Université du Québec
à Rimouski

Étude transcriptomique de l'effet de la température sur le déterminisme du phénotype sexuel chez la plie rouge

Mémoire présenté

dans le cadre du programme de maîtrise en océanographie

en vue de l'obtention du grade de maître ès sciences

PAR

© NINA FLORE ADÉLIE BELLENGER

Août 2023

Composition du jury :

Réjean Tremblay, président du jury, Université du Québec à Rimouski
Céline Audet, directrice de recherche, Université du Québec à Rimouski
Étienne Audet-Walsh, codirecteur de recherche, Université Laval
Marie Vagner, examinatrice externe, CNRS, UMR LEMAR de Brest, France

Dépôt initial le 10 mars 2023 Dépôt final le 23 août 2023

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REMERCIEMENTS

Céline, je souhaitais vivement te remercier. Merci pour ta disponibilité, ta bienveillance et pour tout le temps que m'as consacré au cours de ma maîtrise. J'ai énormément appris à tes côtés et je ne pourrais pas exprimer à quel point je te suis reconnaissante. Je suis fière d'avoir été ton étudiante et de pouvoir entreprendre un doctorat sous ta direction. Merci, Céline Audet, de m'avoir accompagnée tout au long de ce projet qui s'est avéré parfois difficile mais qui en valait tellement la peine.

Étienne Audet-Walsh, j'ai grandement apprécié de t'avoir comme co-directeur durant ma maîtrise. Merci pour ta patience, ta gentillesse et ta disponibilité, notamment pour répondre à toutes mes questions concernant la stéroïdogenèse et les analyses génomiques associées.

Éric Normandeau et Lucas Germain, merci pour tout le temps que vous m'avez consacré, d'avoir été aussi accueillants lors des stages de bioinformatique et d'avoir été si disponibles par la suite.

Je souhaite aussi remercier Maria Angelica Martinez-Silva d'avoir été aussi présente quand mes expériences de laboratoire ne fonctionnaient pas, tu as su me montrer qu'il n'y a pas de problème mais que des solutions, et qu'il faut rester optimiste en tout temps. Ghizlane Banousse, merci pour toute ton aide au cours de mes analyses mais aussi pour toutes les fois où j'ai pu te partager mes doutes et mes avancées. Vous faites toutes les deux partie des plus belles rencontres que j'ai pu faire depuis mon arrivée au Canada.

Merci beaucoup au CRSNG qui a financé ce projet de recherche. Merci au RAQ qui, grâce à ses subventions, m'a permis d'effectuer deux stages à Québec et aussi de participer au « Larval Fish Conference » à San Diego aux États-Unis. Ce voyage constitue une de mes plus belles expériences à ce jour.

Il a été possible de réaliser cette recherche en partie grâce au soutien fourni par Calcul Canada (https://ccdb.computecanada.ca/security/login) et l'Alliance (alliancecan.ca/fr/). Je voudrais aussi remercier la plateforme de séquençage SANGER du CHUL de Québec.

Merci aussi à toutes les personnes que je ne peux pas citer individuellement mais que j'ai rencontré depuis que je suis arrivée ici ou que j'ai laissé en France mais qui ont été très présentes dans cette aventure et qui, je l'espère, le seront aussi pendant la prochaine.

Pour finir, je souhaiterais remercier ma famille et notamment mes parents, pour m'avoir permis de partir au Canada vivre cette expérience et pour leur soutien indéfectible. Je ne pourrais jamais exprimer tout ce que je ressens alors simplement, merci pour tout.

RÉSUMÉ

La plie rouge (*Pseudopleuronectes americanus*) est un poisson plat de l'Atlantique Nord que l'on retrouve essentiellement sur la côte est du Canada et des États-Unis. C'est une espèce dont la détermination du phénotype sexuel semble être influencée par la température, on parle de TSD (« Temperature-dependent Sex Determination »). À cause du réchauffement climatique, il est important de déterminer si ce phénomène est présent ou non chez la plie rouge et à en comprendre les mécanismes sous-jacents. Nous avons émis l'hypothèse selon laquelle la température aurait un impact majeur au cours du développement larvaire et que le stade thermosensible se situerait juste avant la métamorphose en juvénile. De plus, nous avons supposé que la voie de la stéroïdogenèse, qui permet la synthèse des hormones sexuelles (testostérone, 11-cétotestostérone et estrogènes chez les poissons), était impliquée dans ce phénomène. Nous avons donc constitué trois cohortes à partir de pontes effectuées à trois reprises au cours du printemps. Les conditions de température et de salinité correspondaient aux conditions saisonnières du Saint-Laurent et chaque cohorte a ainsi été exposée à un régime de température différent durant tout le développement des œufs puis des larves. Une fois la métamorphose effectuée, tous les juvéniles ont été exposés à une température fixe de 10,7°C. Des larves ont été échantillonnées à 20, 30 et 40 jours post-éclosion et des juvéniles un mois après la métamorphose. L'ARN de ces individus a été extrait, séquencé et analysé afin d'obtenir les niveaux d'expression de l'ensemble des gènes impliqués dans la stéroïdogenèse. L'analyse transcriptomique a permis de bien séparer larves et juvéniles. Les patrons d'expression des gènes ont montré que la voie de la stéroïdogenèse semblait bel et bien être influencée par la température, mais nous n'avons pu mettre en évidence un déséquilibre du sexe-ratio ni une favorisation du phénotype mâle. Cependant, les juvéniles issus des larves élevées au régime de température le plus élevé montrent des différences marquées au niveau de l'expression des gènes de la stéroïdogenèse sans que nous puissions toutefois en déterminer les conséquences. Des analyses complémentaires sur le transcriptome pourraient donc permettre de mieux comprendre ces résultats.

Mots clés : plie rouge, température, phénotype sexuel, TSD, stéroïdogenèse, transcriptome, développement larvaire, juvéniles.

ABSTRACT

Winter flounder (*Pseudopleuronectes americanus*) is a North Atlantic flatfish found on the east coast of Canada and of the United States. It is a species in which the determination of the sexual phenotype seems to be influenced by temperature which is called the TSD phenomenon (Temperature-dependent Sex Determination). Considering the actual global warming, it is important to determine whether or not this phenomenon is present in winter flounder and to determine the underlying mechanisms. We hypothesized that temperature would have a major impact during larval development and that the temperature-sensitive stage would be just before metamorphosis into juveniles. Moreover, we assumed that the steroidogenesis pathway, which allows the synthesis of sex hormones (testosterone, 11-ketotosterone and estrogens in fishes), would be involved in this phenomenon. So, we surveyed three cohorts issued from three different spawns during spring time. Temperature and salinity conditions followed seasonal conditions in the St. Lawrence Estuary and each cohort was thus exposed to a different temperature regime during egg and larval development. Once metamorphosis occurred, all juveniles were exposed to a stable temperature of 10.7 °C. Larvae were sampled at 20, 30, and 40 days post-hatching and juveniles one month after metamorphosis. RNA was extracted from these individuals, sequenced, and analyzed to obtain expression levels of all genes involved in steroidogenesis. Transcriptome analysis allowed clear differentiation between larvae and juveniles. Gene expression patterns showed that the steroidogenesis pathway was influenced by temperature but we were not able to demonstrate a sex-ratio imbalance or a favoring of male phenotype. However, juveniles from larvae reared at the highest temperature regime showed strong differences in the expression of steroidogenesis-related genes, but we were not able to determine the outcome. Complementary analyses of the transcriptome could provide a better understanding of these results.

Key words: winter flounder, temperature, sexual phenotype, TSD, steroidogenesis, transcriptome, larval development, juveniles.

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ADNc	Acide Désoxyribonucléique Complémentaire
ARN / RNA	Acide Ribonucléique / Ribonucleic Acid
DNAse	Deoxyribonuclease
Dpf	Days Post Fertilization
GOid	Gene Ontology Identification
MDS	Multi-Dimensional Scaling
MPO	Pêches et Océans Canada
NOAA	National Oceanic and Atmospheric Administration
PGCs	Primordial Germ Cells
TSD	Temperature-dependent Sex Determination

INTRODUCTION GÉNÉRALE

1.1 Réchauffement climatique et conditions environnementales

Les océans constituent environ 70 % de la planète Terre. Encore largement inexplorés, ils se caractérisent notamment par de vastes écosystèmes côtiers et marins qui sont variés et riches en biodiversité. L'ensemble des espèces qui s'y trouvent coexistent dans un équilibre fragile et forment des écosystèmes complexes. De nombreux facteurs environnementaux caractérisent ces écosystèmes et assurent le bon développement de ces espèces. Malheureusement, l'équilibre peut être fragile et mettre en péril leur bon développement. L'un de ces facteurs, la température, peut avoir un impact conséquent sur le phénotype des poissons. Au cours des dernières années, le réchauffement climatique a eu un effet certain sur les océans. Pour les organismes côtiers qui sont majoritairement ectothermes, la température peut exercer une influence non seulement sur leur environnement, mais aussi sur leur métabolisme, leur développement et leur reproduction. Chez certaines espèces de poissons, ces modifications thermiques risquent même de provoquer des variations phénotypiques majeures (flétan Atlantique — *Hippoglossus hippoglossus*, Jonassen et al. 1999; e.g. Servili et al. 2020).

1.2 Plasticité phénotypique

Le phénotype correspond au résultat de l'expression des gènes. Il s'agit de toutes les caractéristiques morphologiques et physiques telles que la taille, la pigmentation ou encore, le sexe. L'environnement peut affecter l'expression de certains gènes et donc, le phénotype. On parle alors de plasticité phénotypique. Il s'agit de la propriété d'un génotype donné à produire des phénotypes différents en réponse à des conditions environnementales distinctes (Pigliucci 2001). Par exemple, on retrouve chez le poisson cichlidé (*Astatoreochromis alluaudi*) une plasticité de la mâchoire pharyngée dépendante de la dureté de la nourriture

présente dans l'environnement (Pfennig et al. 2014). De même, l'épinoche à trois épines (*Gasterosteus aculeatus*) est une espèce qui se déplace entre des zones d'eau douce et des zones salées et on peut observer des adaptations phénotypiques au niveau de la forme de son corps en fonction de la salinité de son lieu de croissance (Mazzarella et al. 2015). La plasticité phénotypique peut aussi affecter des caractères qui auront des effets secondaires très larges, comme la détermination du sexe indépendamment du sexe génétique.

Chez les poissons, la détermination génotypique du sexe peut prendre diverses formes. Ainsi, chez les poissons plats, les espèces présentent un patron XX-XY et le phénotype sexuel est principalement déterminé par le génotype, bien que l'environnement puisse modifier le sexe phénotypique (Luckenbach et al. 2009). Chez la sole tropicale (*Cynoglossus semilaevis*), ce sont les femelles qui ont des chromosomes sexuels hétérologues, on parle alors de chromosomes ZZ chez les mâles et de chromosomes ZW chez les femelles. Cependant, il a été constaté chez cette même espèce que les facteurs externes tels que la température peuvent modifier la détermination du sexe phénotypique. Les femelles se transforment alors en « pseudo-mâles » et présentent toutes les caractéristiques phénotypiques mâles tout en conservant leurs chromosomes ZW (e.g. Chen et al. 2014). La plasticité phénotypique peut donc jouer un rôle non négligeable sur le déterminisme sexuel et impacter la reproduction et la survie de ces poissons.

1.3 « Temperature-dependent Sex Determination »

Les effets de la température sur le phénotype sexuel ont été observés chez de nombreuses espèces de poissons. Ce phénomène est appelé « Temperature-dependent Sex Determination » ou TSD. Chez le poisson médaka (*Oryzias latipes*), la présence d'un changement de phénotype femelle vers un phénotype mâle a été observé. Lorsque des œufs étaient exposés à une température élevée de 32°C pendant l'incubation puis à 27°C après l'éclosion, 24 % des femelles XX (N = 105) se sont transformées en mâles pour un des groupes et 50 % (N = 38) pour le second groupe. Les mâles ainsi obtenus possédaient des

testicules bien développés et étaient fertiles (Sato et al. 2005). Dans une autre étude menée chez le bar européen (*Dicentrarchus labrax*), deux groupes d'individus ont été exposés à deux conditions de température différentes après la fécondation des œufs. Le premier groupe servait de contrôle et a été exposé à 15°C pendant 60 jours post-fécondation (dpf). Le second groupe a été exposé pendant 10 jours après la fécondation à 15°C avant d'être soumis à une température de 21°C jusqu'à la fin de la croissance (Navarro-Martin et al. 2011). Le groupe soumis au régime de température le plus élevé a montré une favorisation du phénotype mâle. Il semblerait que ce déséquilibre du sexe-ratio soit dû à une inactivation de l'aromatase, une enzyme majeure impliquée dans la synthèse des hormones sexuelles et plus particulièrement dans la transformation de la testostérone en estradiol (Navarro-Martin et al. 2011).

Le phénomène de TSD a aussi été fréquemment observé ou suspecté chez des poissons plats et deux types de modifications ont été répertoriées (e.g. Luckenbach et al. 2009). Le premier, qui est le plus répandu, consiste en une augmentation du nombre de mâles lors d'un réchauffement des eaux Dans le deuxième cas, une température plus élevée provoque à l'inverse une différenciation en femelle. Enfin, le troisième type de modification induit une différenciation en faveur du phénotype mâle soit quand la température est trop basse soit quand elle est trop élevée, mais on conserve un sexe-ratio de 1 : 1 à température intermédiaire. Ces deux derniers cas ont été répertoriés chez très peu d'espèces. Cependant, on retrouve ce phénomène particulièrement chez des poissons plats et semble n'affecter que les femelles. On aurait généralement un génotype femelle qui donnerait un phénotype mâle (cardeau de Floride — *Paralichtys lethostigma*, Luckenbach et al. 2004; plie japonaise — *Paralichthys olivaceus*, Kitano et al. 1999). Cependant, ces poissons présentent des changements phénotypiques uniquement lors d'expériences en milieu contrôlé et il est difficile de savoir si les conditions de températures en milieu naturel peuvent provoquer des changements d'expression du sexe. Ainsi chez la plie japonaise, Yamamoto (1999) a montré que des juvéniles (longueur comprise entre 27 et 37 mm) exposés à une température inférieure à 15°C ou supérieure à 25,0 et 27,5°C présentaient un déséquilibre du sexe-ratio en faveur du phénotype mâle. Cependant, les juvéniles exposés à une température moyenne

comprise entre 17.5 et 22,5 °C présentaient très peu de différenciation en mâles (Yamamoto 1999). Bien que le phénomène de TSD soit de plus en plus observé, les mécanismes de régulation sous-jacents sont encore largement inconnus.

1.4 La voie de la stéroïdogenèse impliquée dans le TSD ?

Une des voies affectée par la température et produisant le TSD pourrait être la voie de régulation des stéroïdes sexuels, considérée comme étant très conservée chez les vertébrés (Ankley & Grey 2013). Chez ces derniers, les hormones sexuelles aussi appelées stéroïdes sexuels sont synthétisées à partir du cholestérol (Fig. 1). Apporté par l'alimentation, celui-ci est d'abord transformé en prégnénolone grâce à une enzyme de clivage. S'en suivent de nombreuses étapes enzymatiques jusqu'à l'obtention de la testostérone, hormone sexuelle importante à la fois chez les mâles et les femelles chez les poissons (Maye et al. 1990). La testostérone est ensuite transformée en estradiol chez les femelles grâce à l'aromatase (e.g. Piferrer & Blázquez 2005) et en 11-cétostérone chez les mâles (Soranganba & Singh 2019). Il a déjà été démontré chez les mammifères, et notamment l'humain, que les hormones stéroïdiennes et leurs récepteurs sont impliqués dans de nombreux processus physiologiques, métaboliques et pathologiques. L'activité de l'aromatase influence très probablement le TSD, puisque celle-ci joue un rôle prépondérant dans le déterminisme sexuel (e.g. Servili et al. 2020; plie japonaise, Kitano et al. 1999).

1.5 Les marqueurs sexuels précoces

Lors de l'étude du déterminisme sexuel chez les poissons, différents marqueurs sexuels précoces permettant de déterminer génétiquement le phénotype sexuel ont été définis. L'aromatase (*cyp19a1a*) est un de ces marqueurs sexuels clés (e.g. Uno et al. 2012; e.g. Piferrer & Blázquez 2005; plie japonaise, Kitano et al. 1999).

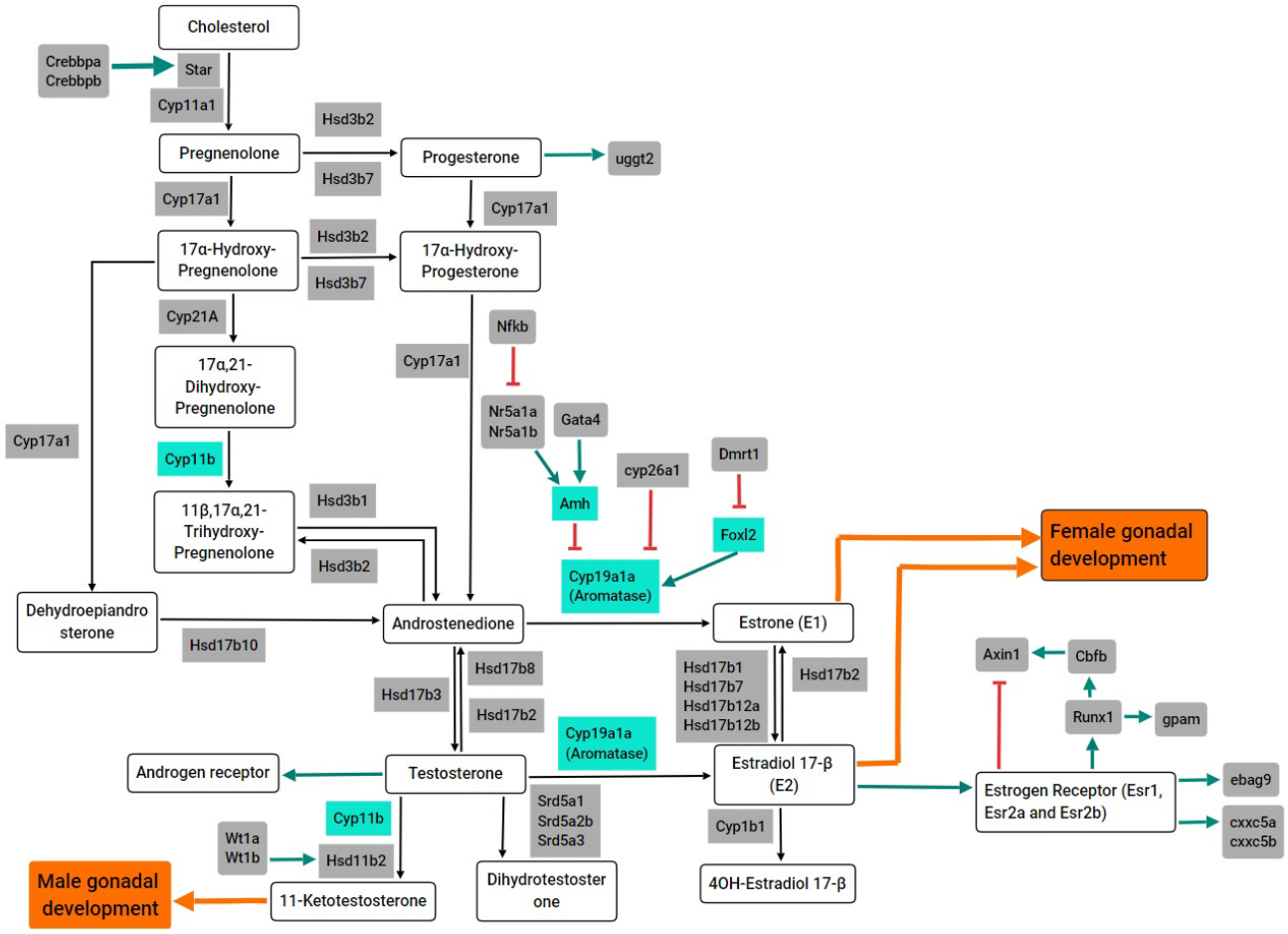


Fig. 1. Représentation de la voie de stéroïdogenèse : étapes de transformations depuis le cholestérol jusqu'à la testostérone et les estrogènes. Les marqueurs précoces sexuels déjà connus sont colorés en vert. Les activations de la voie de la stéroïdogenèse sont représentées par des flèches vertes tandis que les inhibitions sont représentées par des flèches rouges.

Un autre marqueur est le gène « dead end 1 » (*dnd1*) qui permet la migration des cellules germinales primordiales (PGCs) notamment dans les gonades chez les vertébrés. Son rôle majeur dans le développement des gonades femelles a notamment été mis en évidence chez le poisson zèbre (*Danio rerio*, Weidinger et al. 2003) et chez la plie du Pacifique (*Platichthys stellatus*, Yoon et al. 2021). Il a aussi été démontré que les individus devenaient des mâles stériles dans le cas où le gène était inactivé. Le gène « Forkhead Box Protein L2 » (*foxl2*) favorise lui aussi le développement ovarien et son rôle a été largement prouvé chez les mammifères dont l'être humain (Ottolenghi et al. 2005) mais aussi chez certains poissons comme le poisson zèbre (Siegfried et al. 2008). Ce gène intervient de manière précoce et permet l'activation de l'aromatase amenant le développement d'un phénotype femelle (plie japonaise, Yamaguchi et al. 2007). Il existe aussi des marqueurs précoces mâles tels que l'hormone anti-müllérienne (*amh*) qui empêche la formation des ovaires chez l'être humain (e.g. Van Houten et al. 2010). Les poissons comme le poisson zèbre ou encore l'anguille japonaise (*Anguilla japonica*) possèdent un orthologue de l'*amh* qui bloque notamment l'aromatase et s'exprime de manière particulièrement précoce (poisson zèbre, Rodríguez-Marí et al. 2005; e.g. Pfennig et al. 2015; anguille japonaise, Miura et al. 2002). Un autre marqueur connu est la « steroid 11-beta-mono-oxygenase » (*cyp11b*) qui code pour une enzyme clé dans la voie de la stéroïdogenèse et permet la catalyse d'une des dernières étapes permettant la production de testostérone (e.g. Schiffer et al. 2015; e.g. Uno et al. 2012). Son rôle majeur a été mis en évidence chez de nombreuses espèces de poissons tels que la truite arc-en-ciel (*Oncorhynchus mykiss*), le bar commun et l'anguille japonaise (e.g. Schiffer et al. 2015), mais aussi des poissons plats comme la plie japonaise (Meng et al. 2020). Ainsi, les gènes *cyp19a1a*, *dnd1* et *foxl2a* ont été identifiés comme des marqueurs du phénotype femelle et *amh* et *cyp11b* comme des marqueurs du phénotype mâle.

1.6 La plie rouge comme modèle d'étude

La plie rouge (*Pseudopleuronectes americanus*) est une espèce de poisson plat des côtes Atlantique Nord-Ouest que l'on retrouve du sud du Labrador jusqu'à la baie de

Chesapeake (MPO, 2017). C'est la seule espèce de poisson plat qui habite à l'année les zones côtières de l'estuaire du Saint-Laurent grâce aux propriétés antigel de son sang lui permettant de résister aux conditions thermiques hivernales (Sicheri & Yang 1995; Cheng & Merz Jr. 1997). C'est une espèce qui soutient à la fois la pêche commerciale et les pêches récréatives tant au Canada qu'aux États-Unis (MPO, 2017; NOAA, 2022). Malheureusement, une diminution des stocks de plie rouge au cours des dernières années a été observée. Ainsi, le nombre de plies faisant au moins 25 cm est passé de 85 % entre 1971 et 1975 à 30 % entre 2011 et 2016 (MPO, 2017). Les raisons de ces diminutions ne sont pas encore réellement identifiées, mais il semblerait que la pêche ne soit pas suffisante pour l'expliquer. Il y a donc des causes sous-jacentes et le phénomène de TSD pourrait en être une (Fairchild et al. 2010).

Le cycle de vie de la plie rouge se caractérise par plusieurs stades de développement. À l'éclosion, les larves sont pélagiques, mesurent environ 3 mm et présentent une symétrie bilatérale avec, notamment les yeux de part et d'autre de la tête. Entre 30 et 50 jours post éclosion, selon les conditions thermiques, la larve de plie rouge subit une métamorphose pour devenir un juvénile. Cette métamorphose se caractérise par des modifications anatomiques, physiologiques et comportementales et implique notamment une migration des yeux du même côté de la tête et la perte de symétrie bilatérale (Chambers & Leggett 1987; Chambers et al. 1988). Les gonades, quant à elles, se différencient plus tardivement chez des individus faisant au moins 41 mm de long dans des conditions de températures saisonnières variant entre 7 °C et 15 °C (Fairchild et al. 2007). La plie rouge est une espèce chez qui le TSD n'a jamais été mis en évidence ni chez l'adulte ni à de plus jeunes stades de développement, mais sa présence est largement suspectée (Fairchild et al. 2007).

Bien que les connaissances sur la régulation endocrinienne des facteurs de développement chez la plupart des espèces de poissons soient très limitées, je compte m'appuyer sur le fait que la voie métabolique de la stéroïdogenèse est très bien conservée chez les vertébrés (Ankley and Gray 2013), pour tenter de comprendre le phénomène du TSD. Pour cela, j'ai pris comme modèle d'étude la plie rouge, un poisson plat chez qui la

présence de TSD a déjà été suspectée. En effet, en 2003, une étude de la différenciation gonadique chez des populations de plies rouges a été réalisée. Il a alors été observé un déséquilibre du sexe-ratio en faveur des mâles (67 femelles contre 137 mâles). Cette différence de ratio mâle/femelle n'a pas été observée les années suivantes et les résultats suggèrent que ce déséquilibre était dû soit un facteur d'ordre génétique, soit d'ordre environnemental (Fairchild et al. 2007). Si la température océanique venait à augmenter de manière trop significative, on peut s'attendre à des modifications importantes du ratio mâle/femelle chez certaines espèces. Cela a déjà été observé sur la côte est Américaine chez le cardeau de Floride. Des juvéniles ont été capturés dans trois régions différentes : « Pamlico River » (la plus au nord), « Neuse River » (à une longitude intermédiaire) et « South of the New River » (la région la plus au sud). Les juvéniles provenant de « Neuse River » et de la « South of the New River » ont présenté un pourcentage élevé (environ 80 %) de mâles pendant quatre années consécutives (Honeycutt et al. 2019). Dans des expériences complémentaires menées en laboratoire, trois groupes de juvéniles ont été exposés respectivement à des régimes de température autour de 19 °C, 23 °C et 27 °C. Les juvéniles à 19 °C avaient un sexe-ratio contenant 83 % de mâles. Pour ceux à 23 °C, le ratio était de 65 % et enfin, ceux exposés à un régime de température de 27 °C ont donné 100 % de mâles. Le TSD pourrait donc avoir un effet considérable tant pour l'exploitation des ressources aquatiques que pour le maintien des populations de plie rouge (Fairchild et al. 2007).

1.7 Objectifs et hypothèses

D'après ces résultats et la littérature disponible nous avons émis l'hypothèse selon laquelle la température est le facteur qui détermine le sexe phénotypique en favorisant l'expression « mâle ». Dans le cadre de ma maîtrise, j'ai cherché à déterminer le stade de développement pendant lequel la température avec le plus d'impact ainsi que la voie biologique impliquée. J'ai ainsi émis deux sous-hypothèses. La première est que la température prévalente en période d'élevage larvaire a bel et bien un effet sur le développement du sexe phénotypique de la plie rouge et que la période thermosensible se

situe juste avant la période de métamorphose. Ma seconde hypothèse est que le TSD induirait une modification de la régulation de l'activité du génome par les hormones sexuelles et la voie de la stéroïdogenèse.

Pour répondre à ces hypothèses, j'ai réalisé des extractions d'ARN sur des larves et des juvéniles de plie rouge provenant de cohortes exposées à différentes conditions de température. J'ai ensuite utilisé des outils bio-informatiques me permettant d'observer et d'analyser le transcriptome et les niveaux d'expressions de gènes clés de la stéroïdogenèse en fonction de différents régimes de températures.

CHAPITRE 1. STUDY OF THE TRANSCRIPTOME TO UNDERSTAND THE TEMPERATURE EFFECT ON SEXUAL DETERMINISM IN WINTER FLOUNDER (*PSEUDOPLEURONECTES AMERICANUS*)

2.1 INTRODUCTION

Recently, global warming had a huge effect on the oceans. For coastal organisms that are mostly ectotherms, temperature can influence not only their environment but also their metabolism, development, and reproduction. In some fish species, these thermal changes may even cause major phenotypic variations (Atlantic halibut, Jonassen et al. 1999; e.g. Servili et al. 2020). The phenotype corresponds to the result of gene expression but the environment can affect the expression of certain genes and a given genotype can thus produce different phenotypes, it is the phenotypic plasticity (Pigliucci 2001). This plasticity can also affect characteristics that will have side effects, such as sex determination regardless of genetic sex.

Temperature-induced phenotypic sex change is called Temperature-dependent Sex Determination (TSD). This phenomenon has been frequently observed or suspected in many fish species, especially flatfish. The mechanisms underlying the TSD phenomenon are still largely unexplained. However, it is already known that the TSD can cause three different types of changes. The first one is the most common and consists of an increase in the number of males during a warming of the waters. In the second case, a higher temperature causes differentiation in female phenotype. Finally, the third type of modification induces male differentiation when the temperature is too low or too high, with a sex-ratio of 1:1 preserved at intermediate temperature. Those two last types of modification are very rare. In general, the TSD phenomenon seems to be particularly present in flatfish species and seems to affect only females; female genotype expressing a male phenotype (southern flounder, Luckenbach et al. 2004; Japanese flounder, Kitano et al. 1999). However, such observations were mainly the results of controlled experiments and it is difficult to know occurrences in the wild. In the Japanese flounder, Yamamoto (1999) showed that juveniles of total length ranging from

27 mm to 37 mm exposed to temperatures below 15 °C or above 25.0 °C and 27.5 °C exhibited a sex-ratio imbalance in favor of the male phenotype (25 % of females at 15 °C, 50 % at 20 °C and 10 % at 17.5 °C). However, juveniles exposed to an average temperature between 17.5 °C and 22.5 °C showed a ratio around 1:1 (Yamamoto 1999).

In other fish species, like medaka, changes from female to male phenotype was observed when groups of eggs were exposed to high temperatures (32 °C during incubation, or 27 °C post-hatching. A transformation of 24% of the XX females (N = 105) in the first group and 50% (N = 38) in the second group were observed. The resulting males had well-developed testis and were fertile (Sato et al. 2005). In another study on the European seabass (*Dicentrarchus labrax*), two groups of individuals were exposed to two different temperature conditions after fertilization of eggs. The first group served as a control reference and was exposed to 15 °C for 60 days post-fertilization. The second group was exposed for 10 days after fertilization at 15 °C before being submitted to a temperature of 21 °C for 50 days (Navarro-Martin et al. 2011). The group exposed to the highest temperature regime also showed a favoring of the male phenotype. It seems that this sex-ratio imbalance was due to the inactivation of aromatase, a major enzyme involved in the synthesis of sex hormones and more particularly in the transformation of testosterone in estradiol (Navarro-Martin et al. 2011).

The study of sexual determinism in fish identified early sex markers which define the sexual phenotype. The aromatase (coded by the gene *cyp19a1a*) is one of these key sexual markers and is expressed during gonadal differentiation (e.g. Uno et al. 2012; e.g. Piferrer & Blázquez 2005; Japanese flounder, Kitano et al. 1999). Another marker is the "dead end 1" (*dnd1*) gene which allows migration of primordial germ cells (PGCs) especially in the vertebrates gonads. Its role in the development of female gonads has been shown in zebrafish during the embryo development and *dnd* expression is already visible during the one-cell stage embryo (zebrafish, Weidinger et al. 2003; starry flounder, Yoon et al. 2021). The gene “Forkhead Box Protein L2” (*foxl2*) also promotes ovarian development, which was widely demonstrated in mammals, but also in some fish such as zebrafish during gonadal sex

differentiation (around 25 dpf). (Ottolenghi et al. 2005; Siegfried & Nüsslein-Volhard 2008). This gene intervenes before the sex differentiation period (around 50 days post hatching in Japanese flounder) and allows the activation of aromatase leading to the development of a female phenotype (Japanese flounder, Yamaguchi et al. 2007). There are also early male markers such as the anti-müllérian hormone (*amh*) that inhibits ovary formation in human (e.g. Van Houten et al. 2010). Fish such as zebrafish or Japanese eel have an *amh* ortholog that inhibits the aromatase and expresses itself particularly early (around 17 dpf larvae) while the gonads are still undifferentiated (zebrafish, Rodríguez-Marí et al. 2005; e.g. Pfennig et al. 2015; Japanese eel, Miura et al. 2002). Another known marker is the "steroid 11-beta-mono-oxygenase" (*cyp11b*), which codes for a key enzyme in the steroidogenesis pathway and allows the catalysis of one of the last steps in testosterone production (e.g. Schiffer et al. 2015; e.g. Uno et al. 2012). Its major role in testis development has been demonstrated in many fish species such as rainbow trout, the European sea bass and the Japanese eel (e.g. Schiffer et al. 2015), but also flatfish such as Japanese flounder (Meng et al. 2020).

Our study model is the winter flounder in which the presence of TSD has already been strongly suspected. At hatching, winter larvae are pelagic, about 3 mm long and have bilateral symmetry, with the eyes on both sides of the head. Between 30 and 50 days after hatching, depending on the thermal conditions, the winter flounder larva undergoes a metamorphosis which is characterized by anatomical, physiological, and behavioral changes, including eye migration on the same side of the head and loss of bilateral symmetry (Chambers & Leggett 1987; Chambers et al. 1988). In a 2003 study, size at gonadal differentiation was determined and a sex-ratio imbalance in favor of males was observed especially among fishes with a size between 40 mm and 60 mm (67 females vs. 137 males) (Fairchild et al. 2007). If the ocean temperature increases too significantly, significant changes in the male/female ratio can be expected in some species. This has already been observed on the east coast of the United States in southern flounder (*Paralichthys lethostigma*). Juveniles were captured in three different regions: the Pamlico River (most northern), the Neuse River (intermediate longitude) and south of the New River (the most southern region). Juveniles coming from both the Neuse River and the South of the New River showed a high percentage (around

80%) of males during four consecutive years (Honeycutt et al. 2019). In complementary experiments conducted in laboratory, three groups of juveniles were exposed respectively to temperature regimes around 19 °C, 23 °C, and 27 °. The juveniles at 19 °C had a sex ratio of 83% of males. For those à 23 °C, it was a ratio of 65%. Finally, those exposed to a temperature regime of 27 °C gave 100% of males. As a result, TSD could have a significant effect on both the exploitation of aquatic resources and the maintenance of winter flounder populations (Fairchild et al. 2007).

Even though the mechanisms underlying the TSD phenomenon are not quite understood, it seems very likely that they should result from disturbances of the regulatory pathway of sex steroids. This pathway allows the transformation of cholesterol in testosterone (important in both sexes in fishes, Maye et al. 1990), 11-ketotestosterone (males, Soranganba & Singh 2019) and estrogens (females) (Fig. 2). It is highly preserved among vertebrates and have a key role in sexual development in many species (Ankley & Grey 2013). In females, the aromatase is an enzyme that allows to transform testosterone in estradiol. The temperature could affect the genes expression notably by a change in aromatase activity (e.g. Servili et al. 2020; Japanese flounder, Kitano et al. 1999).

In this study, the effect of temperature was studied using RNA-seq analysis on the whole transcriptome of the winter flounder to determine the temperature sensitive stage of life and to prove the involvement of the steroidogenesis pathway via an analysis of the transcriptome. Because juvenile cohorts obtained in our facilities showed constant bias to male phenotype with only one factor being kept stable over experiments (larval rearing at constant 10 °C, Fraboulet et al. 2010; Bélanger et al. 2018; Vagner et al. 2013, 2014) we hypothesized that the thermosensitive period should occur just before metamorphosis. Using different sets of temperature regimes along with transcriptomic study, we assumed that the steroidogenesis pathway would express differences among larval cohorts that may inform us about the TSD phenomenon. Our transcriptomic approach is a real innovation through the analysis over developmental stages of a flatfish species including the study of the whole steroidogenesis pathway which was never looked at in larval and early juvenile fishes.

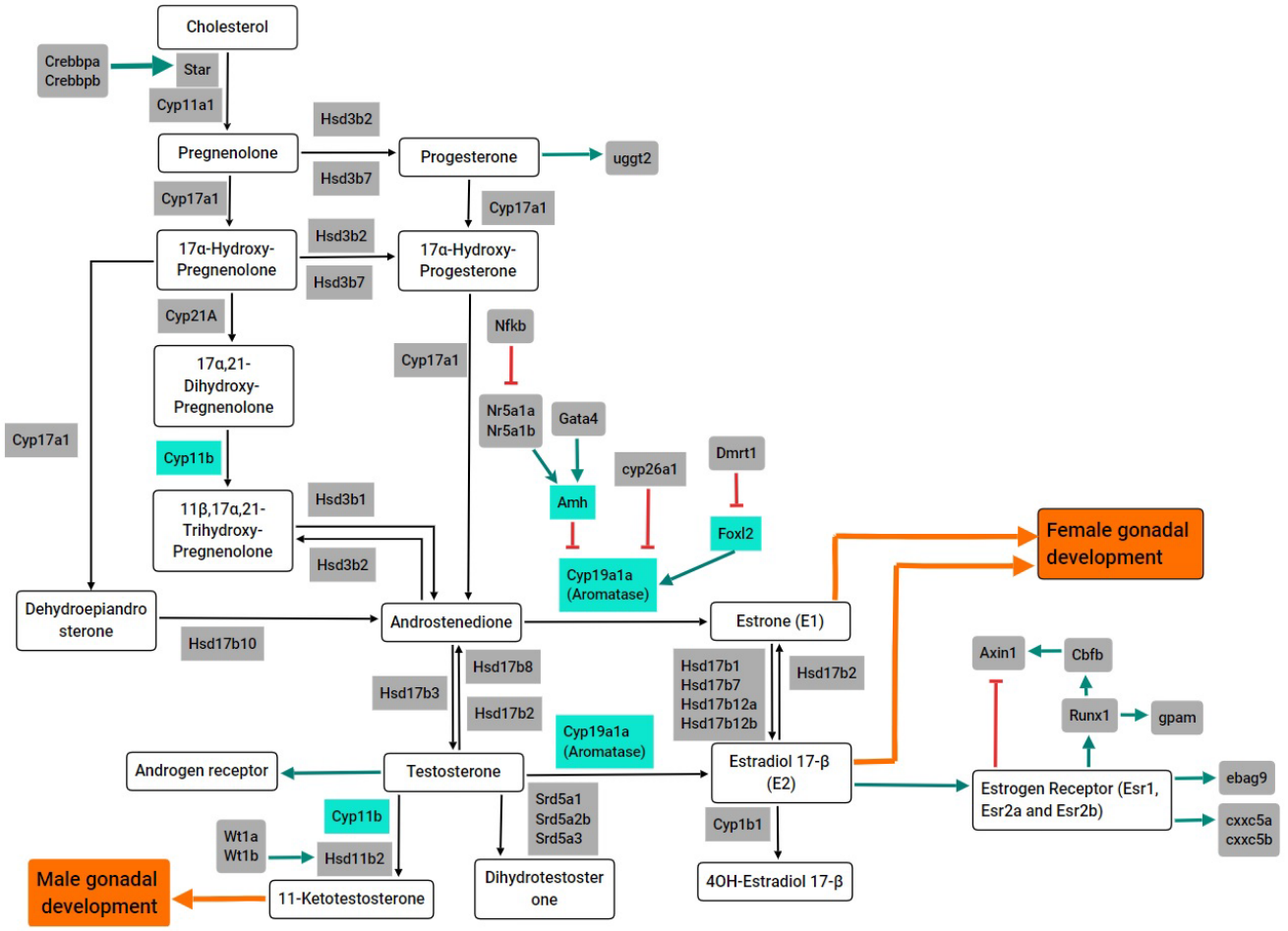


Fig. 2. Representation of the steroidogenesis pathway: transformation steps from cholesterol to testosterone and estrogen. Known early sex markers are in green. The activations of the steroidogenesis pathway are represented by green arrows while inhibitions are represented by red arrows.

2.2 MATERIAL & METHODS

2.2.1 Rearing Experiments

All the rearing experiments were conducted at the Station aquicole of ISMER/UQAR Rimouski (Qué.), Canada; 48 °27=N, 68 °32=W) under stable temperature conditions (10 °C) during the larval development. During almost 15 years, those rearing gave cohorts composed by over 90% of males (Audet, comm. pers.). Thus, four cohorts were surveyed from egg incubation to the juvenile stage under various temperature conditions during the larval stages. Twenty-six females were caught with a trap net in the Baie-des-Chaleurs (Gulf of St. Lawrence) at the end of April 2017. Breeders were driven to our wet lab facilities in the next 12 hours following the capture. The first cohort was obtained on April 27, 2017, from three spawns, and the eggs of three females fertilized with sperm of three males were used for each spawn. The second cohort was obtained on May 3, from 6 spawns, each one combining the eggs of 2 to 4 females fertilized with sperm from 2 to 4 males (2 × 3♀ x 3♂; 1 × 3♀ x 2♂; 1 × 2♀ x 4♂; 1x 4♀ x 4♂). The third cohort was obtained on May 17, 2017, from a spawn using 2 females and 2 males. Fertilization was made according to Ben Khemis et al. (2000). After sperm was added, diatomaceous earth was used to remove adhesiveness of the eggs for 10 min. After iodine treatment for 10 min, each spawn was transferred into two incubators. An upwelling water flow arrived at the bottom of each incubator and the incubators were grouped 3 × 3 inside 250 L water tanks to maintain stable temperature conditions. Salt water was pumped from the St. Lawrence Estuary and temperature and salinity conditions followed natural seasonal variations at this latitude.

Following spring conditions, the temperature regime of the first cohort was colder than the temperature regime of the last one. The last spawn had the highest global temperature exposure (Table 1).

Table 1. Mean egg incubation temperature, number of degree-days (dd), and length (mean ± s.d.) at hatch for the three cohorts.

	(°C)	Fertilization date	Dd	Length (mm)
C1	3.6	26/04	66	2.94 ± 0.26
C2	4.5	03/05	82	3.00 ± 0.21
C3	6.1	17/05	65.5	2.71 ± 0.23

Once hatched, the larvae were transferred in 55 L cylindro-conical tanks with a density of 250 larvae.L-1. An upwelling current was permanently created using an aeration system and the photoperiod was set at 12L:12D (Vagner et al. 2013; Vagner et al. 2014). Larvae were exposed to a flow of filtered ambient sea water under natural temperature and salinity conditions (Table 2) with addition of Nannochloropsis microalgae (final concentration: 0.7 × 106 cells · mL−1) to provide the pseudo-green water conditions required for the larvae. Larvae were fed with enriched rotifers *Brachionus plicatilis* (5 individuals.mL−1), concentrations being adjusted three times during the day (light opening, beginning and end of the afternoon).

Table 2. Degree-days (dd), and larval length (mean ± s.d.) at each day of sampling for each of the three cohorts.

	Day of sampling	Dd	Length (mm)
C1	10	63.0	3.20 ± 0.36
	20	135.0	3.89 ± 0.66
	30	216.5	5.23 ± 0.42
	40	311.5	5.94 ± -.65
C2	10	69.0	3.40 ± 0.62
	20	143.0	3.67 ± 0.29
	30	233.0	5.20 ± 0.65
	40	325.0	6.32 ± 0.43
C3	10	75.0	3.03 ± 0.46
	20	166.0	4.10 ± 0.50
	30	257.5	5.05 ± 0.50
	40	354.5	5.82 ± 0.53

Rotifers were enriched using three microalgae (*Nannochloropsis oculata, Isochrysis galbana, and Pavlova lutheri*), containing a balanced combination of EPA, DHA, and AA (EPA/DHA/AA = 3.8/2.9/1) (Vagner et al. 2014).

After metamorphosis, the settled juveniles from each cohort were collected by gently siphoning twice a day the bottom of the larval tanks and were transferred separately in rectangular tanks (35,5 cm × 65 cm × 6.5 cm) with a density of 400 juveniles per tank with 250 mL of sand on the bottom. (Bélanger et al. 2018). They were reared under natural temperature, salinity, and photoperiod conditions. The juveniles were fed with a mix of enriched rotifers twice a day (Bélanger et al. 2018). The quantity of rotifers depends on the concentration in the tanks. In addition, industrial food was given five times a day.

2.2.2 Samplings

Larvae were sampled at hatch, and on days 10, 20, 30, 40, and 50 and juveniles were sampled one and two months following settlement. All samplings were made prior to the first meal and were made similarly among cohorts. The number of sampled larvae varied among days of samplings taking into account their size. At hatch, 10 larvae were sampled for measurements and transferred in formaldehyde for size measurements (length to the end of the notochord and width) after anesthesia in MS 222, and 3 pools of 50 larvae were sampled from each rearing tank and transferred in RNA-Later following anesthesia and rinsed with filtered salt water. We are not presenting transcriptomic data at hatch and at d 50 in the present paper . On d 10 and d 20, 10 larvae were sampled for size measurements and 3 pools of 20 larvae were sampled as previously described and preserved in RNA-Later for transcriptomy. On days 30 and 40 days, 10 larvae were sampled for measurements and 3 pools of 10 larvae were sampled and preserved in RNA-Later. On day 50 and for juveniles samplings, 10 individuals were used for measurements and 3 pools of 3 individuals were preserved in RNA-Later. Larvae and juveniles immersed in five volumes of RNA later were maintained at 4 °C for 24 hours and then frozen at -80 °C.

2.2.3 RNA extractions

RNA extractions were made using the PureLink™ RNA Micro Scale Kit of Invitrogen™ (Waltham, Massachusetts, USA). Prior to the extraction process, each sample of larvae was divided into two equal groups of larvae to obtain duplicates. The larvae were too small to be extracted one by one. For d 50 larvae and juveniles, each individual was treated separately (triplicates).

The samples were placed in 1.5 mL Eppendorf tubes with 350 µL of lysis buffer and 3.5 µL of β-mercaptoethanol. Samples were lysed using a sonicator set with an amplitude of 70% and 10 pulses of 1 sec. A centrifugation at room temperature was made for 2 min at 12 000 g. The supernatant was collected in a new tube (approximately 350 µL) and 350 µL of 70% ethanol were added. The 700 µL was transferred to a column before being centrifuged at 12 000 g for 1 min at room temperature. The filtrate was thrown and 350 µL of wash buffer were added to the column followed by a centrifugation at 12 000 g for 1 min at room temperature. Once the filtrate was discarded, 10 µL of DNase diluted in 10 µL of DNase Buffer were added to the column and the sample was incubated at room temperature for 15 min. Then 350 µL of wash buffer was added to the column, followed by a centrifugation at 12 000 g for 15 s at room temperature. The filtrate was discarded and 500 µL of wash buffer were added to the column. Centrifugation at 12 000 g for 15 s at room temperature was done and the filtrate was thrown followed by the addition of 500 µL of washing buffer. Centrifugation at 12 000 g for 1 min at room temperature was done and the filtrate was discarded. A 1.5 mL sterile Eppendorf tube was used to replace the collecting tube on the column and 35 µL of RNase-free water was added. The column was incubated for 1 min at room temperature before being centrifuged for 1 min at 12 000 g to elude RNA. RNA was then measured with a Nanodrop ND-1000 Spectrophotometer version 3.3.0 (NanoDrop Technologies, Inc., Delaware, USA) to obtain concentration in ng/µL and the 260/280 ratio. RNA quality was verified by electrophoresis on a 2% agarose gel and migration was visualized using a ChemiDoc XRS+ Imager (BioRad, Inc., California, USA). We selected the samples with the best concentrations and quality score to have the transcriptome

sequenced in replicates or triplicates for d 20, d 30 and d 40 larvae and for 1 and 2 months-old juveniles of each cohort (total 62 samples). Those samples were sent on dry ice at the sequencing platform of CHU de Québec-Université Laval (Quebec City, Canada) where quality was ensured with a Bioanalyzer Tapestation D1000 system and the quality score ranged from 3.9 to 6.3. Because quality scores were below 7, an additional purification of the libraries and an increase in the sequencing coverage were made. The libraries were then set by removing the rRNAs with a poly(A) mRNA enrichment. Finally, only mRNAs were sequenced using the NovaSeq 6000 RNA-Seq method.

2.2.4 Bioinformatics analysis

FastQC (version 0.11.9, Andrews, 2010) was run on the sequenced transcriptomes to obtain the quality score for each sample. MultiQC (version 1.12, Ewels et al. 2016) was used to combine the data of each sample to see all the samples at once. Those two softwares gave all the information about the length of the sequences, their quality, the efficiency, and the depth of the sequencing and if sequences are overrepresented. Then, sequences were aligned using the FASTA sequences of the genome and the transcriptome of the Atlantic halibut as a reference (https://www.ncbi.nlm.nih.gov/assembly/GCA_009819705.1/). Alignment was made using the salmon software (1.4.0 version; Patro et al, 2017) and quantification of each transcript were obtained in the form of counts. A script in R studio was written, using the DESeq2 (Love et al. 2014) and tximport (Soneson et al. 2015) packages to obtain a table with all the samples and their differential expression levels for all expressed genes. The raw expression data were normalized using the DESeq2 function and then were log2 transformed for the further analysis. A global analysis was conducted including all the genes from all the samples and comparing C1, C2, and C3 at each stage of development considering the possible cross effects of the temperature, cohorts, and developments stages factors. The MASS (Venables & Ripley 2002) and the pheatmap (Kolde 2019) packages were used to obtain a representation of these expression levels and the differential expression between the different

cohorts and the temperature conditions by creating Multi-Dimensional Scaling (MDS) and heatmaps. The functions associated to the genes were determined through a gene ontology enrichment using the FASTA sequences of the transcriptome. For each gene, gene ontology identification (GOid) and its associated biological process were obtained using the cluster profiler program (Yu et al. 2012; Wu et al. 2021).

A separate analysis including 138 genes involved in the steroidogenesis pathway (Annexe I) and which have a role in sex determination was performed. Genes were selected using the reactome.org site (https://reactome.org/PathwayBrowser/#/R-HSA-196071). Using the same Bioconductor packages as before, as well as VennDiagram package (Chen 2022) and the result function from DESeq2, differential expression was measured and represented in function of the temperature regimes and the stages of life. Genes considered as significantly differentially expressed had a p-value < 0.05 and no filtration about log2foldchange was used.

2.3 RESULTS

The mRNA transcriptome was analyzed using the DESeq2 package to obtain the counts of mRNAs associated with each gene to compare expression patterns in winter flounder larvae and juveniles obtained from the three different cohorts. After counts normalization, an MDS analysis was performed including the whole dataset. This analysis showed that larvae and juveniles discriminated very well between each other (Fig. 3). The larvae were condensed within a small cluster with limited dispersion, while the juveniles had much more variations among samples. The 1 month-old juveniles issued from the C3 larvae were quite similar to the 2 months-old from all the cohorts.

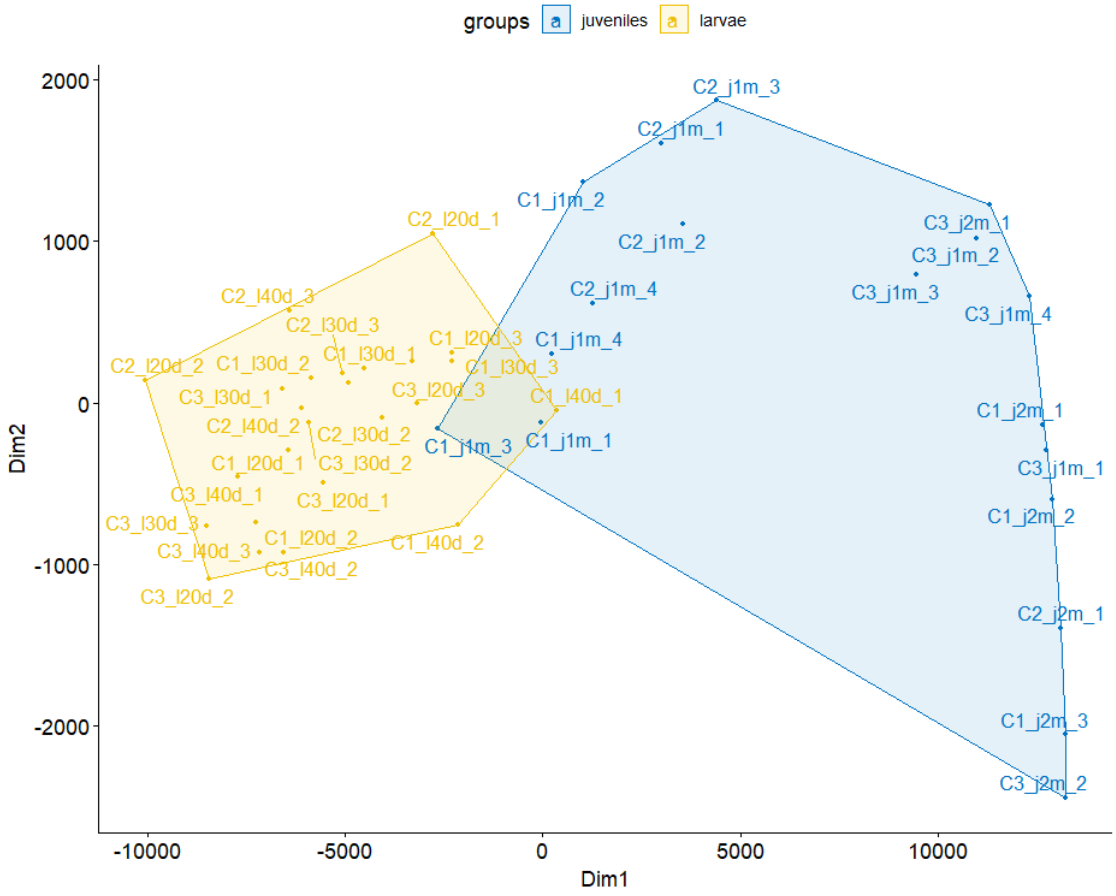


Fig. 3. General MDS (Multi-Dimensional Scaling) of whole mRNA transcriptome. C1 corresponds to larvae at the lowest temperature regime. C2 corresponds to larvae at the medium temperature regime. C3 corresponds to larvae at the highest temperature regime. l refers to larvae and j to juveniles. Axis represents distance among samples.

In the opposite, the 1 month-old juveniles issued from C2 larvae clustered with those issued from C1 larvae. There was also a slight overlap between the C1 juveniles and two C1 larvae showing that, despite the metamorphosis, they had a similar global transcriptomic profile. The results were almost the same when looking at the 500 genes which vary the most (Fig. 4). Within these 500 genes and when comparing C3 vs. C1, the upregulated genes (Fig. 5A) were mostly involved in the mitochondrial function, homoeostasis, cellular organization, some degradation process, and cytoplasmic translation. The downregulated genes (Fig. 5B) were involved in catabolic processes, in detoxification, in visual perception, in structural organization, and in inflammatory response. No genes associated to the steroidogenesis pathway were present in this group of 500. However, within juveniles, those issued from larvae reared at the highest temperature regime (C3) differed from the two others (C1 and C2) and had a similar expression pattern of the 2 months-old juveniles except for one cluster of genes (black square, Fig. 4) which showed many variations in expression levels. When looking at the associated functions of the genes within this cluster, those that were upregulated were all involved in cytoplasmic translation (*rpl12*, *rpl37a*, *rps14*, *rpl23*, *rps15a*, *rpl22*, *rpl18*, *rpl28*, *rps12*, *LOC117761292* (*rps26*), *rpl39*, *rpl38*, *rps27.1*, *rps28*, *rpl6*, *LOC117778826* (*rps8*), and *rpl23a*). Among the downregulated genes, there were *LOC117775520* (*habp2*) which is involved in the proteolysis, and *LOC117772324* (*mmp3-like*) which is also involved in proteolysis as well as in extracellular matrix organization. There were downregulations of *LOC117764105* (*rho*) which is involved in visual perception, the absorption of visible light, and in the rhodopsin mediated signaling pathway, and of *gnat2* which has a role in phototransduction. There was also a lower expression of *LOC117762259* (*amcase-like*) which is involved in the polysaccharide catabolic processes as well as *chia.1* which also has a role in the production of molecular mediator involved in inflammatory response, and in the positive regulation of chemokine production.

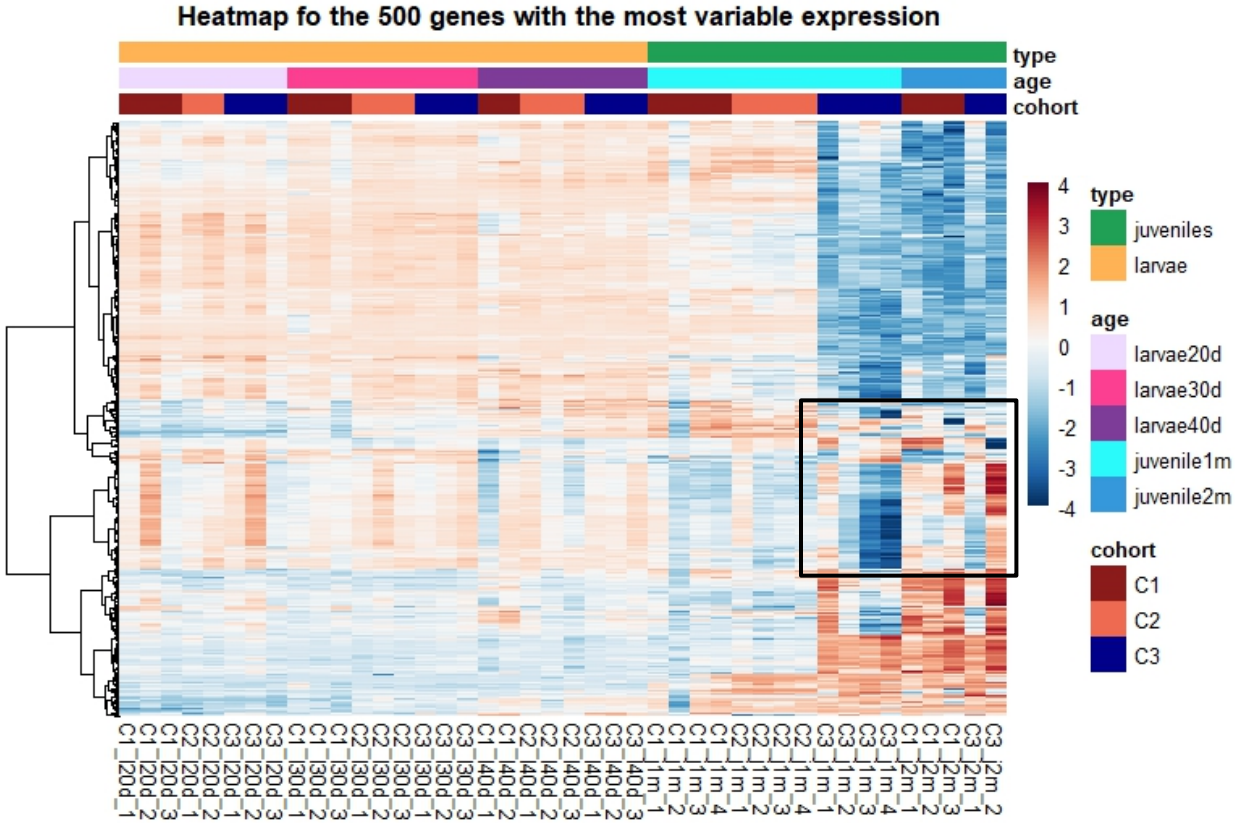


Fig. 4. General heatmap of the 500 genes with the most variable expression. The x-axis corresponds to larvae and juveniles raised at different temperature regimes and the y-axis corresponds to the 500 genes. The red color shows the upregulated genes and the blue shows the downregulated ones. The color scale is defined arbitrarily by choosing the shade range (from -4 to 4). C1 corresponds to larvae at the lowest temperature regime. C2 corresponds to larvae at the medium temperature regime. C3 corresponds to larvae at the highest temperature regime.

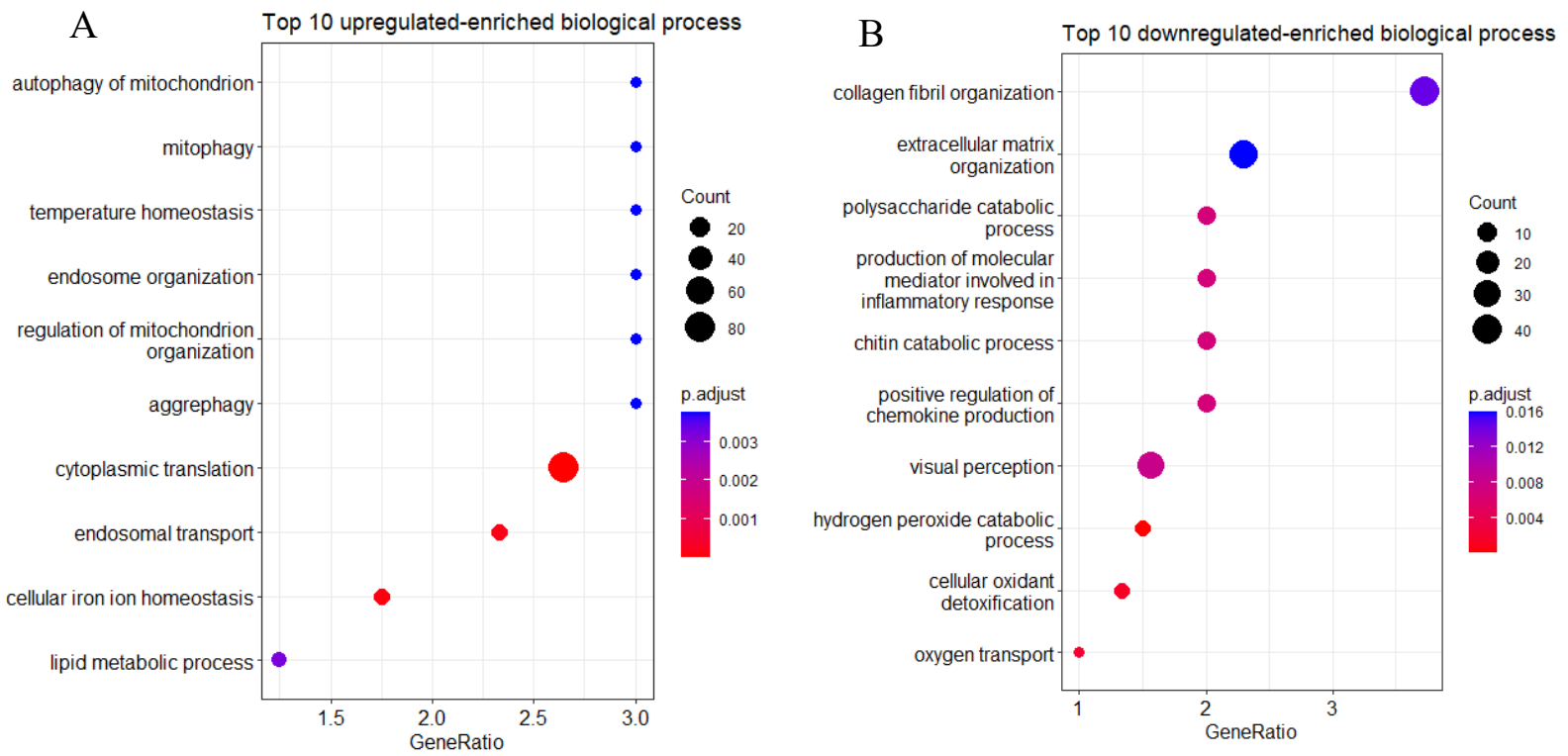
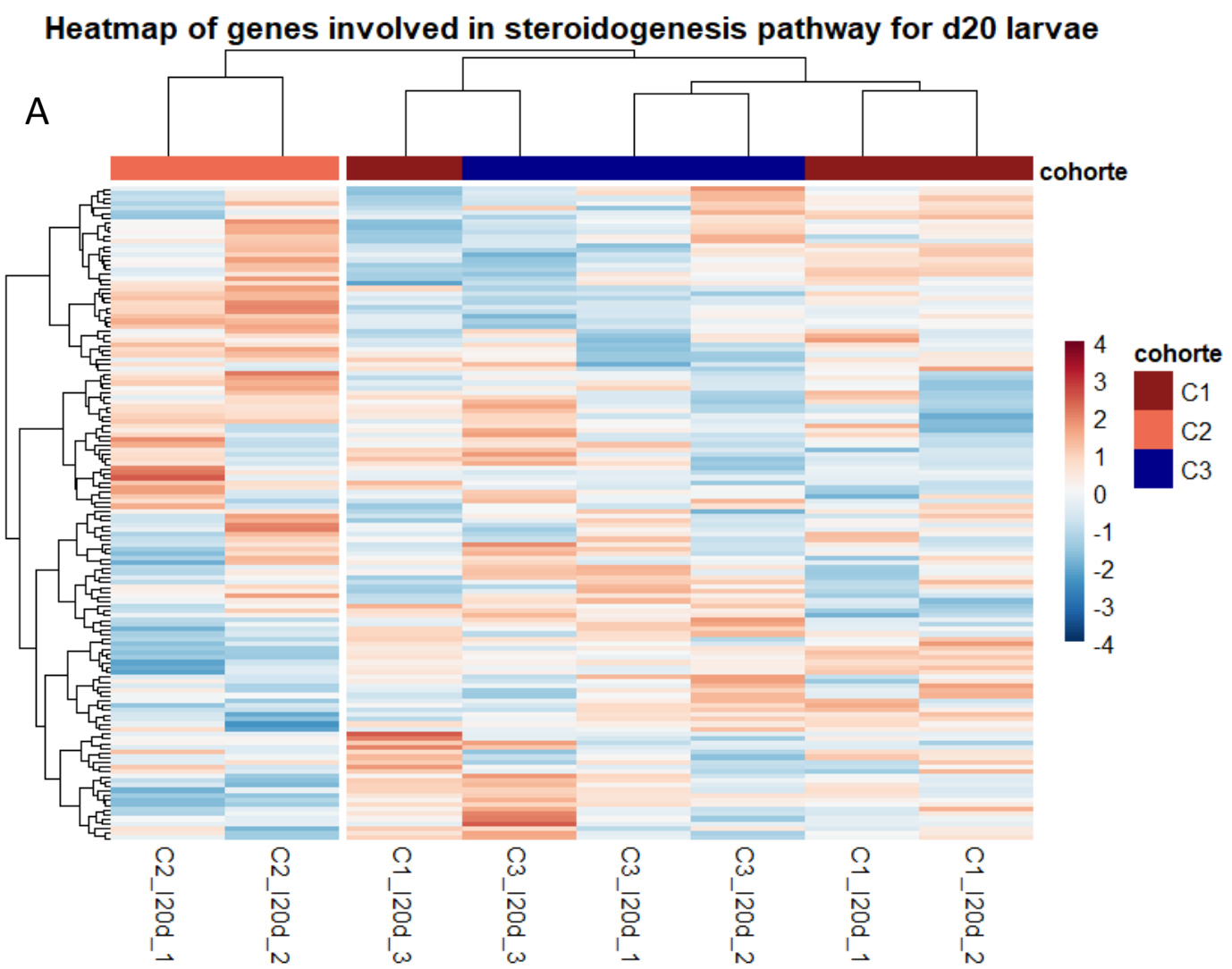


Fig. 5. Dot plot of the functions associated to the 500 genes that showed the most variable expressions in the mRNA transcriptome. The x-axis corresponds to the ratio of genes belonging to a function vs. all the genes. The y-axis corresponds to the main functions, the size of the circles show the number of genes associated with each function and the color scale indicates the p adjust. A) Functions of the upregulated genes. B) Functions of the downregulated genes.

For the genes involved in the steroidogenesis pathway, there were no major differences among 20 days-old larvae raised at different temperature regimes (Fig. 6A). When comparing the different temperature conditions (cohorts), only a few genes were significantly differentially expressed. When comparing C3 vs. C1, two genes showed significant differential expression (Annexe II): in C3, *sts* (steroid sulfatase, for complete names of the different genes, refer to Annexe I) that allows, among other things, the production of estrone sulfate (E1-S), an important form of estrogen. In C1, *ptges3a* (Prostaglandin E synthase 3) that is involved in the female gonadal development was more expressed (Fig. 6B). When comparing C3 vs. C2, two of the upregulated genes in C3 were involved in the modification of histones (*chdl* and *kdm4aa*). Moreover, *LOC117767306* (*cyp2u1*) and *LOC117772382 (cyp2g1-like)* that are very active in steroid synthesis and cholesterol transformation, *fgf13b* that seems to have a role in the testis development, and *hsd17b2* which is involved in the steroidogenesis pathway and allows the conversion of the testosterone and the estradiol were also more expressed. In C2, *fkbp4* that modulates transcription of the androgen receptor (AR) was more expressed as well as *LOC117774338* (*vsig1*) and *mis18bp1* that seems to be involved in the testis maturation. The three last more expressed genes in C2 were promoting the female gonadal development (*ptges3a*, *ptges3b* and *rspo1*) (Fig. 6B). When comparing C2 and C1, *kdm1a* that allows the modification of histone was upregulated as well as *fkbp4*, *ptges3a* and *ptges3b*. In C1, *fsta* (follistatin a) was more expressed. This gene allows ovarian differentiation and is regulated by *foxl2* and *bmp2b*. *LOC117772378*, *LOC117772381* and *LOC117772382*, all corresponding to a *cyp2g1-like gene*, *LOC117760836* that corresponds to *cyp2j2-like* which is also a cytochrome p450 and is activated by estradiol, and *LOC117767306* (*cyp2u1*) and *chdl1* were more expressed too (Fig. 6B).

In 30 days-old larvae, we began to see a differentiation between cohorts (Fig. 7A) but there were still not many genes significantly differentially expressed among them (Fig. 7B). When comparing C3 vs. C1, *gpat2* that acts on cholesterol slightly upstream of steroidogenesis, and *LOC117771222* that codes for *cyp120* which is a cytochrome p450 also involved in sterol metabolic process were more expressed in C1 (Fig. 7B).


Heatmap of genes involved in steroidogenesis pathway for d20 larvae
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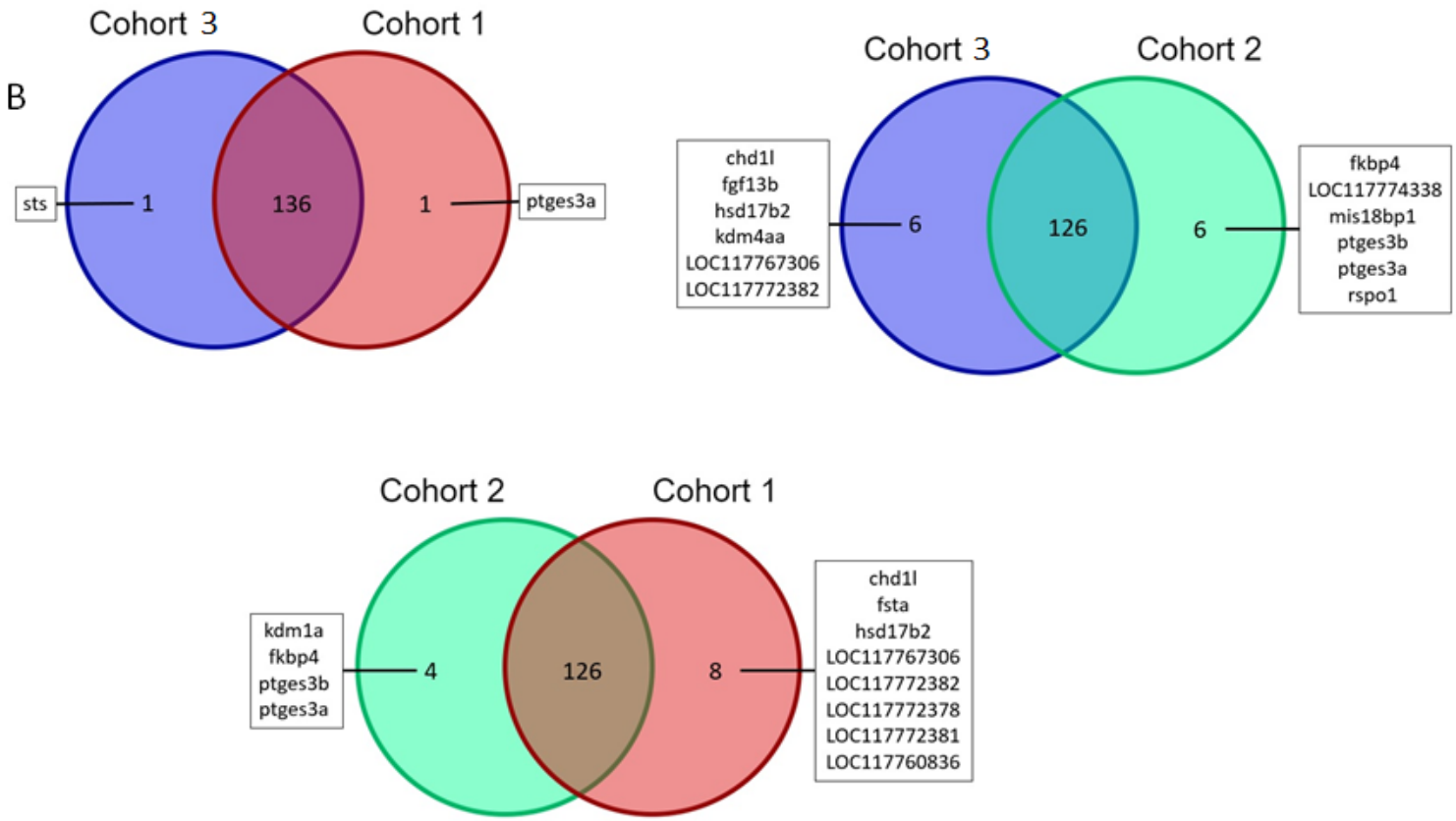
B
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chd1l
fgf13b
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kdm4aa
LOC117767306
LOC117772382
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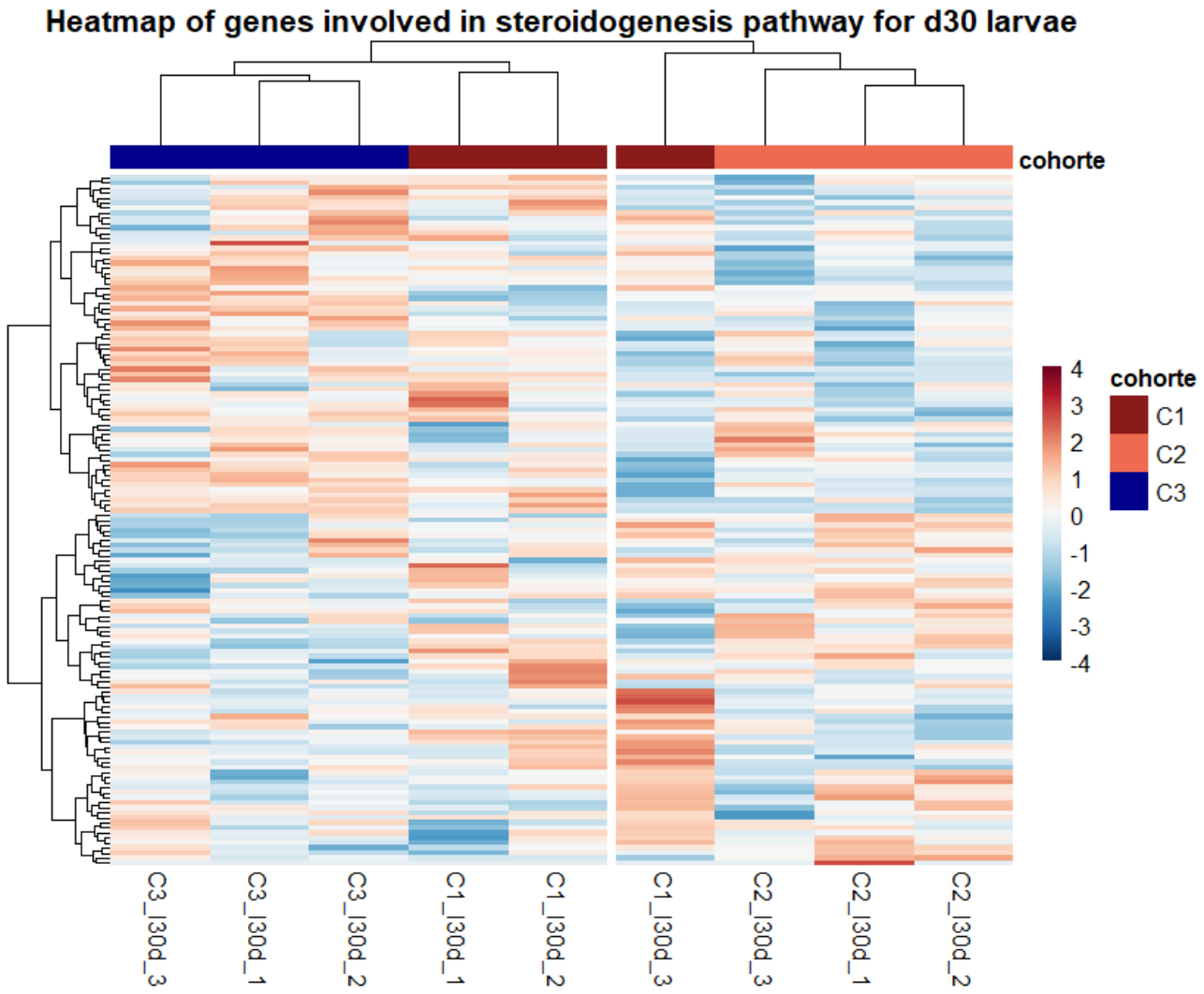
mis18bp1
ptges3b
ptges3a
rspo1
Cohort 2
Cohort 1
kdm1a
fkbp4
ptges3b
ptges3a
4
126
8
chd1l
fsta
hsd17b2
LOC117767306
LOC117772382
LOC117772378
LOC117772381
LOC117760836

Fig. 6. Expression levels of genes involved in the steroidogenesis pathway at 20 days of larval development. C1 corresponds to larvae exposed to the lowest temperature regime (average 7.45 °C). C2 corresponds to the larvae exposed to the medium temperature regime (average 7.80 °C). C3 corresponds to the larvae raised at the highest temperature regime (average 9.44 °C). A) Heatmap where the x-axis corresponding to the 20 days-old larvae raised at different temperature regimes and the y-axis corresponding to the genes involved in the steroidogenesis pathway. The red color shows the upregulated genes and the blue shows the downregulated ones. The color scale is defined arbitrarily by choosing the shade range (from -4 to 4); B) Venn Diagram. Comparisons between the highest temperature regime vs. the lowest (C3 vs. C1), the highest temperature regime vs. the medium one (C3 vs. C2) and the medium temperature regime vs. the lowest (C2 vs. C1). Genes that are significantly more expressed (p value < 0.05) between the different temperature regimes are identified in the corresponding circles (the full name of the genes are identified in Annexe I).


Heatmap of genes involved in steroidogenesis pathway for d30 larvae
cohorte
4
3
2
1
0
-1
-2
-3
-4
cohorte
C1
C2
C3
C3_l30d_3
C3_l30d_1
C3_l30d_2
C1_l30d_1
C1_l30d_2
C1_l30d_3
C2_l30d_3
C2_l30d_1
C2_l30d_2

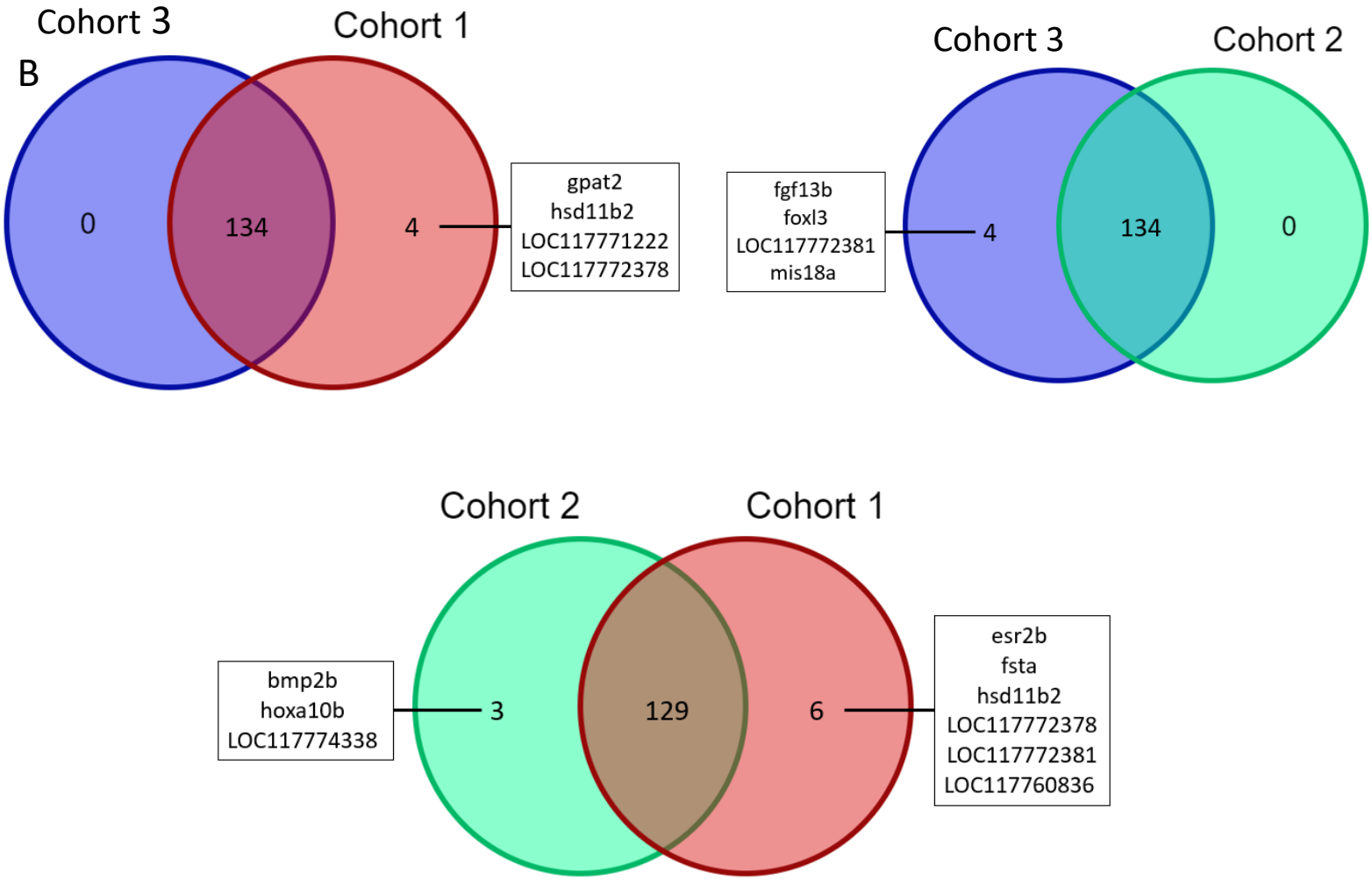
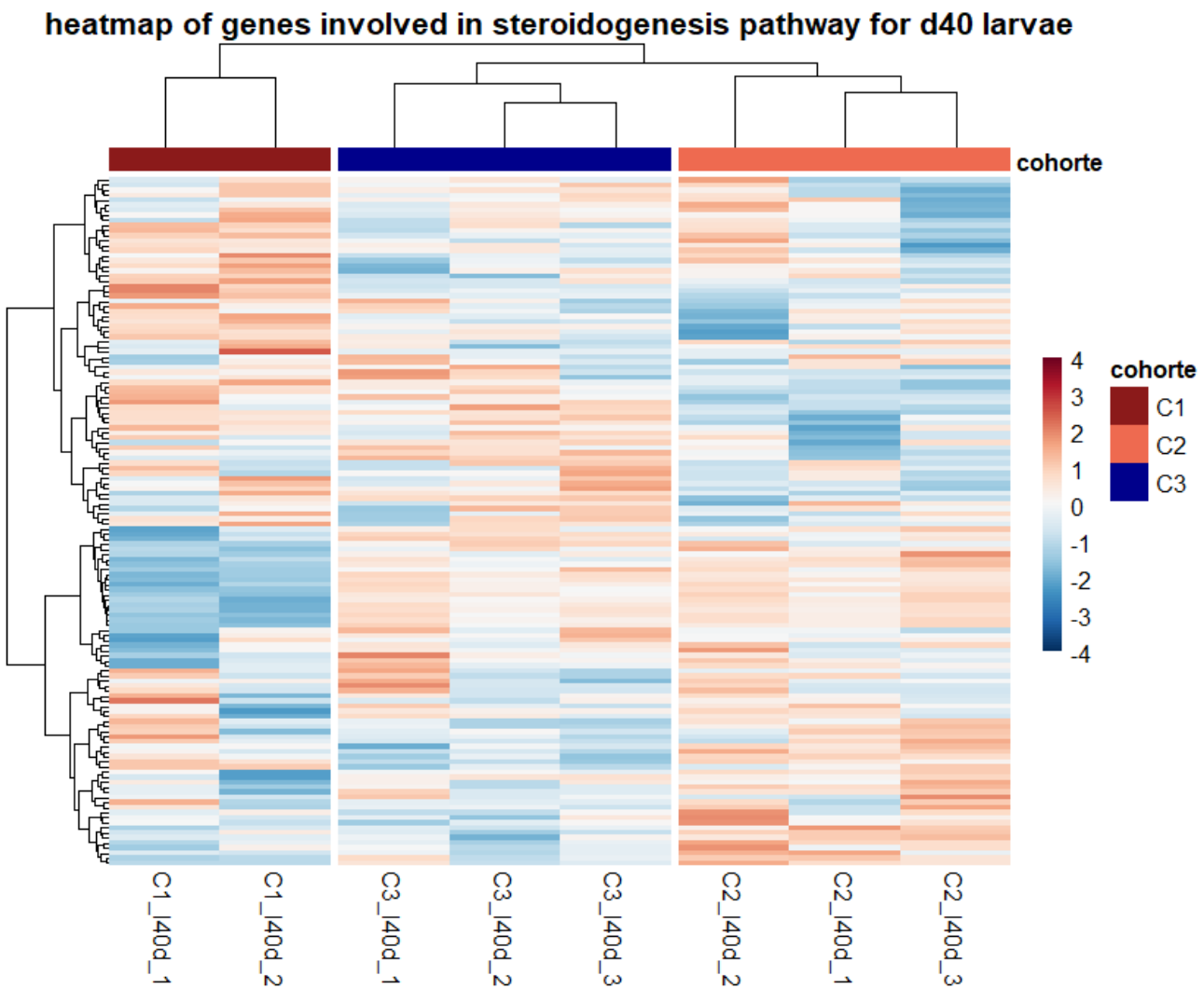

B
Cohort 3
Cohort 1
0
134
4
gpat2
hsd11b2
LOC117771222
LOC117772378
Cohort 3
Cohort 2
fgf13b
foxl3
LOC117772381
mis18a
4
134
0
Cohort 2
Cohort 1
bmp2b
hoxa10b
LOC117774338
3
129
6
esr2b
fsta
hsd11b2
LOC117772378
LOC117772381
LOC117760836

Fig. 7. Expression levels of genes involved in the steroidogenesis pathway at 30 days of larval development. C1 corresponds to larvae exposed to the lowest temperature regime (average 8.46 °C). C2 corresponds to the larvae exposed to the medium temperature regime (average 9.30 °C). C3 corresponds to the larvae raised at the highest temperature (average 9.84 °C). A) Heatmap where the x-axis corresponds to the 30 days-old larvae raised at different temperature regimes and the y-axis corresponds to the genes involved in the steroidogenesis pathway. The red color shows the upregulated genes and the blue shows the downregulated ones. The color scale is defined arbitrarily by choosing the shade range (from -4 to 4); B) Venn Diagram. Comparisons between the highest temperature regime vs. the lowest (C3 vs. C1), the highest temperature regime vs. the medium one (C3 vs. C2) and the medium temperature regime vs. the lowest (C2 vs. C1). Genes that are significantly more expressed (p value < 0.05) between the different temperature regimes are identified in the corresponding circles (the full name of the genes are identified in Annexe I).

In 40 days-old larvae, there was a clear separation between the three temperature regimes (Fig. 8A). The number of genes promoting sex differentiation that were significantly differentially expressed among cohorts increased (about twice as much as in 30 d old larvae) (Fig. 8.B). When comparing C3 vs. C1, *fkbp4*, *mis18a*, *ptges3b* and *sts* were again among differentially expressed genes and were more expressed in C3. There was also a higher expression of *gata4* which, when associated with *nr5a2a* and *nr5a2b*, allows the activation of *amh*. There was a higher expression of *fdft1*, *LOC117770886* (*cyp51a1*), *LOC117776540* (*Cyp4b1*), and of *lss*, *nsdhl* and *sc5d* that are involved in synthesis and modifications of the cholesterol and of *hsd17b7* and *hsd17b12b* both allowing the transformation of estrone in estradiol (Fig. 8B). In C1, *cited1* which is a target of the estrogen receptor, *ep300a* that modifies histones allowing the activation of *crebbpa* and *crebbpb* involved in the first step of the steroidogenesis, *hsd17b12a* involved in 11-ketotestosterone synthesis, *pgd* and *soat2* both allowing transformation of the cholesterol were all upregulated (Fig. 8B). When comparing C3 and C2, C3 showed again a higher expression of *chd1l*, *foxl3*, *LOC117772382* (*cyp2g1-like)*, *LOC117767306* (*cyp2u1*) and *sts*. There was also a higher expression of *nrip1b*, a gene involved in the cholesterol transformation, of *hsd17b8* which is involved in the steroidogenesis pathway and allows transformation of the estrone in estradiol and of *srd5a2b* which promotes the transformation of testosterone in dihydrotestosterone (Fig. 8B). In C2, there were again *ep300a*, *mis18bp1* and *rspo1* that were significantly differentially more expressed. *Emx2* inhibits the ovary development and is negatively regulated by *hoxa10b*, *LOC117751869* (*cyp26b1*) inhibits the aromatase (*cyp19a1a*) and thus the female development, mapk1 is one of the targets of estrogen receptors as well as *znf217* which is a transcription factor and they were all upregulated too (Fig. 8B). For the comparison between C2 and C1, C2 showed a higher expression of several genes seen previously: *fdft1*, *fkbp4*, *gata4*, *hsd17b7*, *hsd17b12b*, *LOC117770886* (*cyp51a1*), *mis18a*, *mis18bp1*, *nsdhl*, *ptges3a* and *sc5d*. Other upregulated genes were *dhcr24* involved in modification of cholesterol before the beginning of the steroidogenesis, *uggt2* which is a target of the progesterone, *stc1* allowing the ovarian development as well as *wnt4* which is inhibited by *dmrt1*.

A


heatmap of genes involved in steroidogenesis pathway for d40 larvae
cohorte
4
3
2
1
0
-1
-2
-3
-4
cohorte
C1
C2
C3
C1_l40d_1
C1_l40d_2
C3_l40d_1
C3_l40d_2
C3_l40d_3
C2_l40d_2
C2_l40d_1
C2_l40d_3

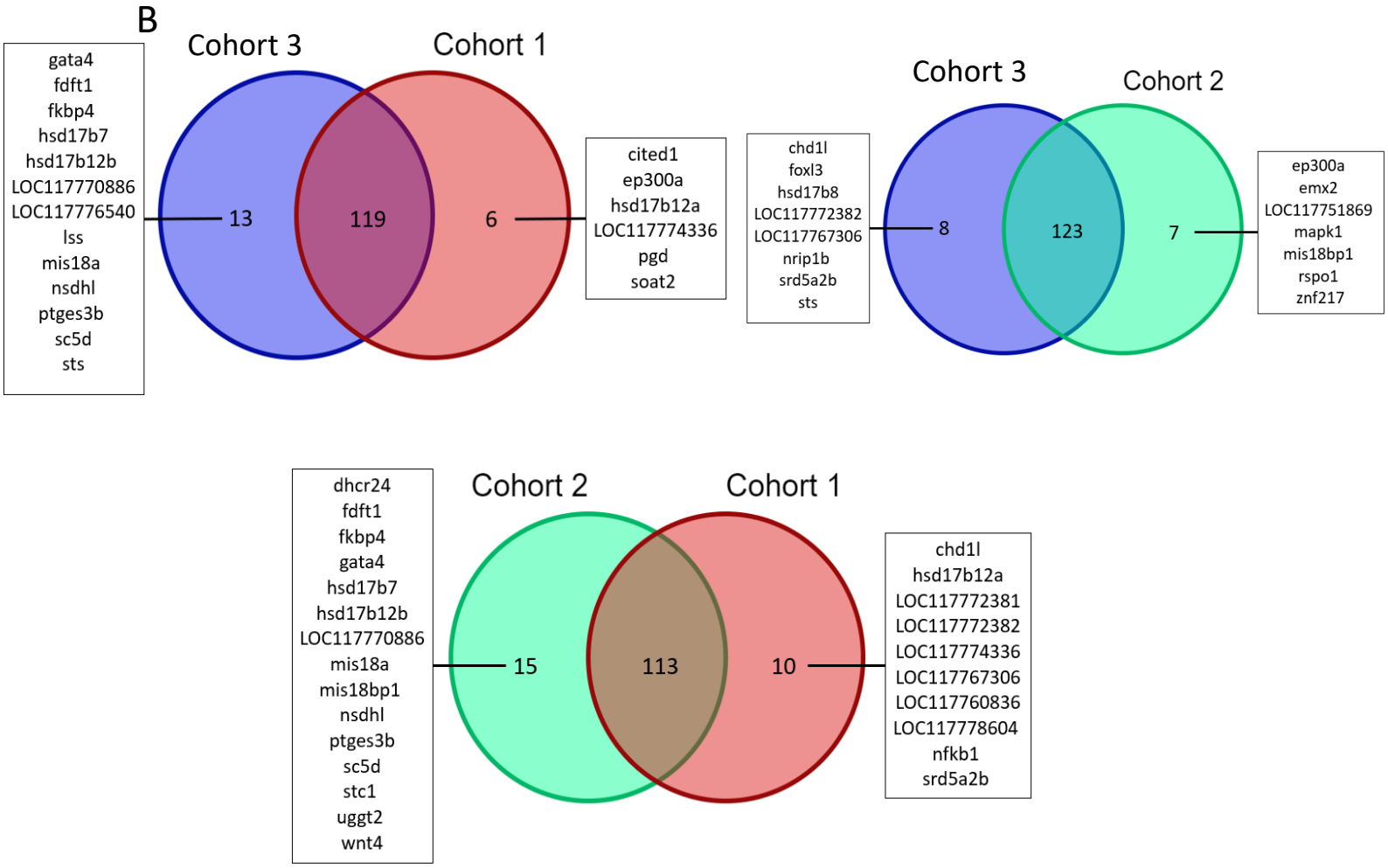
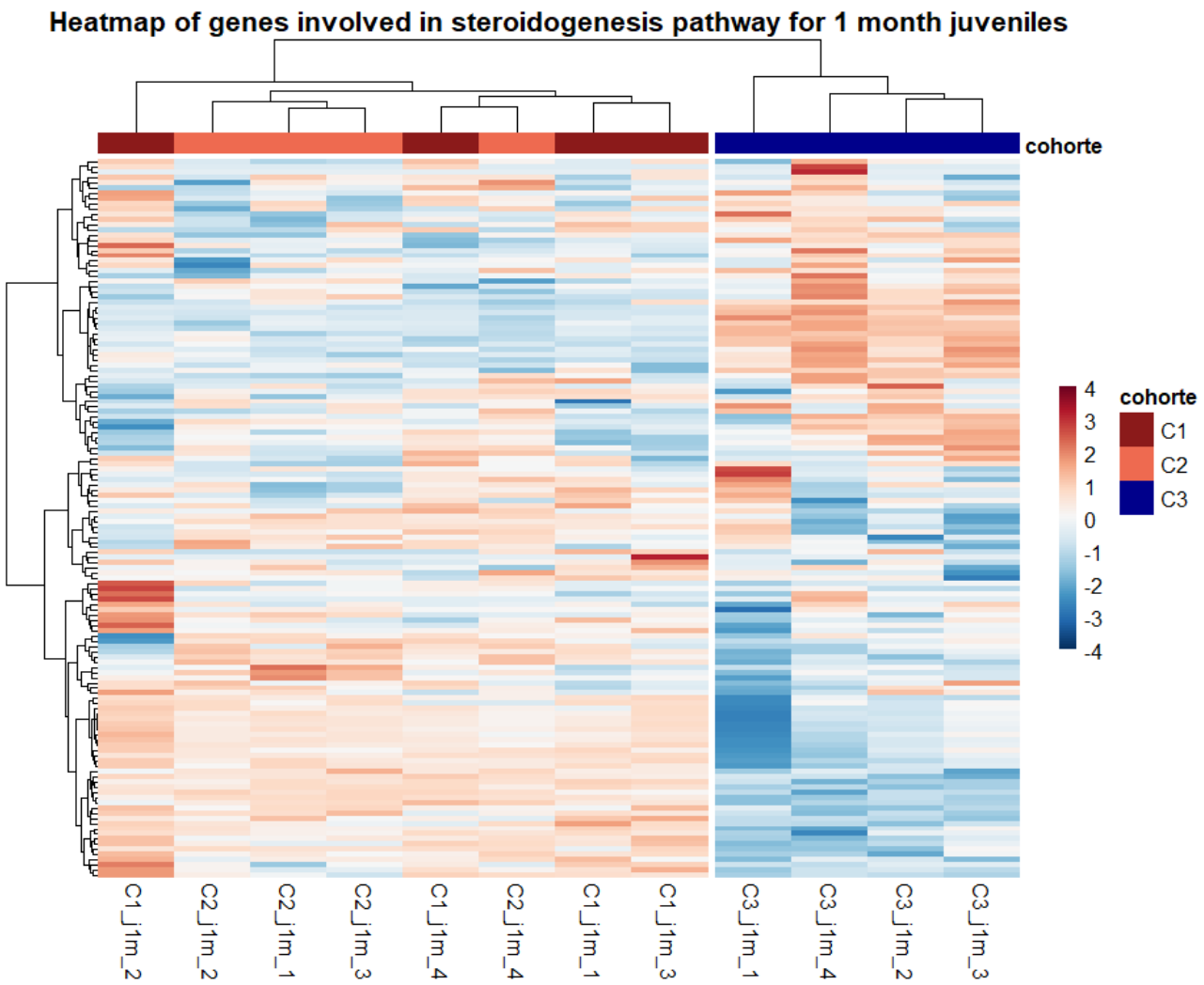

B
Cohort 3
Cohort 1
gata4
fdft1
fkbp4
hsd17b7
hsd17b12b
LOC117770886
LOC117776540
lss
mis18a
nsdhl
ptges3b
sc5d
sts
13
119
6
cited1
ep300a
hsd17b12a
LOC117774336
pgd
soat2
Cohort 3
Cohort 2
chd1l
foxl3
hsd17b8
LOC117772382
LOC117767306
nrip1b
srd5a2b
sts
8
123
7
ep300a
emx2
LOC117751869
mapk1
mis18bp1
rspo1
znf217
Cohort 2
Cohort 1
dhcr24
fdft1
fkbp4
gata4
hsd17b7
hsd17b12b
LOC117770886
mis18a
mis18bp1
nsdhl
ptges3b
sc5d
stc1
uggt2
wnt4
15
113
10
chd1l
hsd17b12a
LOC117772381
LOC117772382
LOC117774336
LOC117767306
LOC117760836
LOC117778604
nfkb1
srd5a2b

Fig. 8. Expression levels of genes involved in the steroidogenesis pathway at 40 days of larval development. C1 corresponds to larvae exposed to the lowest temperature regime (9.86 °C). C2 corresponds to the larvae exposed to the medium temperature regime (9.93 °C). C3 corresponds to the larvae raised at the highest temperature (10.2 °C). A) Heatmap where the x-axis corresponds to the 40 days-old larvae raised at different temperature regimes and the y-axis corresponds to the genes involved in the steroidogenesis pathway. The red color shows the upregulated genes and the blue shows the downregulated ones. The color scale is defined arbitrarily by choosing the shade range (from -4 to 4); B) Venn Diagram. Comparisons between the highest temperature regime vs. the lowest (C3 vs. C1), the highest temperature regime vs. the medium one (C3 vs. C2) and the medium temperature regime vs. the lowest (C2 vs. C1). Genes that are significantly more expressed (p value < 0.05) between the different temperature regimes are identified in the corresponding circles (the full name of the genes are identified in Annexe I).

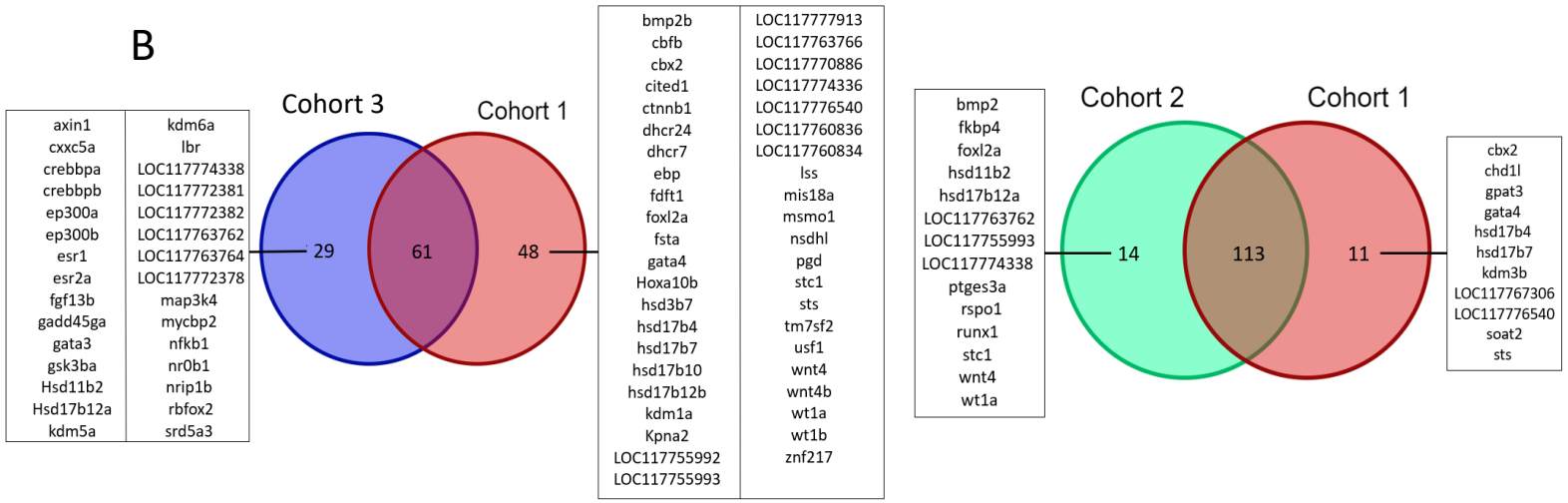
In C1, *chdl1*, *gata4*, *hsd17b7*, *LOC117767306* (*cyp2u1*), *LOC117776540* (*Cyp4b1*), *soat2* and *sts* were upregulated again. A series of other genes were also more expressed, including *cbx2* which is involved in the development of both testis and ovaries following upregulation by other genes such as *foxl2a*, *gpat3* which acts on cholesterol slightly upstream of steroidogenesis, *hsd17b4* which allow the transformation estradiol (E2) in an inactive metabolite and *kdm3a* which allows, as the others *kdm*, the modification of histones and seems to be expressed in the ovary (Fig. 8B).

Finally, the steroidogenesis pattern of gene expression was completely different when looking at juveniles issued from larvae raised at the highest temperature regime (C3) (Fig. 9A). After metamorphosis, the juveniles issued from the different temperature regimes were all exposed to the same temperature conditions (10.7 °C). There is much differentially expressed genes among juveniles (around three times more than for 40 days-old larvae) (Fig. 9B). When comparing C3 vs. C1, 77 genes were differentially expressed. From this, some genes were again more expressed in C3: *ep300a*, *fgf13b*, *hsd11b2*, *hsd17b12a*, *LOC117774338* (*vsig1*), *LOC117772381* (*cyp2g1-like*), *LOC117772382* (*cyp2g1-like*), *LOC117772378* (*cyp2g1-like*) and *nrip1b*. Among others, there is a higher expression of several genes with a key role in the steroidogenesis: *crebbpa* and *crebbpb* which both activate the star gene at the beginning of the steroidogenesis pathway and that are both activated by *ep300b* and *ep300a*, *srd5a3* also involved in steroidogenesis pathway and allowing the conversion of the testosterone in dihydrotestosterone, *map3k4* which activates *gata4* and thus the *amh*, *nfkb1* which inhibits both *nr5a1a* and *nr5a1b* preventing the activation of *amh* (Fig. 9B). In C1, there were again a higher expression of *bmp2b*, *cbx2*, *cited1*, *dhcr24*, *fdft1*, *foxl2a*, *fsta*, *gata4*, *hoxa10b*, *hsd17b4*, *hsd17b7*, *hsd17b12b*, *kdm1a*, *LOC117770886* (*cyp51a1*), *LOC117776540* (*Cyp4b1*), *LOC117760836* (*cyp2j2-like*), *lss*, *mis18a*, *nsdhl*, *pgd*, *stc1*, *sts, wnt4*, and *znf217*. Among the other genes, there was a higher expression of very important genes such as both *LOC117755992* and *LOC117755993* which correspond to *cyp1b1-like* and which hydroxylate estradiol-17-b in 4 OH-estradiol-17b, *cbfb* which is a target of RUNX1 (activated by ERα) and allow the expression of amh, and *wnt4b* which promotes the ovarian development and is inhibited by *dmrt1*.

A


Heatmap of genes involved in steroidogenesis pathway for 1 month juveniles
cohorte
4
3
2
1
0
-1
-2
-3
-4
cohorte
C1
C2
C3
C1_j1m_2
C2_j1m_2
C2_j1m_1
C2_j1m_3
C1_j1m_4
C2_j1m_4
C1_j1m_1
C1_j1m_3
C3_j1m_1
C3_j1m_4
C3_j1m_2
C3_j1m_3

B



axin1	kdm6a
cxxc5a	lbr
crebbpa	LOC117774338
crebbpb	LOC117772381
ep300a	LOC117772382
ep300b	LOC117763762
esr1	LOC117763764
esr2a	LOC117772378
fgf13b	map3k4
gadd45ga	mycbp2
gata3	nfkb1
gsk3ba	nr0b1
Hsd11b2	nrip1b
Hsd17b12a	rbfox2
kdm5a	srd5a3

bmp2b	LOC117777913
cbfb	LOC117763766
cbx2	LOC117770886
cited1	LOC117774336
ctnnb1	LOC117776540
dhcr24	LOC117760836
dhcr7	LOC117760834
ebp	lss
fdft1	mis18a
foxl2a	msmo1
fsta	nsdhl
gata4	pgd
Hoxa10b	stc1
hsd3b7	sts
hsd17b4	tm7sf2
hsd17b7	usf1
hsd17b10	wnt4
hsd17b12b	wnt4b
kdm1a	wt1a
Kpna2	wt1b
LOC117755992	znf217
LOC117755993	

bmp2
fkbp4
foxl2a
hsd11b2
hsd17b12a
LOC117763762
LOC117755993
LOC117774338
ptges3a
rspo1
runx1
stc1
wnt4
wt1a

cbx2
chd1l
gpat3
gata4
hsd17b4
hsd17b7
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LOC117767306
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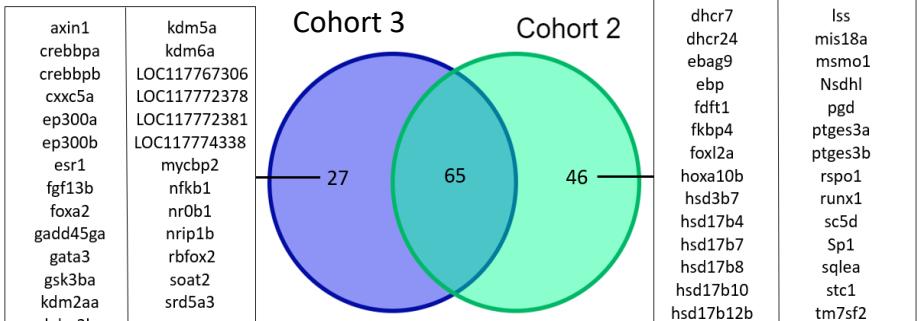
soat2
sts



axin1	kdm5a
crebbpa	kdm6a
crebbpb	LOC117767306
cxxc5a	LOC117772378
ep300a	LOC117772381
ep300b	LOC117774338
esr1	mycbp2
fgf13b	nfkb1
foxa2	nr0b1
gadd45ga	nrip1b
gata3	rbfox2
gsk3ba	soat2
kdm2aa	srd5a3
kdm3b	

bmp2b	LOC117777913
cbfb	LOC117760834
cited1	LOC117755992
ctnnb1	LOC117755993
dhcr7	lss
dhcr24	mis18a
ebag9	msmo1
ebp	Nsdhl
fdft1	pgd
fkbp4	ptges3a
foxl2a	ptges3b
hoxa10b	rspo1
hsd3b7	runx1
hsd17b4	sc5d
hsd17b7	Sp1
hsd17b8	sqlea
hsd17b10	stc1
hsd17b12b	tm7sf2
kpna2	wnt4
LOC117763766	wnt4b
LOC117770886	wt1a
LOC117774336	Wt1b
LOC117776540	znf217

Fig. 9. Expression levels of genes involved in the steroidogenesis pathway in 1 month-old juveniles. C1 corresponds to juveniles obtained from larvae raised at the lowest temperature regime. C2 corresponds to the juveniles obtained from larvae raised at the medium temperature regime. C3 corresponds to juveniles obtained from larvae raised at the highest temperature regime. After metamorphosis, all juveniles were at the same temperature (10.7 °C). A) Heatmap where the x-axis corresponds to the 1 month-old juveniles issued from larvae raised at different temperature regimes and the y-axis corresponds to the genes involved in the steroidogenesis pathway. The red color shows the upregulated genes and the blue shows the downregulated ones. The color scale is defined arbitrarily by choosing the shade range (from -4 to 4); B) Venn Diagram. Comparisons between the highest temperature regime vs. the lowest (C3 vs. C1), the highest temperature regime vs. the medium one (C3 vs. C2) and the medium temperature regime vs. the lowest (C2 vs. C1). Genes that are significantly more expressed (p value < 0.05) between the different temperature regimes are identified in the corresponding circles (the full name of the genes are identified in Annexe I).

There were also a higher expression of *hsd3b7* which is involved in steroidogenesis pathway and allows the transformation of pregnenolone and 17α-Hydroxy-Pregnenolone in progesterone and 17α-Hydroxy-progesterone respectively, *hsd17b10* which promotes the transformation of the dehydroepiandrosterone in Androstenedione, *wt1a* and *wt1b* which both regulate the expression levels of *hsd11b* and thus promote the 11-ketotestosterone formation (Fig. 9B). When comparing C3 and C2, C3 showed many already seen upregulated genes: *axin1, crebbpa, crebbpb, cxxc5a, ep300a, ep300b, esr1, fgf13b, gadd45ga, gata3, gsk3ba, kdm5a, kdm6a, LOC117767306* (*cyp2u1*), *LOC117772378* (*cyp2g1-like*), *LOC117772381* (*cyp2g1-like*), *LOC117774338* (*vsig1*), *mycbp2, nfkb1, nr0b1*, *nrip1b, soat2 and srd5a3*. Among those genes*, crebbpa, crebbpb, Ep300a, Ep300b, Esr1, gsk3ba, mycbp2, nfkb1, nr0b1* and *srd5a3* are very important for the steroidogenesis pathway (Fig. 9B). Among the other genes, there was also a higher expression of *ebag9* which is a target of *esr1* and of *sp1* which is expressed in testis and have a key role in the stimulation of steroidogenesis genes (Fig. 9B). For the last comparison, the C2 upregulated 14 genes and among them *LOC117755993* (*cyp1b1-like*), *foxl2a*, *hsd11b2*, *hsd17b12a* and *wt1a* are key genes in steroidogenesis and sex determination. In the C1, there were eleven genes among which *gata4*, *hsd17b4* and *hsd17b7* were very important too (Fig. 9B).

2.4 DISCUSSION

In this study, we tested whether or not the thermosensitive period for the phenotypic sex determination in winter flounder occurred before metamorphosis in juveniles. When looking at the whole mRNA transcriptome, the main functions which were associated to expression variations were not involved in the steroidogenesis pathway. During the larval development, we did not observe major differences, but several genes associated with steroidogenesis were differentially upregulated according to the temperature treatment (Fig. 10) and their number increased over time.

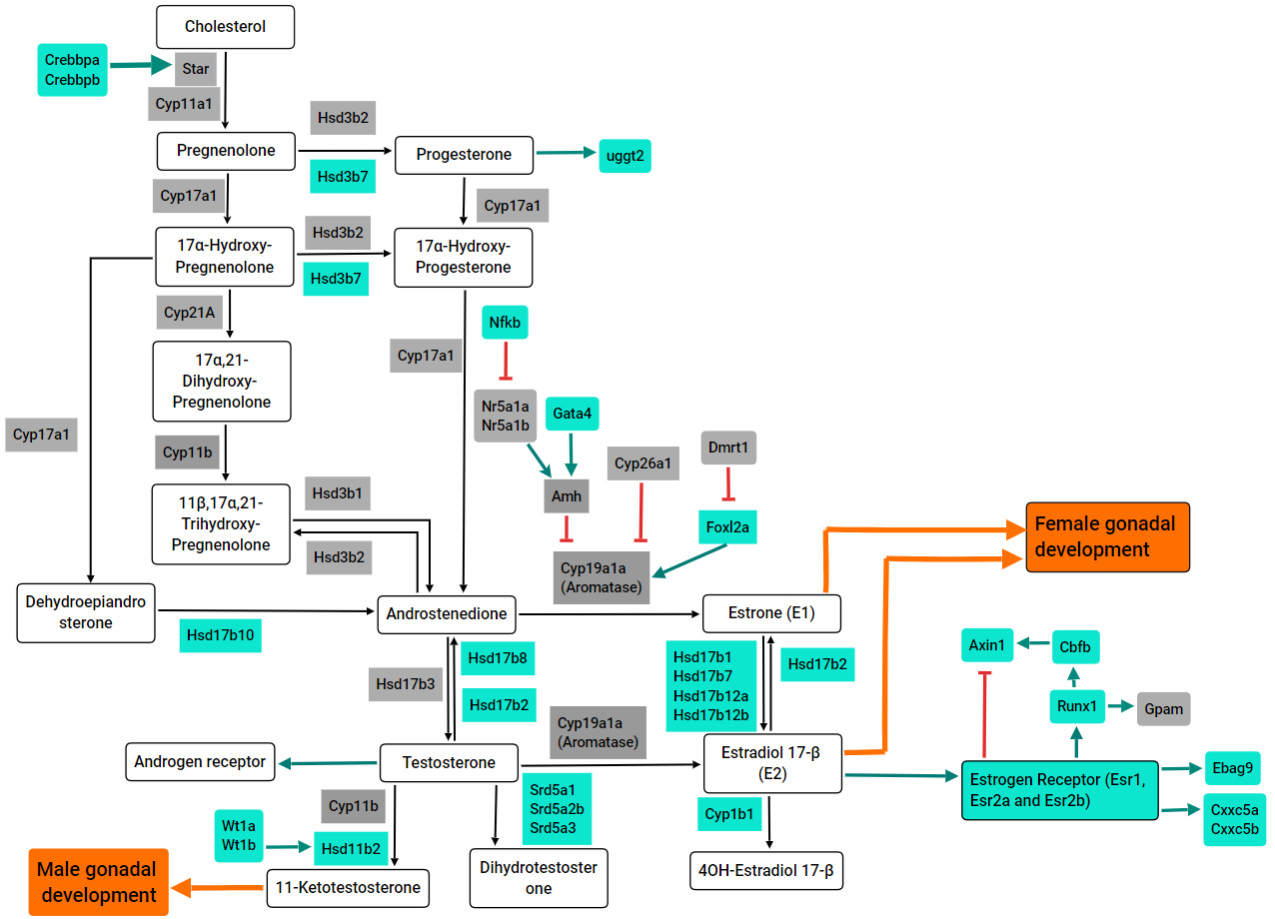


Fig. 10. Representation of the steroidogenesis pathway with all the transformation steps from cholesterol to testosterone and estrogen. Significantly differentially expressed genes are in green. The activations of the genes are represented by green arrows while inhibitions are represented by red arrows.

At the juvenile stage, once the metamorphosis was completed, juveniles issued from the larvae raised at the warmest temperature regime showed a higher expression of 31 steroidogenesis-related genes compared to the two other treatments, indicating an effect of temperature on this metabolic pathway. However, since all cohorts significantly expressed both male- and female-related genes we were not able to determine how this could affect the phenotypic sex.

2.4.1 Laval stage, C1 vs. C2

At the larval stage, we expected that the most differentiated cohorts would be 3 and 1, but the ones which differed the most between each other were cohorts 1 and 2. On days 20 and 40, *ptges3b* which regulates the steroidogenesis pathway and is mainly expressed in the ovary (fathead minnow — *Pimephales promelas*, Martyniuk et al. 2012) and *fkbp4* which has a role in the transfer of steroid hormone receptors between the cell cytoplasm and the nucleus (human, Guiochon-Mantel & Milgrom 1993) were more expressed in C2. After 40 days of development, C2 was also characterized by a higher expression of *hsd17b7* and *hsd17b12b*. These two genes have been shown to be involved in the conversion of the estrone into estradiol in human (Shehu et al. 2008) and zebrafish (Mindnich & Adamski 2009) respectively, suggesting the presence of females among samples. Interestingly, higher expression of *LOC117774338* (*vsig1*) on day 30 which is known to be expressed in testis and allowing spermatogenesis in mouse (*Mus musculus*; Jung et al. 2021) and *gata4* involved in testicular differentiation in fishes (e.g. Nagahama et al. 2021) also distinguished C2. Even though the gonad differentiation is not started yet in flatfish larvae (Japanese flounder, Kitano et al. 1999), those genes might indicate the presence of males among the pools. In C1, *LOC117760836* (*cyp2j2-like*), a gene involved in the estrogen biosynthesis (Japanese medaka; Abdelmoneim et al. 2019), was more expressed on days 20, 30 and 40. *Fsta*, a gene with a key role in early ovarian differentiation (rainbow trout; Nicol et al. 2011) was also more expressed on days 20 and 30.

This and the higher expression of *esrb2* which codes for the estrogen receptor beta (e.g. Amenyogbe et al. 2020) and *nfkb1* which is involved in an activation pathway of aromatase (zebrafish, Pradhan et al. 2012) suggest the presence of females among this cohort too. Moreover, h*sd17b12a* was more expressed in C1 too and is involved in the synthesis of 11-ketotestosterone (Japanese eel, Suzuki et al. 2020) but also in the conversion of the estrone into estradiol (zebrafish, Mindnich & Adamski 2009). However, C1 also showed higher expression of *hsd11b2* a key gene for the production of 11-ketotestosterone (e.g. Yazawa et al. 2008) on day 30, which might be an early indicator of potential male differentiation, even though there is no detectable presence of this hormone before gonad development in adults fishes (e.g. Soranganba & Singh 2019).

2.4.2 Larval stage, C3 vs. C1

When we looked at the two cohorts with the most different temperature regimes (C3 vs. C1), no major differences could be seen before day 40. Indeed, on days 20 and 30, there were only two and four genes significantly differentially expressed respectively between the two cohorts. The *sts* gene (steroid sulfatase) was more expressed on both days 20 and 40, in C3. In the zebrafish, this gene was demonstrated to have a key role in the production of androgens and estrogens (Kurogi et al. 2019). In C1, during this period, we found a higher expression of genes also upregulated when C1 was compared to C2: *hsd11b2* and *LOC117760836 (cyp2j2)*. On day 40, the number of genes more expressed in C3 increased from 0 or 1 to 13. *Hsd17b7*, *hsd17b12b*, *LOC117770886* (*cyp51a1*), and *ptges3b* are all involved in either the production of estrogens or in the ovary development (human, Shehu et al. 2008; zebrafish, Mindnich & Adamski 2009; Chinese sturgeon — *Acipenser sinensis*, Wu et al. 2022; e.g. Goldstone et al. 2010; fathead minnow, Martyniuk et al. 2012) and were among these upregulated genes. Moreover, there was a higher expression of *lss* which is normally activated with the aromatase (zebrafish, Muth-Köhne et al. 2016). However, simultaneously, there was a higher expression of *gata4*, *LOC117776540* (*Cyp4b1*) which

both promote the testicular differentiation (yellow catfish — *Pylodictis olivaris*, Xiong et al. 2015), indicating the presence of male differentiation among the samples. Even if both sexes seemed to be present, we did not have any strong signal that could inform about the sex-ratio.

2.4.3 Juvenile stage

In early juveniles, the number of genes associated with the steroidogenesis pathway which were differentially expressed among cohorts increased by 200 %. It should be reminded that all juveniles were raised under similar temperature, rearing, and feeding conditions, so temperature/cohort effects could only be attributed to events that occurred during the larval stage. The comparisons of C3 vs. C1 and of C3 vs. C2 were very similar, with a high number of significantly differentially expressed genes (77 and 73 genes respectively) which were common to both. This was also twice more genes differentially expressed than when C2 and C1 were compared, indicating that, at this developmental stage, C3 juveniles were the most different. Among genes more expressed in C3, there were *crebbpa*, *crebbpb,* and *ŋfkb1* all allowing the activation of the aromatase (e.g. Sharma et al. 2022; zebrafish, Pradhan et al. 2012). *Axin1* which is involved in the wnt signaling pathway which promotes the regulation and the maturation of the ovarian follicle and the steroid production (zebrafish, Hosseini et al. 2019) was also upregulated. *Ep300b* (e.g. Ortega-Recalde et al. 2020; Nile tilapia—*Oreochromis niloticus*, Xu et al. 2022; e.g. Nagahama et al. 2021), *gata3*, and *nrip1b* (common carp—*Cyprinus carpio*, Wang et al. 2023), and *nr0b1* (black porgy fish—*Acanthopagrus schlegeli*, Wu et al. 2008) are all involved in the ovary differentiation and were also more expressed. These numerous female-related genes suggest the presence of individuals with a female phenotype. However, there was also a higher expression of genes involved in testis differentiation: *axin1*, *fgf13b* (human, Cory et al. 2007; zebrafish, Itoh & Konishi 2007), and *gadd45ga* (e.g. Nagahama et al. 2021) as well as *LOC117774338* (*vsig1*) which is normally expressed in testis. *Cxxc5a* which inhibits *foxl2a* and thus the aromatase (human, L'hôte et al. 2012) and hsd11b2 which are both in favor of a

male phenotype were also more expressed. When comparing C3 vs. C1 and C2, multiple genes promoting the estrogen production were more expressed in both C1 and C2 (*hsd17b12b*, *hd17b7*, *ptges3a*, *LOC117760834* and *LOC117760836* (both corresponding to *cyp2j2-like*, and *foxl2a))* (zebrafish, Siegfried & Nüsslein-Volhard 2008). There were also genes with a role in the ovary differentiation (*LOC117770886 (cyp51a1)*, *bmp2b* (coho salmon, Luckenbach et al. 2011), *ctnnb1, foxl2a*, *wnt4*, and *wnt4b* (e.g. Nagahama et al. 2021)) which were upregulated in both C1 and C2. However, C1 also showed a higher expression of 4 genes involved in the testis differentiation (*cbx2, wt1b* (e.g. Nagahama et al. 2021), *gata4*, and *usf1* (zebrafish, Gautier et al. 2011)), while C2 had only *wt1a* as upregulated gene.

In juveniles from C2, we mainly observed higher expression of genes favoring a female phenotype. However, it should be reminded that there is very few cases among flatfishes of a sex reversal in favor of the female phenotype (Luckenbach et al. 2009). Based on literature and the observations made in our facilities, in absence of an imbalance in favor of males, only a 1:1 sex ratio can be expected.

2.4.4 Early sex markers

In other studies, some genes were set as early sex markers. The aromatase (*cyp19a1a*), which was identified as an early female phenotypic marker (e.g. Piferrer & Blázquez 2005) and allows the conversion of testosterone in estrogens, was never differentially expressed regardless to the temperature regime in our study. Indeed, the level of expression of *cyp19a1a* remained extremely low with 69% of the samples that did not express the aromatase. The same was true for *dnd* which was already identified as an early female sex marker (zebrasifh, Weidinger et al. 2003) with 61% of the samples not expressing this gene. However, *foxl2a* that was associated with female phenotype in young stages (zebrafish, Siegfried & Nüsslein-Volhard 2008) was significantly more expressed in juveniles issued from C1 and C2 vs. C3

which are the two colder temperature regimes (average fold change of 2.64 ± 4.13). *Amh* (average fold change of 1.5 ± 1.01) and *cyp11b* (average fold change of 0.90 ± 0.25) associated with early sex male markers (e.g. Pfennig et al. 2015, e.g. Schiffer et al. 2015), did not appear as significantly differentially expressed during this experiment even if there were much more expressed than for the aromatase and dnd. No a*mh* expression was observed in 16% of the samples while cyp11b was expressed at all the stages with a lot of expression variations (13.20 ± 8.5 counts). However, even though the gene coding for the aromatase was never significantly differentially expressed among cohorts, several genes known as regulators of this enzyme were expressed in the early developmental stages that we surveyed (*folx2a*, *crebbpa*, *crebbpb*, and *nfkb1* as activators and *lss* and *gata4* as inhibitors). All cohorts, including C3, showed both activation and inhibition of the aromatase during the development.

It should be reminded that post-transcriptional control mechanisms may also be involved in the final outcome of the steroidogenesis pathway. Those mechanisms can activate, inactivate, or destroy the RNA. Thus, even if we found gene higher expression, the protein products can be nonexistent. According to Furlan et al. (e.g. 2021), there are RNA highly stable which will last over time and, even if the corresponding gene has a low activity, we can have a relatively high number of copies of mRNA. In contrast, a very active gene can produce unstable RNA which will disappear quickly and then, a small number of copies could be observed. Moreover, there are some mechanisms that reduce the half-life of the RNA such as adenylate- and uridylate-rich elements added in the 3′ untranslated regions, which then allow the fixation of proteins involved in the RNA degradation (Bevilacqua et al. 2003). Those mechanisms can explain the early presence of some genes predicted to be significant far later in the development of winter flounder (like hsd11b2 for the 11-ketotestosterone) or, in the opposite, the absence of some key markers, like the aromatase or the amh.

For the larvae, the RNA extractions were done on pools based on the hypothesis that the temperature would have had a sufficient strong effect on the phenotypic sex determinism to be observed. Our study had for objectives to determine if the temperature had an effect on

the sexual differentiation of young stages of development of winter flounder. Obviously, temperature influenced larval and early juvenile steroidogenesis pathway, but not in a way that could be associated with sex-ratio imbalance. Interestingly, there is an evolution over time of the expression patterns and even between the cohorts but, no clear expression was found in favor of male phenotype, except for four genes among C3 which corresponds to the highest temperature regime and thus, to the group in which we expected a sex-ratio imbalance in favor of males. In nature, larvae live in the water column and are dispersed according to water currents that may be characterized by different temperature conditions. Once metamorphosis occurs, they settle on water bottom where temperature conditions are being considered to be more stable. It will then be important to pursue similar studies during juvenile development but also using different temperature treatments during juvenile rearing.

DISCUSSION GÉNÉRALE

Cette section ne reprend pas la discussion des résultats telle que présentée dans la version de l'article à soumettre, mais vise plutôt à expliciter certaines difficultés rencontrées, les choix expérimentaux qui ont été faits et les perspectives pour des travaux futurs.

Au cours de ma maitrise, j'ai cherché à déterminer l'effet de la température sur le phénotype sexuel chez la plie rouge via une approche transcriptomique novatrice. Nous avons observé les niveaux d'expression de l'ensemble des gènes impliqués dans la stéroïdogenèse durant le développement larvaire et chez des juvéniles, ce qui n'avait jamais été fait auparavant à des stades aussi précoces chez des poissons plats (cardeau de Floride, Luckenbach et al. 2004; plie japonaise, Kitano et al. 1999). Bien que quelques problèmes aient été rencontrés au cours de cette étude, les données de séquençage obtenues pour l'ensemble du transcriptome ouvrent de nombreuses possibilités pour des projets futurs.

Parmi les problèmes rencontrés, nous avons eu des difficultés à extraire l'ARN sur des individus isolés. Les expériences ont donc dû être effectuées sur des pools de larves, puis sur des juvéniles suffisamment gros pour être extraits individuellement. Extraire les larves séparément aurait pu permettre de voir plus clairement les patrons de développement sexuel et d'établir des ratios mâles/femelles individuels pour chaque régime de température. Cependant, au vu des résultats observés chez les autres espèces ainsi qu'à la station aquicole de Pointe-au-Père, nous estimions que la température aurait un effet suffisamment important sur le sexe-ratio pour que cela soit visible sur les patrons d'expression malgré l'utilisation de pools.

Par ailleurs, de l'ARN de juvéniles et de larves à différents stades de développement a été conservés à -80°C et pourra donc par la suite servir à des analyses complémentaires à cette étude. Des duplicatas d'ARN de tous les échantillons séquencés ont aussi été gardés. L'obtention d'ADNc via RT-PCR permettrait une observation plus poussée de l'expression de certains gènes clés de la stéroïdogenèse. Nous avons aussi de l'ARN de larves âgées de 50 jours, ce qui permettrait d'observer les patrons d'expression à un stade intermédiaire à la

transition larves-juvéniles. Du matériel appartenant à des juvéniles plus âgés (2 mois) ainsi qu'à des juvéniles d'un an est aussi disponible et pourrait être utilisé pour des questions portées sur la suite du développement des individus. Enfin, lors des élevages, chaque groupe de juvéniles ayant accompli la métamorphose était séparé en deux bassins distincts, l'un à une température moyenne de 10,7°C et l'autre à 13°C. Cependant, l'analyse des juvéniles exposés à 13°C représentait une quantité de données trop importante pour être traitée au cours de ma maîtrise et nous avons décidé de nous concentrer sur les juvéniles élevés dans des conditions plus similaires aux conditions naturelles. En effet, en plus de tout le matériel génétique encore disponible et stocké à -80°C, la quantité de données générées lors du séquençage des ARNm aux cinq stades de développement était particulièrement importante. Nous nous sommes concentrés sur les gènes de la stéroïdogenèse, mais la masse de données que nous avons en notre possession nous permettrait par exemple de refaire entièrement les analyses transcriptomiques pour des processus autres que la stéroïdogenèse ou encore pour étudier la métamorphose ou la croissance.

Nous avons choisi de suivre trois cohortes exposées à différents régimes de températures similaires aux conditions du Saint-Laurent, typiques de la période de reproduction. Cependant, il aurait aussi pu être intéressant de suivre des larves élevées à des conditions contrôlées et fixes en utilisant des différences de température beaucoup plus tranchées (par exemple 5, 7,5 et 10°C) comme cela a pu être fait dans d'autres études chez des poissons (plie japonaise, Kitano et al. 1999; cardeau hirame, Yamamoto 1999; bar européen, Navarro-Martin et al. 2011; médaka, Sato et al. 2005). Cependant, l'expérience conduite au cours de ma maîtrise a permis de reproduire au mieux les conditions sur le terrain et donc de mieux appréhender les effets du réchauffement des eaux sur les populations de plie rouge. À noter qu'un projet portant sur des échantillonnages de larves de plie rouge en milieu naturel est actuellement en cours dans la Baie-des-Chaleurs (Leblond et al. comm. pers.).

CONCLUSION

En conclusion, notre étude n'a pas permis de trouver un stade thermosensible chez la plie rouge, mais il semble que la température pourrait avoir un effet sur la voie de la stéroïdogenèse notamment avant la métamorphose en juvéniles. Ces résultats ont été observés à la fois pour l'expression des 500 gènes montrant la plus grande variance au sein de l'ensemble des ARNm et à la fois pour les gènes spécifiquement impliqués dans la stéroïdogenèse. Chez les larves, nous avons pu observer à j40 de légères différences entre les cohortes en ce qui a trait aux niveaux d'expression des gènes impliqués dans cette voie. Cette différence est devenue bien plus marquée chez les juvéniles issus des larves exposées au régime de température le plus élevé, avec des patrons d'expression significativement différents de ceux observés pour les deux autres cohortes. Cependant, parmi les gènes significativement différentiellement exprimés, nous n'avons pas retrouvé les marqueurs sexuels précoces reconnus dans la littérature chez les poissons (*cyp19a1a* (aromatase), *dnd* et *foxl2* comme marqueurs femelles et *amh* et *cyp11b* comme marqueurs mâles). Même si les patrons d'expression des autres gènes ont montré que la voie de la stéroïdogenèse semblait bel et bien influencée par la température, nous n'avons pu mettre en évidence un déséquilibre du sexe-ratio ni une favorisation du phénotype mâle et nous n'avons pu en déterminer les conséquences. Malgré tout, la conservation d'une quantité importante de matériel génétique à -80°C ainsi que les nombreuses données transcriptomiques déjà en notre possession pourront permettre de compléter ces analyses, mais aussi de répondre à d'autres questions concernant la physiologie de la plie rouge comme, par exemple, une observation plus poussée des comportements des gènes au cours de la croissance ou au moment de la métamorphose.

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ANNEXES

Annexe I. Liste des noms complets des gènes de la stéroïdogenèse et des abréviations correspondantes

Genes	Complete Names
Amh	Anti-Mullerian Hormone
Axin1	Axin 1
Bmp2b	Bone Morphogenetic Protein 2
Cbfb	Core binding factor beta
Cbx2	Chromobox homolog 2
Ccnd1	Cyclin D1
Chd1l	Chromodomain-helicase-DNA-binding protein 1-like
Cited1	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 1
Cps1	carbamoyl-phosphate synthase 1, mitochondrial
Crebbpa	CREB binding protein a
Crebbpb	CREB binding protein a
Ctnnb1	β-catenin
Cxxc5a	CXXC finger protein 5a
Cxxc5b	CXXC finger protein 5b
Dhcr24	24-dehydrocholesterol reductase
Dhcr7	7-dehydrocholesterol reductase
Dhh	Desert hedgehog signaling molecule
Dmrt1	Doublesex and mab-3 related transcription factor 1
Dnd1	Dead end 1
Ebag9	Estrogen receptor binding site associated antigen 9
Ebp	EBP cholestenol delta-isomerase
Emx2	Empty spiracles homeobox 2
Ep300a	E1A binding protein p300 a
Ep300b	E1A binding protein p300 b
Esr1	Estrogen receptor 1

Esr2a	Estrogen receptor 2a
Esr2b	Estrogen receptor 2b
Fdft1	Farnesyl-diphosphate farnesyltransferase 1
Fgf13b	Fibroblast growth factor 13b
Fgfr2	Fibroblast growth factor receptor 2
Fkbp4	FKBP prolyl isomerase 4
Foxa1	Forkhead box A1
Foxa2	Forkhead box A2
Foxa3	Forkhead box A3
Foxl2a	Forkhead box L2
Foxl3	Forkhead box L3
Fsta	Follistatin a
Gadd45ga	Growth arrest and DNA-damage inducible 45 gamma
Gata3	GATA-binding protein 3
Gata4	GATA-binding protein 4
Gpam	Glycerol-3-phosphate acyltransferase
Gpat2	Glycerol-3-Phosphate Acyltransferase 2
Gpat3	Glycerol-3-Phosphate Acyltransferase 3
Greb1	Growth regulating estrogen receptor binding 1
Gsdf	Gonadal somatic cell derived factor
Gsk3ab	Glycogen synthase kinase 3 alpha-beta
Gsk3ba	Glycogen synthase kinase 3 beta-alpha
Hoxa10b	Homeobox A10
Hsd11b2	Hydroxysteroid (11-beta) dehydrogenase 2
Hsd17b1	Hydroxysteroid (17-beta) dehydrogenase 1
Hsd17b10	Hydroxysteroid (17-beta) dehydrogenase 10
Hsd17b12a	Hydroxysteroid (17-beta) dehydrogenase 12a
Hsd17b12b	Hydroxysteroid (17-beta) dehydrogenase 12b

Hsd17b2	Hydroxysteroid (17-beta) dehydrogenase 2
Hsd17b3	Hydroxysteroid (17-beta) dehydrogenase 3
Hsd17b4	Hydroxysteroid (17-beta) dehydrogenase 4
Hsd17b7	Hydroxysteroid (17-beta) dehydrogenase 7
Hsd17b8	Hydroxysteroid (17-beta) dehydrogenase 8
Hsd3b7	Hydroxysteroid (3-beta) dehydrogenase 7
Jun	Jun proto-oncogene, AP-1 transcription factor subunit
Kctd6a	Potassium channel tetramerization domain containing 6a
Kctd6b	Potassium channel tetramerization domain containing 6b
Kdm1a	Lysine (K)-specific demethylase 1a
Kdm2aa	Lysine (K)-specific demethylase 2Aa
Kdm3b	Lysine (K)-specific demethylase 3b
Kdm4aa	Lysine (K)-specific demethylase 4Aa
Kdm5a	Lysine (K)-specific demethylase 5a
Kdm5c	Lysine (K)-specific demethylase 5c
Kdm6a	Lysine (K)-specific demethylase 6a
Kpna2	Karyopherin alpha 2
Lbr	Lamin B receptor
LOC117751869	Cyp26b1 / Cytochrome P450 family 26 subfamily B member 1
LOC117752562	Cyp26a1 / Cytochrome P450, family 26, subfamily A, polypeptide 1
LOC117754230	Aromatase / Cyp19a1a / Cytochrome P450 family 19 subfamily A polypeptide 1A
LOC117755992	Cyp1b1-like / Cytochrome P450, family 1, subfamily B, polypeptide 1
LOC117755993	Cyp1b1-like / Cytochrome P450, family 1, subfamily B, polypeptide 1
LOC117760834	Cyp2j2-like / Cytochrome P450, family 2, subfamily J, polypeptide 2

LOC117760836	Cyp2j2-like / Cytochrome P450, family 2, subfamily J, polypeptide 2
LOC117763762	Cyp2f2-like / Cytochrome P450, family 2, subfamily F, polypeptide 2
LOC117763764	Cyp2f2-like / Cytochrome P450, family 2, subfamily F, polypeptide 2
LOC117763766	Cyp2f2-like / Cytochrome P450, family 2, subfamily F, polypeptide 2
LOC117767306	Cyp2u1 / Cytochrome P450, family 2, subfamily U, polypeptide 1
LOC117770886	Cyp51a1 / Lanosterol 14-alpha demethylase
LOC117771222	Cyp120 / Cytochrome P450, family 120
LOC117772378	Cyp2g1-like / Cytochrome P450, family 2, subfamily G, polypeptide 1
LOC117772381	Cyp2g1-like / Cytochrome P450, family 2, subfamily G, polypeptide 1
LOC117772382	Cyp2g1-like / Cytochrome P450, family 2, subfamily G, polypeptide 1
LOC117774336	Cldn7-like / Claudin-7-A-like
LOC117774338	Vsig1 / V-set and immunoglobulin domain-containing protein 1-like
LOC117775246	Cyp1b1 / Cytochrome P450, family 1, subfamily B, polypeptide 1
LOC117776041	Cyp26c1 / Cytochrome P450, family, subfamily C, polypeptide 1
LOC117776540	Cyp4b / Cytochrome P450, family, subfamily B
LOC117777191	Cyp11b / Cytochrome P450 family 11 subfamily B
LOC117777913	Cyp7a1 / Cytochrome P450, family 7, subfamily A, polypeptide 1
LOC117778604	Cyp7b1 / Cytochrome P450, family 7, subfamily B, polypeptide 1
Lss	Lanosterol synthase
Map3k4	Mitogen-activated protein kinase kinase kinase 4
Mapk1	Mitogen-activated protein kinase 1
Mis18a	MIS18 kinetochore protein A

Mis18bp1	MIS18 binding protein 1
Msmo1	Methylsterol monooxygenase 1
Mycbp	MYC binding protein
Mycbp2	MYC binding protein 2
Mycbpap	Mycbp associated protein
Nfkb1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
Nr0b1	Nuclear receptor subfamily 0, group B, member 1
Nr5a1a	Nuclear receptor subfamily 5 group A member 1a
Nr5a1b	Nuclear receptor subfamily 5 group A member 1b
Nr5a2	Nuclear receptor subfamily 5, group A, member 2
Nrip1b	Nuclear receptor interacting protein 1b
Nsdhl	NAD(P) dependent steroid dehydrogenase-like
Pgd	Phosphogluconate dehydrogenase
Porb	P450 (cytochrome) oxidoreductase b
Ptges3a	Prostaglandin E synthase 3a
Ptges3b	Prostaglandin E synthase 3b
Rbfox2	RNA binding fox-1 homolog 2
Rspo1	R-spondin 1
Runx1	RUNX family transcription factor 1
Sc5d	Sterol-C5-desaturase
Soat1	Sterol O-acyltransferase 1
Soat2	Sterol O-acyltransferase 2
Sp1	Specificity Protein 1 Transcription Factor
Sqlea	Squalene epoxidase a
Srd5a1	Steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1)
Srd5a2b	Steroid-5-alpha-reductase, alpha polypeptide 2b
Srd5a3	Steroid 5 alpha-reductase 3
Stc2a	Stanniocalcin 2a

Sts	Steroid sulfatase, isozyme S
Tm7sf2	Transmembrane 7 superfamily member 2
Tnfsf13b	TNF superfamily member 13b
Uggt2	UDP-glucose glycoprotein glucosyltransferase 2
Usf1	Upstream transcription factor 1
Usf2	upstream transcription factor 2
Wnt4	Wingless-type MMTV integration site family member 4
Wnt4b	wingless-type MMTV integration site family, member 4b
Wt1a	Wilms' tumor 1 transcription factor a
Wt1b	Wilms' tumor 1 transcription factor b
Znf217	Zinc finger protein 217

Annexe II. Exemple des résultats obtenus concernant la significativité des niveaux d'expression des gènes impliqués dans la stéroïdogenèse : comparaison des larves j20 C3 par rapport aux larves j20 C1.

Genes	Base Mean	Log2 FoldChange	LfcSE	Stat	Pvalue	Padj
Amh	2,7985	1,6093	1,4347	1,1217	0,2619	1
Axin1	20,4020	-0,2147	0,6337	-0,3388	0,7347	1
Bmp2b	97,9442	-0,0125	0,2091	-0,0596	0,9524	1
Cbfb	53,4043	0,1833	0,4061	0,4513	0,6518	1
Cbx2	216,4175	-0,0216	0,2022	-0,1068	0,9149	1
Ccnd1	251,4532	0,2711	0,1526	1,7765	0,07564	1
Chd1l	174.0368	-0.0653	0.3136	-0.2081	0.8351	1
Cited1	43.6429	0.3355	0.2951	1.1368	0.2556	1
Cps1	63.9740	-0.2381	0.4836	-0.4922	0.6225	1
Crebbpa	578.7528	0.0536	0.1404	0.3817	0.7026	1
Crebbpb	619.6851	0.0283	0.1338	0.2115	0.8325	1
Ctnnb1	2873.2157	-0.0244	0.1619	-0.1507	0.8802	1
Cxxc5a	571,0999	0,0497	0,1918	0,2594	0,7953	1
Cxxc5b	943,8261	-0,0028	0,1782	-0,0156	0,9875	1
Dhcr24	1282,7665	0,0105	0,4164	0,0252	0,9798	1
Dhcr7	1127.1910	-0.0943	0.5964	-0.1581	0.8743	1
Dhh	7.3926	0.1212	1.0039	0.1207	0.9039	1
Dmrt1	0.0586	1.8911	7.8800	0.2400	0.8103	1
Dnd1	0.5335	-0.1810	2.9690	-0.0610	0.9513	1
Ebag9	720.9178	-0.0236	0.1612	-0.1466	0.8834	1
Ebp	861.5694	-0.3116	0.4464	-0.6981	0.4851	1
Emx2	74.4527	0.3486	0.2504	1.3922	0.1638	1
Ep300a	562.7380	-0.0415	0.1224	-0.3390	0.7346	1
Ep300b	881.6310	0.0181	0.1667	0.1084	0.9136	1
Esr1	20,5849	-0,0585	0,3820	-0,1531	0,8783	1
Esr2a	9,3892	0,0598	0,9045	0,0661	0,9472	1

Esr2b	8,4527	-0,8940	1,3296	-0,6724	0,5013	1
Fdft1	615,0213	-0,1612	0,3324	-0,4848	0,6277	1
Fgf13b	231,6062	0,0596	0,1374	0,4338	0,6644	1
Fgfr2	261,4433	0,2361	0,4283	0,5512	0,5814	1
Fkbp4	1221,3755	0,0138	0,1459	0,0944	0,9247	1
Foxa1	1,5072	2,3833	1,5988	1,4907	0,1360	1
Foxa2	246,2348	0,0844	0,2629	0,3210	0,7481	1
Foxa3	160,1231	-0,0319	0,3122	-0,1020	0,9187	1
Foxl2a	53,3008	0,1609	0,3669	0,4386	0,6609	1
Foxl3	14,1000	0,8506	0,5809	1,4643	0,1431	1
Fsta	253,1705	-0,1388	0,2780	-0,4995	0,6174	1
Gadd45ga	428,4555	-0,1489	0,4839	-0,3077	0,7583	1
Gata3	142,7125	0,0683	0,1530	0,4460	0,6555	1
Gata4	5,5688	1,2227	0,8193	1,4924	0,1356	1
Gpam	71,4200	0,0368	0,5800	0,0635	0,9493	1
Gpat2	2,3661	1,5793	1,0479	1,5071	0,1317	1
Gpat3	59,3944	0,7188	0,3912	1,8373	0,0661	1
Greb1	6,5679	0,2442	0,7511	0,3251	0,7451	1
Gsdf	1,5858	0,0000	4,8183	0,0000	1	1
Gsk3ab	1012,6006	-0,1144	0,1811	-0,6320	0,5273	1
Gsk3ba	1440,2259	-0,1098	0,1488	-0,7383	0,4603	1
Hoxa10b	159,6361	-0,3146	0,2408	-1,3067	0,1912	1
Hsd11b2	13,1287	-0,1559	0,9100	-0,1713	0,8640	1
Hsd17b1	1,3183	0,1133	2,0636	0,0549	0,9562	1
Hsd17b10	1158,7316	-0,0162	0,1921	-0,0844	0,9327	1
Hsd17b12a	151,5744	-0,4863	0,2632	-1,8475	0,0646	1
Hsd17b12b	607,8443	-0,1062	0,1932	-0,5496	0,5826	1
Hsd17b2	3,3600	-0,8051	1,1385	-0,7071	0,4794	1
Hsd17b3	26,6080	-0,0216	0,6307	-0,0343	0,9726	1

Hsd17b4	535,5472	0,0925	0,2138	0,4328	0,6651	1
Hsd17b7	121,2969	-0,1914	0,2804	-0,6826	0,4948	1
Hsd17b8	209,1847	0,2571	0,2773	0,9271	0,3538	1
Hsd3b7	151.4120	-0.1484	0.2414	-0.6145	0.5389	1
Jun	393.2013	0.2450	0.3511	0.6978	0.4852	1
Kctd6a	73.2687	-0.0579	0.2572	-0.2252	0.8218	1
Kctd6b	101.0001	0.0997	0.2556	0.3902	0.6963	1
Kdm1a	328.7775	0.2293	0.2023	1.1334	0.2570	1
Kdm2aa	341.1696	-0.0588	0.1862	-0.3157	0.7522	1
Kdm3b	1144.9279	0.0568	0.1362	0.4167	0.6769	1
Kdm4aa	305.7031	0.1626	0.1790	0.9085	0.3636	1
Kdm5a	572.2575	-0.0744	0.1612	-0.4616	0.6443	1
Kdm5c	835.2282	-0.0812	0.1329	-0.6107	0.5414	1
Kdm6a	189.7773	-0.0022	0.2300	-0.0097	0.9922	1
Kpna2	1425,8568	0,2659	0,4263	0,6236	0,5328	1
Lbr	42.8601	0.5400	0.5429	0.9948	0.3198	1
LOC117751869	48.4578	0.4561	0.3710	1.2296	0.2188	1
LOC117752562	27.2918	0.1160	0.6679	0.1737	0.8621	1
LOC117754230	0.5982	-2.2989	3.6958	-0.6220	0.5339	1
LOC117755992	171,2214	0,3107	0,3647	0,8517	0,3943	1
LOC117755993	138,8585	0,4385	0,2429	1,8049	0,07108	1
LOC117760834	897,3436	-0,2791	0,4297	-0,6495	0,51599	1
LOC117760836	343,9788	-0,2238	0,3223	-0,6946	0,4872	1
LOC117763762	91,3203	0,6654	1,0044	0,6624	0,5076	1

LOC117763764	977,9847	-0,1187	0,3591	-0,3305	0,7410	1
LOC117763766	32,6655	-0,0571	0,4666	-0,1223	0,9026	1
LOC117767306	48,1553	0,1259	0,2904	0,4335	0,6647	1
LOC117770886	1252,1309	0,0843	0,4712	0,1788	0,8581	1
LOC117771222	23,3107	-0,3587	0,4384	-0,8181	0,4133	1
LOC117772378	102,6732	-1,1165	1,0915	-1,0229	0,3064	1
LOC117772381	41,9857	-1,2750	1,1863	-1,0748	0,2825	1
LOC117772382	39,1909	0,4043	0,4610	0,8769	0,3806	1
LOC117774336	533,3135	-0,1720	0,3076	-0,5594	0,5759	1
LOC117774338	130,8792	-1,2879	0,9089	-1,4170	0,1565	1
LOC117775246	41,9934	-0,0355	0,3956	-0,0898	0,9285	1
LOC117776041	11,2396	0,8149	0,8382	0,9722	0,3310	1
LOC117776540	373,8370	0,0312	0,1920	0,1626	0,8708	1
LOC117777191	13,5596	-0,5530	0,6864	-0,8056	0,4205	1
LOC117777913	373,0430	0,0574	0,5916	0,0969	0,9228	1
LOC117778604	73,4799	-0,0830	0,3245	-0,2557	0,7982	1
Lss	1067,1244	0,0797	0,5561	0,1434	0,8860	1
Map3k4	376,0664	-0,1171	0,1709	-0,6854	0,4931	1
Mapk1	91,2809	0,4711	0,2493	1,8897	0,0588	1
Mis18a	89,5509	0,3426	0,2386	1,4359	0,1510	1
Mis18bp1	78,0043	-0,3152	0,2636	-1,1957	0,2318	1
Msmo1	12067,1123	0,0428	0,4771	0,0897	0,9285	1
Mycbp	251,8199	0,0033	0,2710	0,0122	0,9902	1
Mycbp2	2265,3663	-0,0106	0,1572	-0,0673	0,9464	1
Mycbpap	12,7148	0,0054	0,6225	0,0087	0,9931	1

Nfkb1	254,1338	-0,0030	0,1937	-0,0157	0,9874	1
Nr0b1	48,9292	0,0624	0,3418	0,1826	0,8551	1
Nr5a1a	0,3563	-0,7482	5,4317	-0,1378	0,8904	1
Nr5a1b	0,8161	-0,4276	3,6257	-0,1179	0,9061	1
Nr5a2	208,1191	0,1135	0,1781	0,6372	0,5240	1
Nrip1b	385,2114	-0,1061	0,1433	-0,7409	0,4588	1
Nsdhl	1008,3447	0,0216	0,3668	0,0590	0,9530	1
Pgd	2177,4480	-0,2827	0,1789	-1,5800	0,1141	1
Porb	1519,6684	-0,0111	0,2129	-0,0520	0,9586	1
Ptges3a	386,4442	-0,3697	0,1773	-2,0854	0,0370	1
Ptges3b	3546,5327	-0,0987	0,1325	-0,7444	0,4566	1
Rbfox2	1241.1293	0.0579	0.1512	0.3832	0.7015	1
Rspo1	12.4721	-0.8534	0.5888	-1.4493	0.1473	1
Runx1	32.9027	-0.0196	0.4648	-0.0422	0.9664	1
Sc5d	1015.1695	0.1598	0.3084	0.5183	0.6042	1
Soat1	40.3680	0.1880	0.3477	0.5408	0.5886	1
Soat2	17.0801	-0.0295	0.5182	-0.0570	0.9545	1
Sp1	278.2136	-0.1263	0.1573	-0.8029	0.4220	1
Sqlea	1441.0516	-0.1835	0.6620	-0.2772	0.7816	1
Srd5a1	46.2786	0.0861	0.3536	0.2435	0.8076	1
Srd5a2b	1.9161	-2.1005	1.6581	-1.2668	0.2052	1
Srd5a3	27.4289	-0.3614	0.3045	-1.1871	0.2352	1
Stc2a	118.3256	0.2697	0.3041	0.8870	0.3751	1
Sts	55.3924	1.0761	0.4636	2.3211	0.0203	1
Tm7sf2	1273.9847	-0.0410	0.4733	-0.0867	0.9309	1
Tnfsf13b	8.4613	-1.3596	0.9342	-1.4554	0.1456	1
Uggt2	57.0994	0.3549	0.4029	0.8810	0.3783	1
Usf1	575.6746	0.1680	0.1858	0.9040	0.3660	1

Usf2	172.6044	0.1220	0.1656	0.7371	0.4611	1
Wnt4	30.3133	0.1955	0.3427	0.5706	0.5683	1
Wnt4b	139.5053	0.1174	0.2586	0.4540	0.6499	1
Wt1a	14.4862	0.7906	0.4764	1.6594	0.0970	1
Wt1b	49.0035	-0.1482	0.3209	-0.4619	0.6442	1
Znf217	55.6334	-0.0585	0.2249	-0.2603	0.7946	1