères, l'hormone anti-Müllerienne (amh) joue un rôle dans la régression des canaux de Müller chez les mâles et le développement folliculaire chez les femelles. Au cours du développement testiculaire, l'expression de son gène est induite par *sox9* dans les cellules de Sertoli en voie de différenciation (Sekido & Lovell-Badge, 2009). Les poissons téléostéens ne possèdent pas de canaux de Müller et le rôle exact de l'*amh* n'est pas connu, mais une expression plus élevée chez le mâle dès le début de la différenciation gonadique a été observée chez plusieurs espèces comme le zebrafish (Wang & Orban, 2007) et la truite arc-en-ciel (Vizziano et al., 2007) et laisse supposer un rôle de l'*amh* dans la différenciation testiculaire. Des profils d'expression réciproques entre l'*amh* et *cyp19a1a* suggèrent un possible contrôle négatif de l'*amh* sur le gène de l'aromatase gonadique (Wang & Orban, 2007; Fernandino et al., 2008; Poonlaphdecha et al., 2013).

Chez le tilapia, le gène de l'*amh*, localisé sur le groupe de liaison LG23 (Shirak et al., 2006), est exprimé dans les gonades à partir de 9 jpf. Son niveau d'expression est plus élevé chez le mâle à partir de 19 jpf et continue d'augmenter durant toute la période de différenciation sexuelle, tandis qu'il reste faible chez la femelle (Ijiri et al., 2008) (Figure 2). Il est intéressant de noter que l'*amh* est également exprimé dans le cerveau des tilapias et qu'un dimorphisme

d'expression se manifeste entre 10 et 15 jpf, plus précocement que dans la gonade (Poonlaphdecha et al., 2011).

Dmrt1

Le gène *dmrt1* a suscité beaucoup d'intérêt chez les poissons depuis la découverte du déterminant sexuel majeur chez le médaka, *dmy*, qui est une copie paralogue de *dmrt1* (Matsuda et al., 2002). Alors qu'il ne semble pas impliqué directement dans le déterminisme sexuel chez les mammifères, mais plutôt dans le développement des cellules de Sertoli et la spermatogenèse, il est lié au chromosome Z chez les oiseaux et nécessaire au déterminisme mâle (Smith et al., 2009; Siegfried, 2010). Chez le médaka, *dmrt1* est situé sur un autosome et exprimé tardivement dans le processus de différenciation testiculaire. Seul *dmy* est exprimé dans l'embryon et la larve, déterminant le sexe mâle en régulant la prolifération des cellules germinales primordiales (Kobayashi et al., 2004). Aucun dimorphisme sexuel d'expression n'apparaît durant la différenciation sexuelle chez le zebrafish (Guo et al., 2005). Chez d'autres espèces comme le tilapia (Ijiri et al., 2008), la truite arc-en-ciel (Vizziano et al., 2007) et le bar (*Dicentrarchus labrax*) (Deloffre et al., 2009), *dmrt1* est surexprimé dans les gonades mâles durant la différenciation sexuelle. Son expression différentielle précoce dans les gonades XY en fait probablement un des premiers acteurs de la différenciation testiculaire chez le tilapia. Il est exprimé dans les cellules entourant les cellules germinales à partir de 10 jpf, avant tout signe de différenciation morphologique (prolifération des cellules germinales, histogenèse des canaux efférents ou de la cavité ovarienne) (Ijiri et al., 2008; Kobayashi et al., 2008) (Figure 2). *Dmrt1* pourrait inhiber la différenciation ovarienne en exerçant un contrôle négatif sur l'expression de *foxl2* et *cyp19a1a* et la production d'œstrogènes (Li et al., 2013; Poonlaphdecha et al., 2013).

Cyp19a1

L'aromatase, codée par le gène *cyp19a1*, occupe une position clé dans la stéroïdogenèse en catalysant la synthèse des œstrogènes à partir d'androgènes. Chez tous les vertébrés non-mammaliens, l'aromatase et les œstrogènes jouent un rôle crucial dans le développement ovarien (Lange et al., 2002) (voir § 2.3). Chez les poissons, les reptiles et les oiseaux, une inhibition de l'aromatase peut à elle seule induire une différenciation testiculaire chez des femelles génétiques (Elbrecht & Smith, 1992; Wibbels & Crews, 1994; Guiguen et al., 1999).

Deux copies paralogues du gène de l'aromatase existent chez la plupart des poissons (excepté les anguilles). *Cyp19a1a* code pour l'aromatase dite « gonadique » car il est essentiellement exprimé dans les gonades (principalement les ovaires) durant la période de différenciation et chez l'adulte. La seconde forme appelée « cérébrale » est exprimée par le gène *cyp19a1b* principalement dans le cerveau (Guiguen et al., 2010).

La structure de leurs promoteurs et leurs profils d'expression différents reflètent les spécificités régulatrices et fonctionnelles de chaque gène. Chez le tilapia et d'autres espèces comme le poisson rouge (*Carassius auratus*) et le zebrafish, le promoteur de *cyp19a1a* contient entre autres des motifs de liaison pour *sf1*[13] (*nr5a1/ ad4bp*), *wt1*[14] et *sry* qui sont des facteurs impliqués dans le déterminisme sexuel et la différenciation gonadique chez les mammifères (Callard et al., 2001; Chang et al., 2005; Guiguen et al., 2010). Une séquence de liaison de la protéine *foxl2* a également été identifiée chez le tilapia (Wang et al., 2007). Chez cette espèce, l'aromatase gonadique est exprimée spécifiquement dans les cellules somatiques des gonades XX à partir de 9 jpf et son niveau d'expression augmente progressivement durant toute la période de différenciation sexuelle (Ijiri et al., 2008) (Figure 2). Une expression plus précoce des deux formes a cependant été détectée dans les embryons avant l'apparition des gonades, à partir de 3-4 jpf. Bien qu'une quantification plus précise de son expression soit nécessaire durant les premières phases de développement, la forme cérébrale ne semble pas être exprimée de manière différentielle chez les mâles et les femelles durant la période de différenciation sexuelle (Kwon et al., 2001) comme c'est le cas chez la truite arc-en-ciel (Vizziano-Cantonnet et al., 2011). Le promoteur de c*yp19a1b* contient un élément de réponse aux œstrogènes (Chang et al., 2005) qui permet une autorégulation positive par le produit de synthèse de l'aromatase. La forme cérébrale est exprimée dans les cellules gliales radiaires qui donnent naissance aux neurones et autres cellules gliales et interviennent dans la migration neuronale et sa fonction a donc été largement associée à la neurogenèse (Diotel et al., 2010). Cependant, D'Cotta et al. (2001) ont observé chez le tilapia une activité de l'enzyme plus élevée dans les cerveaux femelles que dans les cerveaux mâles durant la période de différenciation sexuelle, laissant supposer qu'un contrôle post-transcriptionnel ou une régulation différentielle de l'enzyme (Balthazart et al., 2003) pourraient être impliqués dans la différentiation sexuelle du cerveau et peut-être dans celle de la gonade.

[13] steroidogenic factor-1
[14] Wilms tumor 1 protein

Foxl2

Foxl2 code pour un facteur de transcription impliqué dans la différenciation ovarienne chez les vertébrés (Cutting et al., 2013). Il participe à la régulation transcriptionnelle de *cyp19a1a* notamment chez le médaka (Nakamoto et al., 2006), le tilapia (Wang et al., 2007) et la truite arc-en-ciel (Vizziano et al., 2007). Contrairement au médaka chez qui l'expression de *foxl2* apparaît dans les gonades XX après les premiers signes de différenciation morphologique (Nakamoto et al., 2006), son expression est antérieure chez le tilapia (9 jpf) (Ijiri et al., 2008) et il semble donc se situer au plus haut de la cascade de différenciation femelle (Figure 2). Chez cette espèce, une délétion de *foxl2* peut même conduire à une inversion de l'ovaire en testicule (Wang et al., 2007; Li et al., 2013).

Il est particulièrement intéressant de noter que *foxl2* est également exprimé dans le cerveau à l'âge adulte chez le tilapia (Wang et al., 2004) et le poisson-chat africain *Clarias gariepinus* et, chez cette espèce, *foxl2* contrôle également l'expression de l'aromatase cérébrale *cyp19a1b* (Sridevi et al., 2012). Par ailleurs, une expression de *foxl2* a été mise en évidence dans le cerveau au cours de la différenciation sexuelle chez la truite arc-en-ciel, mais sans corrélation directe avec le niveau d'expression de *cyp19a1b* (Vizziano-Cantonnet et al., 2011)

Sf1

Sf1 est un autre facteur de régulation qui semble impliqué dans les voies de différenciation mâle et femelle. Chez les mammifères, il participe à la régulation de l'expression de différentes enzymes stéroïdogéniques, dont l'aromatase, et est également nécessaire à l'activation de l'*amh* (Cutting et al., 2013). Son rôle régulateur de l'aromatase a été mis en évidence chez le médaka et le tilapia durant la vitellogenèse (Watanabe et al., 1999; Yoshiura et al., 2003) et dans le cerveau chez le poisson chat africain (Sridevi et al., 2012). Cependant, son profil d'expression différent de celui de *cyp19a1a* durant la différenciation sexuelle du tilapia suggère qu'il ne joue pas un rôle important dans la régulation de l'aromatase lors de ce processus (Ijiri et al., 2008).

Dax1[15] *(nr0b1)*

Chez les mammifères, *dax1* code pour un récepteur nucléaire jouant un rôle régulateur antagoniste de *sf1* dans le développement gonadal (Iyer & McCabe, 2004). Les profils d'expression dans les gonades de tilapia en développement obtenus par Ijiri et al. (2008) semblaient exclure ce gène d'un rôle important dans la différenciation gonadique mais des résultats récents contradictoires pourraient susciter un nouvel intérêt pour ce gène. D'Cotta et al. (2014) ont en effet mis en évidence une expression plus élevée de *dax1* dans les gonades et le cerveau des individus XY que chez les XX au début de la différenciation sexuelle (10-15 jpf) (Figure 2).

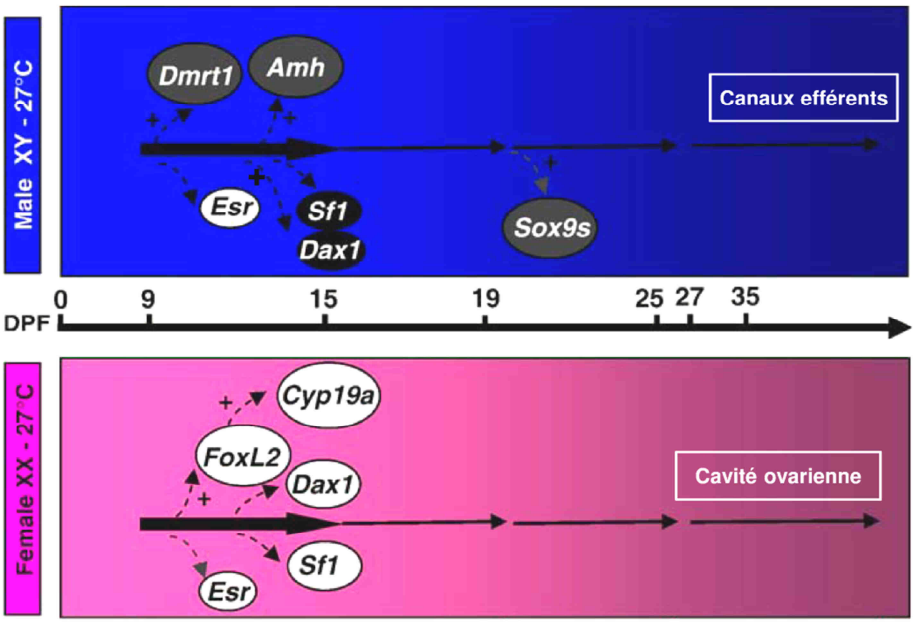

Figure 2. Représentation schématique de l'expression des gènes impliqués dans la cascade de différenciation du sexe dans les gonades au cours du développement mâle (rectangle bleu) et femelle (rectangle rose) chez le tilapia du Nil (modifié d'après Baroiller et al., 2009a; D'Cotta et al., 2014). Les flèches larges représentent la période considérée comme critique pour la différenciation sexuelle (voir § 3.2 et 3.3). L'échelle supérieure représente les jours post-fécondation (dpf). Les cercles blancs représentent les gènes impliqués dans le développement ovarien et les cercles noirs ceux impliqués dans le développement testiculaire. Les signes + indiquent une régulation positive. Les autres gènes sont exprimés à des niveaux similaires après l'initiation de leur expression. Noms des gènes : voir texte. Esr : récepteurs œstrogènes.

[15] Dosage-sensitive sex reversal, adrenal hypoplasia congenital critical region on the X chromosome.

2.3. Contrôle endocrinien de la différenciation sexuelle

Une différence majeure entre les mammifères euthériens et les autres vertébrés est l'importance des stéroïdes sexuels dans la différenciation sexuelle, et particulièrement la nécessité des œstrogènes dans le développement ovarien chez vertébrés non-mammaliens (Herpin et al., 2010; Cutting et al., 2013). Ce rôle majeur se retrouve non seulement chez les espèces gonochoriques à déterminisme sexuel génétique, mais également chez les espèces possédant un déterminisme environnemental (température) et chez les hermaphrodites (Baroiller et al., 1999; Herpin et al., 2010).

Après les premières expériences d'inversion sexuelle par administration de stéroïdes sexuels durant la période de différenciation des gonades chez le médaka (Yamamoto, 1953; Yamamoto, 1958), Yamamoto proposa en 1969 la théorie selon laquelle les œstrogènes étaient les inducteurs naturels du développement ovarien, et les androgènes les inducteurs naturels du développement testiculaires chez les poissons. Le 17β-œstradiol (E2) se retrouve à des niveaux plus élevés chez les femelles et est considéré comme l'œstrogène majeur responsable de la différenciation ovarienne (Lange et al., 2002). A la différence des autres vertébrés chez qui la testostérone est l'androgène principal, les stéroïdes impliqués dans la différenciation testiculaire et les fonctions reproductrices mâles chez les poissons sont des androgènes 11-oxygénés, principalement la 11-cétotestostérone (11KT) (Kime, 1993; Borg, 1994; Baroiller et al., 1999; Devlin & Nagahama, 2002). La testostérone (T) quant à elle joue un rôle majeur puisqu'elle est le substrat d'une part, de l'enzyme 11β-hydroxylase qui produit les androgènes 11-oxygénés, et d'autre part de l'aromatase qui le transforme en 17β-œstradiol (Figure 3).

Le rôle crucial des œstrogènes et de l'aromatase dans la différenciation ovarienne a été mis en évidence expérimentalement par l'utilisation d'inhibiteurs de l'aromatase. Leur administration durant la période sensible de la différenciation sexuelle réduit les niveaux d'œstrogènes et induit une masculinisation entre autres chez le zebrafish (Uchida et al., 2004), le saumon Chinook *Oncorhynchus tshawytschala* (Piferrer et al., 1994), la truite arc-en-ciel et le tilapia (Guiguen et al., 1999; Kwon et al., 2000; Afonso et al., 2001). L'utilisation de traitements similaires a également montré un possible contrôle exercé par les œstrogènes sur l'expression de certains gènes impliqués dans la différenciation sexuelle. L'administration d'un inhibiteur d'aromatase durant la différenciation sexuelle diminue l'expression de *foxl2* chez la truite arc-

en-ciel (Baron et al., 2004) et stimule l'expression de l'*amh* chez le pejerrey *Odonthestes bonariensis*, une espèce avec un fort déterminisme thermique (Fernandino et al., 2008).

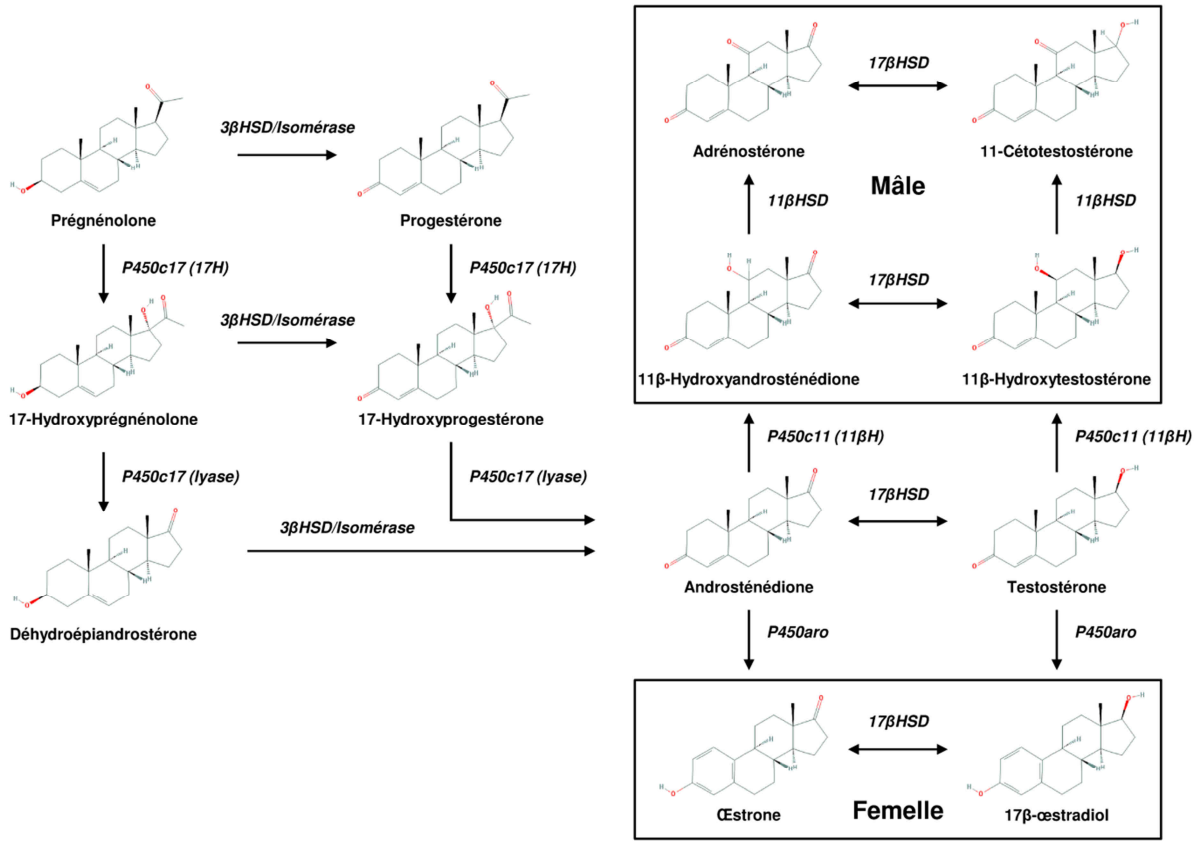

Figure 3. Schéma de la stéroïdogenèse gonadique chez les poissons, établi à partir de résultats récoltés chez le tilapia et la truite arc-en-ciel durant la différenciation sexuelle (d'après Baroiller et al., 1999). P450aro, aromatase ; P450c11 (11βH), 11β-hydroxylase ; P450c17 (17H), 17-hydroxylase ; P450c17 (lyase), 17,20-lyase ; HSD, hydroxysteroid dehydrogenase.

Le contrôle de la différenciation ovarienne par les œstrogènes n'est cependant pas un mécanisme universel chez les poissons puisque les cellules stéroïdogéniques apparaissent après les premiers signes de différenciation histologiques dans les gonades chez plusieurs espèces de poecilidae et chez le médaka (Strüssmann & Nakamura, 2002) et l'administration d'inhibiteur d'aromatase ne modifie pas le développement ovarien chez ce dernier (Kawahara & Yamashita, 2000).

Bien que l'administration d'androgènes exogènes ait montré son pouvoir masculinisant sur la différenciation gonadique chez de nombreuses espèces (Pandian & Sheela, 1995), le rôle des androgènes dans les processus naturels de la différenciation sexuelle a été moins étudié et semble différent selon les espèces. Chez la truite arc-en-ciel, l'enzyme 11β-hydroxylase est uniquement exprimée dans les gonades mâles avant le début de la différenciation histologique, suggérant un rôle important des androgènes 11-oxygénés dans la différenciation sexuelle (Liu et al., 2000). Chez la perche eurasienne (*Perca fluviatilis*), il semblerait que la différenciation gonadique puisse être guidée par le rapport 11KT/E2 (Rougeot et al., 2007). A l'opposé, la 11β-hydroxylase est exprimée dans les gonades de tilapia à partir de 29 jpf, après les premiers événements de différenciation sexuelle (Ijiri et al., 2008) et l'absence d'œstrogènes endogènes plutôt que la présence d'androgènes semble être corrélée avec l'initiation de la différenciation testiculaire (Nagahama, 1999; Strüssmann & Nakamura, 2002). De plus, toujours chez le tilapia, des niveaux plus élevés d'E2 ont été mesurés dans les gonades XX par rapport aux gonades XY durant la période de différenciation sexuelle (D'Cotta et al., 2001b).

Les récepteurs stéroïdiens jouent un rôle important dans la médiation de l'action des hormones androgènes et œstrogènes durant la différenciation sexuelle et une régulation du contrôle hormonal de la différenciation sexuelle pourrait s'opérer au travers d'une expression différentielle de ceux-ci. Cependant, chez le tilapia, les trois récepteurs œstrogènes Esr1, Esr2a, Esr2b et les deux récepteurs androgènes Ar1 et Ar2 sont exprimés à des niveaux semblables dans les gonades XX et XY durant la différenciation sexuelle (Ijiri et al., 2008).

2.4. Développement des structures sexuellement différenciées

2.4.1. Ontogenèse gonadique

Les gonades se développent à partir d'une ébauche bipotentielle qui suit ensuite une voie de différenciation mâle (en testicules) ou femelle (en ovaires). Elle se compose de cellules somatiques et de cellules germinales qui ont des origines embryologiques différentes. Les éléments structuraux somatiques se développent dans la paroi dorso-latérale de la cavité péritonéale tandis que les cellules germinales primordiales (PGCs) se développent ailleurs dans l'embryon et migrent ensuite vers la région gonadique. Elles interagissent alors avec les

cellules somatiques pour former une ébauche gonadique. Après leur migration, les PGCs prolifèrent par divisions mitotiques et se différencient en ovogonies ou spermatogonies, qui entreront par la suite en méiose pour former des ovocytes ou spermatocytes. Avant le début de la méiose, les cellules somatiques entourant les PGCs se différencient également. Dans les testicules, elles formeront notamment les cellules de Sertoli (nourricières) et de Leydig (hormonales). Les cellules de Sertoli entourent les cellules de la lignée germinale, forment les tubules séminifères et supportent la spermatogenèse. Les cellules de Leydig assurent la production de stéroïdes sexuels. Dans les ovaires, les cellules somatiques entourent les cellules germinales pour former de follicules comprenant une couche interne, la granulosa, et une couche externe, la thèque. Les cellules de la thèque produisent des stéroïdes, notamment la T qui est ensuite aromatisée en E2 dans les cellules de la granulosa (Nakamura et al., 1998; Devlin & Nagahama, 2002; Strüssmann & Nakamura, 2002).

Chez les espèces gonochoriques, la différenciation ovarienne précède généralement la différenciation testiculaire qui peut être détectée des jours, voire des semaines ou des mois plus tard selon les espèces. Le développement de la gonade mâle peut être direct, comme chez le tilapia du Nil, ou indirect, comme chez le zebrafish. Dans ce cas, la gonade développe d'abord des caractéristiques femelles, pouvant aller jusqu'à l'apparition d'ovocytes et d'une cavité ovarienne rudimentaire, avant de se masculiniser complètement en testicule (Devlin & Nagahama, 2002; Strüssmann & Nakamura, 2002). L'apparition d'une cavité ovarienne chez les femelles ou des canaux efférents chez le mâle est généralement considérée comme le premier signe histologique de la différenciation gonadique (Nakamura et al., 1998; Kobayashi & Nagahama, 2009). Cependant, au niveau cellulaire, une distinction plus précoce peut généralement être faite entre les gonades mâles et femelles dans le nombre de PGCs. La différenciation ovarienne est généralement associée à un nombre plus élevé et une prolifération plus importante des PGCs que la différenciation testiculaire. Le nombre de PGCs et la régulation de leur activité proliférative par les cellules somatiques environnantes pourraient même être le déclencheur initial de la différenciation gonadique (Braat et al., 1999; Tanaka et al., 2008; Guiguen et al., 2010). Chez le médaka (Kurokawa et al., 2007) et le zebrafish (Siegfried & Nüsslein-Volhard, 2008), une déplétion des PGCs durant les premières phases de développement induit une masculinisation des gonades.

Chez le médaka, il semblerait que le déterminant majeur du sexe, *dmy*, exprimé dans la lignée des cellules de Sertoli, détermine le sexe en contrôlant l'activité proliférative des PGCs

(Kobayashi et al., 2004). Chez certaines espèces thermosensibles (déterminisme environnemental), les hautes températures masculinisantes induisent une réduction du nombre de PGCs par apoptose (Strüssmann et al., 1998; Ito et al., 2003).

Au cours de l'embryogenèse du tilapia, les PGCs sont visibles à partir de 46 h post-fécondation (hpf) (neurula, somitogenèse) (Morrison et al., 2001) (Figure 4). A 4 jpf (éclosion), elles se situent dans le mésoderme entourant la partie postérieure du tube digestif. Elles migrent ensuite et atteignent l'ébauche de gonade à 7 jpf (Kobayashi et al., 2000, 2002). Leur nombre change peu jusqu'à 12 jpf. A partir de ce moment, elles continuent à proliférer dans les gonades XX alors que leur nombre ne change pas jusqu'à 18 jpf dans les gonades XY. La cavité ovarienne et le canal efférent apparaissent dans l'ovaire et le testicule respectivement entre le 25^{e} et le 30^{e} jpf (Kobayashi et al., 2008). A ce moment, les cellules germinales entrent en méiose dans l'ovaire alors qu'elles restent quiescentes dans le testicule avant d'entrer en prophase méiotique vers le 55^{e} jpf (D'Cotta et al., 2001b).

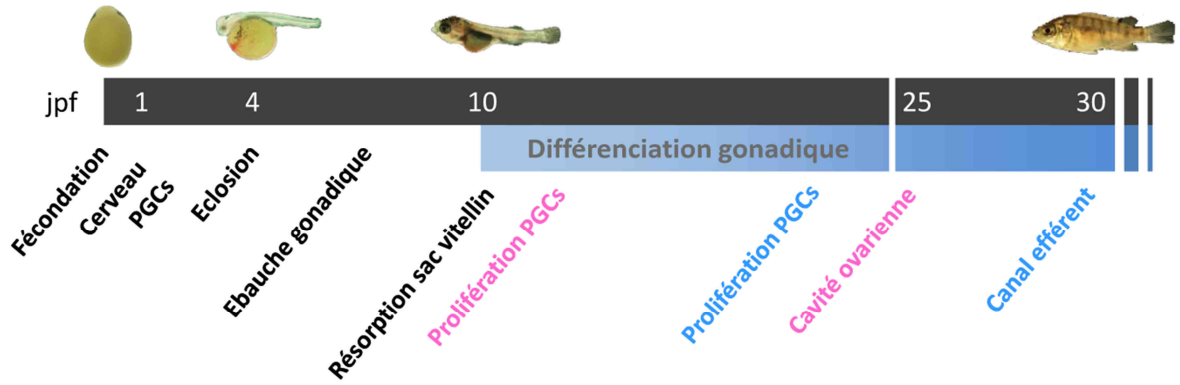

Figure 4. Développement des structures et événements marquants au cours du développement embryonnaire et larvaire et de la période de différenciation des gonades (en rose chez les femelles, en bleu chez les mâles) chez le tilapia du Nil (d'après Nakamura et al., 1998; Kobayashi et al., 2000, 2002, 2008; Morrison et al., 2001).

2.4.2. Rôle du cerveau dans la différenciation sexuelle ?

Selon la théorie classique de la sexualisation chez les mammifères, l'expression du gène *Sry* dans les gonades contrôle leur différenciation sexuelle. Par la suite, la production de stéroïdes sexuels par les ovaires ou les testicules induit le développement de différences sexuelles dans

d'autres structures comme le cerveau (MacLusky & Naftolin, 1981). Chez les poissons, des différences sexuelles dans l'expression de certains gènes apparaissent dans le cerveau durant la différenciation des gonades suggérant une différenciation sexuelle du cerveau au moins concomitante à celle des gonades (Sudhakumari et al., 2005, 2010; Poonlaphdecha et al., 2011; Vizziano-Cantonnet et al., 2011). Pour Francis (1992) et Godwin (2010), le cerveau serait sexuellement différencié avant et jouerait un rôle déterminant dans la différenciation sexuelle des gonades chez les poissons. Cette polarité développementale inversée (par rapport aux mammifères chez qui les gonades déterminent le phénotype sexuel du cerveau) serait à mettre en relation avec la diversité et la plasticité de leurs stratégies sexuelles. Cette notion est particulièrement vraie chez les espèces hermaphrodites dont la différenciation gonadique est guidée par des facteurs sociaux mais pourrait également s'étendre à d'autres espèces avec des systèmes de déterminisme sexuel différents.

Une autre particularité des poissons par rapport aux autres vertébrés est l'importante activité aromatase observée dans leur cerveau chez les adultes et durant le développement (Diotel et al., 2010; Le Page et al., 2010). Il est généralement admis que l'aromatase cérébrale et les œstrogènes ont un rôle stimulateur dans la neurogenèse mais des données récentes semblent plutôt indiquer un rôle inhibiteur dans la prolifération et la migration des cellules cérébrales chez le zebrafish adulte (Coumailleau et al., in press). Quoi qu'il en soit, le rôle de l'aromatase et des œstrogènes dans la neurogenèse semble également associé à la diversité des stratégies sexuelles (Coumailleau et al., in press) et un rôle direct dans la différenciation sexuelle ne peut être exclu (Blázquez & Somoza, 2010; Le Page et al., 2010). Chez le tilapia, l'aromatase cérébrale est exprimée dans l'embryon à 3 jpf (Kwon et al., 2001) et son activité est plus élevée dans le cerveau des femelles que dans celui des mâles durant la période de différenciation sexuelle (D'Cotta et al., 2001b). Chez la truite arc-en-ciel, l'expression des trois récepteurs œstrogènes (*esr1*, *esr2a* et *esr2b*) ainsi que l'expression et l'activité de l'aromatase cérébrale sont (paradoxalement) plus élevées dans les cerveaux mâles durant la différenciation sexuelle (Vizziano-Cantonnet et al., 2011).

Une expression dimorphique (niveau plus élevé chez les mâles) de *sf1* et *dax1* chez la truite arc-en-ciel (Vizziano-Cantonnet et al., 2011), et *amh* (Poonlaphdecha et al., 2011) et *dax1* (D'Cotta et al., 2014) chez le tilapia a également été observée dans le cerveau durant la différenciation sexuelle.

Par ailleurs, un contrôle du cerveau sur la différenciation gonadique pourrait également s'établir via l'axe gonadotrope (hypothalamus-hypophyse-gonade). Son implication dans le contrôle de la gamétogenèse et d'autres fonctions liées à la reproduction a été largement étudiée chez les adultes (voir revue de Zohar et al., 2010). Peu de données existent quant à sa possible implication dans la différenciation sexuelle mais un rôle précoce dans le contrôle de la stéroïdogenèse ne peut être exclu. Chez le tilapia, aucune différence sexuelle n'apparaît dans la production de gonadotropines (LH[16] et FSH[17]) par l'hypophyse durant la période de différenciation sexuelle. Cependant, une augmentation de l'expression du récepteur de la FSH dans les gonades femelles a été observée à partir de 10 jpf et pourrait être nécessaire pour induire l'expression de l'aromatase (Yan et al., 2012).

3. Inversions sexuelles et effets environnementaux sur la différenciation du sexe

3.1. Contrôle du sexe

La plasticité de la différenciation sexuelle est largement exploitée dans la production de populations monosexes visant à améliorer la productivité et la gestion des systèmes aquacoles. Le principal avantage de ce type de production réside dans l'exploitation du dimorphisme sexuel de croissance présent chez de nombreuses espèces. Elle peut également permettre (Beardmore et al., 2001):

- d'améliorer la gestion des stocks de géniteurs ;
- de limiter les dépenses énergétiques liées à la production gonadique (chez les femelles), à l'expression des comportements reproducteurs et à la production non-désirées d'alevins ;
- de réduire les interactions agressives ;
- d'uniformiser les tailles à la récolte ;
- de limiter certains impact négatifs résultant de la maturation sexuelle sur la qualité de la chair ;

[16] Hormone lutéinisante
[17] Hormone folliculo-stimulante

- de réduire les perturbations environnementales dues aux poissons échappés.

Avec 4,5 millions de tonnes (dont 3,2 pour *O. niloticus*) produites en 2012, les tilapias représentent le deuxième groupe d'espèces le plus important dans l'aquaculture mondiale après les carpes (FAO, 2014). L'élevage du tilapia du Nil repose principalement sur la production de populations monosexes mâles qui présentent un meilleur potentiel de croissance que les femelles (± 50 %). Différentes techniques existent pour obtenir des populations monosexes : le sexage manuel, l'hybridation, le contrôle génétique par utilisation de mâles YY (supermâles), les inversions du sexe par administration d'hormones et la masculinisation par les hautes températures (pour le détail de ces techniques, voir: Baroiller & Jalabert, 1989; Baroiller et al., 1995; Mair et al., 1997; Phelps & Popma, 2000; Beardmore et al., 2001). Les deux premières méthodes ont été progressivement abandonnées au profit de l'inversion sexuelle par administration massive d'androgènes (généralement la 17α-méthyltestostérone) qui, par sa facilité de mise en œuvre et son efficacité, représente aujourd'hui la technique la plus largement utilisée en aquaculture (Cnaani & Levavi-Sivan, 2009).

Les inversions sexuelles hormonales et thermiques, décrites aux § 3.2 et 3.3, sont par ailleurs très intéressantes pour l'étude du déterminisme et de la différenciation sexuels. La maîtrise des techniques d'inversion sexuelle hormonales a également conduit au développement de programmes d'élevage assurant la production de mâles YY, qui croisés avec des femelles XX fournissent des descendances 100 % mâles non traitées aux hormones. Outre les mâles YY, les inversions sexuelles fournissent des individus avec d'autres combinaisons de phénotype et génotype sexuels (mâles XX, femelles XY et YY) utilisés comme outils dans les recherches sur le déterminisme sexuel et la biologie reproductive. Les schémas de production de ces individus sont présentés à la Figure 5.

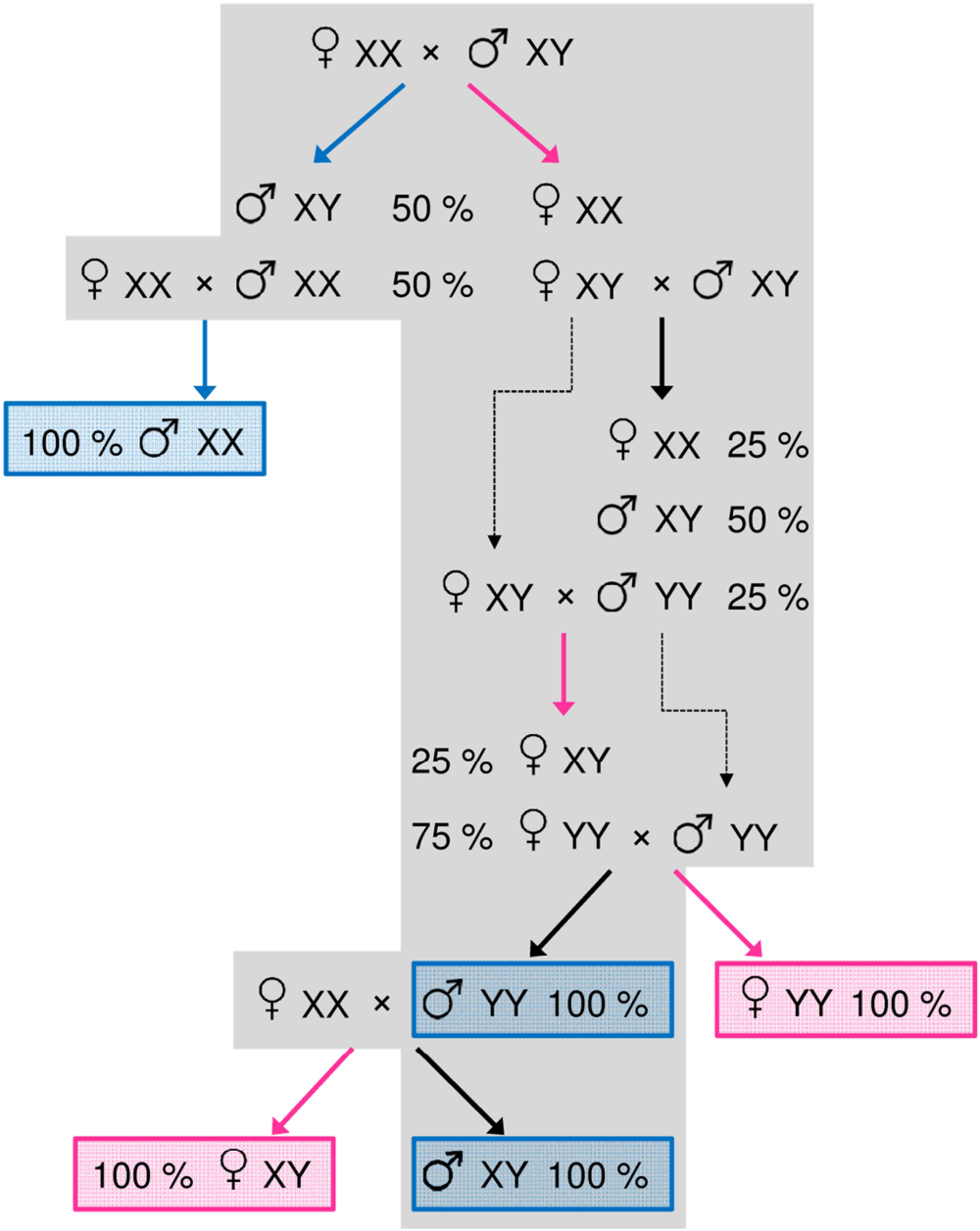

Figure 5. Schéma de production permettant d'obtenir des populations homogènes de toutes les combinaisons de phénotype et génotype sexuels chez le tilapia du Nil. Flèches bleues : masculinisation des progénitures par administration d'androgènes ; flèches roses : féminisation des progénitures par administration d'œstrogènes ; flèches noires : aucun traitement hormonal. Grisé : procédures appliquées en aquaculture pour la production de populations monosexes mâles, soit par masculinisation directe, soit par l'utilisation de mâles YY.

3.2. Stéroïdes exogènes

Même chez les espèces avec un déterminisme génétique fort, la différenciation gonadique reste un processus flexible comme le démontre les nombreuses expériences d'inversions sexuelles réalisées chez les poissons. Des traitements masculinisants ou féminisants par administration de stéroïdes sexuels exogènes (androgènes et œstrogènes respectivement) ont été établis chez plus de 50 espèces de téléostéens (Hunter & Donaldson, 1983; Pandian & Sheela, 1995; Piferrer, 2001; Devlin & Nagahama, 2002). L'efficacité de ces traitements dépend du mode et du moment d'application, de la durée, de la dose et de la nature de l'hormone administrée. De nombreux stéroïdes naturels ou synthétiques peuvent être utilisés et leur administration se fait généralement par inclusion dans la nourriture ou par immersion, et de façon plus anecdotique par injection ou implant (Pandian & Sheela, 1995). Chez le tilapia et de nombreuses autres espèces, l'administration alimentaire est préférée pour sa facilité de mise en œuvre. Une solution alcoolique contenant l'hormone est mélangée à l'aliment qui est ensuite séché pour permettre l'évaporation de l'alcool. Les traitements par immersion (balnéation) présentent l'avantage de pouvoir être appliqués avant le début de l'alimentation exogène chez les espèces dont la période sensible coïncide avec les stades embryonnaires ou larvaires, comme chez certains salmonidés (Piferrer 2001).

La période sensible (ou critique) pour les inversions sexuelles couvre la période de différenciation des gonades et pour être efficaces, les traitements doivent débuter avant les premiers signes histologiques de différenciation gonadique (Hunter & Donaldson, 1983; Hiott & Phelps, 1993). Chez le tilapia, les traitements masculinisants à la 17α-méthyltestostérone (MT) sont généralement appliqués à partir de 10 jpf (premier nourrissage) pour une période de 3 à 4 semaines. A une concentration de 40 à 100 mg kg^{-1} d'aliment, ce type de traitement induit généralement 100 % de mâles chez des individus XX (Baroiller & Jalabert, 1989; Abucay & Mair, 1997; Phelps & Popma, 2000).

Afin de limiter les risques environnementaux et sanitaires liées à l'utilisation massive d'hormones, certains chercheurs ont mis au point des protocoles alternatifs basés sur des traitements par balnéation de courte durée (moins d'un jour). Une exposition d'alevins âgés de 10 à 18 jpf à différents androgènes synthétiques (17α-méthyldihydrotestostérone, acétate de trenbolone, 17α-méthyltestostérone, éthynyltestostérone) en une ou deux immersion(s) de 2 à 4 h induit plus de 80 % de mâles dans des populations mixtes (Contreras-Sanchez et al.,

1997; Gale et al., 1999; Wassermann & Afonso, 2003). S'ils n'ont pas été adoptés par l'industrie aquacole en raison des taux d'inversion plus faibles et de leur mise en œuvre plus laborieuse, ce type de traitements courts pourraient permettre de cibler les périodes les plus précoces de la différenciation sexuelle afin d'en étudier les mécanismes amonts, notamment la cascade d'expression génique pouvant s'établir avant les premiers signes de différenciation sexuelle au niveau cellulaire et histologique et le rôle éventuel du cerveau dans le processus de différenciation du sexe (Rougeot et al., 2008a).

Bien que les traitements d'inversion du sexe aient largement contribué à mettre en évidence le rôle des stéroïdes dans la différenciation et déterminer la période critique de la différenciation sexuelle chez de nombreuses espèces, les mécanismes d'action de ces hormones exogènes, et notamment leur interaction avec le métabolisme stéroïdien naturel et l'action des gènes impliqués dans le déterminisme et la différenciation ne sont pas totalement connus.

Les traitements hormonaux appliqués durant la période sensible de la différenciation du sexe induisent des changements moléculaires et cellulaires importants dans les gonades en développement. Chez le tilapia du Nil, l'administration d'E2 à des alevins XY par immersion entre 8 et 10 jpf induit une augmentation de l'expression de l'aromatase (*cyp19a1a* et *cyp19a1b*) et une diminution de l'expression de *dmrt1* dans les cellules somatiques (Kobayashi et al., 2003), ainsi qu'une activation de l'activité proliférative des PGCs (Kobayashi et al., 2008). A l'inverse, la masculinisation d'individus XX par traitement alimentaire à la MT réprime l'expression d'aromatase (Bhandari et al., 2006) et induit un niveau d'expression de *dmrt1* et un nombre de PGCs caractéristiques des mâles génotypiques (Kobayashi et al., 2008).

Il est particulièrement intéressant de noter que ces traitements hormonaux induisent des modifications de l'expression de certains facteurs impliqués dans la différenciation sexuelle non seulement dans les gonades mais également dans le cerveau. Chez le tilapia du Nil, un traitement alimentaire masculinisant à la MT provoque une diminution d'expression de l'*amh* dans le cerveau à 14 jpf, mais ne modifie pas l'expression de *cyp19a1b* (Ouedraogo et al., manuscrit soumis). Paradoxalement, Tsai et al. (2000) ont rapporté des changements d'activité de l'aromatase cérébrale induits par l'administration d'E2 et de MT chez le tilapia du Mozambique, *Oreochromis mossambicus*, au même âge.

3.3. Température

La température est le principal facteur environnemental pouvant contrôler ou influencer la différenciation du sexe chez les poissons. Une thermosensibilité de la différenciation sexuelle a été mise en évidence pour la première fois chez la capucette (*Menidia menidia*, Atherinidae) (Conover & Kynard, 1981), ensuite chez le tilapia du Nil (Baroiller et al., 1995), et aujourd'hui rapportée chez plus de 60 espèces de téléostéens (Baroiller et al., 2009b). L'importance de la température et des facteurs génétiques (chromosomes sexuels) dans le contrôle de la différenciation est très variable d'une espèce à l'autre. Par exemple, chez le pejerrey *O. bonariensis*, le sexe-ratio est influencé sur une large gamme de température. Des populations monosexes femelles et monosexes mâles sont obtenues à des températures d'incubation respectivement basses (17 °C) et hautes (29 °C), et des températures intermédiaires (24 – 25 °C) induisent des populations dans lesquelles les deux sexes coexistent (Strüssmann et al., 1996; Strüssmann et al., 1997). Par contre, chez le tilapia du Nil, le sexe est génétiquement déterminé en dessous de 32 °C et des températures supérieures (jusqu'à 36 °C) induisent une masculinisation de femelles génotypiques (Baroiller et al., 1995). Chez cette espèce, des températures élevées appliquées durant la période de différenciation des gonades, à partir de 10 jpf et pour une période minimale de 10 jours peuvent induire des taux de masculinisation supérieur à 90 %. L'efficacité de tels traitements est cependant très variable d'une famille à l'autre, certains couples de géniteurs pouvant donner des descendances hautement thermosensibles et d'autres des descendances insensibles avec des sexe-rations équilibrés (Baroiller et al., 1995; Baroiller & D'Cotta, 2001; Baras et al., 2001; Tessema et al., 2006).

Bien que les mécanismes d'action des différents agents masculinisants puissent être partiellement différents chez le tilapia, il est intéressant de constater que la période thermosensible correspond à la période sensible pour les traitements d'inversion sexuelle utilisant des hormones exogènes ou des inhibiteurs de l'aromatase (Baroiller et al., 2009a). L'action des hautes températures sur la différenciation sexuelle passe par la régulation de l'expression de plusieurs gènes clés de la différenciation du sexe dans les gonades en développement. Chez des individus XX soumis à une température de 36 °C à partir de 10 jpf, *dmrt1* et *amh* sont exprimés à un niveau caractéristique des mâles à partir de 13-15 jpf, et pourraient inhiber le développement ovarien en exerçant un contrôle négatif sur *cyp19a1a* et

foxl2, dont l'expression est réprimée à partir de 17-19 jpf, conduisant à un diminution des taux d'œstrogènes (Poonlaphdecha et al., 2013). L'expression de *dax1* est quant à elle stimulée à partir de 11 jpf (D'Cotta et al., 2014).

Les températures masculinisantes induisent également une régulation différentielle des gènes impliqués dans la différenciation sexuelle dans le cerveau. Quatre jours après le début du traitement (14 jpf), l'expression de *dax1* (D'Cotta et al., 2014) et de *cyp19a1b* (Ouedraogo, manuscrit soumis) dans le cerveau augmente par rapport aux individus XX contrôles. Paradoxalement, D'Cotta et al. (2001) ont observé une activité de l'aromatase plus faible chez les individus XX soumis à des hautes températures que chez les XX maintenus à 27 °C, suggérant une régulation post-transcriptionnelle de l'aromatase cérébrale. Chez *O. mossambicus*, une augmentation de l'expression de l'aromatase dans le cerveau est également observée à 13 jpf après 10 jours de traitement à 32 °C (vs contrôle à 26 °C).

Rougeot et al. (2008b) ont mis en évidence une fenêtre de thermosensibilité précoce couvrant la période embryonnaire chez *O. niloticus*. Un traitement des œufs à 36 °C entre 12 hpf et l'éclosion, avant la formation des ébauches de gonades, induit 6 à 27 % de mâles dans des descendances 100 % XX, suggérant un effet de la température sur le développement des PGCs et/ou sur la différenciation sexuelle du cerveau.

Il semblerait donc que la température puisse agir sur la différenciation sexuelle à différents moments du développement, tant sur le cerveau que sur le contrôle de la prolifération des PGCs, ou sur les gonades en développement.

4. Influence des inversions du sexe et du génotype sexuel sur la biologie reproductive

Chez les espèces possédant un déterminisme génétique du sexe, les chromosomes sexuels orientent la différenciation des gonades en testicules ou en ovaires, mais les gènes déterminants le sexe ainsi que d'autres facteurs génétiques portés par les chromosomes sexuels sont également responsables de l'apparition de différences sexuelles d'ordre développemental, morphologique, physiologique ou comportemental qui définiront le

phénotype individuel à l'âge adulte (Mank, 2009). Dès lors, les manipulations du sexe présentées au § 3 soulèvent de nombreuses questions sur leurs conséquences potentielles sur le développement du phénotype sexuel et sur la biologie reproductive chez l'adulte. Les gènes liés aux chromosomes sexuels induisent-ils des modifications biologiques chez les individus présentant des génotypes sexuels particuliers (mâles YY et XX, femelles XY et YY) ? Ces individus sont-ils physiologiquement et comportementalement totalement fonctionnels et adaptés à leur environnement ? Les inversions sexuelles induisent-elles une sexualisation complète de tous les aspects phénotypiques ou certains aspects restent-ils profondément liés au génotype? Les traitements d'inversion du sexe et les chromosomes sexuels affectent-ils de la même manière la différenciation des gonades et celle du cerveau ?

Les réponses apportées à ces questions chez les poissons sont peu nombreuses et concernent généralement des aspects liés au contrôle du sexe en aquaculture. Elles pourraient cependant nous aider également à comprendre comment des systèmes plastiques de déterminisme sexuel sont maintenus ou sélectionnés au cours de l'évolution et évaluer le caractère adaptatif d'individus possédant des systèmes chromosomiques particuliers. Chez le tilapia du Nil, les facteurs génétiques portés par des autosomes (Mair et al., 1991) et pouvant influencer la différenciation sexuelle, ainsi que l'existence de populations naturelles thermosensibles vivant dans des environnements soumis à des températures extrêmes ou des régimes thermiques fluctuant, supposent l'existence naturelle d'individus présentant des génotypes particuliers. Cela a été prouvé par la découverte d'au moins un mâle XX et une femelle XY dans des populations naturelles (Bezault et al., 2007; Baroiller et al., 2009a).

4.1. Performances de reproduction

Etant donné la variété de fonctions attribuées aux hormones sexuelles dans la différenciation sexuelle, la maturation sexuelle, la gamétogenèse (Lubzens et al., 2010; Schulz et al., 2010) et l'expression des comportements reproducteurs (Munakata & Kobayashi, 2010), des modifications du métabolisme stéroïdien pourraient avoir de nombreuses conséquences sur la biologie reproductive. Au cours du développement, la synthèse de T augmente chez les individus XY par rapport aux XX entre 8 et 13 pf chez le tilapia, alors que les larves YY présentent un niveau de synthèse intermédiaire, pouvant conduire à des différences dans le

développement de ces individus (Rowell et al., 2002). En comparant le développement testiculaire de mâles XY et YY, Herrera et al. (2001) ont observé une histogenèse et une spermatogenèse plus rapides chez les mâles YY, conduisant à une puberté précoce et au développement de testicules de plus grandes tailles, suggérant que ces individus pourraient avoir des capacités reproductives supérieures aux mâles XY. Entre 70 et 140 jpf, période d'acquisition de la maturité sexuelle chez les mâles, les mâles XY grandissent plus vite que les mâles YY et les mâles XX ont un taux de croissance intermédiaire. Ces différences pourraient résulter de facteurs génétiques de croissance portés par les chromosomes sexuels et de différences d'investissement énergétique dans le développement des gonades (Toguyeni et al., 2002). Des différences de taille pourraient par ailleurs influencer le succès reproducteur de mâles possédant des génotypes sexuels différents au travers du choix de partenaire réalisé par la femelle.

Différentes caractéristiques pouvant affecter le succès reproducteur ont également été comparées entre des mâles XX sexuellement inversés et des mâles XY normaux chez des espèces pour lesquelles la production de populations monosexes femelles est intéressantes en raison d'une croissance supérieure des femelles. Chez la truite arc-en-ciel et la perche eurasienne, les taux de T, d'E2 et de 11 KT ne sont pas différents au cours du cycle de reproduction (Rougeot et al., 2004; Espinosa et al., 2011), et chez la perche, aucune différence dans la qualité du sperme (densité de spermatozoïdes, motilité et taux de fécondation) n'a été mise en évidence entre les deux types de mâles (Rougeot et al., 2007). Par contre, les mâles XX de truite arc-en-ciel présentent, au moment de la reproduction, un important déficit en 17α,20β-dihydroxy-4-pregnen-3-one (DHP), une hormone stéroïdienne affectant notamment la maturation et la motilité spermatique (Espinosa et al., 2011).

4.2. Comportement

Chez le tilapia bleu (*O. aureus*), espèce à déterminisme génétique ZW, la production d'œufs par les femelles n'est pas affectée par le génotype sexuel. Les femelles ZW et les femelles sexuellement inversée ZZ (pseudofemelles) présentent une fécondité, un poids moyen des œufs et un profil saisonnier de ponte identiques. Cependant, lorsque les deux types de femelles sont en compétition pour la reproduction (10 femelles ZW, 10 pseudofemelles ZZ et

3 mâles ZZ dans le même bassin), le taux de reproduction des pseudofemelles est largement inférieur à celui des femelles. Desprez & Mélard (1998) ont suggéré que les traitements hormonaux d'inversion du sexe n'induisaient pas une féminisation complète de l'individu et qu'une agressivité plus élevée liée à l'expression du génotype sexuel perturberait les comportements reproducteurs, diminuant le succès de la reproduction. Cette hypothèse a été confirmée par Ovidio et al. (2002) qui ont observé une agressivité plus élevée des pseudofemelles par rapport aux femelles ZW lorsqu'elles sont maintenues ensemble. Les pseudofemelles semblent par ailleurs être plus agressives envers les mâles et ne pas exprimer de comportements de parade sexuelle avant de déposer leurs œufs, qui ne sont pas fécondés par le mâle. Aucune donnée comparable n'existe chez une espèce à hétérogamétie mâle.

Ces perturbations comportementales observées chez des individus sexuellement inversés seraient en accord avec l'hypothèse d'une différenciation sexuelle du cerveau précédent celle des gonades, les traitements hormonaux d'inversion du sexe administrés durant la période de différenciation gonadique ne sexualisant pas totalement le cerveau, ou en tout cas avec une influence des gènes portés par les chromosomes sexuels sur le comportement. Chez les mammifères, l'expression de comportements agressifs est associée au chromosome Y, notamment au travers du contrôle exercé par les stéroïdes d'origine gonadique, mais également par une influence directe de gènes portés par les chromosomes sexuels qui ne sont pas impliqués dans le déterminisme sexuel (Gatewood et al., 2006; Cox et al., 2014).

5. Le tilapia du Nil : modèle expérimental

Le tilapia du Nil (*Oreochromis niloticus*, Linnaeus 1758) (Figure 6) appartient à l'ordre le plus diversifié de vertébrés, les Perciformes, et à la famille des Cichlidae qui, avec plus de 1300 espèces répertoriées, est l'une des principales familles de poissons d'eau douce dans le monde (Nelson, 2006). Le terme générique de tilapia regroupe des espèces originaires d'Afrique appartenant à trois genres différents qui se distinguent par le mode d'incubation des œufs et la garde parentale apportées aux progénitures : le genre *Tilapia* regroupe des espèces pondeuses sur substrat, le genre *Sarotherodon* comprend des espèces à incubation buccale et

garde biparentale, et le genre *Oreochromis* est constitué d'espèces à incubation buccale maternelle (Trewavas, 1983).

En conditions naturelles, les mâles *Oreochromis* se regroupent dans des zones peu profondes où ils défendent chacun un territoire de reproduction afin d'y attirer une femelle. Au terme d'une parade nuptiale, la femelle dépose ses ovules sur le substrat dans le nid préparé par le mâle, qui les féconde. La femelle reprend alors les œufs fécondés dans sa bouche et incube sa progéniture au moins jusqu'à la première alimentation exogène des alevins (Philippart & Ruwet, 1982). Le développement ovocytaire est asynchrone[18] chez *O. niloticus* et, en conditions favorables, un nouveau cycle de reproduction peut être initié tous les 25-33 jours. Si les œufs sont retirés de la bouche de la femelle, un nouveau cycle de maturation ovocytaire débute et l'intervalle de ponte est réduit à 12-18 jours (Tacon et al., 1996). En milieu captif, la reproduction est spontanée et, même en absence de mâles, les femelles pondent régulièrement, ce qui permet un contrôle de la reproduction par fécondation artificielle (Myers & Hershberger, 1991).

Figure 6. Le tilapia du Nil, *Oreochromis niloticus*, femelle en incubation (photo : Saïdou Santi).

Une reproduction aisée et fréquente en captivité associée à une maturité sexuelle précoce, une croissance rapide, une tolérance aux densités de population élevées (Mélard, 1986) et à une

[18] Plusieurs stades ovocytaires présents au même moment dans l'ovaire.

large gamme de conditions physico-chimiques (températures naturelles : 13 à 33 °C, températures extrêmes : 8 à 42 °C, oxygène dissous > 1 mg l^{-1}) (Balarin & Hatton, 1979 ; Mélard, 1986) et un régime alimentaire omnivore à tendance phytoplanctonophage (Philippart & Ruwet, 1982) ont fait d'*O. niloticus* une espèce de choix pour l'aquaculture. Le développement de l'élevage et surtout la nécessité de contrôler le sexe ont motivé une recherche importante sur le déterminisme du sexe chez cette espèce. Par la suite, la mise au point des procédures d'inversions hormonales, la découverte d'une thermosensibilité de la différenciation sexuelle et la possibilité de produire des poissons avec différentes combinaisons de phénotype et génotype sexuels ont conforté sa position de modèle remarquable pour l'étude des mécanismes du déterminisme et de la différenciation du sexe.

6. Objectifs et organisation de la thèse

Les objectifs de ce travail sont :

1. De déterminer l'influence du génotype sexuel (XX, XY ou YY) sur certains aspects de la biologie reproductive – ou pouvant altérer le succès reproducteur – chez le tilapia du Nil : qualité du sperme, taux de stéroïdes sexuels et comportements agressifs.

2. D'explorer l'hypothèse d'un rôle du cerveau dans la différenciation sexuelle durant les premiers stades de développement, avant la formation des gonades, et d'étudier les mécanismes d'inversion du sexe au stade embryonnaire chez des individus soumis à un traitement hormonal précoce.

Le travail de recherche est divisé en deux axes. Le premier axe de recherche (partie 2) traite de la biologie reproductive. Le chapitre 1 concerne le contrôle de la reproduction et non l'influence du génotype sexuel sur la biologie reproductive, qui est abordée dans les chapitres 2 et 3. Le premier chapitre traite plus particulièrement de l'effet d'agents stressants sur la maturation ovarienne chez le tilapia. Cette recherche découle d'une démarche méthodologique visant à améliorer la technique de reproduction artificielle en synchronisant les pontes chez plusieurs femelles par exposition à des agents stressants. L'objectif final de la

synchronisation était d'obtenir simultanément une grande quantité d'œufs fécondés utilisés comme matériel biologique dans l'étude des mécanismes d'inversions sexuelles embryonnaires (partie 3). Cette recherche n'a finalement pas abouti à une synchronisation suffisante des pontes chez le tilapia mais a fourni des résultats intéressants sur l'effet du cortisol et du stress sur la maturation ovarienne, ainsi que sur la détermination du moment de la ponte qui a permis de réaliser toutes les reproductions artificielles.

Les résultats d'analyse de la qualité du sperme chez des mâles XX, XY et YY sont présentés au chapitre 2. Plusieurs paramètres ont été mesurés afin de déterminer d'éventuelles différences dans la production de sperme liées au génotype sexuel : indice gonado-somatique, densité spermatique, temps de mobilité des spermatozoïdes et paramètres de motilité.

L'influence du génotype sexuel chez des mâles et des femelles a ensuite été évaluée sur les niveaux de stéroïdes sexuels (T, E2, 11KT) circulants et l'expression des comportements agonistiques (chapitre 3). Huit comportements agonistiques ont été quantifiés dans des confrontations mâle – femelle. Le rôle possible des stéroïdes sexuels dans l'expression de différences comportementales est discuté.

Le second axe (partie 3) s'intéresse au déterminisme et à la différenciation du sexe à des stades précoces du développement chez le tilapia du Nil. Tout d'abord, l'efficacité de différents traitements précoces d'inversion sexuelle utilisant des hormones ou un inhibiteur de l'aromatase a été évaluée sur des pontes XX, XY ou YY (chapitre 4). Ces traitements de courte durée étaient appliqués durant la période embryonnaire, avant le développement des gonades, afin de cibler les mécanismes les plus en amont de la cascade de différenciation sexuelle.

Le traitement le plus efficace (féminisation par administration de 17α-ethynylœstradiol) a ensuite été appliqué à des pontes XY et YY afin de vérifier l'existence d'une période sensible précoce, et d'étudier les mécanismes d'inversion sexuelle aux stades embryonnaires et le rôle potentiel du cerveau dans l'inversion phénotypique du sexe (chapitre 5). Pour cela, les profils de concentration en hormones exogène (17α-ethynylœstradiol) et endogènes (T, E2, 11KT) ont été suivis au cours du développement, et l'expression de *cyp19a1b*, *foxl2* et *amh* a été mesurée dans les têtes des embryons et des jeunes alevins en voie de différenciation sexuelle.

Enfin, les résultats obtenus seront confrontés et discutés dans un contexte plus général du déterminisme et de la différenciation du sexe afin de tirer les conclusions de ce travail et de dégager de nouvelles perspectives de recherche.

Une bibliographie complète est présente à la fin du document. Elle reprend l'ensemble des références citées dans l'introduction et la discussion générale, ainsi que celles citées dans les autres chapitres figurant à la fin de chaque article.

Partie 2

Biologie de la reproduction

Partie 2

1. Contrôle de la reproduction

Chapitre 1

Cortisol is responsible for positive and negative effects in the ovarian maturation induced by the exposure to acute stressors in Nile tilapia, *Oreochromis niloticus*

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Cortisol is responsible for positive and negative effects in the ovarian maturation induced by the exposure to acute stressors in Nile tilapia, *Oreochromis niloticus*

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Abstract The aim of the present study was to evaluate the effect of acute stress and cortisol injection on oocyte final maturation process in female Nile tilapia (*Oreochromis niloticus*). Handling followed by a prophylactic treatment (0.3 mL L^{-1} H_2O_2, 5 g L^{-1} NaCl solution during 30 min) and an environmental change (transfer from a 2 m^3 fibreglass square tank to 50 L aquaria) were used as acute stressors and compared to a single cortisol injection (0.5 or 5 mg kg^{-1} body weight). For both acute stress and cortisol injection (0.5 mg kg^{-1} body weight), serum cortisol level was significantly increased from 2.3 to 134.1 ng mL^{-1} 1 h post-stress/injection and returned to a resting basal value 24 h after the stress/injection. In fish injected with 5 mg kg^{-1} body weight cortisol, mean serum cortisol level reached a peak up to 2500 ng mL^{-1} 1 h after injection. 63 % of the females (mean body weight: 242 ± 4 g) submitted to the acute stress ovulated within 72 h after the stress. In the same way, cortisol injection (5 mg kg^{-1} body weight) at the 10th day of the maturation cycle led to a twofold reduction of the time before ovulation compared to vehicle injected control fish. Relative and total fecundity were significantly decreased in females submitted to an acute stress or cortisol injected at 5 mg kg^{-1} body weight, but not fertilization or hatching rates. In conclusion, acute stress and cortisol induction exert both positive and negative effects on the final reproductive process in *O. niloticus,* and cortisol is the endocrine mediator causing these changes.

Keywords Cortisol · Stress · Fish reproduction · Oocyte maturation · Nile tilapia

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Introduction

Stress, experienced in the wild or in culture conditions, affects all life functions of fish: growth, immunology, disease resistance and reproduction (Iwama et al. 1997; Pankhurst 2011; Schreck et al. 2001). Stress induces three levels of response: a primary physiological response, which consists in the initial

neuroendocrine response, with a release of catecholamine and the stimulation of the hypothalamic-pituitary-interrenal axis inducing the release of corticosteroid hormones; a secondary response, with physiological adjustments linked to metabolism, respiration, acid–base status, immune function and cellular responses; and a tertiary response expressed by a decrease of growth, inhibition of reproduction, modification of behaviour or decrease of disease resistance (Barton 2002; Schreck et al. 2001). Under culture conditions, fish encounter many acute and chronic stressors such as crowding, confinement, handling and other husbandry routines, which may affect these three levels of response.

In fish, the main corticosteroids are cortisol, cortisone, 11-deoxycortisol and corticosterone, cortisol being one of the most commonly used welfare and stress physiological indicator in fish (Barton 2002; Milla et al. 2009). Effects of stress and corticosteroids on fish reproduction have been widely studied (Contreras-Sanchez et al. 1998; Milla et al. 2009; Schreck et al. 2001) and can affect all the levels of the reproductive axis, from neuroendocrine axis to gamete quality and embryo/larval development (Campbell et al. 1992; Iwama et al. 1997; Schreck 2010). Briefly, depending on the intensity/duration of the stressor and the stage of development, the reproductive cycle of females exposed to a stressor or corticosteroid treatments could be negatively or positively affected in term of reproductive performances (Contreras-Sanchez et al. 1998; Milla et al. 2009; Schreck 2010). For examples, negative effects as the occurrence of follicular atresia, disruption or modification of vitellogenesis process (in salmonids), advance or delay in oocyte maturation and ovulation, decrease of fertilization and hatching rates, modification of the spawning behaviour and subsequently low larval quality were reported in many fish species (Schreck et al. 2001; Milla et al. 2009; Schreck 2010). On the other hand, some paradoxical positive effects of stress on reproduction were also punctually reported as the reduction of the interspawning period (Milla et al. 2009; Stratholt et al. 1997) or the enhancement of vitellogenesis (Ding et al. 1994; Shankar and Kulkarni 2006). However, it is not clear whether these stress effects are evoked or not by the transient or prolonged cortisol elevation in the blood because few studies have investigated in parallel the consequences of stress exposure and cortisol administration on the reproductive features.

The Nile tilapia (*Oreochromis niloticus*) is one the most cultured fish species worldwide. It easily reproduces in captivity, without any hormonal stimulation. Under constant photothermal conditions (26–28 °C and 14 h light/10 h dark photoperiod), females spawn regularly throughout the year. However, because of its asynchronous oocyte development, and the failure of hormonal stimulation to trigger spawning, it is difficult to synchronize reproduction in tilapia (Foo and Lam 1993a; Srisakultiew and Wee 1988). Nevertheless, sudden environmental change (mainly a cooling period followed by an increase of water temperature) may improve spawning synchronization in tilapiine species (Coward et al. 1998; Srisakultiew and Wee 1988). Acceleration of the final maturity and the induction of the spawn were previously observed on *Tilapia zillii*, which synchronously spawn within 1–2 days after their transfer into individual aquaria (Coward et al. 1998).

The objectives of the present study were to assess the capacity of acute stress occurrence to synchronize ovulation in Nile tilapia and to determine whether these effects are mediated by cortisol elevation. To address these questions, we investigated the impact of acute stress and single cortisol injection on the induction of ovulation, fertilization and hatching rates in female Nile tilapia.

Materials and methods

Fish

Nile tilapia *O. niloticus* (Lake Manzala strain) originated from the Research and Education Centre in Aquaculture (CEFRA), University of Liège (Belgium). Twenty-month-old broodstock fish (males and females) were maintained in a 2 m^3 (4 m^2) fibreglass square tank in a recirculating system at 27–28 °C, 14 h light/10 h dark and at a stocking density of 30 kg m^{-3}. Fish were fed at satiation with special broodstock commercial tilapia diet (32 % proteins, 6 % lipids, Coppens—The Netherlands). Experiments were carried out according to the guidelines of the University of Liège ethical committee and the European animal welfare recommendations.

Experiment 1: Effect of acute stress on oocyte maturation cycle

Acute stress consisted of netting followed by prophylactic treatment and breeders transfer from the stock tank to another rearing facility. Fish were netted from the stock tank and anaesthetized with 2-phenoxyethanol (Sigma) at 0.4 mL L^{-1}. Sixty females (mean body weight: 242 ± 4 g) were selected after gentle stripping and oocyte emission. Because of their asynchronous oocyte development strategy, females were at different stages of sexual maturity (Foo and Lam 1993a). In order to avoid pathogens transfer between rearing systems, fish were treated in 0.3 mL L^{-1} H_2O_2, 5 g L^{-1} NaCl solution during 30 min before their transfer into individual 50 L aquaria at 27 °C with a 14 h light/10 h dark photoperiod and oxygen above 6 ppm.

After their transfer, female ripeness was checked daily as described by Myers and Hershberger (1991). Ovulation time was assessed by genital papilla distension and behaviour change (nesting activity) just before spawning. Time between stress and ovulation was recorded. Ovulation was also followed in 28 unmanipulated females (second maturation cycle in aquarium) as a control. Eggs were collected by stripping, weighted in order to determine the total and relative fecundity and artificially fertilized with 0.3 mL of sperm originating from three different males. Spermatozoa activation was checked under microscope. Fertilized stripped eggs were incubated in 1.5 L Zug bottle at 28 °C. Egg quality was assessed by fertilization rate (2 h after fertilization), hatching rate and survival rate at 10 days post-fertilization (dpf). Quality of the first stripped eggs, following an acute stress, was compared to quality of eggs stripped after the following maturation cycles.

Blood was sampled from 42 fish for cortisol level analysis before and after the exposure to acute stress. Fish were netted and anaesthetized in 2-phenoxyethanol (Sigma) (0.4 mL L^{-1}), and blood (1 mL) was rapidly (<5 min) sampled by caudal venipuncture on eight stressed fish at 1, 6, 24 h after acute stress application and on eight fish at the time of ovulation following the acute stress. Ten fish from the stock tank were used to determine the basal cortisol level.

Experiment 2: Effect of cortisol injection on the final stages of reproduction

In this experiment, cortisol injection was used to mimic blood cortisol surge that is generally associated with an acute stress. Forty females were transferred into experimental aquaria as described in experiment 1 and allowed to naturally spawn first (Foo and Lam 1993a). After the spawn, eggs were immediately and gently removed from the mouth of the females. Ten days after the first spawn, when secondary oocytes are supposed to complete vitellogenesis (Hussain 2004), fish were anaesthetized with 2-phenoxyethanol (Sigma) at 0.4 mL L^{-1} and weighted. Seven females were injected with 0.5 mg kg^{-1} body weight of cortisol (Sigma, hydrocortisone), and 21 females were injected with 5 mg kg^{-1} body weight of cortisol. Cortisol was previously dissolved in saline solution (0.9 % NaCl) and injected intraperitoneally. Control fish ($n = 11$) received an injection of saline vehicle only. Time between injection and the following ovulation was measured, and quality of stripped eggs was assessed as in experiment 1.

In order to assess the blood cortisol increase after injection, 82 other individuals were used to determine serum cortisol level. Blood was sampled on 10 fish before the cortisol or vehicle injection (0 h, control). Twenty-four individuals were injected with vehicle, 24 received a 0.5 mg kg^{-1} body weight cortisol injection, and 24 other fish received a 5 mg kg^{-1} body weight cortisol injection. For each treatment, eight individuals were blood-sampled at 1, 6 and 24 h after injection.

ELISA cortisol analysis

After centrifugation (4,500 rpm, 20 min, 10 °C), serum was stored at −20 °C until analyses. Serum cortisol assay was carried out by a competitive ELISA following manufacturer instructions (BioSource, Nivelles, Belgium). Twenty microlitres of each cortisol standard, control and serum samples (each in duplicate) were dispensed into different wells of a 96-well plate. Two hundred microlitres of cortisol horseradish peroxidase conjugate were added into each well. After thorough mix for 10 s, 60 min incubation, and three times washing with solution provided in the kit, 100 μL of substrate solution containing tetramethylbenzidine were added. Then, the plate was incubated

for 15 min at room temperature, and the reaction was stopped by adding 100 µL of H_2SO_4 0.5 M. The optical density was read at 450 nm with a microtiter plate reader. The detection limit was 2 ng mL^{-1}, the inter–intra variability was 6.5–7.7, 3.2–7.0 %, and the recovery range was evaluated at 85–111 %, depending on doses.

Statistics

Statistical analysis was performed using Statview 5.0.1 (SAS Institute Inc., USA). Differences among mean values were tested using an analysis of variance (ANOVA) for the reproductive parameters and a Mann–Whitney test for cortisol levels. Data are reported as mean ± standard error of the mean (SEM).

Results

Effect of acute stress

After application of the acute stress, 63 % of the 60 females ovulated within the following 72 h, and 85 % within 1 week. All the females ovulated within 2 weeks (Fig. 1). The time course between exposure to stressor and ovulation ranged from 729 °C h (27 h at 27 °C) to 9,180°C h (14 days at 27 °C) with a mean value of 2,886 ± 341 °C h. In the control group, only 7 % of the 28 females ovulated within 72 h, 53 % within 1 week and 78 % within 2 weeks.

Acute stress (handling followed by prophylactic treatment and transfer) induced a significant ($p < 0.05$) rapid increase of serum cortisol, from 2.6 ± 1.7 ng mL^{-1} before the stress (basal level) to 131.1 ± 63.7 ng mL^{-1} 1 h post-stress (Fig. 2). After this peak, cortisol level significantly decreased and returned to a resting basal value 24 h after the stress. The cortisol level slightly increased at the time of ovulation (28.0 ± 15.5 ng mL^{-1}), but the reached level was not significantly different from the level measured in pre-initial controls or 24 h after the stress.

Relative and total fecundity were significantly ($p < 0.05$) decreased in females submitted to the acute stress by comparison with unstressed control fish (Table 1). Stressed females ovulated significantly ($p < 0.05$) bigger eggs (6.4 ± 0.3 mg) than control females (5.0 ± 0.2 mg). Mean fertilization and hatching rates were not significantly ($p > 0.05$) different between control (93.0 ± 2.0 and 63.2 ± 3.8 %, respectively) and stressed females (94.5 ± 1.2 and 56.4 ± 5.1 %, respectively). Survival rates of larvae at 10 dpf were not significantly different between progenies from both groups (±55 %).

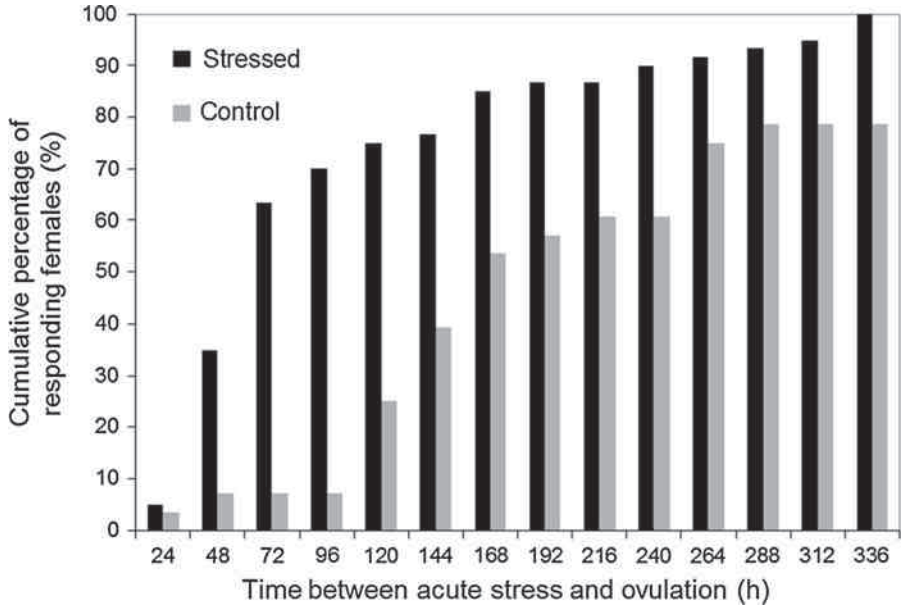

Fig. 1 Cumulative percentage of females ovulating after an acute stress (handling + prophylactic treatment + transfer, $n = 60$) compared to control fish (unmanipulated females, $n = 28$)

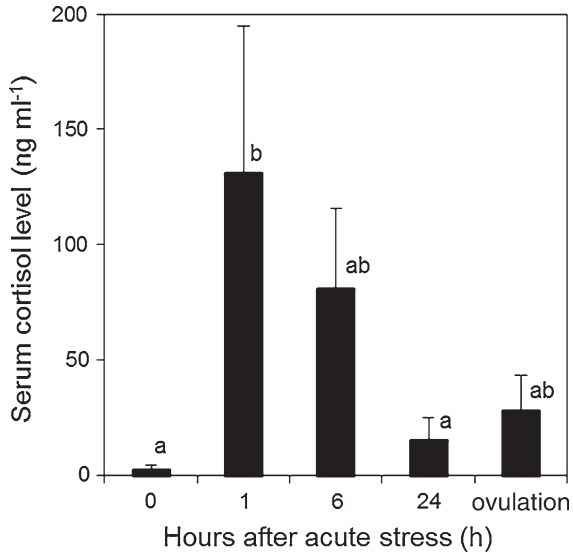

Fig. 2 Serum cortisol concentration (ng mL^{-1}) before, 1, 6 and 24 h after application of an acute stress (handling + prophylactic treatment + transfer) in female Nile tilapia. Values are means + SEM ($n = 6$–10). Values with different letters are significantly ($p < 0.05$) different

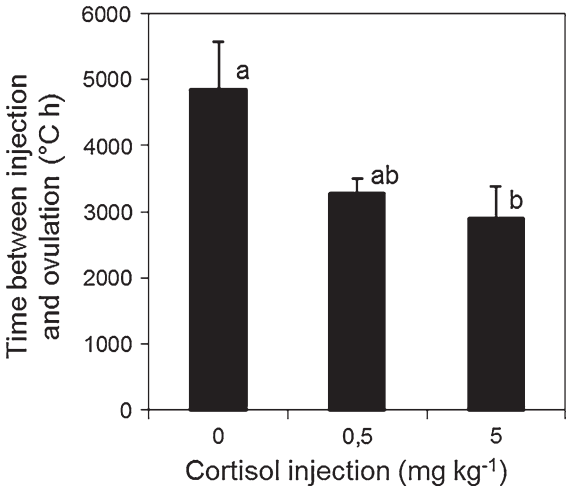

Fig. 3 Time (°C h) between cortisol (0.5 mg kg^{-1}, $n = 5$; 5 mg kg^{-1}, $n = 16$) or vehicle ($n = 11$) injection and ovulation in female Nile tilapia. Fish were injected on the 10th day following the previous ovulation. Values are means + SEM. Different letters indicate significant differences ($p < 0.05$). Fish that did not respond to injection within the following 14 days are not considered in this data set

Effect of cortisol injection

When females were injected on the 10th day of the maturation cycle, ovulation occurred more rapidly in 5 mg kg^{-1} body weight cortisol injected fish than in control fish (after 2,902 ± 473 and 4,861 ± 714°C h, respectively; $p < 0.05$) (Fig. 3). However, injection of 0.5 mg kg^{-1} body weight cortisol did not significantly affect ovulation time. For each treatment, some females did not ovulate within the 14 days following injection: 1 female (9 %) did not ovulate in the control group, and 2 (28 %) and 5 (24 %) females did not ovulate after the cortisol injection at 0.5 and 5 mg kg^{-1} body weight cortisol doses, respectively.

Injection of 0.5 mg kg^{-1} body weight cortisol or saline induced a similar increase in serum cortisol, from 2.3 ± 1.3 ng mL^{-1} before injection to 134.1 ± 33.8 and 90.5 ± 34.7 ng mL^{-1}, respectively, 1 h after injection (Fig. 4). In fish injected with 5 mg kg^{-1} body weight cortisol, mean serum cortisol level reached 2,547.4 ± 471.7 ng mL^{-1} 1 h after injection. This value is significantly higher than the level measured in control fish and fish injected with 0.5 mg kg^{-1} body weight cortisol. Six h and 24 h after injection, all the groups showed similar intermediate serum cortisol levels,

Table 1 Egg characteristics of control females, females submitted to an acute stress (handling + prophylactic treatment + transfer) and saline- (control) and cortisol-injected females

Female groups	Number of females	Egg weight (mg)	Relative fecundity (g eggs kg $female^{-1}$)	Total fecundity (eggs $female^{-1}$)	Fertilization rate (%)	Hatching rate (%)	Survival rate at 10 dpf (%)
Control	12	5.0 ± 0.2[a]	7.6 ± 0.5[a]	2,058 ± 242[a]	93.0 ± 2.0	63.2 ± 3.8	55.6 ± 3.2
Stressed	6	6.4 ± 0.3[b]	5.2 ± 0.6[b]	1,022 ± 126[b]	94.5 ± 1.2	56.4 ± 5.1	55.4 ± 4.5
Control (saline) injected	4	5.0 ± 0.3[a]	8.0 ± 0.4[a]	2,001 ± 136[a]	94.3 ± 1.4	64.9 ± 5.1	52.5 ± 2.1
Cortisol injected (5 mg kg^{-1})	11	6.3 ± 0.2[b]	5.9 ± 0.3[b]	1,249 ± 82[b]	89.1 ± 1.9	65.6 ± 4.8	52.8 ± 3.9

Values are means ± SEM. In each column, mean values with different superscript letters are significantly different ($p < 0.05$)

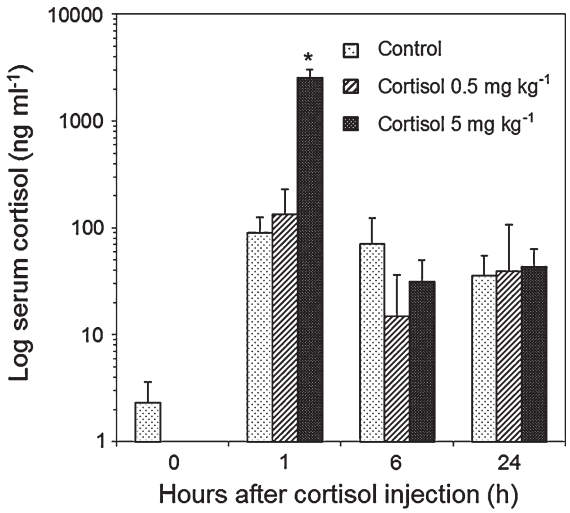

Fig. 4 Serum cortisol concentration after intraperitoneal injection of saline vehicle only (control) or 0.5 and 5 mg kg^{-1} cortisol solution in female Nile tilapia. Values are means + SEM ($n = 8$). *Significantly different from other values

between 14.8 ± 7.5 and 70.4 ± 53.4 ng mL^{-1}. Except from resting values measured before injection, all the values showed a large interindividual variability.

Cortisol-injected females ovulated significantly ($p < 0.05$) bigger eggs (6.3 ± 0.2 mg) than saline-injected ones (5.0 ± 0.3 mg). On contrary, cortisol injection at 5 mg kg^{-1} body weight significantly ($p < 0.05$) decreased the relative and total fecundity (Table 1). Neither fertilization nor hatching rate (89.1 ± 1.9 and 65.6 ± 4.8 %, respectively) was affected by this cortisol injection. Survival rates of larvae at 10 dpf were not significantly different between progenies (±52.5 %). Unfortunately, egg batches from 0.5 mg kg^{-1} cortisol-injected fish were lost during incubation due to a technical problem that occurred in the hatchery.

Discussion

Acute stress advances oocyte maturation in Nile tilapia

Depending on the intensity, the time of application and duration of the stress, the female reproductive process in fish could be negatively or positively influenced by stress (Pankhurst 1998; Schreck 2010). Our results suggest that acute stress accelerates the final stages of oogenesis in Nile tilapia and reduces the time course before ovulation. Schreck et al. (2001) reported that when Nile tilapias are stressed during late vitellogenesis, the spawn occurs immediately. Similar conclusions were drawn by Coward et al. (1998) on *T. zillii* that synchronously spawned within 24–48 h after their transfer into individual aquaria. Such a shortening of the maturation process was also mentioned in salmonids. In coho salmon and rainbow trout, Stratholt et al. (1997) and Contreras-Sanchez et al. (1998) observed that females submitted to a chronic stress during the final maturation process, ovulated on average 2 weeks before unstressed fish. Thus, we suggest that acute stress speeds up the achievement of the final stages of ovarian maturation in all tilapia species and possibly in other fish families.

Cortisol is one of the endocrine mediators of the induction of ovulation post-stress

In our study, the acceleration of the final oocyte maturation soon after the transfer into individual aquaria was associated with an increase of serum cortisol level. This serum cortisol profiles, marked by a significant peak within 1 h after the application of the stress, is a usual response to acute stress widely described in many fish species (Barton 2002; Pankhurst 2011; Schreck et al. 2001). This rapid increase of cortisol after a stress, with comparable concentrations, was also reported in stressed Nile tilapia (Correa et al. 2003) and in *Oreochromis mossambicus* juveniles submitted to an acute stress of handling (Barcellos et al. 1999; Foo and Lam 1993b), confinement (Vijayan et al. 1997) or an acute stress of salinity (Kammerer et al. 2010). Comparative cortisol increase at the time of ovulation and spawning was also reported in salmonids, goldfish and common carp (Milla et al. 2009; Schreck 2010; Schreck et al. 2001; Stratholt et al. 1997). Small (2004) observed an increase of spawn number in cortisol-fed channel catfish and speculated that cortisol could act as a maturation-inducing agent in the channel catfish. In our study, injection of supra-physiological doses of cortisol (5 mg kg^{-1} body weight) accelerated ovulation, suggesting that this cortisol administration could have the same effect on final oocyte maturation than the cortisol elevation observed in fish following acute stress. However, given that the cortisol release was supra-physiological, we cannot rule out any indirect

effect of cortisol on metabolism, which in turns may interfere with reproductive processes. At any case, we can speculate that the effect of acute stress on spawning induction is at least partly explained by the increase of cortisol level and that cortisol may be directly or indirectly implicated in the achievement of maturing process in Nile tilapia.

Which are the physiological mechanisms triggered by exposure to acute stress and cortisol?

In fish, corticosteroids display biphasic (positive and negative) effects on fish reproduction in females mainly during the meiotic oocyte maturation (Milla et al. 2009). In *Oreochromis aureus*, cortisol had an acute effect on hepatic vitellogenin gene expression supporting a direct effect of cortisol on vitellogenesis (Ding et al. 1994). In *Notopterus notopterus*, cortisol administration induced the enhancement of vitellogenesis during the pre-spawning season (Shankar and Kulkarni 2006). This enhancement of vitellogenesis would therefore shorten the interspawning interval (Tacon et al. 2000). In tilapia, the occurrence of oogenesis acceleration after stress or cortisol administration seems to be linked to changes in serum testosterone (T) and estradiol (E2) levels, but the reported data are quite controversial (Foo and Lam 1993a; Coward et al. 1998; Tacon et al. 2000) and mechanisms still unclear. Contreras-Sanchez et al. (1998) suggested that the reduction of ovulation time under stressful conditions late during the maturation process may occur as a switch in reproductive strategy when environmental conditions are not stable. We therefore hypothesise that acute stress in relation to cortisol elevation would accelerate the final oocyte maturation process and ovulation in Nile tilapia. On the other hand, we also observed a slight proportion of female that did not ovulate at all after the injection of cortisol, (28 and 24 % for 0.5 and 5 mg kg^{-1} cortisol, respectively) suggesting that cortisol can also inhibit ovulation or prevent the earlier stage of final oocyte maturation of these females. This difference of response is probably due to differences in stages of oocyte development of the female at the time of cortisol injection. This confirms the biphasic effect of cortisol on the reproductive cycle in Nile tilapia.

Early ovulation could be a direct consequence of the activation of progestogen receptors by corticosteroids. However, if binding of corticosteroids to progestogen receptors was demonstrated for 11-deoxycorticosterone and 11-deoxycortisol in other species, no evidence of such receptor cross-reactivity exists for cortisol (Milla et al. 2009). Considering this effect in an adaptive point of view, early ovulation could be an advantageous reproductive tactic under stressful conditions, as egg retention can be energy consuming. In our study, the acceleration of the ovulation process could also be triggered by the modification of the social structure in the broodstock tanks. Indeed, *O. niloticus* displays a social hierarchy with dominant and subordinated females (Binuramesh et al. 2005). Thus, we also speculate that isolation of females from the social group suppresses the inhibition induced by the presence of the dominant female and allows the subordinated ones to continue their ovulation process, thus reducing the duration of the final stages before ovulation.

Comparison of stress and cortisol effects on reproductive performances

Depending on fish species, fecundity, as well as egg size, hatching rate and survival of new-hatched larvae could be significantly decreased after a stress exposure (Campbell et al. 1992; Contreras-Sanchez et al. 1998; Milla et al. 2009; Soso et al. 2008). In *O. mossambicus*, chronic stress induced by long-term (18 days) cortisol implant caused negative effects on female reproduction, with a significant decrease of GSI and oocyte diameter (Foo and Lam 1993b). In our study, relative and total fecundity were decreased by acute stress and single cortisol injection suggesting that high levels of cortisol are responsible for this loss in fecundity. But surprisingly, egg size was significantly increased for both stressed and cortisol injected females, this discrepancy highlighting the biphasic implications of cortisol in female fish reproduction. Nevertheless, as fertilization, hatching and survival rates of progenies were not significantly affected by the acute stress nor by cortisol injection, we can conclude that even if the weight of the ovary and the eggs were affected, acute stress did not reduce the quality of gametes produced in Nile tilapia.

In conclusion, our results showed that acute stress both positively and negatively affect oocyte final maturation process and ovulation in Nile tilapia, with the acceleration and synchronization of ovulation, a decrease of fecundity and an increase of egg size.

These modifications of the final maturational process seem to be caused by a cortisol elevation.

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Partie 2

2. Influence du génotype sexuel sur la biologie reproductive

Chapitre 2

Sperm quality analysis in XX, XY and YY males of the Nile tilapia (*Oreochromis niloticus*)

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Sperm quality analysis in XX, XY and YY males of the Nile tilapia (*Oreochromis niloticus*)

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Abstract

In Nile tilapia (*Oreochromis niloticus*), individuals with atypical sexual genotype are commonly used in farming (use of YY males to produce all-male offspring), but they also constitute major tools to study sex determinism mechanisms. In other species, sexual genotype and sex reversal procedures affect different aspects of biology, such as growth, behavior and reproductive success. The aim of this study was to assess the influence of sexual genotype on sperm quality in Nile tilapia. Milt characteristics were compared in XX (sex-reversed), XY and YY males in terms of gonadosomatic index, sperm count, sperm motility and duration of sperm motility. Sperm motility was measured by computer-assisted sperm analysis (CASA) quantifying several parameters: total motility, progressive motility, curvilinear velocity, straight line velocity, average path velocity and linearity. None of the sperm traits measured significantly differed between the three genotypes. Mean values of gonadosomatic index, sperm concentration and sperm motility duration of XX, XY and YY males, respectively ranged from 0.92 to 1.33%, from 1.69 to 2.22 $\times 10^9$ cells mL^{-1} and from 18′04″ to 27′32″. Mean values of total motility and curvilinear velocity 1 min after sperm activation, respectively ranged from 53 to 58% and from 71 to 76 $\mu m\ s^{-1}$ for the three genotypes. After 3 min of activity, all the sperm motility and velocity parameters dropped by half and continued to slowly decrease thereafter. Seven min after activation, only 9 to 13% of spermatozoa were still progressive. Our results prove that neither sexual genotype nor hormonal sex reversal treatments affect sperm quality in male Nile tilapias with atypical sexual genotype.

Keywords: *Oreochromis niloticus*; Sperm motility; Sperm quality; CASA; Sexual genotype; Tilapia

1. Introduction

Tilapia culture is widely focused on all-male production because of increased growth rate and avoidance of anarchic reproduction in rearing facilities [1,2]. All-male populations are generally obtained by hormonal masculinizing treatment (with androgens, such as 17α-methyltestosterone) during the sex differentiation period, causing sex reversal of XX individuals. Another efficient technique to produce monosex populations is the use of YY male broodstock [3–5].

Fish with atypical combinations of sexual phenotype and genotype also constitute major tools in sex determinism studies [6–9]. Influence of sex reversal and particular sexual genotype on reproductive biology [10–15] and particularly on sperm characteristics [16–21] is documented in several teleost species. However, data about Nile tilapia (*Oreochromis niloticus*) are lacking.

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In *O. aureus*, normal WZ and sex-reversed ZZ females are characterized by similar fecundity, gonadosomatic index and mean egg weight [10,11]. However, in a mixed group of breeders, spawning rate of ZZ females is lower than normal females because of a high percentage of non-spawning individuals [11,13]. Desprez and Mélard [11] suggested that this lower spawning rate could be related to a higher aggressiveness level in ZZ females. Sex reversal of genotypic males with 17α-ethynylestradiol leads to the development of functional ovaries but would not totally feminize the individual, especially the behavior that could be under control of both sexual phenotype and genotype [11]. This hypothesis is supported by a study of Ovidio et al. [12], that highlighted a higher level of dominance and aggressiveness in ZZ females than in WZ females.

In addition to behavior, reproductive success is also affected by gamete quality. Fertilization rate directly depends on both quality of eggs and sperm [22–24]. Common parameters used to assess sperm quality include sperm concentration, chemical properties and composition of seminal plasma, enzymatic activity, motility, metabolism, morphology, ultrastructure, plasma membrane quality, quality of the nucleus and fertilization capacity [23–25]. All these measurements do not present the same relevance or the same objectivity in evaluating sperm quality and the use of several of them can give a better overview. As motility integrates different cellular and seminal fluid characteristics, it appears as the most objective and reliable estimator to assess sperm quality [22,23].

Computer-assisted sperm analysis (CASA) is a useful tool to objectively and rapidly quantify sperm motility. CASA systems can track a large number of spermatozoa simultaneously and allow measurements of motility parameters in terms of proportion of motile cells, sperm velocity and trajectories. Such analyses generate numerous data that are easily used to study sperm quality [22,23,25]. CASA has found various applications in ecotoxicological studies (adverse effects of pollutants on reproductive biology), broodstock selection and improvement of preservation and fertilization conditions [22,24]. It has also been used to study factors affecting sperm activation and motility.

Many factors affect sperm quality in teleosts: photoperiod, temperature, nutrition, stress, social interactions, age of broodstock, moment of sampling during the breeding season, ionic composition of semen and environment, osmolarity, pH, storage conditions and hormonal induction of spermiation [23,24,26,27]. Most of these factors concern broodstock housing conditions and management; but some play a role after spermiation, affecting activation and fertilization environment.

Little evidence about influence of breeder genetics on gamete quality exists. However, parental genotype (at allelic or genomic level) is a potential strong source of sperm quality variation. In their review, Fauvel et al. [25] pointed out that genetic selection and domestication may alter sperm quality. As examples, Zohar [28] reported a 20-fold reduction of sperm volume produced by salmon after selection and Agnese et al. [29] observed a decrease of fertility in *Heterobranchus longifilis* at the fourth generation of domestication. In the guppy *Poecilia reticulata*, Evans [30] reported a trade-off between sexual ornamentation and milt quality, based on genetic variation. More attractive males that perform courtship behavior invest less in sperm quality and less ornamented sneaker males have better semen motilities. In the same species, inbreeding is also a genetic cause of sperm quality variation, high levels of inbreeding reducing male fertilization success under sperm competition [31]. Change in ploidy level of male broodstock can also modify sperm characteristics. Although no difference between ploidies was observed for other sperm quality descriptors, lower percentage of motile spermatozoa was reported in triploid males of tench (*Tinca tinca*) [32] and Prussian carp (*Carassius gibelio*) [33] than in diploids. In Atlantic cod (*Gadus morhua*), Peruzzi et al. [34] measured lower sperm velocity in triploid than in diploid fish shortly after activation.

Sperm properties of hormonally sex-reversed females have been investigated in perch [19,21] and salmonids [17,20]. In most cases, sexual genotype or sex reversal procedures do not seem to influence sperm quality and fertilization rate. However, comparison between XX and XY males belonging to these species is often difficult because, unlike tilapia, sex-reversed females are generally not functional (i.e., able to spontaneously emit milt). Moreover, sperm quality of YY males has never been analyzed so far in fish.

The aim of this study was to determine if sexual chromosomes or hormonal sex reversal treatment can influence sperm quality in Nile tilapia. XX (sex-reversed) and YY males were compared to normal XY males using different indicators: gonadosomatic index, sperm concentration, sperm motility (using CASA) and duration of sperm motility.

2. Materials and methods

2.1. Fish

Nile tilapia *O. niloticus* (Lake Manzala strain) used in this study originated from the Research and Education Center in Aquaculture (CEFRA), University of Liège (Belgium). All-male XX progeny was sired by XX male mated with normal XX female and fed with 65 mg kg^{-1} diet of 17α-methyltestosterone (Sigma) during the first 30 days of feeding. XY males came from normal cross between XY male and XX female. YY males were obtained by mating YY males and YY females. At the time of analysis, fish were 18 months old. Before the experiment, fish were stocked in 300-L tanks in a recirculating system at 27 to 28 °C with a 14 h light/10 h darkness photoperiodic regime. Feeding was performed once a day with commercial tilapia diet (38% proteins, 7% lipids, Coppens, the Netherlands). XX and YY males were maintained at a density of 200 fish m^{-3} and XY male were held in mixed sex tank at a density of 300 fish m^{-3}. Before sampling, selected broodstock fish were acclimated for 7 days in 250-L aquaria in a recirculating system (temperature: 27 °C; photoperiod: 14 h light/10 h darkness) to standardize their physiological state. Each aquarium was split in 2 compartments by an opaque screen and accommodated 2 individuals. During the acclimatizing period, fish were fed 4 times a day with special broodstock commercial tilapia diet (45% proteins, 5% lipids, Coppens, the Netherlands).

2.2. Sampling

Fish were anesthetized with 2-phenoxyethanol (Sigma) (0.4 mL L^{-1}) and then euthanized by cervical dislocation. After dissection, milt was collected in the sperm duct (to avoid any contamination and subsequent activation of spermatozoa by urine) with a syringe. Once the whole available quantity of sperm was extracted, it was directly diluted in the extender (Bicine solution at pH 7.8 [19,35]) with a 1: 10-v/v dilution and stored on ice.

All experiments were previously submitted to the agreement of the local ethic committee of the University of Liège and were conducted in compliance with the Belgian legislation on animal welfare and experimentation.

2.3. Gonadosomatic index (GSI)

After sperm sampling, gonads were removed and GSI was calculated on 16 individuals of each genotype as 100 × (gonad weight + sperm sample weight)/total body weight.

2.4. Sperm count

Sperm concentration was assessed on 16 fish of each genotype with a Bürker's cell hemocytometer. Diluted milt (1: 10-v/v extender) was rediluted 10-fold with extender. Samples were then re-suspended 20 × with a 4% formalin solution (total dilution 2000 ×). Both chambers of the cell were loaded with 10 μL of diluted sperm solution and spermatozoa were counted in 20 squares of each chamber [36]. Counting was performed using a phase-contrast microscope (400 × magnification). Sperm concentration is expressed as the number of spermatozoa per milliliter (cells mL^{-1}) of undiluted semen.

Total sperm number of each individual was calculated as sperm concentration × sample volume.

2.5. Spermatozoa motility

Spermatozoa motility was quantified by CASA using a Hamilton-Thorne computer-aided semen analyzer IVOS version 12.1 (Hamilthon-Thorne Research, Beverly, USA). CASA settings were optimized to ensure a good detection of tilapia spermatozoa (temperature = 27 °C, frames/s = 60 Hz, number of frames = 30, cell size = 2 pix, minimum cell size = 1 pix, cell intensity = 50, minimum contrast = 20). After determination of sperm concentration, each sample (1: 10-v/v extender) was diluted a second time in extender to obtain a concentration of 100×10^6 cells mL^{-1}. Sperm was activated by a 2-fold dilution in distilled water. Sample was gently homogenized and 10 μL of the semen solution was dropped in the center of the analysis chamber (Makler counting chamber, Sefi-Medical Instruments, Ltd., Haifa, Israel). After final dilution, time necessary for homogenization, pipetting and insertion of the chamber in the IVOS was 1 min and motility analysis started thereafter. Measurements were then carried out every 2 min until 7 min after sperm activation. For each recording time, analysis was performed on 10 consecutive fields of the chamber and collected data were means of 10 values. Motility parameters assessed with CASA were total motility (TM, %), progressive motility (PM, %), curvilinear velocity (VCL, μm s^{-1}), straight line velocity (VSL, μm s^{-1}), average path velocity (VAP, μm s^{-1}) and linearity (LIN, as VSL/VCL × 100, %). Motile spermatozoa are characterized by a VAP > 5 μm s^{-1} and progressive spermatozoa by a VAP > 25 μm s^{-1}. Motility parameters were measured on 16 fish for each genotype. Sperm motility decreases after activation and becomes very low after several minutes. When motility is low, the sensitivity of our IVOS is not sharp enough to properly track spermatozoa movements and the system gives erroneous data. It is able to distinguish a motile gamete from a static one but the velocity

Table 1
Mean (± SD) body weight, gonadosomatic index (%), sperm concentration ($\times 10^9$ cells mL^{-1}), total sperm number ($\times 10^6$ cells) and duration of sperm motility (min s) in XX, XY and YY male Nile tilapias. Different superscript letters indicate significant difference ($P < 0.05$).

	Body weight (g), n = 16	GSI (%), n = 16	Sperm concentration ($\times 10^9$ cells mL^{-1}), n = 16	Total sperm number ($\times 10^6$ cells), n = 16	Duration of motility (min s), n = 8
XX males	262 ± 21[a]	0.92 ± 0.40[a]	1.74 ± 0.79[a]	262 ± 123[a]	27′32″ ± 7′47″[a]
XY males	241 ± 25[b]	1.33 ± 0.48[a]	1.69 ± 0.69[a]	439 ± 325[a]	18′04″ ± 6′56″[a]
YY males	270 ± 25[a]	1.27 ± 0.69[a]	2.22 ± 1.11[a]	446 ± 446[a]	24′52″ ± 10′40″[a]

values (VCL, VSL and VAP) are incorrectly measured, i.e., these values become very high, whereas observed motility decreases. To avoid biased data, we set an efficiency threshold under which velocity data were rejected. Out of our observations (n = 48), the threshold was set as the mean value of TM from which velocity values started to increase and was equal to 25%. In other words, velocity values associated with a TM ≤ 25% were not reliable and were excluded from the analysis. To keep the same number of observations at each recording time and to take into account the motility drop or stop in some samples, rejected or missing values were arbitrary set to 0.

2.6. Duration of sperm motility

Spermatozoa movements were followed on the tracking screen of the IVOS analyzer after motility analysis (temperature = 27 °C; sperm concentration = 50×10^6 cells mL^{-1}). Total duration of sperm motility was measured from initiation of activation (with distilled water v/v) until full stop of all the observed spermatozoa movements. This parameter was determined on 8 fish (within the 16 used for motility analysis) per genotype.

2.7. Statistical analysis

All data were analyzed with ANOVA followed by post-hoc Tukey's test to identify differences between groups. Differences were considered as significant when $P < 0.05$. Data are reported as mean ± standard deviation (SD).

3. Results

The XY males displayed a significantly lower ($P = 0.004$) mean body weight (241 ± 25 g) than the two other genotypes (XX males: 262 ± 21 g; YY males: 270 ± 25 g; Table 1). However, GSI, sperm concentration, total sperm number and duration of sperm motility did not exhibit any significant difference between the three sexual genotypes. Overall means of GSI, sperm concentration, total sperm number and duration of motility for the three genotypes were, respectively, 1.18 ± 0.56%, 1.88 ± 0.90 × 10^9 cells mL^{-1}, 382 ± 331 million cells and 23′29″ ± 9′11″. Means for each genotype are presented in Table 1.

All parameters used to assess spermatozoa motility with CASA significantly and constantly decreased after sperm activation (Fig. 1). For each recording time, motility parameters were never different ($P > 0.05$) between the three genotypes. Motility parameters decreased more severely at the beginning of sperm activation, between 1 and 3 min. Mean values of TM of XX, XY and YY males were, respectively 58 ± 9, 56 ± 13 and 53 ± 15% 1 min after activation and decreased to 38 ± 15, 36 ± 18 and 35 ± 15% in the three respective genotypes 2 min later. Mean PM dropped from 48 ± 9, 44 ± 13 and 42 ± 15% at 1 min for XX, XY and YY males to 24 ± 15, 22 ± 18 and 21 ± 13%, respectively at 3 min. After 7 min, only 9 to 13% of spermatozoa were still progressive. Velocity parameters followed the same profile. Only the mean curvilinear velocity (VCL) of XX and YY male spermatozoa did not exhibit a significant difference between 1 and 3 min after activation. Mean VCL values ranged from 71 to 76 $\mu m\ s^{-1}$ after 1 min and decreased to 13 to 20 $\mu m\ s^{-1}$ after 7 min. Straight line velocity (VSL) and average path velocity (VAP) significantly decreased between 1 and 3 min (from 40-44 to 20–25 $\mu m\ s^{-1}$ and from 45-50 to 24–28 $\mu m\ s^{-1}$, respectively). Between 3 and 7 min, values did not significantly decrease. Mean linearity (LIN) was significantly different between 1 (56–59%) and 5 (23–31%)–7 (14–16%) min after activation.

Globally, none of the motility measurements differed ($P > 0.05$) between XX, XY and YY males.

4. Discussion

Sexual genotype and sex reversal procedures can affect different aspects of fish biology: growth [2,18], mating [18], behavior [12] and reproductive performances [10,11,13]. Although sex-reversed fish some-

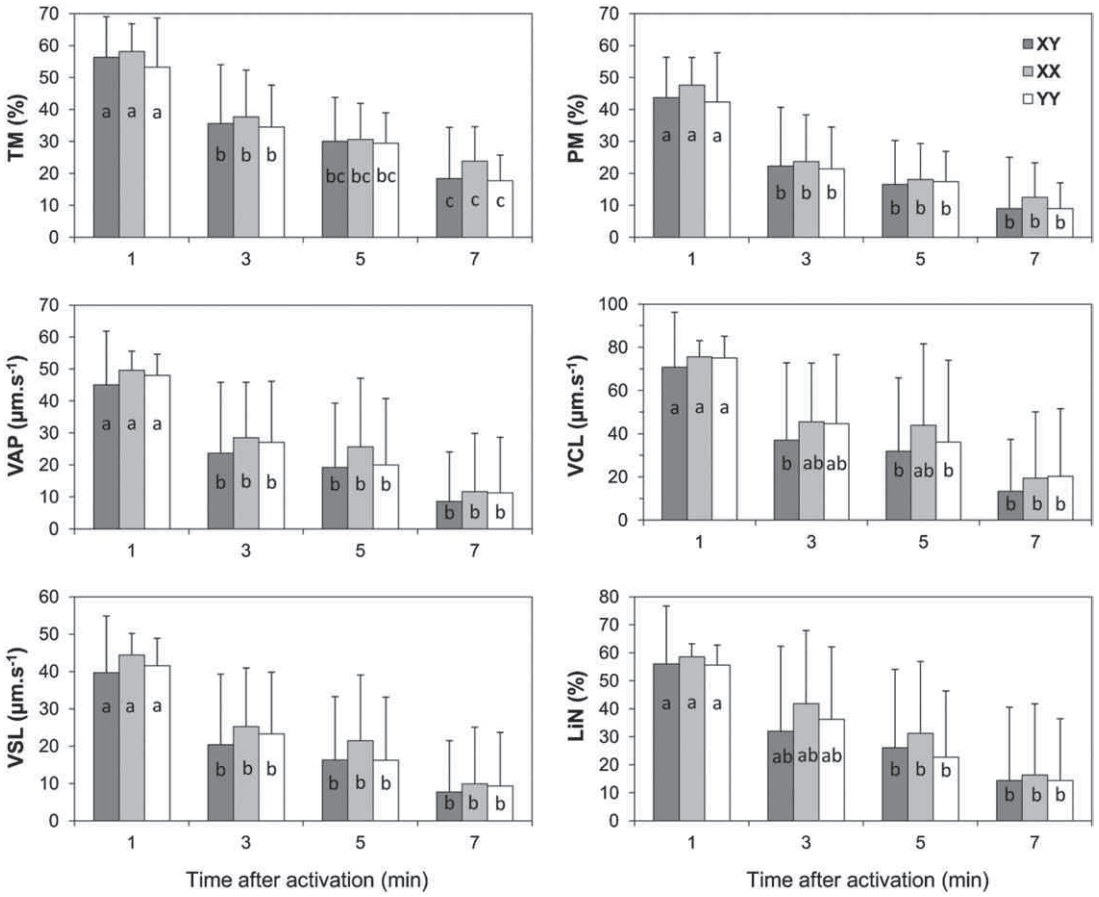

Fig. 1. Comparative motility of spermatozoa from XY, XX and YY male Nile tilapias assessed by CASA. TM, total motility; PM, progressive motility; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; LIN, linearity. Values are means ± SD, n = 16. Different letters indicate significant difference ($P < 0.05$).

times show impairments of genital tract development (e.g., absence of gonoduct, abnormal gonad morphology) or alteration of secondary sexual characteristics, most of them are generally sexually efficient [37–39]. In Nile tilapia, males with atypical sexual genotype (XX and YY) are morphologically undistinguishable from normal XY males. They produce and emit functional sperm and are able to reproduce naturally with females. However, as reported in *O. aureus* by Desprez and Mélard [11] and Desprez et al. [13], sexual genotype could affect reproductive performance of Nile tilapia breeders (Gennotte, unpublished data). Regarding males, reproductive success discrepancies could result from differences in sperm quality or in courtship and mating behavior.

In our study, none of the sperm traits tested significantly differed between XX, XY and YY males, suggesting that sexual genotype or hormonal sex reversal treatment do not affect sperm quality in Nile tilapia. Even though mean body weight of XY males was slightly lower than the other males, GSI did not vary between the three types of male. Mean GSI in the three genotypes were comparable to values reported by Admassu [40] in natural environment (between 0.19 and 1.61% depending on the season) and Fessehaye et al. [41] on normal XY males (0.8–1.4%).

In fish, sperm motility lasts for tens of seconds to tens of minutes, depending on species [42]. In *O. niloticus*, spermatozoa remain motile for 4 to more than 40 min after activation [43–45]. Similar motility duration was also reported in related species *O. mossambicus* with values above 30 min [46].

Our study showed that, among fishes, tilapia is characterized by long sperm motility duration and low sperm concentration. These two features are related to their reproductive strategy and spawning behavior. Tilapia has an asynchronous gametogenesis [47]. If environmental conditions are favorable, tilapia can

spawn throughout the year. After complex courtship and mating behavior, female deposits small batches of eggs in a sequential way and male emits milt over them. Males do not attempt to fertilize eggs after each deposition and it is likely that fertilization is achieved in the mouth of the female after picking up [48,49]. Consequently, fertilization time can be delayed from gamete emission. In *O. niloticus* × *O. mossambicus* hybrids, Myers and Hershberger [49] showed that egg hydratation up to 15 min before fertilization had no influence on fertilization. This suggests that fertilization success is related to sperm motility duration.

In our study, measured sperm concentration (from 1.69 to 2.22 $\times 10^9$ cells mL^{-1}) was similar for the three genotypes and was in the previously reported range for this species: 3.04 $\times$ 10^9 cells mL^{-1} [43] and 1.33 $\times$ 10^9 cells mL^{-1} [50]. Species with synchronous gametogenesis and seasonal spawning produce a more abundant and concentrated milt. For example, sperm concentration of African catfish *Clarias gariepinus* [51], rainbow trout *Oncorhynchus mykiss* [17] and perch *Perca fluviatilis* [19] is at least 10 times higher than tilapia semen.

Sperm motility and velocity decreased progressively after activation. In fish, spermatozoa are immotile in seminal fluid and flagellar movements are rapidly initiated after their release in aquatic environment. Just after activation, sperm velocity and displacement are high and rapidly decrease thereafter, as preaccumulated ATP energy store is consumed by the cell [42]. First measurements of motility and velocity were recorded 1 min after sperm activation and consequently did not reflect maximum values. Values observed in this study were similar to previous studies, Mochida et al. [44] showing around 50% motile spermatozoa 1.5 min after activation. In *O. mossambicus*, sperm has a similar duration of motility and Linhart et al. [52] reported slightly lower values of motility and velocity. After 20 s of activation, percentage of motile spermatozoa was around 48% and velocity around 42 $\mu m\ s^{-1}$. As for the previous parameters assessed in our study, no difference in motility and velocity was observed between the three sexual genotypes.

In addition to motility parameters, the ultimate and most integrative measurement of semen quality is its fertilization capacity. Because of dependence on egg availability and quality, it was not assessed in this study. However, it has been shown in several species that motility strongly correlates with fertilization success [22,23].

Other studies dealing with sperm characteristics of male and sex-reversed female teleosts showed no relationship between sperm quality and sexual genotype or sex reversal treatment. In perch, Rougeot et al. [19] found no difference in terms of GSI, sperm density, motility, velocity and fertilization rate between XY and XX males. In coho salmon (*Oncorhynchus kisutch*), Fitzpatrick et al. [20] showed that when milt was stripped from functional XX males, sperm density, motility and velocity were similar to normal XY males.

Hormonal sex reversal treatments often induce abnormal morphology of the reproductive system in XX males (e.g., gonad deformities, lack of sperm duct or gonopore) [37,39,53]. In this case, intratesticular sperm has to be collected by gonad squeezing, potentially affecting its quality. In salmonids, Geffen and Evans [17] and Fitzpatrick et al. [20] reported some differences in sperm features between males and sex-reversed females but attributed most of them to the collecting method (stripping vs. intratesticular sampling). In our study, the male tilapias of the three sexual genotypes had a functional sexual apparatus with similar morphology and were able to emit milt by stripping.

A study carried out on *Betta splendens* highlighted that males produced by sex reversal of genetic females (by immersion of fry in 17α-methyltestosterone bath) differed from normal males by a decreased GSI, sperm count and sperm motility duration [18]. In this species, impairment of sperm characteristics seemed to be a consequence of the hormonal treatment and not an effect of sexual genotype, since the decrease was correlated to the dose of hormone used in masculinization immersion. Among the two particular sexual genotypes tested for this study, XX males were sex-reversed but YY males did not receive any hormonal treatment. As sperm quality was similar in the three groups of fish, it could be assumed that hormonal treatment had no influence on tested parameters and that the discriminating variable between male groups was therefore restricted to the sexual genotype. Based on the similar quality of sperm from masculinized females and normal males in coho salmon, Fitzpatrick et al. [20] also suggested that sex reversal procedure does not affect sperm development and maturation.

Tilapia could also experience sex reversal in the wild, without any hormonal intervention but by effect of temperature or by influence of minor genetic factors on sex determination [54]. Examining sex-ratios of numerous progenies sired by breeders of different African populations, Bezault et al. [55] highlighted strong

evidences of the existence of natural XX males and XY females. The existence of the latter would imply the existence in their progenies of natural YY males. If males with different genotypes coexist in the wild, we can postulate that their relative fitness would not be affected by sperm competition.

In conclusion, sperm traits assessed in this study showed that XX and YY male Nile tilapias have the same sperm quality as normal XY males. Several comparative studies of sperm quality in other teleost species reported no difference between XY males and XX sex-reversed females, supporting our findings that hormonal sex reversal procedures do not affect sperm quality. Moreover, our study is the first attempt to characterize sperm quality in YY supermales in fish. As YY males did not receive any hormonal treatment and have a similar sperm quality to XX and XY males, we can conclude that sexual genotype do not affect sperm quality in Nile tilapia. In the wild or in an artificial production context, reproductive capacities of males with atypical sexual genotype would not be affected by sperm quality and could be the same as normal males.

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Chapitre 3

Does sexual genotype affect agonistic behaviours and sex steroid levels in the Nile tilapia *Oreochromis niloticus* ?

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Abstract

In Nile tilapia *Oreochromis niloticus*, sex reversal processes using exogenous sex steroids allow to produce fish with different sexual genotypes that constitute major tools to investigate the mechanisms of sex differentiation, from gonad and brain sexual differentiation to behaviour. The aim of this study was to assess the influence of sexual genotype and the role of circulating sex steroids on the expression of agonistic behaviours in Nile tilapia breeders. Eight agonistic behaviours (4 threats and 4 attacks) were quantified in five types of confrontation involving one male (M) and one female (F): MXY×FXX (control), MXY×FXY, MXY×FYY, MXX×FXX and MYY×FXX. Our results showed that phenotypic male and female Nile tilapias with different sexual genotypes have particular behavioural and physiological traits. Compared to natural XX female and XY male, XY and YY females, and XX males expressed higher level of aggressiveness that could be related to higher levels of 17β-oestradiol and 11-ketotestosterone respectively. Our results suggest that the presence of a Y chromosome increases aggressiveness in females. However, since the same relationship between aggressiveness and the Y chromosome is not observed in males, in which the level of aggressiveness is paradoxically higher in XX, we can hypothesize that the differences in aggressiveness are not directly dependent on the genotype but on the sex reversal procedures which young fry were exposed to during their sexual differentiation to produce these breeders. These hormonal treatments could have permanently modified the development of the brain and consequently influenced the behaviour of adults independently of their genotype. In both hypotheses (genotype or sex reversal influence), the causes of behavioural modifications have to be searched in an early modification of the brain sexual differentiation.

Key words: sex differentiation, sex reversal, aggressive behaviour, brain, androgen, oestrogen

1. Introduction

Developmental processes leading to a sexually differentiated organism are noteworthy in fish. From the diversity of their sex determination systems to the lability of their gonad and brain sexual differentiation, the ontogenetic steps of organization of sex differences in fish are singular among vertebrates (Devlin & Nagahama, 2002). In mammals, the gonad phenotype is genetically determined early during development and gonadal hormones subsequently shape irreversible sex differences in the brain that modulate physiology and behaviours in adulthood (MacLusky & Naftolin, 1981). In teleosts, the genetic and endocrine dialog between the developing brain and gonad in the process of sexual differentiation is not fully deciphered but the developmental polarity of these structures could be reversed compared to mammals, the differentiated brain determining the fate of the gonads. This idea was first proposed by Francis (1992) and is now supported by an increasing number of evidence regarding sex dimorphic gene expression and steroid metabolism in the brain (Blázquez & Somoza, 2010; Diotel *et al.*, 2010; Godwin, 2010). The reversed developmental polarity (*i.e.* sexual differentiation of the brain prior to the gonads) may be an adaptive mechanism providing to fish their particular high plasticity of sexual differentiation and the bipotentiality characterizing both gonads and brain (Godwin, 2010; Munakata & Kobayashi, 2010). This concept is well figured in hermaphroditic species in which external factor such as change in social structure can trigger a total phenotypic sex change, with adapted behaviours related to the sexual phenotype (Devlin & Nagahama, 2002). Observations made on hermaphrodites suggest that both male and female characteristics are present in the brain of fish and can be adaptively activated in response to their physiology or environmental influences (Munakata & Kobayashi, 2010). The plasticity of sex differentiation is not restricted to hermaphrodites as exemplified by the numerous sex reversal experiments performed in diverse gonochoristic species. Sex reversal procedures usually rely on the administration of sex steroids or the exposure to environmental factors such as temperature that override the genetic sex determinism (Pandian & Sheela, 1995; Baroiller & D'Cotta, 2001). Even in gonochoristic fish, the sexual plasticity could extend throughout adulthood as suggested by Paul-Prasanth *et al.* (2013) who recently reported the sexual inversion of XX adult medaka *Oryzias latipes* Temminck & Shlegel 1846 and Nile tilapia *Oreochromis niloticus* L. 1758 by long-term oestrogen depletion. In this experiment, sex-reversed XX fish expressed male-typical

behaviour and mated with females, showing that the brain sexual bipotentiality is conserved in adulthood.

In *O. niloticus*, sex reversal procedures allow to produce all the combinations of sexual phenotypes and genotypes (XX, XY and YY males and females). These fish are used in aquaculture for the control of reproduction and the improvement of growth by all-male mass production (Baroiller & Jalabert, 1989; Toguyeni *et al.*, 2002). Fish with atypical combinations of sex chromosomes and sexual phenotype represent also major tools to study the mechanisms of sex determination and the influence of the genotype on biological traits such as reproductive performances and behaviour.

In the African pygmy mouse *Mus minutoides* Smith 1834, one of the rare mammals harbouring an unusual sex determination system with XX and XY females, these latter compensate some loss of fitness by improved reproductive performances, leading to the production of more offspring compared to XX females (Saunders *et al.*, 2014). Differences in behaviour, and especially in aggressiveness level, were also described in XY female laboratory mice *Mus musculus* L. 1758 (Gatewood *et al.*, 2006). In fish, comparison of physiological and reproductive features like sex steroid profiles and sperm quality showed no difference between XX sex-reversed and XY normal males in salmonids (Geffen & Evans, 2000; Fitzpatrick *et al.*, 2005) and European perch *Perca fluviatilis* L. 1758 (Rougeot *et al.*, 2004). Similarly, we observed comparable sperm characteristics in XX, XY and YY males in *O. niloticus* (Gennotte *et al.*, 2012). On the contrary, particular biological traits were reported in sex-reversed females of blue tilapia *Oreochromis aureus* Steindachner 1864, a closely related species with a ZZ/ZW sex determination system. In this species, when ZZ females competed with ZW females for reproduction, the spawning rate of ZZ females was lower (Desprez & Mélard, 1998) and related to a higher level of dominance and aggressiveness than in ZW females (Ovidio *et al.*, 2002). Desprez and Mélard (1998) hypothesized that the phenotypic sex reversal could be incomplete, particularly in the brain, and that the interaction of genotypic and endocrine factors could modify the expression of behaviour.

Hormones, sex steroids especially, are important actors of the expression of social behaviours, including aggressiveness. Differences in profiles of circulating steroids depend on the sexual phenotype but also on the social status, dominant and more aggressive fish generally exhibiting higher androgen levels than subordinates (Oliveira *et al.*, 2002; O'Connell *et al.*,

2013). However, relationships between hormones and behaviour are complex and hormones are considered as behavioural modulators rather than the trigger of agonistic behaviour. Whether they could be the cause of behavioural differences remains uncertain (Oliveira & Gonçalves, 2008).

The aim of this study was to assess the influence of sexual genotype (XX, XY and YY) and the role of circulating sex steroids on the expression of agonistic behaviours in *O. niloticus*. Threatening and fighting behaviours were quantified in pairs involving a male and a female with various sexual genotype combinations. Basal levels of testosterone, 17β-oestradiol and 11-ketotestosterone were measured in a standardized context avoiding influence of reproduction and sexual arousal. These data provide new insights on the influence of sex chromosomes in the process of brain sexual differentiation and on the possible role of sex steroids in translating genotypic differences to the behavioural level.

2. Materials and methods

2.1. Fish

Oreochromis niloticus from the Lake Manzala strain were from the Research and Education Centre in Aquaculture (CEFRA), University of Liège (Belgium). Six groups of fish with different combinations of sexual phenotype and genotype were tested: XX males (MXX; n = 22), XY males (MXY; n = 34), YY males (MYY; n = 22), XX females (FXX; n = 34), XY females (FXY; n = 22) and YY females (FYY; n = 22). All-male XX progenies were obtained by mating XX males with normal XX females and feeding fry with 65 mg.kg^{-1} diet of 17α-methyltestosterone (MT) (Sigma) during the first 30 days of feeding (from 10 days post-fertilization). YY males were obtained by mating YY males with YY females. YY females resulted from the same cross followed by phenotypic sex reversal by alimentary administration of 17α-ethynyloestradiol (EE2) (Sigma, 500 mg kg^{-1}) during the first 30 days of feeding. XY individuals produced by mating YY males with XX females were sex-reversed by a 30-day administration of EE2 (Sigma, 150 mg kg^{-1}) to obtain XY females. XY males and XX females came from normal cross between XY males and XX females. Experiments were conducted on 18 months-old fish. Individual weight was standardized in order to avoid bias in the establishment of dominance and agonistic interactions. Mean body

weights (± S.E.) were: MXX: 274 ± 8 g, MXY: 276 ± 8 g, MYY: 279 ± 6 g, FXX: 227 ± 4 g, FXY: 225 ± 5 g, FYY: 230 ± 7 g. Mean body weight were statistically similar among female groups (ANOVA: $F_{2,98} = 0.177$; $P > 0.05$) and among male groups (ANOVA: $F_{2,95} = 0.099$; $P > 0.05$). Sex-reversed individuals were phenotypically undistinguishable from normal ones. All individuals had a functional sexual apparatus: males were able to emit milt, and females, oocytes by stripping. Before the experiment, fish were stocked in 300-l tanks (density: 50 kg m^{-3}) in a recirculating system at 27-28 °C with a 14 h light / 10 h darkness photoperiodic regime. Feeding was performed once a day at satiation with commercial tilapia diet (38 % proteins, 7 % lipids, Coppens, the Netherlands).

Animal care and the experimental protocol were approved by the local ethic committee of the University of Liège and were conducted in compliance with the European legislation on animal welfare and experimentation.

2.2. Behavioural records and analysis

Agonistic behaviours were observed in fights staged between one male and one female. Five different crosses of broodstock fish with different sexual genotypes were tested: MXY × FXX, MXY × FXY, MXY × FYY, MXX × FXX and MYY × FXX. In each cross, one of the 2 breeders had a normal sexual genotype (MXY or FXX). The pairs MXY × FXX were considered as controls. Each confrontation was repeated with 6 different pairs of fish. In each confrontation, the male displayed a body weight 20 % higher than the female in order to respect the natural sexual dimorphism in *O. niloticus* (Toguyeni *et al.*, 1997).

Prior to observation, fish were acclimated in 250-l aquaria in a flow-through system (temperature: 27 °C; photoperiod: 14 h light / 10 h darkness). Each aquarium was split into two compartments by an opaque partition and accommodated two individuals. Male was on the side of the water inlet and female on the side of the water outlet. During the acclimatization period, fish were fed four times a day with special broodstock commercial tilapia diet (45 % proteins, 5 % lipids, Coppens, the Netherlands). In order to standardize their physiological state, acclimatization was adjusted on the ovarian maturation cycle of the female. After the first spawn of the female, unfertilized eggs were gently removed from her

mouth and behaviour recording was performed two days after egg removal in order to avoid influence of handling stress.

Behaviours were recorded with a video camera (Logitech webcam Pro 9000) connected to a computer and placed behind a screen to prevent any visual contact between the fish and the experimenter. Each pair of fish was video-recorded four times over the day. Sequences were filmed at 0900, 1100, 1300 and 1500 hours. Fish were not fed on the day of observation. Fish were staged by removing the opaque partition 10 min before the first recording. Because aggressive interactions were more intense just after pairing, the first sequence was longer and lasted 10 min; the following recordings were run for 5 min (total recording time: 25 min).

Behavioural analysis was performed using The Observer XT software (Noldus Information Technology, the Netherlands). Quantified agonistic behaviours can be classified in threats and attacks. A detailed description of these behaviours was made by Baerends and Baerends-van Roon (1950) and Falter (1983) and summarized by Longrie *et al.* (2013) (Table I).

Table I. Description and quantification method of the agonistic behaviours observed in Nile tilapia confrontations (1 male vs 1 female), adapted from Baerends and Baerends-van Roon (1950), Falter (1983) and Longrie *et al.* (2013).

Behaviour	Measurement	Description
Threats		
Fin raising	Duration	Dorsal, caudal and anal fins are erected.
Throat swelling	Duration	Buccal cavity puffs up by spreading of the branchiostegal membrane; generally accompanied and emphasized by opercular spreading.
Chasing	Duration	Rapid swim towards the opponent.
Frontal display	Count	Frontal approach of the opponent, usually accompanied by a throat swelling and opercular spreading.
Attacks		
Lateral attack	Count	The attacker violently puts his mouth on the side of the opponent; can ends by a bite.
Tail beating	Count	A sudden or repetitive slap(s) of the tail; fish are side by side.
Mouth fighting	Count	Fish are face-to-face and press their wide open mouth one against the other
Biting	Count	Usually on the side or fins.

Quantification was performed by counting behaviours expressed as punctual events and by measurement of duration for behaviours expressed as a sustained state. The first ones are expressed as frequencies (h^{-1}), the second ones as a proportion (%) of the total recording duration. In this case, the sum of the three concerned behaviours can exceed 100 % as these behaviours can be concomitant.

2.3. Steroid assay

Blood was sampled in XX, XY and YY males and females to measure serum concentration of sex steroids. Fish were transferred from stock tanks to the same facility and held in the same conditions as described above for behavioural records (250-l aquaria split in two compartments). Aquaria were first loaded with males, then with females. Sixteen individuals from each sexual phenotype/genotype combination were sampled. Fish were selected in the same stocks as for behavioural observations but were different individuals. Males were acclimatized one week in aquarium before sampling. Acclimatizing period of females was adjusted on their maturation cycle as described above. Females were sampled two days after their first spawn. Fish were anaesthetized (benzocaine, Sigma, 40 mg l^{-1}) and blood was sampled by caudal venipuncture. After centrifugation (4500 rpm, 20 min, 10 °C), serum was stored at -20 °C until analyses.

Serum concentrations of testosterone (T), 17β-oestradiol (E2) and 11-ketotestosterone (11-KT) were assayed by radioimmunoassay (RIA) after two extractions with cyclohexane-ethyl acetate (1:1) as described in Douxfils *et al.* (2007). All samples and standards were assayed in duplicate. Radioactive hormones were purchased from Amersham Pharmacia (Buckingamshire, England), the T and E2 antibodies from the Laboratoire d'Hormonologie de Marloie (Belgium), and the anti-11KT was provided by Dr. A. Fostier (INRA, Rennes France). Intra-assay coefficients of variation were 4.2, 6.1 and 3.5 % for T, E2 and 11KT respectively. Detection limits ranged from 50 to 80 pg ml^{-1}.

2.4. Data analysis

Expressions of behaviours were separately compared between males and between females. Concentrations of circulating sex steroids were compared between the six groups (XX, XY, YY males and females together). As datasets did not comply with both homoscedasticity and normality, analyses were performed using the non-parametric Kruskal-Wallis test, followed by Mann-Whitney U test for two by two comparisons. Differences were considered as significant at $P \leq 0.05$. Behavioural data are graphically represented using box plots showing the median, the 25 and 75 % percentiles, the minimum and maximum. Results are presented in different boxes for males and females, except for the mouth fighting behaviour that involved the two partners at an identical level. In order to present an overview of the aggressiveness level, we summarized the data collected for all the behaviours in two graphs representing the total agonistic behaviours. Behaviours were grouped according to their quantification method (count vs duration). One graph represents the mean duration of agonistic behaviour expression (% time); the other represents the sum of the countable agonistic behaviours expressed per hour. Steroid concentrations are expressed as mean ± S.E. Statistical analysis was performed using Statistica 10 (StatSoft, France).

3. Results

3.1. Agonistic behaviors

Fin raising (Fig. 1a) was expressed from 0 to 47 % time in pairs with a FXX and from 29 to 76 % time in pairs with a FXY or FYY. These females displayed a significantly higher median expression level (43 and 62 % time for FXY and FYY respectively) than in FXX (9-11 % time). Among MXY, those confronted to FXY and FYY erected their fins at a similar level to females, significantly higher than the other males. Within crosses with FXX, MXX showed a median level (27 % time) significantly higher than MXY and MYY (14 % time).

Throat swelling (Fig. 1b) median expression level was significantly higher for both males and females in crosses with a FXY or FYY (from 29 to 52 % time) than in crosses with FXX (from 8 to 16 % time).

Chasing (Fig. 1c) was observed at a very low level in crosses MXX × FXX, MYY × FXX and MXY × FXX (median level ranged from 0 to 2 % time) and was more frequent in males and females in MXY × FXY (median: 43 and 33 % time respectively) and MXY × FYY (median: 12 and 13 % respectively). The time spent in chasing was very variable in these 2 crosses, depending on the pairs observed (from 0 to 58 % time). The only statistical difference was observed between FXY and all three FXX with a significant higher chasing behaviour for FXY.

Frontal display (Fig. 1d) was expressed in a similar pattern. The median frequency of expression of this behaviour ranged from 0 to 4 h^{-1} in confrontations with a FXX and from 2 to 20 h^{-1} in confrontations with a FXY or FYY.

Lateral attacks (Fig. 1e) were also significantly more frequent in crosses MXY × FXY (median: 23 and 19 h^{-1} for males and females respectively) and MXY × FYY (median: 35 and 48 h^{-1} for males and females respectively) compared to the control crosses MXY × FXX (median: 5 and 4 h^{-1} for males and females respectively). Frequency of attacks in the 2 other crosses with a FXX were not different from the control.

Tail beating (Fig. 1f) median expression levels ranged from 0 to 48 h^{-1} in females and from 22 to 59 h^{-1} in males. Only the males and the females from the MXY × FXY confrontations showed significantly higher level of expression compared to the control group MXY × FXX.

Mouth fighting (Fig. 1g) significantly occurred more frequently in the MXY × FXY (median: 11 h^{-1}) confrontations than in the control (median: 0 h^{-1}). This behaviour was also highly expressed in MXY × FYY confrontations (median: 11 h^{-1}) but in a wide range (from 0 to 46 h^{-1}). Median expression level ranged from 0 to 1.2 h^{-1} in confrontations with FXX.

Biting (Fig. 1h) behaviour was not observed in crosses MYY × FXX and MXY × FXX. Expression was low and not significantly different from 0 in MXX × FXX. It was significantly more frequent in crosses MXY × FYY (median: 12 and 16 h^{-1} for males and females respectively) and intermediate but not significantly different from all the other crosses in MXY × FXY (median: 5 h^{-1} for both sexes).

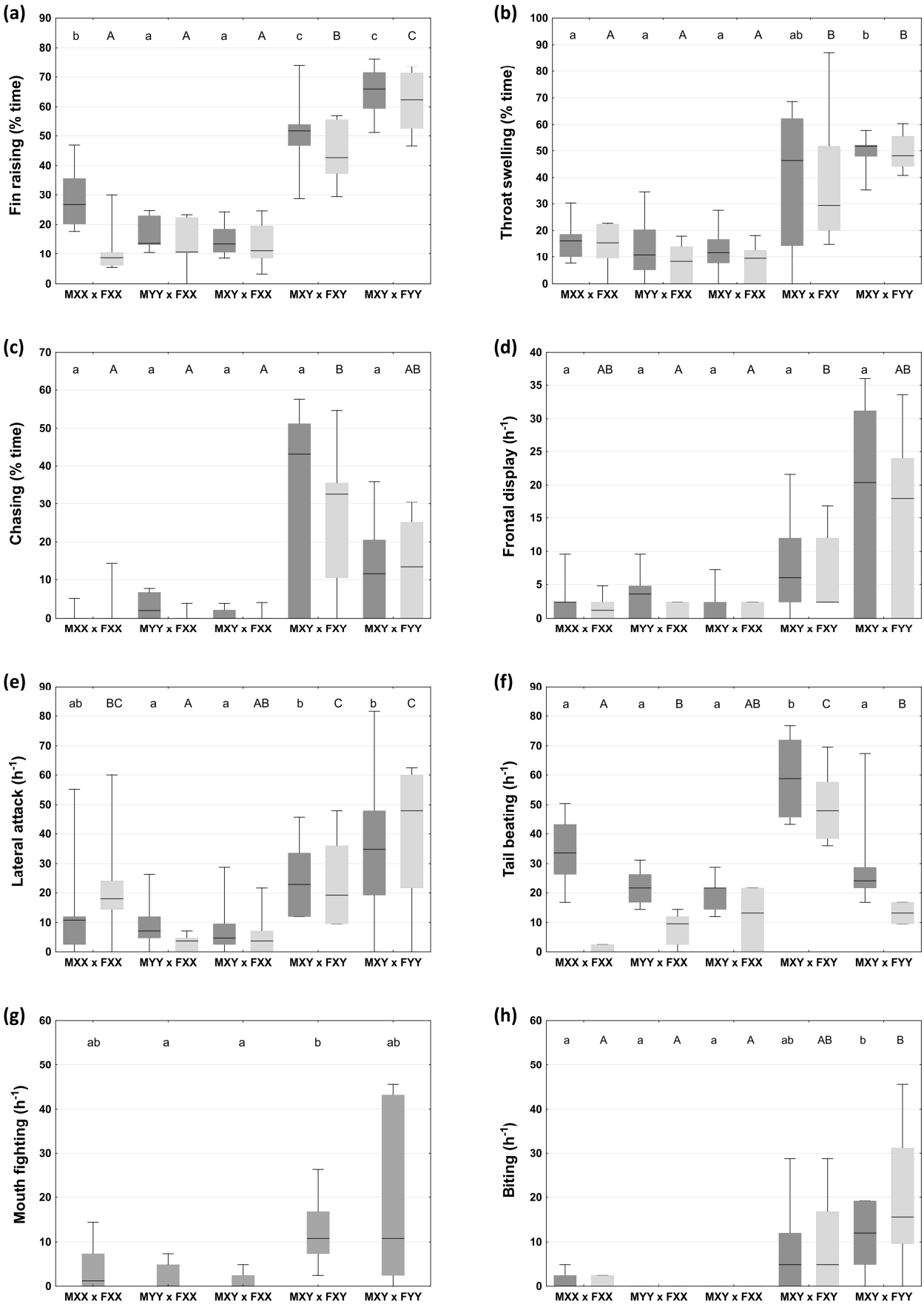
(a)
Fin raising (% time)
b A a A a A c B c C
(b)
Throat swelling (% time)
a A a A a A ab B b B
(c)
Chasing (% time)
a A a A a A a B a AB
(d)
Frontal display (h-1)
a AB a A a A a B a AB
(e)
Lateral attack (h-1)
ab BC a A a AB b C b C
(f)
Tail beating (h-1)
a A a B a AB b C a B
(g)
Mouth fighting (h-1)
ab a a b ab
(h)
Biting (h-1)
a A a A a A ab AB b B
MXX x FXX
MYY x FXX
MXY x FXX
MXY x FXY
MXY x FYY

Fig. 1. Agonistic behaviour levels in five types of confrontation staged between 1 male and 1 female *O. niloticus* with different sex genotypes: MXX × FXX, MYY × FXX, MXY × FXX (control), MXY × FXY and MXY × FYY (n = 6). Fin raising (a), throat swelling (b) and chasing (c) behaviours are expressed as % of observation time. Frontal display (d), lateral attack (e), tail beating (f), mouth fighting (g) and biting (h) behaviours are expressed as number per hour. Bar: median, box: 25 and 75 % percentiles, whiskers: minimum and maximum. Different upper case letters indicate significant differences between females, and different lower case letters indicate significant differences between males. Dark grey: males, light grey: females.

The total time spent in the 3 state behaviours (fin raising, throat swelling and chasing) was significantly higher in the pairs MXY × FXY and MXY × FYY than the pairs with a FXX (Fig. 2a). The % time spent in these behaviours ranged from 17 to 54 % (median: 41 %) for FXY, from 33 to 51 % (median: 42 %) for FYY and from 3 to 17 % (median: 8 %) for FXX. Among males, the expression of these behaviours was significantly higher in MXY staged with FXY and FYY (median: 44 and 39 % respectively) than the other males staged with FXX (median: 15 % in MXX, 10 % in MYY and 9 % in MXY). Expression level in MXX was significantly higher than in MYY and MXY confronted to FXX.

The sum of the event behaviours (lateral attack, frontal display, tail beating, mouth fighting and biting) followed a similar profile with a higher expression level in crosses MXY × FXY and MXY × FYY than in the other ones (Fig. 2b). The total count of these agonistic behaviours reached median values of 95 and 94 h^{-1} in FXY and FYY respectively, and 104 and 112 h^{-1} in MXY staged with them. Agonistic behaviours were expressed at a lower frequency in crosses with FXX (median ranged from 16 to 28 h^{-1} and from 26 h^{-1} to 48 h^{-1} in females and males respectively). Within the crosses with FXX, the number of agonistic behaviours was significantly higher in MXX than MXY and MYY.

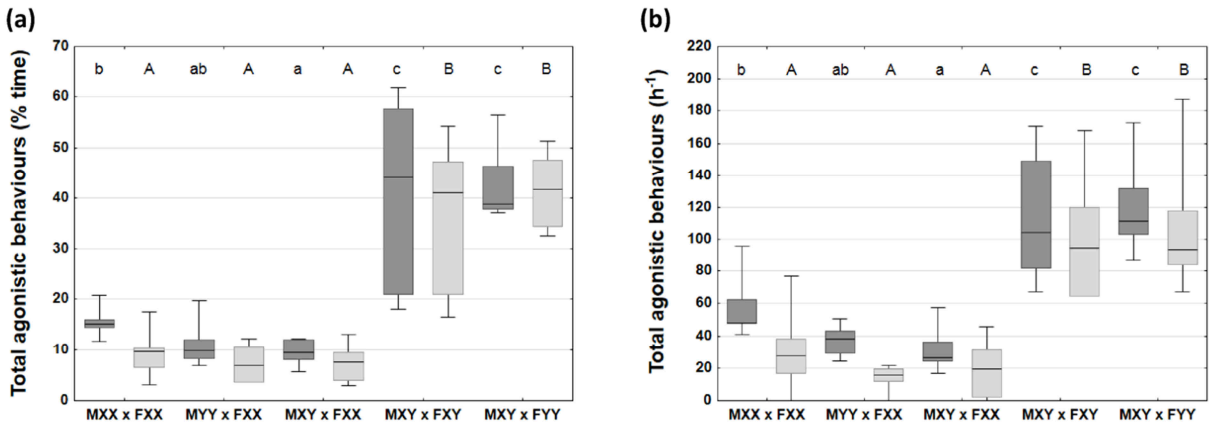

Fig. 2. Summary of agonistic behaviour levels in five types of confrontation staged between 1 male and 1 female *O. niloticus* with different sex genotypes: MXX × FXX, MYY × FXX, MXY × FXX (control), MXY × FXY and MXY × FYY (n = 6). (a) Mean duration (% time) of state behaviours: fin raising, throat swelling and chasing. (b) Total number (h^{-1}) of event behaviours: lateral attack, frontal display, tail beating, mouth fighting and biting. Bar: median, box: 25 and 75 % percentiles, whiskers: minimum and maximum. Different upper case letters indicate significant differences between females, and different lower case letters indicate significant differences between males. Dark grey: males, light grey: females.

3.2. Sex steroids

Mean T serum concentration was significantly higher in males than in females (overall mean: 34.7 ± 6.4 and 4.3 ± 0.6 ng ml^{-1} respectively), but within the same sex phenotype, no statistical difference was observed between the different sex genotypes (Fig. 3a). Mean circulating level of E2 was significantly lower in MXY (2.5 ± 0.2 ng ml^{-1}) than in MXX (4.7 ± 1.1 ng ml^{-1}) and MYY (4.7 ± 0.8 ng ml^{-1}) (Fig. 3b). In females, the highest concentrations of E2 were measured in the YY genotype. Mean value in FYY (14.2 ± 2.2 ng ml^{-1}) was significantly different from the concentration in FXX (6.5 ± 1.4 ng ml^{-1}). FXY were characterized by an intermediate level (9.5 ± 1.7 ng ml^{-1}). No difference in 11KT concentrations was observed between FXX, FXY and FYY (mean: 3.4 ± 0.3 ng ml^{-1}) (Fig. 3c). Higher levels were measured in males with a mean value in MXX (26.5 ± 4.2 ng ml^{-1}) significantly higher than in MXY (16.0 ± 4.1 ng ml^{-1}) and YY (16.4 ± 2.8 ng ml^{-1}).

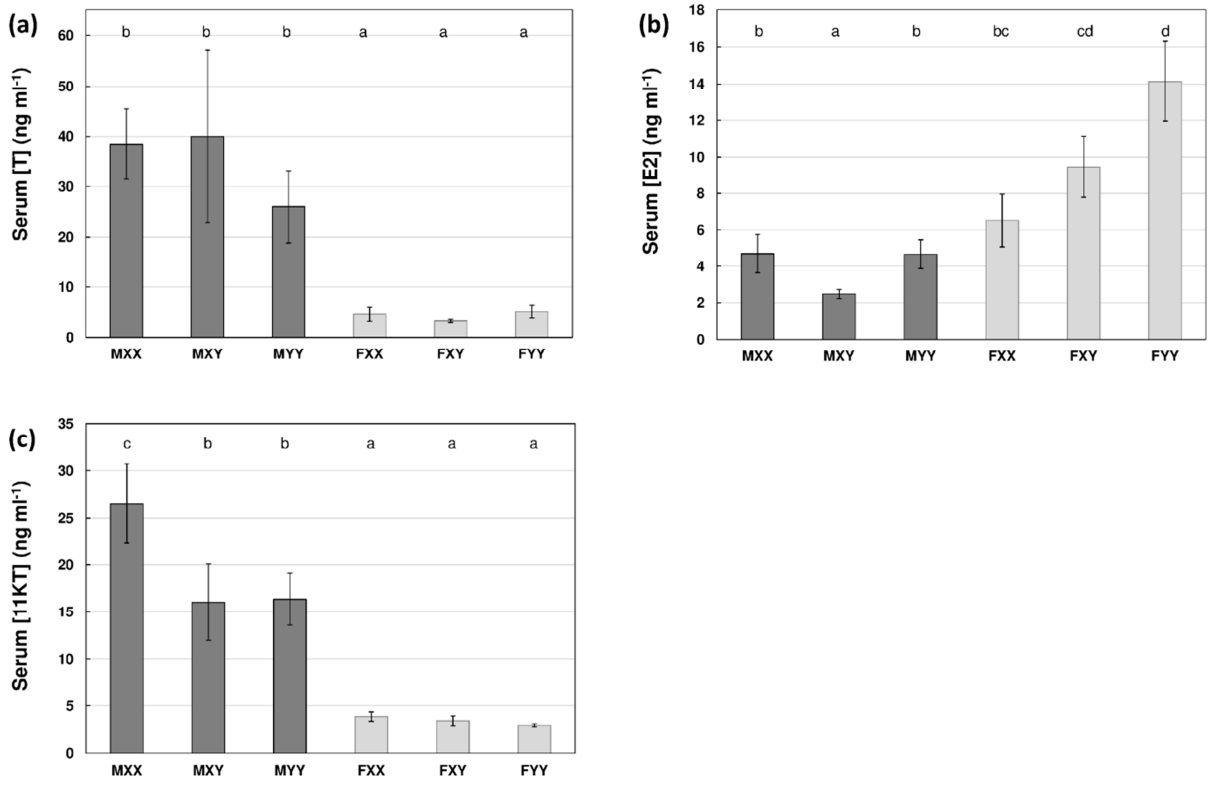

Fig. 3. Circulating levels (mean ± S.E.) of (a) testosterone (T), (b) 17β-oestadiol (E2) and (c) 11-ketotestosterone (11KT) in adult male and female *O. niloticus* with diferent sex genotypes: XX, XY and YY. Different letters indicate significant differences. Dark grey: males, light grey: females.

4. Discussion

The most striking result of the present study was the higher expression of agonistic behaviours in FXY and FYY compared to FXX. From low-intensity (displays and threats) to high-intensity acts (attacks), a similar behavioural expression profile was observed with a higher aggressiveness in MXY × FXY and MXY × FYY confrontations. Although this conclusion can be nuanced when considering behaviours individually, the global pattern emerging from all agonistic behaviours together gives an unequivocal view of the aggressiveness level in these individuals. The higher aggressiveness level in these pairs can be attributed to the female since the intensity of fights was far lower in pairs with normal genotypes MXY × FXX. In every type of confrontation, the male slightly expressed more agonistic behaviours than the female, according to its larger size and the social and territorial behaviour of this species (Baerends & Baerends-van Roon, 1950; Boscolo *et al.*, 2011). *Oreochromis niloticus* has a lek mating system in which male defends a territory to ensure his reproductive success. But even out of a reproductive context, a hierarchical social structure sets up inside a group in which dominant individuals fervently defend a territory against intrusion (Baerends & Baerends-van Roon, 1950). If the intruder is not much aggressive, as the FXX, the interaction rapidly finds an outcome in which the dominant establishes its status against the subordinate. On the contrary, if the intruder is more combative (or more responsive to its opponent's aggressions) and fights to win the confrontation, an aggressive escalation is initiated for the establishment of dominance (Oliveira *et al.*, 2005; Carvalho & Gonçalves-de-Freitas, 2008; Boscolo *et al.*, 2011).

Regarding our main results, two hypotheses can be proposed to explain the higher aggressiveness observed in females carrying Y chromosome. The first one is a direct influence of sexual chromosomes on behaviour through brain sexual differentiation, or on activation of neural mechanisms related to aggressiveness expression, as proposed by Desprez and Mélard (1998) and Ovidio *et al.* (2002) to explain the higher aggressiveness level observed in sex-reversed ZZ females compared to ZW females in *O. aureus*. Brain organizational or activational modifications would be under control of genetic factors linked to the Y chromosome or induced by the absence of a twin copy of factors carried on the X chromosome. This kind of chromosomal influence on sex differentiation is observed in sex reversal experiments. The required dose of EE2 to feminize YY individuals by dietary administration is more than three times higher than the efficient dose for XY (see materials

and methods, unpublished data). Likewise, high temperature treatment (36 °C) applied during the sensitive period for sex differentiation induces different effects in XX (masculinization), XY (none) and YY (feminization) (Kwon *et al.*, 2002). Thermal and hormonal sex reversals evoked here could rely on their own mechanisms, different from that involved in normal brain and behaviour differentiation, but all of them imply that genetic determinants carried by sexual chromosomes in zero, one or two copies can modify the sexual plasticity and differentiation in response to specific stimuli.

In mice, XY females produced by decoupling the major sex determinant *Sry* from the sexual genotype expressed higher aggressiveness compared to XX females. These results showed that aggressive behaviours are influenced by the presence of both *Sry* and other sex-linked genes carried on sex chromosomes that are expressed even in the absence of the major sex-determinant of testis development (Gatewood *et al.*, 2006). Contrary to mammals, sex chromosomes of *O. niloticus* and other fishes are at an early evolutionary stage of differentiation (Volff *et al.*, 2007), but we can assume that such genes linked to the sex chromosomes may influence different aspects of sex differentiation, specially the behaviour. This feature, associated to the reversed developmental polarity of brain and gonad differentiation provide to fish their exceptional sexual plasticity through a decoupling of the different components of sex phenotypical differentiation (gonad, brain, behaviour), as observed in hermaphrodites with social determinism and species with alternative male phenotypes (Godwin, 2010; Almeida *et al.*, 2014). Even if adults of *O. niloticus* with different sexual genotypes are sexually functional, producing fertile gametes and expressing mating and spawning behaviours (Billy & Liley, 1985; Gennotte *et al.*, 2012), the decoupling of components controlling sex expression and the expression of sex-linked genetic factors could explain a genotypic modulation of behavioural aspects like aggressiveness.

Nevertheless, the hypothesis of an increase in aggressiveness linked to the Y chromosome is not supported by the observations made on males in which XY and YY expressed slightly lower levels of aggressiveness than XX. However, our experimental design was not totally adapted to highlight differences in male aggressiveness levels. Male-male confrontations should be studied to solve this issue and definitely rule out the chromosomal influence hypothesis. In this study, FXX were less aggressive and less responsive to male behaviour. Consequently, whatever the genotype, asymmetrical fighting abilities lead the male to rapidly win the confrontation and express few agonistic behaviours.

A second hypothesis, based on an influence of the hormonal sex reversal treatments on the brain sexual differentiation, could also explain the difference in aggressiveness level observed between groups with different genotypes. Only the fish with a sexual genotype opposite to their phenotype (MXX, FXY and FYY), and exposed to a sex reversal treatment during the sensitive period of sex differentiation, displayed modified levels of aggressiveness compared to controls (MXY and FXX). Behavioural effects of steroids as endocrine disrupting chemicals at environmental relevant concentrations are documented (Söffker & Tyler, 2012) but few data exist on the organizational effects of sex reversal treatments at early developmental stages. Androgens and oestrogens influence many aspects of brain development and differentiation (Cooke *et al.*, 1998). In particular, oestrogens play a crucial role in neurogenesis regulation as well as brain sexual differentiation and expression of brain aromatase expression is up-regulated by oestrogens and aromatizable androgens (Diotel *et al.*, 2010). Consequently, the heavy supply of EE2 (to FXY and FYY) and MT (to MXX) during the sensitive period of sex differentiation could have permanently affected the development of the brain.

Tsai and collaborators (Tsai & Wang, 1998, 1999; Wang & Tsai, 1999; Tsai *et al.*, 2000) reported an influence of both E2 and MT, administered in similar sex reversal treatments in *O. mossambicus*, on the production of several neurotransmitters (noradrenaline, γ-aminobutyric acid, glutamate and serotonin) suggesting that these neurotransmission pathways could be involved in brain sexual differentiation or reflect the produced sex difference. In mammals, these neurotransmitters are involved in the development of sexual dimorphism in brain structure and function (Wilson & Davies, 2007) and play regulatory roles in aggressiveness expression (Haller, 2013). Less data exist in fish but serotonin is also known to modulate aggressive behaviours (Winberg & Nilsson, 1993; Summers *et al.*, 2005). Moreover, Billy and Liley (1985) reported an increase in aggressiveness level in adult Mozambique tilapia *Oreochromis mossambicus* (Peters 1852) exposed to a masculinizing treatment of MT during the sensitive period of sex differentiation. Behavioural modifications were not only observed in sex-reversed males (XX), but also in genetic males (XY) submitted to the same hormonal treatment, suggesting that hormonal treatments administered during the sex differentiation period influence behavioural differentiation.

The second question addressed in this study concerned the influence of sexual genotype on the level of circulating sex steroids and their potential regulatory role in agonistic behaviour

expression. In fish, the neural pathways underlying aggressive behaviour expression are modulated by androgens (Oliveira *et al.*, 2002; Oliveira & Gonçalves, 2008). Consequently, the higher level of 11KT in MXX could be linked to their increased agonistic motivation compared to MXY and MYY. Alteration of the steroid metabolism could have been induced by the hormonal treatment used for sex reversal rather than by a genetic influence of sex chromosomes as no difference in 11KT level was observed between MXY and MYY and between female groups. 11KT is synthesized in testes but not in the brain as supported by the absence of 11β-hydroxylase in rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) (Liu *et al.*, 2000) and zebrafish *Danio rerio* (Hamilton 1822) (Diotel *et al.*, 2011). However, the production of gonad steroid is under control of the hypothalamo-pituitary complex by the release of gonadotropins, and the gonadotropic function is itself controlled by diverse neuropeptides and neurotransmitters and by a steroid negative feed-back (Zohar *et al.*, 2010). Developmental alteration of these regulatory pathways could therefore modify the androgen production by the gonads.

This mechanism is also valid for oestrogens. In our study, E2 level increased in females carrying a Y chromosome (from FXX to FYY) but this trend was not observed in males. Similarly to 11KT, sex reversal treatments – made with higher dose of EE2 in YY than in XY – could have permanently modified oestrogen synthesis. Moreover, unlike 11KT, E2 is produced in the gonads and in the brain. Brain aromatase activity is high in fish and correlated to circulating sex steroid levels (Diotel *et al.*, 2010) supporting the idea that differences in E2 level can have a cerebral origin. Disruption of the ontogeny of the gonadotropic system and the expression of brain aromatase was reported in *D. rerio* after exposure to EE2, this steroid inducing an increase in the number of gonadotropin-releasing hormone immunoreactive neurons and a modification of their migration profile as well as an induction of brain aromatase expression (Vosges *et al.*, 2010).

However, beside a differential development of the brain, we have to consider a possible influence of the reproductive cycle on the E2 levels in females. In non-mouthbrooding female of *O. niloticus*, the mean duration of a maturation cycle is 15 days (Tacon *et al.*, 2000). However, it is not known if oocyte maturation speed is the same in XY and YY females, as physiological aspects like growth can be affected by the sexual genotype (Toguyeni *et al.*, 2002). In our study, behavioural measurements and blood sampling were performed on the second day of the maturation cycle and mean E2 levels in XX females increase from 5 to 12

ng ml^{-1} during the first three days of a maturation cycle (Tacon *et al.*, 2000), which is in the range of our measurements in the three different genotypes.

The role of oestrogens in the expression of agonistic behaviours is less documented than the role of androgens and results are sometimes divergent. In birds and mammals, administration of E2 can have a facilitating role in the expression of aggressiveness (Soma et al., 2000; Laredo et al., 2014). In fish, administration of exogenous oestrogens inhibits aggressiveness in different species (Colman *et al.*, 2009; Saaristo *et al.*, 2010). However, in *Astatotilapia burtoni* (Günther 1894), an African cichlid fish, Huffman *et al.* (2013) reported that aromatase activity and high level of E2 promote aggressive behaviours in males, suggesting that a relationship can exist between the higher aggressiveness and the E2 levels in XY and YY female *O. niloticus*.

In conclusion, in *O. niloticus* either sex chromosomes or hormonal sex reversal treatments may have an organizational influence on brain sex differentiation resulting in a differential expression of agonistic behaviours in adulthood. These organizational effects may also have altered sex steroid levels that can modulate aggressiveness expression and could have wider biological influences, particularly on reproductive behaviour. Even if some evidence (asymmetrical effect of sex chromosomes in males and females, and behavioural modification in sex-reversed fish only) provide more support in favour of an hormonal explanation of the origin of behavioural alteration compared to genotype, the question remains open and in both cases, the cause has to be searched in an early modification of brain sexual differentiation. To disentangle the two hypotheses, different types of confrontation (*e.g.* male-male) should be tested and all genotypic variants with a same sexual phenotype should be exposed to an identical hormonal treatment during development (*e.g.* 65 mg MT kg^{-1} for MXX, MXY and MYY; 500 mg EE2 kg^{-1} for FXX, FXY and FYY) in order to standardize the effect of sex reversal procedures among the different genotypes.

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Partie 3

Déterminisme et différenciation précoce du sexe

Chapitre 4

Brief exposure of embryos to steroids or aromatase inhibitor induces sex reversal in Nile tilapia (*Oreochromis niloticus*)

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Abstract

This study aimed to develop sex reversal procedures targeting the embryonic period as tools to study the early steps of sex differentiation in Nile tilapia with XX, XY and YY sexual genotypes. XX eggs were exposed to masculinizing treatments with androgens (17α-methyltestosterone, 11-ketotestosterone) or aromatase inhibitor (Fadrozole), whereas XY and YY eggs were subjected to feminizing treatments with estrogen analog (17α-ethynylestradiol). All treatments consisted of a single or double 4-h immersion applied between 1 and 36 h post-fertilization (hpf). Concentrations of active substances were 1000 or 2000 µg l^{-1} in XX and XY, and 2000 or 6500 µg l^{-1} in YY. Masculinizing treatments of XX embryos achieved a maximal sex reversal rate of 10 % with an exposure at 24 hpf to 1000 µg l^{-1} of 11-ketotestosterone or to 2000 µg l^{-1} of Fadrozole. Feminization of XY embryos was more efficient and induced up to 91 % sex reversal with an exposure to 2000 µg l^{-1} of 17α-ethynylestradiol. Interestingly, similar treatments failed to reverse YY fish to females, suggesting either that a sex determinant linked to the Y chromosome prevents the female pathway when present in two copies, or that a gene present on the X chromosome is needed for the development of a female phenotype.

Keywords: sex determination, sex differentiation, androgen, estrogen, sexual genotype

1. Introduction

Sex reversal techniques are largely used for the control of sex in fish farming and in fundamental studies on sex determinism mechanisms. In tilapia aquaculture, all-male populations are preferred because they achieve higher growth rate and prevent uncontrolled reproduction (Beardmore et al., 2001). Due to its efficiency and simplicity, one of the most common practices used to produce monosex populations of tilapia or to obtain males and females with particular sexual genotypes is the sex reversal through the dietary administration of sex steroids during the sensitive period of sex differentiation, usually from 10 to 38 days post-fertilization (dpf) (Phelps and Popma, 2000). Sex reversal treatments are also useful to explore the role played by genetic and endocrine factors involved in the mechanisms of sex determinism and differentiation.

In fish, a major sex-determining gene similar to the mammalian *Sry* was identified in a few species, but not in the Nile tilapia *Oreochromis niloticus* (Kikuchi and Hamaguchi, 2013). In this species, a sex-specific expression of genes potentially involved in sexual differentiation was reported in the gonad (*foxl2, cy19a1a, dmrt1*) (Ijiri et al., 2008) and in the brain (*amh*) (Poonlaphdecha et al., 2011) from 9-10 dpf but not deeply investigated earlier during the embryonic and larval development. Classically, sex reversal treatments override the natural process of sex differentiation by acting after 10 dpf on mechanisms taking place during the gonad morphogenesis. The masculinizing hormone 17α-methyltestosterone (MT) was reported to up-regulate *dmrt1*, a gene involved in testicular differentiation, from 19 dpf in XX tilapia (Kobayashi et al., 2008). Similarly, administration of 17α-ethynylestradiol (EE2) to XY fry induced the ovarian differentiating pathway by repressing *dmrt1* and promoting the expression of *cyp19a1* (encoding aromatase) at 19 dpf (Kobayashi et al., 2003, 2008).

However, an increasing body of evidence gathered in different species argue for a precocious initiation of the differentiation pathway that could occur in the brain or in the primordial germ cells before the development of the gonad (Tsai et al., 2000; D'Cotta et al., 2001; Kobayashi and Iwamatsu, 2005; Kobayashi et al., 2008; Rougeot et al., 2008a,b; Blázquez and Somoza, 2010). Therefore, it is particularly interesting to target sex reversal treatments on early developmental stages in order to study the upstream mechanisms and trace the sex determinism cascade up to the major determinants. During ontogenesis, the brain starts to morphologically differentiate from 31 h post-fertilization (hpf) and the primordial germ cells

can be identified from 46 hpf in Nile tilapia (Morrison et al., 2001). Then primordial germ cells migrate to the genital ridge to form a gonadal primordium by the 7th dpf (Kobayashi et al., 2003). Based on the assumption that the first steps of molecular sex differentiation would occur during these precocious developmental events, it could be possible to modify the sex determinism by treatments applied during the embryonic period.

In medaka, *Oryzias latipes*, successful feminizing and masculinizing sex inversions were performed by brief exposure of freshly fertilized eggs to 17β-estradiol for 24 h (Iwamatsu et al., 2005; Kobayashi and Iwamatsu, 2005), and preovulatory oocytes to 17α-methyldihydrotestosterone for 10 h (Iwamatsu et al., 2006) respectively. Unlike tilapia, the major sex-determinant (*dmy*) was identified in medaka (Matsuda et al., 2002), but interestingly these two species have similar sex determinism systems and morphogenesis during sex differentiation (Siegfried, 2010), suggesting that such precocious treatments could be efficiently applied in tilapia. In Mozambique tilapia (*Oreochromis mossambicus*), Rosenstein and Hulata ('92) tested various treatment schemes with a double immersion (of 2 to 28 h) of eggs at 2 and 4 dpf in 17β-estradiol solutions (200 to 1500 µg l^{-1}) but did not observe any feminizing effect. Using more prolonged treatments (from fertilization to hatching) with EE2 (500 µg l^{-1}) and MT (1000 µg l^{-1}) in *O. niloticus*, Rougeot et al. (2008a) obtained up to 79 % of feminization and 20 % of masculinization. However, this procedure is not the most appropriate to study the embryonic developmental events as the prolonged exposure led to an important accumulation of hormones that could act by a delayed effect on the process of gonad differentiation in fry. Characterization of the EE2 clearance profile in XY fish submitted to such a feminizing treatment revealed very high extra-physiological concentrations (more than 5000 ng g^{-1}) of EE2 in fry at the onset of the period of gonadal differentiation (10 dpf) (unpublished data).

The objective of the present research was to develop sex reversal procedures in Nile tilapia using brief immersion treatments applied on the first stages of development in order to focus the action of hormones only on embryogenesis. Different masculinizing and feminizing schemes, varying in active substances, concentrations and moments of application, were tested on XX, and XY and YY progenies respectively, to identify the most efficient procedures that might be used to study the early mechanisms of sex differentiation.

2. Material and Methods

2.1. Reproduction and egg incubation

Nile tilapia *O. niloticus* (Lake Manzala strain) originated from the Research and Education Center in Aquaculture (CEFRA), University of Liège (Belgium). Different full-sib families were produced by artificial reproduction (Gennotte et al., 2012). XX progenies were obtained by crossing XX males with XX females, XY progenies by crossing YY males with XX females and YY progenies by crossing YY males with YY females. Broodstock fish were individually stocked in 125-l aquaria at 27 °C with a 14 h light/10 h darkness photoperiodic regime. Fish were fed at satiation with special broodstock commercial tilapia diet (45 % proteins, 5 % lipids, Coppens – The Netherlands). After artificial fertilization, eggs were counted, distributed in different batches according to treatments and incubated in 1.5 L Zug bottle at 27 °C. Fertilization rate was evaluated on 100 eggs after the first mitotic cleavage (2 hpf) (Morrison et al., 2001).

Experiments were carried out according to the guidelines of the University of Liège ethical committee and the European animal welfare recommendations.

2.2. Hormone and aromatase inhibitor solutions

Steroid hormones 17α-ethynylestradiol (EE2), 17α-methyltestosterone (MT), 11-ketotestosterone (11KT) and the aromatase inhibitor Fadrozole (Fa) were purchased from Sigma-Aldrich. Hormones and Fa were dissolved in 100 % ethanol to prepare 1000-fold concentrated stock solutions and stored at 4 °C. For immersion treatments, 1 ml of stock solution was added to 1 l of hatchery water. Control groups were incubated in 1:1000 ethanol.

2.3. Hormonal treatments

Feminizing and masculinizing treatments consisted of a single or a double immersion in hormonal or Fa solutions. All immersions lasted 4 h. Treatments were performed at different stages of development from 1 to 36 hpf. Eggs were transferred from the Zug bottle into a 1-l glass beaker filled with hatchery water and maintained in a thermostatic bath at 27 °C. One ml

of stock solution (ethanol for control groups) was added to the water. During incubation, water was oxygenated by an air diffuser. After 4 h of immersion, eggs were netted, gently soaked up and rinsed in 6 different baths to remove hormone residues. The first 3 baths contained 1:1000 ethanol and the next 3 only hatchery water. Control groups were handled in the same way. After rinsing, eggs were replaced into the hatchery.

A total of 10 different masculinizing treatments were tested on XX progenies, and 6 and 5 feminizing treatments on XY and YY progenies respectively. XX eggs were treated with MT (1000 and 2000 µg l^{-1}), an aromatizable synthetic androgen, 11KT (1000 and 2000 µg l^{-1}), a non-aromatizable natural androgen, and Fa (1000 and 2000 µg l^{-1}), a non-steroidal inhibitor of aromatase, the enzyme that converts androgens into estrogens. XY and YY eggs were treated with EE2 (2000 and 6500 µg l^{-1}), a synthetic estrogen.

The concentrations of hormones used in XX and XY treatments were based on the literature (Gilling et al., '96; Kobayashi et al., 2003; Wassermann and Afonso, 2003) and on our previous experiments of egg immersion (Rougeot et al., 2008a). In YY treatments a first concentration of EE2 equal to the XY treatments was tested, and a second concentration increased in the same proportion as EE2 concentrations used in dietary hormonal treatments (150 mg kg^{-1} for XY and 500 mg kg^{-1} for YY) in our laboratory (unpublished data). As a differential timing of sex differentiation can be suspected in YY compared to XY (Herrera et al., 2001), different moments of treatment application were tested in YY. Hormone and Fa concentrations, and the moment of application of each treatment figure in the result tables (Table 1, 2 and 3).

Each progeny was divided into several batches receiving different treatments and a control group for each moment of treatment application. In YY groups immersed in a 6500 µg EE2 l^{-1} solution at different moments, only one control group immersed at 24 hpf was used. The number of fertilized eggs per batch and the number of progenies per treatment ranged from 78 to 1371 and from 2 to 8 respectively.

MT and EE2 are routinely used in our laboratory for the feminization of XX and the masculinization of XY and YY fry respectively by dietary administration (from 10 to 40 dpf, XX: 65 mg MT kg^{-1} food; XY: 150 mg EE2 kg^{-1} food; YY: 500 mg EE2 kg^{-1} food). These treatments succeed in 94 to 100 % of sex reversal (unpublished data).

2.4. Juvenile rearing and sex-ratio analysis

At 8 dpf, larvae were counted in order to calculate the survival rate (= 100 × n larvae / n fertilized eggs) and transferred into 50-l aquaria (27 °C). From 10 dpf, fish were fed close to satiation 6 times a day with a commercial tilapia diet (47 % proteins, 8 % lipids, Coppens – The Netherlands). Phenotypic sex was determined by the acetocarmine squash method (Guerrero and Shelton, '74) at 90 dpf. Fish were euthanized by overdose (500 mg l^{-1}) of benzocaine (Sigma-Aldrich) and a piece of gonad was microscopically examined after acetocarmine coloration. The number of sexed fish per batch ranged from 22 to 121 (median = 74), from 8 to124 (median = 50) and from 7 to 101 (median = 69) for XX, XY and YY respectively. Adding together the different progenies, the overall number of sexed fish per treatment ranged from 69 to 553.

2.5. Data analysis

Survival (at 8 dpf) and sex reversal rates are expressed as the overall value and the total range (minimum and maximum) of all the progenies exposed to the same treatment. Sex ratios and survival of the treated groups were compared against the values of their corresponding control groups using the 2 × 2 contingency chi-square (χ^2) test (df = 1). Values were considered as significant at $p < 0.05$.

3. Results

3.1. XX masculinization experiments (Table1)

At 8 dpf, survival rates in XX progenies ranged from 22 to 86 % and from 25 to 64 % in treated and control groups respectively. Overall survival values were significantly higher in batches exposed to MT in a single immersion (at 1 or 24 hpf) (from 48 to 61 %) than in their respective controls (39 – 43 %). In fish submitted to a double immersion (at 1 and 24 hpf), survival was similar in control and treated batches (from 35 to 42 %). In 11KT and Fa treatments, overall survival was 59 % in controls and ranged from 51 to 70 % in treated

groups with significantly higher values in fish exposed to 1000 µg l^{-1} of 11 KT and Fa and a lower value in fish exposed to 2000 µg 11KT l^{-1}.

All the XX control fish were females as expected. None of the MT treatments induced a significant deviation of the sex-ratio. In one progeny, immersion in 1000 µg l^{-1} of MT at 1, 24 and 1 + 24 hpf respectively induced 8.1, 8.3 and 9.1 % of males but the numbers of sexed fish were low in these batches ($n < 37$) and deviations were not significant. On the contrary, treatments with 11KT or Fa significantly induced up to 10 % of sex reversal. Overall percentages of males in 11 KT and Fa treatments were similar in 1000 and 2000 µg l^{-1} immersions (3.3 and 3.5 %, 4.4 and 3.9 % for 11KT and Fa respectively) and significantly different from the control groups.

3.2. XY feminization experiments (Table 2)

Survival rates in XY progenies ranged from 26 to 85 % and from 28 to 86 % in EE2-treated and control groups respectively. Overall survival values were above 60 % in groups treated with a single immersion at 1 or 24 hpf and not statistically different from the control groups, except for the group treated in a 1000 µg l^{-1} solution at 1 hpf that displayed a higher survival (72 %). Double immersion at 1 and 24 hpf led to lower survival. In this treatment, survival of hormone-exposed fish (32 – 33 %) was significantly lower than the controls (44 %).

Sex-ratio of the XY control groups was 100 % male as expected. A significant sex reversal effect ($p < 0.001$) was observed in all the EE2 treatments. Fish exposed to EE2 at 1, 24, and 1 + 24 hpf showed increasing overall sex reversal rates: 9.9 – 26.6 % at 1 hpf, 48.0 – 48.6 % at 24 hpf and 65.9 – 65.2 % at 1 + 24 hpf for 1000 and 2000 µg l^{-1} respectively. The efficiency of the treatment was related to the hormone concentration only in the treatment applied at 1 hpf. The maximum sex reversal rate (91 %) was observed in a progeny immersed at 24 hpf in a 2000 µg l^{-1} solution.

3.3. YY feminization experiments (Table 3)

In treatment of YY at 6500 µg EE2 l^{-1}, overall survival was greatly affected in fish immersed at 12 hpf (24 % vs 64 % in the controls). For the treatment applied later (18, 24 and 36 hpf),

survival was similar to the controls. Survival values are missing for the treatment at 2000 µg l^{-1}.

All the YY control fish were males as expected. None of the EE2 treatment significantly skewed sex-ratio in any of the progeny.

Table 1. Effect of androgen and aromatase inhibitor treatments applied in a single or double 4 h-immersion at different moments of the embryonic development (1 and/or 24 hpf) on the survival (at 8 dpf) and the sex ratio of XX Nile tilapia fry (MT: 17α-methyltestosterone; 11KT: 11-ketotestosterone; Fa: fadrozole; * $p < 0.05$).

				% Survival (8 dpf)						% Sex reversal				
Active substance	Concentration (µg l^{-1})	Moment (hpf)	n XX progenies	Min	Max	Overall	χ^2	*p*	Total n sexed	Min	Max	Overall	χ^2	*p*
Control	0	1	3	25	62	39			123	0.0	0.0	0.0		
MT	1000	1	3	29	74	48*	5.9	0.015	173	0.0	8.1	1.7	2.2	0.142
MT	2000	1	3	23	86	60*	31.5	< 0.001	211	0.0	0.0	0.0	0.0	> 0.999
Control	0	24	3	32	56	43			133	0.0	0.0	0.0		
MT	1000	24	3	22	82	53*	6.5	0.011	194	0.0	8.3	1.0	1.4	0.240
MT	2000	24	3	49	74	61*	24.6	< 0.001	220	0.1	2.6	0.9	1.2	0.270
Control	0	1 + 24	2	29	46	36			90	0.0	0.0	0.0		
MT	1000	1 + 24	3	25	61	42	1.5	0.217	145	0.0	9.1	3.5	3.2	0.075
MT	2000	1 + 24	2	35	35	35	0.3	0.592	90	0.0	2.6	1.1	1.0	0.316
Control	0	24	6	37	64	59			453	0.0	0.0	0.0		
11KT	1000	24	3	27	59	63*	4.4	0.036	150	0.0	10.0	3.3*	6.8	0.009
11KT	2000	24	3	25	63	51*	10.2	0.001	200	0.0	8.0	3.5*	7.1	0.008
Fa	1000	24	3	50	77	70*	22.0	< 0.001	250	1.0	7.0	4.4*	9.0	0.003
Fa	2000	24	6	44	71	58	0.0	0.981	464	0.0	10.0	3.9*	17.9	< 0.001

Table 2. Effect of 17α-ethynylestradiol (EE2) treatments applied in a single or double 4 h-immersion at different moments of the embryonic development (1 and/or 24 hpf) on the survival (at 8 dpf) and the sex ratio of XY Nile tilapia fry (* $p < 0.05$).

				% Survival (8 dpf)						% Sex reversal				
Active substance	Concentration (µg l^{-1})	Moment (hpf)	n XY progenies	Min	Max	Overall	χ^2	*p*	Total n sexed	Min	Max	Overall	χ^2	*p*
Control	0	1	3	28	79	62			190	0.0	0.0	0.0		
EE2	1000	1	3	69	73	72*	4.7	0.030	181	5.7	16.7	9.9*	19.9	< 0.001
EE2	2000	1	3	26	85	65	0.5	0.501	154	5.9	29.5	26.6*	57.4	< 0.001
Control	0	24	7	35	86	75			500	0.0	0.0	0.0		
EE2	1000	24	3	38	71	60	3.5	0.063	127	43.6	66.7	48.0*	65.7	< 0.001
EE2	2000	24	8	32	86	76	0.2	0.629	553	2.0	91.0	48.6*	326.7	< 0.001
Control	0	1 + 24	2	44	45	44			96	0.0	0.0	0.0		
EE2	1000	1 + 24	3	31	37	33*	6.1	0.014	88	57.1	84.2	65.9*	92.4	< 0.001
EE2	2000	1 + 24	2	27	35	32*	7.6	0.006	69	57.9	68.0	65.2*	86.1	< 0.001

Table 3. Effect of 17α-ethynylestradiol (EE2) treatments applied in a single 4 h-immersion at different moments of the embryonic development (12, 18, 24, 36 hpf) on the survival (at 8 dpf) and the sex ratio of YY Nile tilapia fry (* $p < 0.05$; - missing data).

				% Survival (8 dpf)						% Sex reversal				
Active substanc	**Concentra-tion (µg l^{-1})**	**Moment (hpf)**	**n YY progenies**	**Min**	**Max**	**Overall**	**χ^2**	***P***	**Total n sexed**	**Min**	**Max**	**Overall**	**χ^2**	***p***
Control	0	24	3	-	-	-	-	-	150	0.0	0.0	0.0		
EE2	2000	24	3	-	-	-	-	-	150	0.0	0.0	0.0	0.0	> 0.999
Control	0	24	4	38	77	64			353	0.0	0.0	0.0		
EE2	6500	12	4	10	42	24*	320.2	< 0.001	128	0.0	1.8	0.8	2.8	0.096
EE2	6500	18	4	25	80	60	3.5	0.062	337	0.0	0.0	0.0	0.0	> 0.999
EE2	6500	24	4	38	78	61	2.5	0.115	344	0.0	1.0	0.3	1.0	0.311
EE2	6500	36	4	38	80	62	0.8	0.369	333	0.0	1.2	0.3	1.1	0.303

4. Discussion

Our results demonstrate that brief 4 h-immersions of fertilized eggs in androgen, aromatase inhibitor, and in estrogen solutions applied between 1 and 24 hpf can induce significant sex reversal in XX and XY Nile tilapias. Feminization of XY with EE2 is 10 to 15-fold more efficient than masculinization of XX with 11KT or Fa. No significant masculinizing effect was observed with MT. Interestingly, estrogen treatments failed to reverse the sex of YY fish, even at higher doses.

Regarding the wide variety of steroid compounds used for sex reversal in fish, different efficiencies arise from different affinities for their receptors, activities of the steroid-receptor complexes, and their metabolism (Devlin and Nagahama, 2002). Since all the steroids and the aromatase inhibitor used in the present study have proven their high potency for sex reversal in fish when administered during gonadal differentiation (Pandian and Sheela, '95; Devlin and Nagahama, 2002), particularly in tilapia (except 11KT that was used for the first time in Nile tilapia in our study) (Jalabert et al., '74; Potts and Phelps, '95; Kwon et al., 2000), the differences in efficiency of the treatments have to be searched in the developmental events targeted by the treatments, the endocrine and genetic mechanisms of sex determination and differentiation taking place during this precocious phase and the genotypic differences of tested fish.

As androgen (Golan and Levavi-Sivan, in press) and estrogen (Kawahara and Yamashita, 2000; Singh, 2013) receptors are involved in hormone-induced sex reversal, their expression is a prerequisite to successfully override genetic sex determination by exogenous hormone exposure. In tilapia, the two androgen receptors (*ar1* and *ar2*) are expressed at a low level at the onset of gonadal development (from 9 dpf) and the three estrogen receptors (*esr1*, *esr2a* and *esr2b*) at a high level, similarly in XX and XY (Ijiri et al., 2008). Before the formation of the gonad, the target of exogenous hormones in the mechanism of sex reversal could be the brain or the primordial germ cells and their environment (Kobayashi and Iwamatsu, 2005; Rougeot et al., 2008a). *Esr1* and *esr2a* are expressed in the brain of both XX and XY tilapia larvae from hatching (4 dpf). In contrast, the expression of androgen receptors is differentially initiated in XX and XY. *Ar1* transcripts are detected from 4 dpf in XY but not before 29 dpf in XX and a*r2* is expressed from 9 dpf in XY and from 4 dpf in XX (Sudhakumari et al., 2005). Even though both androgen and estrogen receptivities seem to be acquired early during

fish development, a quantitative study of the expression of steroid receptors during tilapia embryogenesis and embryonic sex reversal would clarify their role in mediating the action of exogenous hormones, and we cannot exclude that a differential expression of the estrogen and androgen receptors may account for the differences in treatment potencies and genotype sensitivities. More specifically, the absence of one of the two *ar* in the brain of developing XX embryos could be responsible for their low susceptibility to androgen-induced sex reversal.

In natural sexual differentiation in tilapia, androgens do not appear to play an important role (Nagahama, 2005). The most potent natural androgen, 11KT, is not synthesized in the differentiating testis before 29 dpf (Ijiri et al., 2008). This suggests that androgen-signaling pathways may not be fully developed in embryos, explaining the low potency of androgens in embryonic sex-reversing treatment. In contrast, estrogens play a critical role in ovarian differentiation and the lack of estrogens during the period of gonad differentiation is responsible for testis development (Guiguen et al., '99; Kwon et al., 2000; Nagahama, 2005). Given that and the wide diversity of other estrogen functions during ontogenesis (Hao et al., 2013), estrogen-signaling pathways establish early during development and can therefore be used by exogenous hormones to override natural processes.

As the treatment of XX embryos with an aromatase inhibitor induced up to 10 % sex inversion, it is proposed that aromatase and estrogens could play a role in sexual differentiation during embryogenesis. In Nile tilapia, brain aromatase was up-regulated in a dose-dependent manner in EE2-treated XY and YY embryos at 4 dpf (submitted manuscript), suggesting a possible modification of the brain sexual differentiation that could consequently play a role in the development of the sexual phenotype. Both aromatizable (MT) and non-aromatizable (dihydrotestosterone) androgens *in vitro* inhibited aromatase activity in ovarian microsomes of tilapia (Golan and Levavi-Sivan, in press) suggesting that exogenous androgens administered during the differentiation of the gonad exerted an inhibitory action on aromatase activity which could be part of the mechanism of early masculinization. However, since masculinization rates were only 10 % in XX progenies and no sex reversal was observed in YY progenies, changes in the expression or activity of brain aromatase could not be the sole factor involved in the mechanism of embryonic sex reversal.

Results of feminization in XY and the absence of susceptibility to EE2 embryonic treatments in YY can be explained by the presence on the X chromosome of a female inducer or alternatively the presence on the Y of a female repressor (or male inducer). In any case, the mechanism of embryonic sex reversal must rely on a balance between the action of the exogenous steroids and the upstream genetic factors involved in the sex-determining and differentiating cascade. Such a hormonal-genetic balance can be exemplified with *dmrt1* that is a conserved male marker specifically expressed in testes. Evidence of the importance of this gene in testicular differentiation was underlined by the discovery of its homolog *dmy* as the major sex-determinant in medaka (Matsuda et al., 2002). In tilapia, *dmrt1* is expressed specifically in the Sertoli cell lineage from 10 dpf (Ijiri et al., 2008; Kobayashi et al., 2008). It is up-regulated in the differentiating testis of MT-induced XX males and repressed in the differentiating ovary of EE2-induced XY females (Kobayashi et al., 2008), while XX fish carrying extra copies of *dmrt1* as a transgene develop into males (Nagahama, 2005). *Dmrt1* (or an homolog) is not the major sex-determinant in tilapia (Volff et al., 2003) but a sex determinant upstream from *dmrt1* could be expressed during embryogenesis and prevents exogenous estrogen to override the genetic sex determinism when present in two copies.

In conclusion, as shown in a previous study using thermal treatments (Rougeot et al., 2008b), it is possible to artificially reverse the sex differentiation pathway of tilapia during embryogenesis, before the gonad differentiating period. Our results reinforced the hypothesis of an action of exogenous hormones on brain sexual differentiation or on primordial germ cells and their environment, and the important role of aromatase in sex differentiation mechanisms. Nevertheless, as estrogen treatments failed to reverse YY fish to females, the hypothesis of a sex determinant linked to the Y chromosome preventing the female pathway when present in two copies, or a gene present on the X chromosome needed for the development of a female phenotype, is suggested. The investigation of the genetic and endocrine mechanisms involved in embryonic sex reversal would provide valuable insights on sex determination and early sex differentiation in the Nile tilapia.

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Chapitre 5

The sensitive period for male to female sex reversal begins at the embryonic stage in the Nile tilapia and is associated with the sexual genotype

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Abstract

In this study, we applied feminization treatments of 4 h on XY (17α-ethynylestradiol 2000 µg l^{-1}) and YY (6500 µg l^{-1}) Nile tilapia embryos on the first dpf. The mechanism of early sex reversal was investigated by searching for changes in the expression profiles of some sex-differentiating genes in the brain (c*yp19a1b*, *foxl2* and *amh*) and in sexual steroids (testosterone, 17β-estradiol and 11-ketotestosterone) concentrations during embryogenesis and gonad differentiation. No sex reversal was observed in YY but sex reversal rates in XY progenies ranged from 0 to 60 %. These results, together with the clearance profile of 17α-ethynylestradiol confirmed the existence of an early sensitive period for sex determination encompassing embryonic and larval development, prior to any sign of gonad differentiation. The estrogen treatment induced an increased expression of c*yp19a1b* and a higher level of testosterone and 17β-estradiol at 4 dpf in XY and YY. *Foxl2* and *amh* were repressed at 4 dpf and expression levels were not different between treated and control groups at 14 dpf suggesting that *foxl2* did not control *cyp19a1b* in the brain of tilapia embryos. Increased *cyp19a1b* expression in treated embryos could reflect a sign of early brain sexualization but alone cannot account for the sex reversal effect as the treatment was ineffective in YY. The differential sensitivity of XY and YY genotypes to embryo induced-feminization suggests that a sex determinant on the sex chromosomes, either a Y repressor or an X activator may influence the sex reversal during the first steps of tilapia embryogenesis.

Abbreviations: 11KT, 11-ketotestosetrone; *amh*, anti-Müllerian hormone; *ar*, androgen receptor; *cyp19a1*, cytochrome P450 aromatase; *dmrt1*, double-sex mab-3 related transcription factor 1; E2, 17β-estradiol; EE2, 17α-ethynylestradiol; *foxl2*, forkhead box l2 transcription factor; dpf, days post-fertilization; *ef1*, elongation factor 1; *er*, estrogen receptor; hpf, hours post-fertilization; PGC, primordial germ cell; *sf1*, steroidogenic factor 1; sox9, Sry-like HMG-box 9; T, Testosterone.

Keywords: sex determination, brain, aromatase, genes, steroids

1. Introduction

Sex-determining mechanisms are extremely diverse among vertebrates. While the process is relatively conserved and singular in the majority of mammals, characterized by a genetic XX/XY system, in fish there is a wide diversity of sex-determining systems which are part of a continuum ranging from pure genetic determination (chromosomal XY, ZW or polygenic) to environmental sex determination (Devlin and Nagahama, 2002). In fish with genetic sex determination, a master sex-determining gene similar to the mammalian *Sry* (Sinclair et al., 1990) was characterized in a few species: *Dmy* in the medaka *Oryzias latipes* (Matsuda et al., 2002), *Gsdf* in *Oryzias luzonensis* (Myosho et al., 2012), *Amhy* in the Patagonian pejerrey *Odontesthes hatchery* (Hattori et al., 2012), *amhr2* in the tiger pufferfish *Takifugu rubripes* (Kamiya et al., 2012) and *sdY* in the rainbow trout *Oncorhynchus mykiss* (Yano et al., 2012). These recent discoveries of unrelated genes acting as major regulators of sex determination stressed the unconserved nature and rapid turnover of sex chromosomes in fish. In contrast, the downstream genetic factors acting on sexual differentiation, although varying in their regulation and expression profiles, appear more conserved among species with different sex determinism mechanisms (Kikuchi and Hamaguchi, 2013). Within the most important genes, *sox9*, *amh* and *dmrt1* are largely associated with testis differentiation and *foxl2* and *cyp19a1* with ovarian development (Siegfried, 2010).

In tilapia, as in medaka, the expression of *sox9* is initiated early in the ontogenesis but a sexually dimorphic expression appears after the first signs of gonadal differentiation, dismissing it from a key regulator of sex differentiation (Nakamoto et al., 2005; Ijiri et al., 2008; Baroiller et al., 2009). As teleosts do not have Müllerian ducts, the role of *amh* in male differentiation is still unclear. The finding of *amhy*, a duplicated form on the Y chromosome of pejerrey and its receptor *amhr2* as sex determinants reflects the importance of *amh* signaling in certain fish (Hattori et al., 2012; Kamiya et al., 2012). In tilapia, a higher expression of *amh* is observed in differentiating testis around 20 days post-fertilization (dpf) onwards (Ijiri et al., 2008; Poonlaphdecha et al., 2011). Interestingly, a dimorphic expression appears early in the brain of males, at the onset of gonad differentiation (10 dpf), suggesting a role in the brain sexualization (Poonlaphdecha et al., 2011). *Dmrt1* acts early in the process of testis differentiation in non-mammalian vertebrates (Siegfried, 2010). It received much attention in fish since the discovery of its duplicated paralog *dmy* identified as the master sex-determining gene in *O. latipes* (Matsuda et al., 2002). Ijiri et al. (2008) reported a male

specific expression of *dmrt1* in the developing gonad of tilapia from 10 dpf, suggesting an important role in early testis differentiation.

Foxl2 is expressed in a female specific-manner in the developing gonads of mammals, birds, reptiles (Loffler et al., 2003) and fish (Guiguen et al., 2010). In medaka, *foxl2* is up-regulated at the first steps of ovarian differentiation (Nakamoto et al., 2006). In tilapia, a sexually dimorphic expression was reported as early as 9 dpf, before any sign of histological differentiation (Ijiri et al., 2008). An *in vitro* study in tilapia has shown that *foxl2* is an important regulator *cyp19a1* transcription (Wang et al., 2007) and these 2 genes are co-expressed in the differentiating ovaries in rainbow trout (Vizziano-Cantonnet et al., 2011) and tilapia (Wang et al., 2007). Aromatase, encoded by *cyp19a1,* is responsible for estrogen synthesis and therefore plays a key role in the sexual steroidogenesis and gonad differentiation in fish (see reviews by Guiguen et al., 2010 and Diotel et al., 2010). Most teleosts have two distinct forms of aromatase, a gonad form, encoded by *cyp19a1a,* and a brain form encoded by *cyp19a1b* (Tchoudakova and Callard 1998; Kwon et al., 2001). *Cyp19a1a* is up-regulated in the differentiating ovary (D'Cotta et al., 2001b; von Schalburg et al., 2010) and administration of estrogens to genetic males during the sex differentiation period induced feminization of the gonads in different species (Piferrer, 2001; Devlin and Nagahama, 2002). Similarly, treatment of fry with aromatase inhibitor leads to masculinization in genetic females (Piferrer et al., 1994; Afonso et al., 2001; Uchida et al., 2004). In tilapia, expression of *cyp19a1a* is up-regulated in female gonads from 9 dpf onwards (D'Cotta et al., 2001b; Ijiri et al., 2008; Poonlaphdecha et al., 2013) and *cyp19a1b* is expressed in the brain very early during ontogenesis with no difference in expression profiles between sexes (Kwon et al., 2001). Although the regulatory pathways of *cypa19a1a* and *cyp19a1b* have their specificities, several authors have suggested a possible role for brain aromatase in the brain and the gonad sexualization in gonochoristic teleosts (Tsai et al., 2003; Blázquez and Somoza, 2010; Le Page et al., 2010).

Nile tilapia has a XX/XY sex determination system that can be overridden by exogenous steroids and high temperature (masculinizing effect above 32 °C) (Jalabert et al., 1974; Baroiller et al., 1995). The sensitive period extends from 10 dpf (yolk sac resorption) to 25-30 dpf (formation of an ovarian cavity or an intratesticular efferent duct) (Kobayashi et al., 2008). Temperature-induced masculinization was shown to up-regulate the gonad expression of *dmrt1* and *amh,* and repress both *foxl2* and *cyp19a1a* as well as brain aromatase activity

(D'Cotta et al., 2001b; Poonlaphdecha et al., 2013). In contrast, estrogen feminizing treatments up-regulate the expression of both *cyp19a1* genes and down-regulate the expression of *dmrt1* in the gonad (Kobayashi et al., 2003, 2008). In addition to the classical sex reversal procedures, acting during the gonad differentiation, several authors investigated the possible existence of a second sensitive window for sex determination in tilapia, starting earlier in the embryonic phase. Rosenstein and Hulata (1992) attempted to feminize Mozambique tilapia (*O. mossambicus*) by short estrogen (17β-estradiol, progesterone, flutamide) immersions of freshly fertilized eggs but found similar number of females in treated and control groups. More recently, Rougeot et al. (2008a,b) showed that thermal (> 34 °C) and hormonal (17α-methyltestosterone, 17α-ethynylestradiol) treatments of embryos form 12 hpf to hatching induced respectively up to 27 and 68 % sex reversal in *O. niloticus*. The mechanism involved in these phenotypic changes could be different from those involved in later procedures (after 10 dpf) as they are applied before the gonad formation and differentiation. During Nile tilapia embryogenesis, primordial germ cells are apparent as early as 46 hpf (Morrison et al., 2001) and then migrate and reach the gonadal anlagen by the 7th dpf (Kobayashi et al., 2003). Thereafter, morphological changes in the undifferentiated gonad remain discrete until around 20 dpf (D'Cotta et al., 2001b). Rougeot et al. (2008a,b) suggested that early sex reversal treatments could act on the development of primordial germ cells and/or somatic cells of the future gonad, or influence brain sexualization since the brain starts to differentiate from 31 hpf (Morrison et al., 2001). However, another issue raised from the early hormonal treatments regarding the sex reversal mechanism is the possible accumulation of hormones in the embryo and the vitellus, leading to a delayed effect after embryogenesis and a direct action of the hormone on the developing gonad (Piferrer and Donaldson, 1994). Characterization of the uptake and clearance kinetics of 17α-ethynylestradiol (EE2) administered to XY embryos following Rougeot's procedure showed that the whole body concentration of EE2 at the onset of gonad differentiation (10 dpf) was still very high (unpublished data). A delayed and prolonged hormonal supply makes ambiguous the identification of the period of hormone action and target structure.

The present study aimed to verify the existence of a precocious sensitive period for sex reversal in Nile tilapia, before the development of a gonadal primordium tissue, and explore the mechanisms of sex reversal during this period. We used short EE2 immersion treatments applied at early embryonic stages to inverse the phenotypic sex of XY Nile tilapia. EE2

uptake and clearance were followed to confirm the early action of the exogenous hormone. Similar treatments were applied on YY embryos to test a potential influence of the sexual genotype on sex reversal responsiveness and sex determination process. Sex reversal mechanisms and the hypothetical upstream role of the brain in the sex determination process were investigated through the measurements of natural sexual steroids (testosterone, 17β-estradiol and 11-ketotestosterone) and the expression of 3 main sex-differentiating genes (*cyp19a1b*, *amh*, *foxl2*) in the heads of treated embryos and juveniles.

2. Results

2.1. Growth, survival and sex-ratios

In XY progenies, the immersion treatments did not significantly affect the growth during the whole experimental period (Fig. 1A). At 35 dpf, mean body weight of the 3 groups were: 651 ± 14 mg for the group immersed at 1dpf for 4 h in a dose of 2000 µg EE2 l^{-1} (XY2000), 643 ± 12 mg for the controls (XYC) immersed in ethanol 1:1000, and 649 ± 15 mg in the group immersed at 10 dpf for 4 h in a dose of 20 µg EE2 l^{-1} (XY20).

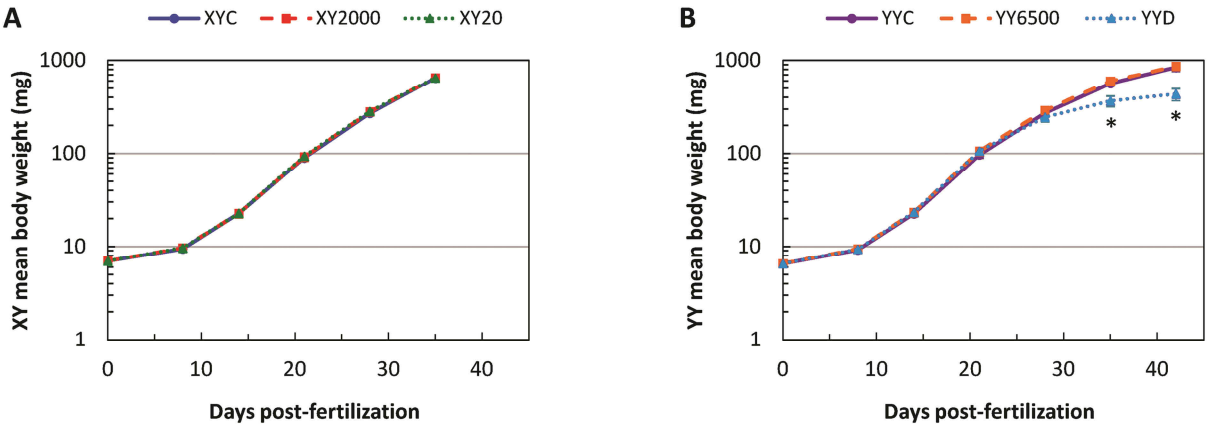

Figure 1. Mean growth curves of tilapia progenies during the experimental period in (A) XY (n = 5) till 35 dpf and (B) YY (n = 4) till 42 dpf. XYC: XY control group; XY2000: XY submitted to an EE2 immersion treatment (2000 µg l^{-1}) for 4 h at 1 dpf; XY20: XY immersed in EE2 at 20 µg l^{-1} for 4 h at 10 dpf; YYC: YY control group; YY6500: YY immersed in EE2 at 6500 µg l^{-1} for 4 h at 1 dpf; YYD: YY submitted to an EE2 dietary treatment of 500 mg kg^{-1} food from 10 to 40 dpf. * indicates significant differences ($p < 0.05$).

In YY fish, growth was very similar between the progenies (Fig. 1B) and immersion treatment did not affect the mean body weight of neither the fish immersed at 1 dpf for 4 h in a 6500 µg EE2 l^{-1} solution (YY6500) nor in the controls (YYC) immersed at 1 dpf for 4 h in ethanol 1:1000. In the group (YYD) fed from 10 to 40 dpf with 500 mg EE2 kg^{-1} food, which served as a positive control for sex reversal, the growth slowed down after 19 days of treatment (28 dpf) and the mean body weight was significantly different from both YYC and YY6500 at 35 and 42 dpf. At the end of the experiment (42 dpf), the mean body weights were 838 ± 73, 864 ± 80 and 433 ± 64 mg in YYC, YY6500 and YYD respectively.

Survival rates in the XY progenies ranged from 11 to 29 % in XYC, from 16 to 34 % in XY2000 and from 24 to 50 % in XY20 groups (table 1). Mean values were not significantly different between XYC and XY 2000, but the mean survival was significantly higher in XY20 than in the controls. In YY progenies, the survival rates ranged from 38 to 58 % in YYC, from 25 to 45 % in YY6500 and from 53 to 71 % in YYD. The mean survival rate was significantly higher in YYD than YYC and YY6500.

Table 1. Survival rates in 5 XY (35 dpf; P1 to P5) and 4 YY (42 dpf; P6 to P9) progenies, and sex-ratios (% females) at 90 dpf of tilapias submitted to EE2 feminization treatments.

		XYC	XY2000	XY20		YYC	YY6500	YYD
Survival (%)		24.5 ± 3.5^{a}	27.7 ± 3.2^{ab}	40.6 ± 4.6^{b}		48.7 ± 4.4^{a}	37.9 ± 4.7^{a}	66.5 ± 4.5^{b}
% females (n sexed)	P1	0.0^{a} (53)	11.8^{b} (68)	0.0^{a} (66)	P6	0.0^{a} (103)	0.9^{a} (106)	99.1^{b} (113)
	P2	0.0^{a} (125)	60.6^{c} (109)	8.6^{b} (93)	P7	0.0^{a} (100)	0.8^{a} (124)	100.0^{b} (100)
	P3	0.0^{a} (110)	6.3^{b} (111)	0.0^{a} (101)	P8	0.0^{a} (100)	0.0^{a} (118)	99.0^{b} (100)
	P4	0.0^{a} (106)	0.9^{a} (106)	0.0^{a} (104)	P9	0.0^{a} (103)	0.0^{a} (58)	83.7^{b} (92)
	P5	0.0^{a} (100)	23.0^{c} (100)	4.0^{b} (101)				
	Mean	0.0 ± 0.0^{a}	20.5 ± 10.7^{c}	2.5 ± 1.7^{b}	Mean	0.0 ± 0.0^{a}	0.4 ± 0.3^{a}	95.5 ± 3.9^{b}

XYC: XY control group; XY2000: XY submitted to an EE2 immersion treatment (2000 µg l^{-1}) for 4 h at 1 dpf; XY20: XY immersed in EE2 at 20 µg l^{-1} for 4 h at 10 dpf; YYC: YY control group; YY6500: YY immersed in EE2 at 6500 µg l^{-1} for 4 h at 1 dpf; YYD: YY submitted to an EE2 dietary treatment of 500 mg kg^{-1} food from 10 to 40 dpf. Different superscript letters indicate significant differences ($p < 0.05$).

Sex-ratio analysis revealed the presence of no female in the control groups (XYC and YYC) (table1). In XY individuals, the efficiency of EE2 immersion treatments differed between progenies. The proportion of females in XY2000 ranged from 0.9 to 60.6 %. Except for the P4 progeny, the immersion treatment with a dose of 2000 µg EE2 l^{-1} for 4 h applied to 1 day-old embryos (XY2000) significantly skewed the sex ratios toward females, with the highest values for P2 (60.6%) and P5 (23.0%) . The immersion treatment of 10 days-old larvae at 20 µg EE2 l^{-1} resulted in a significant sex reversal only in these 2 progenies, with 8.6 % and 4.0 % of females for P2 and P5 respectively. In YY, the EE2 immersion treatment at 1 dpf did not lead to any significant sex reversal. However, the feminization susceptibility of all the YY progenies studied was demonstrated using an EE2 dietary treatment (from 10 to 40 dpf) that achieved from 83.7 to 100.0 % of sex reversal.

2.2. EE2 uptake and clearance

The EE2 used for the feminization treatment of XY embryos (XY2000 group: 2000 µg l^{-1} at 1 dpf) passed through the chorion and rapidly accumulated in the eggs causing a mean concentration of 18929 ± 3565 ng g^{-1} shortly after the immersion (Fig. 2A). This surge represented a 10-fold bioconcentration of the hormone compared to the initial concentration of the hormonal solution. After this peak, the clearance kinetics was rapid. The concentration dropped to 4798 ± 465 ng g^{-1} at 4 dpf and returned to a value (1.41 ± 1.89 ng g^{-1}) statistically similar to the control at 21 dpf (Fig. 2A and 2B). The 20 µg l^{-1} EE2 dose used for the 4-h treatment at 10 dpf (XY20) aimed to reach the same tissue concentration as the XY2000 group, in order to verify if the XY2000 treatment acted before 10 dpf. At this stage, the EE2 tissue concentration of XY20 was 3.5 higher than in XY2000 (Fig. 2B) (3975 ± 995 and 1146 ± 153 ng g^{-1} respectively). However, 1 day after the treatment, the EE2 level of XY20 dropped to 397 ± 39 ng g^{-1} and continued to decrease faster than in XY2000. At 14 dpf, the values of EE2 concentration in XY2000 (28 ± 12 ng g^{-1}) and XY20 (10 ± 5 ng g^{-1}) were not significantly different but still significantly higher than the controls. As in XY2000, the hormone level in XY20 returned to a basal level close to 0 and was statistically similar to the controls at 21 dpf (0.55 ± 0.67 ng g^{-1}).

In YY feminization experiment at a high concentration of 6500 µg l^{-1} (YY6500), the EE2 uptake and clearance followed the same profile as in XY2000, with a 10-fold

bioconcentration of the hormone in the tissue immediately after the immersion (Fig. 2C), the mean concentration peaking at 62755 ± 3691 ng g^{-1}. Afterwards, the EE2 level decreased to 6703 ± 1789 ng g^{-1} at 10 dpf. At 14 dpf, the concentration in YY6500 (28 ± 7 ng g^{-1}) was equal to that measured in XY2000 and returned to a level (0.26 ± 0.03 ng g^{-1}) statistically similar to the control at 21 dpf (Fig. 2D). In the dietary treated groups (YYD) with a dose of 500 mg kg^{-1} food from 10 to 40 dpf, the tissue EE2 concentration slowly increased to 166 ± 44 ng g^{-1} at 14 dpf, 442 ± 40 ng g^{-1} at 21 dpf and reached a maximum of 1792 ± 295 ng g^{-1} at the end of the treatment (40 dpf) (Fig. 2D). As soon as the hormonal treatment ended, the concentration decreased rapidly to 139 ± 21 ng g^{-1} at 42 dpf.

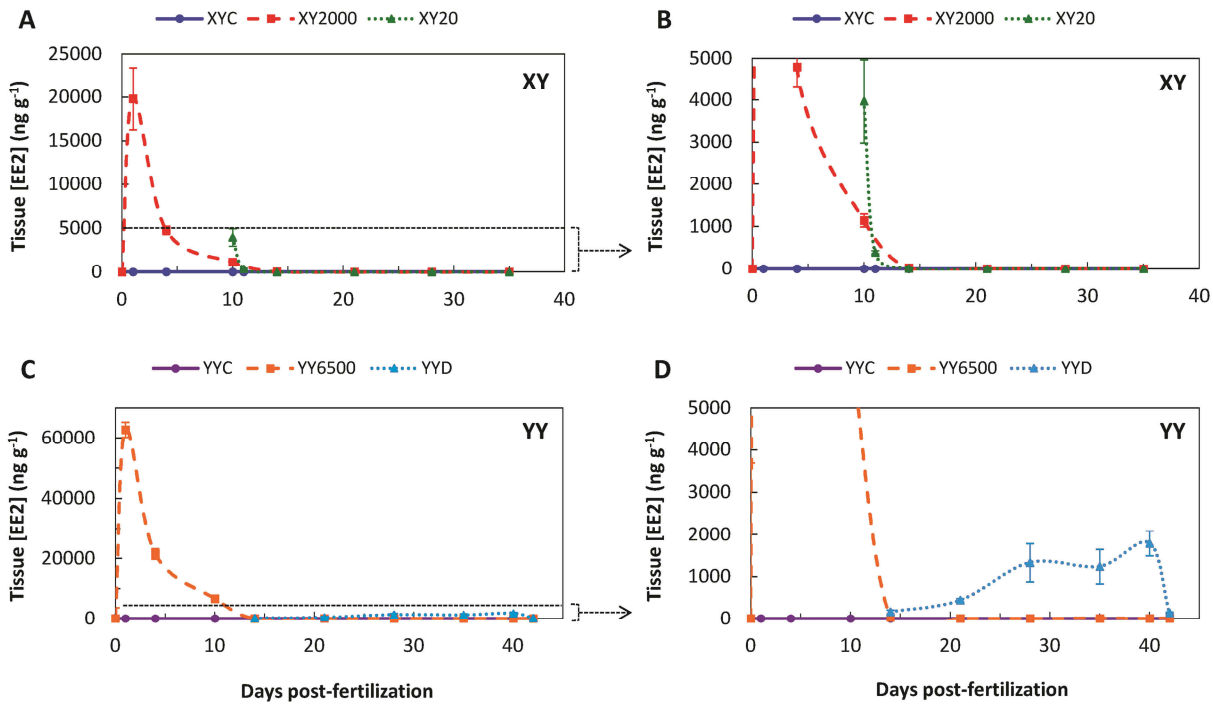

Figure 2. Mean 17α-ethynylestradiol (EE2) tissue concentration in (A, B) XY (n = 5 progenies) and (C, D) YY (n = 4) progenies of Nile tilapia submitted to feminization treatments. A and C: complete profiles; B and D: details between 0 and 5000 ng g^{-1}. XYC: XY control group; XY2000: XY submitted to an EE2 immersion treatment (2000 µg l^{-1}) for 4 h at 1 dpf; XY20: XY immersed in EE2 at 20 µg l^{-1} for 4 h at 10 dpf; YYC: YY control group; YY6500: YY immersed in EE2 at 6500 µg l^{-1} for 4 h at 1 dpf; YYD: YY submitted to an EE2 dietary treatment of 500 mg kg^{-1} food from 10 to 40 dpf.

2.3. T, E2 and 11KT profiles

At 0 dpf, eggs contained high levels of T and E2 in both XY (Fig. 3A and 3C) and YY (Fig. 3B and 3D). The maximal values were measured at 1 dpf (except T in YY): 58 ± 5 ng T g^{-1} and 45 ± 11 ng E2 g^{-1} in XYC; 37 ± 9 ng T g^{-1} and 67 ± 6 ng E2 g^{-1} in YYC. After 1 dpf, concentrations of T and E2 decreased until 10 to 14 dpf. Thereafter, the concentrations remained at a low level (between 5 and 15 ng g^{-1}) until the end of the experimental period.

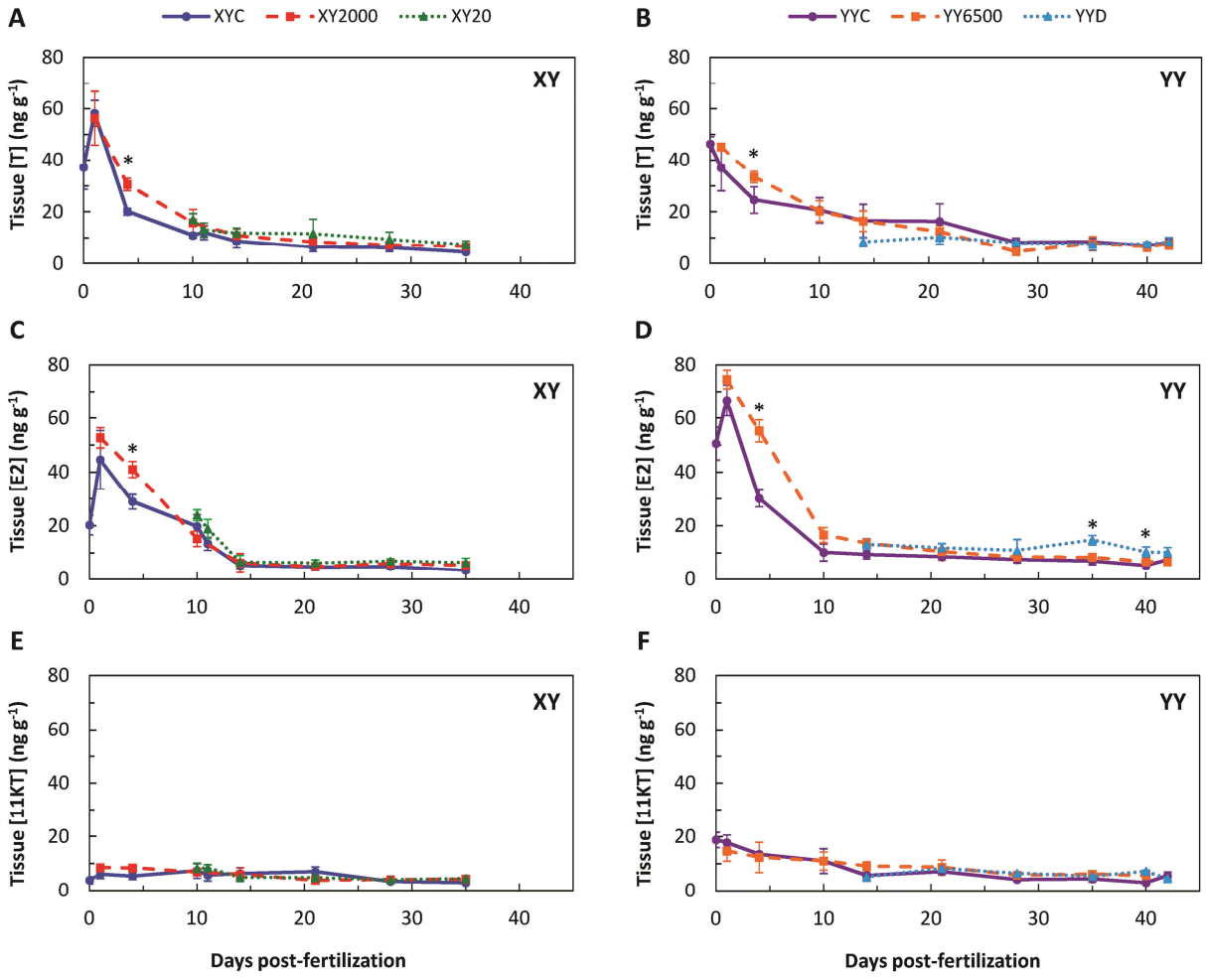

Figure 3. A-B: Mean testosterone (T), C-D: 17β-estradiol (E2) and E-F: 11-ketotestosterone tissue concentration in XY (n = 5) and YY (n = 4) progenies of Nile tilapia submitted to feminization treatments. XYC: XY control group; XY2000: XY submitted to an EE2 immersion treatment (2000 µg l^{-1}) for 4 h at 1 dpf; XY20: XY immersed in EE2 at 20 µg l^{-1} for 4 h at 10 dpf; YYC: YY control group; YY6500: YY immersed in EE2 at 6500 µg l^{-1} for 4 h at 1 dpf; YYD: YY submitted to an EE2 dietary treatment of 500 mg kg^{-1} food from 10 to 40 dpf. * indicates significant differences ($p < 0.05$).

In both XY and YY, groups that received an EE2 immersion treatment at 1 dpf (XY2000 and YY6500) showed a significant increased level of T and E2 at 4 dpf compared to controls (Fig. 3A, 3B, 3C and 3D). No other significant difference was observed between controls and immersed-treated groups. However, E2 concentrations significantly increased in dietary EE2-treated YY at 35 and 40 dpf (Fig. 3D). 11KT showed a different profile. In XY (Fig. 3E), mean levels were low and constant from 0 to 35 dpf and did not exceed 7 ng g^{-1}. YY eggs contained a higher concentration of 11KT at 0 dpf (19 ± 3 ng g^{-1}) (Fig. 3F). It decreased thereafter until 10 dpf to reach a similar level as in XY. No significant difference was observed in 11KT levels between control and treated groups in none of the genotypes.

No correlation was observed between steroid levels in eggs at 0 dpf and sex reversal rates.

2.4. Expression analysis of sex-differentiating genes

Early feminization treatments caused a strong and significant increase in *cyp19a1b* expression in the heads of tilapia embryos 3 days after the treatment (4 dpf) (Fig. 4A and 4B). In the XY2000 EE2-treated group, we observed a 2.4-fold change in relative expression of this gene (39.1 ± 2.7 %) compared to controls XYC (16.1 ± 1.2 %) (Fig. 4A). Brain aromatase expression was even more stimulated in YY (Fig. 4B) with a 3.5-fold increase in relative expression seen in the YY6500 (64.7 ± 5.6 %) compared to controls YYC (18.4 ± 1.3 %). No significant difference was observed between XY and YY controls. At 4 dpf, expression levels of *amh* and *foxl2* were very low (Fig. 4A and 4B) and were not affected by the hormonal immersion treatments or by the genotype ($p > 0.05$).

In the heads of 14 dpf fry, none of the 3 analyzed genes showed a significant difference in expression levels between control and EE2-treated groups or between genotypes (Fig. 4C and 4D). Compared to 4 dpf, relative expression of *cyp19a1b* was lower in all the groups (XYC: 10.2 ± 1.6 %; XY2000: 11.9 ± 3.7 %; YYC: 7 ± 1.3 %; YY6500: 13.2 ± 2.4 %). Both *amh* and *foxl2* expressions were up-regulated. From 4 to 14 dpf, *amh* expression increased from 1.0 ± 0.5 % to 16.2 ± 2.3 %, from 0.5 ± 0.2 % to 12.9 ± 3.1 %, from 1.1 ± 0.3 % to 30.4 ± 15.6 % and from 1.7 ± 0.4 % to 23.4 ± 4.4 % in XYC, XY2000, YYC and YY6500 respectively. *Foxl2* expression increased from 2.0 ± 0.3 % to 33.4 ± 10.7 %, from 1.0 ± 0.3 %

to 35.0 ± 14.3 %, from 3.5 ± 1.7 % to 34.5 ± 25.5 % and from 1.2 ± 0.6 % to 26.8 ± 8.5 % in XYC, XY2000, YYC and YY6500 respectively.

No correlation was observed between changes in gene expression levels between control and treated groups and sex reversal rates.

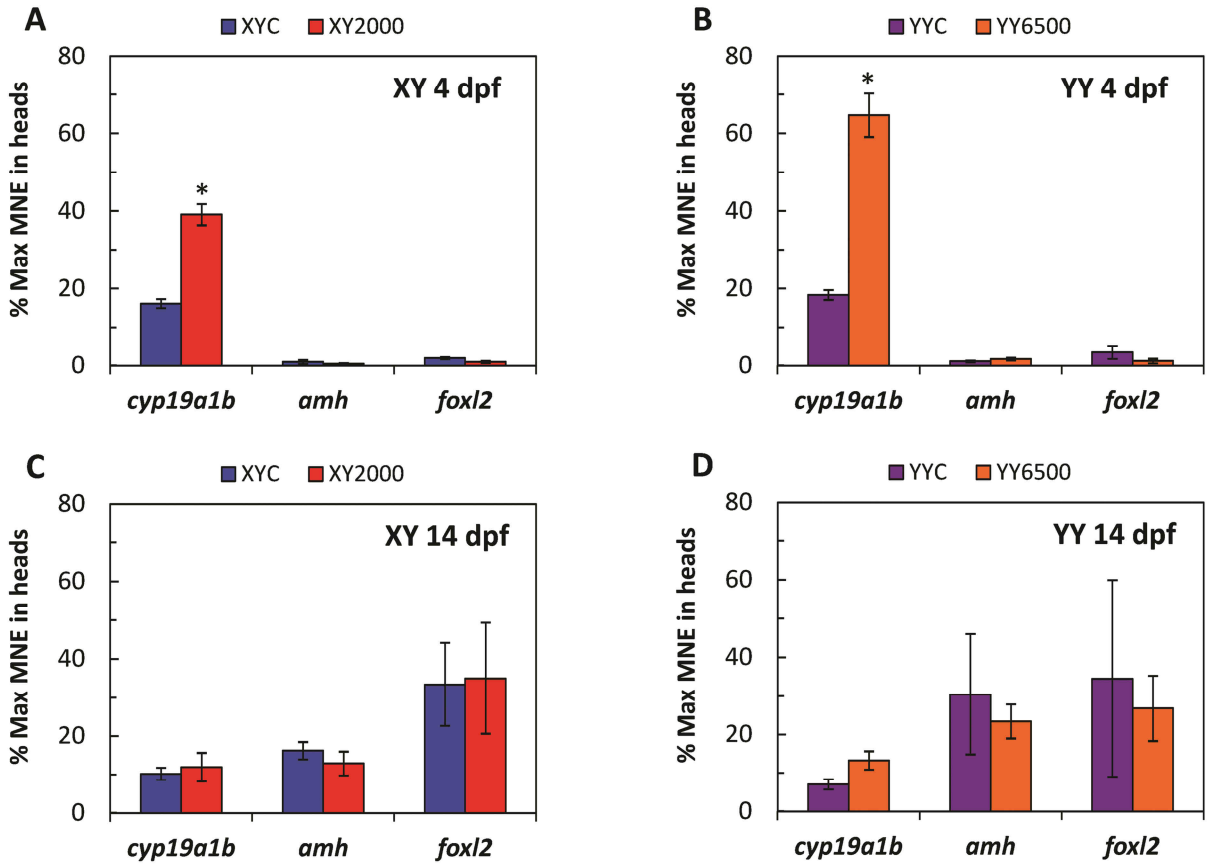

Figure 4. Mean relative expression of *cyp19a1b*, *amh* and *foxl2* at (A, B) 4 dpf and (C, D) 14 dpf in heads of XY (n = 5) and YY (n = 4) progenies of Nile tilapia submitted to early feminization treatments. XYC: XY control group; XY2000: XY submitted to an EE2 immersion treatment (2000 µg l^{-1}) for 4 h at 1 dpf; XY20: XY immersed in EE2 at 20 µg l^{-1} for 4 h at 10 dpf; YYC: YY control group; YY6500: YY immersed in EE2 at 6500 µg l^{-1} for 4 h at 1 dpf; YYD: YY submitted to an EE2 dietary treatment of 500 mg kg^{-1} food from 10 to 40 dpf. * indicates significant differences ($p < 0.05$).

3. Discussion

Our results confirmed the existence of an early sensitive period for sex reversal by exogenous factors during the process of sex determination in the Nile tilapia, covering the embryonic and larval stages. A 4-h immersion treatment in 2000 µg EE2 l^{-1} at 1 dpf induced up to 60 % feminization in XY fish. To our knowledge, this is the first time that such an early hormonal treatment induces sex reversal in tilapia. Similar feminizing treatments were tested on *O. mossambicus* embryos by Rosenstein and Hulata (1992) but failed to induce sex reversal in this species. Other short immersion treatments in hormonal solutions were successfully tested for masculinization and feminization in Nile tilapia but they were all applied later and targeted the gonad differentiating period (Gale et al., 1999; Kobayashi et al., 2003). Our first work (Rougeot et al., 2008a) showed that an immersion treatment in a 100 to 500 µg EE2 l^{-1} solution from 12 hpf to 5 dpf induced 55 to 68 % sex reversal in XY individuals. However, the duration of the treatment and the consequent persistence of exogenous estrogen in the developing fry (more than 5000 ng g^{-1} at 10 dpf; unpublished data) left some doubt about the actual period of action of exogenous estrogens. Here, we shortened the treatment span with a higher EE2 concentration in order to focus the treatment on the embryonic stage and avoid a delayed effect of the accumulated hormone after 10 dpf, during the gonad differentiation. As proven by the low sex-ratio deviation induced by the treatment applied at 10 dpf, the residual hormone could not be responsible for the whole sex reversal effect. Even though the 2 progenies showing the best feminization responsiveness were slightly affected by the treatment at 10 dpf, more than 85 % of the sex reversal effect could be assigned to a mechanism operating before 10 dpf. As the hormone clearance kinetics was very different just after the treatment or several days later, it was difficult to obtain the same EE2 profiles between batches treated at 1 and those treated at 10 dpf. However, the EE2 concentration was 3.5 times higher at 10 dpf in individuals treated at 10 dpf and both groups had a similar concentration at 14 dpf, which means that a brief treatment applied at 10 dpf was appropriate to identify any effect of the residual hormone during the sex-differentiating period. In order to obtain a complete sex reversal of *O. niloticus* XY fry by immersion treatment at the onset of gonad differentiation, Kobayashi et al. (2003) used similar or higher EE2 concentrations (from 10 to 1000 µg l^{-1}) for a prolonged period of 3 days (from 8 to 10 dpf). Since there is an inverse relationship between dose and treatment duration for sex reversal (Piferrer, 2001), it is likely that the necessary estrogen intake at 10 dpf to obtain up to 60 % of sex reversal is far

higher than the residual concentration measured in treated individuals. Moreover, the increase in T and E2 concentrations and the up-regulation of *cyp19a1b* expression induced by the early treatment were observed at 4 dpf and no longer at 14 dpf, suggesting that the hormonal treatment acted early on these parameters. The hormonal-sensitive period for sex determinism in embryos corresponds to the early thermo-sensitive period highlighted by Rougeot et al. (2008b) for masculinization. As temperature could not act through an accumulation and a delayed effect, these 2 studies prove the existence of an embryonic sensitive period where sex can be reversed during sex determination in Nile tilapia.

The brief hormone exposure avoided the drawback of reduced survival rates induced in thermal treatments (Rougeot et al., 2008b; Wessels et al., 2011) or by longer exposures (Rosenstein and Hulata, 1992; Rougeot et al., 2008a). In this study, mean survival rates were low in XY progenies (25-41 %) but were not different between controls and eggs immersed at 1 dpf. As survival was higher in individuals immersed at 10 dpf and YY receiving a dietary treatment, reduced survival rate must be attributed to the mechanical stress induced by early manipulations of eggs rather than to a chemical toxicity. Moreover, short immersions did not present an adverse effect on growth contrary to what was observed in dietary treated YY Nile tilapia where growth showed 50 % reduction during the 30-day treatment period.

Early sex reversal of embryos by brief hormonal exposure was performed in the medaka by Iwamatsu et al. (2005, 2006a) and Kobayashi and Iwamatsu (2005). Since the medaka and the Nile tilapia have similar morphogenesis during sex differentiation (Siegfried, 2010) and both present an early sensitive sex-determining period long before the gonadal differentiation, these 2 species are very interesting models of gonochoristic teleosts to study upstream sex-determining mechanisms. Tilapia has a XX/XY chromosomal system with a masculinizing effect of high temperature and an influence of autosomal factors (Baroiller et al., 1995; Baroiller et al., 2009), but no major sex determinant similar to the medaka *dmy* gene has yet been identified (Cnaani et al., 2008; Palaiokostas et al., 2013). In the medaka, the XY specific expression of *dmy* is initiated in the somatic cells surrounding the primordial germ cells (PGCs), just after their migration to the gonadal anlagen. The onset of *dmy* expression is rapidly followed by a difference in the number of PGCs between genotypic males and females (Kobayashi et al., 2004). Control of proliferative activity and/or apoptosis in PGCs could be part of the first step of the sex-determining pathway and the target of early feminization treatments. This is experimentally supported by Kobayashi and Iwamatsu (2005) who

observed an increased number of PGCs after hatching in XY medaka after a brief exposure of freshly-fertilized eggs to E2.

In Nile tilapia, no information on the involvement of PGCs or PGC surrounding cells in the mechanism of early sex reversal are available but another lead evoked by Rougeot et al. (2008a) and shown by D'Cotta et al. (2001b) considered a role of the brain in the sex differentiation cascade. To explore this hypothesis, we searched in the present study for sex-determining genes differentially expressed in the head of estrogen-immersed embryos. Early estrogen treatments affected the brain differentiation as *cyp19a1b* was clearly up-regulated at 4 dpf in the embryo heads when exposed to EE2. Comparison between XY and YY showed that the increase in expression was dose-dependent. In both genotypes, it was associated with an increase in both T and E2 concentrations. While the increase in E2 synthesis was probably the result of the up-regulation of *cyp19a1b* induced by exogenous EE2, the increase in T suggests that estrogen treatment up-regulated the expression or activity of other steroidogenic enzymes long before the gonad is formed and can synthesize steroids.

Cyp19a1b is very sensitive to exogenous estrogens due to the presence on its promoter of a conserved estrogen responsive element (Diotel et al., 2010). Brain aromatase is expressed in radial glial cells and its activity is very high in the brain of teleost fish. Estrogens and high aromatase activity are now recognized to play an important role in neurogenesis but their possible role in brain sexual differentiation is still unclear (Le Page et al., 2010). *Cyp19a1b* is expressed in the brain early in development in different species with a clear sexually dimorphic expression before the onset of gonad morphological differentiation reported in the rainbow trout (Vizziano-Cantonnet et al., 2011). In the pejerrey, *cyp19a1b* was not differentially expressed before the onset of sex differentiation at neither female nor male-promoting temperatures, but in adults E2 treatments caused *cyp19a1b* up-regulation together with the estrogen receptors *er* and *er* (Strobl-Mazzulla et al., 2008). Similarly, no sex differences for *cyp19a1b* were found in medaka during development but higher levels were observed in adult females (Okubo et al., 2011). In medaka, female-specific expressions were then found during development for the *er* and *ar* genes restricted to certain nuclei, which were activated with E2 treatment (Hiraki et al., 2012). In the Nile tilapia, the expression of the 2 genes coding for aromatase is initiated in the embryos at 3-4 dpf (Kwon et al., 2001). During the gonad differentiation, the high expression level of the ovarian form in the gonad (from 9 dph) is found in females (D'Cotta et al., 2001b; Ijiri et al., 2008) while the brain form is

expressed at the same level in XX and XY brains (Kwon et al., 2001), suggesting a role in neurogenesis rather than in sexual differentiation. However, sex reversal induced by temperature is accompanied by a decrease in brain aromatase activity during the sex-differentiating period (D'Cotta et al. 2001a) with a higher brain aromatase activity in XX female than in XY male tilapias under normal thermal conditions. This observation, together with our results, suggest that a role of the brain and particularly the brain aromatase in the mechanism of gonad sex differentiation cannot be ruled out, and that post-transcriptional control could be responsible for regulating sex-specific aromatase activity (Balthazart et al., 2011). In tilapia, sex-specific expression for *cyp19a1b* may be restricted to certain nuclei and not be readily observable. Nevertheless, the up-regulation of *cyp19a1b* in treated embryos cannot alone be responsible for the sex reversal effect seen in the present study since no sex reversal was observed in YY progenies. If the brain is involved in the mechanism of early sex reversal, and consequently in early sex determinism, this mechanism should be more complex and requires most likely additional factors other than only aromatase.

To investigate one of the possible regulating mechanisms of *cyp19a1b* expression, we measured the early expression of *foxl2* in the brain of normal XY, YY and sex-reversed tilapias. Transactivation studies have shown that *foxl2* together with *sf1* can activate the transcription of *cyp19a1a* by binding to its promoter in tilapia (Wang et al., 2007). In tilapia gonads, the transcription factor *foxl2* is co-expressed at the initiation of the ovarian differentiation suggesting that it regulates *in vivo cyp19a1a* expression (Ijiri et al., 2008; Wang et al., 2007). However, temporal co-expression was not evident in other tilapia strains and temperature-induced masculinization did not suppress both genes simultaneously (Poonlaphdecha et al., 2013). Our present results also suggest that *foxl2* in the brain does not regulate *cyp19a1b* expression, and is not involved in the mechanism of early sex reversal. We found no (or very low) expression of *foxl2* in the heads at the embryonic stage, in XY controls and in EE2 treated embryos, while *cyp19a1b* was markedly and differentially expressed between the 2 groups. As *foxl2* expression is strongly inducible by estrogens in the gonad (Baron et al., 2004; Wang et al., 2007), we can postulate that this gene was totally repressed or alternatively not yet transcribed in the brain of tilapia embryos. It was expressed at 14 dpf, during the gonad differentiation but at a similar level in both XY and YY fish and those treated with EE2. More investigations are still needed to understand the role of this gene in the brain of teleosts. In rainbow trout, *foxl2* is co-expressed with *cyp19a1b* in the brain at the

initiation of gonad differentiation, but a sexually dimorphic expression was only observed for *cyp19a1b* (Vizziano-Cantonnet et al., 2011). The authors concluded that *foxl2* is not the only trigger regulating *cyp19a1b* in the brain. In *Clarias gariepinus* a cooperative regulation of *cyp19a1b* by *foxl2* and the nuclear receptor *ftz-f1* has been demonstrated (Sridevi et al., 2012).

Amh was also a good candidate in the search for cerebral genetic factors involved in early sexual reversal. In different species, *amh* and *cyp19a1a* have reciprocal expression profiles in the differentiating gonad (Fernandino et al., 2008; Wang and Orban 2007). In Nile tilapia, Poonlaphdecha et al. (2013) suggested that *cyp19a1a* inhibits *amh* expression in the gonads of genetic females. This is in contrast to what was found in mammals where amh caused a repression of gonad aromatase activity *via* the suppression of its gene in embryo ovarian cultures (Vigier et al., 1989). *Amh* is down-regulated by estrogens (Fernandino et al., 2008; Schulz et al., 2007) in the gonad and sexually dimorphic expression levels are observed between genetic males and females in tilapia brain at 14 dpf (Poonlaphdecha et al., 2011). In our study, a down-regulation of *amh* expression induced by EE2 was not observed as the gene was totally repressed in embryos, even in XY and YY control. As *foxl2*, its expression was initiated later in the brain and showed no difference between controls and EE2 sex-reversed fish. This suggests that the expression level of *amh* could be related to the sexual genotype and that a factor linked to the Y chromosome could control its expression regardless of the sexual phenotype.

In XY, the sensitivity to EE2 treatments widely varied from one progeny to another as demonstrated by the sex reversal rates ranging from 0 to 60 %. This variability could be related to the differential expression of sex-determining genes or to the endogenous steroid balance during ontogenesis and maternal steroid inheritance. In tilapia as in other fish species, estrogens are required for ovarian differentiation (Kobayashi et al., 2003; Nakamura, 2010). They are also involved in neurogenesis and brain sexualization as suggested by the early expression of brain aromatase (Diotel et al., 2010; Le Page et al., 2010). In tilapia, steroid metabolism is initiated early in embryogenesis since maternal hormones are metabolized during the first days of development (Hines et al, 1999). Sex differences were found for E2 but not for 11KT in tilapia gonads in the mid stage of sex differentiation (D'Cotta et al., 2001a,b) confirming that it is the elevated endogenous estrogen levels that induce ovarian differentiation (D'Cotta et al., 2001b; Devlin and Nagahama, 2002; Guiguen et al., 2010). Our results showed that exogenous sexual steroids can override sex determinism at a very

precocious time before the gonad is formed, similarly to what was reported for medaka embryos by Iwamatsu et al. (2005, 2006b). We could hypothesize that the steroid content of freshly fertilized eggs, is maternally inherited and could influence the sex-determining mechanism. However, we did not find any correlation between steroid (T, E2 and 11KT) contents in freshly ovulated eggs and sex reversal rates of progenies. A deviation from a 1:1 sex ratio in normal crosses (Lester et al., 1989) and heritable differences in temperature sex influences (Baroiller et al., 2009; Wessels and Hörstgen-Schwark, 2011) demonstrated that sex determination is multifactorial in tilapia, with sex chromosomes as well as an influence of minor genetic factors, and we suggest that such factors could influence the responsiveness to early hormonal treatment.

If egg steroid content is not determining in sexual development, we can postulate that a genetic determinant present on the sex chromosomes is responsible for the lack of early sex reversal in YY. There could be a feminizing factor linked to the X chromosome that is necessary to elicit a response to the estrogen treatment, or alternatively a masculinizing factor linked to the Y chromosome and the presence of 2 copies represses or disables the feminizing effect of the exogenous hormone. In any case, the absence of sex reversal in YY is a striking and very interesting result as it suggests that an upstream genetic determinant linked to the sex chromosomes can influence the sex determinism in the first days of the embryogenesis, long before the earliest known evidence of a gonadal differential gene expression (9 dph) (Ijiri et al., 2008).

In summary, we demonstrated for the first time that brief EE2 treatments during embryogenesis are effective in reversing XY tilapias towards a female phenotype, highlighting the existence of an early sensitive period for sex reversal during tilapia sex determination encompassing embryonic and larval development, prior to any sign of gonad differentiation. The estrogen treatment induced an increased expression of brain aromatase at 4 dpf in XY and YY progenies, suggesting that the brain could be sexualized very early during development. Further investigations regarding the expression profiles of other sex-determining genes in the brain and the development of PGCs are needed to elucidate the complex mechanism of early feminization. Because this early embryo induced feminization was ineffective in YY fish, it is suggested that a Y repressor may be modulator or alternatively that an activator on the X chromosome might be needed. Our embryonic

feminizing procedure constitutes a new tool in the search for upstream and/or the major sex determinant in the Nile tilapia.

4. Material and Methods

4.1. Fish stock housing, reproduction and juvenile rearing

Nile tilapia *O. niloticus* from the Lake Manzala strain were from the Research and Education Center in Aquaculture (CEFRA), University of Liège (Belgium). All-male XY progenies were obtained by artificial reproduction of YY males with XX females, and supermale YY progenies from YY males mated with YY females (Gennotte et al., 2012). Broodstock fish were individually stocked in 125-L aquaria at 27 °C with a 14 h light/10 h darkness photoperiodic regime. Fish were fed at satiation with special broodstock commercial tilapia diet (45 % proteins, 5 % lipids, Coppens – The Netherlands). After artificial fertilization, eggs were weighed and counted. Each progeny was divided into 3 batches (controls, a 4-h treated group at 1dpf and a 10 dpf treated group for XY; controls, a 4-h treated group at 1 dpf and a 30-day dietary treated group for YY) and incubated in 1.5 L Zug bottles at 27 °C. Fertilization rates were evaluated on 100 eggs after the first mitotic cleavage (2 hpf) (Morrison et al., 2001). Hatching rates were assessed by counting all fish from each batch at 4 dpf. Larvae were transferred at 8 dpf into 50-L aquaria and feeding started at 10 dpf. In order to determine the food ratio and follow the growth and survival of each batch, the number of individuals and total biomass were recorded every week from 8 dpf to 35 dpf (XY) or 42 (YY) dpf. The homogenization of fish growth between treated and control groups, and amongst progenies, was essential in order to standardize the developmental speed and the post-treatment kinetics of EE2 clearance. Fish were fed close to satiation and food ratios were adjusted to assure the same growth in all batches. Food distribution was performed 6 times a day with a commercial tilapia diet (47 % proteins, 8 % lipids, Coppens – The Netherlands). Experiments were carried out according to the guidelines of the University of Liège ethical committee and the European animal welfare recommendations.

4.2. EE2 solutions

17α-ethynylestradiol (Sigma-Aldrich) was dissolved in 100 % ethanol and then stored at 4 °C. Stock solutions were prepared at different concentrations: 20, 2000 and 6500 mg L^{-1}. For each experiment, 1 ml of stock solution was added to 1 L of water. Control groups were incubated only in 1:1000 ethanol solution. For the YY feminization group treated with EE2-supplemented feed, 500 mg of EE2 was dissolved in 600 ml of 95 % ethanol and mixed with 1 kg of food pellets. It was then allowed to dry and stored at 4 °C.

4.3. Early feminization of XY individuals

To test the sex inversion efficiency of hormonal treatment on developing embryos, feminization treatments were applied to one batch for the 5 XY progenies. It consisted of a single 4-h immersion in a 2000 µg EE2 l^{-1} solution (XY2000 group) at 1 dpf by transferring the eggs from the Zug bottle into a 1 L glass beaker filled with hatchery water and maintained in a thermostatic bath at 27 °C. One ml of stock solution (ethanol for control groups) was then added to the water. During incubation, the water was oxygenated by an air diffuser. After 4 h of immersion, the eggs were netted, the solution gently removed by soaking on paper and rinsed in 6 different baths to remove hormone residues. The first 3 baths contained 1:1000 ethanol and the next 3 had only hatchery water. The batches corresponding to control groups (XYC) were handled in the same way. After rinsing, eggs were replaced into the hatchery.

In order to verify that the 1 dpf feminization treatment really targeted the early developmental stages before 10 days and did not act by a hormonal tissue accumulation and a delayed effect after 10 dpf, we treated the third batch of each progeny at 10 dpf also with a 4-h immersion in a 20 µg EE2 l^{-1} solution (XY20 group). This lower dose was used to reach an EE2 tissue concentration similar to the residual concentration measured in the XY2000 group at 10 dpf. The immersion procedure was essentially the same as the egg treatment.

For steroid analysis, individuals were sampled in XYC, XY2000 and XY20 at 0 (unfertilized eggs, n = 67-100), 1 (n = 80-100), 4 (n = 70-100), 10 (n = 40-60), 11 (n = 20-40), 14 (n = 15-30), 21 (n = 6-10), 28 (n = 6-10) and 35 (n = 6-10) dpf. Samples were weighed and stored at -80 °C until steroid extraction.

Gene expression analysis was performed on individuals sampled at 4 (n = 50) and 14 (n = 20-50) dpf in control and XY2000 groups. Samples were preserved in RNA Later (Ambion, USA) at -20 °C following manufacturer instructions, before RNA extraction.

4.4. Early feminization of YY individuals

Four YY progenies were produced and divided into 3 batches. The treated batches were immersed for 4 h in a 6500 µg EE2 l^{-1} solution at 1 dpf (YY6500). The protocol was the same as described above for the XY treated eggs. Because preliminary results (unpublished) showed that a treatment in a 2000 µg EE2 l^{-1} solution had no effect on YY sex reversal, we increased the EE2 concentration for the YY treatment in the same proportion as the EE2 concentrations used in dietary hormonal treatment (150 mg kg^{-1} for XY and 500 mg kg^{-1} for YY) in our laboratory (unpublished data). The control batches (YYC) were immersed in a solution containing only ethanol (1:1000). The third group (YYD) served as a positive control for sex reversal. The fry from the third batch per progeny were fed *ad libitum* from 10 dpf to 40 dpf with a EE2-supplemented diet (500 mg kg^{-1} food) to verify the susceptibility of each progeny to the feminization treatment applied during the known sensitive period of sex differentiation.

Samplings for steroid analysis were carried out in YYC, YY6500 and YYD at 0 (unfertilized eggs, n = 100), 1 (n = 80-100), 4 (n = 65-150), 10 (n = 30-100), 14 (n = 15-60), 21 (n = 6-15), 28 (n = 6-10), 35 (n = 6-10), 40 (n = 6-10) and 42 (n = 6-10) dpf. Samples were weighed and stored at -80 °C until steroid extraction.

Gene expression analysis was performed on individuals sampled at 4 (n = 40-50) and 14 (n = 20-50) dpf in control and YY6500 groups. Samples were preserved in RNA Later (Ambion, USA) at -20 °C following manufacturer instructions, before RNA extraction.

4.5. Sex-ratio analysis

Phenotypic sex was determined by the acetocarmine squash method (Guerrero and Shelton, 1974) at 90 dpf. Fish were euthanized by an overdose (500 mg l^{-1}) of benzocaine (Sigma-

Aldrich) and a slice of the gonads from 53 to 125 fish per batch was microscopically examined after acetocarmine coloration.

4.6. Steroid extraction

Steroids were extracted following a procedure adapted from (D'Cotta et al., 2001a). Homogenized samples (weight = 0.25 to 1.15 g) were suspended in 100 % ethanol (1 ml g^{-1}) and washed 3 times with 50 % ethanol (1 ml g^{-1}). After the homogenate was centrifuged for15 min at 4000 g, 10 °C, the supernatant was collected. The pellet was resuspended in 80 % ethanol (1 ml g^{-1}) and centrifuged a second time (15 min, 4000 g at 10 °C). Pooled supernatants were partially evaporated and extracted 3 times with 3.5 ml dichloromethane. The organic phase was totally evaporated. The extract was suspended in 300 µl of 100 % ethanol and stored at -20 °C until assays.

4.7. EE2 enzyme immunoassay

EE2 was assayed by EIA following the manufacturer instructions (Europroxima, The Netherlands). Twenty µl of extract was dissolved in 180 µl of dilution buffer provided with the kit. Each sample from treated groups was further diluted to reach a concentration in the detection range (0.02 to 2 ng g^{-1}) of the kit. All samples were assayed in duplicate.

4.8. T, E2 and 11KT radioimmunoassay

Testosterone (T), 17β-estradiol (E2) and 11-ketotestosterone (11KT) concentrations were assayed in developing eggs, larvae and juveniles by radioimmunoassay (RIA). Fifty µl of hormone extract were dried and dissolved in 300 µl of phosphate buffer (0.01 M, pH 7.25) containing 0.1 % gelatin. From this solution, 100 µl were used in duplicates for the assay, as described in Douxfils et al. (2007). Radioactive hormones were purchased from Amersham Pharmacia (Buckingamshire, England). T and E2 antibodies were obtained from the Laboratoire d'Hormonologie de Marloie (CER, Belgium), and the anti-11KT was a gift from A. Fostier (INRA, Rennes, France). Detection limits ranged from 50 to 80 pg ml^{-1}.

4.9. Total RNA extraction and reverse transcription

Heads were dissected from the trunks under a stereo microscope and placed in RNA Later. Each batch sampled was divided in 2 or 3 replicates and each replicate contained from 6 to 17 heads. Total RNA was extracted from pooled heads using the TRIzol reagent (Invitrogen, France) following the manufacturer's protocol after which total RNA was resuspended in 30 to 150 µl of RNase-free water and quantified with a NanoDrop (Thermo Scientific, USA). Extracts were treated to remove genomic DNA with TURBO DNase (Ambion, USA) and single strand cDNA was produced using SuperScript II reverse transcriptase (Invitrogen, Germany) as described in Poonlaphdecha et al. (2011). Reverse transcription was performed with 5 and 4 µg of total RNA for samples aged 4 and 14 dpf respectively. The cDNA was diluted in water to 25 ng μl^{-1} and stored at -20 °C for quantification by real-time PCR.

4.10. Quantitative real-time PCR (qPCR) analysis

Expression levels of *cyp19a1b*, *amh* and *foxl2* were studied in embryo (4 dpf) and fry heads (14 dpf). qPCR was performed on a MX3000P real-time PCR system (Stratagene, USA), except *foxl2* that was amplified on a MX3005P (Stratagene, USA). *Ef1α* was used as a reference to standardize gene expression levels. The primers used are listed in table 2. All biological replicates were analyzed in duplicate in 10 µl volumes using Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies, USA). Reactions were run with 50 ng cDNA and a concentration of 300 nM for each primer. PCR cycling parameters were as follows: enzyme activation at 95 °C for 10 min, 40 amplification cycles at 95 °C for 30 s (denaturation), 60 °C for 30 s (annealing) and 72 °C for 30 s (elongation), followed by a dissociation at 95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s. Annealing temperature for *foxl2* was 64 °C. For each reaction, 2 wells without DNA template served as a negative control. Primer specificity was verified by a final dissociation curve in which a single amplification peak was obtained. For each gene, the amplification efficiency (E) was calculated from gene-specific linear standard curves performed on a serially diluted PCR product and calculated according to the equation $E = 10^{(-1/slope)}$. For each target gene, the median efficiency of the different amplification runs was used. Expression levels of target genes were expressed as mean normalized expression (MNE) using *ef1α* as the reference gene and calculated as follows: $MNE_{target} = (E_{reference})^{mean\ Ct_{reference}} / (E_{target})^{mean\ Ct_{target}}$.

Relative expression levels were then calculated as:

$$\%\, maximum\ MNE = 100 \times MNE_{target}/MNE_{target\ maximum}.$$

Table 2. Primers used for the quantitative real-time PCR.

Gene	Genbank accession n°	Forward primer	Reverse primer
cyp19a1b	AF295761	F957: 5'-GATTCATGAAGCCGAGAAGC-3'	R1184: 5'-TTCAAGATGGTGTTCATCATCTCCT-3'
amh	EF512167	F1577: 5'-AAGCAGCGCAAACATTAACA-3'	R1741: 5'-GTTCCAGTCCACAACCTCCA-3'
foxl2	AY554172	F310: 5'-AAGAGGAGCCGGTTCAGGACAA-3'	R396: 5'-GCTCTCCCGGATAGCCATGG-3'
ef1 α	AB075952	F832: 5'-TGTTGAGACTGGTATCCTGAAGCC-3'	R1072: 5'-GATGATGACCTGAGCGTTGAAGC-3'

4.11. Data analysis

Data are reported as mean ± standard error of the mean (SEM). Mean values of growth, steroid concentrations and relative gene expressions were compared between groups (treated vs control). Normality was analyzed with the Shapiro-Wilk test and homoscedasticity with the Levene test. If data complied with these two conditions, differences between means were searched by ANOVA and multiple comparisons were performed with the Fisher's LSD test. Otherwise, non-parametric Kruskal-Wallis test and Mann-Whitney test were used. Chi^2 test was used to analyze survival and sex reversal rates. Spearman rank correlation coefficients were used to look for correlations between sex reversal rates, steroid concentrations in unfertilized eggs and changes in gene expression levels. Differences were considered as significant at $p < 0.05$. Statistical analysis was performed using Statistica v.10 (StatSoft, France).

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Partie 4

Discussion générale et conclusions

1. Discussion générale

Différents aspects liés au déterminisme du sexe et à la reproduction du tilapia du Nil *lato sensu* ont été investigués au cours de cette thèse (Figure 7). Les deux principales questions adressées dans notre travail étaient d'une part, de déterminer si le génotype sexuel, en l'occurrence les chromosomes sexuels, influencent certaines caractéristiques physiologiques et/ou comportementales de la biologie reproductive, et d'autre part, d'évaluer le possible rôle du cerveau dans les mécanismes de différenciation sexuelle précoce au cours de l'embryogenèse.

La question du rôle du génotype sexuel sur le comportement reproducteur a été abordée par l'étude des comportements agonistiques des géniteurs présentant différentes combinaisons de chromosomes sexuels, complétée par l'étude des taux des principaux stéroïdes sexuels et de la qualité des gamètes des mâles. Les comportements agonistiques ne sont pas impliqués dans la reproduction proprement dite, mais une modification du niveau d'agressivité en dehors de la reproduction peut fortement l'influencer en modifiant le choix du partenaire, en perturbant les comportements reproducteurs – et par conséquent le succès de la fécondation – ainsi que les soins parentaux. Au cours de notre étude, nous avons observé chez les mâles un niveau d'agressivité légèrement supérieur pour les génotypes XX, qui pourrait être lié à leur taux plus élevé de 11KT dans le sang par rapport aux génotypes XY et YY (chapitre 3). Cette différence pourrait, perturber les comportements reproducteurs, ou à l'inverse, favoriser l'accès à la reproduction de ces mâles en leur conférant un statut dominant et une meilleure capacité à défendre leur territoire. Il est intéressant de souligner que bien que la 11KT, qui exerce un contrôle important sur les différentes étapes de la spermatogenèse comme la prolifération des spermatogonies et la maturation des spermatocytes (Nagahama, 1994; Schulz et al., 2010), soit présente en concentration plus élevée chez les mâles XX, l'étude comparative de la qualité du sperme chez des mâles XX, XY et YY (chapitre 2) ne montre aucune différence au niveau de l'indice gonado-somatique, de la densité spermatique et des paramètres caractérisant la mobilité des spermatozoïdes (pourcentage de spermatozoïdes mobiles, durée et vitesse de mobilité), suggérant que les trois types de mâles ont les mêmes capacités de fécondation. Chez les mâles, le génotype sexuel semblerait donc n'influencer que le comportement via une éventuelle augmentation du taux de 11KT. Cependant, l'expression des comportements agressifs mâles ne pourrait être pleinement observée que dans des

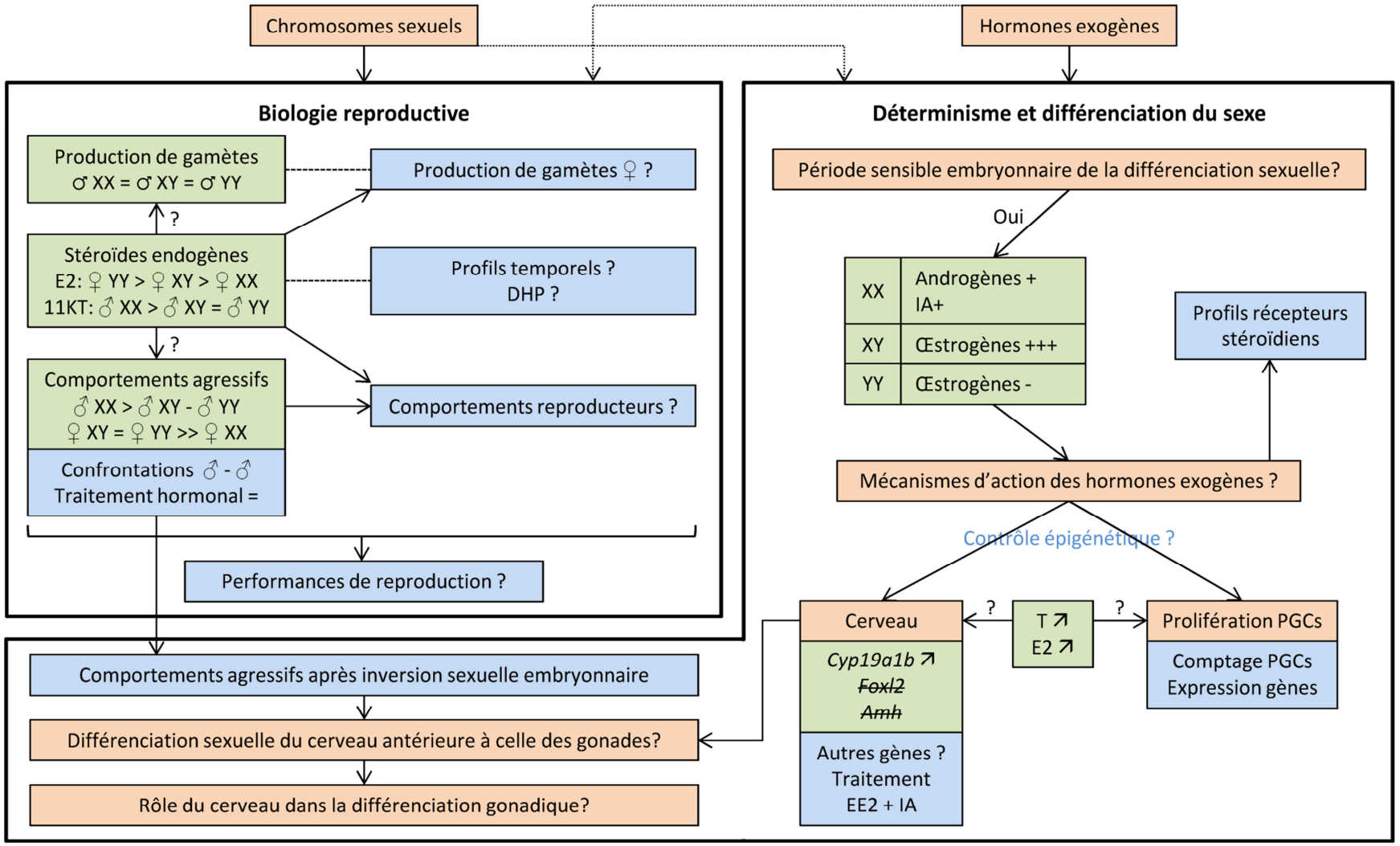

Figure 7. Schéma représentant la démarche scientifique poursuivie dans nos recherches : en orange, les hypothèses de travail et les questions soulevées ; en vert, les éléments de réponses apportés par nos résultats ; en bleu, les perspectives et recherches supplémentaires nécessaires pour compléter ces réponses. Explications détaillées dans le texte (11KT : 11-cétotestostérone ; DHP : 17α,20β-dihydroxy-4-pregnen-3-one ; E2 : 17β-œstradiol ; EE2 : 17α-éthynylœstradiol ; IA : inhibiteur d'aromatase ; PGCs : cellules germinales primordiales ; T : testostérone).

confrontations mâle-mâle dans lesquelles les deux adversaires exhibent des motivations agonistiques plus proches.

De la même manière, notre étude comportementale nous a permis de mettre en évidence un niveau d'agressivité largement supérieur chez les femelles XY et YY par rapport aux femelles XX. Ce taux d'agressivité important, également observé par Ovidio et al. (2002) chez des pseudofemelles *O.aureus* ZZ, pourrait entraver le bon déroulement de la parade nuptiale et de la ponte, et par conséquent diminuer leur succès reproducteur. Parallèlement, nous avons observé des concentrations croissantes en E2 chez les femelles XX, XY et YY. Etant donné que l'E2 joue un rôle crucial dans le contrôle de la vitellogenèse (Nagahama, 1994; Lubzens et al., 2010), nous pourrions nous attendre à observer des différences dans la production de gamètes induites par le génotype sexuel. Dès lors, l'étude de l'impact des chromosomes sexuels sur la production de gamètes, en termes qualitatifs et quantitatifs, doit être étendue aux femelles, bien que chez *O. aureus*, la capacité de production d'ovules (profil saisonnier de ponte, fécondité, taille des ovules) semble similaire entre les femelles ZW et les pseudofemelles ZZ (Desprez & Mélard, 1998).

Notons encore que nos mesures de stéroïdes ne constituent qu'une image ponctuelle et un suivi des fluctuations temporelles des niveaux de stéroïdes, particulièrement sur un cycle complet de reproduction chez les femelles, serait nécessaire pour confirmer les différences observées dans le présent travail. Les mesures stéroïdiennes devraient également être complétées par celle de la DHP, un progestogène fortement impliqué dans la maturation des gamètes, stimulant la production de sperme, la spermiation et la mobilité spermatique chez le mâle (Schulz et al., 2010), induisant la maturation finale des ovocytes et l'ovulation chez les femelles (Lubzens et al., 2010).

Finalement, l'intérêt majeur de notre étude comportementale a été d'étudier l'ensemble des combinaisons de phénotypes et génotypes sexuels, incluant pour la première fois, le génotype YY, ce qui a permis de soulever une nouvelle question. Si les modifications comportementales observées supposent des différences dans la différenciation sexuelle du cerveau, celles-ci sont-elles induites par les chromosomes sexuels ou par les traitements hormonaux d'inversion du sexe administrés pendant la période de différenciation des gonades ? Il est difficile de dénouer ces deux possibilités (voir discussion du chapitre 3), mais une première approche pour tenter d'y répondre, avant d'étudier la nature (neuroanatomique,

génétique, épigénétique) de ces différences sexuelles, serait de réaliser une étude comportementale utilisant des géniteurs ayant tous reçu le même traitement hormonal (même traitement féminisant chez les femelles XX, XY et YY ; même traitement masculinisant chez les mâles XX, XY et YY).

Sur base de l'ensemble des résultats récoltés sur la biologie reproductive et des considérations développées ci-dessus, il peut être suggéré que les différences sexuelles chez ces individus XX, XY et YY peuvent s'exprimer de façon singulière selon le caractère phénotypique considéré et selon que sa différenciation soit influencée par des facteurs génétiques à différents moments du développement, par l'action des hormones exogènes ou par celle des hormones naturelles produites par les gonades différenciées. Jost (1970) émit le concept selon lequel l'état sexuellement différencié d'un individu consistait en différents composants comprenant un sexe génétique, un sexe gonadique, un sexe corporel et un sexe cérébral. Si ces composants semblent intimement liés chez les mammifères, Godwin (2010) formula l'hypothèse d'un découplage de ces aspects chez les poissons, leur conférant cette vaste plasticité observée dans la différenciation sexuelle et les stratégies reproductives.

Un tel découplage pourrait conduire à des individus présentant des caractères phénotypiques sexuels (gonadiques, somatiques, cérébraux et comportementaux) mosaïques chez les tilapias dont le sexe a été manipulé, dépendant de la balance entre les facteurs génétiques portés par les chromosomes sexuels et la période d'action des hormones exogènes au cours du développement. Nos résultats de recherche sur la qualité du sperme (pas de différence physiologique apparente dans la production de gamètes entre les mâles XX, XY et YY) et les comportements agonistiques observés (agressivité plus élevée des mâles XX par rapport aux mâles XY et YY) illustrent ce découplage de la différenciation sexuelle. Si des différences sexuelles liées aux chromosomes sexuels apparaissent dans le cerveau avant l'administration des traitements hormonaux d'inversion sexuelle, ceux-ci pourraient induire une inversion phénotypique du sexe incomplète, notamment au niveau du comportement. Ces considérations rejoignent l'hypothèse d'une différenciation sexuelle du cerveau précédant celle des gonades chez les poissons (Francis, 1992; Godwin, 2010). Afin d'alimenter la discussion de cette hypothèse, il serait particulièrement intéressant d'étudier le comportement agressif des femelles XY sexuellement inversées par traitement embryonnaire pour vérifier si une modification plus précoce de la différenciation sexuelle affecte différemment le cerveau et l'expression des comportements chez l'adulte.

Même chez les mammifères, un certain découplage des mécanismes de différenciation sexuelle existe et la théorie classique selon laquelle la production de stéroïdes sexuels par les gonades différenciées induit la différenciation sexuelle du cerveau et des caractères sexuels secondaires, est aujourd'hui nuancée et revisitée (Craig et al., 2004; Arnold, 2012). En effet, chez les mammifères, des différences sexuelles phénotypiques sont liées à l'expression de gènes complémentaires portés par les chromosomes sexuels, X ou Y, indépendamment de la cascade de différenciation initiée par *Sry* et de la production de stéroïdes gonadiques. Ce type d'influence a été observée dans l'organisation de certains noyaux cérébraux et dans l'expression des comportements agressifs et parentaux chez la souris (Gatewood et al., 2006), ainsi que dans certains comportements sociaux sexuellement dimorphiques chez l'homme (Craig et al., 2004). La preuve la plus évidente de ce découplage des mécanismes de différenciation sexuelle est l'apparition, chez les mammifères et d'autres vertébrés, de différences phénotypiques dépendantes des chromosomes sexuels en amont de la cascade de différenciation sexuelle des gonades initiée par le déterminant génétique majeur du sexe. Par exemple, chez le wallaby de Tammar, la différenciation du scrotum chez le mâle, et des tissus mammaires et de la poche chez les femelles, est contrôlée par le nombre de chromosomes X et initiée avant la différenciation des gonades et la production de stéroïdes sexuels (Renfree & Short, 1988). Chez la souris, Dewing et al. (2003) ont mesuré une expression différentielle de plus de 50 gènes dans les cerveaux des embryons mâles et femelles avant le développement des gonades. Ces gènes, localisés sur le chromosome X ou Y ou différents autosomes, sont notamment impliqués dans la différenciation et la prolifération cellulaire, la régulation transcriptionnelle et la communication cellulaire. Ces auteurs suggèrent que certains de ces gènes portés par le chromosome Y sont des candidats régulateurs des comportements agressifs. De même, dans l'embryon de poulet, une différence sexuelle dans l'expression de certains gènes a été observée dans le cerveau avant la formation des gonades (Lee et al., 2009), mais également beaucoup plus précocement, dans les cellules blastodermiques durant la gastrulation (Zhang et al., 2010). Enfin, une recherche récente a mis en évidence l'expression sexuellement dimorphique d'un gène (*sdgc*[1]) porté par le chromosome Y dans les cellules germinales embryonnaire du médaka, avant la formation des gonades et indépendamment de l'expression du déterminant sexuel majeur *dmy* (Nishimura et al., 2014).

[1] Sex chromosome-dependent differential expression in germ cells

De manière générale – et dans le cadre du déterminisme sexuel génétique – ces données montrent d'une part, que l'expression de certains caractères phénotypiques sexuellement différenciés peut être soit sous contrôle des hormones gonadiques (ou exogènes dans le contexte expérimental des inversions sexuelles), soit directement déterminés par l'expression indépendante de gènes liés aux chromosomes sexuels à différents moments du développement ; et d'autre part, que des différences sexuelles, notamment dans le cerveau, peuvent apparaître aux stades les plus précoces du développement. Une influence précoce des chromosomes sexuels a également été observée dans notre travail, au niveau de la différence de susceptibilité des tilapias XY et YY à l'inversion sexuelle dans les expériences de féminisation embryonnaire induite par un œstrogène exogène (EE2) (chapitres 4 et 5).

Nos recherches sur les inversions sexuelles embryonnaires sont originales à deux égards. D'abord, elles s'intéressent aux trois génotypes sexuels XX, XY et YY, ce qui permet d'aborder, comme dans l'étude de la biologie reproductive, l'étendue de l'influence des chromosomes sexuels sur la différenciation du sexe induite par des hormones exogènes. Ensuite, elles concernent les stades les plus précoces du développement. Que ce soit au niveau expérimental (traitements d'inversion sexuelle) ou analytique (mécanismes génétiques et endocriniens contrôlant la différenciation du sexe), la littérature traitant du déterminisme et de la différenciation du sexe chez le tilapia du Nil compare généralement uniquement des individus XX et XY, et ne s'intéresse qu'à la période de différenciation des gonades (à partir de 9-10 jpf). Très peu de données existent sur les différences sexuelles s'établissant avant cette période. Or, si des différences sexuelles dans l'expression de certains gènes peuvent apparaître, avant la différenciation des gonades, dans le cerveau, les cellules germinales primordiales ou les cellules de la lignée somatique des gonades, la cascade du déterminisme sexuel pourrait être initiée précocement dans l'une de ces structures, comme proposé par Rougeot et al. (2008a,b).

Les différentes méthodes d'inversion du sexe par traitements embryonnaires appliqués à des individus XX, XY et YY, développées dans notre recherche (chapitre 4), nous ont permis d'établir un outil permettant d'exploiter la labilité de la différenciation du sexe aux stades les plus précoces du développement, et d'en étudier les mécanismes. Les traitements courts (4 h) appliqués à 1 jpf se sont montrés beaucoup plus efficaces pour féminiser des individus XY que pour masculiniser des individus XX. Bien que, dans ces expériences, la différenciation du sexe soit artificiellement induite par des doses importantes d'œstrogène ou d'androgène, le

fait que ces hormones puissent outrepasser ou modifier le déterminisme génétique naturel laisse supposer que ce processus est actif à la même période. Les profils d'élimination de l'EE2 administrés aux œufs XY après les traitements féminisants (chapitre 5) ont confirmé l'action précoce de l'hormone exogène durant les stades embryonnaires et larvaires. Nous pouvons donc soutenir l'hypothèse que les premiers événements moléculaires et/ou cellulaires du déterminisme et de la différenciation du sexe chez le tilapia, s'organisent durant ces stades précoces de développement, avant la formation des gonades. De plus, bien qu'elle soit relativement faible, l'action du fadrozole sur des embryons XX suggère que l'aromatase et les œstrogènes pourraient déjà jouer un rôle dans le contrôle naturel de la différenciation du sexe avant le développement des gonades. Cette hypothèse est également soutenue par l'augmentation d'expression de l'aromatase cérébrale et des taux d'E2 observée à 4 jpf dans les embryons XY sexuellement inversés par traitement embryonnaire féminisant (chapitre 5). De plus, une étude partielle de l'activité aromatase dans les têtes de tilapias XX, XY et YY (une famille par génotype, résultats non-présentés) au cours du développement embryonnaire et de la différenciation des gonades (3 à 30 jpf) a montré un pic d'activité entre 4 et 6 jpf, renforçant un peu plus notre hypothèse. Une étude précise et quantitative des profils d'activité et d'expression (des deux formes) de l'aromatase au cours du développement embryonnaire serait nécessaire pour déterminer s'il existe des différences entre individus XX, XY et YY. Le rôle de l'aromatase dans la féminisation induite par l'EE2 des embryons XY pourrait également être vérifié par administration concomitante d'EE2 et d'un inhibiteur de l'aromatase, qui montrerait, si aucune inversion du sexe n'est observée, que l'augmentation d'expression d'aromatase cérébrale observée est impliquée dans le mécanisme d'inversion du sexe.

L'absence d'inversion du sexe des individus YY est un des résultats les plus intéressants de notre étude. Si les œstrogènes exogènes parviennent à supplanter le déterminisme génétique chez les XY, mais pas chez les YY, cela implique deux hypothèses : soit un (ou plusieurs) gène(s) présent(s) sur le chromosome X est (sont) nécessaire(s) à ce stade pour induire la différenciation sexuelle vers un phénotype femelle ; soit un déterminant sexuel mâle présent sur le chromosome Y renforce la voie de différenciation mâle et empêche la voie de différenciation femelle lorsqu'il est présent à l'état homozygote.

La question de savoir si la différenciation sexuelle précoce induite par les œstrogènes passe par une sexualisation du cerveau et/ou par un contrôle de la prolifération de PGCs reste

ouverte. Dans le premier cas, l'augmentation de l'aromatase cérébrale seule ne peut expliquer les résultats et d'autres facteurs génétiques doivent être investigués (notamment les facteurs de transcription *sf1* et *dax1* et les récepteurs stéroïdiens). Dans le second cas, l'augmentation d'expression de l'aromatase pourrait résulter d'une induction directe de son expression par l'EE2 sans conséquence sur la différenciation gonadique. L'étude de cette seconde hypothèse devrait commencer par un suivi précis du nombre de PGCs après le traitement féminisant. Chez le médaka, un traitement d'inversion sexuelle par exposition d'embryons XY à de l'E2 à 1 jpf induit une augmentation du nombre de PGCs qui est, à l'éclosion (9 jpf), semblable à celui des femelles XX (Kobayashi & Iwamatsu, 2005). Cette étude pourrait être encore complétée par les profils d'expression de gènes potentiellement impliqués dans la différenciation sexuelle (*dmrt1*, *amh*, *sf1*, *dax1*, *foxl2*, *cyp19a1a*, récepteurs stéroïdiens) dans les troncs durant les stades embryonnaires et larvaires. L'utilisation complémentaire de la qPCR et de l'hybridation *in situ* fournirait à la fois des données quantitatives et spatiales sur l'expression de ces gènes.

D'un point de vue technique, remarquons que les méthodes d'analyse d'expression de gènes connaissent des évolutions rapides et que l'étude « gène par gène » fait de plus en plus place à des approche génomiques plus puissantes permettant la quantification simultanée de l'expression de nombreux gènes. Par exemple, l'utilisation de microarray (voir Douglas, 2006), dans l'une des rares études recherchant des différences sexuelles aux stades embryonnaires, à mis en évidence chez la truite arc-en-ciel, une différence sexuelle dans l'expression de plus de 800 gènes avant la différenciation des gonades, notamment des gènes impliqués dans la différenciation du sexe comme *sox9*, *dmrt1*, *dax1*, *wt1*, *foxl2* et *cyp19a1a* (Hale et al., 2011). Aujourd'hui, des techniques encore plus performantes de séquençage de nouvelle génération, comme le RNA-seq, permettent une caractérisation quantitative de l'ensemble du transcriptome (voir Qian et al., 2014). Tao et al. (2013) ont appliqué cette technique à l'étude du transcriptome des gonades de tilapias XX et XY pendant et après la différenciation sexuelle des gonades (de 9 à 184 jpf). Sur plus de 21000 gènes analysés, 259 étaient exprimés de manière spécifique dans les gonades XY, et 69 dans les gonades XX ; parmi eux de nombreux gènes sont impliqués dans la stéroïdogenèse, soulignant le rôle des stéroïdes sexuels dans la différenciation sexuelle et le maintien du phénotype sexuel. Sur base de ces différences, ces auteurs ont émis l'hypothèse que l'expression précoce d'un déterminant sexuel majeur dans les gonades XY pourrait déclencher la cascade génétique de

différenciation mâle avant l'émergence d'un schéma d'expression génique femelle. Bien qu'aucune analyse semblable n'ait été menée avant 9 jpf, durant les stades embryonnaires et larvaires chez le tilapia, tous ces résultats récents renforcent l'hypothèse des mécanismes précoces de détermination sexuelle différentiellement exprimés en fonction du génotype sexuel.

L'étude fonctionnelle des gènes impliqués dans le déterminisme et la différenciation du sexe bénéficie quant à elle du développement de nouvelles techniques d'édition du génome (ZFNs[2], TALENs[3], CRISPR/Cas[4]) permettant de réaliser des knockdowns spécifiques par l'action ciblée de nucléases induisant des mutations dans les gènes d'intérêts (voir Gaj et al., 2013). L'utilisation de ces techniques a récemment mis en évidence le rôle antagoniste de *dmrt1* et *foxl2* dans le contrôle de *cyp19a1a* et la production d'œstrogènes durant la différenciation gonadique chez le tilapia (Li et al., 2013), et devrait prochainement permettre de décoder la fonction et les mécanismes de régulation d'autres gènes (Li et al., 2014). L'application combinée d'analyse étendue du transcriptome (RNA-seq) et d'édition génomique (ZNFs) aux stades les plus précoces de la différenciation sexuelle moléculaire a récemment permis la découverte du déterminant génétique majeur du sexe chez la truite arc-en-ciel (Yano et al., 2012)

L'étude fonctionnelle de la régulation des gènes impliqués dans les inversions embryonnaires, et plus largement dans le déterminisme du sexe nécessite également la compréhension des processus épigénétiques contrôlant leur expression. Notre connaissance actuelle du contrôle épigénétique de l'expression des gènes inclut trois types de mécanismes: la méthylation de l'ADN, la modification des histones et un contrôle post-transcriptionnel par des ARNs non-codant comme le microARNs (miARNs) (Piferrer, 2013). Ces mécanismes régulateurs sont fortement impliqués dans le déterminisme et la différenciation du sexe chez les vertébrés. Chez le poulet, Bannister et al. (2011) ont mis en évidence un miARN (miR-202*) associé à la différenciation testiculaire. Lorsque des embryons de poulet sont féminisés par injection d'E2, une diminution de l'expression de miR-202* est observée et corrélée à une diminution de l'expression de *dmrt1 et sox9* ainsi qu'une augmentation de l'expression de *cyp19a* et *foxl2*. La masculinisation des embryons par administration d'un inhibiteur de l'aromatase

[2] Zinc-finger nucleases
[3] Transcription activator-like effector nucleases
[4] Clustered regulatory interspaced short palindromic repeat/Cas-based RNA-guided DNA endonucleases

provoque l'effet inverse. Chez le tilapia, la caractérisation de l'expression de miARNs dans les ovaires et les testicules entre 30 et 165 jpf a révélé près de 240 miARNs exprimés de manière différentielle entre les deux sexes (Xiao et al., 2014). Chez la souris, le niveau de méthylation des histones du locus *Sry* contrôle l'expression du déterminant sexuel majeur et donc le déterminisme du sexe (Kuroki et al., 2013). Chez le bar, la masculinisation induite par des hautes températures, appliquées avant la formation des gonades, implique un contrôle de l'expression du gène de l'aromatase par méthylation de son promoteur (Navarro-Martín et al., 2011). La persistance de cet effet à long terme (dans les gonades différenciées de poissons âgés d'un an) est très intéressante et ce type de contrôle pourrait être impliqué dans les mécanismes d'inversion embryonnaire induite par des stéroïdes chez le tilapia. Finalement, si les mécanismes de contrôle épigénétique jouent un rôle crucial dans le déterminisme du sexe et la différenciation des gonades, chez les mammifères, ils contribuent également à l'établissement et la maintenance de différences sexuelles induites par les stéroïdes sexuels dans le cerveau (McCarthy & Nugent, 2013). Ce type de contrôle pourrait rendre compte des modifications comportementales observées chez les géniteurs sexuellement inversés ou présentant des génotypes sexuels particuliers chez le tilapia.

Les nouvelles possibilités offertes par des techniques d'analyses génétiques et moléculaires toujours plus puissantes et les nouvelles approches fonctionnelles permettant la compréhension des mécanismes de la différenciation sexuelle (épigénétique) doivent être exploitées pour poursuivre l'étude de l'apparition des différences sexuelles dans le cerveau et les gonades et les interactions entre les deux aux stades les plus précoces du développement, ainsi que leurs influences sur la biologie des adultes. Outre les différents avantages que présente le tilapia du Nil en tant que modèle pour l'étude du déterminisme du sexe (déterminisme génétique et environnemental, facilité de production de populations monosexes, génome séquencé), les inversions embryonnaires fournissent un nouvel outil pour étudier les mécanismes génomiques, génétiques et endocriniens les plus précoces de la différenciation sexuelle du cerveau et des gonades.

L'étude du déterminisme du sexe chez le tilapia en particulier, et chez les poissons et les autres vertébrés en général, n'a pas fini de nous apprendre et de nous surprendre.

2. Conclusions

L'étude de l'influence du génotype sexuel (XX, XY et YY) sur certains aspects physiologiques et comportementaux lié à la biologie de la reproduction du tilapia du Nil a montré que : 1) la qualité du sperme des mâles est semblable quelque soit le génotype sexuel et n'affecterait donc pas les capacités de reproduction de ces trois types de mâles ; et 2) le métabolisme stéroïdien (E2, 11KT) ainsi que l'expression des comportements agressifs sont modifiés chez les femelles XY, YY et les mâles XX, ce qui pourrait perturber les comportements reproducteurs de ces poissons.

Nous ne savons pas si les différences observées sont liées à l'expression du génotype sexuel ou à des perturbations engendrées par les traitements hormonaux d'inversion sexuelle administrés durant la période de différenciation des gonades. Nos résultats suggèrent que différents déclencheurs (génétiques, hormonaux) peuvent être à l'origine de différences sexuelles phénotypiques et que celles apparaissant dans le cerveau, conduisant à des modifications comportementales, sont initiées avant ou pendant la différenciation des gonades.

Grâce aux techniques d'inversion du sexe par traitements hormonaux courts appliqués durant la période embryonnaire que nous avons développés, nous avons pu confirmer l'existence d'une période sensible de la différenciation sexuelle durant les 10 premiers jours de vie, avant la formation des gonades. Les processus génétiques et endocriniens déterminant le sexe semblent donc s'établir précocement chez le tilapia. L'augmentation de l'expression de l'aromatase cérébrale dans les embryons XY traités aux œstrogènes pourrait être impliquée dans le mécanisme de différenciation sexuelle induit par ce traitement, mais la question du rôle du cerveau dans l'initiation de la différenciation des gonades reste ouverte.

Il est nécessaire de continuer à investiguer les différences sexuelles apparaissant le plus en amont dans le développement (notamment à la lumière des nouveaux développements scientifiques dans les domaines de la génétique et de l'épigénétique), pour comprendre tant les mécanismes du déterminisme et de la différenciation sexuels, que l'influence que ceux-ci peuvent avoir sur la physiologie et les comportements reproducteurs chez les adultes différenciés.

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