Le déterminisme sexuel de l'huître crassostrea gigas : du phénotype aux facteurs moléculaires sous-jacents

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Normandie Université

THÈSE

Pour obtenir le diplôme de doctorat

Spécialité PHYSIOLOGIE ET BIOLOGIE DES ORGANISMES - POPULATIONS - INTERACTIONS

Préparée au sein de l'Université de Caen Normandie

Le déterminisme sexuel de l'huître Crassostrea gigas : du phénotype aux facteurs moléculaires sous-jacents

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Thèse soutenue publiquement le 12/12/2019 devant le jury composé de		
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UNIVERSITÉ CAEN NORMANDIE

ED 497 NBISE
Normande de Biologie Intégrative, Santé, Environnement

BOREA
Biologie des organismes et écosystèmes aquatiques

Avant-propos

Ces travaux de recherche ont été réalisés au sein de l'Ecole Doctorale Normande de Biologie Intégrative, Santé, Environnement (EdNBISE) sous la direction du Dr Anne-Sophie MARTINEZ et de l'encadrement scientifique du Dr Lionel DEGREMONT.

Ils se sont déroulés au laboratoire BOREA "Biologie des ORganismes et Ecosystèmes Aquatiques" (MNHN, CNRS-7208, IRD-207, Sorbonne Université, UCN, UA) de l'Université de Caen Normandie et au laboratoire " Génétique et Pathologie des Mollusques Marins" LGPMM / SG2M de l'IFREMER La Tremblade. Cette thèse a aussi bénéficié des infrastructures des stations IFREMER La Tremblade et Bouin.

Ce travail de recherche a été financé par l'IFREMER et la Région Normandie.

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Ecole doctorale Normande
Biologie Intégrative,
Santé, Environnement

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Remerciements

Je tiens à remercier vivement la disponibilité des membres du jury : le Docteur Jean-François Baroiller, Directeur de Recherche au CIRAD et le Docteur Yann Guiguen, Directeur de Recherche à l'INRA qui ont accepté de lire et de critiquer ce manuscrit en qualité de rapporteurs, ainsi que le Docteure Caroline Fabioux, Maître de Conférence à l'Université de Bretagne Occidentale et le Docteur Pierre Boudry, Directeur de Recherche à l'IFREMER qui ont bien voulu examiner ce travail. Je leur suis extrêmement reconnaissante d'avoir pu se rendre disponible afin de siéger dans mon jury de thèse.

Ce travail a été mené conjointement au sein de l'unité Biologie des Organismes et Ecosystèmes Aquatiques de l'Université de Caen et du Laboratoire Génétique et Pathologie des Mollusques Marins de l'Ifremer. Je remercie Monsieur Jean Prou et Madame Bénédicte Charrier, responsables de site, de m'avoir accueillie pendant ces trois années à la station de La Tremblade. Je tiens également à remercier le Docteure Sylvie Lapègue et le Docteur Christophe Stavrakakis, responsables de l'unité SG2M ainsi que Monsieur Christian Béchemin, responsable du LGPMM. Je remercie de la même manière le Professeur Pascal Sourdaine et le Docteure Céline Zatylny-Gaudin, responsables de l'unité BOREA sur le site caennais pour m'avoir permis d'effectuer une partie de mes travaux dans les locaux universitaires.

Après trois années passées sous sa houlette, il est à présent temps pour moi de remercier infiniment le Docteure Anne-Sophie Martinez. Ta très grande disponibilité -et cela malgré les kilomètres-, ton raisonnement scientifique, tes corrections & tes encouragements m'ont été d'une aide indéfectible. Merci infiniment pour ton implication et ta présence dans la dernière ligne droite ! Merci également de m'avoir fait confiance, de m'avoir accueillie chaleureusement lors de mes allers-retours caennais, d'avoir toujours trouvée un moment pour nos discussions scientifiques mais aussi d'avoir su prendre le temps de me connaître en tant que personne. Je te remercie d'avoir été une directrice scientifique remarquable et résolument humaine.

L'ensemble de mes travaux de recherches repose également sur la présence d'un second mentor sans qui cette thèse n'aurait pas eu lieu. C'est pourquoi je remercie très chaleureusement le Docteur Lionel Dégremont. Merci de m'avoir initiée à l'ostréiculture, de m'avoir confié « tes » huîtres par milliers, d'avoir été patient lors de mes erreurs et surtout d'avoir pris chaque année du temps pour les sexer. Tu m'as appris à relativiser face aux pertes de matériel biologique, d'accepter les imprévus & d'être organisée tout en gardant de la flexibilité. Merci pour tes conseils, corrections et relectures à chaque étape de ce travail de thèse. Ta passion pour la génétique et ton amour du travail bien fait m'ont accompagnée tout au long de cette thèse.

Je remercie l'équipe génétique du LGPMM. Un grand merci à toi JB, pour ton aide dans mes travaux, pour m'avoir initiée à la modélisation, pour nos nombreuses conversations scientifiques et pour avoir toujours trouvé un créneau disponible (et mémorisé) pour me recevoir (chose ô combien difficile !). Merci Abdellah, pour nos échanges passionnants aussi bien à la paillasse que dans les couloirs. J'aurai aimé prendre le temps d'en savoir un peu plus sur la cytogénétique... Un grand merci Elise ; tu as été un appui indéfectible tant sur les parcs ostréicoles qu'à l'écloserie. Je te souhaite d'obtenir plus de moyens (humain & matériel) pour les sorties sur l'estran et en mer. Merci Christophe pour ta bonne humeur. Merci Serge pour ton aide dans mes déboires informatiques, j'ai cru être un chat noir plus d'une fois. Merci également pour la préparation du séquenceur. Cela m'amène à remercier vivement Florence pour m'avoir appris à m'en servir mais aussi avoir pris du temps pour me former et être toujours disponible lors de mes questionnements et manipulations. Joël et Cyril, merci pour nos discussions.

Un grand merci à l'(ancienne) équipe écloserie du LPGMM : Fred, pour avoir été à l'écoute de mes besoins, Pascal P, Stéphane & JC. Je tiens tout particulièrement à remercier Jean-Pierre ; ma première année n'aurait pas été la même sans toi ! Merci de m'avoir accompagnée lors de très nombreuses sorties sur le terrain, pour m'avoir aidée dans le puçage et pour avoir réalisé des biométries. Merci d'avoir répondu présent quand j'en avais besoin. Une autre personne a donné de son temps et de son énergie dans le marquage des huîtres, il s'agit de Pascal S : Un grand merci à toi !

Sortir sur le parc inclut parfois l'utilisation du challand, je remercie ainsi Jean-Luc et Philippe de m'avoir acceptée à bord avec bonne humeur et convivialité. Merci également aux autres membres (dont des anciens) du LERPC : Patrick, Olivier L, Jean-François, Dimitri, Stéphane R, Stéphane G, Olivier C & Sylvie pour les superbes moments passés ensemble.

Merci à toute l'équipe de Bouin pour s'être occupé de « mes » huîtres à un moment ou à un autre de leur vie.

Merci à l'ensemble de l'équipe Pathologie. Grâce à l'emplacement du bureau des doctorants, j'ai pu interagir quotidiennement avec vous pour mon plus grand plaisir. Vous avez su prendre du temps pour m'expliquer les recherches et tâches qui vous occupaient et cette interaction a été très enrichissante. En particulier, merci Benjamin pour tes conseils, merci Nicole pour le partage de tes connaissances et protocoles, et également pour les commandes ! Agnès, Delphine T, Isabelle ; merci pour nos échanges. Cyrielle : tu as rendu certaines de mes journées moins grises par ta bonne humeur communicative et ton sourire alors merci ! Merci aussi pour les commandes d'azote liquide. Merci également à l'ensemble du LNR : Céline, Bruno, Estelle, Mathilde, Delphine S, Lydie & Coralie, pour nos rencontres.

Véronique et Florence, merci pour votre aide respective dans les méandres administratifs et les recherches d'articles souvent « préhistoriques » !

Je tiens également à remercier les personnes avec qui j'ai interagi à Caen. Pour commencer, merci aux membres de l'équipe Emerge : Aude, Christophe, Guillaume, Kristell, Clothilde, Floriane, Lorane. Merci à Myriam & à Catherine pour avoir régler des tâches administratives chronophages. Merci également à Nathalie, Juliette, Karine, Jean-Paul, Emilie, Marie-Pierre, Sandra, Fabienne, Christophe pour m'avoir aidée sur des questions statistiques ou logistiques ou permis de prendre du recul. Bonne continuation et bon courage à Laura, Aurore, Julie, Stéphanie, Floriane, Lorane, Maëva, Charles, Louis & Quentin pour la suite de vos carrières.

Je remercie les membres de mon CSI et ainsi que ceux qui ont siégés à mon comité de thèse pour leur disponibilité, leur critique et leur soutien.

Une thèse n'est viable qu'avec des financements c'est pourquoi je remercie la région Normandie et l'Ifremer pour ma bourse doctorale. Je remercie également la DS de l'Ifremer, l'école doctorale NBISE, la Malacological Society of London et le GDR Repro pour les bourses qu'ils m'ont accordée. Cela m'a permis d'effectuer deux congrès internationaux (Angleterre et Espagne) et une mobilité internationale (Australie). Un Grand Merci !

I truly thank Professor Abigail Elizur for her welcome at the GeneCology centre. Thank you to Dr Suwansa-ard Saowaros for the initiation to CLC software and for all the time you gave to me during my stay. Thanks to all the other student I met there and with whom I shared the office. It was a really nice and effective collaboration and I was very lucky to interact and see how research are conduct in your country. Thanks a lot !

Parce qu'une thèse est autant une expérience professionnelle qu'une aventure humaine riche en rencontres, je remercie les « jeunes » de passage à la station pour les moments de complicité, d'entraide et de solidarité : Alice, Agathe, Camille, Mélodie, Yoann, Paul, Marion, Bapsy, Nastasia, Tom, Gabriel, Paul & Mirna.

Je remercie « mes miss » du fond du cœur : Sandy, Leïla & Ophélie. Vous m'avez montré la voie en partageant vos hauts & vos bas, vos échecs & vos victoires. Ainsi je me suis sentie entourée et comprise. Vous voir terminer l'une après l'autre m'a démontré que c'était possible (même si vos états respectifs en fin de rédaction m'ont fait redouter d'autant plus mon tour) ! Merci pour votre amitié, votre soutien et pour le partage de vos expériences.

Je pense que pour être performant dans son travail, l'ambiance joue un rôle majeur. Ainsi ce fut un plaisir de partager mes journées avec vous mes colloc' de bureau. Merci pour votre écoute, nos pauses, nos débats, nos rires... Ces moments sont passés bien trop vite à mon goût. Merci Clément pour ta vision parfois différente mais intéressante des choses, pour le partage de références, d'informations, de bons plans & pour nos pauses détentes (car oui il en faut !). Nous voici presque arrivés, courage à toi pour ce sprint final ! Nicolas, je te remercie pour ta gentillesse, ta disponibilité et ta générosité. Milles merci d'avoir bien voulu partager ton appartement, je n'aurai pu rêver mieux ! Aurélie, je suis ravie de t'avoir rencontrée et d'avoir pu te connaître

avant nos mobilités respectives. Ton avis m'a souvent été précieux, tout comme les moments partagés ensemble loin du bureau. Merci pour ton amitié. Je tiens à vous souhaiter bon courage pour la suite et fin de vos thèses, vous y arriverez brillamment. Ma porte sera toujours ouverte et le téléphone allumé, surtout n'hésitez pas.

Petit mot pour Camille : Ca y est, à toi le flambeau ! Merci pour tous les moments passés ensemble tant à la station qu'à l'extérieur. Bonne chance dans cette aventure qu'est une thèse !

J'ai eu la chance de rencontrer des gens passionnés et passionnants au cours de ces trois années, je tiens encore une fois à tous les remercier ici. Certains de mes collègues d'hier sont devenus mes amis d'aujourd'hui : merci à vous.

Voici dix ans que je suis « sur la route » en changeant régulièrement de ville, de région voir de pays. Je n'ai oublié aucune de mes rencontres et elles m'ont toutes apporté quelque chose faisant de moi qui je suis aujourd'hui. Je voudrai ici les remercier car je ne vous oublie pas.

Merci à ma famille, ma marraine, à Léo et particulièrement à Guin's pour les longues soirées de travail passées ensemble. Surtout, je remercie mes parents pour m'avoir toujours soutenue dans mes choix, pour les conseils prodigués depuis tant d'années et pour votre amour. C'est grâce à vous que j'en suis là aujourd'hui ! Je termine ces remerciements en pensant à Louis, merci pour ton soutien, ta confiance et ton amour. Prête pour de nouvelles aventures ! On the road again...

« Pour réaliser ses rêves, il importe d'en avoir,

et de créer les conditions pour comprendre les obstacles

sur sa propre route. » Jacques Attali

« La mer. Il faut l'imaginer, la voir avec le regard d'un homme de jadis : comme une barrière

étendue jusqu'à l'horizon, comme une immensité obsédante omniprésente merveilleuse

énigmatique… A elle seule, elle est un univers, une planète. » Fernand Braudel

Publications et communications

Publications présentées dans le manuscrit :

Broquard C., Martinez AS., Maurouard E., Lamy JB., Dégremont L. (2020) Sex determination in the oyster *Crassostrea gigas* – a large longitudinal study of population sex ratio and individual sex changes. *Aquaculture,* 515

Broquard C., Martinez AS., Maurouard E., Berthelot JP., Dégremont L. Relationship between sex and growth of the Pacific oyster, *Crassostrea gigas* (En préparation)

Broquard C., Suwansa-ard S., Adeline B., Lepoitevin M., Dégremont L., Lamy JB., Morga B., Elizur A., Martinez AS. Gonadal transcriptomes associated to sex phenotypes reveal relevant genes of the time window of sex determination in *Crassostrea gigas*, a sequential hermaphrodite mollusc (En préparation)

Communications orales & affichée :

Broquard C., Dégremont L., Martinez AS (2017). Protandric sex in *Crassostrea gigas* : myth or reality? First answers from a large and long-term monitoring of sex changes. Physiomar17, 18-21 Septembre 2017, Cambridge, Angleterre (Communication orale).

Broquard C., Martinez AS., Dégremont L. (2019). Is there a link between sex/sex changes and weight in the Pacific oyster *Crassostrea gigas*? JEDNBISE19, 21-22 Mars 2019, Caen, France (Communication affichée).

Broquard C., Suwansa-ard S., Dégremont L., Lamy JB., Morga B., Elizur A., Martinez AS. (2019) Gonadal transcriptomic analysis in the oyster *Crassostrea gigas* – How to unravel the locks of a sequential hermaphrodite in order to identify potential male and female sex-determining genes. SEB Seville 2019, 2-5 Juillet 2019, Séville, Espagne (Communication orale).

Liste des illustrations

Liste des figures

Dans l'article 1 :

Dans l'article 2 :

Dans l'article 3 :

Liste des tableaux

Dans l'article 1 :

Dans l'article 2:

Dans l'article 3:

Liste des abréviations

ADAR: RNA-specific adenosine deaminase

BMP-2: Bone morphogenetic protein 2

CDA: Cytidine deaminase

ChIP: Chromatin ImmunoPrecipitation

CNA: Calcineurin A

CRISPR/Cas9:Clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9

DEA: Differential expression analysis

Dmrt: Doublesex and Mab3-related transcription factor

DUI: Doubly uniparental inheritance

EMSA: Electrophoretic mobility shift assay

ER: Estrogen receptor

FAO: Food and Agriculture Organization

Fgf9: Fibroblast growth factor-9

Foxl2: Forkhead boc L2

Fst: Follistatine

GO: Gene ontology

OsHV-1: Ostreid herpes virus 1

Opv : Ovocyte en pré-vitellogenèse

Ov: Ovocyte en vitellogenèse

Ovp: Ovocyte vitellogénique pédonculé

Rspo: R-spondin

SCA: Spermatogenesis and centriole associated

SD: Sex determination

SE: Standard error

SF1: Steroidogenic factor 1

SNP: Single nucleotide polymorphism

Sox: Sry-box

Sptr: Spermatide ronde

Sptm: Spermatide mature

Sry: Sex-determining region on Y chromosome

Sxl: Sex lethal

T: Tubule gonadique

Tr: Tissue de reserve

Tra: Transformer

TSSK: Testis-specific serine/threonine-protein kinase

Vg: Vitellogenin

Wnt4: Wingless-type MMTV integration site family, member 4

Xol-1: XO lethal protein 1

Table des matières

Introduction générale

L'huître creuse *Crassostrea gigas*

A. Sa classification biologique

L'huître creuse *Crassostrea gigas* (Thunberg, 1793) est un mollusque bivalve marin dont la position phylogénétique a été remise en question récemment par Salvi *et al.* (2014), Salvi & Mariottini (2017). Ces auteurs suggèrent la création d'un nouveau genre nommé *Magallana*. Ainsi l'espèce *C. gigas* serait renommée pour devenir *Magallana gigas.* Bien que cette modification taxonomique ait été acceptée dans le « world register of marine species » (WoRMS), son utilisation est aujourd'hui contestée par de nombreux scientifiques (Bayne *et al.,* 2017 ; Backeljau, 2018). Approuvant les remarques faites par ce consortium de scientifiques spécialistes de l'espèce quant aux limites des études menées par Salvi & Mariottini (2017), l'appellation *Crassostrea gigas* a été gardée dans l'ensemble des travaux présentés dans ce manuscrit. Sa classification biologique est définie comme suit :

Règne : *Animalia*

Sous-règne : *Bilateria*

Infra-règne : *Protostomia*

Super-Embranchement : *Lophotrochozoa*

Embranchement : *Mollusca*

Classe : *Bivalvia*

Sous-classe : *Pteriomorphia*

Ordre : *Ostreoida*

Super-Famille : *Ostreoidea*

Famille : *Ostreidae*

Sous famille : *Crassostreinae*

Genre : *Crassostrea* ou *Magallana*

Espèce : *Crassostrea gigas* ou *Magallana gigas*

Cette espèce possède également divers noms vernaculaires tels qu'huître creuse, huître japonaise ou huître creuse du Pacifique.

B. Son anatomie

L'huître creuse possède les caractéristiques anatomiques des mollusques à savoir un corps mou recouvert d'un manteau dont les cellules bordantes sécrètent du carbonate de calcium permettant la formation d'une coquille calcaire autour de l'animal. L'espacement entre la masse viscérale et le manteau est appelé cavité palléale. La présence de terminaisons nerveuses au niveau du repli médian du manteau confère à l'huître une perception sensorielle tactile. Son système circulatoire est lacunaire, ainsi l'hémolymphe, fluide incolore, circule dans les vaisseaux et immerge les divers tissus anatomiques. Cette circulation est permise grâce à la présence d'un cœur composé d'un ventricule unique et de deux oreillettes. L'hémolymphe oxygénée aux niveaux des branchies est envoyée vers la masse viscérale et le muscle adducteur par un réseau artériel, puis une fois désoxygénée, elle est propulsée à nouveau aux branchies. Le système digestif est constitué par un court œsophage, un estomac dans lequel se trouve le sac du stylet cristallin intervenant dans le broyage mécanique et chimique des aliments, un intestin et un rectum. Une gonade assure sa reproduction sexuée.

En tant que bivalve, *C. gigas* possède un corps aplati latéralement et une coquille constituée de deux valves distinctes reliées au niveau d'une charnière et pouvant s'ouvrir et se fermer par la contraction d'un muscle adducteur. Le renflement induit par la soudure des lobes du manteau au niveau de la charnière est appelé capuchon céphalique. L'huître creuse ne possède qu'une bouche et quatre palpes labiaux. Ces derniers ont une fonction de tri des particules et d'acheminement des nutriments contenus dans l'eau de mer filtrée vers l'orifice buccal. Une fois ingérées par la bouche, les particules planctoniques cheminent à travers tout le système digestif c'est-à-dire dans l'œsophage, l'estomac contenant un stylet cristallin assurant leur broyage, la glande digestive et l'intestin jusqu'à être éliminées par le rectum. Les particules à faible valeur nutritionnelle ou toxiques sont écartées du tractus digestif par les palpes labiaux, s'agrègent en pseudofèces puis sont rejetés à l'extérieur de l'animal. Le système nerveux est réduit à trois paires de ganglions regroupés près de l'œsophage et difficilement observable.

Ce lamellibranche se nourrit d'algues phytoplanctoniques présentes en suspension dans l'eau de mer. Pour ce faire, il crée un courant d'eau continu au sein de sa cavité palléale en agitant

les cils présents sur le bord de son manteau, ses palpes labiaux et les cils de sa double paire de branchies lamellaires, présentes des palpes labiaux jusqu'à l'anus. La filtration de l'eau de mer permise par les branchies permet la nutrition de l'animal par l'apport de particules organiques ainsi que sa respiratoire par l'apport d'oxygène dissous. Lorsque l'apport en nutriments et oxygène par filtration de l'eau de mer environnante n'est pas possible (fermeture des valves suite à un stress ou à une exondation), l'huître peut utiliser l'eau de mer contenue dans la cavité palléale puis puiser de l'énergie dans les réserves de glycogène contenues dans les cellules du bord du manteau.

Pour finir, l'appartenance de *C. gigas* à l'ordre des Ostreida lui confère une valve gauche (inférieure) creuse et courbe vers la charnière tandis que la valve droite (supérieure) est plane voire légèrement convexe.

C. Sa reproduction

L'huitre creuse se reproduit par fécondation externe de gamètes femelles par des gamètes mâles. La production de ces cellules sexuées au sein de l'animal nécessite la présence d'une gonade. Celle-ci se développe autour de la glande digestive à proximité du cœur et du muscle adducteur. Elle est composée d'un tissu mixte formé de tubules gonadiques et de tissu conjonctif vésiculeux appelé également tissu de réserve car les cellules vésiculeuses qui le constituent sont une source en glycogène (Li *et al.*, 2000). Cette ressource énergétique est mobilisée lors de la gamétogenèse qui a lieu au sein des tubules gonadiques. Ces derniers sont séparés du tissu de réserve par des cellules myoépithéliales et contiennent les cellules germinales et les cellules somatiques associées (Franco *et al.*, 2008). Ainsi, la formation des tubules gonadiques et la gamétogenèse se font au dépend du tissu conjonctif vésiculeux qui régresse proportionnellement (Berthelin *et al.*, 2000 ; Heude-Berthelin *et al.*, 2001 ; Franco *et al.*, 2008). Les tubules gonadiques s'ouvrent sur des canaux collecteurs composés par un épithélium germinatif (face interne) et un épithélium cilié (face externe) (Heude-Berthelin *et al.*, 2001). Par ces canaux, les gamètes sont transportés jusqu'aux gonoductes puis sont expulsés dans le milieu extérieur au niveau du gonopore situé en arrière du muscle adducteur. Après la libération des gamètes dans le milieu marin (ponte), la gonade subit une forte

modification. En effet, les tubules gonadiques qui occupaient la majorité de son aire régressent, les cellules germinales différenciées restantes dans les tubules sont phagocytées par des hémocytes infiltrés et le tissu conjonctif vésiculaire se développe (Berthelin *et al.,* 2000 ; Heude-Berthelin *et al.,* 2001 ; Franco *et al.,* 2008). Pendant cette phase de repos sexuel, un nombre limité de tubules gonadiques se maintient et possède des cellules, parmi lesquelles des cellules souches permettant l'initiation d'une prochaine gamétogenèse (Fabioux *et al.,* 2004).

L'organe reproducteur de l'huître creuse est ainsi labile, il se développe puis régresse en fonction de l'avancée de la gamétogenèse. Son évolution est cyclique et annuelle (en milieu naturel). A partir d'observations histologiques, la division de la gamétogenèse en stades a été proposée par Quayle (1969), Marteil (1976), Yakovlev (1977), Mann (1979), Robinson (1992), Steele (1998), Heude-Berthelin *et al.* (2001) et Lango-Reynoso *et al.* (2000). La majorité d'entre eux s'accordent sur un cycle débutant par le repos sexuel, puis la gamétogenèse qui aboutit à l'expulsion des gamètes. Cependant selon les auteurs, l'ensemble du cycle de reproduction peut être découpé en un nombre plus ou moins important de stades. Dans le cas des travaux présentés dans ce manuscrit, la classification utilisée est celle proposée par Heude-Berthelin *et al.* (2001) et définie comme suit :

Stade 0 : phase de repos sexuel où l'aire gonadique est très restreinte, avec un tissu conjonctif vésiculaire qui commence à se reformer et quelques tubules gonadiques peu volumineux contenant un nombre limité de cellules, germinales et somatiques associées, probablement souches. Le sexe de l'huître n'est pas identifiable à ce stade (Figure 1).

Stade 1 : phase d'initiation de la gamétogenèse, avec la prolifération des gonies. Les tubules gonadiques se développent et sont enchassés dans le tissu de réserve. A la fin de ce stade, la différentiation des cellules germinales en spermatogonies ou en ovogonies a eu lieu, permettant l'identification du sexe de l'animal (Figure 1).

Stade 2 : phase de méiose et de maturation des gonies. Elles se différencient alors en spermatocytes voire certaines en spermatides chez les mâles ou en ovocytes à divers stades de mise en réserve chez les femelles. Les tubules gonadiques sont volumineux, le tissu de réverse régresse et l'aire gonadique s'accroît encore (Figure 1).

Stade 3 : phase de maturité sexuelle où les spermatozoïdes et les ovocytes matures sont majoritaires au sein des tubules gonadiques. La gonade est alors l'organe le plus volumineux de l'huître. Le tissu conjonctif vésiculeux est très limité. S'en suit la libération des gamètes par ponte, qui peut être partielle ou totale. Les gamètes résiduels sont ensuite phagocytés et le tissu de réserve commence à nouveau à reconstituer des réserves en glycogène avant le début d'un nouveau cycle de reproduction (Figure 1).

Une fois expulsés, les gamètes mâles et femelles se rencontrent dans l'eau de mer permettant la fécondation des ovocytes par les spermatozoïdes. L'embryon qui en résulte passe rapidement par les stades morula, blastula et gastrula puis entame son développement larvaire. Lorsque la larve atteint le stade pédivéligère, son pied lui permet de se fixer à un substrat. La métamorphose qui s'en suit la transforme en naissain/juvénile puis une fois la première maturité sexuelle atteinte, l'huître est considérée comme adulte.

Chez *C. gigas*, il est impossible de prédire le sexe d'un animal au cycle gamétogénétique suivant car cette espèce peut changer de sexe au cours de sa vie. En effet, des suivis individuels ont permis de valider la présence d'hermaphrodisme séquentiel au sein de l'espèce *C. gigas* (Amemiya, 1929 ; Lannan, 1971 ; Guo *et al*., 1998 ; Lango-Reynoso, 1999 ; Park *et al*., 2012, Yasuoka & Yusa, 2016). La période du déterminisme sexuel de l'huître *C. gigas* adulte se situe entre le stade 3 d'un cycle gamétogénétique et le stade 0 du cycle suivant (Naimi *et al.,* 2009*a et b* ; Dheilly *et al.,* 2012 ; Santerre *et al.,* 2012, 2014). Il y aurait également en moyenne moins de 1% d'hermaphrodites simultanés au sein de sa population (Amemiya, 1929 ; Guo *et al*., 1998 ; Steele & Mulcahy, 1999 ; Normand *et al.,* 2009 ; Yasuoka & Yusa, 2016).

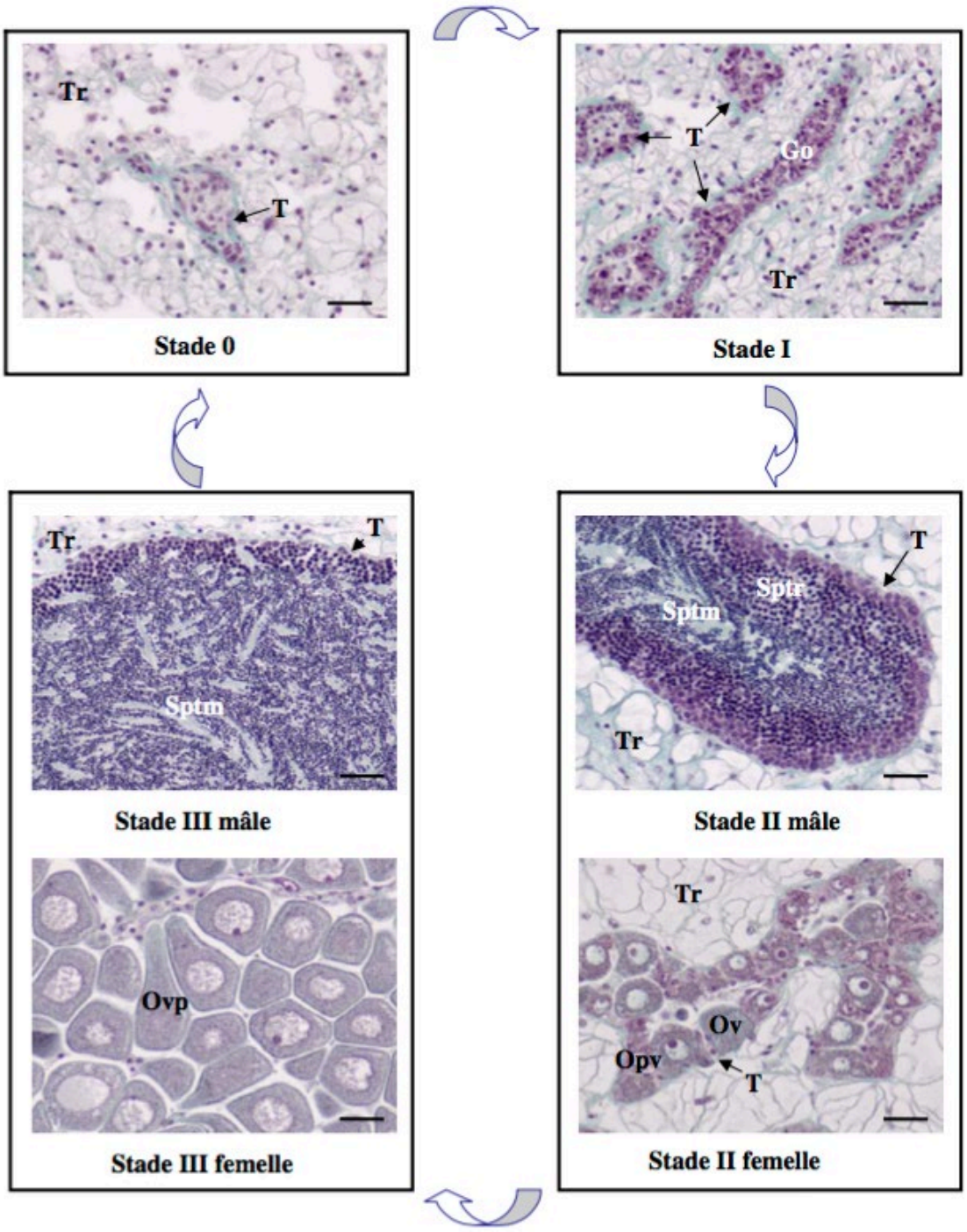

Figure 1: Cycle de reproduction de *Crassostrea gigas* adulte (Photos: AS Martinez)

Stade 0 de repos sexuel ; Stade I de prolifération des gonies ; Stade II de différenciation des gamètes et Stade III de maturité sexuelle. Opv : ovocyte en pré-vitellogenèse ; Ov : ovocyte en vitellogenèse ; Ovp : ovocyte vitellogénique pédonculé ; Sptr : spermatide ronde ; Sptm : spermatide mature ; T : tubule gonadique ; Tr : tissu de réserve. Barre d'échelle : 20µm.

D. Son génome

Crassostrea gigas a été le second mollusque à avoir son génome séquencé (Zhang *et al*., 2012), juste après celui de *Pinctada fucata* (Takeuchi *et al.,* 2012). Cependant, la caractérisation du génome de l'huître creuse est toujours en cours et demeure difficile, due d'une part à un nombre important de régions répétitives (62%), et d'autre part à un niveau élevé de polymorphisme (Zhang *et al.,* 2012). De très nombreuses études ont utilisé des approches transcriptomiques afin d'étudier l'expression des gènes dans divers tissus. Ainsi, la base de données GigaTON, accessible au public, regroupe 114 banques de séquences d'ARN de *Crassostrea gigas.* Les transcriptomes ont été réalisés en utilisant tous les stades du développement embryo-larvaire, les organes adultes, différents facteurs de stress environnementaux dont les métaux lourds, la température, la salinité et l'exondation (Rivière *et al.,* 2015). Les transcriptomes en lien avec la reproduction de l'huître creuse *C. gigas* sont en nombre plus limité (Dheilly *et al.,* 2012, 2014 ; Zhang *et al.,* 2014 ; Tong *et al.,* 2015 ; Yue *et al*., 2018). *Crassostrea gigas* possède également 10 paires de chromosomes, cependant aucun chromosome sexuel n'a été identifié (Ahmed & Sparks, 1967 ; Thiriot-Quiévreux, 1984). De plus, quatre paires de chromosomes (n°1, 5, 9 et 10) peuvent être affectés par la perte de l'un des deux homologues induisant des caryotypes aneuploïdes (Leitao *et al.,* 2001).

E. Son habitat

L'huître creuse *Crassostrea gigas* vit dans les écosystèmes marins et estuariens. A l'état embryonnaire jusqu'à larvaire pédivéligère, elle est planctonique vivant librement dans la colonne d'eau. Après la métamorphose de la larve pédivéligère, elle devient benthique en se fixant à des substrats, de préférence solides, de la zone intertidale à subtidale de faible profondeur – jusqu'à 40 mètres. Cependant, s'adaptant facilement à son environnement, elle peut également coloniser des fonds vaseux et sablo-vaseux (FAO, 2019). Etant un organisme marin, elle vit dans une eau de mer dont la salinité varie généralement entre 32 et 35‰, bien qu'elle supporte des variations plus importantes, faisant d'elle une espèce euryhaline. De plus, sa capacité à supporter de grandes amplitudes de températures -de 3,5 et 34°C-, la définit

comme une espèce eurytherme. Espèce poïkilotherme, sa température corporelle interne varie avec celle de son environnement. *C. gigas* consomme des producteurs primaires par filtration et est à son tour la proie d'espèces diverses (téléostéens, crustacés, échinodermes, gastéropodes, oiseaux et humaine). Sa place au sein de l'écosystème fait d'elle une espèce ingénieure. Originaire d'Asie, ces caractéristiques ont permis son introduction dans de nombreuses régions du monde au cours du XXème siècle notamment aux Etats-Unis et au Canada dès 1902, en Australie en 1947, en Nouvelle-Zélande en 1958, en France en 1966 et au Chili en 1982 (Grizel & Héral, 1991 ; Shatkin *et al.,* 1997 ; Troost, 2010). Au-delà de son implantation volontaire, des bancs sauvages se sont établis dans divers endroits du globe et impactent négativement des espèces endémiques en induisant une modification des écosystèmes (Ruesink *et al.,* 2005 ; Ruesink, 2007 ; Troost, 2010 ; Herbert *et al.,* 2016). Sa grande capacité de colonisation du milieu et la menace potentielle qu'elle représente lui valent d'être inscrite sur la liste des espèces invasives (Global Invasive Species Database).

F. Son importance économique

L'huître creuse *Crassostrea gigas* est le mollusque le plus utilisé en aquaculture avec une production mondiale de 639 030 tonnes en 2017 pour un budget de 1,2 milliard de dollars américains (hors Chine, FAO, 2019). En France, elle était l'espèce aquatique la plus vendue en 2016 avec 118 900 tonnes (FranceAgriMer, 2019). La valeur monétaire des ventes de *C. gigas* réalisées en France pour l'année 2016 représente 486 millions d'euros (FranceAgriMer, 2019). Cela fait d'elle l'espèce aquacole la plus marchande de l'hexagone cette année-là, loin devant la mytiliculture (55 200 tonnes, 149 millions d'euros) et la pisciculture, toutes espèces confondues (40 730 tonnes vendues pour 168 millions d'euros) (FranceAgriMer, 2019). Ces données montrent l'importance de *C. gigas* dans l'économie aquacole mondiale.

Les paramètres biologiques importants pour l'élevage de *C. gigas* en lien avec le sexe ou le déterminisme sexuel

L'approvisionnement en juvéniles est l'un des piliers de l'ostréiculture. La production française d'huîtres creuses *Crassostrea gigas* repose majoritairement sur du naissain provenant de captage naturel (70%) (Robert *et al.,* 2013). Deux aires géographiques constituent les principales zones de captage français, le Bassin Marennes-Oléron et le Bassin d'Arcachon (Robert *et al.*, 2013 ; Muehlbauer *et al.*, 2014). Cependant, le captage comporte des contraintes techniques pour les ostréiculteurs (possession d'un parc dans une zone de captage, adéquation entre le type de collecteur et la concession ostréicole…) ainsi qu'une instabilité de rendement inhérente à la variabilité de la reproduction de l'espèce dans son milieu naturel. Ainsi, 30% du naissain utilisé par les exploitations ostréicoles sont produit en écloserie (Robert *et al.*, 2013).

A. Le sexe-ratio des géniteurs

Les écloseries produisent du naissain d'huître *C. gigas* à partir de stocks de géniteurs. Afin de disposer de géniteurs matures et procéder à des pontes la plus grande partie de l'année, ces animaux sont souvent maintenus en eau de mer chauffée (favorise la gamétogénèse) ou refroidie (évite la ponte des animaux matures), puis nourrie afin de maintenir l'état physiologique optimal jusqu'aux croisements. Les croisements nécessitent souvent quelques dizaines ou centaines et peu de mâles (1 à quelques dizaines d'individus duploïdes pour la production d'huîtres diploïdes, 1 à 5 mâles tétraploïdes pour la production d'huîtres triploïdes). Hors, l'huître creuse *C. gigas* étant un hermaphrodite séquentiel (Amemiya, 1929 ; Lannan, 1971 ; Guo *et al.,* 1998 ; Lango Reynoso, 1999 ; Park *et al.,* 2012 ; Yasuoka & Yusa, 2016), son sexe-ratio est amené à varier chaque année. Ainsi, la maîtrise du sexe-ratio des géniteurs constitue un enjeu pour les écloseries commerciales d'huîtres creuses. Il faut aussi noter la présence d'hermaphrodisme simultané chez cette espèce (Amemiya, 1929 ; Guo *et al.,* 1998 ; Steele & Mulcahy, 1999 ; Normand *et al.*, 2009 ; Yasuoka & Yusa, 2016), cependant

sa fréquence très limité (<1%) permet de l'exclure des réflexions liées à la profession. Pour prévenir un sexe-ratio déséquilibré, il faut également une diversité d'âge au sein des géniteurs afin de prendre en compte la tendance protandre (individu mâle lors de la 1ère maturité et qui devient femelle au cours de sa vie) observée chez cette espèce (Guo *et al.*, 1998 ; Enriquez-Diaz *et al.*, 2009 ; Yasuoka & Yusa, 2016). Toutefois cette protandrie demeure sujette à question car d'autres travaux réalisés chez *C. gigas* indiquent un sexe-ratio à 1ère maturité sexuelle à dominante femelles (Amemiya, 1929 ; Lango Reynoso, 1999 ; Santerre *et al.*, 2013) ou encore équilibré (Fabioux *et al.*, 2005 ; Park *et al*., 2012).

La compréhension des facteurs moléculaires, génétiques et environnementaux influençant les sexe-ratios est nécessaire afin de réaliser les élevages dans des conditions optimales. Cependant, peu de travaux existent à ce sujet. D'un point de vue moléculaire, des profils d'expression de gènes potentiels du déterminisme sexuel ont été identifiés chez des juvéniles de *C. gigas* (Santerre *et al.*, 2013). Les profils d'expression en faveur d'un sexe, mesurés sur un échantillon de la population, étaient toujours en accord avec le sexe-ratio observé pour la population, suggérant ainsi une influence de ces facteurs moléculaires sur le déterminisme sexuel et donc sur le sexe-ratio. D'un point de vue génétique, la ploïdie de *C. gigas* ne semble pas influencer son sexe-ratio. En effet, les sexe-ratios de populations diploïdes et triploïdes -élevées dans les même conditions- étaient similaires d'après Allen & Downing (1990) et Normand *et al.* (2009). Néanmoins, Guo *et al.* (1998) démontrent une forte variation du sexe-ratio entre des familles d'huîtres creuses *C. gigas* variant de 10% à 71% pour les femelles, suggérant une base génétique du sexe-ratio. Concernant les facteurs environnementaux, les travaux de Fabioux *et al.* (2005) ont mis en évidence une influence de la température et de la luminosité sur le sexe-ratio de l'huître creuse *C. gigas* adulte. En effet dans leur étude, la population conditionnée à faible température (8°C) et faible luminosité (8 heures par jour) présentait un sexe-ratio en faveur des mâles, tandis que les populations élevées en conditions naturelle ou accélérée (température et luminosité supérieures) présentaient un sexe-ratio équilibré. Par la suite, l'influence de la température sur le sexe-ratio a également été testée dans une population d'huîtres juvéniles (Santerre *et al.*, 2013). Les résultats ont alors indiqué un sexe-ratio en faveur des femelles lors d'un conditionnement à 18°C et 22°C et un sexe-ratio à dominante mâle pour les températures supérieures (25°C et 28°C). De plus, l'exposition au nonylphenol induirait une féminisation d'huîtres âgées de 10 mois, ainsi qu'une augmentation

du nombre d'hermaphrodites simultanés chez *C. gigas* (Nice *et al.,* 2003). Précédemment, un sexe-ratio en faveur des femelles avait également été obtenu lors de l'injection d'estradiol chez des huîtres creuses *C. gigas* adultes (Mori *et al.,* 1966).

D'autres facteurs environnementaux, telle que la richesse nutritive du milieu, ont été décrits comme ayant un effet sur la gamétogenèse de *C. gigas* (Soletchnik *et al.,* 1997 ; Langoe-Reynoso, 1999), cependant, leur influence sur le déterminisme du sexe n'a pas été étudiée.

B. La croissance

La croissance est un paramètre important dans l'activité ostréicole car elle conditionne la durée du cycle de production. C'est pourquoi de nombreuses études ont porté sur la recherche de facteurs améliorant la croissance des espèces ostréicoles dont celle de *Crassostrea gigas*.

Il a été démontré dans différentes études que le taux de croissance n'était pas linéaire mais variait en fonction des saisons et des zones géographiques (Imai & Sakai, 1961 ; Askew, 1972 ; Malouf & Breese, 1977 ; Sumner, 1980, 1981 ; Brown, 1988 ; Hyun *et al.,* 2001 ; Dégremont *et al.,* 2005 ; Costil *et al.,* 2005 ; Enriquez-Diaz *et al.,* 2009 ; Mondol *et al.,* 2016). La température et la photopériode sont également des facteurs influençant les paramètres morphologiques (Fabioux *et al.*, 2005). De plus, dans l'étude réalisée par Sumner (1981), le naissain élevé en zone subtidale présentait une croissance supérieure à celui de la zone intertidale. L'étude menée par Azéma *et al.* (2017*a*) a confirmé qu'un temps d'exondation plus important (25% contre 12% et 2%) induisait une meilleure croissance pour du naissain. La richesse nutritive du milieu, apparentée à la quantité de phytoplancton, a été indiquée comme étant un autre facteur environnemental impactant la croissance de *C. gigas* (Langdon & Waldock, 1981 ; Brown & Hartwick, 1988 ; Brown, 1988 ; Rico-Villa *et al.*, 2009). Une faible salinité (<20%) combinée à un milieu pauvre en nutriments ont ralenti la croissance des huîtres adultes suivies par Brown & Hartwick (1988). De plus, l'étude sur la pollution aux métaux dans le milieu aquatique réalisée par Brereton *et al.* (1973) a montré un ralentissement de la croissance larvaire lors d'une exposition au zinc.

L'ensemble des connaissances acquises ont permis de modéliser la croissance de *Crassostrea gigas* en fonction de l'apport alimentaire du milieu (Kobayashi *et al.,* 1997 ; Hyun *et al.,* 2001). Des modèles de type « bilan dynamique d'énergie » (DEB) ont également été créés spécialement pour *Crassostrea gigas,* en tenant compte des connaissances acquises sur sa physiologie. Ils permettent de décrire la distribution de l'énergie au sein d'un organisme à travers ses fonctions physiologiques (reproduction, croissance, nutrition et stockage de l'énergie). Le premier de ce modèle a été réalisé en prenant comme milieu trophique le Bassin Marennes Oléron (Bacher *et al.*, 1991). Par la suite, un modèle a été désigné pour l'étang de Thau (Gangnery *et al.,* 2003), puis d'autres modèles ont été développés en ajoutant des données nouvellement acquises sur l'huître creuse *C. gigas* (Van der Veer *et al.,* 2006 ; Pouvreau *et al.,* 2006 ; Ren & Schiel, 2008 ; Bourlès *et al.,* 2009 ; Alunno-Bruscia *et al.,* 2011 ; Barillé *et al.,* 2011).

Plusieurs estimations des paramètres génétiques (héritabilité et corrélations génétiques) relatifs à la croissance chez *C. gigas* ont été réalisées. Ainsi chez les huîtres juvéniles, l'héritabilité calculée pour la croissance était très faible en conditions contrôlées (0,05 ± 0,18 ; Ernande *et al.,* 2003) ainsi qu'en milieu naturel (entre 0,15 ± 0 ,08 et 0,07 ± 0,07 selon le site Dégremont *et al.,* 2007). Cependant au sein de populations adultes, les héritabilités calculées étaient de 0,36 ± 0,19, 0,49 ± 0,25, 0,45 ± 0,23 et 0,35 ± 0,17 pour la largeur, la longueur, l'épaisseur de la coquille respectivement et 0,35 ± 0,17 pour le poids total (Kong *et al.,* 2015). De plus leur étude a trouvé une corrélation génétique importante (0,79 ± 0,25) entre la longueur de la coquille et le poids de l'animal.

Plusieurs programmes de sélection génétique sur les caractères morphologiques de l'huître creuse *C. gigas* ont vu le jour (Dekkers & Hospital, 2002 ; Langdon *et al.,* 2003 ; He *et al.*, 2008 ; Li *et al.,* 2011 ; Wang *et al.,* 2012 ; Zhang *et al.*, 2019). Ainsi, dans l'étude de Li *et al.* (2011), les descendants issus de géniteurs sélectionnés pour leur forte croissance possèdaient une coquille de 7,9% à 12,2% plus grande que les individus de lignées non sélectionnées (héritabilité calculée entre 0,15 ± 0,03 et 0,40 ± 0,02 selon la lignée de sélection). Utilisant cette génération, une seconde sélection génétique a été réalisée par Wang *et al.* (2012). Elle a permis de confirmer l'héritabilité modérée pour la longueur de coquille (de 0,31 ± 0,07 à 0,46 ± 0,14 selon la lignée).

La ploïdie est également connue comme un facteur impactant la croissance de l'huître, avec des individus triploïdes présentant des paramètres morphologiques supérieurs à ceux des individus diploïdes (Shpigel *et al.,* 1992 ; Normand *et al.*, 2009 ; Ibarra *et al.*, 2017). Ainsi des populations d'huîtres triploïdes ont été utilisées dans des programmes de sélection génétique visant à améliorer la résistance à des agents pathogènes dans le but de réduire le temps d'exposition à ces agents (Hand *et al.,* 1998 ; Dégremont *et al.,* 2012).

L'étude comparative d'huîtres diploïdes et triploïdes réalisée par Normand *et al.* (2009) a permis de mettre en évidence l'effet de la ploïdie –ainsi que de la technique d'induction de la triploïdie- sur le poids frais (masse viscérale sans coquille) d'individus juvéniles. Les huîtres triploïdes issues d'un croisement de géniteurs diploïdes avec la rétention du 2nd globule polaire avaient un poids frais supérieur à celui des triploïdes issues d'un croisement entre un géniteur diploïde et un géniteur tétraploïde. Les masses viscérales de ces deux catégories d'huîtres triploïdes étaient plus lourdes que celles des huîtres diploïdes. Au sein de chaque catégorie, les femelles présentaient un poids frais significativement plus élevé que celui des mâles. L'absence de mesures répétées dans le temps n'a cependant pas permis de calculer des taux de croissance.

Alors que de nombreuses études se sont intéressées à la croissance chez *C. gigas,* très peu d'entre elles ont étudié l'influence du sexe sur ce paramètre. De tels travaux nécessiteraient le suivi temporel d'individus d'âge et d'environnement identiques. Or, des études précédentes ont étudié le sexe-ratio par catégorie de taille d'individus, sur des populations sauvages et sans tenir compte de l'âge des individus (Buroker, 1983 ; Yasuoka & Yusa, 2016). L'unique étude ayant pris en compte ces prérequis a démontré l'existence d'un dimorphisme sexuel, les femelles présentant un poids supérieur ainsi qu'une coquille plus longue que les mâles (Baghurst & Mitchell, 2002). Cependant, dans leurs travaux, la croissance a été calculée à l'échelle de la population et non individuellement, car les biométries ont été réalisées sur des individus différents. De plus, malgré la connaissance de l'hermaphrodisme séquentiel chez *C. gigas* depuis 90 ans (Amemiya, 1929), à notre connaissance, aucune étude ne s'est intéressée à l'influence des changements de sexe sur sa croissance.

C. Les mortalités

Des mortalités massives affectent régulièrement l'huître creuse *Crassostrea gigas et i*mpactent fortement la filière ostréicole.

Des études ont permis de mettre en évidence la composante multifactorielle des mortalités estivales (Elston, 1993 ; Cheney *et al.,* 2000 ; Paul-Pont *et al.,* 2013 ; Pernet *et al.,* 2014). En effet, la saison, la qualité de l'environnement ainsi que les pratiques culturales et la présence d'agents pathogènes influencent la survie des populations d'huîtres. Aussi, le virus *OsHV-1* a été identifié comme étant la principale cause de mortalité dans les populations d'huîtres juvéniles (Nicolas *et al.*, 1992 ; Sauvage *et al.,* 2009 ; Oden *et al.*, 2011). Tandis que les bactéries du genre *Vibrio*, et notamment *Vibrio aesturianus*, ont été détectées lors de phénomènes de mortalités touchant les huîtres adultes (François *et al.*, 2013 ; Travers *et al.,* 2015 ; Barbosa Solomieu *et al.,* 2015). L'incidence de la température a aussi été testé et des lignées issues de sélection massale à la résistance aux mortalités ont été réalisées sur des individus adultes (Elston *et al.*, 1987, Friedman *et al.*, 1991). La composition génétique de la résistance aux mortalités estivales du naissain de *C. gigas* (Dégremont *et al.*, 2005 ; Dégremont *et al.*, 2007) a également été confirmé pour la résistance aux infections par *OsHV-1* (Dégremont, 2011 ; Dégremont *et al.,* 2015 ; Azéma *et al.*, 2017*a*). Des gains de survie importants à *OsHV-1* ont ainsi pu être obtenus après quatre générations de sélection massale sur estran au stade naissain (Dégremont *et al.*, 2015).

L'effet de la ploïdie sur les mortalités de *C. gigas* a, quant à lui, été peu évalué. Ainsi, les études menées par Garnier-Géré *et al.* (2002) et celles d'Ibarra *et al.* (2017) ont indiqué un taux de mortalité identique au sein des populations diploïdes et triploïdes suivies. Pour *OsHV-1*, les deux ploïdies présentent des susceptibilités identiques, celles-ci dépendant uniquement du niveau de sélection des géniteurs utilisés (Dégremont *et al.,* 2016). Au contraire, les taux de mortalités étaient supérieurs pour les triploïdes comparativement aux diploïdes dans le cas d'infections expérimentales à *V. aesturianus* (Azéma *et al.,* 2016).

A notre connaissance, aucune étude ne s'est intéressée à l'effet du sexe et/ou du changement de sexe sur la capacité de survie de l'huître creuse *C. gigas.*

Facteurs moléculaires du déterminisme sexuel chez l'huitre creuse *C. gigas*

A. Les modes de déterminisme du sexe dans le règne animal, chez les Mollusques et chez *Crassostrea gigas*

Le déterminisme sexuel est un ensemble de mécanismes moléculaires et cellulaires par lesquels les cellules germinales souches d'un organisme vont être engagées dans la différenciation cellulaire aboutissant à un organe reproducteur mâle (testicule) ou femelle (ovaire). Dans le règne animal, trois modes de déterminisme du sexe ont été définis : le déterminisme sexuel génétique, le déterminisme sexuel environnemental et le déterminisme sexuel mixte intégrant des facteurs à la fois génétiques et environnementaux (Ghiselin, 1974 ; Bell, 1982 ; Bull, 1983 ; Wilkins, 1995 ; Valenzuela *et al.,* 2003 ; Mittwoch, 2005 ; Haag & Doty, 2005 ; Gamble & Zarkower, 2012 ; Bachtrog *et al.,* 2014).

Le déterminisme sexuel génétique…

Ce type de déterminisme du sexe est basé sur l'existence de chromosomes sexuels, dont l'expression des gènes qu'ils portent détermine le sexe de l'animal. Il est le déterminisme sexuel majeur du règne animal. Il comprend trois sous-catégories : i) le déterminisme basé sur l'hétérogamétie d'un sexe, ii) le déterminisme « dose-dépendant » et iii) le déterminisme « haplo-diploïde ».

…Basé sur l'hétérogamétie

Ce déterminisme sexuel repose sur la présence d'hétérochromosomes. Cette hétérogamétie peut induire la voie de différenciation mâle (XY) ou la voie femelle (ZW). En effet, chez la plupart des Mammifères, les organismes possédant une homogamétie XX sont femelles et une hétérogamétie XY sont mâles. Tandis que chez les Oiseaux, l'hétérogamétie ZW induit des individus femelles et l'homogamétie ZZ des individus mâles. Ce mode de déterminisme sexuel peut être étendu à trois chromosomes sexuels, comme c'est le cas par exemple chez le guppy *Xiphophorus maculatus* avec des hétérogaméties mâle (XY) et femelle (WY et WX) (Veith *et al.*, 2003) ou chez la souris pygmée africaine *Mus minutoides* qui possède un chromosome X,

un Y et un X mutant appelé X*, dont la particularité est d'inhiber le chromosome Y (Veyrunes *et al*., 2010).

… « Dose-dépendant »

Ce déterminisme sexuel dépend de la valeur du ratio entre le nombre de chromosomes sexuels (X) et le nombre de chromosomes autosomaux (A). Par exemple chez le nématode *Caenorhabditis elegans*, lorsque ce ratio X/A est inférieur à 1 l'individu est mâle, tandis que lorsqu'il est égal ou supérieur à 1 l'individu est alors femelle (Madi & Herman, 1979).

… « Haplo-Diploïde»

Ce déterminisme sexuel est possible chez un certain nombre d'Insectes (hyménoptères, coléoptères et thysanoptères en particulier) (Aron & Passera, 1999). Chez ces espèces, telle que l'abeille, capables de parthénogenèse, la détermination du sexe est fonction de la fécondation (ou non) de l'œuf (Heimpel & De Boer, 2008). Ainsi l'œuf non fécondé donnera un individu mâle (parthénogenèse arrhénotoque) tandis qu'une femelle sera la résultante d'un œuf fécondé.

Un second processus aboutit au déterminisme génétique « haplo-diploïde » ; il s'agit de l'élimination du génome paternel post-fécondation induisant des mâles haploïdes, retrouvés chez certains Insectes comme, par exemple, la cochenille (Herrick & Seger, 1999).

Le déterminisme sexuel environnemental

Le déterminisme sexuel peut être influencé par des facteurs sociaux tel que la densité de population ou des facteurs physico-chimiques du milieu. Ce type de déterminisme est répandu chez les Protostomiens mais également chez certains poissons Téléostéens et Squamates ou Chéloniens (Korpelainen, 1990). Par exemple, chez le ver marin *Bonellia viridis*, l'identité sexuelle d'un individu dépend de l'occupation de son habitat : si la niche est vide, la larve devient femelle tandis que si son habitat est occupé par un congénère femelle, elle devient mâle (Leutert, 1975). Chez les tortues *Testudo graeca* et *Emys orbicularis* en revanche, c'est la température d'incubation des œufs qui détermine le sexe des embryons, une température élevée étant en faveur des femelles chez ces 2 espèces (supérieure à 30-31°C et 28,5°C respectivement) (Pieau, 1971).

Le déterminisme sexuel mixte (génétique + environnemental)

Dans certains cas, bien que des chromosomes sexuels soient présents, l'environnement influence également le futur sexe de l'animal dans une période labile, entraînant un sexe phénotypique qui ne correspond pas au sexe génétique. De nombreux poissons Téléostéens (Baroiller & D'Cotta, 2001 ; Ospina-Alvarez & Piferrer, 2008 ; Baroiller *et al.,* 2009) ainsi que certains Amphibiens (Eggert, 2004 ; Manolakou *et al*., 2006 ; Matsuba *et al.,* 2007) et Squamates et Chéloniens (Shine *et al.,* 2002 ; Sarre *et al.,* 2004 ; Quinn *et al.,* 2007 ; Warner *et al.,* 2008) ont ainsi un déterminisme sexuel thermosensible. Chez le tilapia *Oreochromis niloticus* par exemple, une élévation de la température (32°C) provoque une masculinisation phénotypique d'embryons génétiquement femelles (XX) (Baroiller *et al.*, 1995). A l'inverse, chez l'iguane australien *Pogona vitticeps*, une incubation des œufs à 32°C induit une féminisation d'embryons génétiquement mâles (ZZ) (Quinn *et al*., 2007).

Modes de déterminisme sexuel chez les Mollusques

Les Mollusques présentent des modes de déterminisme du sexe variés et les travaux à ce sujet sont encore peu nombreux. Ainsi par un exemple, (i) un système X/Y est retrouvé chez de nombreux Gastéropodes (Vitturi *et al*., 1998) ou chez la mactre naine *Mulinia lateralis* (Guo & Allen, 1994), (ii) un système X/0 est retrouvé chez les escargots *Theodoxus* et *Littorina* sp (Vitturi & Catalano, 1988 ; Vitturi *et al.*, 1988) ou encore (iii) un système dose-dépendant X/A est retrouvé pour la mye commune *Mya arenaria* (Allen *et al*., 1986). Chez *Crassostrea virginica*, un système multi-loci a été suspecté il y a déjà quelques années mais n'a pas fait l'objet de plus d'études (Haley, 1977, 1979). De nombreux Bivalves, dont les membres des ordres *Mytiloida, Unionoida, Veneroida* et *Nuculanoida*, présentent un système inhabituel de transmission de l'ADN mitochondrial connu sous le nom de '*doubly uniparental inheritance*' (DUI), qui implique la transmission des génomes mitochondriaux des deux parents (Kenchington *et al.,* 2002, 2009 ; Breton *et al*., 2007, 2009, 2011 ; Passamonti & Ghiselli, 2009 ; Boyle & Etter, 2013 ; Zouros, 2013). Cependant, chez plus de 150 espèces de Bivalves, aucun chromosome sexuel hétéromorphe n'a été caractérisé en cytogénétique (pour revue, Breton *et al*., 2017).

Chez les Mollusques, le déterminisme sexuel pourrait aussi être influencé par des facteurs environnementaux abiotiques (température, pollution, acidification des océans, stéroïdes

exogènes) ou biotiques (disponibilité de la nourriture) (Breton *et al.*, 2017 ; Boulais *et al.*, 2017 ; Parker *et al.*, 2018) (Tableau 1). Les travaux qui suggèrent cette hypothèse sont essentiellement basés sur des observations de sexe-ratios chez des Bivalves.

Tableau 1 : ***Facteurs environnementaux biotiques et abiotiques ayant une influence sur le sexe-ratio de Mollusques***

Facteurs/espèces	Traitement et/ou résultats	Références bibliographiques
Stéroïdes exogènes *Crassostrea gigas* *Mulinia lateralis* *Placopecten magellanicus* *Pinctada margaritifera*	17β-estradiol: féminisation Methyltestostérone: masculinisation 17β-estradiol, testost., progest. DHEA: masculinisation 17β-estradiol: féminisation	Pour revue Breton et al. 2017 Teaniniuraitemoana et al. 2016
Température *Crassostrea corteziensis* *Crassostrea gigas* *Crassostrea virginica* *Pinctada margaritifera*	♂ à 18°C ; ♀ à 9°C ♂ à T° extrême (< 8°C ; > 25°C) ♀ à T° élevée ♂ à T° élevée de 28°C	Chávez-Villalba *et al.* 2008 ; Rodriguez-Jaramillo *et al.* 2008 ; Fabioux *et al.* 2005 ; Lango-Reynoso *et al.* 2006 ; Santerre *et al.* 2013 ; Coe 1936 ; Teaniniuraitemoana *et al.* 2016
Acidification des océans *Saccostrea glomerata*	16 % de ♀ en + / ♂	Boulais *et al.* 2017 ; Parker *et al.* 2018
Nourriture *Argopecten irradians* *Crassostrea gigas* *Mytella charuana* *Pinctada margaritifera*	♂ - peu de nourriture ♂ - peu de nourriture ♂ - peu de nourriture ♂ - peu de nourriture	Sastry 1968 ; Lango-Reynoso 1999 ; Stenyakina *et al.* 2010 ; Teaniniuraitemoana *et al.* 2016 ; Chávez-Villalba *et al.* 2011
Pollution *omphina veneriformis* *Mya arenaria*	♂ avec tributylétain ♂ avec tributylétain	Pour revue Breton *et al.* 2017

Des facteurs sociaux pourraient également jouer un rôle dans le déterminisme sexuel, comme chez la crépidule *Crepidula fornicata*, chez qui les petits individus qui se fixent sur des gros deviennent mâles alors que les solitaires deviennent femelles (Hoagland, 1977). De même, le stress induit par les greffes, chez l'huître perlière *Pinctada mazatlanica*, pourrait être responsable des sexe-ratios biaisés en faveur des femelles (Saucedo *et al.*, 2001).

Mode de déterminisme du sexe chez l'huître creuse C. gigas

A la genèse de l'étude du déterminisme sexuel chez l'huître creuse *C. gigas*, ce mécanisme physiologique était considéré, chez cette espèce, comme dépendant exclusivement de facteurs environnementaux tels que son alimentation et la température de l'eau de mer (Coe, 1936 ; Quale, 1988). L'influence de facteurs génétiques a longuement été suspectée sans pouvoir être démontrée (Coe, 1932 ; Needler, 1942). De plus, jusqu'à présent, aucun hétérochromosome sexuel n'a pu être identifié en cytométrie chez *Crassostrea gigas* (Ahmed & Sparks, 1967 ; Thiriot-Quievreux, 1984, 2002). L'étude menée par Guo *et al.* (1998), sur des populations d'huîtres creuses issues de croisements, a permis de mettre en évidence un effet paternel important sur le sexe des descendants. Ces auteurs ont ainsi pu conclure à l'existence d'une influence génétique sur le déterminisme sexuel de *C. gigas.* A l'issue de leurs travaux, ils ont proposé un modèle génétique du déterminisme sexuel basé sur un seul locus avec un allèle mâle dominant (M) et un allèle protandrique (F). Ainsi les huîtres présenteraient deux génotypes possibles : des vrais mâles (MF) ne changeant pas de sexe et des individus (FF) étant mâles à la première maturité sexuelle et pouvant évoluer vers des femelles sous l'effet de gènes secondaires ou de l'environnement (Guo *et al.*, 1998). Ce modèle n'expliquant pas toute l'hétérogénéité des sex-ratios mentionnés dans la littérature, Hedrick & Hedgecock (2010) ont formulé un second modèle. Dans ce dernier, la population d'huîtres *C. gigas* présenterait 3 génotypes distincts (MM, FM et FF), toujours basés sur un seul locus. Les mâles présenteraient toujours deux génotypes ; (MM) pour les vrais mâles ne changeant pas de sexe et (FM) pour les mâles pouvant devenir femelles. Par contre, cette fois, les femelles seraient également issus de deux génotypes : (FF) pour les vraies femelles ne changeant pas de sexe et (FM) pour les femelles issus de mâles ou évoluant vers des mâles. A nouveau, la capacité d'un individu à changer de sexe serait également sous l'influence de facteurs externes.

Les deux modèles proposés supposent l'existence d'un gène majeur du déterminisme sexuel chez *Crassostrea gigas.* Cependant, à l'heure actuelle, il n'a pas été identifié.

B. Les gènes du déterminisme sexuel chez les Mollusques et chez *C. gigas* : homologues de facteurs connus des cascades moléculaires et candidats potentiels issus des approches transcriptomiques globales

Le déterminisme génétique du sexe peut impliquer plusieurs gènes (oligogénique ou polygénique) comme cela a été montré chez le poisson zèbre *Danio rerio* (Liew *et al*., 2012) ou le bar *Dicentrarchus labrax* (Vandeputte *et al*., 2007). Cependant, il apparaît que chez de nombreuses espèces, c'est un gène majeur qui initie le déterminisme sexuel. Le mode d'action d'un tel gène est soit binaire (présence/absence) –par exemple le gène *Sry* chez la souris (Sinclair *et al*., 1990)- soit « dose-dépendant » -c'est le cas de *Dmrt1* chez le poulet (Smith *et al*., 2009). En plus de ce gène principal, la mise en place d'une gonade différenciée nécessite la présence d'une cascade de facteurs moléculaires activant ou inhibant certains gènes.

Les réseaux de gènes du déterminisme sexuel dans le règne animal

Parmi les organismes modèles, la souris *Mus musculus*, le nématode *Caenorhabditis elegans* et la drosophile *Drosophila melanogaster* possèdent chacun un gène majeur induisant la différenciation sexuelle. Il s'agit respectivement des gènes *Sry* (Sex-determining region on Y chromosome) (Sinclair *et al.,* 1990 ; Koopman *et al*., 1991), *Xol-1 (*XO lethal protein 1*)* (Miller, 1988) et *Sxl (*Sex lethal*)* (Zhu *et al*., 1997).

…chez la souris *Mus musculus*

Le gène *Sry* a été identifié chez la souris par Sinclair *et al*. (1990). Sa présence chez des souris génétiques femelles (XX) a induit la mise en place de testicules fonctionnels, donc phénotypiquement mâles (Koopman *et al*., 1991). Ainsi, en partenariat avec le gène *SF1 (*Steroidogenic Factor 1*)*, le gène *Sry* active la transcription du facteur *Sox9 (*Sry-bOX 9*)* qui à son tour active l'expression du gène *Fgf9 (*Fibroblast growth factor-9*).* Ce dernier agit avec un rétro-contrôle positif sur *Sox9.* De nombreux autres gènes et leurs produits géniques, tels que *Gata-4, Fog2, Wt1, Nr5a1, Pgsd, Fgfr1, CBX2, Sox8, Amh, Dax1 et Dhhh*, sont aussi nécessaires pour les régulations (positives et négatives) et le maintien de cette voie de détermination du sexe mâle. *Dmrt1* (Doublesex and Mab3-related transcription factor) est aussi nécessaire pour le maintien du sexe gonadique mâle, en particulier pour prévenir la reprogrammation

féminine (Eggers & Sinclair, 2012). Chez les femelles, deux voies moléculaires indépendantes permettent la différenciation de la gonade en ovaire, par l'activation de *Fst (*follistatine*) :* la voie *Wnt-4/Rspo1/β-caténine (*Wingless-type MMTV integration site family, member 4/R-spondin 1/β-caténine*)* et la voie *Foxl2/BMP-2/aromatase (*Forkhead bOX L2/Bone Morphogenetic Protein 2/aromatase*)* (Nef & Vassalli, 2009 ; Pannetier & Pailhoux, 2010). Ces deux voies inhiberaient l'expression des facteurs de la différenciation testiculaire tels que *Sox9 et Fgf9* lors de la différenciation femelle et, à l'inverse, elles seraient inhibées par *Sox9, Fgf9* et probablement *Sry* lors de la différenciation mâle (Ottolenghi *et al.,* 2007 ; Uhlenhaut *et al.,* 2009 ; Pannetier & Pailhoux, 2010). Le rôle de *Sry* en tant que déterminant de la voie mâle serait conservé chez tous les Mammifères placentaires et marsupiaux (Veyrunes *et al*., 2008).

… chez le nématode, *Caenorhabditis elegans*

Chez le nématode, l'inhibition de l'expression du gène *Xol-1* chez des embryons génétiquement mâles (XO) induit leur féminisation (Miller *et al*., 1988). Quand il est actif, ce gène permet l'inhibition de l'expression de gènes *Sdc* (*Sdc-1, Sdc-2*) qui étaient considérés jusqu'alors comme des gènes candidats du déterminisme sexuel mâle (Nusbaum & Meyer, 1989 ; Villeneuve & Meyer, 1990 ; Meyer, 2005). L'inhibition des protéines Sdc rend possible l'expression du gène *her-1* qui inhibe à son tour l'expression de *Tra-2*. L'expression de ce dernier étant inhibée, les gènes *Fem-1*, *Fem-2* et *Fem-3* peuvent s'exprimer et réguler négativement le gène *Tra-1*, conduisant ainsi la différenciation gonadique mâle (Kuwabara & Kimble, 1992). Cette différenciation mâle est rendue possible par l'expression du gène *Mab-3*, homologue du gène *Dmrt1* des Mammifères (Yi *et al*., 2000).

… chez la drosophile Drosophila melanogaster

Chez *D. melanogaster*, *Sxl* est retrouvé sur le chromosome X et sa transcription est activée uniquement chez des individus diploïdes génétiquement femelles (XX) (Zhu *et al*., 1997). La protéine Sxl, en plus d'activer l'expression de *Sxl* chez la femelle (rétro-contrôle positif), induit l'épissage du gène *Tra* (*Transformer*) (Zhu *et al.,* 1997). En découle la production d'une protéine Tra fonctionnelle qui, avec l'aide de la protéine Tra2 non spécifique du sexe, se lie à *Dsx* -gène homologue de *Dmrt1*- (Boggs *et al.,* 1987 ; Amrein *et al*., 1988 ; Inoue *et al.,* 1990). Cette liaison entraîne la production, par épissage, d'une protéine Dsx spécifique de la femelle (Hoshijima *et al*., 1991).

Alors que le gène *Tra* est un facteur d'épissage tardif de la cascade de différenciation chez *D. melanogaster*, chez la mouche *Ceratitis capitata*, il aurait un rôle clé dans la détermination du sexe (Pane *et al.*, 2002). En effet, chez cette dernière, le gène homologue *CcTra* est retrouvé naturellement chez des embryons génétiquement femelles (XX) et l'inhibition de son expression conduit à des mouches phénotypiquement mâles (XX) (Pane *et al.*, 2002).

Des homologues de facteurs connus des réseaux de gènes du déterminisme sexuel, conservés chez les Mollusques

Plusieurs gènes connus et conservés du déterminisme sexuel des Mammifères ont été retrouvés en approches globales, chez les Mollusques Bivalves et Gastéropodes, comme notamment *Gadd45γ, MAP3K4, Gata-4, LHX9, Wt1, CBX, Fgf18, Rspo1, Wnt-4, β-Caténine, Fst, Dax1* ou *ATRX* mais aussi des gènes à domaine DM (dont *Dmrt1*), des gènes de la famille *Sox* (dont *Sox9 et Sox30*) et le gène *Foxl2* (Ghiselli *et al.*, 2011 ; Dheilly *et al.*, 2012 ; Teaniniuraitemoana *et al.*, 2014, 2015 ; Zhang *et al.*, 2014 ; Shi *et al.*, 2015 ; Tong *et al.*, 2015 ; Chen *et al.*, 2017 ; Yue *et al.*, 2018 ; Mulyana *et al.*, 2018 ; Tableau 2). Quelques gènes conservés des cascades des Protostomiens ont aussi été retrouvés chez des Mollusques, comme les gènes *Sxl, Tra* ou *Fem* (Zhang *et al.*, 2014 ; Teaniniuraitemoana *et al.*, 2014, 2015 ; Shi *et al.*, 2015 ; Tong *et al.*, 2015 ; Chen *et al.*, 2017).

Les expressions gonadiques des transcrits des homologues de *Sox9/SoxE, Sox30/SoxH, FoxL2 et Dmrt1 (et autres Dmrt)* ont aussi été mesurées chez certains Mollusques en approches globales. Pour les homologues de *Dmrt1* (et autres *Dmrt*), une surexpression a été relevée dans la gonade mature mâle chez la coquille Saint Jacques (Ns-*Dmrta2* ; Galindo-Torres *et al.*, 2018), l'huître perlière (*Pmag-dmrt* ; Teaniniuraitemoana *et al.*, 2014, 2015), le pétoncle (*Pydmrt* ; Li *et al.*, 2016), la moule (*Dmrt1* ; Shi *et al.*, 2015) et l'ormeau (*Hadmrt1* ; Valenzuela-Munoz *et al.*, 2014). De même, l'expression des homologues de *Sox9/SoxE* a été retrouvée plus élevée dans la gonade mâle de la coquille Saint Jacques (Galindo-Torres *et al.*, 2018) et chez la moule (Shi *et al.*, 2015) mais pas chez le pétoncle (Li *et al.*, 2016) et le clam (Chan *et al.*, 2017). Le gène homologue de *Sox30/SoxH* était quant à lui fortement exprimé dans les gonades mâles et plus limité en expression voire absent des gonades femelles chez le pétoncle (Li *et al.*, 2016) et le clam (Ghiselli *et al.*, 2011). Enfin, les homologues de *Foxl2* ont présenté une expression dimorphique entre les sexes en faveur des femelles chez *P. margaritifera*

(Teaniniuraitemoana *et al*., 2014, 2015), *P. yessoensis* (Li *et al.,* 2016), *T. granosa* (Chen *et al*., 2017) et *H. schlegelii* (Shi *et al.,* 2015). L'expression de l'homologue chez *C. hongkongensis* était également supérieure dans la gonade femelle comparativement aux autres tissus -dont la gonade mâle- (Tong *et al*., 2015).

Tableau 2 : ***Homologues de gènes conservés du déterminisme sexuel des Mammifères retrouvés chez les Mollusques Bivalves et Gastéropodes***

Mammifères	Mollusques	Références bibliographiques
Dmrt1* et famille *Dmrt	*Chlamys farreri (Cf-dmrt4-like); Pinctada martensii (pmDmrt2, pmDmrt5); Haliotis asinina (dmrt1); P. fucata (Pifuc-Dmrt2, Pifuc-Dm-like A et Pifuc-Dm-like B), ; Nodipecten subnodorus (Ns-dmrt1, Ns-dmrta2)*; *P. margaritifera (pmargdmrt2, pmargdmrt) ; Patinopecten yessoensis (Pydmrt); Tegillarca granosa (DmrtA2)*; *Reishia clavigera (Dmrt*); *Cristarias plicata (Dmrt1) ; Hyriopsis* schlegelii (Dmrt1/2) ; *H. rufescens (HaDmrt1)*	Feng *et al*., 2010 ; Yu *et al*., 2009, 2011 ; Klinbunga *et al.,* 2009 ; Matsumoto *et al.*, 2013 ; Llera-Herrera *et al.,* 2013 ; Teaniniuraitemoana *et al*., 2014, 2015 ; Li *et al*., 2016 ; Chen *et al*., 2017 ; Ip *et al.,* 2015 ; Patnaik *et al*., 2016 ; Shi *et al*., 2015 ; Valenzuela-Munoz *et al*., 2014 ; Galindo-Torres *et al*., 2018 ; Shi *et al.*, 2015 ; Valenzuela-Munoz *et al.,* 2014
Sox9* et *Sox30	*Sox9/SoxE : N. subnodorus ; P. margaritifera ; P. fucata* ; *P. yessoensis ; T. granosa ; C. plicata* ; *H. schlegelii Sox30/SoxH* : *P yessoensis* ; *Ruditapes philippinarum*	Galindo-Torres, 2018 ; Teaniniuraitemoana *et al.,* 2014 ; Li *et al*., 2016 ; Ghiselli *et al*., 2011 ; Patnaik *et al*., 2016 ; Shi *et al*., 2015
FoxL2	*C. farreri (Cf-foxl2) ; A. californica* ; *L. gigantea ; P. margaritifera (Pmarg-foxl2)* ; *C. honkongensis (ChFoxL2)* ; *P. yessoensis (Pyfoxl2)* ; *T. granosa (Foxn2 et Foxe) ; C. plicata (Foxl2)* ; *H. schlegelii (Foxl2)*	Liu *et al*., 2012 ; Matsumoto *et al.,* 2013 ; Teaniniuraitemoana *et al*., 2014, 2015 ; Tong *et al*., 2015 ; Li *et al.*, 2016 ; Chen *et al*., 2017 ; Patnaik *et al*., 2006 ; Shi *et al*., 2015

Des candidats potentiels du déterminisme sexuel issus des approches transcriptomiques globales chez les Mollusques

Chez les Mollusques Bivalves et Gastéropodes, les approches transcriptomiques globales ont permis de mettre en évidence des facteurs conservés du déterminisme sexuel (*cf.* § ci-dessus) et des facteurs de la différenciation gonadique. Parmi ces derniers, certains pourraient présenter une expression dimorphique entre les sexes, (i) précoce en accord avec un rôle dans le déterminisme sexuel ou (ii) plus tardive en accord avec un rôle dans le maintien du sexe.

Ainsi, chez des Mollusques gonochoriques des approches ont mis à jour des gènes différentiellement exprimés selon les sexes, comme chez la moule *Hyriopsis schlegelii* sur des gonades mâtures mâles et femelles (Shi *et al*., 2015), chez le clam *Tegillarca granosa* (Chen *et al.,* 2017) et chez la moule *Perumytilus purpuratus* (Briones *et al*., 2018). De même, ce type d'études faites chez le clam *Ruditapes philippinarum* sur des individus entiers mâles et femelles ont mis en évidence 35 gènes connus pour leur implication dans la reproduction, dont l'homologue de *Sox30* (Ghiselli *et al*., 2011). Une seconde étude, réalisée en RNA-Seq sur cette espèce, à partir de gonades mâles et femelles, a permis d'identifier 1,284 gènes exprimés différentiellement entre les sexes, sans analyse plus approfondie de chacun (Ghiselli *et al*., 2018). Chez l'ormeau *Haliotis rufescens*, un séquençage de gonades mâtures mâles et femelles a permis de mettre en évidence l'existence de '*single nucleotide polymorphism'* (SNP) associés à certains gènes tels que la *Vitellogénine* (Valenzuela-Munoz *et al.,* 2014). Enfin, chez le pétoncle japonais *Patinopecten yessoensis,* l'analyse des transcriptomes de testicules et d'ovaires, ont mis en évidence 4394 gènes s'exprimant différentiellement selon le sexe (Li *et al.,* 2016).

En ce qui concerne les Mollusques hermaphrodites simultanés, chez le pétoncle *Argopecten purpuratus*, une banque d'ADNc de gonades mâtures mâles et femelles et immatures a permis de mettre en évidence 7 gènes connus comme étant impliqués dans des processus de la reproduction chez d'autres organismes, *TSSK* (Testis-specific serine/threonine-protein kinase), *Vg* (vitellogenin), *CDA* (cytidine deaminase), *ADAR* (RNA-specific adenosine deaminase), *CNA* (calcineurin A), *SCA* (spermatogenesis and centriole associated 1) et le gène codant la centrine (Boutet *et al*., 2008). En RT-qPCR, les transcrits présentaient une expression plus importante dans la gonade comparativement aux autres tissus et dimorphique entre les sexes et entre les stades (gonades immatures *versus* gonades matures), à l'exception de la

centrine. Chez la coquille Saint-Jacques *Nodipecten subnodosus*, les gènes impliqués dans la différenciation gonadique ont été étudiés par analyse transcriptomique de gonades mâles et femelles à différents stades de gamétogenèse (Llera-Herrera *et al*., 2013). Les auteurs ont identifié 3 gènes possiblement impliqués dans la méiose chez les mâles. Une seconde étude réalisée sur des juvéniles de cette espèce a permis de mettre en relation l'expression de transcrit dans des gonades indifférenciées, par rapport à d'autres tissus (glande digestive, muscle adducteur) et aux stades de développement (larvaire, embryonnaire) (Galindo-Torres *et al*., 2018).

Enfin, en ce qui concerne les Mollusques hermaphrodites séquentiels, chez l'huître perlière *Pinctada fucata*, Matsumoto *et al*. (2013) ont recherché dans le génome (Takeuchi *et al*., 2012), des homologues de gènes connus de la reproduction Ils en ont identifiés plus de 40, dont des homologues de la vitellogénine *(Pifuc-Vtg)* et d'ER *(Estrogen receptor) (Pifuc-ER).* Chez une autre espèce d'huître perlière (*P. margaritifera*), l'analyse différentielle à partir de transcriptomes de gonades mâles et femelles à différents stades de gamétogenèse, a permis d'identifier 87 gènes impliqués dans le déterminisme sexuel et/ou la différenciation gonadique (Teaniniuraitemoana *et al*., 2014). Douze d'entre eux ont été validés en qPCR et étaient plutôt exprimés dans les gonades mâtures, soit femelles (dont *vit-6, fasn1, dicer1, sodA* et *dnmt1*), soit mâles (dont *tssk1*, *fbxo39, ccnb3* et *dyi3*). Une seconde étude transcriptomique réalisée par Teaniniuraitemoana *et al.* (2015) a identifié 1 937 contigs impliqués dans les voies de différenciation gonadique mâle et femelle. Chez l'huître *Crassostrea hongkongensis*, des transcriptomes de gonades mâtures mâles et femelles ont mis en lumière plus de 1,910 gènes identifiés comme en lien avec la reproduction (lignée germinale/déterminisme sexuel/différenciation gonadique) (Tong *et al*., 2015).

Les candidats au déterminisme sexuel chez C. gigas

Lors des premières études visant la caractérisation de facteurs moléculaires du déterminisme sexuel chez l'huître creuse *Crassostrea gigas,* aucune donnée génomique n'était disponible. Aussi, l'identification des premiers gènes candidats a résulté d'une approche ciblée utilisant des tissus gonadiques d'huîtres adultes prélevés aux différents stades de gamétogenèse (Naimi *et al*., 2009*a et b*). Après obtention d'ADNc, l'utilisation d'amorces ciblant des séquences homologues aux gènes conservés a permis la caractérisation de deux homologues :

Cg-Dmrl et *Cg-Foxl2*. L'expression ubiquiste de *Cg-Dmrl* et son homologie avec *Dmrt4/5* plutôt qu'avec *Dmrt1* ont suggéré un rôle de ce facteur dans la différenciation sexuelle, mais pas dans le déterminisme sexuel (Naimi *et al.,* 2009*a*). Le second gène homologue retrouvé chez l'huître creuse, *Cg-Foxl2*, avait une expression croissante au cours du cycle gamétogénétique adulte, biaisée en faveur des femelles, ne permettant pas de discriminer un rôle dans la vitellogenèse, la différenciation gonadique ou le déterminisme sexuel du cycle suivant (Naimi *et al*., 2009*b*). Cependant, l'hypothèse de l'existence d'un ARN anti-sens naturel de *Cg-Foxl2* (*Cg-Foxl2os*) a été avancée par Naimi *et al.* (2009*b*) puis confirmée par Santerre *et al.* (2012). Il inhiberait l'expression de *Cg-FoxL2* chez les mâles en stade 3, en formant des duplex cytoplasmiques avec le transcrit de *Cg-FoxL2*. *Cg-FoxL2* et *Cg-FoxL2os* joueraient ainsi un rôle clé dans le déterminisme sexuel femelle chez *C. gigas* en stade 3 (Santerre *et al*., 2012). Par une approche ciblée, deux autres facteurs potentiels du déterminisme sexuel ont été caractérisés chez *C. gigas*. L'un (*Cg-SoxE*) présentait un pic d'expression dans la gonade indifférenciée, tandis que l'autre (*Cg-β-caténine*) présentait une expression élevée et dimorphique en faveur des femelles, dès le stade 2, surtout marquée en stade 3 (Santerre *et al.,* 2014). L'ensemble de ces travaux a aussi permis de proposer une fenêtre temporelle au déterminisme sexuel adulte de *C. gigas*, entre le stade 3 d'un cycle et le stade 0 du cycle suivant.

Des approches transcriptomiques globales ont également permis d'apporter des connaissances nouvelles sur les facteurs moléculaires liés au sexe chez *Crassostrea gigas*. Une première étude menée en microarray sur des gonades avait pour objectif de mettre en évidence des transcrits à différents stades de gamétogenèse, spécifiques des mâles et des femelles (Dheilly *et al*., 2012). L'analyse de l'expression différentielle des transcrits entre les sexes a mis en lumière 77 gènes, 68 surexprimés dans les gonades femelles (dont *Cg-Foxl2*, déjà identifié) et 9 chez les mâles, certains en stade 0.

Une seconde étude transcriptomique globale (RNA-Seq) menée par Zhang *et al.* (2014) a ensuite eu pour objectif d'identifier des gènes du déterminisme sexuel chez l'huître creuse, dans des gonades mâtures (stade 3). Ils ont retrouvé *Cg-foxl2* dans les transcriptomes avec, à nouveau, une expression marquée dans la gonade mature femelle, non-spécifique de la gonade comme précédemment mentionné (Naimi et al, 2009b). Un autre facteur de la famille Fox a été mentionné, *FoxN5* ; il a été présenté comme ayant une expression gonadique mâle-

spécifique. Ces mêmes auteurs ont aussi mis en évidence un homologue du gène *Sox30* et de trois gènes à domaine DM, *Cg01830, Cg15952* (*Cg-DMl* - Naimi *et al.*, 2009*a*) et *Cg19568* nommé *Cg-Dsx* (*Dmrt1-like*). Les expressions spécifiques de *Cg-Dsx/Dmrt1-like* et *Cg-SoxH*, uniquement gonadiques et uniquement dans les gonades mâles matures, ont alors été associées à un rôle dans le déterminisme sexuel mâle. En l'absence de validation par RT-qPCR et en l'absence de profils d'expression sur l'ensemble du cycle de gamétogenèse, cette information reste une hypothèse.

Récemment, une nouvelle étude transcriptomique a été conduite par Yue *et al.* (2018) sur des tissus somatiques (manteau, branchie, muscle adducteur et masse viscérale) et sur des gonades aux différents stades de gamétogenèse. Ces auteurs ont mis en évidence six clusters regroupant des gènes différentiellement exprimés au cours du cycle de gamétogenèse et/ou selon le sexe. Seuls *Cg-Dsx, Cg-SoxH* et *Cg-Foxl2* ont été identifiés au sein des clusters en tant que gène potentiel du déterminisme du sexe. Leurs profils d'expressions au cours du cycle gamétogénétique n'ont pas été mentionnés. Aucun gène exprimé en stade 0, période partielle du déterminisme sexuel, n'est ressorti de l'étude.

Ainsi, l'ensemble de ces travaux chez les Mollusques et chez *C. gigas* ont permis l'identification de quelques gènes conservés du déterminisme sexuel et candidats dont l'expression serait en accord avec un rôle direct ou indirect dans le déterminisme sexuel, mais qui ne sont pas connus comme tels dans le règne animal. Cependant, ces études présentent des limitent et les profils d'expression de beaucoup de ces gènes sont incomplets, limitant ainsi l'interprétation sur leur rôle possible dans le déterminisme sexuel.

C. Les limites des études du déterminisme sexuel chez les Mollusques et chez les hermaphrodites séquentiels

Les types d'hermaphrodismes

L'hermaphrodisme caractérise un individu capable de se reproduire en tant que mâle et femelle au cours de sa vie. Dans le règne animal, les hermaphrodites sont présents chez des Protostomiens et Deutérostomiens de divers clades (Atz, 1964 ; Reinboth, 1970 ; Ghiselin,

1969 ; Charnov, 1979). Dans ce mode de reproduction, deux types d'hermaphrodisme se distinguent : l'hermaphrodisme simultané et l'hermaphrodisme séquentiel.

L'hermaphrodisme simultané, ou hermaphrodisme vrai, caractérise un individu produisant lors d'un même cycle de reproduction à la fois des gamètes mâles et femelles. Ce mode de reproduction est présent chez des espèces phylogénétiquement éloignées telles que le poisson Téléostéen *Serranus tortugarum* (Fischer, 1984), les Mollusques *Navanax inermis* (Leonard & Lukowiak, 1984) et *Argopecten purpuratus* (Disalvo *et al*., 1984) ou l'Arthropode Malacostracé *Lysmata wurdemanni* (Bauer & Holt, 1998). Chez les Mollusques qui ont fait l'objet d'études moléculaires de la différenciation gonadique/du déterminisme sexuel, on compte par exemple le pétoncle *Argopecten purpuratus* (Boutet *et al*., 2008) et la coquille Saint-Jacques *Nodipecten subnodosus* (Gallindo-Torres *et al*., 2018).

L'hermaphrodisme séquentiel ou successif, fait référence à un individu capable de changer au moins une fois de sexe au cours de sa vie. Ce changement peut être protandre (mâle devenant femelle) comme c'est le cas, par exemple, chez des poissons clown du genre *Amphiprion* (Moyer & Nakazono, 1978), des poissons perciformes de la famille des *Sparidae* (Atz, 1964) ou chez le pétoncle géant *Hinnites giganteus* (Lauren, 1982). Ce changement peut aussi être protogyne (femelle évoluant en mâle) comme observé par exemple chez la dorade grise *Spondyliosoma cantharus* (Reinboth, 1962) ou les poissons perroquets de la famille des *Scaridae* (Reinboth, 1970). Ainsi, la spécificité des espèces hermaphrodites séquentielles est la détermination du sexe répétée au cours de leurs vies. Chez les Mollusques qui ont fait l'objet d'études moléculaires de la différenciation gonadique/du déterminisme sexuel, on compte par exemple les huîtres perlières *Pinctada fucata* (Matsumoto *et al*., 2013) et *P. margaritifera* (Teaniniuraitemoana *et al*., 2014, 2015) et l'huître *Crassostrea hongkongensis* (Tong *et al*., 2015).

L'huître creuse *Crassostrea gigas* a été décrite comme une espèce présentant à la fois des individus hermaphrodites simultanés –en proportion très limitée (<1%)- (Amemiya, 1929 ; Guo *et al*., 1998 ; Steele & Mulcahy, 1999 ; Normand *et al.*, 2009 ; Yasuoka & Yusa, 2016) et des individus hermaphrodites séquentiels, capables de changer de sexe au cours de leur vie (Amemiya, 1929 ; Lannan, 1971 ; Guo *et al*., 1998 ; Lango Reynoso, 1999 ; Park *et al*., 2012 ;

Yasuoka & Yusa, 2016). La tendance protandre du changement de sexe a été avancée (Guo *et al*., 1998 ; Enriquez-Diaz *et al.,* 2009 ; Yasuoka & Yusa, 2016), bien que d'autres études réalisées sur cette espèce indiquaient un sexe-ratio à 1ère maturité sexuelle équilibré ou à dominante femelle (Amemiya, 1929 ; Lango Reynoso, 1999 ; Fabioux *et al*., 2005 ; Park *et al*., 2012 ; Santerre *et al*., 2013).

Les limites des études du déterminisme sexuel chez les Mollusques et chez les hermaphrodites séquentiels

Jusqu'à présent, les travaux sur la reproduction et/ou le déterminisme sexuel chez *C. gigas* et chez les Mollusques ont été confrontés à plusieurs limites. Les études ciblées ont certes, permis une identification précise des homologues, mais elles limitent, de fait, le nombre de facteurs étudiés. Les approches globales, en séquençant le transcriptome entier, présentent un plus grand potentiel de découverte. Parmi les études précédentes, peu ont étudié l'expression de gènes sur toute la période du déterminisme sexuel (stade 3 d'un cycle et stade 0 du cycle suivant chez *C. gigas* par exemple ; Naimi *et al*., 2009*a et b*, Santerre *et al*., 2012, 2014 ; Dheilly *et al*., 2012 ; Yue *et al*., 2018). Les approches globales se sont essentiellement focalisées sur des gonades mâtures ou sans aucune précision (Shi *et al*., 2015 ; Chen *et al*., 2017 ; Ghiselli *et al*., 2011, 2018 ; Valenzuela-Munoz *et al*., 2014 ; Boutet *et al*., 2008 ; Tong *et al*., 2015 ; Zhang *et al.,* 2014). Hors, chez des organismes gonochoriques ou hermaphrodites simultanés, le déterminisme sexuel a lieu bien avant, au cours du développement, par exemple chez le juvénile précoce chez l'huître *C. gigas* (Naimi *et al*., 2009*b* ; Santerre *et al.,* 2013). Chez des espèces hermaphrodites séquentielles, le problème est tout autre. Quel que soit le moment d'étude au cours d'un cycle de reproduction, le futur sexe de l'animal est inconnu car l'animal est susceptible de changer de sexe au cycle de reproduction suivant. Le lien entre le profil d'expression d'un facteur et le futur phénotype du sexe est donc impossible, limitant ainsi l'interprétation des résultats et donc les hypothèses sur le rôle potentiel dudit facteur dans le déterminisme sexuel.

Dans le règne animal, le déterminisme sexuel a été moins étudié chez les espèces hermaphrodites, alors que ce mode de reproduction est bien conservé (Ghiselin, 1969), notamment du fait de limites semblables à celles mentionnées ci-dessus. Les études moléculaires réalisées ont surtout porté sur les poissons Téléostéens, parmi lesquels on compte de nombreuses espèces hermaphrodites séquentielles protandres ou protogynes (Bobe *et al*., 2014). Comme chez les Mollusques, des homologues de gènes du déterminisme sexuel ont cependant été identifiés. Ainsi, *Dmrt1* a été retrouvé chez des espèces protandres comme *Acanthopagrus schegeli* (He *et al.,* 2003) et protogynes comme *Halichoeres poecilopterus* (Miyake *et al*., 2012). Son expression était alors corrélée à la différenciation ou à la régression du testicule lors du changement de sexe, respectivement. De même, des homologues de *Sox*9 ont été décrits chez des Téléostéens hermaphrodites séquentiels comme le mérou *Epinephelus coïoides* (Luo *et al*., 2010) et chez l'anguille *Monopterus albus* (Zhou *et al*., 2003). Chez le mérou, le profil d'expression de *Sox9* a suggéré un rôle dans l'initiation de la différenciation gonadique mâle, en amont de l'expression de *Dmrt1* (Luo *et al*., 2010). Chez l'anguille, l'expression des formes dupliquées *Sox9al et Sox9a2* dans les testicules et les ovaires suggère des rôles similaires dans la différenciation gonadique au cours du changement sexuel chez cette espèce (Zhou *et al*., 2003). Enfin, *Foxl2* a également été caractérisé chez plusieurs espèces hermaphrodites séquentielles, avec des profils d'expression différents selon les espèces. Ainsi, par exemple, son expression lors du changement de sexe augmentait chez le pagre protandre *Acanthopagrus schlegeli* (Wu *et al*., 2010) et diminuait chez le mérou protogyne *Epinephelus merra* (Alam *et al*., 2008). Chez le poisson clown protandre *Amphiprion bicinctus* et chez la girelle protogyne *Thalassoma bifasciatum*, *FoxL2* est surexprimé dans les gonades femelles (Casas *et al*., 2016 ; Liu *et al*., 2015). Par contre, aucun dimorphisme d'expression n'a été observé durant le changement de sexe chez *Halichoeres tenuispinis* (Kobayashi *et al*., 2010). De tels travaux chez des Téléostéens hermaphrodites séquentiels ont été facilités car ces espèces ont un déterminisme sexuel sous influence environnementale et que le changement de sexe ainsi provoqué était rapide (par exemple Liu et al, 2015). De fait, ces études ont permis de mettre en relation des profils d'expressions moléculaires avec des phénotypes du sexe « induits ».

Objectifs de la thèse

Dans ce contexte, afin d'approfondir les connaissances sur le déterminisme du sexe chez *Crassostrea gigas*, plusieurs objectifs ont été fixés dans le cadre de cette thèse :

i) L'identification des phénotypes du sexe et du changement de sexe sur les 6 premières années de vie de l'huître

ii) La détermination de l'influence du sexe et du changement de sexe sur les paramètres morphologiques de l'animal

iii) La caractérisation de facteurs moléculaires exprimés lors du déterminisme sexuel, chez des individus à phénotypes du sexe et du changement de sexe connus

Pour répondre à ces objectifs, deux cohortes d'huîtres creuses ont été produites en écloserie en mars 2013 et en juin 2014, puis testées dans un site d'élevage du bassin Marennes-Oléron depuis novembre 2013 et novembre 2014, respectivement. Les huîtres ont été marquées individuellement, puis sexées et mesurées chaque année (poids, longueur, largeur et épaisseur). Des individus à phénotypes sexuels contrastés ont également été choisis parmi les individus de l'une des cohortes, à savoir des femelles et des mâles n'ayant pas changé de sexe durant les 5 premières années de leur vie. Un séquençage en RNA-Seq a ensuite été réalisé sur des tissus gonadiques de ces individus choisis, à différents stades de gamétogenèse couvrant toute la période du déterminisme sexuel.

Ces éléments nouveaux permettront d'approfondir les connaissances sur le déterminisme sexuel et le mode de reproduction de *Crassostrea gigas*. Une meilleure compréhension de la physiologie reproductive de cette espèce optimisera les techniques de production en écloserie et enrichira le savoir relatif à la biologie de la reproduction au sein des Lophotrochozoaires et des Mollusques

Chapitre 1

Déterminisme sexuel chez l'huître creuse *Crassostrea gigas* - étude pluriannuelle du sex-ratio et du changement de sexe au sein de populations à larges effectifs

Déterminisme sexuel chez l'huître creuse *Crassostrea gigas* – étude pluriannuelle du sexe-ratio et du changement de sexe au sein de populations à larges effectifs

Questions : Les populations de *Crassostrea gigas* présentent-elles des **sexe-ratios équilibrés** ? Quelles sont les **fréquences de changement de sexe** au cours de la vie d'un animal ? L'huitre privilégie-t-elle un **type de changement de sexe** ?

Méthode : L'étude repose sur **deux populations**. La première a été produite en écloserie en 2013 à partir d'huîtres **sauvages** prélevées sur le bassin de Marennes-Oléron, zone où les stocks d'huîtres sauvages sont les plus importants. La seconde a été produite en écloserie en 2014 à partir d'individus **issus de la première**. Les deux populations ont été testées dans le **bassin Marennes-Oléron** à partir de novembre 2013 pour la 1ère et de novembre 2014 pour la 2nde. L'effectif initial était respectivement de **7488** et **4320** individus. Ces huîtres ont été suivies **individuellement** dès 2014 pour la 1ère population et 2015 pour la 2nde. Chaque année, l'identification du sexe a été déterminée par observation des **gamètes,** soit à l'**œil nu** lors de l'induction de la **ponte** par chocs thermiques, soit à l'aide d'une **loupe binoculaire** lors de **biopsie** de la gonade. Les changements de sexe ont été constatés par l'intermédiaire de **l'historique des phénotypes sexuels** collectés sur l'ensemble des 6 années pour la population 1, et des 5 années pour la population 2.

Résultats : Lors de la 1ère année de sexage, la **majorité** des huîtres étaient **femelles** (69% et 54% pour la population 1 et 2 respectivement). Un **sexe-ratio biaisé en faveur des femelles** a également été observé lors des sexages suivants (allant de 61% à 71% et de 54% à 67% pour la 1ère et 2nde population respectivement). Entre les **deux premières années** de sexage, **34%** des huîtres ont **changé de sexe** pour la population 1 et **47%** pour la population 2. La **proportion d'huîtres changeant de sexe** entre deux saisons de reproduction a **diminué au fil des années** au sein des deux populations, avec seulement 9% entre la 5ème et la 6ème année de sexage pour la population 1, et 11% entre la 4ème et la 5ème année de sexage pour la population 2. Au sein de la population 1, **1386 huîtres ont pu être sexées chaque année**. Parmi elles, **58% ont changé de sexe au moins une fois** entre la 1ère et la 6ème année de sexage dont 32% n'ont effectué qu'un seul changement. Ces changements uniques de sexe ont eu lieu dans les **deux**

sens, c'est-à-dire de mâle vers femelle (**protandre**) et de femelle vers mâle (**protogyne**) concernant respectivement **19%** et **13%** de la population 1. De plus, au sein des 1386 huîtres, 19% ont changé de sexe deux fois, 5% trois fois, 1% quatre fois et 0,1% cinq fois –soit tous les ans-. Un nombre important (**42%**) **d'huîtres n'ont jamais changé de sexe** au cours de la période d'étude, dont 34% étaient toujours femelles et 8% toujours mâles. Au cours des cinq années de suivi de la population 2, **333 huîtres ont pu être sexées chaque année** et parmi elles **24% n'ont jamais changé de sexe** dont 17% étaient toujours femelles et 7% toujours mâles. Au sein des huîtres ayant changé de sexe au cours de l'étude (76%), 44% ont présenté un changement unique, 26% ont changé de sexe deux fois, 5% trois fois et 1% quatre fois. A partir des données collectées dans la population 1, une régression logistique a été réalisée permettant de **prédire le taux d'hermaphrodites** chez l'espèce d'après sa durée de vie. Il apparaît que 95% de la population présenterait au moins un changement de sexe au cours des 19 premières années de vie, **plus précocement** chez les huîtres nées **mâles** que celles nées femelles (11 ans contre 27 ans respectivement).

Conclusions : Dans le cas de notre étude, l'huître creuse *Crassostrea gigas* présente un **sexe-ratio en faveur des femelles**. De plus, d'après la régression logistique et la durée de vie de l'huître, présumée de 20 ans, cette dernière serait bien un **hermaphrodite séquentiel**, bien que sur les six années de suivi, un nombre important d'individus n'ait pas changé de sexe. Elle est capable de changer plusieurs fois de sexe, cependant la **fréquence de changement de sexe décroît** lorsque le nombre des années de vie augmente. Cette espèce ne semble toutefois **pas** avoir **de tendance à un type de changement en particulier**, car la protandrie, tout comme la protogynie, ont été observées. Toutefois, les huîtres identifiées **mâles en première année** de sexage auraient **tendance à changer de sexe plus précocement**.

Ma participation : J'ai participé au sexage des 2 populations en années 4 et 5, effectué le suivi des lots en mer, créé les bases de données relatives à chaque population et les ai maintenues à jour. J'ai également participé aux traitements statistiques et réalisé l'interprétation ainsi que la mise en forme des résultats.

Ces travaux ont fait l'objet d'une valorisation par communication orale :

C Broquard, L Dégremont, AS Martinez. 2017 Protandric sex in *Crassostrea gigas*: myth or reality? First answers from a large and long-term monitoring of sex changes**.** Physiomar, 18-21 Septembre 2017, Cambridge, Royaume Uni.

Aquaculture 515 (2020) 734555

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Sex determination in the oyster *Crassostrea gigas* - A large longitudinal study of population sex ratios and individual sex changes

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ARTICLE INFO

Keywords:
sex-ratio
Sex change
Oysters
Hermaphroditism
Crassostrea gigas

ABSTRACT

Understanding sex determination in the Pacific oyster *Crassostrea gigas,* a sequential hermaphrodite, can provide prospective on the evolution of sex-determining systems for comparative reproduction from an evolutionary perspective. Surprisingly, this mechanism is still poorly understood. To date, sex ratio and sex change have never been studied at the individual level for a large size group and long-term monitoring. To this purpose, we performed an ambitious individual long-term follow-up (6 years) on a large population (cohort 1: 7488 oysters) produced from wild oysters, as well as for a second population produced from the cohort 1 (cohort 2: 4320 oysters). All oysters were individually sexed from 2014 to 2019. For the cohort 1, our results showed a significantly female-biased sex ratio each year, ranging from 61 to 73% for the cohort 1. The proportion of oysters exhibiting sex change between the first two breeding seasons was 34% and decreased each year, ending at 9% between years 5 and 6. From the initial population, 1386 oysters were sexed six years in a row. Among them, 58% were sequential hermaphrodites, within which 32% changed sex once (19% protandric and 13% protogynic), 19% twice, 5% three times, 1% four times and 0.1% five times. In contrast, 42% never exhibited a sex change, within which 34% were potentially true females and 8% potentially true males. However, a logistic regression model indicates that those oysters could experience one sex reversal in subsequent years resulting that all oysters of our population of *C. gigas* would be sequential hermaphrodites. Similar results were observed for the cohort 2, although the proportion of sequential hermaphrodite was higher than the one observed for cohort 1. It is supposed that a genetic basis exist for sex change in *C. gigas*. Our work participates to unravel the barriers existing about the sequential hermaphroditism, the protandry and the sexual system in *C. gigas,* still currently debated.

1. Introduction

As sex determination is of major importance to sexual reproduction, it is the subject of many studies across the animal kingdom. Although the mechanisms of sex determination are remarkably diverse among organisms, they can be grouped into three main modes: (i) genotypic sex determination where sex is established by the genotype (gonosomes or autosomes), (ii) environmental sex determination where sex is influenced by environmental cues, and (iii) a mix of genotypic and environmental sex determination (Bachtrog et al., 2014).

The sex-determining mechanisms observed across the tree of life are very diverse because they can evolve rapidly (Bachtrog et al., 2014). A striking example of diversity in sex determination is freshwater crustaceans in the family Limnadiidae (Weeks et al., 2006). Consequently, different modes of sex determination are found among closely related species or populations of the same species and in contrast, the same mode may evolve independently in distant clades (Bachtrog et al., 2014). This diversity of sex-determining mechanisms is associated with two modes of sexual reproduction in animals: gonochorism (only one distinct sex in any individual organism) and hermaphroditism (simultaneous when individuals function as male and female at the same time; sequential when individuals first function as one sex and then switch to the other sex). Gonochorism appears more widespread than hermaphroditism, which is only observed in approximately 5% of all animal species (Bachtrog et al., 2014).

Sex determination defines individual sex and is therefore closely

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https://doi.org/10.1016/j.aquaculture.2019.734555
Received 30 July 2019; Received in revised form 19 September 2019; Accepted 29 September 2019
Available online 14 October 2019

related to the sex ratio of a population and its variation. Fisher (1930) theorized that the sex ratio within a population should be balanced (1:1) under the hypothesis that producing males or females requires an equal cost. This balance is the "evolutionarily stable strategy" and is maintained by natural selection, which promotes the rarer sex. However, in the animal kingdom, biased sex ratios are commonly observed. This may be induced by differential mortality related to sex (Arendt et al., 2014), inbreeding and local competition for mates and food (Hamilton, 1967), endocrine-disrupting environmental pollutants (Mills and Chichester, 2005), or adaptive maternal effects that result in differential investment in male or female offspring (Trivers and Willard, 1973).

Mollusca, the phylum to which oysters belong, provides a rich source of material to better understand the evolution of sex and sex determination (Breton et al., 2017). Indeed this phylum (i) is the second largest in the animal kingdom, (ii) belongs to Lophotrochozoa, a clade poorly understood in terms of reproduction, (iii) provides a richness of species with highly diverse modes of sexual reproduction ranging from functional hermaphroditism (simultaneous and sequential) to gonochorism, suggesting diverse underlying sex-determining mechanisms, and (iv) includes species of economic and nutritional importance, which makes knowledge of their sex determination highly necessary to provide useful tools for the control of their sex in aquaculture. Within molluscs, gonochorism appears as the most common sexual system, occurring in seven of the eight extant classes (Collin, 2013), while approximately 40% of the 5600 genera are classified as hermaphrodites (Heller, 1993). Among bivalves, only approximately 4% of the 10,000 extant species have been determined to not be strictly gonochoric (Coe, 1943); indeed, hermaphroditism has been identified in 13 out of the 117 families (Heller, 1993). However, this number of hermaphroditic bivalve species is probably an underestimation because of (i) its determination based on a limited number of individuals and groups that sometimes lack sexual dimorphism, (ii) the misidentification of sex in simultaneous hermaphrodites based on the study of gonad fragments, and (iii) the misidentification of sex change in sequential hermaphrodites observed at a population scale and not at an individual scale (Yusa, 2007).

Concerning the Pacific oyster *Crassostrea gigas*, its sex-determination system has not been established and there are two longstanding paradigms concerning hermaphroditism in this species:

i) Oysters are sequential hermaphrodites (encountering sex changes at some point in their lifespan). However, few studies provide direct observations of individual sex changes, and these observations have been limited to two years of life in *C. gigas* (Amemiya, 1929; Lango Reynoso, 1999; Lannan, 1971; Park et al., 2012; Yasuoka and Yusa, 2016).
ii) Oysters are protandrous hermaphrodites (born male and change sex to a female) with a striking example provided by Guo et al. (1998), suggesting a higher proportion of males in younger oysters. Nevertheless, five independent studies reported primary sex ratios that were biased in favor of females or were well-balanced (1:1) (Amemiya, 1929; Fabioux et al., 2005; Lango Reynoso, 1999; Park et al., 2012; Santerre et al., 2013).

None of the above studies has investigated the mode of reproduction in individual *C. gigas* for more than two years and/or used a large number of oysters, leading to a lack of consensus among them. Direct observations are crucial and are a mandatory component of experimental design for better understanding of sex determination in *C. gigas*.

In this study, we aimed to assess the temporal variation of the sex ratio for a *C. gigas* population (cohort 1) over the six first-years to identify potentially true males and potentially true females, as well as sequential hermaphrodites. Thus, 7488 oysters were tagged and then sexed from 2014 to 2019 to clarify the sex determination in this major species used in aquaculture. In addition, the sex ratio and the sex change from 2015 to 2019 was also recorded for a second cohort using 4320 *C. gigas*.

2. Materials and methods

2.1. First cohort using wild oysters

2.1.1. Biological material

Twenty half-sib families, each consisting of two full-sib families of *C. gigas,* were produced at the Ifremer hatchery in La Tremblade (France) on 27 March 2013 from a wild oyster population sampled from the Marennes-Oléron Bay (France). The parents were opened to determine their sex as well as their level of maturity by microscopic observation of gonad samples spread on a slide (presence of spermatozoa or oocytes). Twenty males and forty females were kept for mating, each male being crossed with two females. The gametes were collected from each parent by stripping the gonad. After fertilization, the larvae were raised in 30-L tanks at 25 °C in UV-treated, filtered, and aerated seawater. All families were raised separately. The water was changed three times per week. Larvae were fed daily with *Isochrysis galbana* (30,000 cells/mL) until they reached 140 µm, after which the diet was supplemented with *Skeletonema costatum* (30,000 cells/mL). Two weeks after fertilization, larvae were placed on cultch in flow-through raceways at 20 °C supplied with UV-treated seawater enriched with *S. costatum*. Oyster spat were reared under standard hatchery conditions until they reached a size of 2 mm. In May 2013, 5000 oysters per family were transferred to the Ifremer nursery in Bouin (France) (Baud and Bacher, 1990). Density was reduced during the nursery period as some oysters were used in studies to determine the genetic basis for resistance to pathogens (Azéma et al., 2017a, 2017b). Meanwhile, each family was kept in one sieve at high density to reduce the growth until November 2013 and they were protected within the facility under biosecurity control to avoid contamination with major oyster pathogens such as OsHV-1 and *Vibrio aestuarianus*. Further details on these families are provided elsewhere (Azéma et al., 2017a, 2017b).

2.1.2. Field study

The field study lasted from November 2013 to July 2019. In November 2013, 38 families were transferred to the field study site at La Floride in the Marennes-Oléron Bay, which is the main area for shellfish culture in Europe (Goulletquer and Le Moine, 2002). This site is located in the intertidal area and the mean immersion time is around 50%, which is low in comparison to growing leases. This choice was based to avoid a second gametogenesis within a year. Each family was grown separately throughout the study. Approximately 14,000 oysters were deployed (Table 1) (average individual weight 8.0 g); the mean number of oysters per family was 367 and ranged from 150 to 964 among families. Each family was placed into a single labelled sealed oyster bag, except for eight families for which two bags were needed because of the high number of oysters. All bags were randomly attached to racks. Every month, bags were checked to make sure that they were well-attached to the racks and that they were free of defects that would

Table 1
Number of oysters deployed in the field in year 0 for cohorts 1 and 2, and then sexed male or female each year.

Year	2013	2014	2015	2016	2017	2018	2019
Cohort 1[a]	Y0	Y1	Y2	Y3	Y4	Y5	Y6
	13946	7488	4851	3440	2699	2093	1426
Cohort 2[a]		Y0	Y1	Y2	Y3	Y4	Y5
		6090	4320	2519	1541	685	421

[a] Y for year. Some oysters (< 1%) were not sexed for a particular year (any gametes observed by biopsy/spawn). So they did not appear for that year while they did for the others. For example, an oyster of the cohort 1 could have been sexed in Years Y1, Y2, Y3, Y5 and Y6, but not in Y4.

cause loss. The seawater temperature was recorded every hour throughout the study using two probes (ThermoTrack; supplementary data 1). For this study, data are presented without distinguishing the families to have a broad view of sex ratio and sex change for the studied population of *C. gigas*.

2.1.3. Sex observation

All oysters were checked annually at the time of sexual maturity in June from 2014 to 2019. At the beginning of June, oysters were transferred from the field to the laboratory and held in a flow-through system. Seawater was chilled to 15 °C until sex was determined to avoid unintentional spawning events. The number of oysters sexed each year is indicated in Table 1; it decreased throughout the study mainly because of mortality and to a much lesser extent, sampling for molecular analyses (data not shown). After the first sexing (June 2014), male and female oysters were separated into two labelled oyster bags until individual labelling. In April 2015, all live oysters were individually marked with a plastic-laminated number glued with epoxy resin (Sader©) on the upper valve. After labelling, males and females were mixed in one oyster bag per family. Two non-destructive methods were used to determine oyster sex: induced spawning and gonad biopsy. For years 1, 2, 3 and 5, gonad biopsy concerned less than 5% of the oyster population, while it was 37% at year 4 in 2017 and 100% at year 6 in 2019 due to technical reasons (12,000 additional oysters sexed in 2017, data not shown, and hatchery closed in 2019). The biopsy method did not induce higher mortality than oysters that spawned (data not shown). To visualize the emission of the gametes during induced spawning, oysters were placed in a single layer with sufficient distance from each other in a black-bottomed 200-L tank filled with seawater. Thermal shocks in the form of alternating ambient (20 °C) and warm (30 °C) water were used to trigger spawning. Oyster gametes were also added to the tank as a stimulant. Males emit their spermatozoa as a long, opaque white mesh. Females are identifiable by the emission of their oocytes in the form of repeated, dense, and granular clouds.

After spawning commenced, each oyster was placed in a transparent 300-mL beaker containing seawater at 25 °C to ensure that the observed gametes were from the suspected oyster and to confirm the nature of the gametes. When massive spawns occurred, seawater was removed, and all oysters were individually placed into beakers.

The thermal shock cycle was repeated up to 10 times, but some oysters did not respond to induction. For non-responding individuals, a biopsy of the gonad was performed and sex was determined by microscopic observation of gonad smears. Oysters were placed in a 5-L tray with a muscle relaxant solution consisting of seawater (3/5), freshwater (2/5), and magnesium chloride (50 g/L). As soon as the shells opened, a smear of gonad was taken using a needle (0.9 × 38 mm; Terumo©) and a 1-mL syringe (Terumo©). Mature gametes were visualized microscopically (40 ×) to determine the sex. Oysters with oocytes were identified as females and those with spermatozoa were classified as males. Oysters with both mature oocytes and spermatozoa were identified as simultaneous hermaphrodites (represented less than 1% per year). For some oysters, sex could not be determined, and they were categorized as “empty”. These two categories were excluded from the results presented below. After spawning and biopsies, male and female oysters were placed in separate trays until all data was recorded. Males and females from the same family were placed into culture bags and the bags were returned to the study site.

2.2. Second cohort

The first cohort was produced in March 2013, kept in high density until November 2013, and then sexed for the first time in June 2014. There is a chance that the primary sex ratio could have been missed. Consequently, a second cohort was produced in June, then deployed in the field in November and sexed for the first time in June of the following year. This protocol is also close to the life cycle of oysters in the Marennes-Oléron region, with spawning occurring in June/July. The second cohort was produced on June 16th, 2014 using four families of the cohort 1 that were selected for their higher resistance to OsHV-1 and *Vibrio aestuarianus*. Thus, 14 females and seven males, all sibling of the cohort 1 (i.e. those oysters were not followed in the longitudinal study), were used producing 15 full-sib families and 5 half-sib families. The same hatchery and nursery protocols used for the cohort 1 were applied for the cohort 2. Spat were transferred on the same site used for the cohort 1 in November 2014 (mean individual weight 2.6 g, one bag of 1 kg per family, i.e. around 406 oysters per family and 6090 oysters deployed). Each family was grown in separate bag until individual tagging (Pit-tag, Biolog-ID, BERNAY France) in April 2016. Then, oysters were mixed and grown using standard field on-growing method. Sex was recorded as described above, in June of each year from 2015 to 2019 (Table 1). As for cohort 1, data are presented without distinguishing the families to have a broad view of sex ratio and sex change for a second cohort of *C. gigas*.

2.3. Statistical analyses

All statistical analyses were conducted using R® (version x64 3.4.1, RCore Team) with significance set at $\alpha = 0.05$. No data required transformation before analysis.

Sex ratio was calculated each year from year 1 to year 6 as the number of females divided by the number of females and males sexed at year Y. The standard error (SE) was calculated such $SE = \sqrt{(p * q)/n}$ where p is the proportion of females, q = 1-p the proportion of males, and n the sample size. The sex ratio of each cohort for years 1 to 6 was compared to the suggested “ideal” ratio of 1:1 using χ^2 tests. Sex ratio was compared among years by logistic regression and a logit transformation, and pairwise comparisons among years were conducted using least-squares means.

Sex was recorded from year 1 to year 6, leading to five sets of data recording the sex change between two consecutive years, defined as sets Y1/2, Y2/3, Y3/4, Y4/5, and Y5/6 for the cohort 1. Similarly, four sets were available for the cohort 2 defined as sets Y1/2, Y2/3, Y3/4, and Y4/5. For each set, the percentage of sex change was calculated from the ratio of the number of oysters that exhibited sex change between year Y and year Y + 1 and the total number of oysters sexed in year Y + 1. Sex change was compared among sets by logistic regression and a logit transformation.

Finally, the estimated regression equations were obtained for the cohort 1, as well as for males and females sexed at year 1 to compute the predicted cumulative percentage of sequential hermaphrodites at the desired age (in years) from year 1 to year 30.

3. Results

3.1. First cohort

3.1.1. Sex ratio

The sex ratio of the population every year is shown in Fig. 1. The mean percentage of females among years was 67% ranging from 61% in year 2 to 73% in year 4. The sex ratio was significantly different from 1:1 every year ($P < 0.0001$). Similarly, the sex ratio was significantly different among years ($P < 0.0001$). All pairwise comparisons were significant ($P < 0.01$) except between year 1 and year 5, between year 1 and year 6, and between year 5 and 6.

3.1.2. Sex change between two consecutive years

For set Y1/2, 66% of the population did not change sex (Fig. 2). This proportion significantly increased for the subsequent sets ($P < 0.0001$) with 84% for Y2/3, 82% for Y3/4, 89% for Y4/5 and 91% for Y5/6.

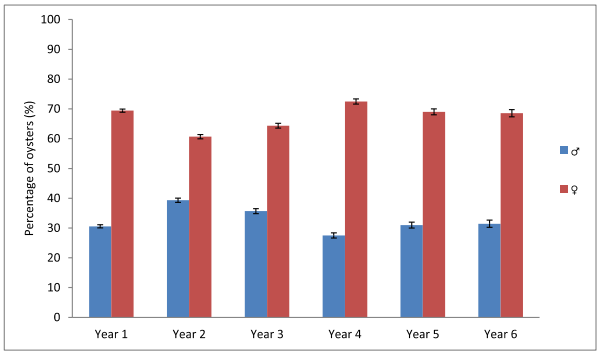


Fig. 1. Sex ratio (± SE) for the cohort 1 from year 1 to year 6. The number of oyster sexed each year is reported in Table 1.

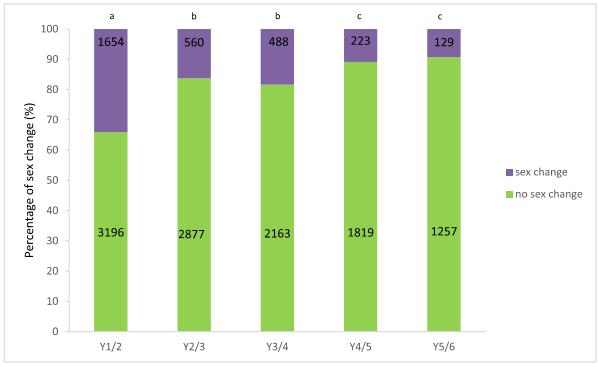


Fig. 2. Percentage of the oyster population for the cohort 1 experiencing or not a sex change between two consecutive years for each set (Y1/2 to Y5/6, Y being the year). The numbers of oysters that experienced or not a sex change are reported inside the bar. Oysters without any observable gametes and simultaneous hermaphrodites at year Y were excluded. The letters a, b, and c indicate significant differences among sets ($P < 0.0001$).

3.1.3. Percentage of sequential hermaphrodites at year 6

For oysters sexed each year from year 1 to year 6 (n = 1386), 42% never exhibited any sex change (Fig. 3). Among them, 34% were potentially true females and 8% were potentially true males. The percentages of oysters undergoing sex changes were 32%, 19%, 5%, 1% and 0.1% for 1, 2, 3, 4 and 5 sex changes, respectively (Fig. 3).

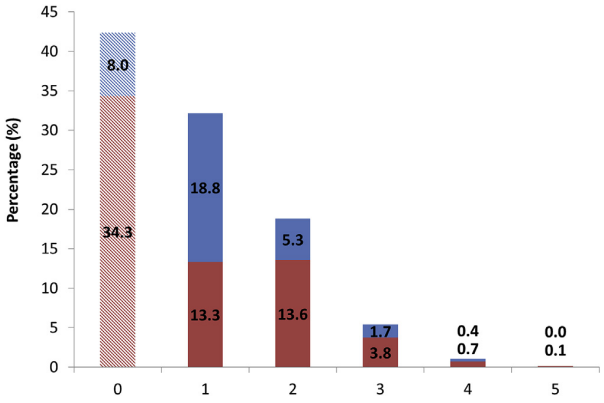
3.1.4. Prediction of the percentage of sequential hermaphrodites during the lifespan in C. gigas

The regression equations to predict the cumulative percentage of sequential hermaphrodites according to the age of the oysters are given in Table 2. Although 42% of the oysters were identified as potentially true females and potentially true males at year 6 (Figs. 3), Fig. 4 predicts that almost all oysters should experience at least one sex change during their lifetime, occurring at any time, even if the probability for sex change decreased in older oysters. Thus, 95% of the population are predicted to exhibit at least one sex change between year 1 and year 19. It may occur significantly earlier for the males (in the first 11 years) compared to the females (in the first 27 years) (Fig. 4). The percentages of new sequential hermaphrodites each year are given in supplementary data 2.

3.2. Second cohort

3.2.1. Sex ratio

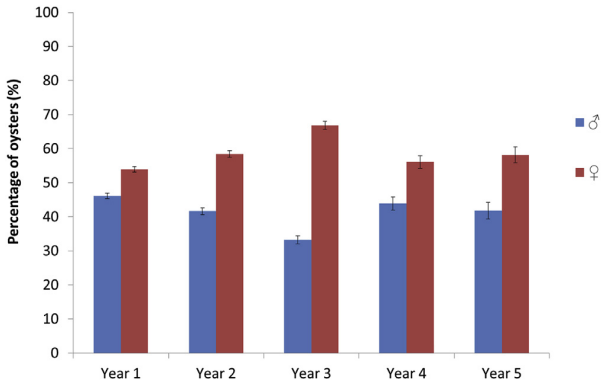
The sex ratio of the population every year is shown in Fig. 5. The mean percentage of females among years was 59% ranging from 54% in year 1 to 67% in year 3. The sex ratio was significantly different from 1:1 every year ($P < 0.01$). Similarly, the sex ratio was significantly different among years ($P < 0.0001$). Pairwise comparisons were significant ($P < 0.01$) between year 1 and year 2, and between year 3 and the other years.



eir age (in years), as well as for oysters sexed either male or female at year 1.

3.2.2. Sex change between two consecutive years

For set Y1/2, 53% of the population did not change sex (Fig. 6). This proportion significantly increased for the subsequent sets ($P < 0.0001$) with 62% for Y2/3, 86% for Y3/4, and 89% for Y4/5.



3.2.3. Percentage of sequential hermaphrodites at year 5

For oysters sexed each year from year 1 to year 5 (n = 333), 24% never exhibited any sex change. Among them, 17% were potentially true females and 7% were potentially true males. The percentages of oysters undergoing sex changes were 44%, 26%, 5% and 1% for 1, 2, 3, and 4 sex changes, respectively.

4. Discussion

This study aimed to investigate, for the first time, the time-course of the sex ratio and the ability to change sex during the first six years of the life of *C. gigas*. It allows us to estimate the proportion of sequential hermaphrodites in the population, and to clarify three milestones that are still debated regarding sex determination in this species: sequential hermaphroditism, protandry, and the sexual system. As a consequence, this study will also provide useful information for comparative reproductive biology as it concerns (i) a representative of Lophotrochozoa, which is poorly documented in this aspect of its biology, (ii) an organism with a very plastic reproductive system, and (iii) an invertebrate with sex-determining genes, something more in common with vertebrates than with other invertebrates (Santerre et al., 2012, 2014; Zhang et al., 2014).

4.1. Sequential hermaphroditism in C. gigas

Simultaneous hermaphroditism was observed during our study, with an annual frequency of less than 1% (data not shown). This small proportion is similar to that previously reported in *C. gigas* (Amemiya, 1929; Guo et al., 1998; Normand et al., 2009; Steele and Mulcahy, 1999; Yasuoka and Yusa, 2016). In contrast, sequential hermaphroditism describes animals that first function as one sex and then switch to the other sex. From the handful of studies on sex determination in oysters, sequential hermaphroditism has been poorly characterized at the individual scale and the distinction between individuals that undergo a sex change and those that do not is rarely achieved. For this reason, our study showed an accurate identification of sequential hermaphrodites based on the number of sex changes observed during the five or six years recorded and the evolution of sex change by age.

Thus, 66% of oysters of the cohort 1 did not change sex during the two first years (Fig. 2) which is in agreement with the results reported in *C. gigas* after two breeding seasons by Amemiya (1929) (66%) and Park et al. (2012) (60%). For the cohort 2, a lower proportion was observed with 53% of the oysters that did not change sex during the two first years (Fig. 6) matching with the results reported by Lango Reynoso (1999) (45–52%). Although environment might play a role, this could be explained by the parents of the cohort 2. Indeed, one the four families used to produce the cohort 2 exhibited the highest tendency for sex change among the 38 families of the cohort 1. This family contributed to 9 of the 15 families of the cohort 2 suggesting that genetic factors might be involved for sex change in *C. gigas*.

From the 1386 oysters sexed six years in a row for the cohort 1, 42% did not change sex which is within the range for similar studies in *C. virginica* (33–57%) (Haley, 1979; Needler, 1942). Within the 42% of oysters that did not experience sex change, 34% were potentially true females and only 8% were potentially true males. This contrasts with the two studies in *C. virginica* that found 45% true males and 12% true females (Haley, 1979) and 30% males and 4% females (Needler, 1942). Although Coe (1932) introduced the idea of true males and Hedrick and Hedgecock (2010) added true females, this is the first time that the proportions of these groups are reported in *C. gigas*. Again, the lower proportion of true females (17%) and true males (7%)(Fig. 7) for the cohort 2 than those reported in cohort 1 could be explained due to the inherence of genetic factors through the families used to produce the cohort 2.

8%) and protogynic (20.7%) oysters. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Consequently, 58% of the oyster population for the cohort 1 were sequential hermaphrodites after six breeding seasons. The percentages of oysters encountering 1, 2, 3, 4 or 5 sex changes in our study were 32, 19, 5, 1 and 0.1%, respectively for the cohort 1 (Fig. 3). Our study demonstrates that most of the sex-changing oysters exhibit only one or two sex changes (51%), while only 6% of the population had at least three sex changes. Similar results were found for the cohort 2 (Fig. 7). This is also in agreement with the results observed in *C. virginica* after five years with 59% and 7%, respectively, although this study was only based on 57 oysters (Needler, 1942). Also, 25% of the oyster population experienced bidirectional changes and that true alternating sexuality, with a sex change encountered each year, only involved a very limited proportion of the population (0.1% at year 6 for the cohort 1 and 0.9% at year 5 for the cohort 2).

Among the sequential hermaphrodites, older animals exhibited less frequent sex change, even if sex change was observed over the whole study. Thus, 34% of the oysters (n = 4850) changed sex between the two first breeding seasons, while it decreased to 9% between the fifth and sixth breeding season (n = 1386) (Fig. 2). Similar tendency was observed for the cohort 2 from 47% of the oysters (n = 2465) that changed sex between the two first breeding seasons to 11% between the fourth and fifth breeding season (n = 339) (Fig. 7). There is a lack of information on sex change at the individual level in the literature for *C. gigas,* as previous studies have only recorded the sex ratio for two years (Amemiya, 1929; Lango Reynoso, 1999; Park et al., 2012). In *C. virginica*, no distinct pattern was apparent in the rhythm of changes from younger to older oysters with 12, 15, 18, 18 and 6%, respectively (Galtsoff, 1937, 1964), and with 39, 12, 28 and 35%, respectively (Needler, 1942). The variability in the rates of sex change with oyster age cannot be explained, as the cues that control sex change in oysters remain poorly understood. Meanwhile, several factors might control sex change as demonstrated for hermaphroditic fishes with environmental cues (temperature, pH, hypoxia), density, social structure, or attainment of a critical age or size (reviewed in Todd et al. (2016)). Thus, it could be assumed that younger oysters could be more sensitive to the factors triggering a sex change in our *C. gigas* populations.

Even if our collected data showed the existence of potentially true males and potentially true females after six years of follow-up, the predicted cumulative percentage of sequential hermaphrodites was up to 95% over their life period. It suggests that all oysters of our population of *Crassostrea gigas* could be potentially sequential hermaphrodites. Nevertheless, results obtained in the first six years could be useful for aquaculture and research purposes, to control the conditioning in hatchery by optimizing the number of adults (Helm et al., 2004), to produce inbreed lines (Lannan, 1971; Yang et al., 2015) or to improve sex-specific growth (Baghurst and Mitchell, 2002).

4.2. Protandry in C. gigas

Previous studies considered *Crassostrea* oysters as protandrous hermaphrodite (Coe, 1934; Galtsoff, 1964; Guo et al., 1998). Protandrous animals are defined here as those (i) exhibiting a primary sex ratio within the population distorted toward males (first-maturing sex in sex-changing animals as suggested by Charnov (1982)) that evolves toward females, and also (ii) exhibiting one sex change.

Our populations of *C. gigas* exhibited a primary sex ratio significantly biased toward females with 69% for the cohort 1 (n = 7409) (Fig. 1) and in a lesser extent, 54% for the cohort 2 (n = 4320)(Fig. 5). However, previous studies did not reach a consensus concerning the primary sex ratio in *C. gigas.* The sex ratio was biased in favor of females in some studies (Amemiya, 1929; Lango Reynoso, 1999; Santerre et al., 2013), while some observed 1:1 primary sex ratios (Fabioux et al., 2005; Park et al., 2012), and others observed sex ratios biased toward males (Enriquez-Diaz et al., 2009; Guo et al., 1998; Yasuoka and Yusa, 2016). In other oyster species, male-biased sex ratios have been reported in *C. virginica* (Coe, 1936; Galtsoff, 1937; Haley, 1979; Kennedy, 1983; Powell et al., 2013) and *Saccostrea cucullata* (Morton, 1991), while there is a large predominance of females in *C. rhizophorae* (Littlewood and Gordon, 1988) and no significant dominance of either sex in *C. madrasensis* (Mohan Joseph and Madhyastha, 1984) and *C. gasar* (Ramos et al., 2013). In bivalves, Morton (1991) proposed that a pronounced female bias could optimize the reproductive success, by maximizing resource allocation into the more energy-demanding process of oogenesis. However, this diversity of primary biased sex ratio falls in line with the high phenotypic plasticity of the oyster due to complex genotype-environment interactions. In this respect, many biological mechanisms are proposed to affect the primary sex ratio of organisms, which is expected to be 1:1 under heterogamety, including genes and cytoplasmic factors, the sexual system, and the mode of sex determination (Yusa, 2007). Cross-generational plasticity may also induce bias. Thus, the ecological conditions experienced by the mother could influence life-history trade-offs in offspring and result in the production of more of the sex that provides greater fitness returns (Wade et al., 2003). Sex ratio may also be distorted to survive in heterogeneous environments (Ghiselin, 1969), especially for organisms with low mobility such as the oyster. However, according to Yusa (2007), several other factors may explain the existence of bias in sex ratios, such as the misidentification of sex, sampling size bias, sex-related differences in mortality, and age differences at the time of sexual maturity. The design of our study limited such bias as follows: (i) hatchery-produced oysters were the same age and were individually monitored on our experimental oyster farm; (ii) a large number of oysters (7409 and 4320 individuals for the cohorts 1 and 2, respectively) at the beginning of the survey make the results robust; (iii) mortalities were not significantly correlated with sex (results not shown); and, (iv) the time of sexual maturity is well-known for both sexes (Berthelin et al., 2000) and was accurately checked annually by spawning and/or gonad biopsy.

The significant bias in the primary sex ratio toward females in the first year was maintained over the following five years (Figs. 1 and 5). This tendency is explained by the (i) higher proportion of true females (34% and 17%) than the proportion of true males (8% and 7%) that did not change sex during the five/six years of the study, (ii) high percentage of females among the oysters showing two sex reversals (74% and 63% for cohorts 1 and 2, respectively Figs. 3 and 7), and (iii) protandrous males (19% and 23%) (Figs. 3 and 7). Female-biased sex ratios that were maintained over the second year have also been previously reported in *C. gigas* (Amemiya, 1929; Lango Reynoso, 1999), while reports of primary male-biased sex ratios showed an increase over time of the proportion of females in *C. gigas* (Guo et al., 1998) and *C. virginica* (Haley, 1979).

During our study, 19% of the oyster population underwent protandrous sex change while 13% underwent protogynous sex change for the cohort 1 (Fig. 3), while it was 23% and 21% for the cohort 2 (Fig. 7). Although many previous studies suggested that protandry is the typical form of sexuality in oysters (Coe, 1934; Galtsoff, 1964; Guo et al., 1998; Parker et al., 2018; Powell et al., 2011), protogynous sex changes have also been observed in *C. gigas* for 70% of the animals changing their sex only once (calculated from Park et al. (2012)) and in *C. virginica* (Haley, 1979).

Our results strongly encourage the scientific community to consider the oyster as a very flexible sex-changer, undoubtedly experiencing both protandrous and protogynous sex changes, as well as multiple sex changes (Figs. 3 and 7).

4.3. Hypotheses for the sexual system of C. gigas

As our study involved long-term monitoring of a large population of oysters that were individually sexed each year for six years, it allowed us to gather a large amount of reliable data related to the sex ratio and the ability to change sex in *C. gigas*. These data highlight the plasticity of reproduction in *C. gigas*, as previously mentioned for instance by Guo

et al. (1998) who discussed “protandric sex change, dioecy and hermaphroditism” and Hedrick and Hedgecock (2010) who discussed “dioecious, sequential hermaphrodites and some rare simultaneous hermaphrodites”. From our data, the mode of reproduction of *C. gigas* could only involve sequential hermaphrodites and some rare simultaneous hermaphrodites. Our work highlights a plasticity for the mode of reproduction at the individual level in *C. gigas* by proposing robust percentages of potentially true males and true females and sequential hermaphrodites and the temporal variation for sex change among the hermaphrodites after 6 years as well as the simulated percentage of hermaphrodites during the lifespan of one *C. gigas* population.

Based on these results, we propose a hypothesis involving changes in the mode of reproduction in *C. gigas* in France. When environmental conditions change or when a species occupies a new habitat, selection may favor a transition from hermaphroditism to gonochorism or vice versa (Weeks et al., 2006). In France, the production of *C. angulata* collapsed, and to sustain the oyster production, *C. gigas* was massively introduced during the 1970s from Japan and British Columbia (Grizel and Héral, 1991). Although there was no genetic differentiation or decrease in diversity between the population of *C. gigas* from Japan (the origin of European populations) and those from France (Rohfritsch et al., 2013), the latter may have experienced selection for the mode of sex determination to adapt to its new habitat along the French coast. Similarly, global warming has increased seawater temperature, a parameter known to be involved in environmental sex determination, as well as ocean acidity. Recently, it was found that ocean acidification altered sex determination in *Saccostrea glomerata* leading to a significant change in the population sex ratio by increasing the proportion of females (Parker et al., 2018). A similar trend was also observed in *C. hongkongensis* concerning trace metal pollution (Weng and Wang, 2015). Thus, this phenotypic plasticity could be an adaptive response to spatially heterogeneous and/or temporally varying environments (Ernande et al., 2004), and such variation may switch the mode of reproduction of *C. gigas* from hermaphroditism to gonochorism or vice versa. This modulation could involve three transitionary sexual systems (i) trioecy (mix of males, females and simultaneous hermaphrodites), (ii) androdioecy (mix of males and simultaneous hermaphrodites), and (iii) gynodioecy (mix of females and simultaneous hermaphrodites) (Charlesworth and Charlesworth, 1978; Charnov, 1982). However, these modes of reproduction do not take into account the sequential hermaphroditism that was very evident in *C. gigas* in this study. This particularity can be an intermediate strategy developed by the oyster to quickly cope with environmental variations.

In conclusion, the results of the present study showed a sex ratio distorted in favor of females each year for the two cohorts for five and six years. Among the oysters sexed six years in a row, 42% didn't change sex, while changing sex more than two times was scarce (7%). Similar trends were observed for the cohort 2, although sex reversal was higher. This could be explained by a genetic basis for sex change, as one of the family used as parents showed the highest proportion of sex changer oysters (i.e. sequential hermaphrodites). For the first time in *C. gigas*, we found that sex changes decreased with the age of the oyster. Finally, it appears that the entire population of oysters should be sequential hermaphrodites. Our study provides valuable information for designing future studies to (i) better understand genetic control of sex-determining mechanisms in *C. gigas*, (ii) manage production in hatcheries (control sex ratios and implement breeding programs) and assist in fisheries management, (iii) study comparative reproductive biology as very little information is available regarding this topic in molluscs, Lophotrochozoa, and other species exhibiting hermaphroditism (a well-conserved mode of reproduction in the animal kingdom), and (iv) advance evolutionary perspectives on the sexual system.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Anthony Bourgeau, Hugo Koechlin and Agathe Leveque for their help to phenotype the oysters in year 2, 3 and 5, respectively. We also thank the hatchery team of the LGPMM (Ifremer-La Tremblade) for their help in the oyster production as well as the nursery team of the LSPC (Ifremer-Bouin). Finally, we warmly thank Patrick Azéma for its help for the production of the oysters used in this study. We thank Ifremer and Région Normandie for Ph.D. scholarship.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.734555.

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Supplementary data

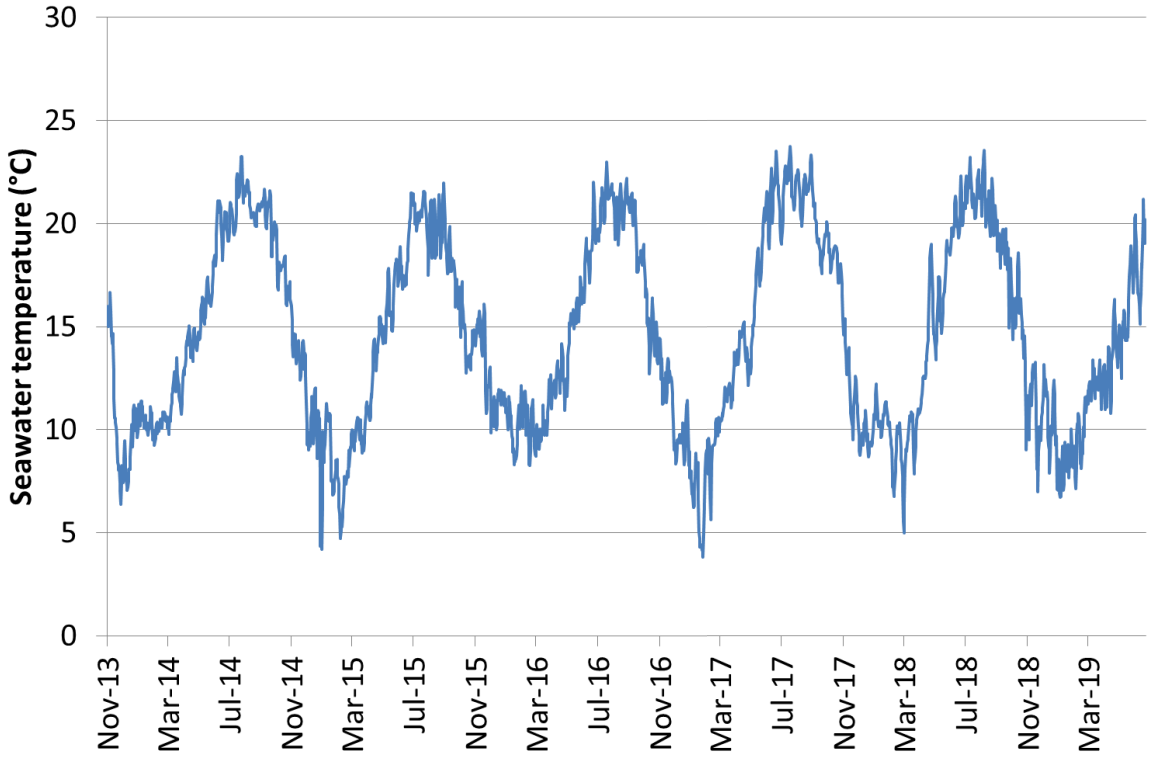

Figure S1: Seawater temperature (°C) from September 2013 to June 2019.

Seawater temperature ranged from 3.8°C in January 2016 to 24.2 °C in June 2019 as shown above (Supplementary Figure 1). Each year, the period for sex determination in *C. gigas* is suspected to occur between September and January. The seawater temperature during this period is shown in black frames. It decreased from 21.7 to 6.4 °C (means = 13.6 °C) in 2013/2014, from 21.7 to 4.2 °C (means = 14 °C) in 2014/2015, from 21 to 8.3 °C (means = 13.5 °C) in 2015/2016, from 21.5 to 3.8 °C (means =12.5 °C) in 2016/2017, from 21.5 to 8.7 °C (means = 14 °C) in 2017/2018 and from to 20.1 to 6.7°C in 2018/2019 (mean =12.6°C).

Chapitre 2

Relation entre le sexe et la croissance chez l'huître creuse *Crassostrea gigas*

Relation entre le sexe, le changement de sexe et la croissance chez l'huître creuse *Crassostrea gigas*

Questions : Existe-t-il un **dimorphisme sexuel** pour le poids total, la longueur, la largeur et l'épaisseur de la coquille chez *Crassostrea gigas* ? Quel est **l'effet du changement de sexe** sur ces caractères en lien avec la croissance ?

Méthode : Une population d'huîtres creuses a été produite en 2013 à partir de géniteurs sauvages. Cette population a ensuite été testée dans le bassin Marennes-Oléron de novembre 2013 à juin 2019. Ainsi 7488 huîtres ont été **sexées** à partir de 2014 puis **marquées** et suivies **individuellement** jusqu'en 2019 (*cf.* Chapitre 1 pour la description du sexe-ratio et du changement de sexe). Au premier sexage en 2014, le **poids total** des huîtres par sexe a été enregistré, puis à partir du printemps 2015, chaque huître a été pesée (poids total) et sa coquille mesurée (**longueur**, **largeur** et **épaisseur**).

Résultats : Dès la 1ère année de collecte, les **femelles** présentaient des valeurs morphologiques (poids, longueur, largeur, épaisseur de coquille) **significativement supérieurs** aux mâles (14 *vs* 13g, 59 *vs* 56mm, 39 *vs* 37mm, 21 *vs* 20mm pour les femelles et mâles respectivement). Lors de **deux cycles de reproduction consécutifs**, les huîtres **restant femelles** et celles effectuant un changement de sexe **protandre** (mâle vers femelle) présentaient des **gains de poids supérieurs** à ceux des individus restant mâles ou passant de femelles à mâles et cela de façon significative ($P < 0,05$) excepté pour les couples d'années *Year*2-3 et *Year*4-5. Parmi les individus mesurés et sexés **chaque année pendant 6 ans**, les huîtres **protandres** (un seul changement de sexe, de mâle vers femelle) présentaient le **meilleur taux de croissance** [poids total final (pf) = 73g], suivi par les individus mâles à la première maturité sexuelle puis changeant 3 fois de sexe (pf = 72g) et les huîtres restant toujours femelles (pf = 72g), puis les huîtres effectuant des changements bidirectionnels (mâle en 1ère année pf = 71g, femelle en 1ère année pf = 69g), les huîtres mâles n'ayant jamais changé de sexe (pf = 65g) et enfin les huîtres protogynes (un seul changement, femelle vers mâle) (pf = 64g). Pour finir, la **croissance la plus faible** concernait les huîtres **femelles en 1ère année** et **changeant 3 fois** de sexe au cours des 6 années de suivi (pf = 60g).

Conclusions : À âge et environnement identiques, les femelles présentent des biométries supérieures à celles des mâles, chaque année et au cours de plusieurs années de vie, **confirmant un dimorphisme sexuel chez *Crassostrea gigas*.** Le **nombre** de changements de sexe ainsi que le **sens** du changement **influence la croissance** des huîtres. Pour les individus présentant un **sexe primaire femelle**, **tout changement de sexe ralentit** sa croissance tandis que pour les huîtres à **1ère maturité mâle**, **tout changement de sexe augmentera** son taux de croissance.

Ma participation : J'ai effectué la collecte des données morphologiques de l'année 4 et participé à celle de l'année 5, créé la base de données et l'ai maintenue à jour. J'ai également participé aux traitements statistiques et réalisé l'interprétation ainsi que la mise en forme des résultats.

Ces travaux ont fait l'objet d'une valorisation par communication affichée :

C Broquard, AS Martinez, L Dégremont. 2019 Is there a link between sex/sex changes and weight in the Pacific oyster *Crassostrea gigas*? JEDNBISE19, 21-22 Mars 2019, Caen, France

Remarque : Pour des raisons logistiques, les co-auteurs de l'article scientifique n'ont pas encore pris connaissance de la version présentée ci-après.

Article 2

Relationship between sex and growth of the Pacific oyster, *Crassostrea gigas*

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Abstract

The Pacific oyster *Crassostrea gigas* is a sequential hermaphrodite, being male or female during one annual reproductive cycle and potentially changing sex between two consecutive cycles. Very few studies have highlighted sex-specific asymmetries in length and weight in natural or hatchery-produced oyster populations, and none has examined the effect of sex change on biometric parameters neither at the individual level nor at the level of the population. To this purpose, we performed an individual long-term follow-up (6 years) on a large population (7,488 animals). All oysters were individually sexed from 2014 (year 1) to 2019 (year 6), and individually weighted and measured for shell length, width and thickness from year 2 to year 6, while whole weight per sex was estimated at year 1. From year 2 to year 6, female oysters had greater whole weight (+8-13%), shell length (+3-5%), shell width (+4-6%) and shell thickness (+3-5%) than males. Although this sexual dimorphism appears low at year 6, it might be related to the low growing environment where the oysters grew for 6 years. Interestingly, the way of the sex change between two consecutives reproductive cycles also had a significant effect on the weight growth, and in a lesser extent for shell growth traits with an advantage for males becoming females in comparison with females that showed a sex change toward males. From the oysters sexed during six years and showing from 0 to 3 sex changes (1,369 oysters), we found that the frequency of sex change along with its directions influenced the growth. Thus, the difference in final weight at year 6 increased with the number of sex changes (0, 1, and 3) with true females having a higher final weight (71.8g) than true males (65.1g)(+10%), as protandric oysters (only one sex change from male to female) (73.3g) in comparison with protogynic oysters (64.2g) (only one sex change from female to male) (+14%), as well as males at year 1 changing sex three times (71.8g) in comparison with those being females at year 1 (60.5g) (+18%). In contrast, oysters with bidirectional sex changes regardless of their first sex showed similar final weights (69 and 70.8 g). Thus, sexual dimorphism in *C. gigas* exists between males and females for weight and shell traits, which is amplified throughout the sex changers highlighting the importance of the successive hermaphrodites for growth. Our study brings a new milestone to know the potential for genetic improvement on sex ratio and sex reversal for growth in addition to selective breeding programs focusing on weight/shell traits and triploïdization.

1. Introduction

Native from Asia, the Pacific oyster is now cultivated worldwide with a global production of 573 617 tons in 2016 (FAO, 2019). In France, the species was introduced in the early 1970s in order to support the oyster industry. As a result, its domestic production has increased to 135 629 tons in 1996. However, periods of massive mortality have reduced the national production: down to 96 203 tons in 2008 due to the presence of pathogen *OsHV-1* and down to 73 177 tons in 2012 linked to the increase of detection of *Vibrio aestuarianus* in the environment (FAO, 2019). To cope with these losses, breeding programs have been put in place to improve the resistance of this species to diseases and so increase their survival rates (Segarra et al., 2010; Travers et al., 2017).

Another lever to increase yield and support the profession is to select oysters with good growth potential. For centuries, selective breeding has been an important contributor to huge advances in genetic improvement and productivity of farmed animals (Dégremont, 2011; Dégremont et al., 2016; Dégremont et al., 2015). Some studies performed on *C. gigas* using mass selection have shown good performance. For example, after one generation of mass selection, an average response to selection of 9.5% in yield has been obtained by Dekkers and Hospital (2002) and two stocks were 12.2% larger than control lines in the experiment done by Langdon et al. (2003).

Many fish species show sexual dimorphism in size (Li et al., 2011). For some, females are bigger as it is the case for the rainbow trout (Bye and Lincoln, 1986) while others have bigger males, like for the Nile tilapia (Beardmore et al., 2001). Nowadays, only one study has been done on the effect of sex on the growth of *C. gigas* (Baghurst and Mitchell, 2002). The study was conducted over seven months, with a monthly sample of 100 individuals. The authors found the existence of asynchronous sex-specific growth rates where females grew faster than males.

Crassostrea gigas is known to be a hermaphrodite species with an annual gametogenetic cycle (Amemiya, 1929). However, up to date, no study was performed to establish if its ability of changing sex has an influence on its growth performance. Moreover, except the study conducted by Baghurst and Mitchell (2002), no others study looked at the relation between age, sex and growth. Therefore, in this study, a detailed examination of the effects of sex and

sex change on the growth performance of *C. gigas* was conducted over six years on a large population. The aim of the study was to generate reference data on the relation between the sexual system and the growth in order to assure a better yield for growers.

2. Material and methods

2.1. Biological material

The broodstock were sampled from a wild oyster population from the Marennes-Oléron Bay (France). They were hold in the conditioning room at the Ifremer hatchery in La Tremblade (France). Seawater temperature was gradually increased to 21 °C during one week in February 2013. Seawater flow was 400 L/h, and a cultured phytoplankton diet (*Isochrysis galbana, Tetraselmis suecica, and Skeletonema costatum*) was provided ad libitum (50,000 cells/mL). Twenty males and forty females were used, each male being crossed with two females producing 40 full-sib families of *C. gigas* on March 27th, 2013. The parents were opened and sexed by microscopic observation of gonad samples spread on a slide (presence of spermatozoa or oocytes). The gametes were collected from each parent by stripping the gonad. After fertilization, the larvae were raised in 30-L tanks at 25°C in UV-treated, filtered, and aerated seawater. All families were raised separately. The water was changed three times per week. Larvae were fed daily with *Isochrysis galbana* (30,000 cells/mL) until they reached 140 µm, after which the diet was supplemented with *Skeletonema costatum* (30,000 cells/mL). Two weeks after fertilization, larvae were placed on cultch in flow-through raceways at 20°C supplied with UV-treated seawater enriched with *S. costatum*. Oyster spat were reared under standard hatchery conditions until they reached a size of 2 mm. In May 2013, 5,000 oysters per family were transferred to the Ifremer nursery in Bouin (France). Density was reduced during the nursery period as some oysters were used in studies to determine the genetic basis for resistance to pathogens (Azéma et al., 2017b). Each family was kept in one sieve until November 2013 and they were protected within the facility under biosecurity control to avoid contamination with major oyster pathogens such as OsHV-1 and *Vibrio aestuarianus*. Further details on these families are provided elsewhere (Azéma et al., 2017a; Azéma et al., 2017b).

2.2. Field study

During all the study, families were kept separately. Nevertheless, data are presented without distinguishing the families in order to have a broad view of sex ratio, sex change and growth in our oyster population of *C. gigas*. The field study lasted from November 2013 to July 2019. At deployment, the mean individual weight was approximately 3g. Around 14,000 oysters were deployed to the field study site at La Floride in the Marennes-Oléron Bay, which is the main area for shellfish culture in Europe (Azéma et al., 2017a; Azéma et al., 2017b). This site is located in the intertidal area and the mean immersion time is around 50%, which is low in comparison to surrounding growing leases. This choice was based to avoid a second gametogenesis within a year. Oysters grew in bags which were randomly attached to racks. Every month, bags were checked to make sure that they were well-attached to the racks and that they were free of defects that would cause loss. The seawater temperature was recorded every hour throughout the study using two probes (ThermoTrack; supplementary data).

2.3. Sex observation

All oysters were checked annually at the time of sexual maturity in June from 2014 to 2019. At the beginning of June, oysters were transferred from the field to the laboratory and held in a flow-through system. Seawater was chilled to 15°C until sex was determined in order to avoid unintentional spawning events. The number of oysters sexed each year is indicated in Table 1; it decreased throughout the study mainly because of mortality and to a much lesser extent, sampling for molecular analyses (data not shown). Non-destructive methods were used to determine oyster sex. Firstly, spawn was induced using thermal shocks (20-30°C). In order to visualise the emission of the gametes, oysters were placed in a single layer with sufficient distance from each other in a black-bottomed 200-L tank filled with seawater. Some oyster gametes were also added to the tank as a stimulant. Males emit their spermatozoa as a long, opaque white mesh. Females are identifiable by the emission of their oocytes in the form of repeated, dense, and granular clouds. When the spawn was triggered, each oyster was placed in a transparent 300-mL beaker containing seawater at 25°C to confirm the nature of the gametes. When massive spawns occurred, seawater was removed, and all oysters were

individually placed into beakers. The thermal shock cycle was repeated up to 10 times, but some oysters did not respond to induction.

Secondly, for non-responding individuals, a biopsy of the gonad was performed and sex was determined by microscopic observation of gonad smears. Oysters were placed in a 5-L tray with a muscle relaxant solution consisting of seawater (3/5), freshwater (2/5), and magnesium chloride (50 g/L). As soon as the shells opened, a smear of gonad was taken using a needle (0.9 × 38 mm; Terumo©) and a 1-mL syringe (Terumo©). Mature gametes were visualised microscopically (40×) to determine the sex. Oysters with oocytes were identified as females and those with spermatozoa were classified as males. Oysters with both mature oocytes and spermatozoa were identified as simultaneous hermaphrodites (represented less than 1% per year). For some oysters, sex could not be determined, and they were categorised as “empty”. These two categories were excluded from the results presented below. After spawning and biopsies, male and female oysters were placed in separate trays until all data was recorded. Males and females from the same family were placed into culture bags and the bags were returned to the study site.

2.4. Labelling the oysters

After the first sexing in June 2014, male and female oysters were separated into labelled oyster bags. In April 2015, all live oysters were individually marked with a plastic-laminated number glued with epoxy resin (Sader©) on the upper valve, and then, males and females were mixed until July 2019.

2.5. Growth

After the first sexing in June 2014 and for each family, the total weight of the males was divided by the number of males to obtain the mean individual weight at year 1. Same approach was done for the females. Then, the whole weight (in grams) of each oyster was recorded using a 0,01g precision scale in April from 2015 to 2019. The shell of each oyster was also measured (length, width and thickness) using an electronic calliper since 2015. After measurements, oysters returned to the study site.

2.6. Data analyses

All statistical analyses were conducted using R® (version x64 3.4.1, RCore Team) with significance set at $\alpha = 0.05$.

The sex recorded in June was already determined two months earlier when measurements occurred. As a result, the whole weight (g), the shell length (mm), the shell width (mm) and the shell depth (mm) recorded in April were compared between female and male at each year using one-way analysis of variance.

Then, the growth of oysters sexed two years in a row defined as the increment of weight, shell length, shell width and shell depth during this period was compared among four groups, which were oysters showing sex reversal from ♀ to ♂ and from ♂ to ♀, as well as those that did not change sex, either male (♂♂) or female (♀♀). The growth was calculated for the weight between years 1 and 2, and for all traits between years 2 and 3, and so on until years 5 and 6.

During the study, 1,386 oysters were sexed six years in a row. Among them, 17 oysters had more than three sex changes, and were then removed from the subsequent analyses due to the low number in comparison with 0 (n=587), 1 (n=446), 2 (n=261) and 3 (n=75) sex changes. For each year, the weight was compared between males and females sexed at year 1 according to their number of sex change (i.e. true males and true females, protandric and protogynic oysters, oysters that changed twice, and oysters that changed tree times).

Finally, the gain of weight between the year 1 and the year 6 and the gain of shell length between years 2 and 6 (no data at year 1) for all oysters sexed six years in a row were analysed according to the following model ():

$$Y_{ijk} = \mu + sex\ change_i + sex\_Year\_1_j + sex\ change_i \times sex\_Year\_1_j + \varepsilon_{ijk}$$

where μ is the intercept, $sex\ change_i$ is the number of sex change from year 1 to year 6 (0, 1, 2, and 3), and $sex\_Year\_1_j$ is the sex recorded at year 1 (male or female) and ε_{ijk} is the error term.

3. Results

3.1. Relationship between sex and whole weight, shell length, width and thickness for each year

Mean individual whole weight per sex recorded every year is shown in Fig. 1. For females, the weight was 14g in 2014 and increased with age up to 72g in 2019 (Fig. 1) (Table 1). Males weighed 13g in 2014 and increased to 65g in 2019. Each year, females were significantly heavier than males, ranging from +8% at year 3 to 13% at year 4 ($P < 0.0001$) (Table 1).

The mean length was 59mm for females and 56mm for males at year 2 and they grew to 74mm and 71mm, respectively by year 6 (Fig. 2). There were significant differences in shell length between males and females each year ($P < 0.0001$), the females being longer than males (+3% to 5% depending of the year) (Table 1). Similar patterns were found for the width (+4-6%) and the thickness of the shell (+3-4%) (Table 1).

3.2. Relationship between growth and sex change between two consecutive years

Between years 1-2, the weight growth was significantly different among the four groups ($P < 0.0001$), with the highest growth for the females both years (15.8g), intermediate for the oysters showing a sex reversal from male to female (14.7g) and the lowest for those showing a sex reversal from female to male and the males two years in a row (12.1 and 12.5g, respectively) (Fig. 3) (Table 3). In a lesser extent, a similar pattern was observed between years 2-3, although weight growth was not significantly different among the four groups ranging from 9.4 to 10.1g ($P = 0.14$) (Fig. 3) (Table 3). In contrast, significant differences of weight growth among groups were observed again between years 3-4 ($P < 0.0001$), between years 4-5 ($P < 0.01$), and between years 5-6 ($P < 0.0001$). Overall, oysters having a sex reversal from male to female had the highest growth, while those showing a sex reversal from female to male had the lowest (Fig. 3).

For the shell growth, significant differences among groups were observed between years 2-3 and between years 3-4 for the length, width and depth ($P < 0.05$) while it was not significant between years 4-5 and between years 5-6 ($P > 0.05$), except for width at years 4-5 ($P = 0.04$) and depth at years 5-6 ($P = 0.04$). Overall, the highest shell growth was observed for oysters

with a sex reversal from male to females and the lowest was observed for oysters with a sex reversal from female to males (Table 3).

3.3. Weight and growth of oysters sexed each year from year 1 to year 6

The mean individual weight for oysters sexed each year according to their first sex at year 1 and the number of sex change are reported in Fig. 4. At the end of the study, the final weight was 71.8g for true females (i.e. no sex change from years 1 to 6) and 65.1g for true males with significant differences from year 2 to year 6 (Fig. 4a). For the protandric and the protogynic oysters (i.e. one sex change), the protandric oysters had significant higher weight than protogynic oysters from year 3 to year 6 ending to 73.3g and 64.2g, respectively (Fig. 4b). For oysters changing sex twice; males and females at year 1 had a similar final individual weight reaching 70.8g and 69.0g, respectively (Fig. 4c). Finally, among oysters that subsequently had three sex changes, males at year 1 were significantly heavier than those that were females at year 1, since year 3, ending respectively at 71.8g and 60.5g (Fig. 4d).

A significant interaction between the sex at year 1 and the number of sex change was found for the gain of weight between year 1 and year 6 ($P < 0.0001$). Within sex at year 1, significant difference of growth was observed for females with the highest growth for oysters that had 0 and 2 sex changes, intermediate for those showing one sex change, while those having 3 sex changes had the lowest growth ($P < 0.0001$) (Fig. 5). For the oysters sexed males at year 1, oysters that never changed sex during the study showed a significant lowest growth than those exhibiting 1, 2, and 3 sex changes ($P < 0.01$) (Fig.5). Within sex change, males and females at year 1 that had two sex changes during the study showed a similar growth ($P = 0.82$), while females had a significant higher weight gain than males when they did not exhibit a sex change during the study (Fig. 5). In contrast, males at year 1 showed a significant higher growth than females at year 1 when oysters had one and three sex changes throughout the study (Fig. 5).

Regarding the growth for the shell length between year 2 and year 6, a significant interaction between sex at year 1 and the number of sex change was found ($P < 0.01$). Within sex at year 1, a significant difference of growth was observed for females ($P < 0.01$) while it was not significant for males ($P = 0.051$). For the females at year 1, the highest shell growth was

observed for oysters showing 0 and 2 sex changes (14.8 and 15.5mm, respectively), while it was 12.5 and 13,5mm for those having 3 and 1 sex change, respectively (Fig. 6). The opposite trend was found for males at year 1, with the best shell length growth for individuals having 1 and 3 sex changes (17.6 and 17.1mm, respectively) and the lowest for the ones with 0 and 2 sex changes (15.5 and 16.1mm, respectively) (Fig. 6). Within sex change, males and females that had 0 or 2 sex changes showed a similar growth (P = 0.37 and 0.53, respectively), while males had a significant higher shell length growth than females when they exhibited 1 or 3 sex changes throughout the study ($P < 0.01$) (Fig. 6).

4. Discussion

Sexual growth dimorphism occurs frequently in the animal kingdom (Anderson and Vitt, 1990; Greenwood and Wheeler, 1987; Isaac, 2005; Kupfer, 2009; Leimar et al., 1994; Rensch, 1950; Richter, 1983; Székely et al., 2004). In teleost fish, various species exhibit sex-specific growth (Parker, 1992), such as perch (Craig, 1987; Guti, 1993), tilapia (Toguyeni et al., 1997), halibut (Ghinter et al., 2019), turbot (Imsland et al., 1997) and sea bass (Saillant et al., 2001), but it remains very limited in oyster species. Although some studies showed that larger oysters are predominantly females (Buroker, 1983; Harding et al., 2013; Powell et al., 2013; Yasuoka and Yusa, 2016), only a few studies in our knowledge report sex-specific growth using hatchery-produced oysters (Baghurst and Mitchell, 2002; Normand et al., 2009). The latter had a known and identical age, which is an advantage over wild populations that are composed by several year classes confounding age, and size, to which sex reversal also occurs, making difficult to disentangle the relationship between the growth and sex in *C. gigas*.

This study reported the growth of a large population of *C. gigas* recorded each year during six years from 7,488 oysters at year 1 to 1,426 oysters at year 6. In addition, sex ratio and sex change of this population, both described in Broquard et al. (2020), were also used to determine if a sex-specific growth advantage exists in our population. Clearly, females always showed a higher whole weight, shell length, shell width and shell depth than males each year (Table 1), which is in agreement with the results observed for the whole weight and the shell length by Baghurst and Mitchell (2002). The advantage of the females over the males was observed as soon as the year 2 which represented around +8% at year 3 to +13% at year 4, but only increased from 3.3g at year 2 to 6.7g at year 6 for the whole weight (Fig.1) (Table 1).

In a lesser extent, similar trends were observed for shell traits ranging from +3% to 6% depending of the year (Table 1), which is much lower than results found by Baghurst and Mitchell (2002) with a gain of 18% of shell length for females against males. It could be supposed that the growth difference in favour of the females may have decreased in our study due to the immersion time and/or food level. Indeed, the experimental farming site was located in high intertidal area, and so, reducing the emersion times in order to prevent a second gametogenesis within a year. It was found that weight difference between male and female perch depends on feeding level, with high food level favouring a sexual growth dimorphism (Fontaine et al., 1997). Similarly, growth difference between diploid and triploid mussels was evident in a high growth site while it was not detectable in a low growth site (Brake et al., 2004). Thus, environmental factors may interact with sex-specific growth.

Oysters are sequential hermaphrodites, meaning that some of the females and males observed from the year 2 to the year 6 experienced one or several sex changes as described in Broquard et al. (2020), which could counteract the finding of a better growth of the females over the males. Nevertheless, females that never experienced a sex change had a better growth than males (Fig. 4). One hypothesis proposed by Baghurst & Mitchell (2002) to explain this advantage is that females are better able to retain glycogen reserves. Such behaviour was already described for *C. gigas* in the Marennes-Oléron Bay with higher reserves of lipids and glycogen for females than males during the gametogenesis (Deslous-Paoli and Heral, 1988; Mann, 1979; Soletchnik et al., 1997). Such ability to accumulate more reserves may support the higher reproductive effort for females than males in *C. gigas* as demonstrated for the spat stage by Normand et al. (2009).

For the first time in our knowledge, growth of sequential hermaphrodites in oysters was compared to the growth of true males and true females. Surprisingly, the number of sex-reversal combined to the primary sex had a significant impact on growth. Thus, true females had a similar weight growth than protandric oysters (males at year 1 and that had only one sex change) and males at year 1 that underwent three sex changes (Fig. 5). Although data were not available at year 1, similar trend was observed for the shell length growth between years 2-6 except that true females had a lower growth than sequential hermaphrodites showing one and three sex changes that end up to females (Fig. 6). This suggests the absence of a negative impact of sex reversal on growth in *C. gigas* and so, no additional energy cost for sex reversal.

In contrast, a female at year 1 that had one sex reversal (protogynic) showed a lower growth than a true female, and in a lesser extent, a true male (Fig. 5,6). This was magnified for females at year 1 showing three sex changes; two from female to male and one from male to female. Thus, growth was significantly reduced when the number of sex change from female to male increased. Nevertheless, care must be taken for oysters showing three sex changes due to a relative low number of oysters (n=75) in comparison with 0 (n=587), 1 (n=446), and 2 (n=261) sex changes. This deleterious effect of changing sex from female to male is related to maleness, and could be partly explained due to the difference in reproductive effort, or energy reserves (Baghurst and Mitchell, 2002; Deslous-Paoli and Heral, 1988; Normand et al., 2009) with lower energy into whole weight and shell growth for males than females. Our study suggests that the size sexual dimorphism demonstrated throughout the true females and the true males is also observed for sequential hermaphrodites showing one or three sex changes with a better growth for oysters being or becoming a female.

For oysters underwent two sex changes, they had a similar growth rate whatever their sex at year 1. This would mean that the deleterious effect of becoming male for a female was compensated by the previous or the following sex reversal from male to female.

From our results, care must be taken when selective breeding focused on growth in *C. gigas*. Indeed, mass selection program have been developed in *C. gigas* for this trait, (Li et al., 2011; Wang et al., 2012; Zhang et al., 2019). Such breeding program might select true females or successive hermaphrodites that exhibit sex change from male to female, and might exclude the true males. Consequently, the allele frequencies for the sex determination may change in the subsequent selected population. This must await further investigatation.

Further studies are required to investigate the potential growth advantage for the females, in particular in environment favouring the growth (temperature and food availability). If proven, then farmers will have a new way to improve their production in addition to the other methods favouring the oyster growth currently available using triploids (Dégremont et al., 2012; Dégremont et al., 2019; Hand et al., 1998), selective breeding for the weight/length/yield (He et al., 2008; Langdon et al., 2003; Toro and Newkirk, 1990; Wang et al., 2012), and combining breeding and triploids (Hand et al., 2004)

Acknowledgments

We thank the hatchery team of the LGPMM (Ifremer-La Tremblade) for their help in the oyster production as well as the nursery team of the LSPC (Ifremer-Bouin). Our thanks also go to Pascal Schwertle for his punctual collection of measures in 2017, to Anthony Bourgeau, Hugo Koechlin, Agathe Lévêque, Nastasia Belle and Paul Martel for their involvement in sexing and measurements done in 2015, 2016, 2018 and 2019. Finally, we warmly thank Patrick Azéma for its help for the production of the oysters used in this study. We thank Ifremer and Région Normandie for Ph.D. scholarship.

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Figure captions

Figure 1. Mean individual weight (g) for males (♂) and females (♀) from year 1 to year 6. Bars represent standard error (SE).

Figure 2. Mean shell length (mm) for males (♂) and females (♀) from year 1 to year 6. Bars represent standard error (SE).

Figure 3. Gain of weight (g) between two consecutive years for oysters that did not change sex (♂->♂ and ♀->♀) and for oysters that exhibited sex reversal from ♀->♂ and from ♂ -> ♀. Bars represent standard error (SE), and different letters are significantly different ($P < 0.05$). ♀ and ♂ mean female and male respectively.

Figure 4. Mean (±SE) individual weight (g) from year 1 to year 6 for oysters sexed male (♂) or female (♀) at year 1 and that exhibited 0, 1, 2, and 3 sex changes (Panels a, b, c and d respectively). (* significant difference between males and females; $P < 0.05$). Sex change may occur anytime from year 2 to year 6; as example for an oyster changing twice, sex reversal could happen between year 2 and year 3, and then between year 3 and year 4, while for another oyster, it could be between year 2 and year 3, and then between year 5 and year 6.

Figure 5. Mean (±SE) gain of weight (g) from year 1 to year 6 for oysters sexed male or female at year 1 and that exhibited 0, 1, 2, and 3 sex changes. (* significant difference between males and females; $P < 0.05$)

Figure 6. Mean (±SE) gain of shell length (mm) from year 2 to year 6 for oysters sexed male or female at year 1 and that exhibited 0, 1, 2, and 3 sex changes. (* significant difference between males and females; $P < 0.05$).

Table captions

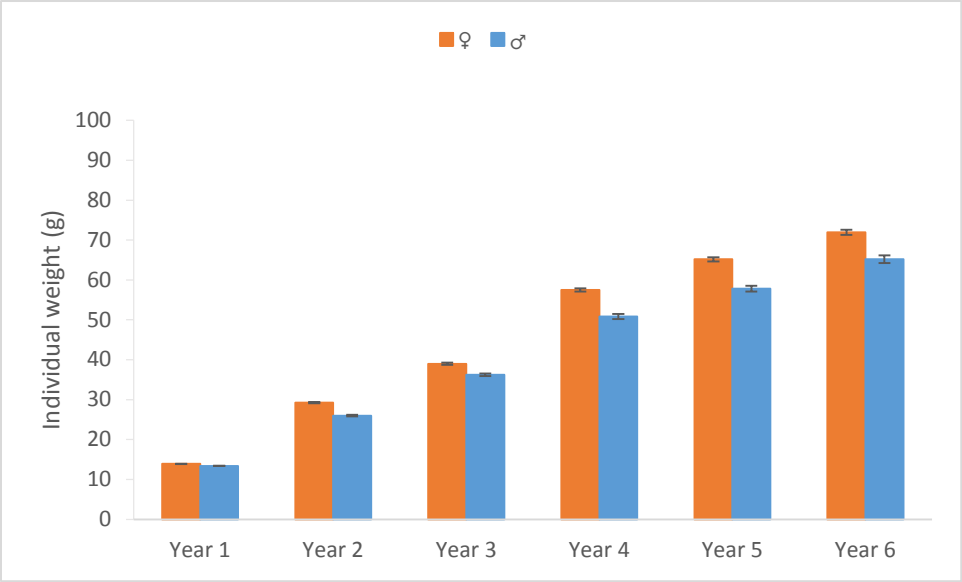
Table 1. Number of oysters measured and sexed male or female each year

Table 2. Mean (± SE) whole weight (g), shell length (mm), shell width (mm) and shell thickness (mm) for females (♀) and males (♂) from year 1 to year 6.

Table 3. Mean (± SE) whole weight (g), shell length (mm), shell width (mm) and shell thickness (mm) gains between two consecutive years for each group.

Supplementary data caption

Figure S1. Seawater temperature (°C) from September 2013 to June 2019.



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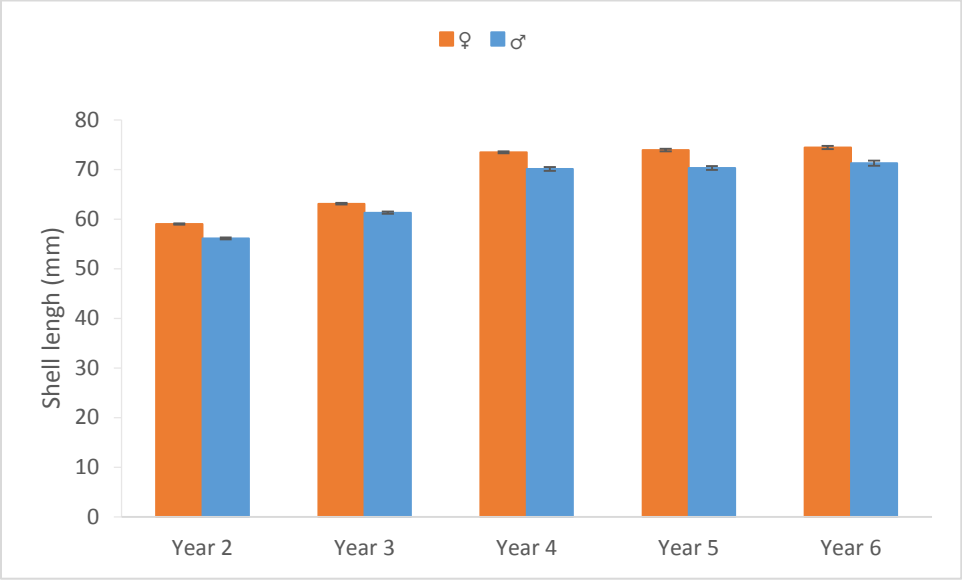
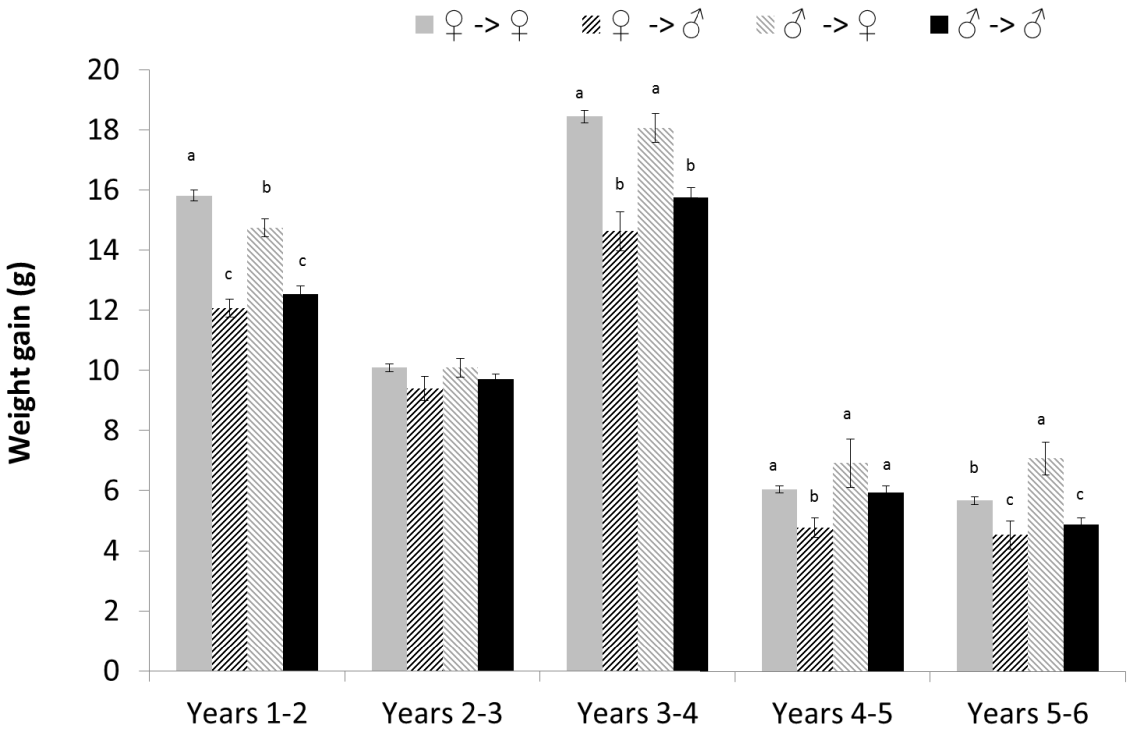


Figure 2. Mean shell length (mm) for males (♂) and females (♀) from year 1 to year 6. Bars represent standard error (SE).



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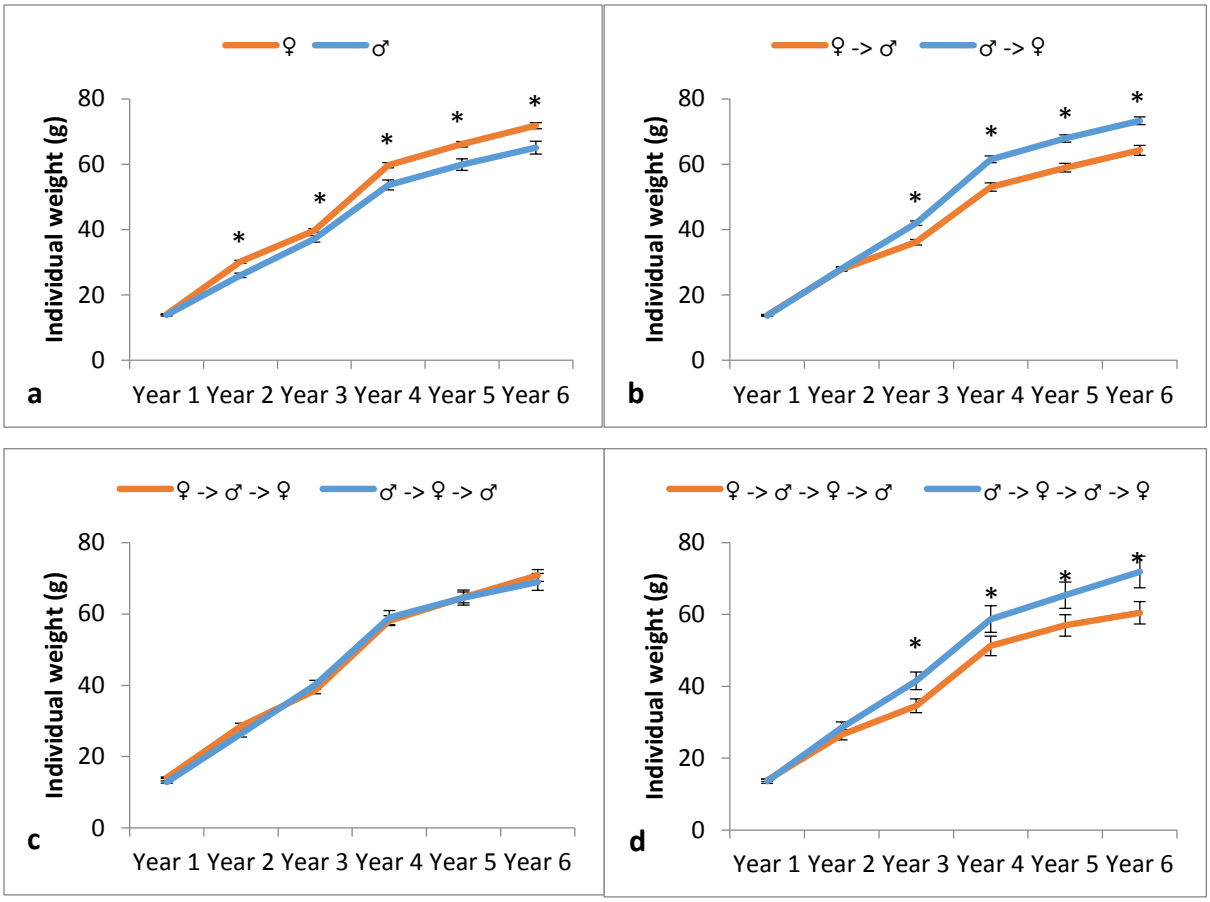

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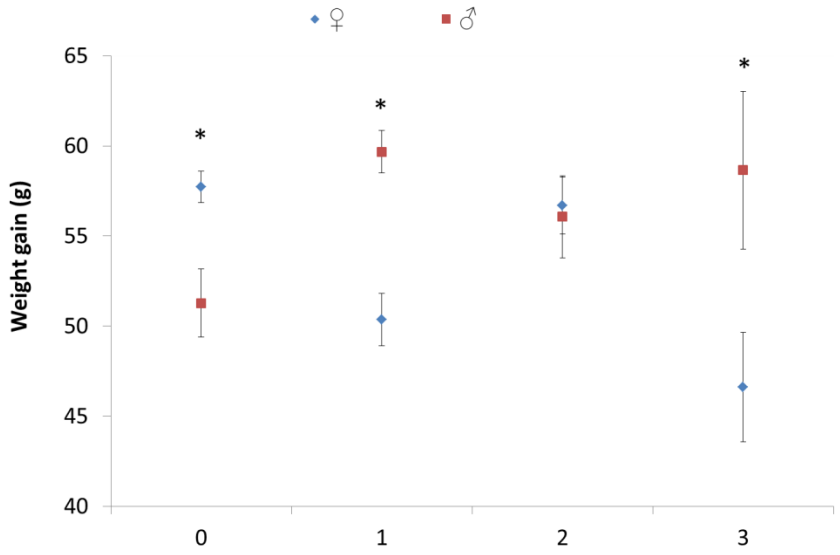

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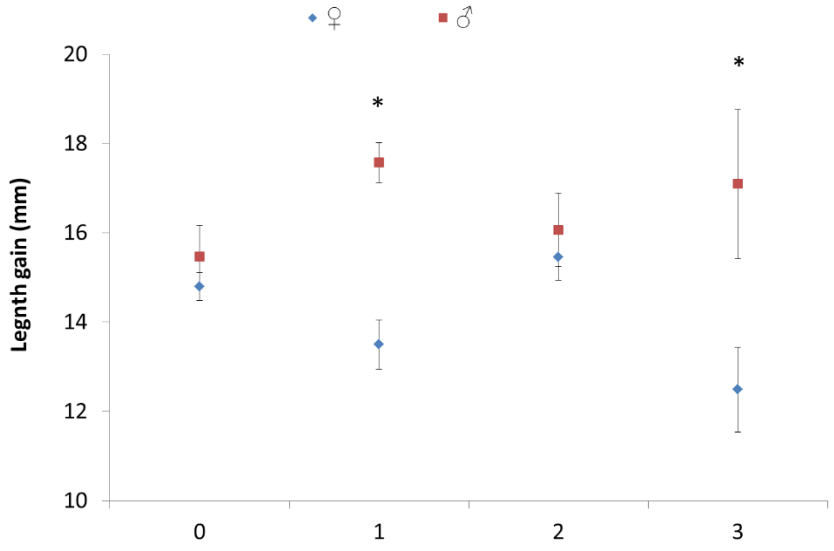

Figure 6. Mean (±SE) gain of shell length (mm) from year 2 to year 6 for oysters sexed male or female at year 1 and that exhibited 0, 1, 2, and 3 sex changes. (* significant difference between males and females; $P < 0.05$).

Table 1. Number of oysters measured and sexed male or female each year

Year	2014	2015	2016	2017	2018	2019
	Year 1	Year 2	Year 3	Year 4	Year 5	Year 6
Number	7488[1]	4846[2]	3416[2]	2687[2]	2081[2]	1426[2]

[1]In Year 1, the mean individual weight per sex and per family was defined as the total weight of all females divided by the number of females or the total weight of all males divided by the number of males

[2] From Year 2 to Year 6, whole weight, shell length, shell width and shell thickness were measured individually.

Table 2. Mean (± SE) whole weight (g), shell length (mm), shell width (mm) and shell thickness (mm) for females (♀) and males (♂) from year 1 to year 6.

		Year 1	Year 2	Year 3	Year 4	Year 5	Year 6
Whole Weight (g)	♀	**13.9 ± 0.0***	**29.3 ± 0.2***	**39.0 ± 0.3***	**57.5 ± 0.4***	**65.2 ± 0.5***	**71.9 ± 0.6***
	♂	**13.4 ± 0.1***	**26.0 ± 0.2***	**36.2 ± 0.3***	**50.9 ± 0.6***	**57.8 ± 0.7***	**65.2 ± 1.0***
Shell Length (mm)	♀	**NA[1]**	**59.0 ± 0.1***	**63.1 ± 0.2***	**73.5 ± 0.2***	**73.9 ± 0.2***	**74.4 ± 0.3***
	♂	**NA[1]**	**56.1 ± 0.2***	**61.3 ± 0.2***	**70.1 ± 0.4***	**70.3 ± 0.4***	**71.3 ± 0.5***
Shell Width (mm)	♀	**NA[1]**	**39.4 ± 0.1***	**41.1 ± 0.1***	**48.3 ± 0.1***	**47.4 ± 0.1***	**46.2 ± 0.2***
	♂	**NA[1]**	**37.3 ± 0.1***	**39.7 ± 0.2***	**45.6 ± 0.2***	**45.1 ± 0.2***	**44.4 ± 0.3***
Shell Thickness (mm)	♀	**NA[1]**	**21.2 ± 0.0***	**23.6 ± 0.1***	**26.4 ± 0.1***	**27.1 ± 0.1***	**29.0 ± 0.1***
	♂	**NA[1]**	**20.3 ± 0.1***	**22.8 ± 0.1***	**25.2 ± 0.1***	**26.0 ± 0.1***	**28.1 ± 0.2***

[1] NA means not available

* Significant difference between females and males; $P < 0.0001$

Table 3. Mean (± SE) whole weight (g), shell length (mm), shell width (mm) and shell thickness (mm) gains between two consecutive years for each group.

	Groups[1]	Years 1-2	Years 2-3	Years 3-4	Years 4-5	Years 5-6
Whole Weight (g)	♀ -> ♀	15.8 ± 0.2^{a}	10.1 ± 0.1	18.4 ± 0.2^{a}	6.0 ± 0.1^{a}	5.7 ± 0.1^{b}
	♀ -> ♂	12.1 ± 0.3^{c}	9.4 ± 0.4	14.6 ± 0.6^{b}	4.8 ± 0.3^{b}	4.5 ± 0.5^{c}
	♂ -> ♀	14.7 ± 0.3^{b}	10.1 ± 0.3	18.1 ± 0.5^{a}	6.9 ± 0.8^{a}	7.1 ± 0.5^{a}
	♂ -> ♂	12.5 ± 0.2^{c}	9.7 ± 0.2	15.7 ± 0.3^{b}	5.9 ± 0.2^{a}	4.9 ± 0.2^{c}
Shell Length (mm)	♀ -> ♀	NA[2]	4.4 ± 0.1^{b}	10.3 ± 0.1^{a}	-0.3 ± 0.1	0.1 ± 0.1
	♀ -> ♂	NA[2]	4.0 ± 0.1^{c}	9.8 ± 0.5^{ab}	-0.7 ± 0.3	-0.6 ± 0.4
	♂ -> ♀	NA[2]	5.0 ± 0.2^{a}	10.2 ± 0.1^{a}	0.3 ± 0.1	0.6 ± 0.4
	♂ -> ♂	NA[2]	4.8 ± 0.1^{a}	9.3 ± 0.2^{b}	-0.4 ± 0.2	0.2 ± 0.1
Shell Width (mm)	♀ -> ♀	NA[2]	1.8 ± 0.1^{b}	7.2 ± 0.1^{a}	-1.4 ± 0.1^{b}	-1.3 ± 0.1
	♀ -> ♂	NA[2]	1.3 ± 0.2^{c}	6.7 ± 0.5^{ab}	-1.5 ± 0.3^{b}	-2.0 ± 0.3
	♂ -> ♀	NA[2]	2.3 ± 0.2^{a}	6.6 ± 0.3^{b}	-0.6 ± 0.3^{a}	-0.8 ± 0.4
	♂ -> ♂	NA[2]	2.2 ± 0.1^{a}	6.3 ± 0.2^{b}	-1.0 ± 0.1^{a}	-1.1 ± 0.1
Shell Thickness (mm)	♀ -> ♀	NA[2]	2.4 ± 0.0^{b}	2.8 ± 0.0^{b}	0.5 ± 0.0	1.7 ± 0.1^{b}
	♀ -> ♂	NA[2]	2.0 ± 0.1^{c}	2.5 ± 0.2^{b}	0.4 ± 0.1	1.7 ± 0.2^{b}
	♂ -> ♀	NA[2]	2.5 ± 0.1^{ab}	3.0 ± 0.1^{a}	0.8 ± 0.3	2.3 ± 0.2^{a}
	♂ -> ♂	NA[2]	2.5 ± 0.1^{a}	2.6 ± 0.1^{b}	0.5 ± 0.1	1.7 ± 0.1^{b}

[1] ♀ -> ♀ is a female at year n which remained a female at year n+1; ♀ -> ♂ is a female at year n which became a male at year n+1; ♂ -> ♀ is a male at year n which became a female at year n+1; ♂ -> ♂ is a male at year n which remained a male at year n+1,

[2] NA means not available,

^{a,b,c,d} : different letters indicate significant differences between the groups within a couple of years (Years 1-2, Years 2-3…), for each kind of measurement ($P < 0.05$).

Supplementary data

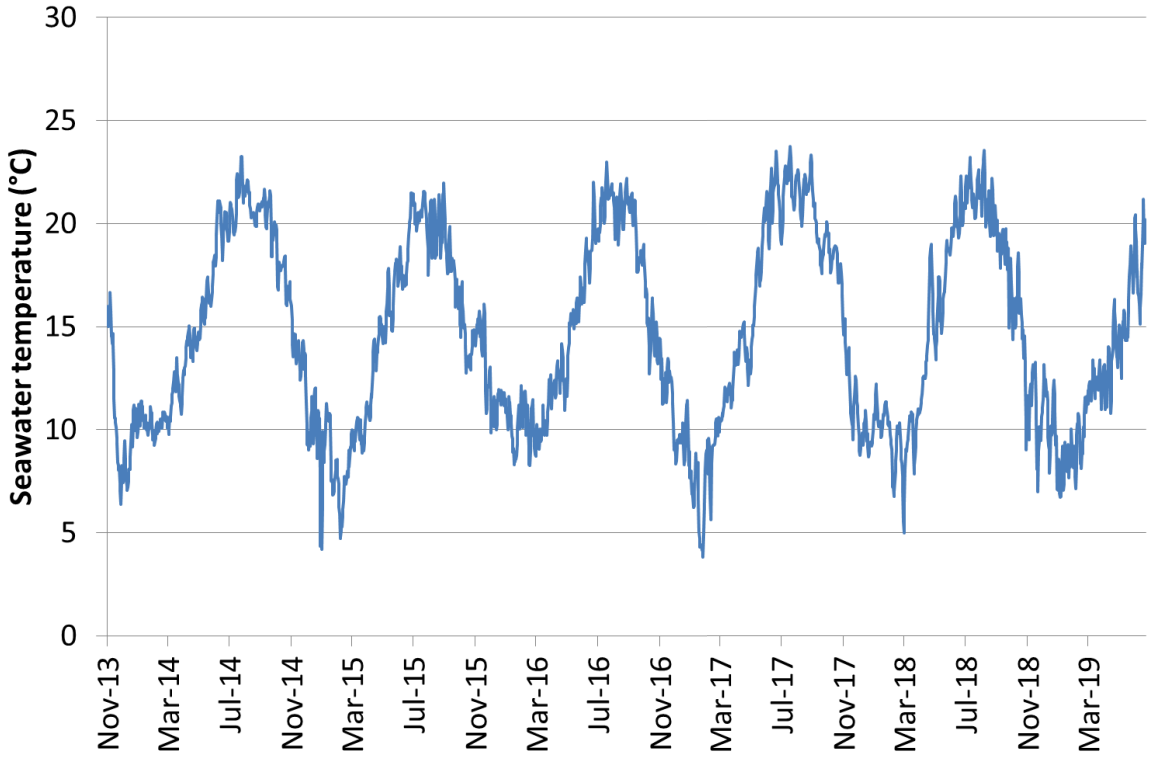

Figure S1. Seawater temperature (°C) from September 2013 to June 2019.

Seawater temperature ranged from 3.8°C in January 2016 to 24.2 °C in June 2019 as shown above (Supplementary Figure 1). Each year, the period for sex determination in *C. gigas* is suspected to occur between September and January. The seawater temperature during this period is shown in black frames. It decreased from 21.7 to 6.4 °C (means = 13.6 °C) in 2013/2014, from 21.7 to 4.2 °C (means = 14 °C) in 2014/2015, from 21 to 8.3 °C (means = 13.5 °C) in 2015/2016, from 21.5 to 3.8 °C (means =12.5 °C) in 2016/2017, from 21.5 to 8.7 °C (means = 14 °C) in 2017/2018 and from to 20.1 to 6.7°C in 2018/2019 (mean =12.6°C).

Chapitre 3

Les transcriptomes gonadiques associés aux phénotypes du sexe révèlent des gènes pertinents de la fenêtre temporelle du déterminisme sexuel chez *Crassostrea gigas*, un mollusque hermaphrodite séquentiel

Les transcriptomes gonadiques associés aux phénotypes du sexe révèlent des gènes pertinents de la fenêtre temporelle du déterminisme sexuel chez *Crassostrea gigas*, un mollusque hermaphrodite séquentiel.

Questions : Quels sont les gènes qui présentent un pic d'expression à la **période du déterminisme sexuel** et sont alors **différentiellement exprimés** entre les mâles et les femelles ? Quels homologues de gènes connus du déterminisme sexuel sont **retrouvés** chez l'huître creuse *Crassostrea gigas* ? Leurs **profils d'expression** sont-ils en accord avec un **rôle** possible dans le déterminisme sexuel chez *C. gigas* ?

Méthode : Après cinq années de sexage de la population 1 d'huîtres creuses diploïdes (*cf.* Chapitre 1), **neuf** huîtres identifiées **femelles chaque année** et **neuf** huîtres identifiées **mâle chaque année** ont été choisies pour l'étude du transcriptome gonadique. Ces « vrais » males et "vraies" femelles devaient permettre de « prédire » le futur sexe de l'animal en stade 0 alors que la gonade est indifférenciée, mais aussi en stade 3, afin de mieux interpréter les profils d'expression moléculaires. Trois huîtres par sexe et par stade ont été sacrifiées, aux **stades gamétogénétiques 0 et 3** couvrant la **période du déterminisme sexuel** et au stade 1 pour comparaison. Après obtention des transcriptomes gonadiques par RNA-Seq, une **analyse d'expression différentielle** (AED) a été réalisée. Des RT-qPCR ont été effectuées afin de valider le RNA-Seq et pour obtenir les **profils d'expression de onze gènes** issus de l'AED, sur la **totalité du cycle gamétogénétique adulte** incluant également le stade 2. Les profils d'expression de 22 gènes homologues de gènes connus des cascades du déterminisme sexuel d'organismes modèles (Protostomiens Ecdysozoaires et Deutérostomiens) ont également été extraits des données de RNA-Seq.

Résultats : Après séquençage des dix-huit gonades, 1,424,810,382 *raw reads* ont été obtenus avec 724,160,832 et 700,649,550 *reads* issus respectivement des gonades femelles et mâles. Les PHRED score Q30 des transcriptomes variaient de 96,20 à 97,98% et en moyenne 97,48% des *reads* ont été alignés sur le génome. Sur les 10,061 gènes exprimés dans les gonades, **9,582** étaient différentiellement exprimés entre les **stades** gamétogénétiques et **141 entre les sexes** (dont 98 surexprimés chez les femelles et 43 chez les mâles). Lors du recoupement des

deux analyses, **84 gènes** ont été désignés comme ayant une expression à la fois **stade- et sexe-spécifique**. Parmi les gènes étudiés en RT-qPCR, **quatre** d'entre eux présentaient un pic d'expression **dimorphique** à la période du **déterminisme sexuel** (stade 0 ou 3) ; ils codent **Protein PML-like** (CGI 10018971), **Protein singed-like** (CGI 10016132), **PREDICTED: paramyosin** (CGI 10028666) et **Trophoblast glycoprotein-like** (CGI 10006800). **Vingt-deux homologues** connus de gènes du déterminisme sexuel, recherchés dans les données transcriptomiques issues de ce travail, y ont été **retrouvés**. *FoxN5* (CGI_10023645) et *LOC105342716* (Sex-Determining Region Y protein; CGI_10003991) ont aussi été recherchés dans les données de RNA-Seq car (i) le 1er appartient à la famille des facteurs Fox comme *FoxL2* et est connu pour son expression dans les gonades mâles matures et (ii) le 2nd est annoté dans NCBI comme gène appartenant à la famille *Sox*, à laquelle appartiennent aussi *SoxH* et *SoxE*. Seuls 10 gènes parmi les 22 présentaient une expression significativement différentielle au cours de la gamétogenèse pour l'un des sexes ou pour les deux. Parmi eux, seuls 2 [codant **FoxL2** (CGI 10011004) et la **FST** (CGI_10024194)] présentaient une expression significativement **dimorphique** entre les sexe **à la période du déterminisme sexuel.**

Conclusions : Cette analyse en RNA-Seq a ainsi permis de mettre en évidence 84 gènes différentiellement exprimés entre les sexes et les stades, dont **57** étant **surexprimés à la période du déterminisme sexuel**. Parmi eux, **quatre** gènes nouveaux sont ressortis en qPCR comme ayant des profils d'expression en accord avec un **rôle dans le déterminisme sexuel**, codant **Protein PML-like** (CGI 10018971), **Protein singed-like** (CGI 10016132), **PREDICTED: paramyosin** (CGI 10028666) et **Trophoblast glycoprotein-like** (CGI 10006800). Des gènes homologues de gènes connus du déterminisme sexuel ont aussi été identifiés, certains pour la 1ère fois chez l'huître. Parmi eux, seuls ceux codant **FoxL2** (CGI 10011004) et la **FST** (CGI_10024194) ont montré une expression en accord avec un **rôle potentiel dans le déterminisme sexuel** chez *C. gigas*. Des approches ciblées sur tous ces gènes (phylogénies, expressions tissulaires et cellulaires gonadiques, approches fonctionnelles …) seront indispensables pour affiner les hypothèses concernant leurs rôles.

Ma participation : J'ai participé à l'élaboration du plan d'échantillonnage, prélevé et conditionné les gonades ainsi que les branchies, extrait les ARN, vérifié leur qualité, participé à l'analyse cytométrique de la ploïdie, participé à la réalisation des lames histologiques, démarché la plateforme de séquençage, géré l'envoi des échantillons ainsi que la collecte des données de séquençage, réalisé les analyses bioinformatiques, interprété les données, participé à la sélection des gènes utilisé en RT-qPCR, participé au design des amorces, testé et validé les amorces, réalisé les RT-qPCR, recherché les homologues des gènes conservés au sein des transcriptomes, interprété les résultats et participé à leur mise en forme.

La majeure partie des analyses bioinformatiques a été effectuée lors de mon séjour au *GeneCology Research Centre* de *l'University of the Sunshine Coast* (Australie), rendue possible grâce à l'obtention d'une bourse de mobilité pour jeunes chercheurs offerte par la Direction Scientifique de l'Ifremer.

Ces travaux ont fait l'objet d'une valorisation par communication orale :

C Broquard, S Saowaros, L Dégremont, JB Lamy, B Morga, A Elizur, AS Martinez. 2019 Gonadal transcriptomic analysis in the oyster *Crassostrea gigas* – How to unravel the locks of a sequential hermaphrodite in order to identify potential male and female sex-determining genes. SEB, 2-5 Juillet 2019, Séville, Espagne.

Remarque : Pour des raisons logistiques, les co-auteurs de l'article scientifique n'ont pas encore pris connaissance de la version présentée ci-après.

Article 3

Gonadal transcriptomes associated to sex phenotypes reveal relevant genes of the time window of sex determination in *Crassostrea gigas*, a sequential hermaphrodite mollusc.

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Abstract

Molluscs are an important phylum in the animal kingdom, as a representative of the Lophotrochozoa and because they include a large number of species. However, few studies have investigated the molecular cascade of sex determination within this phylum. The Pacific oyster *Crassostrea gigas* is a Bivalve Mollusc of economic interest and of physiological and phylogenetic importance, because of its global production and its mode of reproduction (sequential irregular hermaphrodite). Although some studies identified genes of the sex-determining pathway in this oyster, knowledge still needs to be deepened, notably about the expression patterns. Indeed, these patterns need to cover the entire sex-determining period and they have to be associated to a sex phenotype, usually impossible to know in this sequential irregular hermaphrodite. To this purpose, we performed a gonadal RNA-Seq analysis of chosen diploid oysters, sampled during the entire time-window of sex determination (stages 0 and 3), among males and females which never changed sex during the 5 first years of their life. This individual and long-term monitoring of the sex phenotypes allowed us to explain the molecular expression patterns in the light of the most statistically likely future sex of each oyster. Homologs of conserved sex-determining genes were also searched within the oyster's transcriptome data. Differential expression analysis of gonadal transcriptomes revealed that 9,723 genes exhibited a significantly different expression between gametogenetic stages and 141 between sexes (98 overexpressed in females; 43 in males). Eighty-four genes were identified as having both stage- and gender-specific expression, 57 being over-expressed at the time of sex determination. Among them, four novel genes emerged also in qPCR as having a dimorphic peak of expression at the time of sex determination, coding Trophoblast glycoprotein-like, Protein PML-like, Protein singed-like and PREDICTED: paramyosin. Twenty-two homologs of known sex-determining genes were also found in the present transcriptome data, some, for the first time in this oyster. Of these, only those coding FoxL2 and FST showed an expression consistent with a potential role in sex determination. Our work should provide additional information on the molecular mechanisms of sex determination in *C. gigas* and their evolution in the animal kingdom.

Key words: RNA-Seq, gonad transcriptome, sex determination, oyster

1. Introduction

Molluscs are a branch of Lophotrochozoa super-phylum which include a large number of species -around 85,000- (Rosenberg, 2014). Many studies have been done on organisms of this phylum, especially about their reproduction as various reproductive strategies exist, dioecy, simultaneous and sequential hermaphroditism (Ruppert *et al.,* 2004, Breton *et al.*, 2017). Although whole-genome sequenced studies have been increasing this last decade in Molluscs, especially for Bivalves and Gastropods (For more information about genomes, see the Genome Online Database GOLD, https://gold.jgi.doe.gov), still only a few of them concern the understanding of the molecular mechanisms of reproduction and especially of sex determination (Boutet *et al.*, 2008 ; Ghiselli *et al.*, 2011, 2018; Dheilly *et al.*, 2012, 2014 ; de Sousa *et al.*, 2014 ; Shi *et al.*, 2015 ; Tong *et al.*, 2015 ; Teaniniuraitemoana *et al.*, 2014 ; Zhang *et al.*, 2014 ; Li *et al.*, 2016 ; Patnaik *et al.*, 2016 ; Chen *et al.*, 2017 ; Yu *et al.*, 2017 ; Briones *et al.*, 2018 ; Galindo-Torres *et al.*, 2018 ; Yue *et al.*, 2018). Yet, information at the molecular scale is necessary to understand the evolution of sex determination patterns in the animal kingdom but also to control the sex of species of aquaculture interest.

As part of the Molluscs, *Crassostrea gigas* is the most produced bivalve organism, with 639 030 tons in 2017 (FAO, 2019). This oyster is originally from East Asia but was introduced in many countries around the world for aquaculture production. This species is described as a mix of dioecy, simultaneous and sequential hermaphroditism (Coe, 1943; Guo *et al.,* 1998; Broquard *et al.,* 2020) with a genetic and environmental sex determination (Guo *et al.*, 1998; Chavez-Villalba *et al.*, 2003; Fabioux *et al.*, 2005; Naimi *et al.*, 2009b; Santerre *et al.*, 2013). Two genetic models of sex determination have been proposed for this species free of identified sex chromosomes (Leitão *et al.*, 1999); they are based on a single locus involving either two (Guo *et al.*, 1998) or three genotypes (Hedrick & Hedgecock, 2010). A time window of sex determination has also been defined during the adult gametogenetic cycle (Naimi *et al.*, 2009a and b; Santerre *et al.*, 2012, 2014; Dheilly *et al.*, 2012), between the end of a cycle when animals are mature (stage 3 according to Heude-Berthelin *et al.*, 2001) and after spawning, at the beginning of a new cycle (stage 0 according to Heude-Berthelin *et al.*, 2001). Few orthologs of classical genes involved in sex determination or differentiation have also been identified in *C. gigas* by targeted or more recently by large-scale studies, such as *Foxl2, Fem, Wnt-4, Gata-4, Run, β-catenin, SoxH/30, Dsx/Dmrt1-like, SoxE* (Naimi *et al.*, 2009a and

b; Dheilly *et al.*, 2012, 2014; Santerre *et al.*, 2012, 2014; Zhang *et al.*, 2014; Yue *et al.*, 2018). Most of these genes are also found in other bivalves, Ecdysozoa or in Vertebrate organisms, suggesting that sex-determining/differentiating pathways share common genes among Vertebrates and 'invertebrates' (Santerre *et al.*, 2013, 2014; Zhang *et al.*, 2014; Li *et al.*, 2016; Yue *et al.*, 2018). Other more unexpected /unknown genes like *Cg-Sh3kbp1, Cg-Malrd1-like, Cg-FoxN5* as well as a long non-coding RNA (*LOC105321313*) and uncharacterized *LOC105345697* have also been found in *C. gigas* as having a dimorphic expression in mature gonads, in agreement with a potential role in sex determination / differentiation (Zhang *et al.*, 2014; Yue *et al.*, 2018).

While almost no functional approach is available in *C. gigas*, large-scale molecular studies are very powerful tools to study sex determination/differentiation as they increase molecular knowledge about this physiological mechanism. A microarrays-based analysis (Dheilly *et al.*, 2012) mainly identified sex-related genes and few sex-determining homologs, among which *Foxl2* already identified (Naimi *et al.*, 2009b) and *Wnt-4, Gata-4* and *Fem* which do not exhibit a sexually dimorphic expression as expected for a sex-determining gene. This work also highlighted 511 genes expressed in stage 0, without distinguishing the sex to which it can be associated. Later on, a genomic analysis on mature animals (stage 3) (Zhang *et al.*, 2014) allowed to identify three new homologs of sex-determining genes, two of them *(*Dsx/*Dmrt1-like* and *SoxH)* with a male-specific expression and one (*Run*) without any sex-specific expression. A male specific gene, *Cg-FoxN5*, was also mentioned. More recently, a gonadal RNA-Seq analysis (Yue *et al.*, 2018) highlighted genes clustered in five expression profiles: more expressed in stage 1, decreasing or increasing expression throughout oogenesis and spermatogenesis, specific expression in female gonads, expression more in male gonads and increasing throughout spermatogenesis. These authors also found the sex-determining genes *Dsx/Dmrt1-like, FoxL2* and *SoxH* already identified (Naimi *et al.*, 2009b; Zhang *et al.*, 2014) and found that *Dsx/Dmrt1-like* is more expressed in stage 1, while Zhang *et al.* (2014) mentioned a high and dimorphic expression in stage 3. In the end, in most of these previous works, no gene of interest has been mentioned in stage 0, which is part of the sex-determining period of *C. gigas*. When they did (Dheilly *et al.*, 2012), they could not associate the expression patterns to one sex in any case. Whether at this stage or in stage 3, the expression patterns could not be associated to the future sex (sex phenotypes) as it is usually unknown in this

sequential hermaphrodite. At last, the ploidy has not been determined/mentioned while it is naturally variable and it is known to modify the oyster's gametogenesis (Jouaux *et al.*, 2010 ; Dheilly *et al.*, 2014).

In this context, in the present study, we performed a gonadal RNA-Seq analysis of *C. gigas* and we compared it with the available genome data (Zhang *et al.*, 2012). For this purpose, we chose diploid oysters, sampled during the entire time-window of sex determination (stages 0 and 3; stage 1 for comparison) among males and females which never changed sex during the 5 first years of their life. This individual and long-term monitoring of the sex phenotypes allowed us to explain each molecular expression pattern obtained in the light of the most statistically likely future sex of each oyster. In this way, we were also able to filter more drastically genes with high and dimorphic expression during the sex-determining time-window in this sequential irregular hermaphrodite. Our results should provide further insights into the molecular mechanisms of sex determination, reproduction and hermaphroditism in *C. gigas* and into their evolution in the animal kingdom.

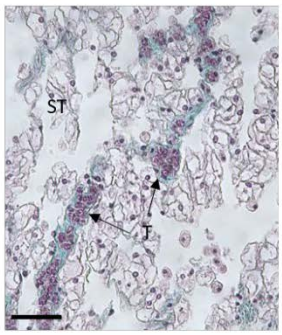
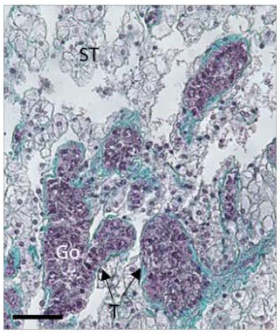
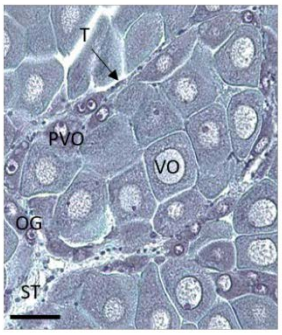
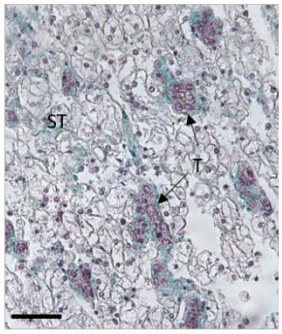
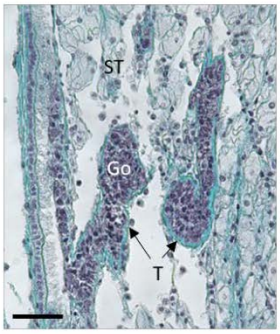
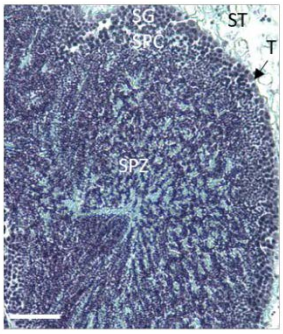
2. Materials and Methods

2.1. Sampling and strategy applied for the choice of animals

The oysters used for these experiments were sampled among a population of *Crassostrea gigas* produced at the Ifremer facilities from a wild broodstock (for more information see Broquard *et al*, 2020). Animals were individually selected according to several criteria: their sex phenotype, their gametogenetic stage and their ploidy. For this purpose, they were sampled in January, February and June 2018 and their gonads were collected for RNA extractions (frozen in liquid nitrogen) and for histology (fixed in a Davidson solution). Gills were also collected and conserved in 70° ethanol for analysis by flow cytometry. It allowed us to be sure of selecting only diploid oysters, while ploidy can be very plastic (Leitão *et al.*, 2001). The gametogenetic stages were determined *a posteriori* based on criteria described by Heude-Berthelin *et al.* (2001): Stage 0 (resting stage), stage 1 (re-initiation of gametogenesis illustrated by gonia proliferation), stage 2 (maturation stage with active spermatogenesis and growing oocytes) and stage 3 (mature stage with ripe gonads before spawning). For RNA-Seq itself, nine males and nine female oysters were chosen in stages 0, 3 and 1 (3 individuals per

stage), the first two stages because they correspond to the time-window of sex determination in this species (Naimi *et al*., 2009a and b; Santerre *et al.*, 2012, 2014; Dheilly *et al.*, 2012) and the last one for comparison (Table 1). For the validations by qPCR, twenty-four animals (3 individuals per stage and per sex) were selected as covering the entire gametogenetic cycle *ie* stages 0, 1, 2 and 3. Traditionally, by histology, sex can be determined from the end of stage 1, knowing that gonad is undifferentiated in stage 0. However, during one cycle, the sex at the next cycle cannot be predicted, because *C. gigas* is a sequential irregular hermaphrodite. An asset of our study was to work on oysters whose sex phenotypes never changed over the 5 past years of their life (Broquard *et al.*, 2020), allowing us to assign a "very likely" future sex phenotype to these "true" males and "true females".

Table 1: Basic description and histological illustrations of sex and stages of Crassostrea gigas used for RNA-sequencing, based on criteria described by Heude-Berthelin et al. (2001) and on determination of sex phenotypes by Broquard et al. (2020). Stage 0, resting stage; stage I, re-initiation of gametogenesis; stage III, mature stage with ripe gonads. Go: gonia, OG: oogonia, PVO: pre-vitellogenic oocytes, SG: spermatogonia, SPC: spermatocytes, SPZ: spermatozoa, ST: storage tissue, T: gonadal tubule, VO: vitellogenic oocytes. Bars: 25 µm.

Gametogenetic stages by histology associated to sex phenotypes	Stage 0	Stage I	Stage III
Females			
Males			

2.2. RNA preparation, cDNA library construction and Illumina sequencing

Total RNA of eighteen gonads (3 males and 3 females per stage; stages 0, 1 and 3) were individually extracted using TRI Reagent (Sigma®), treated with TURBO™ DNAse (Invitrogen) to remove genomic DNA and purified with Direct-zol™ RNA MiniPrep (Zymo Research) following the manufacturer's protocol. RNA concentration, integrity and purity were assessed on agarose gels (1%) and using NanoDrop 2000 (Thermo Scientific), Qubit® 2.0 Fluorometer (Invitrogen) and Bioanalyzer 2100 (Agilent Technologies). The cDNA libraries were made from the total RNA of the eighteen individual samples, nine of each sex, conformed to the required purity criteria (A260/A230 and A260/A280 > 1.8) and quality levels (RIN > 8) for cDNA library preparation. The cDNA libraries were constructed by the platform LIGAN-PM (Lille, France) using TruSeq RNA Library Prep kit v2 (Illumina) following the manufacturer's recommendations. Briefly, per library, approximately 1 µg of total RNA sample was purified using oligo-dT beads, followed by fragmentation with Elute, Prime, Fragment Mix. First-strand cDNA was generated by reverse transcription with First Strand Master Mix (25 °C for 10 min, 42 °C for 50 min and 70 °C for 15 min) and the synthesis of second-strand cDNA was performed in the presence of Second Strand Master Mix and dATP, dGTP, dCTP, dUTP mix (16 °C for 1 h). The purified fragmented cDNA was incubated in presence of End Repair Mix (30 °C for 30 min), then adenylated (3' end) by addition of A-Tailing Mix (37 °C for 30 min) and mixed to RNA index adapter and ligation mix (30 °C for 10 min). The final purification step was performed with AMPure XP beads (Beckman Coulter). Several rounds of PCR amplification were performed to enrich the cDNA fragments, prior to the purification of PCR products. The libraries quality was assessed by checking the distribution of the fragments size using the Agilent bioanalyzer DNA 1000 (Agilent Technologies) and the libraries were quantified by RT-qPCR (KAPA Library Quantification Kit, Roche). The resultant cDNA libraries were paired-end sequenced (150bp paired-end reads generated) on an Illumina HiSeq™ 4000 at the platform LIGAN-PM (Lille, France).

2.3. Sequence and differential expression analysis (DEA)

All the analyses were conducted using the CLC Genomics Workbench 11.0.1 software (Qiagen bioinformatics). First, the removing of reads containing adapter, reads containing poly-N and low-quality reads, from raw data generated, allowed to obtain clean data. Then, the reads were aligned to the reference genome of *Crassostrea gigas (*genome v9; Zhang *et al.*, 2012)

and those mapped to each reference gene were counted. A differential gene expression analysis between the sexes and the gametogenetic stages was also performed using a model based on the negative binomial distribution. Only genes with a false discovery rate (FDR) < 0.05, a fold change > 1.5 and a p-value < 0.05 were assigned as differentially expressed. All the genes were functionally annotated by searching sequence similarities in the Swiss-Prot and Nr (NCBI) databases with an e-value cut-off at $1e^{-5}$. Results were imported into Blast2GO, and Gene Ontology (GO) terms at level 2 as well as eggNOG annotation with hit max at 20 were assigned. This "blind" approach was completed by a "targeted" approach to search for homologs of known and conserved sex-determining genes and to assess their expression patterns by RNA-Seq. For this purpose, the CGI numbers of these oyster's homologs were searched on NCBI, which then allowed us to extract them from the excel file resulting from the DEA, regardless of their expression profiles by RNA-Seq.

2.4. Quantitative Real-Time PCR

In order to validate RNA-Seq data and to provide annual expression patterns for some relevant genes, RT-qPCR were performed on eleven of them selected from the DEA, on samples covering the entire gametogenetic cycle for both sexes. FoxL2, whose expression has already been mentioned by RT-qPCR (Naimi *et al.*, 2009b; Santerre *et al.*, 2012) was also taken as a control. Gene-specific primers were designed using Primer3 (v. 0.4.0) and ordered at Eurogentec. Their sequences are listed in Table 2. Total RNA of twenty-four gonads (3 males and 3 females per stage; stages 0, 1, 2 and 3) were used for this experiment. They were first individually treated with DNAse I RQ1 (Promega) and purified with NucleoSpin® RNA clean-up kit (Macherey-Nagel) following the manufacturer's instructions. Then cDNA was synthesized using M-MLV Reverse Transcriptase (Promega) with oligo$(dT)_{15}$ Primer (Promega) follow ing the manufacturer's advices. The amplifications were conducted on a CFX96 Real-Time PCR Detection System (Biorad) using GoTaq® qPCR Master Mix (Promega). Cycling parameters were 95°C for 5min, then 45 cycles of 95°C for 15 s and 60°C for 45s. PCR efficiency of each primer pair was determined based on a five-point standard curve generated from a two-fold dilution series. Negative controls (total RNA not reverse-transcribed), blank controls (sterile water) and inter-plate controls were added on each microplate. All PCR reactions were performed in triplicates. Melting curve analysis was performed to verify the specificity of each primer. The Elongation Factor 1-alpha (EF1α, GenBank Accession Number: BQ426516) was

used as a reference gene to normalize gene expression levels of the targeted genes. Relative gene expression was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001).

Table 2: List and sequences of primers used for Quantitative Real-Time PCR

Name or CGI of the corresponding gene	Name of the primer	Sequence of S and AS primers
Elongation Factor 1 α	EF1α S EF1α AS	ACCACCCTGGTGAGATCAAG ACGACGATCGCATTTCTCTT
FoxL2	FoxL2 S FoxL2 AS	AATATCAGGGATGGGCACAA TCCTTGGGTGCAGGAACTA
CGI_10006800	CGI_10006800 S1 CGI_10006800 AS1	GGTCTATCTTCGCTGGTTGC AGCAAATGCATGTTGATGGA
CGI_10016132	CGI_10016132 S2 CGI_10016132 AS2	GGGTCTAACGGGAAACCATT AACCAAAGTCACACCGGAAG
CGI_10026009	CGI_10026009 S2 CGI_10026009 AS2	ACCTCCTATGCCCATGACAG ACCATTGTCGGGCATTATGT
CGI_10018971	CGI_10018971 S1 CGI_10018971 AS1	TGCCACTGAACGAGTCTTTG GGTCCTCTTGCGTTCTTCTG
CGI_10008094	CGI_10008094 S2 CGI_10008094 AS2	CATGGCATTCAAAGGGAGAT TTCTCTTTAGTCCGCCTGGA
CGI_10025872	CGI_10025872 S2 CGI_10025872 AS2	CGACGGAGAATCCAGGACTA CAAGCGGTTGTAAGGTCCAT
CGI_10021158	CGI_10021158 S1 CGI_10021158 AS1	CTGGAAGACTCCAGCCAGAC GGCGATTGCAGATACTCCTC
CGI_10028666	CGI_10028666 S2 CGI_10028666 AS2	GACGACGGAGATCGCTAAAG TCTGGAACTGCGTGAGATTG
CGI_10023199	CGI_10023199 S2 CGI_10023199 AS2	GTTACGAGATGCCCCATTGT GGTAGGACTCGCTGTTGAGG
CGI_10011342	CGI_10011342 S1 CGI_10011342 AS1	AAATCGGTTCATCCGACAAG CGTGATTCGCACCAACTCTA
CGI_10001090	CGI_10001090 S1 CGI_10001090 AS1	GGTGTTCGGAATTGATGGAC CCTTTGGTGGCACTTAGGAA

3. Results

3.1. Transcriptomic analysis

The transcriptomes sequencing generated a total of 1,424,810,382 raw reads (724,160,832 and 700,649,550 reads from gonads considered as female and male respectively) (Table 3). The average PHRED score Q30 varied from 96.20 to 97.98%. After trimming and quality filtering, 1,388,380,771 reads were successfully mapped to the *Crassostrea gigas* genome with an average efficiency of 97.48% (ranging from 96.44 to 98.52%). Among the 26,101 genes from the reference genome, 10,061 genes were detected in the present gonad transcriptomes.

Table 3: Summary statistics of Crassostrea gigas gonad transcriptomes sequencing. Sample id were designed as follows: sex (F=female, M=male), number of biological replicates (1 to 3), gametogenetic stage (S0, S1, S3).

Sample id	Mapped reads (count)	Mapped reads (%)	Average length of mapped reads (bp)	Average PHRED score 20 (%)	Average PHRED score 30 (%)
F1S0	103,017,875	96.91	140.15	99.4	96.7
F2S0	92,804,237	96.44	140.75	99.45	96.94
F3S0	105,541,018	96.82	141.33	99.66	97.08
M1S0	84,283,633	96.44	140.50	99.4	96.88
M2S0	68,444,100	97.07	140.10	99.63	97.25
M3S0	76,120,564	97.51	139.14	99.49	97.33
F1S1	42,539,142	96.68	140.39	98.92	96.2
F2S1	51,467,467	97.42	138.93	99.58	97.21
F3S1	57,539,741	98.05	139.44	99.86	97.92
M1S1	68,411,348	98.19	141.55	99.8	97.93
M2S1	81,529,233	97.23	141.39	99.83	97.82
M3S1	64,087,363	98.08	141.64	99.79	97.43
F1S3	75,387,566	98.31	141.85	99.81	97.78
F2S3	104,268,494	97.61	142.49	99.89	97.98
F3S3	72,514,064	98.52	142.85	99.77	97.62
M1S3	86,192,288	97.85	142.01	99.87	97.87
M2S3	68,983,407	97.89	142.32	99.73	97.5
M3S3	85,249,231	97.71	142.14	99.78	97.73

A principal component analysis (PCA) was performed on all 10,061 genes of the 18 gonads sampled to assess the homogeneity of the whole dataset and the degree of correlation of gene expression patterns with sex and gametogenic stages. The first two principal components explained 43.1% of the total variance. The scatter plot (Figure 1) showed that transcriptional profiles are rather clustered by gametogenetic stage (determined by histology) than by sex (determined by long-term monitoring of sex phenotypes).

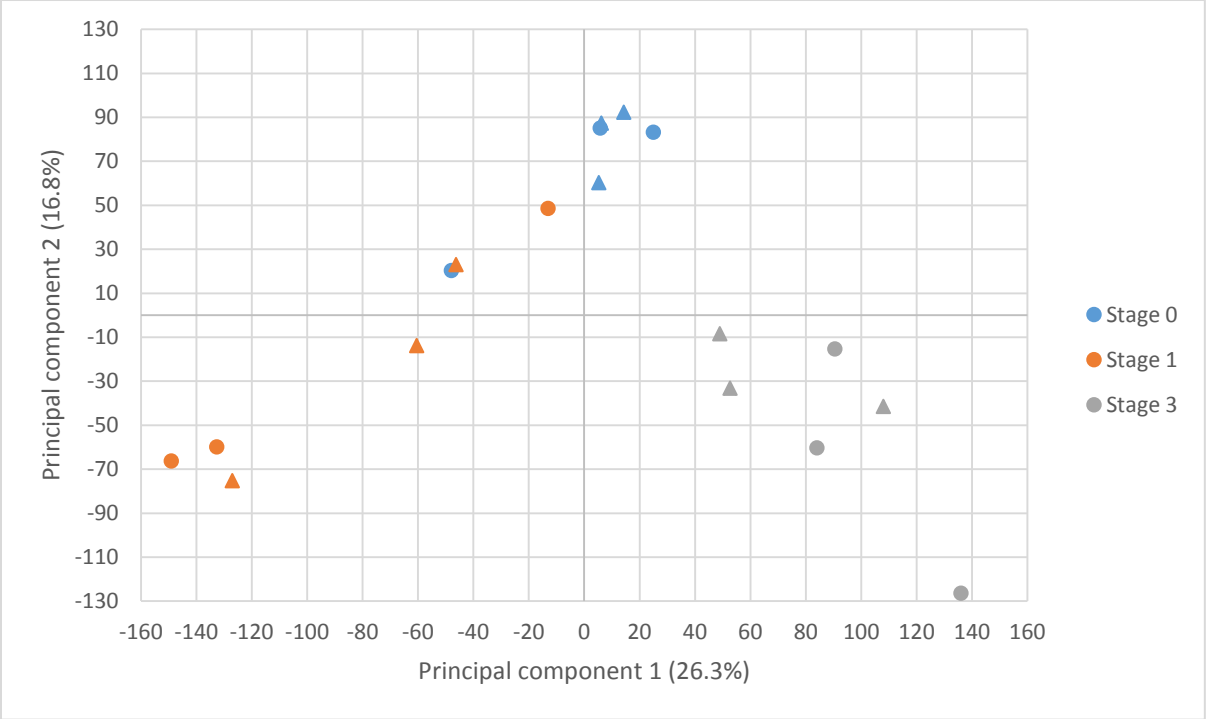


Figure 1. Principal component scatter plot of the 10,061 genes expressed in the eighteen gonad transcriptomes. Triangle symbols are for females and round symbols for males.

3.2. Differential gene expression analysis

A differential expression analysis (DEA) was conducted in order to identify sex- and stage-specific gene expression patterns. It revealed a total of 9,723 genes (96.64% of the 10,061 gonadic genes) significantly differentially expressed either between gametogenetic stages (9,582 genes; 95.24%) or sexes (141 genes; 1.40%) with a FDR < 0.05 (Figure 2). Among genes with a sex-specific expression, 98 (69.50%) were significantly more expressed in females and 43 (30.50%) in males. When data from both DEA were coupled, it appeared that 84 genes (0.86%) were significantly differentially expressed between both gametogenetic stages and

sexes (Supplementary data; S1). These genes were classified by stage with 13, 27 and 44 genes more expressed in stage 0, 1 and 3 respectively and by sex with 46 and 38 genes more expressed in females and males respectively (Table 4).

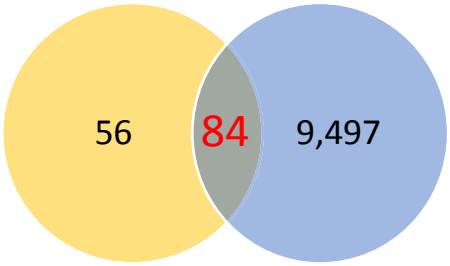


Figure 2. Venn diagram for DEA within C. gigas gonad transcriptome for genes significantly differentially expressed between gametogenetic stages (in blue) and sexes (in orange). In blue 9,723 genes significantly differentially expressed between gametogenetic stages. In orange, 141 genes significantly differentially expressed between sexes.

Table 4: Distribution of the 84 genes (numbers and % in brackets) significantly differentially expressed between genders and gametogenetic stages.

Sex	*Stage 0*	*Stage 1*	*Stage 3*	*Total*
Female	12	16	18	46 (54.8%)
Male	1	11	26	38 (45.2%)
Total	13 (15.5%)	27 (32.1%)	44 (52.4%)	84

3.4. Gene ontology analysis

A gene ontology (G0) annotation was performed at level 2 among the 84 genes resulting from the DEA (Figure 3). A total of 47 genes (56%) had eggNOG assignations and 49 genes (58.3%) were assigned with at least one GO term. Within the "biological process" category, the main GO terms were grouped in metabolic process (21%) and cellular process (18%). Binding (23%) and catalytic activity (19%) were predominantly assigned to "molecular functions". In the category "cellular components", the largest proportion of GO terms referred to membrane (21%) and membrane part (19%). A GO enrichment analysis was conducted to identify over-representation of GO terms based on sex- and stage-biased differentially expressed genes, but no enrichment of GO terms was found.

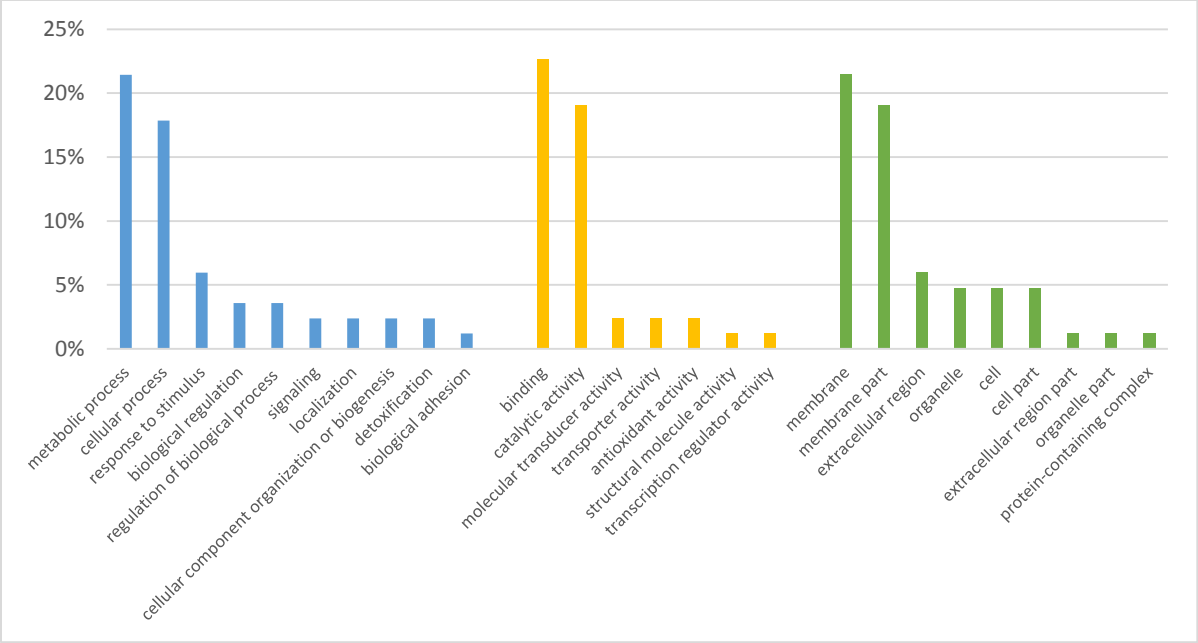


Figure 3. Gene ontology (GO) annotation (at level 2) of sex- and stage-biased genes of C. gigas. Blue: biological process; yellow: molecular functions; green: cellular components.

3.5. Expression patterns of oyster's homologs of known sex-determining genes, evaluated by DEA

A targeted approach was also undertaken in addition to the overall approach. The purpose was to focus on oyster's homologs of known sex-determining genes, in order to highlight their expression patterns by RNA-Seq even if they did not emerge from the DEA as significantly differentially expressed between both gametogenetic stages and sexes. For some of these genes (*Gadd45γ, Wnt-4, FST*), two numbers of CGI, maybe corresponding to 2 isoforms, were assigned to one name of gene in NCBI; they will be arbitrarily called "(a)" and "(b)" for a better understanding. A total of 22 genes were annotated, in the *C. gigas* gonad transcriptomes, as genes related to sex-determining genes of Mammals (18 genes) and drosophila (4 genes). *FoxN5* (CGI_10023645) and *LOC105342716* (Sex-Determining Region Y protein; CGI_10003991) were also taken into account because (i) the 1st one belongs to the Fox gene family, like *FoxL2* and previously exhibited an expression in mature male oysters (Zhang *et al.*, 2014) and (ii) the 2nd is annotated in NCBI as a gene of the *Sox* family to which *SoxH* and *SoxE* belong. Among the mammalian homologs, 2 are known to be involved in the bipotential

gonad, 9 are known as male sex-determining genes, 7 as female sex-determining and 2 are unknown (*FoxN5* and *LOC105342716*). Four drosophila homologs were also found in *C. gigas*. All were divided in 7 clusters according to their highest expression along the gametogenetic cycle: cluster 1 - in early male gonad; cluster 2 - in early female gonad; cluster 3 - in early gonad whatever the sex; cluster 4 - in mature male gonad; cluster 5 - in mature female gonad; cluster 6 - in mature gonad whatever the sex and cluster 7 - no differential expression at all.

The first group included *FST(a)* (CGI_10024194), *Wnt4(a)* (CGI_10019644) and *LOC105342716* (Sex-Determining Region Y protein), 3 genes with a significant peak of expression in early male gonads (stage 0 or 1). This expression was also significantly higher from that of females for *FST(a). Gadd45ɣ(a)* (CGI_10022728) appeared to be the only representative of the 2nd group, exhibiting a significantly high expression in early female gonads (stage 1), without being significantly different between genders. The 3rd cluster included *SoxE* (CGI_10022931), *RSPO3* (CGI_100021271) and *FST* (b) (CGI_10014129), significantly more expressed in early gonads (stage 1) in both males and females, without being significantly different between genders. *Dsx/Dmrt1-like* (CGI_10019568) appeared to be the only representative of the 4th group exhibiting a significantly high expression in mature male gonads (stage 3), without being significantly different between genders. Only *FoxL2* (CGI_10011004) belonged to the 5th group, showing a significantly higher expression in mature female gonads (stage 3), also significantly higher compared to males. *FoxN5* (CGI_10023645), *SoxH* (CGI_10006950) and the drosophila homolog *CBX1* (CGI_10028470) were grouped in the 6th cluster, the one of genes significantly highly expressed in mature gonads for both genders, without being significantly different between both. At last, genes expressed over the entire gametogenetic cycle without any sex-dimorphic and stage-specific peak of expression were grouped in cluster 7. It included the mammalian homologs *β-Catenin* (CGI_10026479), *ATRX* (CGI_100027273), *EMX2* (CGI_100025052), *FGF18* (CGI_100017045), *Gadd45ɣ(b)* (CGI_10012922), *LHX9* (CGI_100015423), *MAP3K* (CGI_100017027), *SF1* (CGI_100022774), and *Wnt-4(b)* (CGI_10028941) and the drosophila homologs *CBX8* (CGI_10026606) / *HP1* (CGI_10028772) and *Run* (CGI_10005366).

3.6. Expression patterns of genes resulting from the DEA, measured by real-time qPCR during the entire gametogenetic cycle

RT-qPCR were performed on eleven genes (Table 5) selected from the 84 relevant ones (on top of *FoxL2*), in order to validate the RNA-Seq and to explore their temporal expression during an entire gametogenetic cycle (stages 0, 1, 2 and 3 for males and females). Most of them were chosen because they exhibited by RNA-Seq a significantly higher expression during the sex-determining period (*i.e* stages 0 or 3) which was then also dimorphic between sexes. Some of them were chosen for their high dimorphic expression just after the sex-determining period, in stage 1. FoxL2 was taken as a control.

Table 5: Eleven genes identified in the C. gigas transcriptome and selected for RT-qPCR

Gene name	Due to stage FDR p-value	Due to sex FDR p-value	Blasted in the oyster's genome to	Blast e-value
CGI_10006800	4,44E-11	2,85E-04	Trophoblast glycoprotein-like	0.0
CGI_10028666	2,47E-14	1,51E-04	PREDICTED: paramyosin	0.0
CGI_10023199	1,03E-06	1,90E-02	Peroxidase-like protein	0.0
CGI_10011342	2,22E-02	3,61E-02	Complement C1q-like protein 4	2.39E-116
CGI_10016132	0,00E+00	1,51E-04	Protein singed-like	0.0
CGI_10021158	8,69E-11	4,23E-02	PI-PLC X domain-containing protein 2	0.0
CGI_10001090	5,08E-06	2,59E-02	Ephrin type-A receptor 2	3.09E-78
CGI_10026009	0,00E+00	3,66E-02	Hypothetical protein	0.0
CGI_10018971	1,05E-02	8,93E-04	Protein PML-like	0.0
CGI_10008094	5,88E-05	8,79E-05	GATA zinc finger domain-containing protein 14-like	0.0
CGI_10025872	3,34E-03	1,62E-03	MAM and LDL-receptor class A domain-containing protein 1-like	0.0

Seven genes exhibited a dimorphic expression in stage 3 (Figure 4). Four of them increased their expression over the course of gametogenesis in females (by a factor of 3 to 150; CGI 10011004-FoxL2, CGI 10018971-Protein PML-like, CGI 10025872-MAM and LDL-receptor class A domain-containing protein 1-like and CGI 10008094-GATA zinc finger domain-containing protein 14-like) and 3 in males (by a factor of 40 to 2000; CGI 10006800-Trophoblast glycoprotein-like, CGI 10016132-Protein singed-like and CGI 10026009-Hypothetical protein). For the "female genes", the expression of CGI 10011004 (FoxL2) and CGI 10018971 (Protein PML-like) sharply increased between stages 2 and 3, while the increase was more progressive for CGI 10025872 (MAM and LDL-receptor class A domain-containing protein 1-like) and CGI 10008094 (GATA zinc finger domain-containing protein 14-like). For the "male genes", the expression (i) was always dimorphic in favour of the males for CGI 10006800 (Trophoblast glycoprotein-like) and (ii) sharply increased from stage 2 for CGI 10016132 (Protein singed-like) and from stage 1 for CGI 10026009 (Hypothetical protein). On the other hand, two genes were more expressed in stage 0 with a sharp decrease from stage 1 (by a factor 7 to 3900; Figure 3), one with an expression in favour of females (CGI 10028666-PREDICTED: paramyosin), the other with a similar profile for both sexes (CGI 10011342-Complement C1q-like protein 4). One other gene (CGI 10001090-Ephrin type-A receptor 2) exhibited a decrease of expression over the course of gametogenesis, with a peak in stage 0 and a 125 fold-decrease in stage 1 for females and a peak of expression (by a factor 60) in stage 1 for males. The two last genes (CGI 10021158-PI-PLC X domain-containing protein 2 and CGI 10023199-Peroxidase-like protein) exhibited a moderate peak of expression in stage 1 for both sexes, with a higher level of expression in males compared to females.

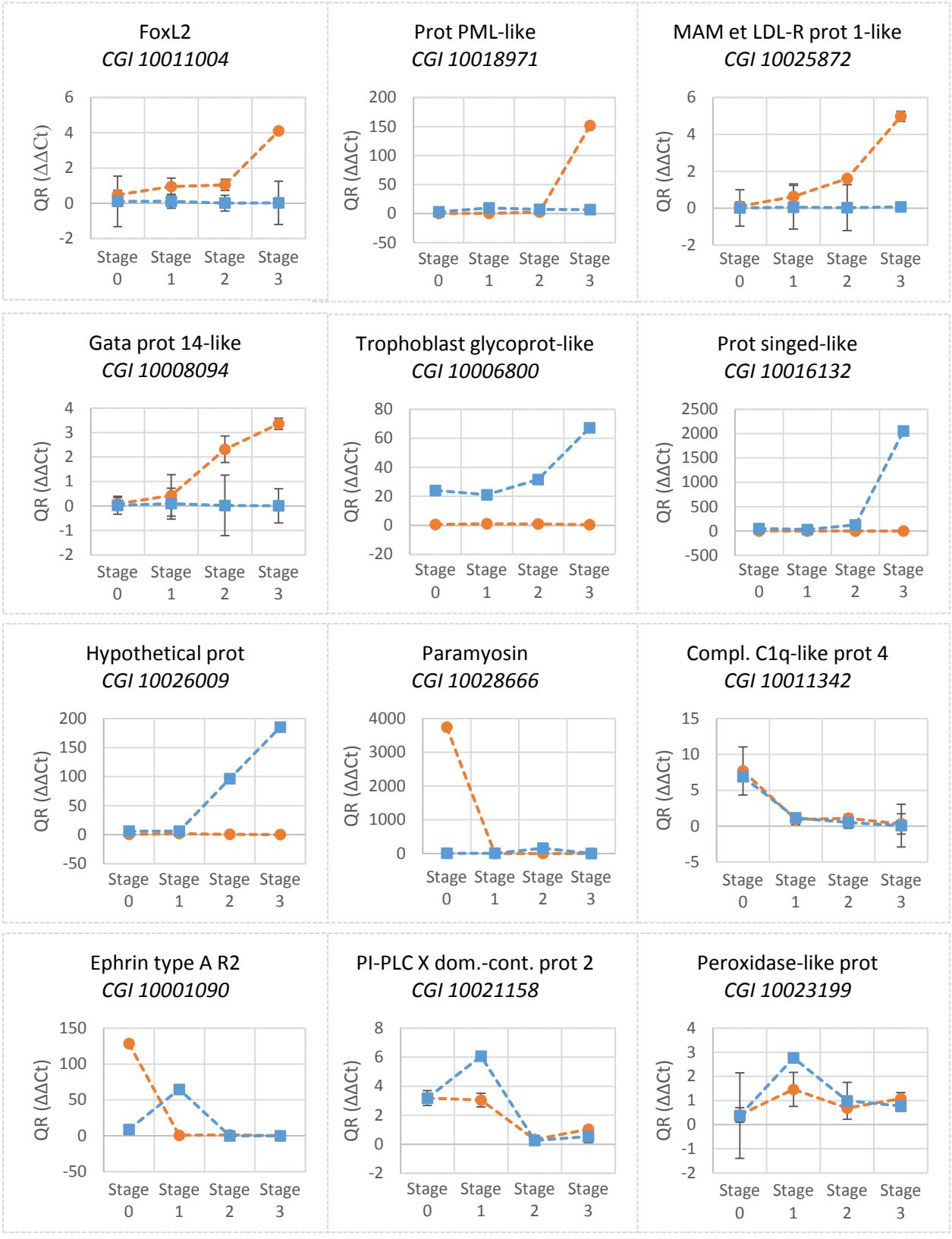


Figure 4. Expressions of eleven relevant genes selected, relatives to EF1α, measured by Real-Time qPCR in gonads along the oyster's gametogenetic cycle (stages 0, 1, 2 and 3 for males and females). FoxL2 was also taken as a control. Females: orange lines, males: blue lines. N=3 for each stage and sex. QR: Relative Quantity. Values are means + SEM of triplicates.

4. Discussion

The aim of our study was to identify genes significantly differentially expressed between males and females at the time of sex determination in oysters *C. gigas*.

4.1. Choice of animals - Associate, for the 1st time, molecular expression patterns over the entire time-window of sex determination to future sex phenotypes in a sequential hermaphrodite

The challenge was double: - (1) To highlight relevant genes covering the entire sex-determining period, which extends from stage 3 of one gametogenetic cycle when animals are matures to stage 0, just after spawning when animals start a new cycle (Naimi *et al.,* 2009a and b, Santerre *et al*., 2012, 2014; Dheilly *et al.*, 2012). - (2) To link each molecular expression pattern to a sex phenotype, *i.e* to the most statistically likely future sex of the animal, while this future sex is usually impossible to know because *C. gigas* is a sequential irregular hermaphrodite.

Previous works done on this topic in *C. gigas* are rather scarce. Moreover, very few have focused on stage 0 (Dheilly *et al.*, 2012); they rather focused on mature animals (Zhang *et al.*, 2014 and Yue *et al.*, 2018). In both case, the expression patterns could not be associated to the future sex of the animals. It is the same limit for the very few large-scale molecular studies existing on this topic in other sequential hermaphrodite bivalves (Tong *et al.*, 2015; Teaniniuraitemoana *et al.*, 2014 and 2015). Thus, studies on oysters *C. hongkongensis* (Tong *et al.*, 2015) and *Pinctada margaritifera* (Teaniniuraitemoana *et al.*, 2014, 2015) were done on sexable or sexually mature animals and their future sex was unknown. Some of these limits can also exist for studies made in simultaneous hermaphrodites such as *Nodipecten subnosus* (Galindo-Torres *et al.*, 2018) and *Argopecten purpuratus* (Boutet *et al.,* 2008). Indeed, the 1st one was done on undifferentiated juvenile gonads without discriminating against future sexes. The authors adopted therefore a targeted approach looking for known homologs or genes containing key words “sex determination/differentiation”, “development”, “germ line”, “spermatogenesis” or “oogenesis”. The 2nd one was done on mature male and female gonads

and on immature gonads but rather focused on genes of the mature gonads, *i.e* long after sex determination

In the present study, transcriptomes were made on gonads of individuals (i) in stage 0 and 3, the time-window of sex determination in *C. gigas* and in stage 1, just after, for comparison and (ii) identified as males and females never changing sex during the first years of their life (Broquard *et al.*, 2020). These "real" males and "real" females were designed to "predict" the future sex of the animal in stage 0 while the gonad is undifferentiated, but also in stafe 3, in order to better interpret molecular expression profiles. For this last point, the challenge was to be able to assign the most likely future sex phenotype to each oyster and to explain therefore expression patterns as if animals were gonochoric individuals. To this purpose, individuals were picked within a population whose (i) 42% of oysters never changed sex during the 5 first years of their life; (ii) and proportion of oysters changing sex decreased over the years (9% at the end of the study). Moreover, by a predictive logistic regression, individuals without any sex change during 6 years, may encounter a 1st sex change within the next 5 or 21 years according to their 1st sex (Broquard *et al*, 2020).

On top of that, we made sure that oysters were diploids while it was never determined/mentioned in the papers cited above. Yet, it is naturally variable and known to modify gametogenesis and transcriptomes, at least in *C. gigas*, (Jouaux *et al.*, 2010; Dheilly *et al.,* 2014).

4.2. The safeguards of transcriptome quality

The clean reads obtained presented high values of Q20 (from 98.92 to 99.89%) and Q30 (from 96.20 to 97.98%). This quality of sequencing is similar, although superior, to that found for other Mollusc transcriptomes. Indeed, Yue *et al.* (2018) obtained Q20 ranging from 94.74 to 98.14% for *C. gigas.* Chen *et al.* (2017) reported Q30 of at least 89.46% for their eight transcriptomes of *Tegillarca granosa*. In mussels, the Q20 was equal to 98.31% for *Cristaria plicata* (Patnaik *et al.*, 2016) and ranging from 97.36 to 97.67% for *Hyriopsis schlegelii* (Shi *et al.*, 2015). Finally, transcriptomes of the gastropod *Reishia clavigera* provided Q30 ranging from 88.02 to 90.90% (Ip *et al.*, 2015). The transcriptomes made in the present study had higher rates of alignment to *C. gigas* genome (from 96.44 to 98.52%) than the one found by

Yue *et al.* (2018) (from 57.07 to 68.37%). No information on this aspect was mentioned by Zhang *et al.* (2014). Finally, the DEA we performed showed 9,723 differentially expressed genes (DEG) within the gonads. This number is close to the 9,343 DEGs reported by Yue *et al.* (2018) in the same species. A lower number (1,937) of DEGs was found in the gonad transcriptomes of the oyster *Pinctada margaritifera* (Teaniniuraitemoana *et al.*, 2015).

4.3. Targeted approach - Conservation of sex-determining (SD) genes in Bilaterians but what about their expression patterns in C. gigas?

Presence of SD gene homologs in *C. gigas* gonad transcriptomes

A total of 22 genes were annotated in the *C. gigas* gonad transcriptomes, as genes related to sex determination, on top of *FoxN5* and *LOC105342716* (Sex-Determining Region Y protein). Eighteen of these genes were related to mammalian homologs and four to drosophila homologs. These results suggest that sex-determining genes are more conserved between the oyster and Vertebrates than with Ecdysozoa, an idea already mentioned by Santerre *et al.* (2012 and 2014) and Zhang *et al.* (2014). Most of these genes were also found in other Bivalve Molluscs (Ghiselli *et al.*, 2011 ; Dheilly *et al.*, 2012, 2014 ; de Sousa *et al.*, 2014 ; Teaniniuraitemoana *et al.*, 2014 ; Zhang *et al.*, 2014 ; Shi *et al.*, 2015 ; Tong *et al.*, 2015 ; Li *et al.*, 2016 ; Patnaik *et al.*, 2016 ; Chen *et al.*, 2017 ; Yu *et al.*, 2017 ; Galindo-Torres *et al.*, 2018 ; Yue *et al.*, 2018). These results are in line with previous works which suggest that sex-determining/differentiating pathways share common genes among Vertebrates (Deuterostomes) and Ecdysozoa (Protostomes) (Santerre *et al.*, 2012, 2014; Zhang *et al.*, 2014; Li *et al.,* 2016 and Yue *et al.*, 2018). However, further studies need to be done to verify if these oyster's genes have kept the sex-determining function of their homologs.

Stage-specific peak of expression of SD gene homologs in *C. gigas* gonad transcriptomes

In *C. gigas*, the above genes showed 7 different expression profiles, according to their highest expression along the gametogenetic cycle: 1 - in early male gonad ; 2 - in early female gonad ; 3 - in early gonad whatever the sex ; 4 - in mature male gonad ; 5 - in mature female gonad ; 6 - in mature gonad whatever the sex and 7 - no differential expression at all.

The first group included *FST(a)* (CGI_10024194), *Wnt-4(a)* (CGI_10019644) and *LOC105342716* (Sex-Determining Region Y protein). In *C. gigas*, *Wnt-4* and *FST* showed a peak of expression in stage 0 during the sex determination time frame, in potential future males. The *FST* expression was also significantly higher in males compared to females. This contrasts with Mammals, in which FST is required for female sex determination (Kashimada *et al.,* 2011). However, Xia *et al.* (2004) found the presence of a FST-like 3 (FSTL3) most highly expressed in mammalian testes and suggested its role as local regulator of male gonadal development and gametogenesis. Amongst Bivalves, *FST* was found in the clam *T. granosa* (Chen *et al*., 2017) and in the mature gonad of oyster *C. hongkongensis* (Tong *et al*, 2015) but the latter authors suggest that it may not be involved in reproduction. In mice, *Wnt-4* is expressed in the undifferentiated XX and XY gonads and is then down-regulated in the male gonad (Bernard & Harley, 2007) but it has also been shown to have a function in the testis determination pathway (Sertoli cell differentiation) (Jeays-Ward *et al.*, 2004). Amongst Bivalves, the presence of *Wnt-4* was reported in *C. gigas*, *C. hongkongensis* and *T. granosa* (Zhang *et al.*, 2014; Tong *et al.*, 2015; Chen *et al.*, 2017) without exhibiting any sex-specific difference, in contrast with the higher female expression found in the mussel *H. schlegelii* (Shi *et al*., 2015). *LOC105342716* (Sex-Determining Region Y protein) encodes a factor annotated as *Sry* (Sex determining Region on Y), the master gene, specific to Mammals (Kashimada & Koopman, 2010). However, the non-dimorphic expression of this factor in *C. gigas* does not allow it to be considered as a factor of sex determination, like *Wnt-4*, by the way. In contrast, further investigations are essential to clarify the role of FST in the oyster's gonad. A direct or indirect role in the sex determination still cannot be excluded.

In *C. gigas* transcriptomes, *Gadd45γ(a)* (CGI_10022728) was the only representative of the 2nd group, exhibiting a significantly high expression in early female gonads (stage 1) without being significantly different between genders. Such a factor has already been mentioned in *C. gigas*, without any dimorphic expression (Dheilly *et al.*, 2012 ; Zhang *et al.*, 2014) as in *C. hongkongensis* and in the clam *T. granosa* (Tong *et al.,* 2015 ; Chen *et al*., 2017). While it is essential for primary male sex determination in Mammals (Johnen *et al.*, 2013), its non-dimorphic expression, what's more in stage 1 after the sex-determining time-window, excludes it from candidates potentially involved in this mechanism.

The 3rd cluster included *SoxE* (CGI_10022931), *RSPO3* (CGI_100021271) and *FST (b)* (CGI_10014129), significantly more expressed in early gonads (stage 1) in both males and females, without being significantly different between genders. In previous works done in *C. gigas*, SoxE showed a peak of expression earlier, in stage 0, with a variable expression according to individuals (Santerre *et al.*, 2014) and also an expression in mature gonads without any difference between testis and ovary (Zhang *et al.*, 2014), as in the scallop *P. yessoensis* (Li *et al*., 2016). A *SoxE* was also found in other Bivalves such as *C. hongkongensis* (Tong *et al.*, 2015), *P. margaritifera* (Teaniniuraitemoana *et al.*, 2014) or *T. granosa* (Chen *et al.*, 2017). One single *RSPO* has previously been found in *C. gigas* and named *RSPO3* (Zhang *et al.*, 2012). In contrast with the present study, in the mussel *H. schlegelii*, *RSPO1* was more expressed in female gonads (Shi *et al.*, 2015). Thus, the non-dimorphic expression of *RSPO* in *C. gigas,* what's more after the period of sex determination, excludes it from candidates potentially involved in this mechanism as for *SoxE* and *FST* (b) (CGI_10014129).

Dsx/Dmrt1-like (CGI_10019568) appeared to be the only representative of the 4th group, exhibiting a significantly high expression in mature male gonads (stage 3), without being significantly different between genders. In contrast to our results, Yue *et al.* (2018) found *Dsx/Dmrt1-like* clustered with genes more expressed in stage 1 but Zhang *et al.* (2014) found the same high expression in mature gonads. However, the latter authors mentioned a male specific expression by measuring the RPKM, without statistical analyses. Still, they found some non-neglectful expression in ovary and a huge variability between individual testes, probably according to the future sex of the animals. It makes therefore the difference between both sexes most likely not significant, in agreement with the present study. Thus, the non-dimorphic peak of expression of this gene in *C. gigas* at the period of sex determination seems to exclude it from candidates potentially involved in this mechanism.

Only *FoxL2* (CGI_10011004) belonged to the 5th group, showing a significantly higher expression in mature female gonads, also significantly higher compared to males. These results are in agreement with previous studies carried out on *C. gigas* and within Molluscs (Naimi *et al*., 2009b; Santerre *et al.*, 2012 ; Dheilly *et al.*, 2012 ; Zhang *et al.*, 2014 ; Yue *et al.*, 2018 ; Teaniniuraitemoana *et al.*, 2014, 2015 ; Tong *et al.*, 2015 ; Shi *et al.*, 2015 ; Li *et al.*, 2016 ; Chen *et al.*, 2017). Our results confirm again the very likely role of FoxL2 as a female sex-determining gene.

At last, *FoxN5* (CGI_10023645), *SoxH* (CGI_10006950) and *CBX1* (CGI_10028470) were grouped in the 6th cluster, the one of genes significantly highly expressed in mature gonads for both genders, without being significantly different between both. Among Molluscs, *FoxN5* was only reported once, by Zhang *et al.* (2014), in mature gonads of *C. gigas*. As for *Dsx/Dmrt1-like*, in contrast with our results, the authors only mentioned a male specific expression. Again, it may not be significantly different with the ovarian expression, considering the huge variability between individual testes. Concerning *SoxH,* although Yue *et al.* (2018) observed the same increase of expression along the gametogenetic cycle in *C. gigas*, in contrast to our results, they reported a sex-biased expression in favour of males. The same male-bias expression of this gene was found by Zhang *et al.* (2014), Li *et al.* (2016) and Ghiselli *et al.* (2011) in mature gonads. Such expression in mature male gonads is in agreement with the expression of its mammalian homolog in spermatocytes and spermatids and its role in the regulation of spermiogenesis (Roumaud *et al.*, 2018; Zhang *et al.*, 2018), rather than sex determination. Concerning the female expression of this factor found in the present study, an expression in the mouse foetal and adult ovaries has also been found by Zhang *et al.* (2018), with levels 10% of that in adult testis and 2- to 4-fold higher than in foetal testis. Concerning *CBX1*, to our knowledge, it is the 1st time that this drosophila homolog was mentioned in *C. gigas* gonads. Other genes of the same family (*CBX5* and *CBX8*) were also found in *T granosa* gonad transcriptome by Chen *et al.* (2017). To conclude, the non-dimorphic peak of expression of all these genes in *C. gigas* at the period of sex determination seems to exclude them from candidates potentially involved in this mechanism.

Stable and non-dimorphic expression of SD gene homologs in *C. gigas* gonad transcriptomes

According to our results, homologs of mammalian *β-Catenin* (CGI_10026479), *ATRX* (CGI_100027273), *EMX2* (CGI_100025052), *FGF18* (CGI_100017045), *Gadd45γ(b)* (CGI_10012922), *LHX9* (CGI_100015423), *MAP3K* (CGI_100017027), *SF1* (CGI_100022774), and *Wnt-4(b)* (CGI_10028941) and the drosophila homologs *CBX8* (CGI_10026606), *HP1* (CGI_10028772) and *Run* (CGI_10005366) were expressed in the gonads over the entire gametogenetic cycle without any sex- or gender-specific peak of expression.

All these homologs have been reported at least once in previous studies in oyster species or in others Mollusc (Stephano & Gould, 2000 ; Santerre *et al.*, 2014 ; Zhang *et al.*, 2014 ; Li *et al.*, 2014 ; Shi *et al.*, 2015 ; Tong *et al.*, 2015 ; Patnaik *et al.*, 2016; Chen *et al.*, 2017; Liu *et al.,* 2017), except the homolog of *HP1*. Concerning their expression in the present study, the one of *Run, LHX9, EMX2* and *FGF18* is in agreement with the one found in other oyster and clam species (Zhang *et al.*, 2014; Tong *et al.*, 2015; Chen *et al.*, 2017). In contrast, the non-dimorphic expression patterns of *ATRX, MAP3K* and *CBX8* are not consistent with previous works which mention an expression higher in female gonads, except in the clam *T. granosa* (Tong *et al.*, 2015; Chen *et al.*, 2017). The present pattern of expression of *β-Catenin* is consistent with the one previously obtained by Zhang *et al.* (2014) in *C. gigas*. However, it contrasts with the work of Santerre *et al.* (2014) who reported an increase of expression in female mature gonads of *C. gigas* and Tong *et al.* (2015) who obtained a higher expression in female gonad than in other tissues for *C. hongkongensis*. *Wnt-4* and *Gadd45γ* expressions have previously been discussed for the 2 other CGI numbers (see above). Although disagreements in the expression of some of these factors still remain to be explained, their lack of differential expression during gametogenesis and between the sexes suggest that they may not have conserved their role in sex determination in *C. gigas*.

4.4. Large-scale approach - Genes differentially expressed between sexes during the sex-determining time window in C. gigas

The genes resulting from the DEA and studied in qPCR were divided according to the gametogenetic stage presenting their highest expression. To our knowledge, most of them had not been previously identified in *C. gigas* or in other Molluscs.

In stage 1, both sexes presented a peak of expression of CGI 10021158 (PLCXD2, PI-PLC X domain-containing protein 2) and CGI 10023199 (Peroxidase-like protein). In gonads, a PLCXD3 gene was detected in mouse foetal Leydig cells without any role assignment (McClelland *et al*, 2015). A human *PLCXD2* gene was also mentioned as being expressed in testes and ovaries with a 10 x-higher expression in testes (Fagerberg *et al.*, 2014), in agreement with the pattern found in this study. A female germline-specific expression was found for chorion peroxidase transcripts from 0-1-day-old ovaries and 0-1h embryos in the

mosquito *Anopheles stephensi* (Biedler *et al.*, 2015). The role of both enzymes still needs to be elucidated in the gonads of *C. gigas*, while just after sex determination (Naimi *et al.*, 2009a and b; Santerre *et al.*, 2012, 2014; Dheilly *et al.*, 2012), in stage 1, gonia proliferate among other gonadic processes (Heude-Berthelin *et al.*, 2001).

Among the genes most expressed at mature stage (stage 3), two different kind of patterns were observed.

The first one showed a regular increase of expression from stage 1 till stage 3, in males for CGI 10026009 (Hypothetical protein) and CGI_10006800 (Trophoblast glycoprotein-like) and in females for CGI 10008094 (GATA zinc finger domain-containing protein 14-like) and CGI 10025872 (MAM and LDL-receptor class A domain-containing protein 1-like). The expression of CGI 10006800 (Trophoblast glycoprotein-like) was also always dimorphic in favour of the males. A homolog of the Trophoblast glycoprotein, called Waif1a, was reported, in zebrafish and xenopus male embryos as well as in mammalian cells, to inhibit the canonical Wnt/β-catenin signalling involved in female sex-determination (Kagermeier-Schenk *et al.*, 2011). The clear male dimorphic expression of this gene in *C. gigas* is in agreement with a likely role in inhibition of the Wnt/β-catenin signalling, notably in stage 3, during the sex determination time-window. The hypothetical protein (CGI 10026009) blasted with the mammalian protein TESK2, a testis-specific protein kinase 2. Its expression pattern in rat testes suggests a role in meiotic stages and/or early stages of spermiogenesis, therefore long after sex determination (Fagerberg *et al.*, 2014). Its expression in *C. gigas* is in agreement with such role. The female-specific expression increasing over gametogenesis observed in *C. gigas* for CGI 10025872 (MALRD1, MAM and LDL-receptor class A domain-containing protein 1-like) was similar to the one obtained by qPCR for the so called ovarian gene *Cg-MALRD1-like* by Yue *et al.* (2018). Its role within *C. gigas* female gonads will have to be clarified, knowing that in Mammals, it is rather expressed in the digestive tract. Concerning GATA zinc finger domain-containing protein 14-like (CGI 10008094), other isoforms/CGI numbers are available on NCBI, suggesting maybe different isoforms. This CGI blasted with the mammalian *Gata-4*. This latter protein was detected in bipotential primitive gonads of both XX and XY mouse embryos and was markedly down-regulated shortly after the histological differentiation of the ovary, suggesting an

involvement in early gonadal development and possibly sexual dimorphism (Viger *et al.*, 1998). Its role in *C. gigas* still needs to be elucidated.

The 2nd kind of pattern observed in *C. gigas* in the present study showed a sudden peak of expression between stages 2 and 3, in males for CGI 10016132 (Protein singed-like) and in females for CGI 10018971 (PML-like-protein) and CGI_10011004 (FoxL2), the control, previously studied (Santerre *et al.*, 2012). This female expression of the gene encoding PML-like-protein is in agreement with previous studies. Indeed, in *C elegans*, SUMO is required for gonadal and uterine-vulval organogenesis (Broday *et al.*, 2004) and it may act by affecting nuclear and subnuclear localization of PML proteins. In mice, *PML* transcript isoform II was found in mature sperm and the isoforms I and II in oocytes, suggesting that the mature gametes may carry the transcripts to the embryo (Ebrahimian *et al.,* 2010). In mice again, Hadjimichael *et al.* (2017) showed that over-expression of the *PML* gene in embryonic stems cell lines delays cell differentiation, suggesting its essential role as regulator of stem cell pluripotency and somatic cell reprogramming. In *C. gigas*, in the state of knowledge, it is impossible to discriminate, for PML-like-protein, a role in late gametogenesis (spermiogenesis) or in very early events preparing the new cycle (sex determination), both occurring in mature gonads in stage 3. Concerning now the protein singed-like, whose gene is homologous to the mammalian *fascin*, it is required, in drosophila, for actin filament bundle formation in the cytoplasm of nurse cells during oogenesis (Cant, 1996). In mice, Tubb *et al.* (2002) reported a testis *fascin* (*FSCN3*), expressed specifically in the elongated spermatids and remaining in mature spermatozoa. The expression found in the present work in *C. gigas* does not fit exactly with a localization in spermatids and spermatozoa as it sharply increased after stage 2, while many elongated spermatids are already present at this stage. To conclude, further investigations need to be done on these 2 genes to elucidate their role in *C. gigas* gonads, but an involvement in sex determination cannot be ruled out. Foxl2 appears again as a very likely candidate for the female sex determination in *C. gigas*.

Finally, three genes showed a peak of expression in stage 0 followed by a sharp decrease, CGI 10028666 (PREDICTED: paramyosin) in females only, CGI 10011342 (Complement C1q-like protein 4) for both sexes and CGI 10001090 (Ephrin type-A receptor 2) in females also, but with a peak of expression in stage 1 in males. In *C elegans*, Miller *et al.* (2003) reported that

EphR could participate to promote sperm-sensing control mechanism for oocyte meiotic maturation. Somatic gonad sheath cells *via* Eph receptor signalling could also promote germ-cell death in this species (Li *et al.*, 2012). The role of this receptor in *C. gigas* gonad is still unknown based on its expression. About now Complement C1q-like protein, its transcripts were mentioned in pathogen-resistant oysters *C. gigas* and it was considered as an immune factor (Fleury & Huvet, 2012) as well as other C1q domain proteins present in this oyster (Gerdol *et al.*, 2015 ; Jiang *et al.*, 2015 ; Lv *et al.*, 2018) and in other Bivalve Molluscs such as the scallop *Chlamys farreri* (Wang *et al*., 2012). In the fish *Carassius auratus,* a C1q-like factor participates in regulating primordial germ cell development in early embryos (Mei *et al.*, 2014). In mice, a C1qtnf4 factor was upregulated in 11.5 dpc female somatic cells of the gonads (Beverdam & Koopman, 2006). In human cell lines, a C1q Domain-Containing Protein 1 (Caprin-2) promotes activation of the canonical Wnt signaling pathway by regulating LRP5/6 phosphorylation (Ding *et al.*, 2008), a signalling also involved in ovarian sex determination. Although immunity could play a role in the oyster's sex determination as demonstrated in drosophila (Zhao *et al.,* 2015), no clear role can be assigned to the Complement C1q-like protein 4 found in this study and whose transcripts were highly expressed in both sexes in stage 0. At last, paramyosin is a protein found in many muscles of Molluscs (Cohen, 1971). In drosophila, it was suspected to up-regulate the expression of *Tra2* in males, outside the sex determination pathway, as an off-target effect (Argue & Neckameyer, 2014). In Cephalobidae nematods and in *C. elegans*, paramyosin is a structural component of the female gonad which has an essential role in ovulation (Ono *et al.*, 2007; Bert *et al.*, 2017). Both roles do not fit with the expression found in the present study, in females and at the very beginning of the gametogenesis. Interestingly, paramyosin of *Schistosoma mansoni* appeared as homologous to *Taenia solium* antigen B, an immunogenic protein with anti-complement activity (Laclette *et al.*, 1991). In Vertebrates, a H-Y antigen was expressed in the heterogametic sex regardless of whether this is male or female. Its role, although still controversial, is notably suggested to be associated with a common denominator underlying the development of mammalian testes and avian ovaries, enhanced growth rate of the dominant heterogametic gonad at a critical stage of development (Mittwoch, 1977). In Mammals, such serologically detectable male antigens could also be associated with testis activity or spermatogenesis and may be antigenic when expressed in females (Sutou *et al.*, 2001). Although it appears clearly that the role of

paramyosin has to be elucidated in *C. gigas*, its dimorphic expression in stage 0 during the oyster's sex determination time-window and in females is promising.

To conclude, 6 genes emerged from the present work, with a promising expression profile during the time window of sex determination of the oyster *C. gigas*; they encode FoxL2 (CGI_10011004), FST (CGI_10024194), PREDICTED: paramyosin (CGI 10028666), Trophoblast glycoprotein-like (CGI_10006800), protein Singed-like (CGI 10016132) and PML-like-protein (CGI 10018971). The 2 first ones were obtained by the targeted approach while the 4 last ones emerged from the large-scale approach. Most of them (except FoxL2) are candidates with little information available in *C. gigas* and within Molluscs. Further investigations will be essential to clarify their homologies, especially when 2 CGI (isoforms?) are available and their roles in the oyster's gonad. Amongst them, some may have direct effects in the sex determination pathway and others, rather off-target effects during the sex determination time-window in *C. gigas*.

Acknowledgements

The authors thank the UMR 8199 LIGAN-PM Genomics platform (Lille, France) which belongs to the 'Federation de Recherche' 3508 Labex EGID (European Genomics Institute for Diabetes; ANR-10-LABX-46) and was supported by the ANR Equipex 2010 session (ANR-10-EQPX-07-01; 'LIGAN-PM'). The LIGAN-PM Genomics platform (Lille, France) is also supported by the FEDER and the Region Nord-Pas-de-Calais-Picardie. The authors also thank the platforms of the *SF4206 ICORE* of the University of Caen Normandy, Proteogen (qPCR) and Flux Cytometry (ploidy). This work was supported by *Région Normandie and Ifremer for C. Broquard's Ph.D scholarship. An Ifremer travel grant allowed C. Broquard to go to the GeneCology research unit (Australia) to perform RNA-Seq analyses.*

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Supplementary data

Table S1: List of sex- and stage-specific expressed genes

Name	Max group mean	Fold change	P-value	FDR p-value	Largest fold change	M vs. F - Max group mean	M vs. F - Fold change	M vs. F - P-value	M vs. F - FDR p-value
CGI_10006800	19,37	-33,45	1,84E-12	4,44E-11	1 vs. 3	11,85	9,53	3,38E-07	0,00
CGI_10013174	10,79	7,42	9,47E-03	2,91E-02	1 vs. 3	9,36	-23,74	4,91E-09	0,00
CGI_10021480	5102,18	-2155,65	0,00E+00	0,00E+00	0 vs. 3	2616,29	11,78	1,94E-09	0,00
CGI_10014625	71,07	-12534,86	0,00E+00	0,00E+00	0 vs. 3	35,96	12,12	1,06E-06	0,00
CGI_10017957	46,81	-674,78	0,00E+00	0,00E+00	0 vs. 3	22,06	5,87	1,43E-04	0,03
CGI_10014722	0,41	-6,38	1,18E-02	3,49E-02	1 vs. 3	0,41	-6,33	1,96E-04	0,04
CGI_10028682	78,75	-428,45	0,00E+00	0,00E+00	0 vs. 3	46,08	9,05	3,09E-06	0,00
CGI_10028666	120,52	1438,10	5,55E-16	2,47E-14	0 vs. 3	79,85	-24,75	1,74E-07	0,00
CGI_10012057	10,76	-1153,07	0,00E+00	0,00E+00	0 vs. 3	7,06	55,73	1,82E-09	0,00
CGI_10015725	7,84	623,87	7,24E-09	9,46E-08	0 vs. 3	9,15	-10,26	7,90E-05	0,02
CGI_10027551	0,33	-32,51	4,71E-03	1,61E-02	0 vs. 3	0,34	14,73	1,24E-04	0,03
CGI_10021674	1448,01	-43,22	1,43E-12	3,53E-11	0 vs. 3	797,88	4,80	4,29E-05	0,01
CGI_10010524	195,93	-441,21	0,00E+00	0,00E+00	0 vs. 3	96,82	4,80	8,75E-05	0,03
CGI_10007477	39,43	-122,24	0,00E+00	0,00E+00	0 vs. 3	20,43	11,20	1,70E-07	0,00
CGI_10023199	0,71	62,00	9,71E-08	1,03E-06	0 vs. 3	0,46	-18,82	5,89E-05	0,02
CGI_10021858	174,87	-22,18	4,39E-09	5,98E-08	0 vs. 3	105,58	4,81	3,13E-05	0,01
CGI_10016132	256,94	-345,45	0,00E+00	0,00E+00	0 vs. 3	124,38	10,99	1,73E-07	0,00
CGI_10011342	139,67	8,49	6,86E-03	2,22E-02	0 vs. 3	107,28	-6,51	1,73E-04	0,04
CGI_10013147	7,55	13,86	1,02E-05	7,19E-05	0 vs. 3	4,98	-14,79	4,00E-09	0,00
CGI_10017735	19,97	64,32	1,09E-09	1,64E-08	1 vs. 3	15,21	-5,40	1,15E-04	0,03
CGI_10018392	150,97	-9741,65	0,00E+00	0,00E+00	0 vs. 3	78,67	10,34	2,01E-05	0,01
CGI_10019797	51,63	-841,42	0,00E+00	0,00E+00	0 vs. 3	25,09	8,10	1,54E-05	0,01
CGI_10015188	1584,84	-1428,88	0,00E+00	0,00E+00	0 vs. 3	720,03	9,48	6,25E-06	0,00
CGI_10016091	15,53	14,03	1,49E-04	7,95E-04	1 vs. 3	12,81	-6,78	1,23E-05	0,01
CGI_10003290	113,32	-909,36	0,00E+00	0,00E+00	0 vs. 3	58,81	6,77	3,53E-05	0,01
CGI_10021396	1726,50	-1153,74	0,00E+00	0,00E+00	0 vs. 3	828,00	7,40	6,77E-06	0,00
CGI_10005367	16,70	26,11	4,96E-09	6,67E-08	1 vs. 3	14,17	-4,31	2,38E-04	0,04
CGI_10013256	3,85	49,81	8,20E-08	8,84E-07	1 vs. 3	2,89	9,82	8,26E-07	0,00
CGI_10018380	12,31	-8,18	1,29E-04	6,98E-04	1 vs. 3	9,40	-5,72	1,40E-05	0,01
CGI_10010156	16,03	-150,97	0,00E+00	0,00E+00	0 vs. 3	8,55	5,76	1,49E-04	0,03
CGI_10026009	137,76	-104,35	0,00E+00	0,00E+00	0 vs. 3	67,29	4,62	1,79E-04	0,04
CGI_10024233	6,12	20,13	1,07E-05	7,48E-05	1 vs. 3	4,75	6,28	9,54E-05	0,03
CGI_10027220	15,51	15,74	5,36E-06	4,00E-05	0 vs. 3	10,53	-62,68	2,36E-10	0,00
CGI_10003082	12,51	-3,22	1,34E-02	3,86E-02	0 vs. 3	12,99	4,48	4,15E-06	0,00
CGI_10015499	26,04	76,27	3,72E-11	7,22E-10	1 vs. 3	23,96	4,85	1,44E-04	0,03
CGI_10020939	907,59	-2963,95	0,00E+00	0,00E+00	0 vs. 3	451,44	5,51	3,18E-05	0,01
CGI_10019677	37,61	175,79	4,12E-13	1,13E-11	0 vs. 3	23,79	-5,31	2,17E-04	0,04
CGI_10017434	9,37	-4,35	1,72E-02	4,75E-02	1 vs. 3	8,81	-4,80	8,97E-05	0,03
CGI_10021259	138,28	-2561,34	0,00E+00	0,00E+00	0 vs. 3	74,32	10,88	2,79E-05	0,01
CGI_10003550	3,74	-379,79	1,21E-13	3,59E-12	0 vs. 3	2,49	164,85	4,82E-11	0,00
CGI_10023296	28,23	17,41	9,06E-05	5,08E-04	1 vs. 3	22,91	-7,69	7,02E-05	0,02
CGI_10008058	6,24	-17,57	7,52E-07	6,65E-06	0 vs. 3	4,35	4,44	1,75E-04	0,04
CGI_10009276	27,18	39,11	1,67E-15	6,86E-14	1 vs. 3	24,16	3,28	1,15E-04	0,03
CGI_10021158	5,26	12,59	3,80E-12	8,69E-11	1 vs. 3	4,30	2,79	2,15E-04	0,04
CGI_10001090	893,13	40,22	5,61E-07	5,08E-06	0 vs. 3	894,35	6,45	9,25E-05	0,03
CGI_10009567	0,17	-4,90	7,03E-03	2,26E-02	1 vs. 3	0,15	4,79	1,19E-04	0,03
CGI_10014592	4,06	-50,80	1,14E-12	2,87E-11	0 vs. 3	2,62	-5,90	1,52E-04	0,03
CGI_10022857	1,40	6,86	5,88E-05	3,46E-04	1 vs. 3	1,15	-3,47	1,14E-04	0,03
CGI_10022324	55,75	43,59	1,04E-05	7,31E-05	0 vs. 3	37,74	-6,55	1,48E-04	0,03

Name	Max group mean	Fold change	P-value	FDR p-value	Largest fold change	M vs. F - Max group mean	M vs. F - Fold change	M vs. F - P-value	M vs. F - FDR p-value
CGI_10022326	37,09	35,72	2,03E-07	2,01E-06	0 vs. 3	26,42	-7,23	5,60E-06	0,00
CGI_10009738	0,55	-11,34	1,33E-02	3,84E-02	0 vs. 1	0,47	29,89	1,77E-04	0,04
CGI_10014837	19,87	22,41	3,39E-04	1,65E-03	1 vs. 3	17,87	-10,66	1,08E-05	0,00
CGI_10025113	2,06	-127,97	4,34E-07	4,02E-06	0 vs. 3	1,41	-10,39	9,45E-05	0,03
CGI_10004155	4,88	16,23	2,67E-05	1,71E-04	1 vs. 3	5,15	5,57	5,03E-05	0,02
CGI_10012110	6,06	-9,96	5,56E-06	4,14E-05	0 vs. 3	5,07	-12,76	2,93E-12	0,00
CGI_10015666	3,87	-6,73	1,68E-05	1,12E-04	0 vs. 3	2,94	-4,17	6,60E-05	0,02
CGI_10002141	0,60	18,51	8,86E-04	3,85E-03	1 vs. 3	0,72	6,84	1,66E-04	0,04
CGI_10012308	25,60	9,28	5,97E-04	2,72E-03	1 vs. 3	22,19	-9,33	2,17E-05	0,01
CGI_10010209	33,26	9,13	1,09E-05	7,63E-05	1 vs. 3	25,57	-5,76	8,19E-07	0,00
CGI_10010210	18,79	25,33	2,24E-03	8,58E-03	0 vs. 3	12,74	-22,84	5,27E-06	0,00
CGI_10006159	1,14	-120,39	1,86E-05	1,23E-04	0 vs. 3	0,80	-31,59	1,89E-05	0,01
CGI_10002771	0,73	32,74	1,47E-02	4,18E-02	1 vs. 3	0,58	33,46	1,72E-04	0,04
CGI_10011118	324,59	138,53	5,14E-08	5,73E-07	0 vs. 3	216,58	-32,45	1,36E-08	0,00
CGI_10018971	6,54	-7,16	2,85E-03	1,05E-02	0 vs. 3	5,10	-6,48	1,47E-06	0,00
CGI_10016777	20,78	18,85	1,29E-08	1,62E-07	1 vs. 3	16,96	-4,93	5,73E-06	0,00
CGI_10002595	84,99	31,56	2,58E-05	1,66E-04	0 vs. 3	60,70	-6,62	8,78E-05	0,03
CGI_10004317	9,91	12,70	2,00E-07	1,98E-06	1 vs. 3	7,97	3,90	2,33E-04	0,04
CGI_10006066	0,81	-7,34	6,58E-05	3,84E-04	1 vs. 3	0,61	4,64	1,55E-04	0,03
CGI_10016764	3,62	-10,43	1,61E-09	2,34E-08	0 vs. 3	2,45	-3,09	2,68E-04	0,05
CGI_10026227	2,05	11,98	7,91E-05	4,52E-04	1 vs. 3	1,54	-7,99	1,14E-06	0,00
CGI_10020453	10,20	-7,43	2,47E-03	9,30E-03	0 vs. 3	8,09	-6,18	1,95E-05	0,01
CGI_10008094	0,94	-13,88	8,17E-06	5,88E-05	0 vs. 3	0,70	-18,30	8,56E-08	0,00
CGI_10025872	21,72	-14,58	7,54E-04	3,34E-03	0 vs. 3	15,87	-8,81	2,97E-06	0,00
CGI_10025875	5,58	-33,70	7,22E-05	4,18E-04	1 vs. 3	3,88	-7,94	6,79E-05	0,02
CGI_10026350	2,28	45,16	1,35E-08	1,69E-07	1 vs. 3	1,66	-8,37	2,31E-04	0,04
CGI_10019606	31,16	16,15	1,38E-03	5,66E-03	1 vs. 3	25,59	-7,09	1,54E-04	0,03
CGI_10023444	5,78	-4,25	7,96E-03	2,52E-02	0 vs. 3	5,14	-7,10	3,61E-07	0,00
CGI_10019043	2,23	-22,96	1,38E-03	5,68E-03	0 vs. 3	1,62	-28,19	5,03E-06	0,00
CGI_10028128	10,36	18,48	9,84E-05	5,48E-04	1 vs. 3	7,46	-10,41	2,80E-06	0,00
CGI_10000017	0,98	-33,13	2,49E-03	9,39E-03	0 vs. 3	0,83	-45,81	6,40E-05	0,02
CGI_10000111	2,59	-13,93	8,30E-05	4,71E-04	0 vs. 1	3,23	-6,97	1,07E-04	0,03
CGI_10000794	3,19	-10,70	6,16E-03	2,03E-02	0 vs. 3	2,71	-15,94	6,42E-08	0,00
CGI_10007856	3,10	14,13	2,26E-07	2,22E-06	1 vs. 3	2,78	-5,99	1,81E-06	0,00
CGI_10028757	27,66	23,44	1,60E-13	4,66E-12	1 vs. 3	20,12	3,02	1,59E-04	0,03

Conclusion générale et perspectives

Le déterminisme du sexe est un événement majeur de la reproduction sexuée animale. Toutefois, les mécanismes génétiques et moléculaires sous-jacents ont été très peu étudiés au sein des Mollusques et des Lophotrochozoaires. L'appartenance de l'huître creuse *Crassostrea gigas* à ces phyla ainsi que son hermaphrodisme séquentiel en font un modèle d'étude particulièrement intéressant pour cette thématique. Ainsi, cette thèse s'inscrit dans une volonté d'approfondir les connaissances relatives au déterminisme sexuel chez ce Mollusque Bivalve.

L'hermaphrodisme séquentiel de l'huître creuse *C. gigas*

L'objectif premier de cette thèse était d'identifier les phénotypes sexuels de l'huître lors des 5 à 6 premières années de vie, afin de déterminer le sexe-ratio ainsi que l'occurrence des changements de sexe.

A cet effet, deux populations ont été produites en écloserie et sexées individuellement depuis 2014 pour la première et depuis 2015 pour la seconde. Les résultats de cette étude ont mis en avant un sexe-ratio biaisé en faveur des femelles dès la première maturité sexuelle (69% et 54% pour la 1ère et 2nde population respectivement) et maintenu les années suivantes. Une régression logistique, à partir des données de sexage des 6 premières années de la population 1, et basée sur le premier sexe déterminé, a suggéré qu'il n'y aurait pas d'huîtres gonochoriques au sein de la population suivie, même si 42% des individus n'avaient jamais changé de sexe. Tous les individus seraient des hermaphrodites séquentiels, même s'ils connaissent un changement de sexe plus tardif. Enfin, ce travail a montré que cette espèce ne semble pas avoir de tendance forte à la protandrie (<23%). Toutefois, les huîtres identifiées mâles en première année de sexage auraient tendance à changer de sexe plus précocement, ce qui pourrait expliquer la tendance protandre qui était jusqu'alors attribuée à *C. gigas*.

La mise en perspective d'un hermaphrodisme exclusif (séquentiel ou simultané selon les individus) chez *Crassostrea gigas* remet en question les deux modèles génétiques actuels du déterminisme sexuel de cette espèce. En effet, ces deux modèles proposent la présence de génotypes gonochoriques : MF chez Guo *et al.* (1998) et MM et FF chez Hedrick & Hedgecock

(2010). Ainsi, l'ensemble des huîtres suivies lors de cette thèse serait de génotype FF selon Guo *et al*. (1998) ou FM selon Hedrick & Hedgecock (2010). Or, la seconde population étant produite à partir de géniteurs présents dans la première, cela signifierait :

- d'après le modèle de Guo *et al.* (1998) : un croisement de deux géniteurs FF donnant une descendance théorique à 100% FF, signifiant que 100% des huîtres seraient des mâles lors de la 1ère maturité sexuelle. Or dans la population 2 du présent travail, le sexe-ratio en 1ère année était à 54% en faveur des femelles.

- d'après le modèle de Hedrick & Hedgecock (2010) : un croisement de deux géniteurs FM produisant une descendance théorique de 50% FM, 25% FF et 25% MM, *càd*, 50% d'huîtres changeant de sexe au cours de leur vie et 50% ne changeant jamais. Or dans le cadre de cette thèse, après 5 ans de suivi, la population 2 ne présentait déjà plus que 24% d'individus qui n'ayant pas changé de sexe.

L'étude des phénotypes sexuels a également permis de souligner l'irrégularité de l'hermaphrodisme de *C. gigas*, tant dans le temps (changement de sexe parfois tardif) qu'en nombre (changements de sexe répétés). Dans les précédents modèles génétiques, une des assomptions formulées était le caractère unique du changement de sexe. Ainsi, des individus hermaphrodites séquentiels (FF ou FM) ne subiraient qu'un seul changement de sexe au cours de leur vie. Or les résultats de cette thèse ont montré que les huîtres sont capables de plusieurs changements de sexe au cours de leur vie, allant même jusqu'à changer chaque année pour un nombre très limité d'entre elles (0,1% et 0,9% pour les populations 1 et 2 respectivement). De plus, ces modèles envisageaient l'hermaphrodisme uniquement protandre, alors que ce travail a démontré, au sein des deux populations suivies, que des individus de 1er sexe femelle changent de sexe une à plusieurs fois en 5 à 6 ans.

A partir de ce constat, il serait maintenant intéressant de proposer un nouveau modèle génétique du déterminisme sexuel de *C. gigas*, plus en accord avec les observations de terrain. Pour cela, il pourrait être envisagé d'étudier pendant plusieurs années consécutives le sexe-ratio au sein de familles issues de croisements utilisant des phénotypes contrastés (fortes tendances mâles ou femelles ; à forte capacité à changer de sexe ou non), dont l'historique des phénotypes du sexe des géniteurs est connu sur plusieurs années consécutives. En complément, d'autres méthodes pourraient être utilisées pour essayer d'identifier les

génotypes du sexe, comme la gynogenèse pour déterminer le sexe hétérogamétique s'il existe (XY chez les mâles ; ZW chez les femelles), les croisements produits par autofécondation utilisant des hermaphrodites simultanés ou encore les triploïdes produites par croisements d'huîtres diploïdes et rétention d'un des deux globules polaires. Ce nouveau modèle génétique serait ainsi alimenté par les proportions de sexe-ratios obtenues par ces diverses approches.

Le dimorphisme sexuel de croissance chez *C. gigas*

La détermination de l'effet du sexe et du changement de sexe sur les paramètres morphologiques de l'huître *C. gigas* représentait le second objectif de cette thèse.

Ainsi, des mesures biométriques individuelles ont été effectuées chaque printemps, sur la première population sexée (*cf.* Chapitre 1), afin de mettre en relation les paramètres morphologiques et les phénotypes sexuels. Notre étude a alors montré que les individus femelles présentaient des caractéristiques morphologiques (poids, longueur, largeur et épaisseur de la coquille) supérieures à celles des mâles et cela, chaque année. De même, les croissances entre deux années étaient généralement plus élevées chez les huîtres étant mâle l'année n puis changeant de sexe l'année n+1. Au contraire, celles changeant de sexe de femelle vers mâle présentaient les plus faibles croissances. Parmi la population suivie les 6 années, i), les biométries des femelles étaient supérieures à celles des mâles pour des individus ne changeant jamais de sexe et ii) au sein des hermaphrodites détectés, l'huître protandre (changement unique mâle vers femelle) et l'huître avec un sexe primaire mâle changeant trois fois de sexe, présentaient les meilleures croissances. Cette croissance était également supérieure à celle des huîtres restées femelles durant la totalité de l'étude.

L'existence d'un dimorphisme sexuel, ainsi que d'un effet de l'hermaphrodisme (nombre et sens du changement de sexe) sur la croissance, présentent un intérêt pour la filière ostréicole. *De facto*, une meilleure croissance conduirait à un meilleur rendement de production et ainsi un cycle d'élevage plus rapide avant la commercialisation. D'après ces données de thèse, une lignée de mâles protandres serait à privilégier en sélection génétique, suivies par une de mâles changeant régulièremetn de sexe (3 fois sur 6ans) ou de femelles ne changeant pas de sexe

(si elles existent *cf.* Chapitre 1). Avant de sélectionner de telles lignées, il faudrait en premier lieu s'assurer de l'héritabilité de l'ensemble des caractères concernés (sexe, changement de sexe et poids *a minima*) et des corrélations génétiques entre tous. Chez *Crassostrea gigas*, l'héritabilité du poids et de la longueur de coquille ont déjà fait l'objet d'études antérieures, sans jamais y associer le sexe ou la capacité (et fréquence) à changer de sexe (Langdon *et al.*, 2003 ; Wang *et al*., 2012). Une telle sélection de mâles protandres, ou changeant trois fois de sexe, ainsi que de femelles ne changeant pas de sexe reposerait donc sur une meilleure connaissance des facteurs génétiques mais aussi sur celle des facteurs moléculaires du déterminisme sexuel. De plus, si ces caractères génétiques étaient transmis entre générations, cela suggèrerait la présence de gènes liés à la croissance, au sexe et au changement de sexe. Ainsi, les données phénotypiques et de RNA-Seq acquises ici (*cf.* Chapitres 1, 2 et 3), combinées aux données qui seront extraites d'un DNA-Seq gonadique réalisé dans le cadre de cette thèse, seront une base précieuse pour poursuivre les travaux en génétique sur le déterminisme sexuel de *C. gigas*.

Les facteurs moléculaires exprimés lors du déterminisme sexuel chez *C. gigas*

Le dernier objectif de ce travail de thèse était de caractériser des facteurs moléculaires potentiels du déterminisme sexuel.

Le challenge a consisté ici à associer, pour la 1ère fois, les patterns d'expression moléculaires, au futur sexe de l'animal, afin (i) de mieux les expliquer chez cet hermaphrodite séquentiel irrégulier et (ii) de couvrir toute la période du déterminisme sexuel. Ainsi, l'étude pluriannuelle du sex-ratio et du changement de sexe, effectuée sur des populations à larges effectifs (*cf.* Chapitre 1), nous a permis de choisir des individus mâles et femelles n'ayant jamais changé de sexe en 5 ans de vie. Nous avons émis l'hypothèse qu'à l'instar du passé, ils resteraient mâles et femelles, respectivement, dans le futur.

Après séquençage de leurs gonades en période de déterminisme sexuel (stades 0 et 3 de gamétogenèse), l'analyse des transcriptomes a révélé 84 gènes exprimés à la fois de façon

stade- et sexe-spécifique. Quatre d'entre eux (codant Trophoblast glycoprotein-like, Protein PML-like, Protein singed-like and PREDICTED: paramyosin) ont aussi présenté en RT-qPCR des profils d'expressions en accord avec un rôle possible dans le déterminisme sexuel. Les homologues de gènes connus du déterminisme sexuel ont également été recherchés et parmi eux, seuls ceux codant Foxl2 et FST présentaient des profils d'expressions en accord avec un rôle possible dans le déterminisme sexuel. Ces gènes n'ont, jusqu'à présent, fait l'objet d'aucune étude approfondie chez l'huître creuse *C. gigas*, excepté *FoxL2* (Santerre *et al*., 2012 ; Zhang *et al*., 2014).

Ainsi, ces travaux ont permis de caractériser 6 gènes connus et inconnus qui pourraient être des candidats pertinents ayant un rôle direct ou indirect dans le déterminisme sexuel de *C. gigas*. Cependant, 46 autres gènes présentaient en RNA-Seq un pic d'expression en stade 0 ou 3, dimorphique entre les mâles et les femelles. Des gènes qui ressortiraient d'une analyse approfondie des transcriptomiques déjà réalisés chez l'huître (Dheilly *et al.,* 2012, 2014 ; Zhang *et al*., 2014 ; Yue *et al*., 2018) pourraient aussi être pris en compte. Ainsi, dans un premier temps, les profils d'expression prometteurs des 46 gènes du présent travail et ceux extraits des travaux précédents devraient être confirmés en RT-qPCR sur tout le cycle gamétogénétique adulte. Les patterns d'expression des plus pertinents pourraient aussi être évalués dans divers tissus somatiques (manteau, branchies, muscle adducteur, palpes labiaux). Cela permettrait de vérifier s'ils sont gonade-spécifiques, même si des facteurs clés du déterminisme sexuel des Mammifères, comme par exemple Sox9, ne le sont pas forcément (Jo *et al*., 2014). Dans un 2ème temps, il serait nécessaire de vérifier, pour les facteurs les plus pertinents, les homologies par des études phylogénétiques ciblées, notamment quand les gènes semblent présenter plusieurs isoformes. Ensuite, des analyses d'expression spatiale de leurs transcrits et/ou protéines pourraient être réalisées en hybridation *in situ* et/ou immunofluorescence, afin de déterminer dans quels types cellulaires gonadiques (cellules germinales plus ou moins différenciées, cellules somatiques dans les tubules gonadiques, cellules somatiques hors tubules gonadiques) ils sont présents. Pour aller plus loin, l'étude des régulations au sein de la cascade moléculaire, pour des facteurs choisis, pourrait être abordée par ImmunoPrécipitation de la CHromatine (ChIP) ou par Retard sur gel (EMSA). Enfin, rien de mieux qu'une approche fonctionnelle, comme l'ARN interference (RNAi) (Fabioux *et al*., 2009) ou le CRISPR/Cas9 (Yu *et al*., 2019), déjà testés chez l'huître, pour discriminer sans équivoque, un rôle des facteurs

les plus prometteurs dans le déterminisme sexuel, d'un autre rôle dans la différenciation gonadique de *C. gigas*.

Dans tous les cas, il reste indispensable d'interpréter les résultats quels qu'ils soient, à la lumière du futur sexe des animaux chez cet organisme hermaphrodite séquentiel sans chromosomes sexuels identifiés. L'approche choisie dans ce travail a en partie permis de de lever ce verrou technique Cependant, malgré les divers garde-fous choisis, elle reste critiquable car elle ne permet pas d'être absolument sûrs du futur sexe des animaux. Seules des approches non invasives, comme des biopsies réalisées en stade 0 et 3 (cycle n), permettraient d'associer un pattern d'expression moléculaire au futur sexe d'un animal (évalué en stade 3 du cycle n et stade 3 du cycle n+1, respectivement). Dans le cadre de cette thèse, des biopsies ont été réalisées sur plusieurs cycles de reproduction successifs. Certes, elles n'ont pas permis de récupérer suffisamment de gonade pour réaliser des RNA-Seq, mais elles seront indispensables dans de futurs travaux, afin d'obtenir des profils d'expressions en RT-qPCR et les associer de façon certaine, au futur sexe de chaque individu.

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Le déterminisme sexuel de l'huître *Crassostrea gigas* : du phénotype aux facteurs moléculaires sous-jacents

Le savoir relatif au mode de reproduction et au déterminisme sexuel de l'huître creuse *Crassostrea gigas* demeure limité. Etant une espèce hermaphrodite séquentielle, la détermination de son sexe a lieu plusieurs fois au cours de sa vie. Dans le cadre de cette thèse, les phénotypes sexuels des 6 premières années de vie de l'huître ont été identifiés au sein de deux cohortes. Ainsi, le sexe-ratio était biaisé vers les femelles dès la première maturité sexuelle, ainsi que pour toutes les années suivantes. Après six années de sexage, 42%, 32%, 19%, 5%, 1% et 0,1% des huîtres ont montré 0, 1, 2, 3, 4 et 5 changements de sexe. La fréquence des changements de sexe décroissait aussi avec l'âge des individus (34% entre les années 1-2 à 9% entre les années 5-6). Ces travaux de thèse ont également cherché à déterminer l'influence du sexe et du changement de sexe sur les paramètres morphologiques des individus. Ainsi, un dimorphisme sexuel a été identifié pour le poids total ainsi que la longueur, largeur et épaisseur de la coquille, en faveur des huîtres femelles, dont celles ayant changé de sexe dans le sens male vers femelle. Enfin, une analyse transcriptomique de la gonade d'individus à phénotypes contrastés (« vraies » femelles et « vrais » mâles) devait permettre d'approfondir les connaissances sur les facteurs moléculaires du déterminisme sexuel. Ainsi, cette approche a permis (i) d'identifier les profils d'expression d'homologues du déterminisme sexuel sur toute la période de ce mécanisme et (ii) d'identifier de nouveaux acteurs moléculaires d'intérêt surexprimés spécifiquement dans un sexe lors du déterminisme sexuel. Chaque pattern d'expression obtenu chez de « vrais » mâles ou de « vraies » femelles a ainsi pu être interprété à la lumière du plus probable futur phénotype du sexe, malgré l'hermaphrodisme séquentiel. Cette approche a permis d'affiner les hypothèses concernant le rôle des divers facteurs dans le déterminisme du sexe de l'huître creuse.

Mots clés : déterminisme sexuel, hermaphrodisme, croissance, facteurs moléculaires, C. gigas

The sex determination of the oyster *Crassostrea gigas* : from phenotype to underlying molecular factors

Knowledge about the mode of reproduction and the sex determination of the cupped oyster *Crassostrea gigas* remains limited. As a sequential hermaphroditic species, sex determination takes place several times during its lifetime. As part of this thesis, the sexual phenotypes of the first 6 years of life of oysters were identified in two cohorts. Thus, the sex ratio was biased towards females from the first sexual maturity, as well as for all subsequent years. After six years of sexing, 42%, 32%, 19%, 5%, 1% and 0.1% of oysters showed 0, 1, 2, 2, 3, 4 and 5 sex changes. The frequency of sex changes also decreased with age (34% between years 1-2 to 9% between years 5-6). This thesis work has also sought to determine the influence of sex and sex change on the morphological parameters of individuals. Thus, a sexual dimorphism was identified for the total weight as well as the length, width and thickness of the shell, in favour of female oysters, including those that have changed sex in the male to female direction. Finally, a transcriptomic analysis of the gonad of individuals with contrasted phenotypes ("true" females and "true" males) was to provide further knowledge on the molecular factors of sex determination. Thus, this approach made it possible (i) to identify the expression patterns of homologs of sex-determining genes, over the entire period of this mechanism and (ii) to identify new molecular actors of interest, overexpressed specifically in one sex during sex determination. Each expression pattern obtained in "true" males or "true" females could thus be interpreted in the light of the most probable future phenotype of the sex, despite sequential hermaphroditism. This approach allowed to refine hypotheses about the role of various factors in determining the sex of this cupped oyster.

Key words : sex determination, hermaphroditism, growth, molecular factors, C. gigas