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Sources de variation intra-populationnelle de la morphologie des otolithes: asymétrie directionnelle et régime alimentaire

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« C'était très bien, c'était très bien

vous c'était bien là bas,

vous c'était bien… enfin comme ci comme ça

[…]

Ecoutez, j'ai une conception personnelle de l'ouvrage,

ce n'est pas assez triomphal,

de l'orgueil bon sang,

c'est de la bouillie tout ça,

c'était pas mauvais, c'était très mauvais voilà »

Stanislas Lefort, chef d'orchestre à l'Opéra de Paris (joué par Louis de Funès)

La Grande Vadrouille de Gérard Oury, 1966.

RESUME

Les otolithes sont des pièces calcifiées de l'oreille interne des Ostéichtyens impliquées dans les fonctions d'audition et d'équilibration. Leur morphologie est utilisée comme indicateur de divers processus ou propriétés écologiques. Cette application nécessite d'identifier les facteurs endogènes et exogènes agissant simultanément comme sources de variation. Cette thèse porte sur la détection et la quantification des contributions relatives de l'asymétrie directionnelle et du régime alimentaire à la variation morphologique des otolithes à l'échelle intra-populationnelle. Une asymétrie directionnelle entre les otolithes droit et gauche est montrée chez les poissons plats, l'otolithe le plus long et large étant toujours du côté aveugle, contrairement aux espèces de poissons ronds pour lesquels l'asymétrie est moindre. Cependant, l'asymétrie n'excède pas 18 % d'amplitude suggérant une canalisation de la symétrie de forme d'origine évolutive. Une corrélation entre le régime alimentaire et la forme des otolithes est détectée chez 4 espèces de poissons *in situ*. La composition du régime contribue plus fortement à la variabilité morphologique que la quantité ingérée et impacte la forme des otolithes à la fois globalement et localement. Une étude expérimentale sur le bar commun (*Dicentrarchus labrax*) montre que la composition du régime alimentaire en acides gras essentiels au stade larvaire affecte la morphogenèse des otolithes pendant le stade juvénile sans affecter la croissance somatique des individus. Ceci suggère un effet direct et non indirect via la croissance somatique. Cet effet disparait aux stades ultérieurs ce qui évoque une canalisation de la morphologie des otolithes.

Mots clés : Otolithes, Oreille interne, Ostéichtyens, Symétrie, Alimentation, Analyse de forme, Analyse de Fourier, Morphométrie géométrique

ABSTRACT

Otoliths are calcified structures located in Osteichthyes' inner ear that are involved in audition and balance. Their morphology is used as an indicator of various ecological processes or properties. This application requires identifying the endogenous and exogenous factors that act simultaneously as sources of shape variation. This thesis aims at detecting and quantifying the relative contributions of directional asymmetry and diet to otolith shape variation at the intra-population level. Directional asymmetry between left and right otoliths was found in flat-fishes, the blind-side otolith being always longer and larger, whereas it was negligible in round-fishes. However, asymmetry amplitude never exceeded 18 %, which suggests evolutionary canalization of otolith shape symmetry. A correlation between global diet and otolith was detected in 4 species studied *in situ*. Diet composition contributed more than food amount to morphological variation and affected otolith shape both globally and locally. An experimental study on sea bass (*Dicentrarchus larbrax*) showed that diet composition in terms of essential polyunsaturated fatty acids at larval stage affects otolith morphogenesis during juvenile stage without impacting on individuals' somatic growth. This result suggests a direct effect of diet on otolith shape and not an indirect one through the somatic-otolith growth relationship. This effect disappeared at later stages, morphogenetic trajectories converging back to a similar shape, which suggests ontogenetic canalization of otolith shape.

Keywords: Otoliths, Inner ear, Osteichthyes, Symmetry, Diet composition, Shape analysis, Fourier analysis, geometric morphometrics

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Introduction générale

Contexte général et problématique de la thèse

L'homme pêche depuis les temps préhistoriques pour se nourrir. Mais durant les six dernières décennies, la production mondiale de poissons n'a cessé d'augmenter de manière constante (Figure 1 A). L'augmentation de la population humaine, et donc de la demande, mais aussi le développement des performances des engins de pêche, de l'aquaculture et des technologies qui facilitent le transport et la vente des marchandises, expliquent en grande partie cette augmentation. Dans ce contexte de demande croissante, un état préoccupant des ressources halieutiques mondiales est rapporté où les captures de poissons sauvages semblent plafonner depuis la fin des années 80 et où plus de 80% des stocks de poissons marins sauvages gérés à l'échelle mondiale sont en situation de pleine ou de surexploitation (Figure 1 B) (FAO 2014). Ces stocks nécessitent donc des plans de gestion stricts pour une productivité équilibrée et donc durable avec comme point de mire l'atteinte du Rendement Maximum Durable (RMD ou Maximum Sustainable Yield, MSY) pour une majorité de stocks (Sommet Mondial du Développement Durable de Johannesburg 2002, Politique Commune des Pêches européenne).

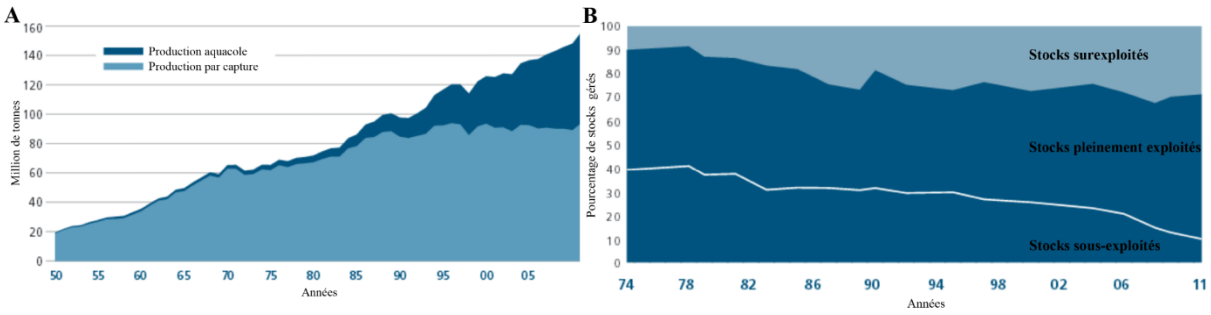

Figure 1: Tendances mondiales de la production aquacole et des captures de pêche entre 1950 et 2005 (A) et de l'état des stocks gérés de poissons marins entre 1974 et 2011 (B). Redessinée d'après les données de la FAO (Food and Agriculture Organization of the United Nations ; 2014).

Une gestion durable des ressources halieutiques nécessite des connaissances sur la biologie des espèces, sur la dynamique de leurs populations et de leurs écosystèmes sous forçages naturels et anthropiques. Notamment, l'identification des populations et/ou stocks de

poissons est une composante importante de la gestion des pêcheries (Begg & Waldman 1999). Un stock est défini généralement comme la partie exploitable de la population (Laurec & Le Guen 1981a) c'est-à-dire un ensemble d'individus de la même espèce présentant des caractéristiques biologiques similaires (homogénéité) qui se maintient lui-même à travers le temps et qui présente des caractéristiques biologiques similaires (homogénéité). Selon les champs disciplinaires cette définition peut être précisée. Les stocks de poissons peuvent être génétiquement déterminés, c'est-à-dire que les individus d'un même stock présentent une homogénéité génétique (le plus souvent au niveau des marqueurs neutres), ou encore définis sur la base de variations phénotypique qui résultent à la fois du génotype et de l'influence environnementale. On utilise dans ce dernier cas la notion de stock phénotypique, regroupant ainsi des individus phénotypiquement similaires en termes de croissance, reproduction et de mortalité par exemple (Cadrin et al. 2005). De ce fait, les différences entre les traits d'histoire de vie des individus sont fréquemment utilisées comme indicateurs pour l'identification et la délimitation des aires géographiques des stocks de poissons (Begg et al. 1999, Begg & Waldman 1999). De plus, l'étude de la croissance des individus est une composante importante des procédures classiques d'évaluation analytique des stocks qui permet ainsi d'estimer avec plus de précision leur biomasse exploitable (Haddon 2010). L'un des outils les plus populaire pour estimer la croissance des individus est l'otolithe (Campana 2005).

Qu'est-ce un otolithe ?

L'oreille interne des Ostéichtyens est une structure paire située sous le cerveau dans le crâne du poisson. Chaque oreille interne (droite et gauche) est composée de trois canaux semi-circulaires et de trois organes otolithiques (Figure 2). Ce système typique des vertébrés Gnathostomes permet la double fonction de l'audition et du maintien de l'équilibre de l'animal. Les canaux semi-circulaires, positionnés de manière orthogonale entre eux, sont

associés à des ampoules (Figure 2) permettant la détection des accélérations angulaires à l'aide du déplacement du liquide remplissant complétement le lumen du système, l'endolymphe. Chaque canal débouche sur un sac otique formé par un épithélium cellulaire délimitant une chambre otolithique remplie d'endolymphe dans laquelle baigne une concrétion calcaire appelée otolithe (oto : oreille ; lithos : pierre). Les trois sacs otolithiques nommés *sacculus*, *utriculus* et *lagena* contiennent respectivement la *sagitta*, le *lapillus* et l'*asteriscus* (Figure 2). Même si la fonction de chaque sac otolitique n'est pas clairement définie, il semblerait que chaque sac soit impliqué à la fois dans la fonction vestibulaire et celle de l'audition (Popper & Lu 2000).

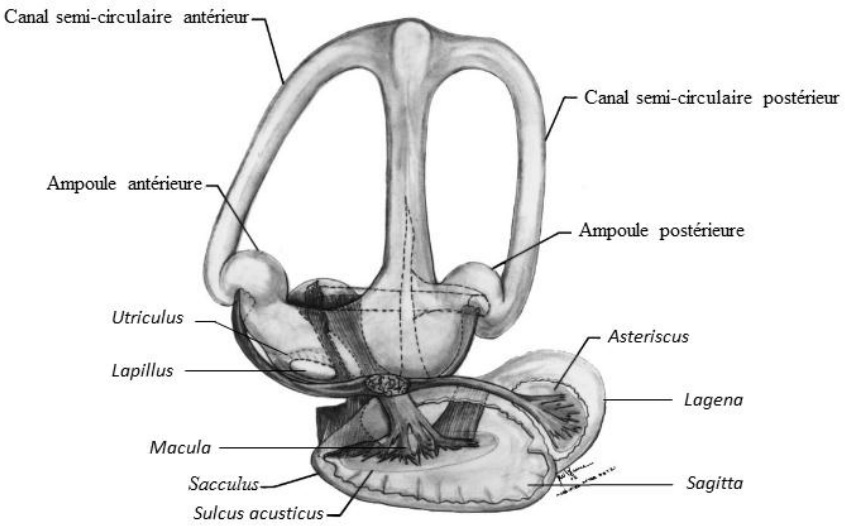

Figure 2 : Illustration de l'oreille interne du hareng (*Clupea harengus*). Redessinée d'après Retzius 1881.

L'otolithe des poissons appartient à un système de mécanoréception où ses changements de position sont détectés par la stimulation des cils apicaux des cellules sensorielles qui sont directement à son contact. La transmission de l'information au système nerveux se fait sous forme d'un message électrique traduisant les déformations des cils par l'otolithe. Les cellules sensorielles appartiennent au tissu sensoriel nommé la *macula* (Figure 2). Cette dernière est située au niveau du *sulcus acusticus* sur la face proximale de l'otolithe et

est reliée à l'otolithe par la membrane otolithique que traversent les cils sensoriels pour être au contact de l'otolithe (Figure 2). Ce système de mécanoréception est réceptif à une grande variété de stimuli sensoriels tels que l'oscillation liée à des fréquences auditives, la gravité, l'accélération et les vibrations. Les *sagittae* sont en général les otolithes les plus volumineux ; de ce fait, c'est la paire la plus analysée et étudiée. Ainsi, le mot otolithe désignera les otolithes sagittaux dans la suite de cette thèse.

Les propriétés uniques des otolithes

Contrairement aux mammifères, la taille et la morphologie des otolithes changent tout au long de la vie du poisson puisque leur formation est accrétionnelle, c'est-à-dire qu'elle se fait par dépôt de couches successives. Celles-ci sont constituées de carbonate de calcium principalement sous forme d'aragonite qui précipite sur une matrice organique principalement composée de protéines et de protéoglycans (Payan et al. 2004). Ce processus est appelé biominéralisation des otolithes. La succession de ces couches crée généralement des marques successives observables dans la structure de l'otolithe dont la rythmicité peut aller de l'infradien au circannuel, ce qui permet une réelle reconstruction temporelle de la vie de l'animal.

Comparativement aux homéothermes (mammifères, oiseaux, etc.), les patrons de croissance chez les ectothermes comme les poissons sont d'avantage soumis aux influences environnementales. De ce fait, la composition chimique de l'otolithe reflète l'environnement expérimenté par l'animal même si cette relation est très complexe. En effet, l'empreinte environnementale ne s'inscrit par directement dans la pièce calcifiée mais suit plutôt une série d'étapes successives de l'eau jusqu'à la formation l'otolithe. Les éléments et les ions présents dans l'eau et dans l'alimentation vont traverser plusieurs compartiments liquides (le plasma sanguin et l'endolymphe), ce qui implique la traversée de plusieurs barrières physiologiques

(système branchial de l'animal, paroi intestinale, etc.) et donc d'entrer dans des processus de régulation physiologique (e.g. osmorégulation) plus ou moins importants selon les éléments avant de précipiter pour former l'otolithe (Campana 1999). Ainsi, la formation de l'otolithe est soumise à l'influence de facteurs à la fois environnementaux et endogènes.

L'otolithe est métaboliquement inerte. Aucune réabsorption minérale et/ou dégradation n'est possible après la formation du biominéral contrairement aux autres pièces calcifiées du poisson, telles que les écailles et les vertèbres qui peuvent être des réservoirs de calcium.

Grâce à ces trois propriétés − la croissance acrétionnelle tout au long de la vie du poisson, la composition sous contrôle de facteurs endogènes, exogènes et ontogénétiques, et la non réabsorption et dégradation du biominéral −, l'ensemble de l'otolithe est considéré comme une véritable archive biologique individuelle révélatrice de l'historique de l'animal. Chaque couche offre un potentiel unique de reconstruction de la vie de l'animal et de son environnement au moment de son dépôt (Campana & Thorrold 2001). Ainsi, l'otolithe est utilisé comme outil et est exploité de différentes manières (structure, chimie, forme) afin d'extraire de nombreuses informations et ce à différentes échelles d'organisation biologique.

Structure de l'otolithe

En 1899, la découverte par Reibisch, des anneaux réguliers (série d'accroissements) formant la structure de l'otolithe a suscité une attention particulière qui s'est portée sur la rythmicité des marques d'accroissement successives de l'otolithe et l'estimation de l'âge de l'animal. Ainsi, différents types d'anneaux sont distingués selon leur rythmicité (Figure 3).

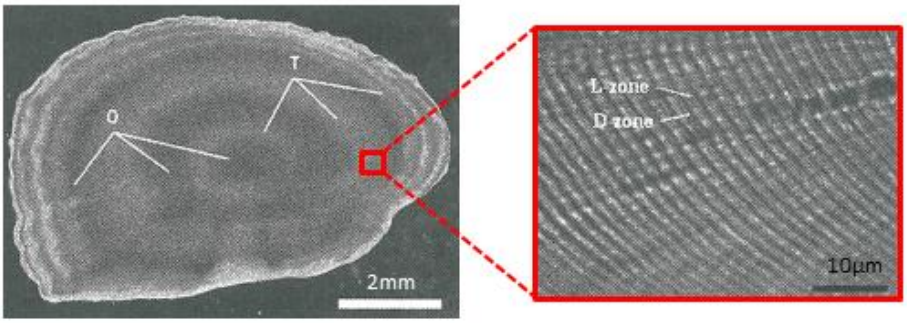

Figure 3 : Macrostructure d'un otolithe de Plie (*Pleuronectes platessa*) sous lumière transmise (à gauche) et une coupe transversale d'un otolithe de *Vinciguerria nimbaria* sous lumière transmise afin d'observer la microstructure de l'otolithe (à droite). Redessinée d'après Allemand et al. 2007.

Les accroissements primaires sont ceux qui possèdent une résolution journalière et sont formés par la succession de couches zone -D et zone -L autour du nucléus qui sont respectivement riche en matrice organique et riche en minéral. La microstructure de l'otolithe apparait donc comme une succession de zones sombres et claires sous lumière transmise (Figure 3 à droite). La macrostructure de l'otolithe a une résolution saisonnière et est caractérisée par l'alternance de zones opaques sous lumière transmise (O) plus riches en matière organique déposées en été, et translucides (T) déposées en hiver sous lumière transmise (Figure 3 à gauche). Elles diffèrent par leur largueur d'accroissement qui est plus importante pour les zones opaques que pour les zones translucides. La succession de deux anneaux saisonniers permet de définir les anneaux annuels. Enfin, des discontinuités structurales nommé « check » peuvent interrompre la succession régulière des anneaux. Ils correspondent à des stress variés que rencontre l'individu (Panfili et al. 2002), certains liés à des changements ontogénétiques extrêmes tels que la métamorphose ou la maturation sexuelle pour certaines espèces.

Ainsi, la gamme de rythmicité de l'infradien au circannuel que présente les micro- et macrostructure des otolithes est une aubaine pour estimer l'âge des poissons ainsi que leurs patrons de croissance. Ceci est d'autant plus intéressant que la structure en âge au sein des populations de poissons commerciaux est utilisée pour évaluer leurs dynamiques et que la

croissance individuelle est un trait d'histoire de vie important pour l'identification des stocks (Begg et al. 1999, Begg & Waldman 1999, Cadrin et al. 2005). Ces applications expliquent pourquoi l'estimation de ces paramètres, âge et croissance, ont longtemps dominé le champ des études portant sur l'otolithe, suivie par l'étude de sa chimie (Campana & Thorrold 2001).

Chimie de l'otolithe

Les otolithes sont composés d'une fraction minérale de carbonate de calcium $CaCO_3$ (généralement sous forme d'aragonite) et d'une fraction organique mineure représentant entre 0.1 à 10 % de l'otolithe (Carlström 1963, Degens et al. 1969). Ainsi, les otolithes sont très purs et composés majoritairement de calcium, de carbone et d'oxygène. Pourtant, l'analyse de la composition chimique de l'otolithe révèle la présence d'autres composés dont la concentration est mineure (>100 ppm) comme par exemple l'azote, le strontium, ou le potassium et d'autres à l'état de trace (<100 ppm) tels que le baryum, le magnésium, le fer, ou le cuivre. Tous les éléments proviennent principalement de l'eau et vont se retrouver piégés lors de la précipitation des carbonates de calcium (Panfili et al. 2002). De ce fait, la composition chimique de l'otolithe reflète celles des masses d'eau dans lesquelles le poisson a séjourné à condition de trouver la relation qui relie la concentration de l'élément dans l'eau à celle de l'otolithe. En effet, cette relation n'est pas directe puisque les éléments traversent plusieurs barrières physiques entre l'eau et l'endolymphe et subissent les effets de facteurs endogènes, ce qui peut brouiller, diminuer ou amplifier le signal.

Néanmoins, certains éléments vont pouvoir être utilisés comme traceurs naturels des individus. Par exemple, le rapport Sr/Ca des otolithes est un indicateur de la salinité de l'eau dans laquelle le poisson a séjourné. A l'aide de cette information, le comportement migratoire et les chemins de migration des espèces, particulièrement des espèces diadromes, peuvent être identifiés (Secor 1992). L'analyse du ratio des isotopes de l'oxygène ^{18}O et ^{16}O ($\delta^{18}O$) montre

une corrélation négative entre le $\delta^{18}O$ mesuré dans l'otolithe et la température de l'eau. En effet, une augmentation de la température engendre un enrichissement en isotope lourd et donc une diminution du $\delta^{18}O$ (Kalish 1991, Hoie et al. 2004). De plus, la concentration en oxygène précipité dans l'otolithe apparaît en équilibre avec celle l'eau. Ainsi, le $\delta^{18}O$ dans l'otolithe est un indicateur de la température de l'eau dans laquelle vit l'individu, et, de ce fait, un véritable outil pour reconstruire l'histoire environnementale de l'individu (Kalish 1991, Patterson et al. 1993). Le ratio des isotopes du carbone ^{13}C et ^{12}C ($\delta^{13}C$) dans le muscle blanc des poissons est quant à lui connu pour identifier les différentes sources d'alimentation des individus et donc indirectement les habitats dans lesquels ils vivent (à condition que les $\delta^{13}C$ des sources soient différents et connus). Une forte corrélation entre le $\delta^{13}C$ des acides aminés du muscle et celui des acides aminés de l'otolithe est observée (McMahon, Fogel, et al. 2011, McMahon, Berumen, et al. 2011). C'est pourquoi la composition protéique de la matrice organique des otolithes est un véritable traceur des sources de nourriture. Des combinaisons de concentrations de différents éléments dans l'otolithe peuvent aussi être utilisées comme une empreinte individuelle et donc comme marqueur biologique pour identifier les différentes zones de nourriceries d'une espèce ou d'une population (Campana et al. 1994, de Pontual et al. 2000). Il en est de même avec les ratios isotopiques de différents éléments tels que la combinaison du $\delta^{13}C$ et du $\delta^{18}O$ qui permet d'identifier les nourriceries mais aussi d'étudier leur connectivité (Rooker et al. 2008).

Forme des otolithes

La forme des otolithes est également un outil très utilisé en halieutique et ce à différents niveaux d'organisation biologique (Figure 4).

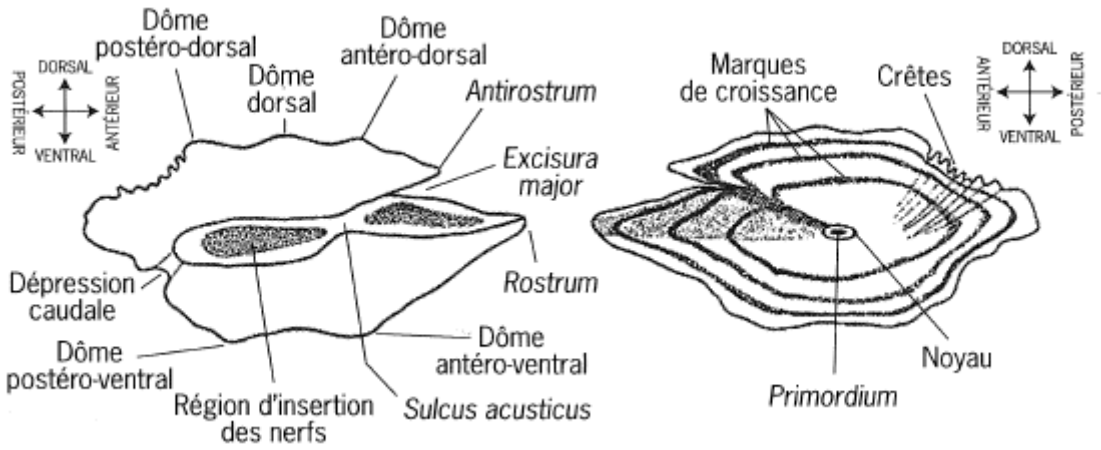

Figure 4 : Les différentes parties de la face proximale (à gauche) et de la face distale (à droite) d'une *sagitta* typique. Tirée de Panfili et al. 2002.

La forme des otolithes est tout d'abord spécifique. Son analyse permet donc l'identification des espèces (Figure 5), particulièrement pour celles appartenant à un même genre dont la variabilité morphologique du corps n'est pas suffisante pour les différencier (Tuset et al. 2006, Stransky & MacLellan 2011). Cette spécificité permet aussi l'identification des espèces lors de fouilles archéologiques dans des couches sédimentaires (paléontologie) ou encore lors de l'analyse de contenus stomacaux de mammifères, d'oiseaux ou de poissons piscivores (écologie trophique) (Boström et al. 2012). A l'échelle individuelle, la forme des otolithes peut être utilisée afin d'estimer l'âge des individus chez certaines espèces de poissons, ce qui permet d'étudier par la suite les traits d'histoire de vie individuels (âge à maturité, croissance) mais aussi à une l'échelle d'organisation biologique supérieure telle que la population (structure en âge) (Figure 5; Steward et al. 2009, Beyer & Szedlmayer 2010). Depuis ces vingt dernières années, l'analyse de la forme des otolithes est aussi un outil très utilisé en dynamique des populations. Grâce à l'analyse de la variabilité de forme des otolithes à l'échelle intra-spécifique, les populations de poissons peuvent être identifiées par le regroupement des individus dont la morphologie des otolithes est homogène suggérant une base génétique, un développement et un passé environnemental similaires. Ceci permet d'identifier les différentes populations locales (Mérigot et al. 2007), les différentes zones de

frai (Galley et al. 2006, Burke et al. 2008b) et principalement les différents stocks d'une espèce (Figure 5; Begg & Brown 2000, Bolles & Begg 2000, Cardinale et al. 2004, Turan 2006, Burke et al. 2008a, Stransky et al. 2008, McAdam et al. 2012, Cañás et al. 2012, ANNEXE I, ANNEXE II). Les résultats obtenus sont souvent comparés et/ou complétés par ceux provenant d'autres méthodes : marqueurs génétiques et parasitaires, microchimie et microstructure des otolithes, morphologie du poisson, ou analyse des traits d'histoire de vie (Cadrin et al. 2005). L'analyse de la forme présente l'avantage d'être une méthode peu coûteuse (Cadrin & Friedland 1999), moins chronophage et surtout non destructive, donc sans biais dû à la méthode de préparation.

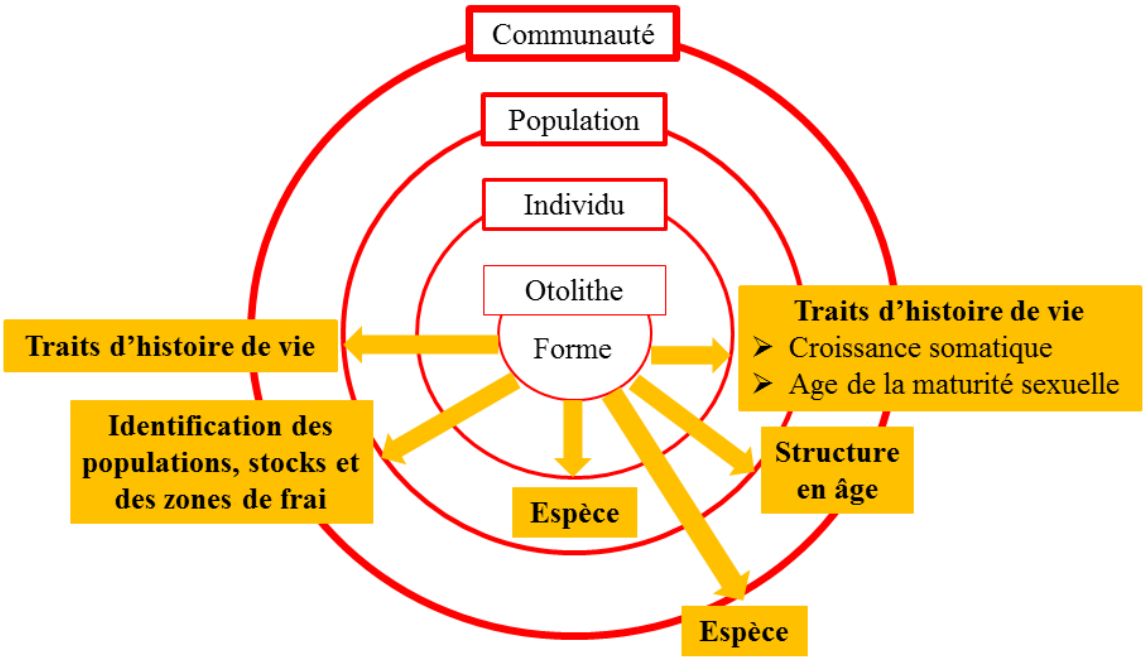

Figure 5 : Illustration des diverses applications basées sur la forme des otolithes aux différentes échelles d'organisation biologique. Redessinée d'après de Pontual, 2009.

L'utilisation rigoureuse et non biaisée de la morphologie des otolithes comme indicateur de diverses propriétés biologiques ou processus écologiques nécessite de comprendre les mécanismes sous-jacents à l'origine de la variabilité morphologique. En effet, la forme des otolithes résulte de l'action d'un ensemble de facteurs agissant simultanément

sur les processus de biominéralisation de l'otolithe. Il est donc nécessaire d'identifier les facteurs qui agissent comme sources de variabilité morphologique, d'évaluer leur importance relative et leurs modes d'action (localisation des effets et hypothèses sur les processus sous-jacents). Ceci est d'autant plus important à l'échelle intra-populationnelle où la variabilité morphologique des otolithes est très peu étudiée et pourrait être confondue avec celle aux échelles supérieures. Cette thèse s'inscrit dans une problématique d'identification et de quantification des sources et de compréhension des mécanismes de variabilité morphologique des otolithes à l'échelle intra-populationnelle.

Etat des connaissances sur la forme des otolithes

Biominéralisation des otolithes

L'étude des variations morphologiques des otolithes nécessite tout d'abord la compréhension de leur biominéralisation et plus particulièrement des mécanismes qui régissent la forme des otolithes. Le sac otique est un environnement clos délimité par l'épithélium sacculaire. Il est rempli d'endolymphe dans laquelle baigne l'otolithe. Ainsi, ce sont uniquement les composés de l'endolymphe qui interviennent dans la formation de l'otolithe, la biominéralisation des otolithes étant donc un processus acellulaire. La précipitation du carbonate de calcium est gouvernée par la réaction suivante :

$$Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+$$

Deux facteurs permettent la régulation du processus de calcification :

- le coefficient de saturation $Sa^2 = [Ca^{2+}]\ [HCO_3^-]/Ks$, où Ks est le produit de solubilité, doit être supérieur à 1 pour que la précipitation ait lieu ;
- le pH de l'endolymphe doit être plus alcalin (~8) que le pH sanguin : de ce fait, les ions H^+ produits lors de la formation du cristal doivent être éliminés afin d'éviter que la calcification s'arrête par acidose.

La composition et l'agencement des composés de l'endolymphe sont déterminés par les différentes cellules qui composent l'épithélium sacculaire. Celui-ci peut être subdivisé en quatre zones (Pisam et al. 1998) (Figure 6) :

- La *macula* contient les cellules sensorielles intervenant dans la fonction audio-vestibulaire de l'animal et des cellules de soutien dont le réticulum endoplasmique est très développé. Ceci leur confère une fonction sécrétrice, particulièrement de protéines collagènes, qui pourrait jouer un rôle dans la composition de l'endolymphe et la formation de l'otolithe et de sa membrane.
- La « meshwork area » se situe de part et d'autre de la macula. Elle est composée d'ionocytes de grande taille dont le cytoplasme renferme de nombreuses mitochondries et possèdent de nombreuses Na^+/K^+ ATPase. Cette zone est donc impliquée dans le transport actif des ions entre le plasma et l'endolymphe et dans le maintien des différences de concentration ionique entre ces deux compartiments.
- La « patches area » se situe au pôle opposé des 2 précédentes zones. Elle est composée d'ionocytes de petite taille qui sont aussi riches en mitochondries dont la fonction est la sécrétion de K^+. La concentration de cet ion est très importante dans l'endolymphe pour permettre la dépolarisation des cellules sensorielles.
- La zone intermédiaire se situe entre la « meshwork area » et la « patches area ».

Cette hétérogénéité spatiale des cellules au niveau de l'épithélium sacculaire crée une hétérogénéité spatiale de la concentration de certains composés dans l'endolymphe (Payan et al. 1999, Borelli et al. 2001). En effet, des gradients de type proximal-distal ont été mis en évidence au sein de l'endolymphe sacculaire notamment pour les éléments qui interviennent dans la formation de l'otolithe (Figure 6). Ces gradients régissent donc la formation de l'otolithe, ce qui explique sa forme convexe puisque les fortes concentrations des ions et des protéines intervenant dans la biomineralisation se trouvent dans la zone proximale de sorte

que l'otolithe croît d'avantage au niveau de sa face proximale qu'au niveau de sa face distale (Payan et al. 1999).

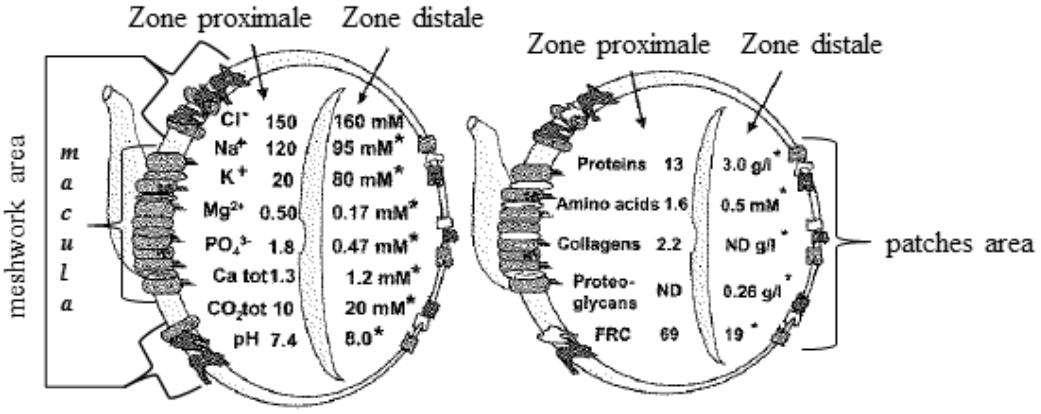

Figure 6 : Hétérogénéité spatiale des cellules de l'épithélium sacculaire et des composés ioniques (à gauche) et des composés organiques (à droite) de l'endolymphe. Les * indiquent des gradients de concentrations significatifs entre les zones proximale et distale. Redessinée d'après Allemand et al. 2007.

Des variations nycthémérales des concentrations des éléments dans la zone proximale ont également été mises en évidence (Edeyer et al. 2000, Borelli et al. 2003). Les composés organiques présentent une plus forte concentration pendant le jour contrairement aux ions dont la concentration est maximale pendant la nuit. Ceci soulève l'hypothèse d'une opposition de phase entre le dépôt des composés organiques de la matrice et la précipitation du carbonate de calcium. La matrice organique se forme à la fin de la journée lorsque les concentrations des précurseurs organiques dans l'endolymphe sont à leur maximum alors que la formation du cristal se réalise au début de la journée. Ce mécanisme serait responsable de l'observation des incréments primaires sur la microstructure de l'otolithe.

Le rôle de la matrice organique

Même si la matrice organique ne représente qu'une fraction mineure du biominéral, c'est-à-dire entre 0.1 à 10 % de l'otolithe (Carlström 1963, Degens et al. 1969), elle joue un rôle important qui s'étend du contrôle de la formation du *nucleus* − par sa cristallisation, son

orientation et sa morphologie − jusqu'au polymorphisme des unités de cristal − aragonite ou vatérite − qui composent l'otolithe (Nagasawa 2013). La matrice protéique est composée de 48 % de protéines, 23 % de collagène et 29 % de protéoglycanes (Borelli et al. 2001). Toutes ces molécules organiques sont formées à partir de précurseurs synthétisés par l'épithélium sacculaire. Seules quelques protéines sont à la fois présentes dans l'endolymphe et dans l'otolithe, ce qui suggère que les précurseurs protéiques sont modifiés durant la formation de la matrice organique (Borelli et al. 2001). Les différentes protéines connues retrouvées dans l'otolithe peuvent être regroupées en trois groupes selon leurs fonctions dans la biominéralisation de l'otolithe et leurs méthodes d'extraction (Figure 7).

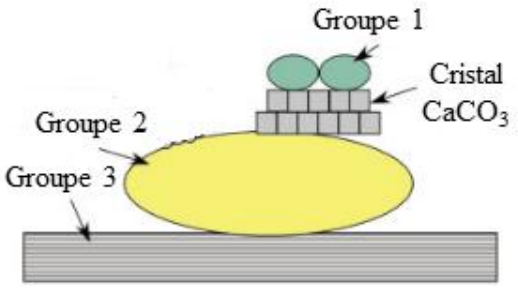

Figure 7 : Modèle de classification des molécules de la matrice organique des otolithes d'après Nagasawa, 2013.

Starmaker (Söllner et al. 2003) et l'OMM-64 (Otolith Matrix Macromolecule-64) (Tohse et al. 2009) sont des protéines solubles dans l'eau (et dans l'acide ou encore l'EDTA) intervenant dans le contrôle de la nucléation ainsi que dans le polymorphisme et l'orientation du carbonate de calcium grâce à une région riche en glutamate qui facilite l'interaction avec le cristal (groupe 2, Figure 7). L'OMP-1 (Otolith Matrix Protein-1) est une autre protéine soluble dans l'eau nécessaire dans le contrôle de la croissance de l'otolithe (groupe 1, Figure 7) et la déposition de l'otolin-1, protéine structurelle, appartenant à la famille des collagènes (groupe 3, Figure 7), qui intervient dans la stabilisation entre le minéral et les fractions

organiques de l'otolithe et assure la bonne disposition de l'otolithe sur l'épithélium sensoriel (Murayama et al. 2005).

Ainsi, la composition et la quantité des composés de la matrice organique sont à l'origine de la variabilité morphologique des otolithes et sont sous l'influence de facteurs endogènes et exogènes comme le sont les éléments ioniques.

Sources de variation de la forme

Parce que chaque espèce possède une forme d'otolithes qui lui est propre, la variabilité de la forme des otolithes reste bien plus élevée à l'échelle inter-spécifique qu'à l'échelle intra-spécifique, même entre espèces proches d'un point de vue taxonomique au sein d'un même genre ou d'une même famille par exemple (Monteiro et al. 2005). La morphologie des otolithes est donc déterminée génétiquement (Figure 8). Il n'en reste pas moins que la variabilité intra-spécifique observée entre populations est importante et résulte de l'action d'une somme de facteurs qui agissent simultanément. De ce fait, les conséquences de chacun de ces facteurs sur la variabilité sont peu connues et difficiles à appréhender (Cadrin & Friedland 1999). En effet, la variabilité génotypique entre individus n'est pas le seul facteur engendrant de la variabilité morphologique puisque l'existence d'une corrélation entre variabilité de forme des otolithes et variabilité environnementale a aussi été mise en évidence. L'influence de facteurs abiotiques tels que la température de l'eau (Bolles & Begg 2000) ou de facteurs biotiques tels que la quantité de nourriture (Gagliano & McCormick 2004, Hüssy 2008) a notamment été démontrée (Figure 8). C'est pourquoi l'analyse de la variation phénotypique de la forme des otolithes est si suscitée pour l'identification des stocks et des populations de poissons (Figure 8).

S'il existe des facteurs qui engendrent des variations morphologiques des otolithes, d'autres processus s'opposent à cette action dans le but de conserver le bon fonctionnement

de l'oreille interne. En effet, l'otolithe étant impliquée dans les processus d'audition et d'équilibre du poisson (Krysl et al. 2012), il est donc nécessaire que la variation morphologique engendrée par les différents facteurs décrits précédemment ne vienne pas impacter le bon fonctionnement de l'oreille interne et donc la bonne perception de l'environnement par le poisson. Les travaux de modélisation de Lychakov & Rebane (2005) ont suggéré que l'asymétrie de masse entre les otolithes droit et gauche doit être la plus minime possible et que sa valeur ne doit pas excéder 20% de différence afin d'éviter des incompatibilités et des mouvements incongrus engendrant des défaillances dans les fonctions audio-vestibulaires de l'animal. Il en est de même sur les premiers stades de vie des individus (post-larvaire et juvénile) où la trajectoire ontogénétique des individus et la forme des otolithes sont fortement uniformes et singulières (Capoccioni et al. 2011, Vignon 2012). L'hypothèse soulevée à propos de cette faible instabilité développementale est l'existence d'une contrainte biologique posée sur la fonction de l'otolithe dans l'oreille interne puisque certaines de ses protéines interviennent dans le contrôle de sa forme et que la composition de l'endolymphe permettant sa biominéralisation est régulée et sous contrôle génétique (Morales-Nin 2000, Borelli et al. 2003). Cette réduction de la flexibilité développementale d'un caractère, comme la morphologie de l'otolithe, est appelée canalisation (Waddington 1940) et est définie comme la capacité de produire un phénotype unique malgré la variabilité génétique ou environnementale rencontrée au cours de l'ontogenèse (West-Eberhard 2003).

Du fait de ces influences multifactorielles, un des principaux problème est de pouvoir identifier et quantifier les parts respectives des principaux facteurs dans la variation morphologique des otolithes (Begg & Brown 2000; Mérigot et al. 2007; Burke et al. 2008a). Vignon et Morat (2010) ont montré que la part génétique affecte directement la forme de l'otolithe et de manière localisée − notamment au niveau du rostre et de l'anti-rostre chez le vivaneau à raies bleus, *Lutjanus kasmira* − alors que l'environnement influence indirectement

sa forme globale au travers de la relation entre croissance somatique et croissance de l'otolithe. En effet, en affectant la croissance somatique des individus, l'environnement affecte également la croissance des otolithes et donc leur forme (Campana & Casselman 1993; Cardinale et al. 2004; Vignon & Morat 2010 ; Curin-osorio et al. 2012). En plus des facteurs génétiques et environnementaux, certains facteurs ontogénétiques, souvent mesurés par des variables d'état individuelles, sont aussi une source importante de variation de la forme de l'otolithe (Figure 8). En effet, celle-ci varie selon le sexe de l'individu (Bolles & Begg 2000), son âge (Mérigot et al. 2007), sa taille (Bolles & Begg 2000) mais aussi en fonction de ses trait d'histoire de vie tels que la vitesse de croissance de l'individu ou encore sa maturité sexuelle (Mérigot et al. 2007). Ces phénomènes ontogénétiques ne sont pas identiques sur toutes les parties de l'otolithe et affectent donc souvent la direction de ses axes de croissance. En effet, l'axe antéro-postérieur de l'otolithe se développe bien plus rapidement que l'axe dorso-ventral (Galley et al. 2006). Autrement dit, en vieillissant, l'otolithe du poisson s'allonge et devient plus elliptique. Selon la vitesse de croissance des individus, des changements plus minimes peuvent être détectés (Galley et al. 2006). Par exemple, chez l'anchois (*Engraulis encrasicolus*), les individus dont la croissance est plus lente possèdent des otolithes plus larges que ceux dont la croissance est plus rapide (Pecquerie et al. 2012). Il en est de même avec la maturité sexuelle de l'individu. L'arrivée de la maturité sexuelle influence la répartition de l'énergie entre la croissance, la reproduction et la maintenance et affecte donc le métabolisme. L'énergie allouée à la croissance somatique de l'individu diminue et par conséquent la croissance de l'otolithe et sa forme en sont impactées (Tuset et al. 2003; Mérigot et al. 2007).

Au-delà de sa variabilité inter-spécifique et inter-populations, la forme des otolithes varie également à l'échelle intra-populationnelle. Néanmoins, peu d'études de la variabilité morphologique des otolithes ont été conduites à cette échelle. Les principales concernent

l'asymétrie fluctuante (Palmer et al. 2010; Díaz-Gil et al. 2015), l'influence de l'habitat (Vignon 2012) et celle de la quantité de nourriture (Hüssy 2008) (Figure 8).

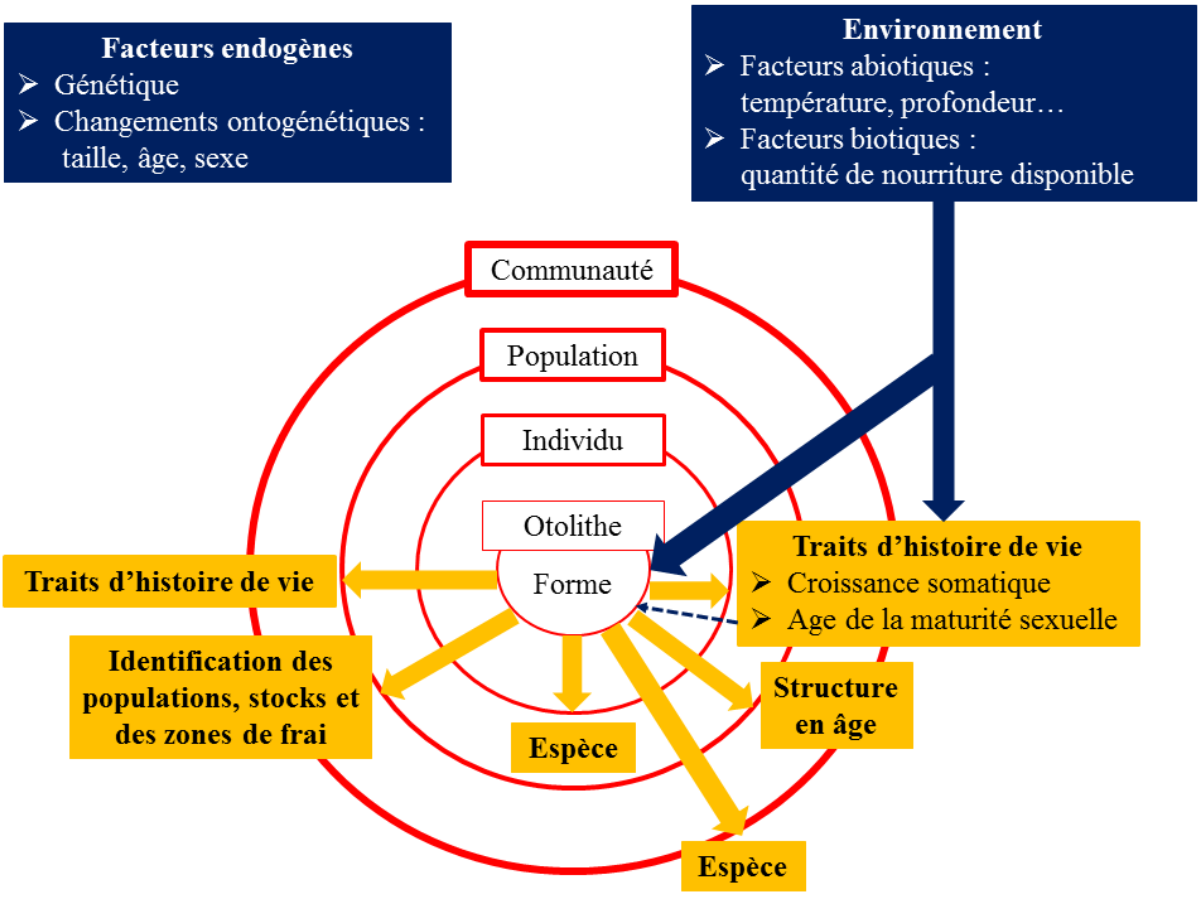

Figure 8 : Illustration regroupant à la fois les applications basées sur la forme des otolithes (en jaune) selon les échelles d'organisation biologique ainsi que les différentes sources de variation morphologiques connues des otolithes (en bleu). Redessinée d'après de Pontual (2009).

Des questions sans réponses

La variabilité intra-populationnelle de la morphologie des otolithes peut découler de variations inter-individuelles mais aussi intra-individuelles. Ces dernières ont été majoritairement étudiées en termes d'asymétrie fluctuante des otolithes (Palmer et al. 2010; Díaz-Gil et al. 2015). L'asymétrie fluctuante est mesurée comme la déviation aléatoire par rapport à la forme idéale des structures paires des organismes (telles que les otolithes) caractérisés par une symétrie bilatérale (Palmer 1994). Cette déviation représente l'instabilité du développement, c'est-à-dire l'inhabilité d'un organisme à produire un phénotype régulier

sous contraintes environnementale et/ou génétique. Cette mesure est devenue un outil très populaire pour examiner les effets du stress sur le développement des organismes à symétrie bilatérale (Palmer 1994). L'asymétrie fluctuante de la forme entre les otolithes droit et gauche est donc un outil qui peut permettre de mesurer cette instabilité développementale. Par exemple, elle peut être mesurée par des paramètres morphologiques tels que le diamètre ou le périmètre et est utilisée comme un indicateur de condition des larves d'anchois (Somarakis et al. 1997) mais aussi comme un indicateur de bonne santé d'un point de vue nutritionnel chez les larves de morue (*Gadus morhua*) (Grønkjær & Sand 2003).

Les espèces appartenant à l'ordre des Pleuronectiformes possèdent un corps asymétrique unique adapté à leur style de vie benthique. Ce comportement latéralisé est sous le contrôle d'une hormone thyroïdienne qui induit des changements vestibulaires (Schreiber 2006). En effet, pendant la métamorphose durant laquelle l'asymétrie s'établit, l'axe des yeux de la larve et l'axe des canaux semi-circulaires qui étaient parallèles au stade larvaire (et qui le reste pour les poissons ronds) deviennent perpendiculaires (Graf & Baker 1983). Cette métamorphose s'accompagne de plus d'une déformation de la boîte crânienne ainsi que de la migration d'un œil vers l'autre flanc due à une prolifération de cellules suborbitales (Bao et al. 2011). Ces bouleversements au cours de la métamorphose sont à l'origine d'une différence de biominéralisation des otolithes entre les oreilles internes droite et gauche. En effet, la vitesse d'accrétion du carbonate de calcium de l'otolithe est généralement plus rapide dans l'oreille interne située côté flanc aveugle que dans celle du flanc oculaire résultant en une asymétrie de masse entre les deux otolithes (Sogard 1991, Fischer & Thompson 2004, Helling et al. 2005). Cependant aucune étude ne s'est intéressée à la différence de forme des otolithes droit et gauche qui pourrait en découler d'un point de vue systématique. Les résultats précédents soulèvent pourtant la question de l'importance de la localisation de l'otolithe dans l'oreille interne droite ou gauche comme source potentielle de variation de forme des

otolithes. L'asymétrie de forme systématique des structures paires des organismes est appelée asymétrie directionnelle et pourrait être une source de variation intra-individuelle de la forme des otolithes qui s'ajoute à l'asymétrie fluctuante. Plus encore, la variation de forme engendrée par l'asymétrie directionnelle pourrait être confondue avec celle de l'asymétrie fluctuante et donc induire un biais dans la mesure de cette dernière.

La variabilité inter-individuelle de la morphologie des otolithes peut, comme la variabilité inter-populationnelle, découler de la variabilité génétique, ontogénétique et environnementale mais à l'échelle des individus d'une même population. Parmi les facteurs environnementaux, l'influence des facteurs biotiques reste relativement peu étudiée. Pourtant, il a été montré que la quantité de nourriture ingérée par l'animal peut affecter la forme de l'otolithe et donc constituer une source de variation morphologique inter-individuelle des otolithes. En effet, une étude en milieu contrôlé sur les jeunes morues a montré que plus la quantité de nourriture était importante plus le nombre et la taille des lobes secondaires des otolithes augmentait (Hüssy 2008). De plus, Gagliano et McCormick (2004) ont montré chez des poissons tropicaux récifaux que la forme des otolithes droit et gauche reflétait l'histoire alimentaire en termes de fréquence d'alimentation et de quantité ingérée des individus, ainsi que leur condition. La variation morphologique intra-populationnelle observée entre individus vivants dans différents habitats suite à un changement ontogénétique (Vignon 2012) pourrait être notamment en partie liée à un changement de régime alimentaire concomitant. De nombreuses études se sont intéressées aux conséquences de la nutrition du poisson à travers une restriction alimentaire ou une période de jeûne sur la croissance de l'otolithe (Mosegaard et al. 1988; Fablet et al. 2011), son opacité (Høie et al. 2008) et sur sa structure notamment sur l'apparition de faux anneaux appelés aussi 'check' (Fablet et al. 2011). Ainsi, seul l'effet de la quantité de nourriture a été étudié, ce qui soulève la question de l'importance de la composition du bol alimentaire sur la variabilité morphologique des otolithes. Cette hypothèse

est soutenue par le fait que la composition élémentaire des otolithes peut refléter celle des proies (Sanchez-Jerez et al. 2002, Buckel et al. 2004, Elsdon 2010). Ainsi un transfert trophique des éléments est possible, ce qui fait des otolithes un tissu d'accumulation des éléments (Thorrold et al. 1997). Etant donné l'influence de la quantité de nourriture sur la forme des otolithes et celle du régime alimentaire sur leur composition chimique, la question de l'importance du régime alimentaire de l'animal et particulièrement la composition du bol alimentaire dans la variabilité morphologique des otolithes peut être soulevée.

Méthodes quantitatives de description de la forme des otolithes

Afin d'étudier sa variabilité, il est nécessaire de décrire la forme des otolithes de manière quantitative. Il existe deux grands types de méthodes : les méthodes univariées et les méthodes multivariées. Les méthodes univariées reposent sur des paramètres morphologiques ou descripteurs tels que l'aire, le périmètre, la longueur – mesurée comme le plus grand axe de l'otolithe –, la largeur – le plus grand axe perpendiculaire à la longueur –, l'épaisseur et la masse. Ce sont des variables univariées, mesurées à partir d'une image de l'otolithe et de l'utilisation d'un logiciel d'analyses d'images excepté pour la masse et l'épaisseur pour lesquelles une balance et un pied à coulisse sont respectivement utilisés. Á partir de ces descripteurs morphologiques, des indices de forme peuvent être calculés. Ce sont également des variables univariées dont le but est de caractériser la forme de l'otolithe par une forme géométrique simple. Les otolithes peuvent donc être décrits comme des cercles ce qui donne l'indice de circularité, des rectangles d'où l'indice de rectangularité ou encore une ellipse d'où l'indice d'ellipsité. Les indices de forme étant construits à partir des mêmes paramètres morphologiques, un test de corrélation permet d'identifier les liens potentiels entre les indices et d'éviter ainsi des redondances (Mérigot et al. 2007).

Concernant les méthodes multivariées, les deux principalement utilisées sont l'analyse des ellipses de Fourier et la morphométrie géométrique (géomorphométrie) basée sur les points (semi-)homologues (« semi-landmarks » en anglais). Plus rarement, et c'est pourquoi leur principe ne sera pas exposé ici, la dimension fractale peut être aussi utilisée pour quantifier la complexité du contour de l'otolithe (Duarte-Neto et al. 2008) ou la géodésie pour caractériser localement la variation de forme (Benzinou et al. 2013). L'analyse des ellipses de Fourier en 2 dimensions permet de décrire le contour fermé de n'importe quel objet à partir d'ellipses (Kuhl & Giardina 1982). Le contour de l'otolithe est projeté dans un repère orthonormé arbitraire en 2 dimensions (x et y ; Figure 9A). Le résultat de cette projection est un système de 2 fonctions paramétriques x(t) et y(t), soit une fonction par axe (Figure 9B), qui peuvent être décrites par une somme de fonctions trigonométriques, ou harmoniques, correspondant à autant d'ellipses (Equation 1). Chaque harmonique *i* est elle-même décrite par deux sommes paramétriques d'une fonction cosinus et d'une fonction sinus (une somme par axe du repère orthonormé) dont les paramètres sont les descripteurs ou coefficients de Fourier. Ces fonctions sont données par :

$$\begin{cases} x(t) = \dfrac{A_0}{2} + \sum_{i=1}^{m} (A_i \cos(i\omega t) + B_i \sin(i\omega t)) \\ y(t) = \dfrac{C_0}{2} + \sum_{i=1}^{m} (C_i \cos(i\omega t) + D_i \sin(i\omega t)) \end{cases} \quad (1)$$

où :

- A_i, B_i, C_i, D_i sont les coefficients de Fourier de l'harmonique *i*.
- *t* est la distance de l'arc le long du contour mesurée à partir d'un point de départ arbitraire jusqu'au point considéré. t ϵ [0, T], T étant le périmètre du contour fermé (approximation par le polygone équivalent). T est aussi la période des fonctions x(t) et y(t), ce qui permet de définir la longueur d'onde $\omega=2\pi/T$.
- *m* est le nombre total d'harmoniques utilisées pour approximer x(t) et y(t).

- (A_0,C_0) sont les coordonnées du barycentre de l'otolithe.

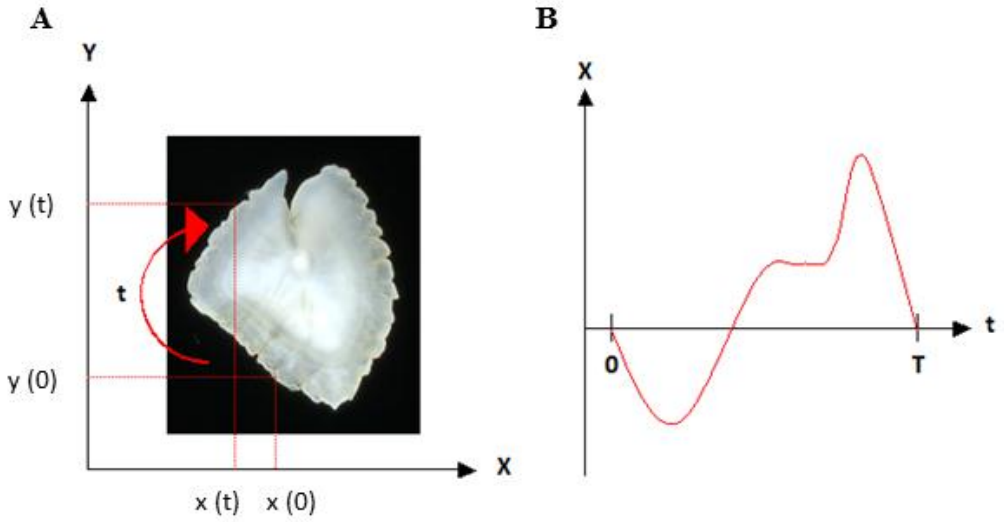

Figure 9 : Projection des points du contour d'un otolithe de grondin perlon (*Chelidonichthys lucerna*) pour l'obtention des descripteurs de Fourier. Projection des points du contour dans un repère en deux dimensions (A). Fonction x(t) obtenue après la projection des points du contour de l'otolithe sur l'axe des x (B).

Ainsi chaque harmonique est caractérisée par 4 descripteurs de Fourier. Une standardisation des descripteurs de Fourier par rapport à la première harmonique est réalisée. Les descripteurs standardisés sont alors invariants par rapport à la taille et l'orientation de l'otolithe ainsi que le point de départ choisi pour la projection du contour (Kuhl & Giardina 1982). Il en résulte la dégénération des trois premiers descripteurs de Fourier dans la description de la variabilité de forme puisqu'ils sont respectivement égaux à 1, ~ 0 and ~ 0. Les descripteurs standardisés de l'ensemble des harmoniques supérieures à 1 peuvent alors être utilisés comme variables descriptives de la forme de l'otolithe.

Plus le nombre d'harmoniques utilisées augmente, plus la précision du contour reconstitué est importante et ce jusqu'à un seuil correspondant à la reconstitution parfaite du contour de l'otolithe lorsque le nombre d'harmoniques tend vers l'infini. La puissance de Fourier cumulée (p_F) mesure la précision de la reconstruction obtenue avec un nombre d'harmoniques *m*. Elle correspond plus précisément à la somme cumulée de la proportion de

variance des coordonnées des points du contour que représente chaque harmonique et est calculée par l'équation suivante :

$$p_F(m) = \sum_{i=0}^{m} \frac{A_i^2 + B_i^2 + C_i^2 + D_i^2}{2}. \quad (2)$$

La puissance de Fourier cumulée est généralement utilisée pour déterminer le nombre d'harmoniques nécessaires pour reconstruire le contour de l'otolithe moyen (au sens moyenné sur l'ensemble de l'échantillon) de manière satisfaisante en fixant un pourcentage minimal de variance à expliquer (Figure 10). La même procédure peut être appliquée à chaque otolithe séparément plutôt qu'à l'otolithe moyen.

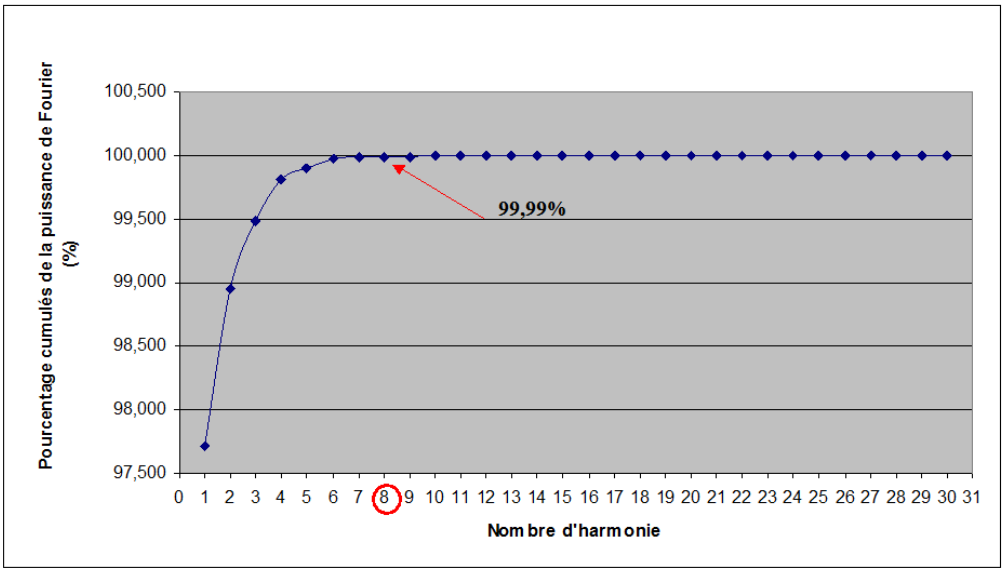

Figure 10 : Exemple de la représentation de la puissance de Fourier cumulée (%) pour déterminer le nombre d'harmoniques à utiliser, ici 8 harmoniques suffisent pour reconstituer 99.99 % de la forme de l'otolithe moyenné sur l'ensemble de l'échantillon.

La géomorphométrie basée sur des points homologues permet de résumer toute forme étudiée en un certain nombre de points de référence ou d'intérêt dits homologues en ce sens qu'ils peuvent être identifiés de manière non équivoque et se correspondent chez tous les individus. Les coordonnées des points homologues de l'ensemble des individus sont ensuite soumises à une analyse procrustéenne généralisée qui permet de réaliser une superposition de l'ensemble des points de l'échantillon au travers de 3 transformations − une transposition, une

rotation et une mise à l'échelle – en utilisant comme référence la forme moyenne dans l'échantillon. La minimisation des distances Procrustes entre individus, c'est-à-dire la racine carrée de la somme des distances au carré entre les paires de points homologues, est utilisée comme critère pour cette analyse (Figure 11). Les distances Procrustes résiduelles entre les individus après superposition sont appelés résidus de Procruste et représentent la variabilité de morphologique intrinsèque, i.e. non liée à la position, l'orientation ou la taille, au sein de l'échantillon. Les résidus de Procrustes peuvent alors être utilisés comme variables décrivant la variabilité morphologique.

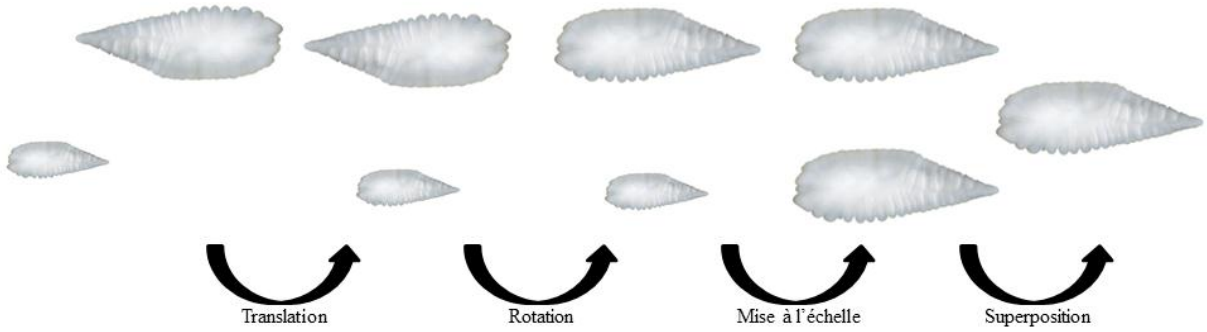

Figure 11 : Illustration des étapes réalisée lors d'une analyse procrusténne sur un otolithe de merlan (*Merlaengius merlangus*).

Cependant, un problème spécifique aux otolithes est que le nombre de caractéristiques morphologiques précises pouvant jouer le rôle de points homologues présents sur le contour de l'otolithe est souvent insuffisant pour décrire la forme et surtout les courbes caractéristiques. C'est pourquoi la méthode des points semi-homologues (« semi-landmarks ») est privilégiée. Les points semi-homologues sont obtenus par construction géométriques à partir des points homologues et ne correspondent donc pas à des caractéristiques morphologiques particulières. Un nombre arbitraire de points semi-homologues sont généralement positionnés de manière équidistante sur le contour des otolithes à partir d'un point homologue situé sur le rostre. L'utilisation de points semi-homologues ajoute une étape par rapport à l'analyse procrustéenne généralisée classique à partir des points homologues.

Durant cette étape, les points semi-homologues sont glissés le long de la courbe de l'otolithe jusqu'à ce qu'ils correspondent le plus possible à leurs homologues le long de l'otolithe de référence et donc jusqu'à ce que ces paires de points deviennent le plus homologues possible. Cette étape peut être réalisée sur la base de deux critères de minimisation différents : soit la minimisation des distances Procrustes entre les paires de points semi-homologues, soit la minimisation de l'énergie de déformation (bending energy) nécessaire pour produire le changement de courbe nécessaire pour reproduire la courbe de référence.

Le choix entre ces deux méthodes multivariées dépend généralement de la question posée. Une étude modélisant les trajectoires de croissance chez le sar à museau pointu (*Diplodus puntazzo*) a montré une grande similarité entre les résultats obtenus par la géomorphométrie et ceux obtenus par l'analyse de Fourier (Loy et al. 2000). L'analyse de Fourier permettrait d'obtenir plus d'informations sur la variabilité morphologique puisqu'elle est basée sur tout le contour de l'objet, ce qui permet une description et une reconstruction des formes plus précise. La principale limitation de cette méthode est le nombre important de descripteurs de Fourier nécessaires. L'avantage de la géomorphométrie est la facilité avec laquelle elle permet de localiser des effets grâce à la construction de grilles de déformation entre paires de formes. Quelle que soit la méthode employée, le choix du nombre de points homologues ou d'harmoniques est important car la précision de la description de la forme et donc les résultats peuvent en dépendre.

Le choix entre l'utilisation de méthodes uni- ou multivariées dépend également de la question scientifique posée. Les descripteurs morphologiques et les indices de forme permettent surtout de différencier les traits d'histoire de vie tels que la croissance, c'est-à-dire des processus liés à la physiologie du poisson puisque ces méthodes ont tendance à résumer l'information biologique. L'avantage de l'utilisation des méthodes multivariées est de décrire plus précisément les variations de forme, particulièrement celles résultants d'effets à de plus

petites échelles telles que celles générées par les effets environnementaux (Mérigot et al. 2007). L'avantage de l'utilisation des méthodes univariées réside essentiellement dans la facilité de comparaison des différences observées mais aussi de leur interprétation biologique (Ponton 2006), contrairement aux méthodes multivariées où les différences observées sont moins intuitives et biologiquement plus difficiles à interpréter (Cadrin & Friedland 1999; Tuset et al. 2006). Néanmoins, le couplage de méthodes à la fois univariées et multivariées pour l'identification des stocks permet d'augmenter le pouvoir discriminant des analyses (Casselman et al. 1981; Galley et al. 2006; Canas et al. 2012).

Objectifs et plan du mémoire

En résumé, du fait de sa variabilité, la forme des otolithes est utilisée comme indicateur naturel biologique et ce à plusieurs niveaux d'organisation biologique. Cependant, cette variabilité résulte de l'interaction de nombreux facteurs notamment génétiques, ontogénétiques (état individuel) et environnementaux (biotiques et abiotiques) qui agissent sur la biominéralisation et donc la forme. L'utilisation robuste et non biaisée de la forme des otolithes comme indicateur requiert donc d'identifier, de dénouer, de quantifier et de localiser l'effet des différents facteurs qui impactent leur biominéralisation et engendrent de la variabilité morphologique. Il convient de noter que les facteurs impliqués et/ou l'importance relative de leurs effets diffèrent selon l'échelle à laquelle est observée la variabilité de forme. La variabilité de forme à l'échelle intra-populationnelle reste très peu étudiée. Il est cependant important de l'appréhender et d'en comprendre les sources puisque qu'elle pourrait être confondue ou noyée dans la variabilité aux échelles supérieures et de ce fait biaiser l'utilisation de la forme de l'otolithe comme outil discriminant à ces échelles. Au-delà de cet aspect appliqué, la forme de l'otolithe peut être considérée comme un proxy de sa biominéralisation puisque la matrice organique et le gradient proximal-distal des composés de

l'endolymphe sont responsables de la variabilité morphologique des otolithes. De ce fait, étudier les facteurs qui influencent la forme des otolithes à l'échelle intra-populationnelle permet d'identifier les facteurs qui influencent leur biominéralisation, chose que les études aux échelles supérieures ne permettent pas ou indirectement seulement.

Cette thèse se propose donc d'analyser deux sources potentielles de variation morphologique à l'échelle intra-populationnelle, l'asymétrie directionnelle et le régime alimentaire, tout en prenant en compte les autres sources de variations déjà connues dans les travaux (Figure 12). Dans le cas où les deux facteurs étudiés agissent significativement sur la variabilité de forme des otolithes, leurs effets sont localisés sur la morphologie de l'otolithe, mais aussi démêlés des autres facteurs et quantifiés de manière relative.

Le **Chapitre 1** présente une étude de l'asymétrie directionnelle comme source de variation intra-individuelle de la forme des otolithes (Figure 12). La question posée est de savoir si le côté d'origine de l'otolithe (cavité otique droite ou gauche) est un facteur de variation de sa forme. En effet, une asymétrie directionnelle des otolithes a déjà été mise en évidence chez les pleuronectiformes en termes de masse (Sogard 1991, Fischer & Thompson 2004, Helling et al. 2005) et de composition isotopique et élémentaire (Loher et al. 2008, Kajajian et al. 2014). Puisque l'asymétrie fluctuante de la forme des otolithes est étudiée comme indicateur de bonne santé de l'individu ou de stress, il est nécessaire de dénouer la part respective de variabilité de forme liée à ces deux types d'asymétrie afin d'éviter qu'elles ne soient confondues ce qui pourrait engendrer des mesures biaisées de l'asymétrie fluctuante. L'étude a été réalisée à la fois chez 4 espèces de poissons marins plats (1 sénestre et 3 dextres) et de 4 espèces de poissons marins ronds. La forme des otolithes, décrite par analyse de Fourrier, et leur longueur selon l'axe antéro-postérieur ont été modélisées statistiquement en fonction de leur localisation dans la cavité otique droite ou gauche tout en prenant en compte les effets ontogénétiques, i.e. âge, effet allométrique de la taille corporelle et

dimorphisme sexuel. Pour chacune des espèces étudiées, l'asymétrie directionnelle de la forme des otolithes a été quantifiée et illustrée au moyen de reconstructions de formes et de longueurs moyennes.

Les **Chapitres 2 et 3** présentent deux études complémentaires s'intéressant à l'existence potentielle d'une corrélation entre le régime alimentaire des poissons, particulièrement sa composition, et la variabilité de forme des otolithes (Figure 12). De nombreuses études ont montré que la quantité de nourriture affectait la croissance de l'otolithe (Mosegaard et al. 1988; Fablet et al. 2011), son opacité (Høie et al. 2008), sa structure (Fablet et al. 2011) et sa forme (Hüssy 2008) . De plus, la composition élémentaire des otolithes des poissons reflète en partie celle de leurs proies et, donc, la composition de leur régime alimentaire (Sanchez-Jerez et al. 2002, Buckel et al. 2004, Elsdon 2010). Au-delà de la quantité de nourriture, la question de l'importance de la composition du bol alimentaire pour la variabilité morphologique des otolithes se pose. Le but du **Chapitre 2** est d'analyser tout d'abord la corrélation entre le régime alimentaire et la forme des otolithes à l'échelle intra-populationnelle. Cette étude a été réalisée *in situ* chez cinq espèces de poissons d'intérêt commercial en Manche Orientale. Au-delà de l'effet du régime global, cette partie s'attache également à identifier les catégories de proies affectant la morphologie des otolithes, décrite par analyse de Fourier, puis à estimer la contribution respective de la composition et de la quantité du bol alimentaire à la variabilité morphologique. Comme pour le chapitre précédent, les effets ontogénétiques (au travers des variables d'état individuelles) et de l'environnement abiotique ont été pris en compte dans les modèles statistiques. Des reconstructions de forme à partir des prédictions de ces modèles ont permis de localiser les effets de ces deux facteurs sur la forme des otolithes.

Enfin le **Chapitre 3** vise à compléter l'étude *in situ* de l'influence de la composition du régime alimentaire sur la morphologie des otolithes du chapitre précédent, par une étude

expérimentale en milieu contrôlé. A partir de marquages de l'otolithe à l'oxytetracycline (OTC), la croissance et la morphogénèse des otolithes de bar commun (*Dicentrarchus labrax)* aux stades larvaires et juvéniles ont été étudiées pour des régimes alimentaires variant en termes de concentration en acides gras polyinsaturés essentiels. Le choix de ce régime provient du fait que les acides gras polyinsaturés sont connus pour intervenir dans la croissance, le développement du système nerveux et la survie des larves de poissons (Sargent et al. 1999). De plus, certains d'entre eux sont essentiels, c'est à dire qu'ils ne peuvent être synthétisés par les poissons qui doivent donc se les procurer par la nourriture. Or il s'avère que, selon certains auteurs, le changement climatique pourrait diminuer la croissance des cellules phytoplanctoniques qui alimentent le réseau tropique en acides gras essentiels, telles les diatomées, et/ou réduire la concentration d'acides gras essentiels au sein de ces mêmes cellules (Gómez & Souissi 2008, Pahl et al. 2010, Chen 2012). Une fois de plus, l'influence des effets ontogénétiques a été prise en compte et les effets sur la forme des otolithes et leur trajectoire morphogénétique ont été localisés en utilisant une approche de morphométrie géométrique.

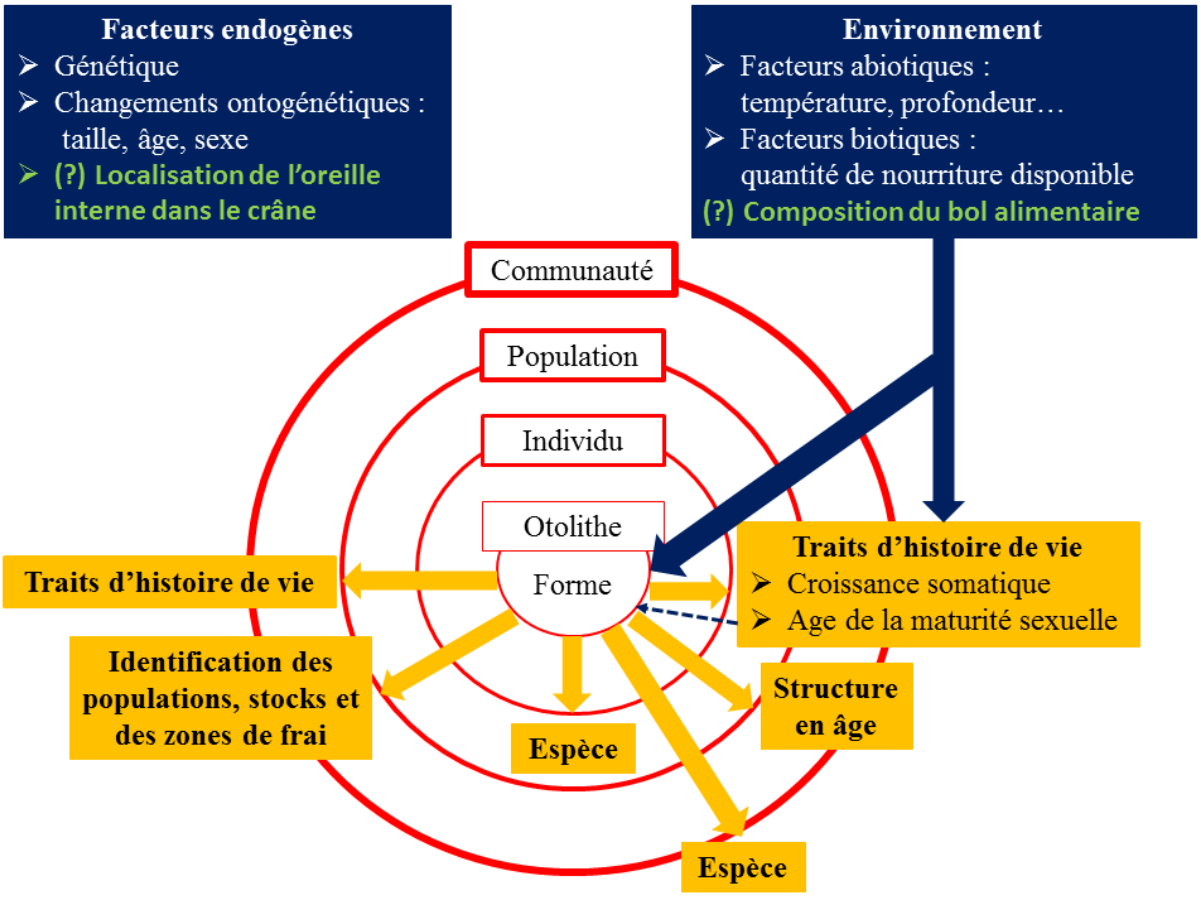

Figure 12 : Illustration regroupant à la fois les applications basées sur la forme des otolithes (en jaune) selon les échelles d'organisation biologique ainsi que les différentes sources de variation morphologiques connues des otolithes (en bleu) regroupées avec celles étudiées au travers de cette thèse (en vert). Redessinée d'après de Pontual (2009).

Chapitre 1
Sagittal otolith morphogenesis asymmetry in marine fishes

Valorisation de ce chapitre:

- Publication :

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- Colloque National de Sclérochronologie « Structures dures ou calcifiées chez les organismes aquatiques : leur utilisation en écologie halieutique » en Juillet 2013 à Rennes.
- Journées de la société française d'ichtyologie en Avril 2014.
- The 8th French Symposium *"Morphometrics and Evolution of Shape" en Juin 2014.*
- Journée des étudiants de l'IFREMER Centre Manche Mer du Nord en Juin 2013 et 2014

ABSTRACT

This study investigated and compared asymmetry in sagittal otolith shape and length between left and right inner ears in 4 roundfish and 4 flatfish species of commercial interest. For each species, the effect of ontogenetic changes (individuals' age and/or total body length), sexual dimorphism (individuals' sex) and the side of the head of the otolith's location on the shape and length of paired otoliths (between 143 and 702 pairs according to species) were evaluated. Ontogenetic changes in otolith shape and length were observed for all species. Sexual dimorphism, either in otolith shape and length or in their ontogenetic changes, was detected for half of the species, be they round or flat. Significant directional asymmetry in otolith shape and length was detected in one roundfish species each, but its inconsistency across species and its small average amplitude (6.17 % for shape and 1.99 % for length) suggested that it has barely any biological relevance. Significant directional asymmetry in otolith shape and length was found for all flatfish species except otolith length for one species. Its average amplitude varied between 2.06 and 17.50 % for shape and between 0 and 11.83 % for length and increased significantly throughout ontogeny for two species, one dextral and one sinistral. The longer (length) and rounder otolith (shape) appeared to be always on the blind-side whatever the species. These results suggest differential biomineralization between the blind and ocular inner ears in flatfish species that could result from perturbations of the proximal-distal gradient of otolith precursors in the endolymph and/or the otolith position relative to the geometry of the saccular epithelium due to body morphology asymmetry and lateralized behavior. The fact that asymmetry never exceeded 18% even at the individual level suggests an evolutionary canalization of otolith shape symmetry to avoid negative effects on fish audition and balance. Technically, asymmetry should be accounted for in future studies based on otolith shape.

Key words: otolith shape analysis, interspecific, symmetry, marine species

RESUME

L'asymétrie de forme entre les otolithes droit et gauche d'un individu a été analysée chez 8 espèces de poissons marins d'intérêt commercial. Les effets ontogénétiques, représentés par la longueur totale et l'âge du poisson, le dimorphisme sexuel et la localisation de l'oreille interne dans le crâne ont été évalués sur des paires d'otolithes, prélevées chez 4 espèces poissons ronds et 4 espèces de poissons plats, comme source potentielle de variation de la forme des otolithes et de leurs longueurs. Des changements ontogénétiques et du dimorphisme sexuel sur la forme et/ou la longueur des otolithes ont été détectés significatifs respectivement chez toutes les espèces étudiées et chez la moitié d'entre elles (poissons ronds et poissons plats).

Une asymétrie directionnelle sur la forme des otolithes et sur leurs longueurs a été détectée significative chez une espèce de poisson rond mais la faible amplitude de son effet ne suggère aucune pertinence biologique contrairement à celles observées chez les espèces de poissons plats. En effet, une asymétrie directionnelle significative dont l'amplitude moyenne de l'effet variait entre 2.06 et 17.50 % de différence entre la forme de l'otolithe droit et gauche, a été mise en évidence chez toutes les espèces de poissons plats. De plus, une augmentation de cet effet au cours de l'ontogénie a été observée chez deux espèces ; une espèce dextre et une espèce sénestre. En ce qui concerne l'asymétrie de longueur des otolithes, des résultats similaires ont été observés puisqu'une asymétrie directionnelle significative a été détectée chez toutes les espèces de poissons plats à l'exception d'une. L'amplitude de son effet variait entre 0 et 11.83 % et une augmentation de cette différence avec le développement ontogénétique de l'individu a été aussi observée chez les 2 mêmes espèces que pour l'asymétrie de forme. L'otolithe le plus large et le plus long était toujours situé dans l'oreille interne du flanc aveugle du poisson suggérant une biominéralisation différentielle entre les deux otosacs. Cette différence de biominéralisation entre l'otolithe droit et l'otolithe gauche résulte probablement de différences entre les gradients proximal-distal des précurseurs de l'otolithe situés dans l'endolymphe survenant après la métamorphose responsable de l'asymétrie du corps de l'animal et de son comportement latéralisé. Du fait que l'otolithe soit un composant important au système de mécanoréception de l'oreille interne, l'asymétrie de forme entre les deux oreilles n'excède jamais plus de 18 % de différence soulevant l'hypothèse de l'existence d'une pression de sélection pour le bon fonctionnent du système audio-vestibulaire de l'animal. D'un point de vue technique, l'asymétrie de forme des otolithes doit être prise en compte dans les études qui portent sur la forme des otolithes.

Mots-clés : Analyse de forme des otolithes, étude interspécifique, symétrie, espèces marines

Introduction

Sagittae are one of the three otolith pairs found in Teleosteans' inner ears. They are calcified structures involved in audition and balance systems. They grow by accretion, i.e. deposition of successive calcium carbonate layers on an organic matrix, which allows recording life-history events over the fish lifetime. Contrary to bones and scales, they are metabolically inert such that any material deposited remains unaltered and cannot be resorbed (Campana & Neilson 1985). Due to these two properties, otoliths are veritable black boxes (Lecomte-Finiger 1999) containing reliable fingerprints that are considered as an invaluable source of information for reconstructing a fish's entire life-cycle (Campana & Thorrold 2001). In the last two decades, an increasing number of studies have used otolith shape as an indicator to discriminate fish stocks and populations (Bolles & Begg 2000, Mérigot et al. 2007, Stransky, Murta, et al. 2008). Shape analyses were initially based on comparison of linear distances (e.g. length, width) and, more recently, on geometric outline methods of shape variation (Cadrin et al. 2013). Using otolith shape as a discriminating tool requires the identification and the understanding of factors that affect otolith morphology and could thus act as confounding factors. Indeed, otolith biomineralization and, thus morphogenesis results from multi-causal processes due to the interaction of many internal (physiological) and external (environmental) factors. Consequently, *sagittae* are characterized by high morphological variability which is under a dual regulation (Vignon & Morat 2010). On the one hand, otolith shape is genetically determined (L'Abée-Lund 1988) and is highly species-specific and thus useful in revealing phylogenetic relationships between species (Lombarte & Lleonart 1993). On the other hand, environmental factors act on metabolism that, in turn, affects somatic growth and consequently the quantity of material deposited on otoliths (Cardinale et al. 2004, Galley et al. 2006, Stransky, Baumann, et al. 2008). Individual parameters also affect otolith morphology. Otolith shape changes according to ontogenetic

stage as represented by size (Hüssy 2008), age (Castonguay et al. 1991) or sexual maturity status (Mérigot et al. 2007). It also varies with sex (Castonguay et al. 1991, Bolles & Begg 2000).

Over the years, most studies investigating the sources of otolith shape variation have dealt with the influence of external factors, such as environmental conditions, or individual characteristics, such as individuals' genotype or state. However, to our knowledge, no study has systematically investigated a potential intra-individual source of variation, namely otolith shape difference between the right and left inner ears (referred to as otolith location side hereafter). From a structural point of view, the two parts of the vestibular system are strictly similar with 3 orthogonal semicircular canals that allow detecting angular accelerations and 3 otolithic organs dedicated to audition and balance (Panfili et al. 2002). Although there are some interspecific differences in the size and shape of these elements, otoliths are bilaterally symmetrical in roundfishes (Popper & Lu 2000). One can however question otolith bilateral symmetry in flatfishes as these species undergo deep asymmetric morphological changes during their metamorphosis including a cranial deformation and the migration of one eye to the other side caused by cell proliferation in suborbital tissue (Bao et al. 2011). Some consequences are observed such as the change of eye shape during metamorphosis (Li et al. 2013). It is known that some adaptive changes in the vestibulo-ocular reflex system exist to cope up with the change of orientation between the axis of flatfish eyes and the horizontal semicircular canals of their inner ear that become oriented perpendicular to each other during metamorphosis (Graf & Baker 1983). It has been shown that this behavior lateralization affects otolith biomineralization. Carbonate accretion rates are skewed between left and right inner ears in flatfishes as otoliths from the blind side grow generally faster thus resulting in an asymmetry of otolith mass (Sogard 1991, Fischer & Thompson 2004, Helling et al. 2005). In addition, an asymmetry in carbon and oxygen isotopic ratios, as well as, in some trace metal

elements between right and left otoliths has been observed in some flatfish species (*Hippoglossus stenolepis* by Loher et al. 2008, *Paralichthys dentatus* by Kajajian et al. 2014). These observations suggest that biomineralization is affected by flatfish asymmetric morphology and lateralized behavior, which may induce asymmetry in otolith shape.

The objective of the present study is to investigate whether morphogenesis patterns of left and right sagittal otoliths (referred to as otoliths thereafter) are similar in a series of species, using their shape as a proxy for biomineralization. An original approach relying on an inter-specific comparison between several roundfish and flatfish species is reported in this study. Additionally, both sinistral and dextral flatfish species were selected in order to assess the influence of the ocular side. The asymmetry in otolith length and shape described by Elliptic Fourier Descriptors (EFDs) were tested. In order to avoid confounding effects, sources of variation related to individual state, namely sex and ontogenetic stage (represented by age and body size), were accounted for. The latter allowed an assessment of whether right and left otolith lengths and shapes diverged along development.

Materials and methods

Sample collection

Four roundfish and four flatfish species of commercial interest were sampled (Table I). Roundfishes belonged to three different families: *Gadidae* represented by whiting *Merlangius merlangus* (Linnaeus 1758; 264 individuals) and haddock *Melanogrammus aeglefinus* (Linnaeus 1758; 142 individuals), *Clupeidae* with herring *Clupea harengus* (Linnaeus 1758; 204 individuals), and *Mullidae* represented by red mullet *Mullus barbatus barbatus* (Linnaeus 1758; 279 individuals). Flatfishes were selected according to their ocular side: three dextral species, 702 European plaice *Pleuronectes platessa* (Linnaeus 1758), 361 common dab *Limanda limanda* (Linnaeus 1758), and 209 common sole *Solea solea* (Linnaeus

1758); and one sinistral species, megrim *Lepidorhombus whiffiagonis* (Walbaum 1762) with 151 otolith pairs sampled. A total of 2 313 fish were captured in 2013 by bottom trawling. Depending on species, individuals were sampled either during scientific surveys (International Bottom Trawl Survey in the North Sea, IBTS; MEDiterranean International bottom Trawl Survey in the Gulf of Lions, MEDITS; and the French *Nephrops* Survey in the Bay of Biscay, LANGOLF) or from commercial landings of vessels fishing in the eastern English Channel in Boulogne-sur-Mer (France)**.** For a given species, samples came from the same eco-region in order to limit the environmental impact on otolith shape variation. Following sampling, individuals' total length (L) was measured to the nearest centimeter and their sex was determined by gonad observation. Sagittal otolith pairs were removed from each individual to estimate its age by interpreting macrostructure and were further used for shape analysis.

Table I: Roundfish and flatfish samples. The number of individuals collected and separated between females and males are given for each species together with indicators of the age and total length distributions in the sample (mean ± standard deviation and minimum-maximum range). The location (General Fisheries Commission for the Mediterranean, GFCM, or International Council for the Exploration of the Sea, ICES, divisions), month and origin (survey or market) of capture are also given.

Species	Number (Females/Males)	Age (years) Mean± SD (Min-Max)	Total length (cm) Mean± SD (Min-Max)	ICES & GFCM divisions	Catch month	Survey & market
M. aeglefinus	143 (58/85)	3,78 ± 1.92 (1 - 8)	33.72 ± 6.87 (17 - 46)	VIId & IVc	January	IBTS
M. merlangus	264 (141/123)	3 ± 1.60 (1 - 7)	26.74 ± 6.39 (10 - 44)	VIId & IVc	January	IBTS
M. barbatus barbatus	279 (140/139)	1.51 ± 0.71 (1 - 3)	14.75 ± 3.62 (9 - 24)	37.1.2	June	MEDITS
C. harengus	204 (115/89)	2.51 ± 1.43 (1 - 7)	20.50 ± 4.43 (12 - 31)	VIId & IVc	January	IBTS
P. platessa	702 (324/378)	3.88 ± 1.22 (1 - 8)	27.15 ± 5.94 (10 - 49)	VIId & IVc	January	IBTS
L. limanda	361 (261/100)	4.29 ± 1.42 (2 - 8)	21.73 ± 4.80 (10 - 37)	VIId & IVc	January	IBTS
S. solea	209 (200/9)	4.58 ± 1.25 (3 - 8)	29.81 ± 3.60 (24 - 42)	VIId	April	market in Boulogne sur Mer
L. whiffiagonis	151 (117/34)	3.54 ± 1.73 (1 - 7)	30.17 ± 9.08 (14 - 48)	VIIIa & VIIIb	May	LANGOLF

Otolith shape analysis

In the following, otolith shape analysis and statistical analyses are described as they were conducted for each species separately.

Each otolith was cleaned by an ultrasonic bath with water at room temperature during 10 minutes, then brushed to remove residual tissues and stored dry in tubes. Batches of otoliths were automatically digitized at a high resolution (3200 dpi) using a scanner EPSON V750 and individual images were extracted using the software TNPC 7.0 (www.tnpc.fr). In order to compare left and right otolith shapes, a mirror image of the left otoliths was used. The length of each otolith (O_{L}), defined as the largest distance along the antero-posterior axis, was measured on its numerical image.

An elliptical Fourier series analysis was performed on each otolith contour delineated and extracted by image binarization. This method reconstructs any type of shape with a closed two-dimensional contour (Kuhl & Giardina 1982). In this case, each point of the otolith contour was projected on the axes of a two-dimensional coordinate system, and the projection was reconstructed using ellipses named harmonics described by a couple of trigonometric equations (one per axis). These equations describe projected contour coordinates as a combination of cosine and sine waves that in turn represent bends in the otolith contour. Each harmonic (H_{i}) was characterized by 4 coefficients (A_{i}, B_{i}, C_{i} and D_i) corresponding to the parameters of the trigonometric equations (2 per equation, 1 for cosine and 1 for sine waves), the so-called Elliptic Fourier Descriptors (*EFD*s). The number of harmonics n used to reconstruct each otolith outline in the sample was determined as follows. The cumulated Fourier power ($P_{\mathrm{F}}(n_k)$) was calculated for each individual otolith k as the sum of the proportion of variance in contour coordinates accounted for by each harmonic and is equal to:

$$P_{\mathrm{F}}(n_k) = \sum_{i=1}^{n_k} \frac{A_i^2 + B_i^2 + C_i^2 + D_i^2}{2}. \qquad (1)$$

The number of harmonics n_k was then chosen such that $P_F(n_k)$ reaches 99.99% of variance in contour coordinates or, in other words, such that shape is reconstructed at 99.99% (Lestrel 2008). A majority of studies compute the cumulated Fourier power P_F using EFDs averaged across the full sample or some part of it, so that the harmonics selected describe the average otolith shape. In this study, in order to insure that each individual otolith in the sample was reconstructed with a precision at least 99.99 %, $P_F(n_k)$ and n_k were calculated for each individual otolith k. The maximum number of harmonics $n = \max_k(n_k)$ across individual otoliths was then used to reconstruct each individual otolith of the sample.

After extracting the n harmonics for each individual otolith, their EFDs were normalized by the first harmonic providing EFDs invariant with respect to size, rotation and starting point (Kuhl & Giardina 1982), and resulting in the degeneration of the first three EFDs (A_1, B_1, C_1) for each individual, respectively equal to 1, ~ 0 and ~ 0.

All morphometric parameters (O_L and *EFD*s) were obtained by using TNPC v7 software.

Statistical analyses

A principal component analysis (*PCA*) was carried out on the *EFD*s matrix of the sample (*EFD*s as columns and individual otoliths as rows) and a subset of the resulting principal components were selected as otolith shape descriptors according to the broken stick model (Borcard et al. 2011) (Figure 1). This allowed us to decrease the number of variables used to describe otolith shape variability while ensuring that the main sources of shape variation were kept and to avoid collinearity between shape descriptors (Rohlf & Archie 1984).

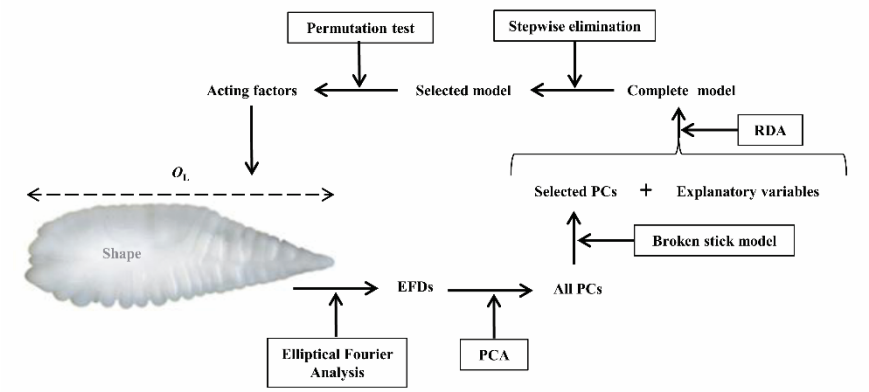


Figure 1: Schematic representation of the sequence of steps to describe otolith shape and statistical analysis performed to identify factors affecting the otolith shape.

Otolith length (O_L) and the selected principal component matrix representing otolith shape (S) were modeled as depending on a series of potentially influential variables (eq. 2): fish age (A) and total length (L) as a factor and a continuous effect, respectively, in order to represent a potential ontogenetic effect on otolith shape; *s*ex (S_e) of the individual as a factor potentially affecting physiology and metabolism, and thus indirectly otolith biomineralization; and the otolith location side (S_i, thereafter called "location side effect") as a factor to evaluate the potential asymmetry between right and left otoliths. In addition, the second and third-order interactions between variables were included in the model.

$$\begin{aligned} y \sim\ & A + L + S_e + S_i + A\times L + A\times S_e + A\times S_i + L\times S_e + L\times S_i + S_e\times S_i \\ & + A\times L\times S_e + L\times S_e\times S_i + A\times S_i\times S_e + L\times A\times S_i \end{aligned} \quad (2)$$

with $y = O_L$ or $y = S$.

Regarding O_L, model (2) was fitted using a linear effect model. The model was reduced by a bidirectional elimination procedure based on the Akaike Information Criterion (Borcard et al. 2011). In order to determine active factors, significance of effects in the reduced model was tested by *F* tests between nested models respecting marginality of the effects (type 2 tests; Fox & Weisberg 2011). The assumption of normality and homogeneity of variance were checked on residual data. A Box-Cox transformation with a species-

dependent parameter λ (Box & Cox 1964) on otolith length data proved necessary to achieve normality (eq 3).

$$O_{\mathrm{L}}^{'} = \frac{O_{\mathrm{L}}^{\lambda} - 1}{\lambda} \quad (3)$$

Regarding the shape matrix (S), model (2) was adjusted using a redundancy analysis (RDA; Legendre & Legendre 2012). The model was reduced by a bidirectional elimination procedure based on significance of the effects determined by permutation tests. The significance of the remaining effects in the reduced model was eventually tested by permutations. In case of significant location side effect, the percentage of non-overlapping surface ($\%NOS$) between the right and left otolith shapes was calculated by reconstructing the shape of the left and right otoliths on the basis of the EFDs for each individual. The $\%NOS$ was computed relative to the total area and averaging across individuals. Normalization of the *EFD*s ensured that the left and right reconstructions were automatically superimposed as they are invariant with respect to size, orientation and starting point.

All statistical analyses were performed using the 'vegan'(Oksanen et al. 2013), 'MASS' (Venables & Ripley 2002), 'sp' (Pebesma & Bivand 2005), 'rgeos' (Bivand & Rundel 2014) and 'car'(Fox & Weisberg 2011) packages in the statistical environment R (R Development Core Team 2013).

Results

Roundfishes

As expected, an ontogenetic effect was observed on both otolith length (Table II) and shape (Table III) for each species. The ontogenetic effect was sex independent in the form of an age and a total length effect on otolith shape in *M. aeglefinus* and an age-total length interaction affecting otolith length in *M. aeglefinus* and *M. barbatus* as well as otolith shape in *M. merlangus*. In other cases, the ontogenetic effect varied across sexes as testified by the

significant interaction between age and sex and/or total length and sex affecting otolith length and shape in *C. harengus* and the significant interaction between age, total length and sex affecting otolith length in *M. merlangus* and otolith shape in *M. barbatus*. A main sex effect was identified on otolith length in *M. barbatus* only (Table II). A location side effect was found for otolith length in *C. harengus* and otolith shape in *M. merlangus*. However, differences between left and right otoliths were negligible, be it for otolith length in *C. harengus* as the left otolith, on average, was only 1.99 % smaller than the right one [Figure 2(a)] or the otolith shape in *M. merlangus* where the average $\%NOS$ between the right and left otolith shapes was only 6.17 % [Figure 3(a)].

Table II. Variables and factors (A: age, L: total length, S_e: sex, S_i: side) and O_L their interactions acting on otolith length of the species studied. The first column contains lambda values (λ) used for the box-cox transformation of O_L data (normality and homoscedasticity verified on residuals). For each species, effects eliminated during the AIC-based bidirectional elimination are indicated by white background, whereas effects kept in the selected model are highlighted by grey shading. Dark-grey shading indicates a length difference between left and right otolith whereas light-grey shading indicates other effects. The F statistic of each tested effect (while respecting marginality of the effects, type 2 tests) is presented with numerator degrees of freedom as exponent and denominator degrees of freedom as index together with the corresponding P-value. Given that marginality of the effects was respected, lower order effects were not tested when corresponding higher order effects were significant.

Species	λ	A	L	S_e	S_i	$A\times L$	$A\times S_e$	$L\times S_e$	$L\times S_i$	$A\times L\times S_e$
Roundfishes										
M. aeglefinus	-0.8			2.59^{1}_{258} 0.108		**5.67^{7}_{258}** **0.001**	1.34^{6}_{258} 0.239	3.03^{1}_{258} 0.083		1.83^{4}_{258} 0.123
M. merlangus	0.5									**4.61^{5}_{501}** **0.001**
M. barbatus barbatus	0.1			**5.24^{1}_{546}** **0.022**		**3.73^{2}_{546}** **0.025**	0.78^{2}_{546} 0.459	2.15^{1}_{546} 0.143		2.44^{2}_{546} 0.088
C. harengus	0.1				**104.41^{1}_{383}** **0.001**	**4.10^{6}_{383}** **0.001**	**4.87^{5}_{383}** **0.001**	**9.74^{1}_{383}** **0.002**		2.16^{3}_{383} 0.092
Flatfishes										
P. platessa	0.1								**6.67^{1}_{1373}** **0.100**	**2.29^{5}_{1373}** **0.043**
L. limanda	0.1								**6.56^{1}_{694}** **0.011**	**3.35^{4}_{694}** **0.009**
S. solea	0.6			0.01^{1}_{401} 0.969	2.91^{1}_{401} 0.089	**6.62^{5}_{401}** **0.001**	2.36^{3}_{401} 0.071			
L. whiffiagonis	-0.5			2.30^{1}_{285} 0.130		**9.71^{6}_{285}** **0.001**			**10.46^{1}_{285}** **0.001**	

Table III. Variables and factors (A : age, L : total length, S_e : sex, S_i : side) and their interactions acting on otolith shape of the species studied. For each species, effects eliminated during the significance-based bidirectional elimination are indicated by white background, whereas effects kept in the selected model are highlighted by grey shading. Dark-grey shading indicates a shape difference between left and right otolith whereas light-grey shading indicates other effects. The permutation-based *F* statistic of each significant effect (while respecting marginality of the effects, type 2 tests) is presented with numerator degrees of freedom as exponent and denominator degrees of freedom as indices together with the corresponding P-value. Given that marginality of the effects was respected, lower order effects were not tested when corresponding higher order effects were significant.

Species	A	L	S_e	S_i	$A\times L$	$A\times S_e$	$L\times S_e$	$L\times S_i$	$A\times L\times S_e$
Roundfishes									
M. aeglefinus	$\mathbf{3.95^{7}_{277}}$ **0.001**	$\mathbf{4.81^{1}_{277}}$ **0.002**							
M. merlangus				$\mathbf{4.61^{1}_{513}}$ **0.008**	$\mathbf{2.60^{6}_{513}}$ **0.001**				
M. barbatus barbatus									$\mathbf{4.77^{2}_{546}}$ **0.001**
C. harengus					$\mathbf{4.21^{6}_{392}}$ **0.001**		$\mathbf{3.59^{1}_{392}}$ **0.016**		
Flatfishes									
P. platessa			$\mathbf{3.74^{1}_{1385}}$ **0.009**		$\mathbf{1.92^{7}_{1385}}$ **0.012**			$\mathbf{6.65^{1}_{1385}}$ **0.001**	
L. limanda					$\mathbf{2.03^{6}_{704}}$ **0.016**		$\mathbf{5.49^{1}_{704}}$ **0.006**	$\mathbf{7.66^{1}_{704}}$ **0.002**	
S. solea				$\mathbf{91.92^{1}_{407}}$ **0.001**		$\mathbf{4.41^{3}_{407}}$ **0.001**			
L. whiffiagonis					$\mathbf{1.59^{6}_{286}}$ **0.032**			$\mathbf{19.28^{1}_{286}}$ **0.001**	

Flatfishes

Again, an ontogenetic effect was found on both otolith length (Table II) and shape (Table III) for all species. The ontogenetic effect was sex independent in the form of an age-total length interaction affecting otolith length in *S. solea* and *L. whiffiagonis*, and otolith shape in *P. platessa* and *L. whiffiagonis*. In other cases, the ontogenetic effect varied according to sex as shown by the total length-sex interaction acting on otolith shape in *L. limanda*, the age-sex interaction acting on otolith shape in *S. solea* and the interaction between age, total length and sex affecting otolith length in *P. platessa* and *L. limanda*. A location side effect was identified for all flatfishes either on both otolith length (Table II) and shape (Table III) or on otolith shape alone. For *S. solea*, a location side effect was observed on otolith shape only and its amplitude was larger than for *M. merlangus*, the $\%NOS$ between the right and left otolith shapes being 7.94 % [Figure 3(b)]. The main difference between normalized otoliths was in terms of width (taken as the distance perpendicular to otolith length, i.e. along the dorso-ventral axis), the left one being wider. In other flatfishes, a location side-total length interaction affected otolith shape (Table III). The ratio between the anterior-posterior and the dorso-ventral axes increased with fish size and these morphogenesis patterns differed significantly between the left and right otoliths [Figure 3(c, d, e)].

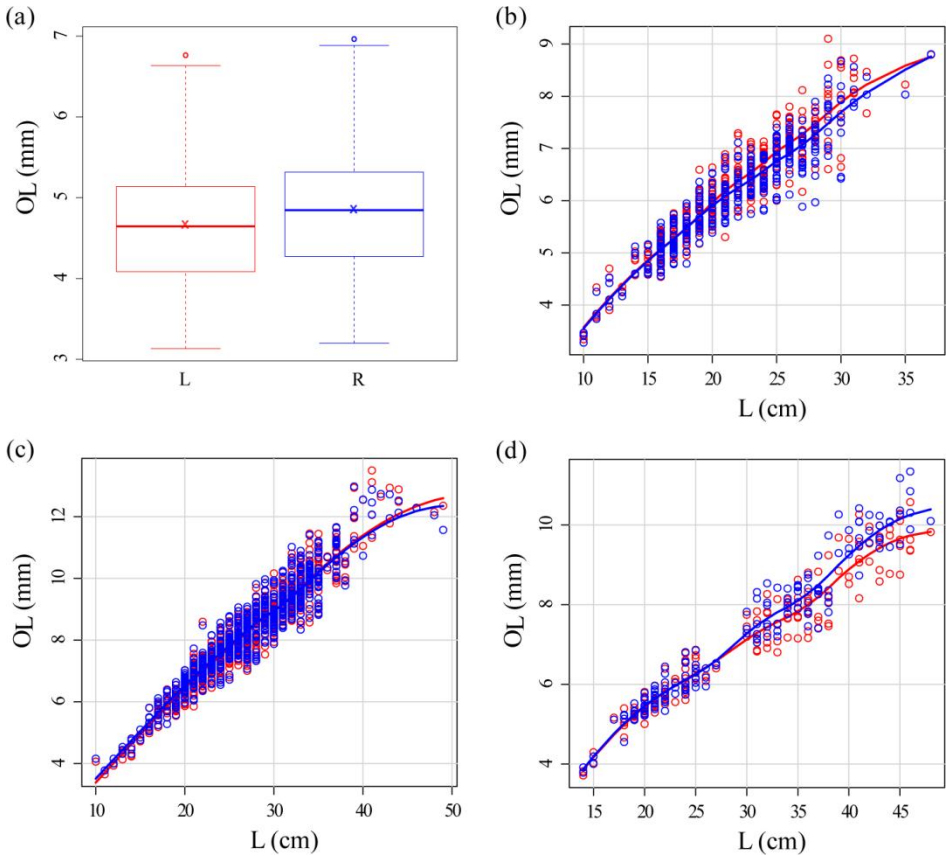


Figure 2: Difference between right and left otolith lengths. (a) Boxplot of otolith length O_L according to its location side, left L (in red) or right R (in blue) in *C. harengus*. The bottom and top of the box are the first and the third quartiles of the data distribution, the horizontal segment and the cross inside the box are the median and the mean, respectively, whiskers represent the most extreme data point within 1.5 interquartile range, and circles are data points out of this range. Change of left (in red) and right (in blue) otolith length O_L according to fish total length L for (b) *L. limanda*, (c) *P. platessa*, and (d) *L. whiffiagonis*. Open circles are data points and continuous curves are loess smoothers fitted to the data points with a span of 0.5.

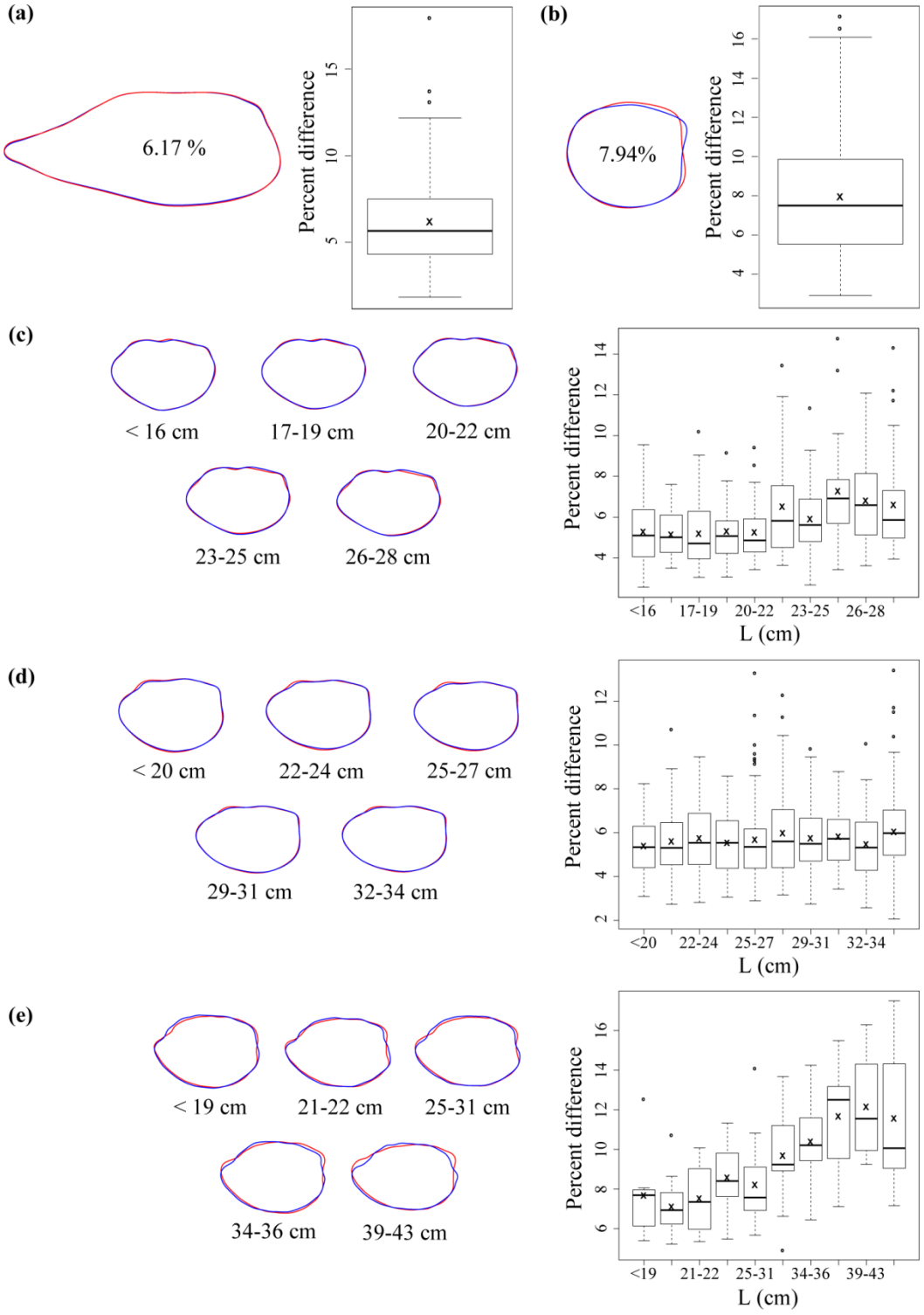


Figure 3: Difference between right and left otolith shapes. Representation of overlap between the right (blue line) and left (red line) otolith mean contours (left side), accompanied by a boxplot of percent difference of surface between normalized right and left otolith shapes (right side). For species presenting a significant location side×total length interaction, boxplots and mean contours are shown for increasing total length classes (1 in 2 only for mean contours). Percentage in mean contours give the average of percent difference of surface between normalized right and left otolith shapes. See Figure 2 caption for how boxplots describe the data distribution. (a) *M. merlangus*; (b) *S. solea*; (c) *L. limanda;* (d) *P. platessa*; and (e) *L. whiffiagonis*

In the two dextral flatfishes (*L. limanda*, [Figure 3(c)] and *P. platessa*, [Figure 3(d)]), the average $\%NOS$ between the right and left otolith shapes was 5.88 % and 5.71 %, respectively and increased significantly according to fish total length for *L. limanda* ($\%NOS = 0.008\,L + 0.967$, $P < 0.05$, $n = 361$) but not *P. platessa*. Due to its small amplitude, the difference between the right and left otolith shapes was difficult to observe similar to *M. merlangus*. In contrast, in the sinistral flatfish (*L. whiffiagonis*) the difference between the right and left otolith shapes was larger [Figure 3(e)]. The $\%NOS$ was equal to 9.57 % on average and increased significantly according to fish total length ($\%NOS = 0.016\,L + 1.518$, $P < 0.05$, $n = 151$). In this species, the right otolith was wider than the left one contrary to *S. solea*. Results on otolith length were qualitatively similar (Figure 2). A difference between otolith lengths was observed in dextral flatfish species [Figure 2(b, c)] that increased with fish total length in *L. limanda*, the left otolith being longer, but not in *P. platessa*. Otolith length also differed between location sides in the sinistral flatfish species [Figure 2(d)], the right otolith being longer contrary to *L. limanda*, and this difference increased with fish total length.

Discussion

Ontogenetic changes and sexual dimorphism in sagittal otolith shape

The use of otoliths in stock management studies has been advocated because otoliths are routinely collected and used for age estimation (Cardinale et al. 2004). As the otolith growth rate varies more widely between stocks than within a stock and given that it affects otolith shape, otolith shape analysis is a natural and quick method for identifying stocks (Campana & Casselman 1993). However, several confounding effects have been pointed out and must be accounted to avoid introducing bias into the analyses.

For instance, otolith shape variation is well known to be also related to ontogeny: otoliths grow faster along the anterior-posterior axis than along the dorsal-ventral axis, thus becoming increasingly elongated as individuals develop (Galley et al. 2006). Consequently, otolith growth patterns are characterized by a negative allometric relationship between length and width (Simoneau et al. 2000). The present study also revealed such ontogenetic changes in both otolith length and shape. For most species, a positive relationship between otolith length and both fish total length and estimated age is identified. The average otolith shapes reconstructed by Fourier analysis showed that otoliths became more elliptic as fish total length increased. Sex is also known as potentially affecting otolith shape depending on species (e.g. Begg & Brown 2000, Simoneau et al. 2000). The present study confirmed that for some species there is a sexual dimorphism in otolith shape and/or length. The differences in otolith shape and length observed between sexes, ages and body total lengths are likely to result from differences in body growth rate but also physiology and metabolism for the sex effect (Campana & Casselman 1993, Begg & Brown 2000, Simoneau et al. 2000, Cardinale et al. 2004). In this study, fish total length and sex have been forced into the model as potential confounding factors to avoid introducing bias into the asymmetrical analysis. It results that sometimes their effects were not correctly estimated (for example, we found a significant sex effect for *S. solea* but sample sex-ratio was highly unbalanced). This, however, was not problematic as they were only covariates and our primary interest was in estimating asymmetry.

Asymmetry in sagittal otolith shape between right and left inner ears

In the present study, the otolith location side had been identified as a new factor affecting the otolith shape. Depending on species, the morphogenesis patterns were not

similar between inner ears. In roundfishes, only 2 species, *M. merlangus* and *C. harengus* exhibited a significant difference in their otolith morphogenesis patterns between location sides. However, the right and left reconstructed otolith shapes were hardly discernible in *M. merlangus.* Similarly, the right and left otolith lengths in *C. harengus* were very close, the right otolith being only 0.2 mm, i.e. roughly 4 %, longer than the left one. These results raise the question of the trade-off between an effect's statistical significance and its amplitude, which determines its biological significance. In this present study, each sample size was relatively large (≥143, see Table I) so that statistical tests had high statistical power and could detect any small difference. Moreover, the otolith shape reconstruction procedure, based on Fourier power calculated at the individual level in order to keep the maximum number of harmonics, allowed describing the otolith outline with very fine details. In other words, the high power of statistical tests combined with the fine description of otolith shape allowed the detection of very small differences between right and left otolith shapes but the biological importance of such small-amplitude effects may be questioned.

In flatfishes, a significant location side effect was observed on otolith shape and length for all species. Globally, right and left otolith shapes and lengths were more asymmetric in flatfishes than in roundfishes. These results are complementary to those of Lychakov *et al.* (2008) who showed that otolith mass asymmetry in marine flatfishes was significantly larger than in marine roundfishes. Moreover, these authors also showed that otolith mass asymmetry is directional, the heaviest otolith being on the blind side for the majority of flatfishes. The present study confirmed the directional asymmetry between otoliths in flatfishes; the widest and longest otolith was almost always located on the blind side. This present study also showed that otolith shape and length asymmetry could vary during ontogeny (as indicated by significant interactions between location side and fish total length) for 3 flatfish species: *P. platessa*, *L. limanda,* and *L. whiffiagonis*. Among these, the degree of asymmetry was found

to increase with fish total length in *L. limanda* and *L. whiffiagonis*. This suggests that, at least for these species, otolith shape and length asymmetry is built over individuals' lifetime, which is consistent with the accretional growth of otoliths. The absence of ontogenetic trend in otolith shape and length asymmetry for *S. solea* and *P. platessa* in this paper as well as in otolith mass observed by Lychakov *et al.* (2006) may be related to the fact that otolith asymmetry is built at different speeds according to species after metamorphosis and that rather old individuals (relative to the timing of these processes) were observed in this study (Sogard 1991).

Asymmetry in sagittal otolith shape and biomineralization

The larger asymmetry in otolith shape and length observed in flatfish species and its ontogenetic increase for some of them suggest differences in otolith biomineralization between left and right inner ears that are stronger in flatfishes than in roundfish species. Biomineralization of sagittal otolith is an acellular process that takes place in the saccule (otic sac). Otoliths grow by accretion and precipitation of organic and ionic precursors contained in the saccular endolymph in which they are bathing. Otolith biomineralization is therefore totally dependent on the endolymph composition in terms of precursors, which are either synthesized (organic ones) or transported (ionic ones) by the saccular epithelium (Payan et al. 2004). The organic matrix, although present in minute amounts, is thought to play a key role in otolith formation as in all biomineralization process (Nagasawa 2013). OMP1 and otolin 1 OMP-1, two proteins that are co-localized in otolith microstructures (Murayama et al. 2005), are produced by most of the saccular epithelium for the former but by a limited part of the marginal zone of the sensory epithelium (macula zone) for the latter (Murayama et al. 2004). Another protein found in zebrafish otolith, starmaker is thought to regulate crystal formation (Söllner et al. 2003). Beside its organic composition, the endolymph ionic composition

depends on the location and the activity of some mitochondria-rich cells called ionocytes in the saccular epithelium that transport ions from plasma to endolymph either passively and/or actively (Pisam et al. 1998, Payan et al. 1999). In teleosteans, the general structure of the saccular epithelium is characterized by the presence of large ionocytes in the area contiguous with the macula and small ionocytes on the opposite side (Mayer-Gostan et al. 1997, Pisam et al. 1998). This spatial cellular organization of the epithelium creates a proximal-distal gradient of precursors in the endolymph, which is assumed necessary for the otolith formation (Payan et al. 1999, 2004, Borelli et al. 2001).

Any difference in the proximal-distal gradient of precursors and/or the spatial cellular organization of the saccular epithelium between the right and left saccules will likely generate asymmetric otolith biomineralization. Three phenomena could generate such a difference. Firstly, structural constraints linked to the considerable morphological changes that fish undergo during metamorphosis could affect differentially the shape of the right and left saccules and thus the geometry of the saccular epithelium relative to otolith position within the endolymph. Such asymmetric structural constraints could result from the cranial deformation during metamorphosis in flatfishes, notably the associated relocation of the anterior part of the frontal bones from the blind side to the ocular side (Brewster 1987). One can see here a parallel with the asymmetry in eye shapes in flatfishes, the blind-eye being larger than the ocular-eye contrary to roundfishes for which the difference in eye shapes is not significant (Li et al. 2013). The present study showed that the blind-side was the location of the widest otolith for both dextral and sinistral flatfish species.

Secondly, the asymmetric body shape and the related lateralized behavior of flatfishes (Schreiber 2006) will likely induce an asymmetric effect of gravity on otoliths and the precursors within the endolymph as the ocular-side saccule will be located above the blind-side one. Gravity will push the otolith and precursors towards the macula in the ocular-side

saccule and away from the macula in the blind-side saccule. This will create an asymmetry in the proximal-distal gradient of precursors in the endolymph as well as in the position of the otolith relative to the saccular epithelium and might therefore generate asymmetric biomineralization.

Thirdly, any difference in the spatial distribution of protein secreting cells and/or of ionocytes between the left and right saccules is likely to generate an asymmetry in biomineralization. However, no study that compares the spatial cellular organization of the saccular epithelium between right and left saccules in flatfish could be found in the literature. Pisam *et al.* (1998) investigated differences in the cellular organization of the saccular epithelium between *Salmon trutta* (trout) and *Scophthalmus maximus* (turbot) but without comparing right and left saccules.

Functional aspects of sagittal otolith shape asymmetry

The shape asymmetry observed in this study never exceeded 20 % even in flatfish species that exhibited a clear directional asymmetry. This is in agreement with the modeling study of Lychakov & Rebane (2005) who have shown that otolith mass asymmetry must be lower than 20 % and must be kept at the lowest possible level to avoid issues in acoustic and vestibular functions due to incompatibility and incongruity of the right and left otolith movements. Likewise, it can be assumed that shape asymmetry must be low to avoid reducing hearing capacities because otolith shape is also implicated in its response movements following a sound wave. Because of the irregularity of its shape, the otolith will not only move according to a simple back and forth translation in the direction of the sound wave but will also have a rocking motion in several directions, which produces additional stimuli that the fish may be able to process for additional cues on the characteristics and direction of the oncoming sound (Krysl et al. 2012). Such a complex answer should be similar between the

two inner ears for a good environmental perception. This is likely to generate a selection pressure that favors individuals characterized by a low shape asymmetry, even in flatfish species characterized by a directional asymmetry, because they have a better audition and a better reception of environmental signals and thus, a better survival. One can see here a parallel with selection on fluctuating asymmetry (Brown & Brown 1998). The strength of this selection pressure could vary according to species. The fact that, in this study, otolith shape asymmetry was found to be lower in dextral than in sinistral flatfishes suggests that such a selective pressure could be stronger in the former.

Differences in otolith mass asymmetry between sinistral and dextral flatfishes have also been observed. In the dextral *P. platessa*, no significant difference in mass was found between the left and right sagittal otoliths contrary to the sinistral *S. maximus* for which the right sagittal otolith was found to be significantly heavier (Helling et al. 2005). In this study, the sinistral species *L. whiffiagonis* exhibited the greatest shape asymmetry and, within dextral species, *S. solea* presented a larger shape asymmetry than *P. platessa* and *L. limanda*. Taken together, these results suggest that the degree of directional otolith shape asymmetry in flatfishes is determined primarily by the ocular side and secondarily by the taxonomic family. However, Lychakov (2013) found opposite results as they observed that families of sinistral flatfish species, namely *Bothidae* and *Citharidae*, presented a lower otolith mass asymmetry than a dextral family, namely *Soleidae*. They concluded that otolith mass asymmetry was correlated with mobility rather than with the ocular side as the more mobile species, within the families *Bothidae* and *Citharidae*, had more symmetric otoliths than the less mobile ones, in the *Soleidae*. On the same line, the results of this study could be interpreted as suggesting a correlation between the degree of otolith shape asymmetry and hunting behavior. The widest and longest otolith, thus the heaviest one, which is always located on the blind side, allows a higher acoustic sensitivity and resolution capability but at low frequencies and with a lower

operation speed (Lychakov & Rebane 2000). This involves an asymmetry in the audition function between inner ears, which may be related to fish predatory behavior. *Scophthalmidae*, notably *L. whiffiagonis*, are mainly fish consumers that are actively stalking fish contrary to *Soleidae*, including *S. solea*, and *Pleuronectidae*, including *P. platessa* and *L. limanda*, that always hunt on the substratum and feed mainly on crustaceans, gastropods, bivalve and polychaete prey (Braber & de Groot 1973, Holmes & Gibson 1983, Besyst et al. 1999, Pinnegar et al. 2003).

Implications for studies based on otolith shape

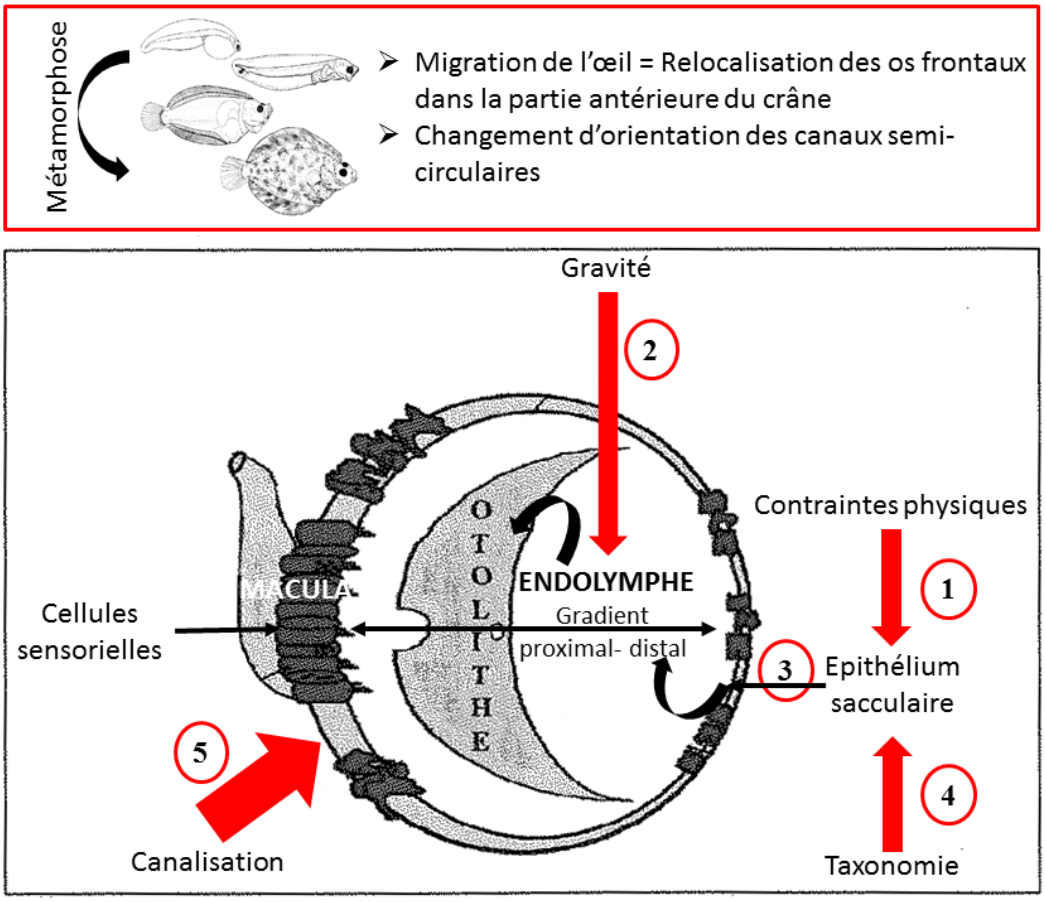
Studies based on otolith shape need whole otolith samples without damages. Ideally, such studies should be based either on otoliths coming from the same location side or on otolith pairs. In practice, otoliths are regularly damaged or even lost during sampling so that some otolith pairs are incomplete with an alternation between right and left otolith. In order to maximize sample size and statistical power, one may be tempted to use mirror images of undamaged otoliths to replace the damaged ones hoping that this procedure will produce limited statistical bias in the results. The results of this study suggest that such a procedure could be used for roundfish species, since these present a rather symmetrical pattern of morphogenesis between inner ears. For flatfishes, such a procedure should be avoided, given the observed directional otolith shape asymmetry, or be limited to species for which a preliminary analysis shows a very weak degree of asymmetry. More generally, it is recommended that studies based on otolith shape should use otoliths coming always from the same location side throughout the study and to be careful when comparing studies which have used otoliths sampled on different location side.

Conclusion

Using otolith shape as a proxy of otolith biomineralization, this study has revealed an asymmetry in otolith morphogenesis patterns between the right and left inner ears in flatfish species, while roundfish species exhibit more symmetrical morphogenesis patterns. This result is most likely related to the important morphological and behavioral changes occurring during the metamorphosis of flatfishes that could affect differentially right and left otolith biomineralization. However, the degree of otolith shape asymmetry never exceeded 20 %, which may be due to an evolutionary canalization of otolith shape symmetry to avoid negative impacts on fish environment perception (audition and balance). In this study, a maximum shape asymmetry of 17.50 % was found. In addition to investigating the consequences of otolith asymmetry on fish's audition and balance, it would be also interesting to study further otolith biomineralization and specifically how it differs between inner ears in order to understand the processes that drive the otolith shape asymmetry observed. From a technical point of view, asymmetry should be taken into account in studies based on otolith shape.

Schéma conceptuel des hypothèses soulevées dans le chapitre 1 :

Hypothèses soulevées pouvant expliquer la différence de biominéralisation et, donc, de forme des otolithes entre les saccules droit et gauche chez les pleuronectiformes.



1 Les contraintes structurelles liées aux changements morphologiques considérables que les poissons plats subissent pendant la métamorphose pourraient affecter différemment la forme des saccules droit et gauche. Ainsi, la géométrie de l'épithélium sacculaire pourrait être affectée engendrant des conséquences sur le gradient proximal-distal ainsi que la position de l'otolithe dans l'endolymphe.

2 La forme du corps asymétrique et le comportement latéralisé des poissons plats induisent une asymétrie de l'effet de la gravité sur les otolithes et les précurseurs dans l'endolymphe provoquant une asymétrie du gradient proximal-distal des précurseurs et de position de l'otolithe dans l'endolymphe.

3 Toute différence dans la répartition spatiale des cellules composant l'épithélium sacculaire est susceptible de générer une asymétrie dans la disposition des précurseurs dans l'endolymphe et donc provoquer une asymétrie au niveau du gradient proximal-distal.

4 Selon le côté oculaire de l'espèce et de sa famille taxonomique, le degré d'asymétrie directionnelle des otolithes varie et suggère une corrélation avec son comportement de chasse.

5 L'asymétrie de forme ne doit pas excéder un certain seuil pour le bon fonctionnement audio-vestibulaire de l'oreille interne et donc la bonne perception de l'environnement par l'animal

Chapitre 2
Is diet correlated with otolith shape in marine fish?

Valorisation de ce chapitre:

- Publication soumise une fois dans le journal *Marine Ecology Progress Series* dont la décision revenue est une correction majeure.

- Présentations orales:
- 5th International Otolith Symposium en Octobre 2014.
- Journée des étudiants de l'IFREMER Centre Manche Mer du Nord en Juin 2015.

ABSTRACT

Previous studies have shown that food amount affects fish otoliths' structure, opacity and shape and that diet composition impacts otoliths' chemical composition. The purpose of this study was to investigate the potential correlation between diet and otolith shape in five wild marine fish species by addressing three complementary questions. First, is there a global relationship between diet and otolith shape? Second, which prey categories are involved in this relationship? Third, what are the respective contributions of food quantity and relative composition to otolith shape variation? For each species, we investigated how otolith shape, described by Fourier descriptors, varies with diet, determined as weight per prey category by stomach contents analysis. The three questions were tackled by describing diet in the analysis in three different ways. In order to remove potential confounding effects, individual-state variables and environmental factors were also included in the analysis. First, a significant correlation between diet and otolith shape was detected for all species studied except one. Second, both main and secondary prey categories explained partially otolith shape variability and otolith shape reconstructions showed that both the otoliths' global shape and finer details were affected. Third, the contribution of diet relative composition to otolith shape variation was much higher than that of ingested food quantity. These effects may result from diet influence on the quantity and composition of saccular endolymph proteins used for the organic matrix synthesis that plays an important role in otolith biomineralization. Our results emphasize the lack of understanding of otolith biomineralization, especially 3D spatial processes.

Key words: Morphometric analysis, English Channel, Interspecific, Otolith growth, Saccular otolith

RESUME

Plusieurs études ont révélé l'influence de la quantité de nourriture ingérée par l'animal sur la mircrostructure, l'opacité et la forme de l'otolithe ainsi que l'influence de la composition du bol alimentaire sur la composition chimique de l'otolithe. Dans cette étude, la corrélation entre le régime alimentaire (déterminé par le poids des proies au sein de différentes catégories) et la forme des otolithes (décrite par les descripteurs de Fourier) a été étudiée chez 5 espèces de poissons marins analysées séparément, afin de répondre à 3 questions complémentaires. Premièrement, existe-t-il une corrélation globale entre la variation du régime alimentaire des individus et la variation de forme de leurs otolithes ? Deuxièmement, quelles sont les catégories de proies impliquées dans cette corrélation ? Troisièmement, quelle est la part d'effet respective de la quantité et de la composition du bol alimentaire sur la variation de forme des otolithes ? Pour chacune de ces 3 questions, la description du régime alimentaire a été abordée de trois manières différentes. De plus, des variables d'état individuel et des variables environnementales ont été ajoutées au model afin de retirer leurs effets confondants sur la variation de la forme des otolithes. Premièrement, une corrélation entre la variation de forme des otolithes et le régime alimentaire, décrit à la fois comme la quantité et la qualité du bol alimentaire, a été détectée chez quatre espèces étudiées sur cinq. Deuxièmement, la variation de forme des otolithes était corrélée à des catégories de proies principales mais aussi secondaires. Des reconstructions de formes ont été réalisées et ont révélé que la composition alimentaire impactait à la fois la forme globale de l'otolithe ainsi que ces détails. Troisièmement, la composition du bol alimentaire expliquait davantage de variation de forme des otolithes que celle expliquée par la quantité de nourriture ingérée par l'animal. Les effets observés du régime alimentaire sur la variation de forme des otolithes pourraient être expliqués par leurs effets sur la quantité et la composition des protéines de l'endolymphe ; précurseurs de la matrice organique qui possède un rôle important dans la biominéralisation de l'otolithe. Nos résultats soulignent le manque d'études concernant la biominéralisation des otolithes et particulièrement l'effet des facteurs endogènes et exogènes sur cette dernière, ainsi que le manque de connaissances sur les processus en trois dimensions.

Mots-clés : Analyse morphométriques, Manche Orientale, étude interspécifique, croissance de l'otolithe, otolithe sacculaire

Introduction

Otoliths are calcified concretions organized in 3 pairs found in Teleostean fishes' inner ears and that are involved in their audition and balance. The *sagittae* (the most studied otolith pair because of their large size) are mainly composed of calcium carbonate in aragonite form deposited on an organic matrix representing only 0.1 to 10 % of total material (Degens et al. 1969). The organic matrix, although present in minute amounts in otoliths, is thought to play a key role in its formation as in all biomineralization processes (Nagasawa 2013). Biomineralization of sagittal otolith (referred to as "otolith" thereafter) is an acellular process that takes place in the saccule (otic sac). Otoliths grow by accretion and precipitation of organic and ionic precursors contained in the saccular endolymph in which they are bathing. Otolith biomineralization is therefore totally dependent on the endolymph composition and precursors are either synthesized (organic ones) or transported (ionic ones) by secretory cells and ionocytes, respectively, belonging to the saccular epithelium (Payan et al. 2004). Moreover, the spatial distribution of ionocytes and secretory cells in the saccular epithelium induces concentration gradients of both ions and organic precursors in the endolymph that are involved in otolith biomineralization process (Pisam et al. 1998, Payan et al. 1999, Borelli et al. 2001). Otolith biomineralization results from multi-causal processes due to the interaction of many internal (physiological) and external (environmental) factors (Allemand et al. 2007), which generates high morphological variability in otolith shape.

First, otolith shape is genetically determined (L'Abée-Lund 1988) and species-specific. It is thus useful in revealing phylogenetic relationships between species (Lombarte & Lleonart 1993). Second, factors or processes acting on fish metabolism and physiology have an impact on otolith morphology, such as ontogenetic development (size: Hüssy, 2008 and age: Castonguay et al. 1991), sexual maturation (Mérigot et al. 2007) or sex (Castonguay et al. 1991, Bolles & Begg 2000). Third, environmental factors such as water temperature

(Cardinale et al. 2004) produce otolith growth variation and thus shape variability. Food amount also impacts otolith shape in that it has both an indirect effect through otolith growth generating variation in the "global" otolith shape as an ontogenetic effect and a direct effect on the otolith's crenulations (Gagliano & McCormick 2004, Cardinale et al. 2004, Hüssy 2008).

The consequences of fish nutrition on otolith structure and growth, especially the impact of starvation or food restriction and satiation, have been highly studied (Molony & Choat 1990, Molony & Sheaves 1998, Hüssy & Mosegaard 2004, Fernandez-Jover & Sanchez-Jerez 2015). Several studies have indicated a close relationship between otolith growth and standard metabolic rate (Mosegaard et al. 1988, Fablet et al. 2011). Others indicated that otolith accretion is regulated by feeding-induced thermogenesis (Huuskonen & Karjalainen 1998). A decrease in the otolith increments' width and thus in otolith growth was observed after reduced feeding periods (Massou et al. 2002). More translucent otolith material is deposited in response to severe (long period and low ration) food restriction, and could be responsible for the formation of translucent otolith structural discontinuities that do not conform to the seasonal opaque and translucent layers of annuli (Høie et al. 2008). The identification of such secondary growth structures, named "checks", is important to avoid bias in age estimation (Panfili et al. 2002). Otolith growth and opacity have been modeled depending on food and temperature (Fablet et al. 2011) whereas backward mode of the model can be used to reconstruct a fish's food history given opacity and temperature histories (Pecquerie et al. 2012). Changes in opacity are the consequences of variation in the composition of inorganic and organic otolith compounds (Jolivet et al. 2013) and precursors. A starvation period leads to change in blood plasma composition, which generates a decrease in the acid-base equilibrium in the saccular endolymph and thus, induces a reduction of aragonite precipitation rate. As a consequence, a reduction of daily growth rate due to

starvation could be observed even if calcium concentration was not affected (Payan et al. 1998). Concerning the organic precursors, only the protein «factor retarding crystallization» (FRC) concentration decreased during the starvation period especially in the proximal zone. Authors supposed that this change plays a key role in the intensity of aragonite deposition and thus otolith growth (Guibbolini et al. 2006).

Beyond the food amount, diet composition can also affect the otolith composition (Sanchez-Jerez et al. 2002). For instance, Barium (Ba) and strontium (Sr) concentrations in *Pomatomus saltatrix* otoliths were related to the concentration of these elements in their prey (Buckel et al. 2004). Authors made the assumption that the diet effect on Ba and Sr concentration in otoliths could be either direct or indirect through diet-based growth rate variation inducing element incorporation rate variation in otoliths. Even if around 80 % of Sr and Ba in otoliths came from water and not from diet (Walther & Thorrold 2006), a trophic transfer may be considered as a potential source of element accumulation in fish otolith. The concentration of manganese (Mn) in the habitat and prey items was also related to its accumulation in otoliths (Sanchez-Jerez et al. 2002). The fact that the Mn:Ca ratio in otoliths is not correlated to the same ratio in water suggests a trophic transfer of metallic elements, such as Mn (Thorrold et al. 1997). Moreover, variations in $\delta^{13}C$ otolith values were observed to correlate with variations in muscular $\delta^{13}C$ values among diet treatment (Elsdon et al. 2010). However, carbon in otoliths comes from several sources and integration depends on several factors and processes, mainly diet preferences as well as metabolic rate (Kalish 1991) and ambient dissolved inorganic carbon (Solomon et al. 2006). It should be noted that the use of otolith $\delta^{13}C$ to reconstruct individual fishes' diet history requires the knowledge of the influence of every other sources/processes on otolith $\delta^{13}C$ signature (Elsdon et al. 2010).

In summary, previous experimental works revealed that the food ration level affects otolith structure, opacity and shape, and studies in laboratory and wild conditions have shown that diet composition impacts otolith chemical composition. In the present study, we investigated the potential effect of diet, described as a combination of both food composition and quantity, on the otolith shape of five fish species, three roundfishes and two flatfishes sampled in the wild. More specifically, we addressed three different questions logically related. We first tested for a global correlation between diet and otolith shape. Second, in case a significant correlation was found, prey categories involved in this correlation were identified. Third, we quantified the respective contributions of food quantity and composition to otolith shape variation. For all questions, the effect of confounding factors, such as individual and environmental factors, on otolith shape was quantified and removed to obtain unbiased estimates of food effects.

Materials and methods

Sample collection

Five fish species of commercial interest, three roundfish species and two dextral flatfish species were sampled: 47 striped red mullets, 28 tub gurnards, 32 red gurnards and 42 European plaices and 36 common soles (Table 1).

All fish were caught in the eastern English Channel (Figure 1), the marine corridor between France and England resting on a shallow continental shelf, during the annual Channel Ground Fish Survey (CGFS) operated on board the R.V. "Gwen Drez" in October 2009. The fishing gear was a GOV bottom trawl with a 10-mm stretched mesh size in the codend, that was towed during 30 min at an average speed of 3.5 knot (Coppin et al. 2002). No selection has been realized according to individuals' life stage during sampling. Fishes were caught based on the fishing gear selectivity and the seasonality. Following their capture,

fish were identified at the taxonomic level and sampled individuals were frozen in liquid nitrogen for their conservation on board.

Table 1: Characteristics of the samples studied. Number of samples analyzed (N), number of females (N_F), number of males (N_M), the number of undetermined sex (N_U), proportion of mature individuals (% Mat), age and total length distributions of the samples.

Species	N	N_F / N_M / N_U	% Mat	Age (years) Mean ± SD Min-Max	Total length (cm) Mean ± SD Min-Max
Striped red mullet	47	8/16/23	48.94	0.62 ± 0.79 0-3	16.51 ± 5.6 9-32
Tub gurnard	28	10/18/0	32.14	1.75 ± 0.84 0-3	25.86 ± 6.61 16-40
Red gurnard	32	12/19/1	37.50	2.69 ± 0.74 1-4	24.91 ± 2.85 20-31
European plaice	42	26/14/2	76.19	1.81 ± 1.38 0-7	27.33 ± 7.45 9-43
Common sole	36	18/17/1	91.67	2.44 ± 1.61 1-6	25.94 ± 5.81 17-38

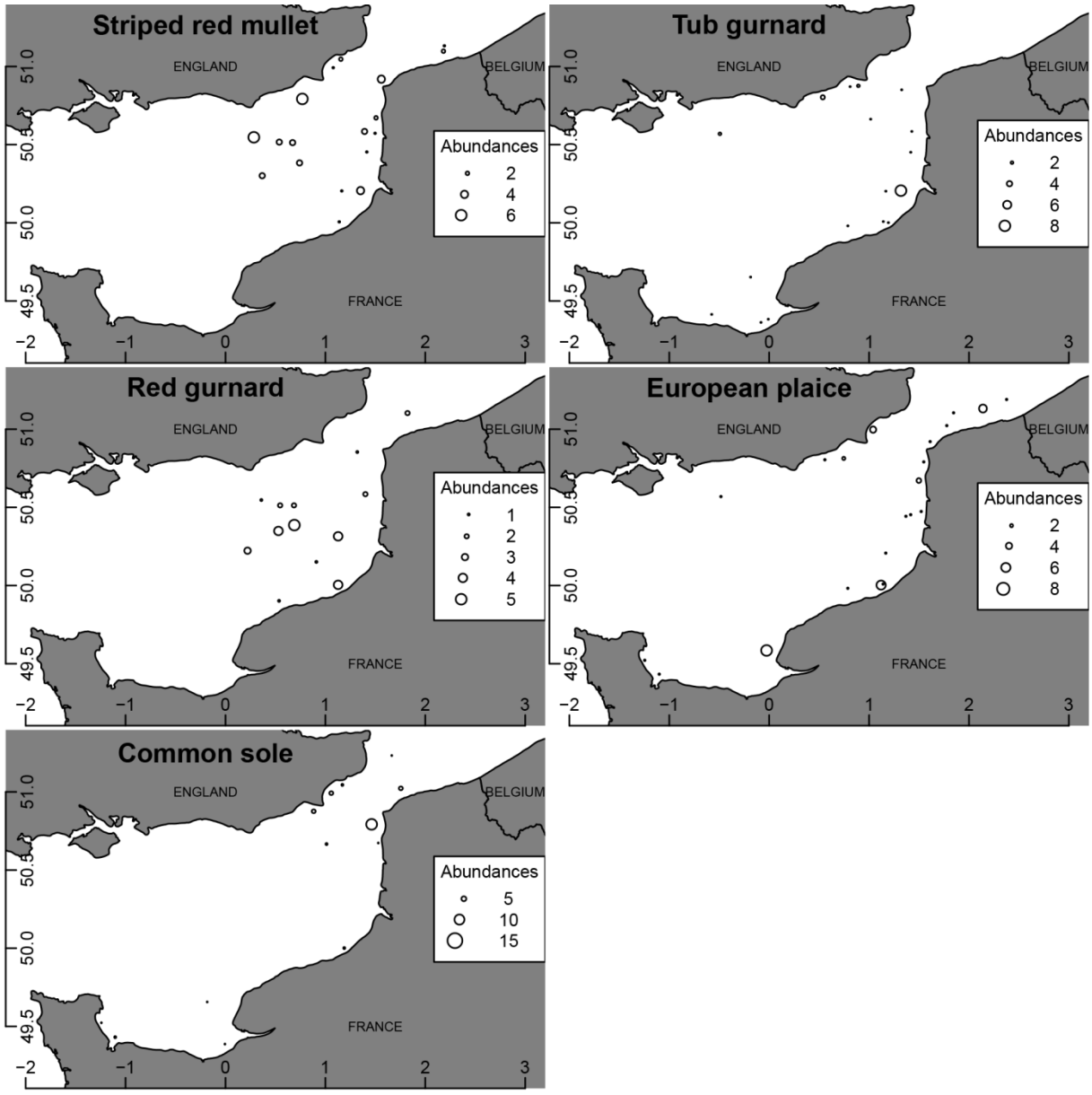


Figure 1: Map of sampling areas in the eastern English Channel according to species. Each circle represents a fishing site and circle size gives the relative sampling abundance.

Back in the laboratory, individuals were defrosted, measured (total length, L_T) to the nearest centimeter and their sex and maturity status were determined by gonads observation according to the recommendations of international expert groups (ICES 2014). Digestive tract was extracted and its contents removed and stored in a Petri-dish for analysis. Sagittal otoliths were also removed from each individual, one of them being used to estimate the individual's age by interpreting macrostructures according to the recommendations of international expert

groups and the second one for shape analysis (Table 1). For striped red mullet, age was estimated by reading macrostructures on the sagittal otolith pair.

The left otolith was red under transmitted light while the right one under reflected light before being burned to confirm the age estimation done previously (ICES 2012). The left otolith (not burned) was then used for shape analysis. For tub gurnard and red gurnard, even if there is no recommendation from ICES expert groups, the same methods proved to be effective and were used. For flatfishes, only the whole left otolith was used to estimate age as well as for shape analysis of European plaice (ICES 2010), whereas for common sole a section of the left otolith were necessary for age reading (Mahé et al. 2012) so that the right otolith was used for shape analysis.

Hereafter, we describe otolith shape analysis, diet analysis and statistical analyses as they were conducted for each species separately.

Otolith shape analysis

Each otolith was cleaned by an ultrasonic bath in water at room temperature during 10 minutes, then brushed to remove residual tissues and stored dry in tubes. Batches of otoliths were automatically digitized at a high resolution (3 200 dpi) using a scanner EPSON V750 and individual images were extracted. An Elliptical Fourier analysis was performed on each otolith contour delineated and extracted after image binarization. This method reconstructs any type of shape with a closed two dimensional contour (Kuhl & Giardina 1982). In this case, each point of the otolith contour is projected on the axes of a two-dimensional coordinate system and the projection is reconstructed using ellipses named harmonics described by a couple of trigonometric equations (one per axis). These equations describe projected contour coordinates as a combination of cosine and sine waves that in turn represent bends in the otolith contour. Each harmonic (H_i) is characterized by 4 coefficients (A_i , B_i ,

C_i and D_i), called Elliptic Fourier Descriptors (*EFD*s), which correspond to the parameters of the trigonometric equations (2 per equation, 1 for cosine and 1 for sine waves). The number of harmonics n used to reconstruct each otolith outline in the sample was determined as follows using the cumulated Fourier power ($P_F(n_k)$). This parameter was calculated for each otolith k as the sum of the proportion of variation in contour coordinates accounted for by each harmonic and it is equal to:

$$P_F(n_k) = \sum_{i=1}^{n_k} \frac{A_i^2 + B_i^2 + C_i^2 + D_i^2}{2}. \quad (1)$$

The number of harmonics n_k was then chosen such that $P_F(n_k)$ reaches 99.99 % of variation in contour coordinates or, in other words, such that shape is reconstructed at 99.99 % (Lestrel 2008). A majority of studies compute the cumulated Fourier power P_F using EFDs averaged across the full sample or part of it, so that the selected harmonics describe the average otolith shape. In order to insure that each individual otolith in the sample was reconstructed with a precision of 99.99 % for this study, $P_F(n_k)$ and n_k were calculated for each individual otolith k. The maximum number of harmonics $n = \max_k(n_k)$ across all otoliths was then used to reconstruct each individual otolith of the sample.

After extracting the n harmonics for each individual otolith, their EFDs were normalized by the first harmonic providing EFDs invariant with respect to size, rotation and starting point (Kuhl & Giardina 1982), and resulting in the degeneration of the first three EFDs (A_i, B_i and C_i) for each individual, respectively equal to 1, ≈ 0 and ≈ 0. EFDs were then gathered in a matrix $\mathbf{F}$ with EFDs as columns and individuals as rows.

All otolith images and morphometric measurements were obtained by the use of the TNPC 7.0 software (www.tnpc.fr).

Diet analysis

For each fish, taxonomic identification of prey items in the stomach content was carried out using a binocular loupe. Prey items were identified to the lowest possible taxonomic level before being weighed (g, wet weight). In view of their high diversity, preys were grouped into 22 categories based on their main *taxonomic level. First, preys were categorized at least according to their* Phylum in the taxonomic hierarchy (e.g. *annelida, cnidaria*). If further taxomonic determination was possible, categories were based on Class (e.g. *cephalopoda, gastropoda*), Order (e.g. *amphipoda, isopoda*) or Infra-Order (e.g. *brachyoura, anomoura*). Teleosts were split in two categories depending on their energetic value, lean and fatty species corresponding to an energetic value inferior and superior to 100 kcal.100g^{-1}, respectively. For each studied species, stomach content data were grouped in a diet composition matrix in terms of weight $\mathbf{W}$, the weight w_{ij} of each prey category j (columns) in the digestive tract of each individual i (rows). In addition, the relative contribution of prey categories to diet composition by weight ($\%W_j = \sum_i w_{ij} / \sum_i \sum_{j'} w_{ij'}$) and the relative frequency of occurrence ($\%F_j$) of each prey category (Godfriaux 1969), were computed for each studied species (Figure 2).

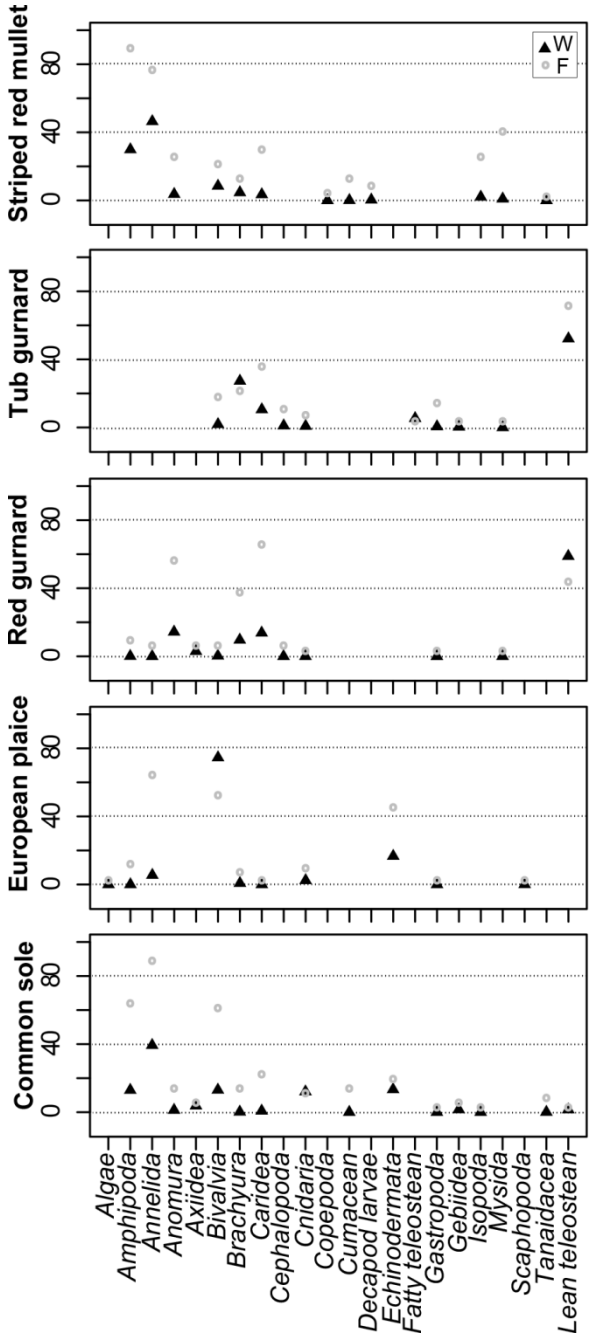


Figure 2: Contribution of prey categories to species specific diet measured in terms of relative weight (W, black triangle) and relative frequency of occurrence (F, grey open circle).

Statistical analysis

A principal component analysis (PCA) combined with broken stick principal component selection (Borcard et al. 2011) was performed on the EFDs matrix $\mathbf{F}$, with the aim to decrease the number of dimensions used to describe otolith shape variability while ensuring that the main sources of shape variation were kept and to avoid collinearity between

them (Rohlf & Archie 1984). The selected principal components were gathered in an otolith shape matrix S representing inter-individual otolith shape variation.

The otolith shape matrix $\mathbf{S}$ was modeled using redundancy analysis (RDA) as depending on three explanatory matrices: an individual matrix $\mathbf{I}$ grouping individual-state variables, an environmental matrix $\mathbf{E}$ grouping external environmental factors, and a diet matrix $\mathbf{D}$ derived from the diet composition $\mathbf{W}$ matrix (Eq. 2). The first two matrices were included in the model to disentangle and remove the effect of individual-state and environmental conditions as possible confounding factors on otolith shape.

$$\mathbf{S} \sim \mathbf{I} + \mathbf{E} + \mathbf{D} \quad (2)$$

The individual matrix $\mathbf{I}$ was composed of fish age A as a factor and total length $L_{\mathbf{T}}$ as a continuous effect to represent the ontogenetic effect on otolith shape, sex $S_{\mathbf{e}}$ and maturity status M of the individual as factors potentially affecting fish physiology and metabolism, and thus indirectly otolith biomineralization.

The environment matrix $\mathbf{E}$ contained four variables to describe environmental conditions that may also affect otolith biomineralization: temperature T and salinity $S_{\mathbf{a}}$ which were extracted from the hydrodynamic model MARS 3D (Lazure & Dumas 2008) and averaged over the month of October 2009 and, the depth $D_{\mathbf{p}}$ which was measured at each sampling station during the survey, and the longitude and latitude points ($L_{\mathbf{o}} \times L_{\mathbf{a}}$) of the catching sampling station (Eq. 3).

$$\mathrm{S} \sim A + L_{\mathbf{T}} + S_{\mathbf{e}} + M + T + S_{\mathbf{a}} + D_{\mathbf{p}} + L_{\mathbf{o}} \times L_{\mathbf{a}} \quad (3)$$

The complete model described by Eq.3 was reduced by a bidirectional elimination procedure based on significance of the effects determined by permutation tests (Borcard et al. 2011). After model reduction, a variation partitioning was performed to estimate the percent contribution of the three reduced matrices $\mathbf{I}$, $\mathbf{E}$, and $\mathbf{D}$ to otolith shape variation. The strict contribution of each reduced matrix $\mathbf{I}$, $\mathbf{E}$, $\mathbf{D}$ to variation was tested using partial redundancy

analysis (pRDA) followed by a permutations test, with the matrix tested as explanatory matrix and the two other matrices as covariables.

In order to answer the three main questions of the study, the previously described analysis was performed with the diet matrix **D** constructed in three different ways (Figure 3) as described below.

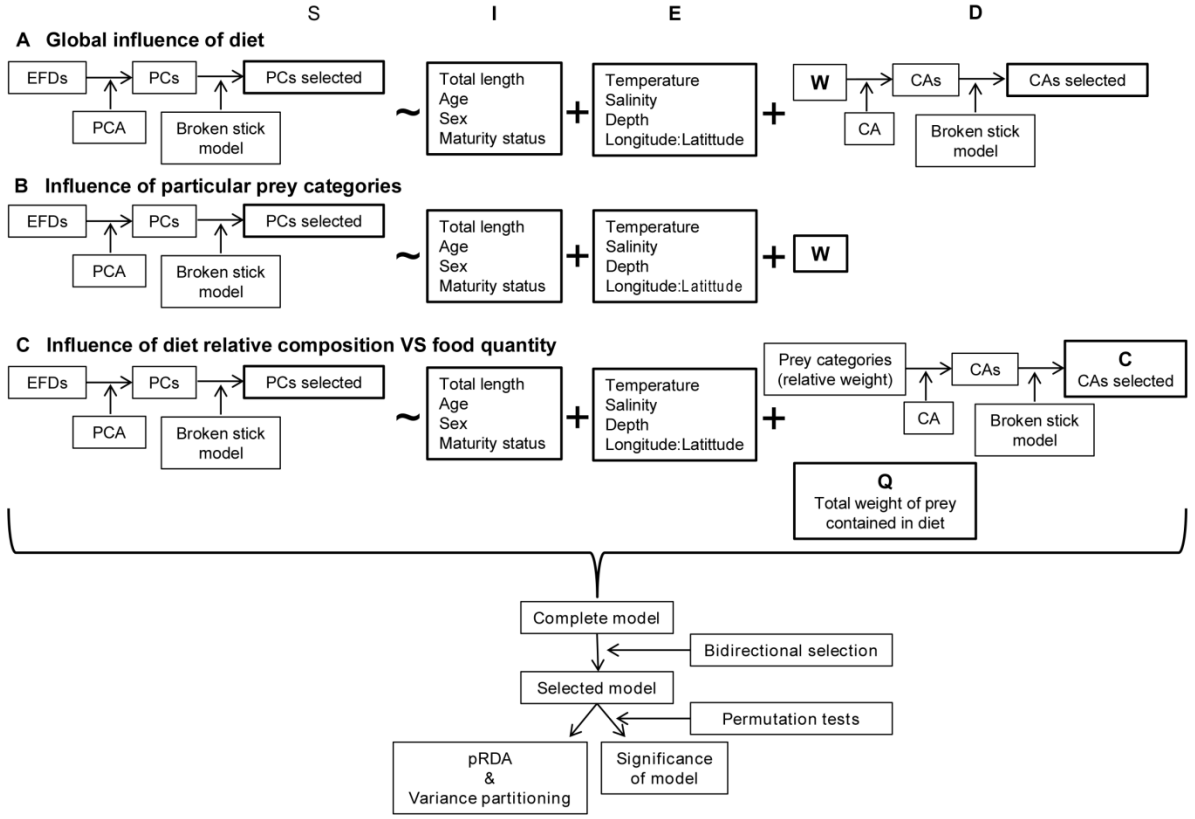


Figure 3: Schematic representation of the sequential steps of the three RDA statistical analyses performed to identify the potential influence of (A) diet (model 1), (B) the prey categories (model 2) and(C) the relative effects of food composition and quantity (model 3).

Global influence of diet (model 1)

In order to estimate the potential global effect of diet on otolith shape (Figure 3A), matrix **D** was composed of a number of selected correspondence axes resulting from a correspondence analysis (CA) applied to the diet composition matrix **W**. Correspondence axes were selected according to the broken stick method. As for the PCA applied to EDFs, this analysis was chosen to decrease the number of dimensions used to describe fish diet variation and to remove collinearity between prey categories. Moreover, CA is a method

adapted to the analysis of species abundance data without pre-transformation because abundance data within prey categories are not normally distributed (Borcard et al. 2011). In order to ensure that matrix **D** was kept in the reduced model, model reduction was based on a pRDA where the otolith shape matrix **S** was explained by matrices **I** and **E** while matrix **D** was considered as covariables. Hence, matrices **I** and **E** used in variation partitioning were reduced, while matrix **D** was not.

Influence of particular prey categories (model 2)

In this analysis, matrix **D** (Figure 3B) was simply set equal to the diet composition matrix **W**. Model reduction was directly performed on Eq. 3 (with D = W) and hence affected individual-state and environmental variables but also prey categories. Hence, matrices **I**, **E** and **D** used in variation partitioning were all reduced. In order to identify among the selected prey categories those really related to otolith shape, permutations tests were performed for each selected prey categories to test their significance. Moreover to illustrate the effects of the prey categories consumed on otolith shape, 8 predicted otolith shapes were produced for each species in the following way. A pRDA was performed on the otolith shape matrix **S** with the selected prey categories as explanatory variables and the selected individual-state and environmental variables as covariables. From this model, 8 sets of coordinates in the matrix **S** space were predicted at the 8 combinations of ±1 standard deviation (sd) along the two first axes of the pRDA $\{(+sd_1,0),(-sd_1,0),(+sd_1,+sd_2),(-sd_1,+sd_2),(+sd_1,-sd_2),(-sd_1,-sd_2),(0,+sd_2),(0,-sd_2)\}$ representing variation in linear combinations of the selected prey categories. Predictions in the matrix **S** space were then projected back to the original space of *EFDs* and predicted *EFDs* were then used to produce predictions of otolith shape. Predicted otolith shapes were then drawn on the biplot of the model (Figure 5).

Influence of diet relative composition VS food quantity (model 3)

In this last analysis, matrix $\mathbf{D}$ was decomposed into a matrix representing the relative diet composition $\mathbf{C}$ and a vector representing food quantity $\mathbf{Q}$ (Figure 3C). The relative diet composition matrix $\mathbf{C}$ was obtained by performing a CA on the matrix of relative contribution of prey categories to diet composition $\%W_j$ and selecting correspondence axes according to the broken stick method. The vector $\mathbf{Q}$ gathered the total weight of the stomach content of each individual, in other words the sums along rows $\sum_j w_{ij}$ of the diet composition matrix $\mathbf{W}$. In order to ensure that matrices $\mathbf{C}$ and $\mathbf{Q}$ were kept in the reduced model (with the ultimate aim to estimate their relative contribution to otolith shape variation), model reduction was based on a pRDA where the otolith shape matrix $\mathbf{S}$ was explained by matrices $\mathbf{I}$ and $\mathbf{E}$ while matrices $\mathbf{C}$ and $\mathbf{Q}$ were considered as covariables. Hence, matrices $\mathbf{I}$ and $\mathbf{E}$ used in variation partitioning were reduced, while matrices $\mathbf{C}$ and $\mathbf{Q}$ were not.

All statistical analyses were performed using the package ‘vegan’ (Oksanen et al. 2013) in the statistical environment R (R Core Team 2014).

Results

The effects of individual state and environmental conditions on fish otolith shape have already been studied previously in detail by Hüssy (2008) and Capoccioni et al. (2011). They were accounted for in our analyses to avoid potential confounding effects but were not discussed in this paper as these were not the main focus of this study. Consequently, their effects are described in Annex 1.

Global influence of diet on otolith shape (model 1)

The selected models explained between 15.63 % and 25.61 % of otolith shape variability for roundfish species. For flatfish species, the percentages of explained variation were higher: 31.94 % and 37.50 % for plaice and sole, respectively (Table 2).Variation partitioning revealed that the individual matrix **I** explained the greatest part of variation in otolith shape for all species, between 19 % and 27 %, except for red gurnard for which it was not significant (Figure 4, first column). A significant relationship between the environmental matrix **E** and otolith shape was detected in European plaice only, with matrix **E** explaining 14 % of otolith shape variation. For all species, a significant diet contribution to otolith shape variability was detected at a threshold of 5 %, except for tub gurnard for which it was significant at a threshold of 10 % (Table 2). Matrix **D** explained between 10 % and 16 % of otolith shape variability (Figure 4).

Influence of particular prey categories on otolith shape (model 2)

According to species, 2 to 7 prey categories were kept in model 2 (Table 2) except for tub gurnard for which no prey categories was selected and, hence, no selected model was tested. Explained variation by model 2 varied between 22.08 % and 35.11% according to species. As in model 1, the individual matrix **I** contributed significantly to otolith shape variability for all species except for red gurnard (Figure 4, second column). It explained between 15% and 19% of shape variability, which was slightly less than in model 1.

Table 2. Results of the three RDA models (as detailed in figure 3) estimated for the different species. The otolith shape describes the response matrix with the number of principal components (N PCs) used to describe otolith shape and the explained variance (%). Environment, Individual and Diet correspond to explanatory matrixes. Environment and Individual show the selected environmental and individual variables, respectively. Diet indicates the variables used in the diet matrix; the number of correspondence axis (N CAs) and the explained variance (%) used to describe the diet and the food composition, respectively, for models 1 and 3 and the prey category for model 2. The F statistic and the variability explained by each model is presented with the degrees of freedom (df) and the corresponding P-value.

Species	Otolith shape		Environment	Individual	Diet		Model selected			
	N PCs	%					df	F	P-value	% explained
Global influence of diet (model 1)					N CAs	%				
Striped red mullet	4	77		Size Sex	10	99.85	13	2.22	0.001	25.61
Tub gurnard	2	70.18	Longitude : Latitude	Age Sex	4	90.59	9	1.77	0.052	20.43
Red gurnard	3	77.84	Longitude : Latitude Temperature		4	94.01	6	1.96	0.032	15.63
European plaice	3	74.02	Temperature Salinity	Age Sex Maturity	4	93.53	15	2.28	0.001	31.94
Common sole	3	78.23		Age Size Sex	8	95.32	16	2.31	0.003	37.50

Table 2 (continued)

Influence of particular prey categories (model 2)				Prey category				
Striped red mullet	4	77		Size Sex	Anomura Bivalvia Brachyura Caridea Isopoda Annelida Decapod larvae	10	3.49	0.001	35.11
Tub gurnard									
Red gurnard	3	77.84	Longitude : Latitude Temperature Depth	Size Age Maturity	Annelida Axiidea Mysida Gastropoda Teleostean Caridea Cephalopoda	15	1.59	0.072	22.08
European plaice	3	74.02	Depth	Age	Amphipoda Brachyura Echinodermata	10	2.85	0.001	31.10
Common sole	3	78.23	Temperature	Age Size	Anomura Cnidaria	9	2.72	0.002	30.67
Influence of diet relative composition *vs* food quantity (model 3)					N CAs	%			
Striped red mullet	4	77		Size Sex	10	99.87	14	2.19	0.001	26.66
Tub gurnard									
Red gurnard	3	77.84	Longitude : Latitude Temperature	Maturity	4	94.23	8	1.88	0.021	18.55
European plaice	3	74.02	Temperature Salinity	Age Sex Maturity	4	92.64	16	2.12	0.005	30.44
Common sole	3	78.23	Temperature Longitude : Latitude	Size	9	96.78	16	2.31	0.003	25.37

A significant relationship between environment and otolith shape was detected for common sole and matrix **E** explained 6 % of otolith shape variation. Concerning prey categories, a significant contribution to otolith shape variability was detected for all species (except tub gurnard, see above). The selected prey categories explained between 10 % and 21 % of otolith shape variability, which was slightly higher than the global effect of diet in model 1. According to species, the prey categories impacting the otolith shape were different in terms of relative frequency of occurrence $\%F_j$ and relative contribution of prey categories to diet $\%W_j$. For striped red mullet, the prey categories with an effect on otolith shape (Figure 5) were either primary ones characterized by a high $\%F_j$ and small $\%W_j$ such as *annelida*, or intermediate ones characterized by a small $\%W_j$ with respect to $\%F_j$ such as *caridea*, or secondary ones with a low $\%F_j$ and small $\%W_j$ such as *bivalvia*, *brachyoura*, and *decapoda* larvae (Figure 2). Likewise, for European plaice, influential prey categories were either primary ones such as *echinodermata* or secondary ones such as *brachyoura* and *amphipoda*. For red gurnard and common sole, only secondary prey categories influenced otolith shape: *gastropoda* and *annelida* for red gurnard and *cnidaria* and *anomura* for common sole.

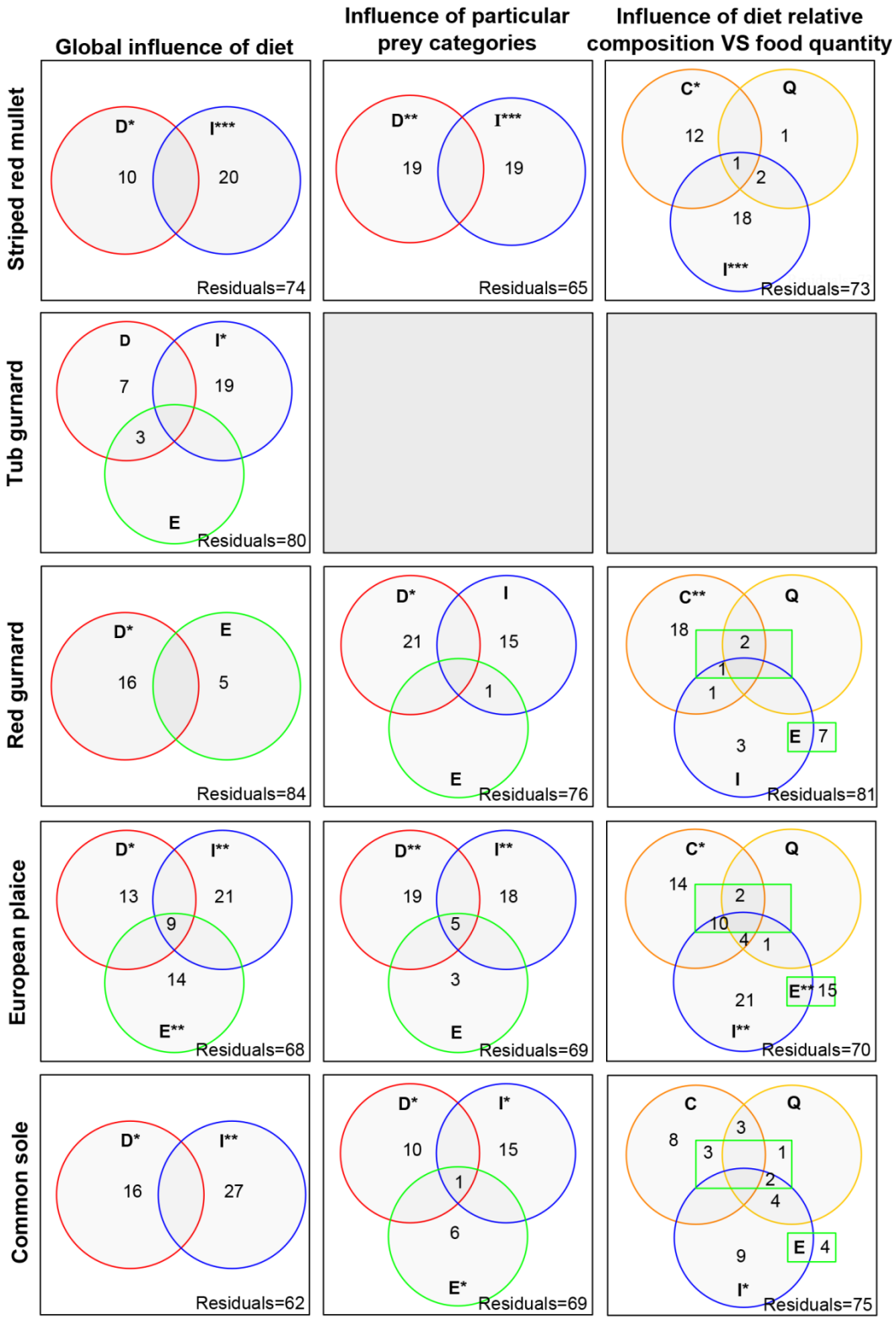


Figure 4: Variance partitioning between selected variables according to three models (columns) and the studied species (lines) : diet variables matrix (D), individual variables matrix (I), environment variables matrix (E), food composition (C) and food quantity (Q). Corresponding P-values are indicated by the following symbols: <5 %*, <1 %, <0.1 %***. The number in each circle represents the variance explained in the model by each variables matrix and the correlations between them. The lack of numerical value means that the variance explained was equal or inferior to zero.**

Predicted otolith shapes as reconstructed in Figure 5 revealed that diet influenced global otolith shape through the length/width ratio and thus otoliths' ellipticity, but also finer details. For roundfishes, variations occurred in the otolith crenations, the width of the *excisura major* (the indentation between the rostrum and the antirostrum), and the length of the rostrum and the antirostrum, whereas for flatfishes, diet appeared to impact rather the posterior and dorsal parts of the otolith shape.

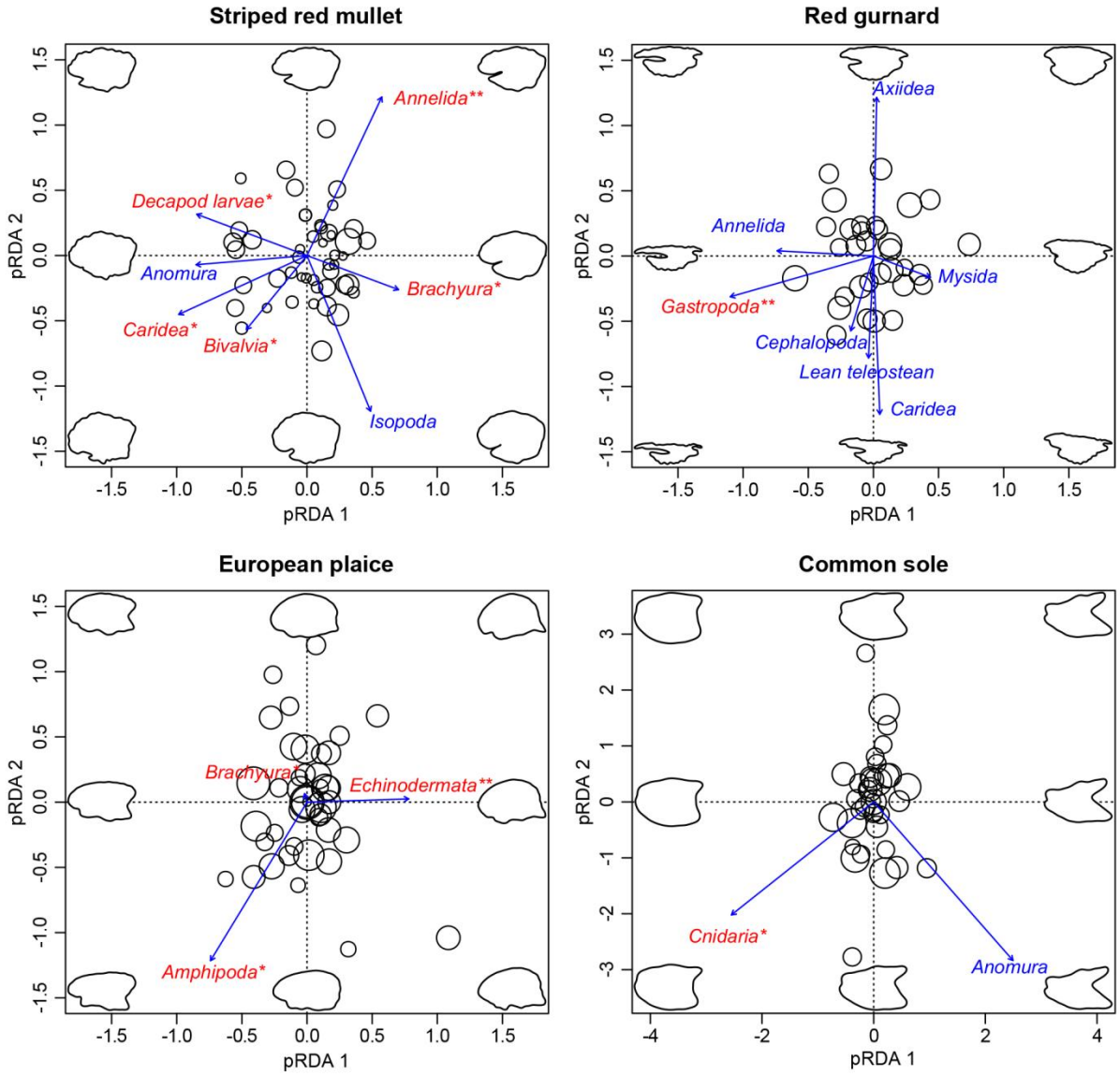


Figure 5: pRDA biplot of otolith shape variability constrained by selected prey categories and conditioned by selected individual and environmental variables (model 3) according to species. For each species, eight otolith shapes have been reconstructed from model predictions illustrating the relationship between diet and otolith shape. Explanatory variables with a significant effect (permutation test) on otolith shape are in red, the corresponding P-value being indicated by the following symbols: <5%*, <1%, <0.1 %***. Each circle represents an individual and its size represents its total length.**

Influence of relative diet composition *vs* food quantity on otolith shape (model 3)

For tub gurnard, model 3 was not estimated given the absence of diet effect in model 1 (and subsequently model 2). For other species, model 3 explained between 18.55 % and 30.44 % of otolith shape variability (Table 2). Variation partitioning gave similar results in terms of individual and environmental effects to those obtained with model 1 except for common sole for which the explained variation by the individual matrix **I** decreased (Figure 4, third column). Concerning diet, relative composition **C** was detected as contributing significantly to otolith shape variability for striped red mullet, red gurnard and European plaice. Its effect explained 12 % to 18 % of otolith variation. No significant relationship between **C** and otolith shape was found for common sole even if variation partitioning attributed 8 % of otolith shape variation to this matrix. Contrary to diet relative composition **C**, food quantity **Q** was not significantly related to otolith shape whatever the species and explained at very low percent any variation of otolith shape.

Discussion

In this study, we showed an intra-population relationship between diet and otolith shape for all fish species studied, although a less robust link was observed for tub gurnard. For the latter, this may be due to the number of samples analyzed which was smaller compared to the other species studied which may explain a lower power of signal detection since diet was significantly related to otolith shape at a 10 % threshold. Then we were able to relate some prey categories, be they primary or secondary ones, to otolith shape variations. Moreover, otolith reconstructions showed that these variations could affect both global shape and finer details. Lastly, by comparing the contributions of food composition and quantity, we showed that food composition was a larger source of otolith shape variation than the quantity of food ingested by fish.

The role of organic matrix composition in otolith biomineralization

Otoliths are mostly composed of calcium carbonate in aragonite form deposited on an organic matrix that represents a minor part of the total material (Carlström 1963, Degens et al. 1969). However, the otolith matrix plays an important role in its formation since it controls the nucleation, the crystallization, the orientation and the morphology as well as the polymorphism of crystal units it is composed of (Nagasawa 2013). Organic matrix is mainly composed of proteins, amino acids (AAs), collagens and proteoglycans which precursors are secreted by the saccular epithelium in the endolymph (Payan et al. 2004). However, only 3 major proteins are present in their definite form both in the endolymph and the otolith. This suggests that the organic matrix is not directly composed of compounds present in endolymph but also of proteins derived from the modification of precursors during their deposition onto the otolith (Borelli et al. 2001). McMahon et al. (2010) observed that AAs' $\delta^{13}C$ values in fish muscle and in their diet co-varied, with significant differences diet treatments. Moreover, they showed that AAs' $\delta^{13}C$ values in muscles and in otoliths were correlated with a slope around 1 and thus recorded an identical dietary information (McMahon et al. 2011). Consequently, the AAs found in otolith proteins come from the food consumed by fish.

Otolith shape is determined by its crystalline architecture (calcium carbonate $CaC0_3$). Several proteins are known to control the $CaC0_3$ polymorphism (aragonite, calcite or vaterite) and its morphology of crystal units. Starmaker (Söllner et al. 2003) and Otolith Matrix Macromolecule-64 (OMM-64) (Tohse et al. 2009) are water-soluble and acidic (due to a calcium-binding region rich in glutamate) glycoproteins involved in the control of crystal polymorphism (Nagasawa 2013). The Otolith Matrix Protein-1 (OMP-1) is another water-soluble protein required for normal otolith growth and for the deposition of another otolith protein, otolin-1. The latter is a collagenous protein that makes up the structural network for

subsequent calcification, and thus stabilizes the otolith's mineral and organic fractions and insures the correct arrangement of otoliths on the sensory epithelium (Murayama et al. 2005).

Potential mechanisms of diet composition influence on otolith shape

In the present study, a significant relationship between diet taxonomic composition and otolith shape was detected for all species except for one. According to the prey categories consumed, otolith shape presented some variations in both global shape, such as the degree of ellipticity, and finer details, such as otolith crenations or the width of the *excisura major*. Two hypotheses, or a combination of both, could explain the correlation between otolith shape and food composition variation. First, the total quantity of proteins in the saccular endolymph could vary according to the quantity of proteins in the prey consumed, which would affect the rate of organic matrix synthesis and thus $CaCO_3$ deposition and ultimately otolith growth. Protein consumption has been known to have the highest regulatory impact on protein synthesis (Houlihan et al. 1988). Consequently, food composition could influence "global" otolith shape through effects on otolith growth. Secondly, the proteic composition of the organic matrix, i.e. the relative quantity of water-soluble, water-insoluble and insoluble proteins, may change according to food composition, which would impact the crystal structure (orientation, morphology, and polymorphism) of precipitated $CaCO_3$ and, thus, otolith shape. More precisely, food composition varies in terms of proteins or even AAs, especially the essential AAs, such as leucine, that are necessary for the synthesis of some otolith matrix proteins involved in the control of crystal structure. Consequently, food proteic composition could have a direct contribution to variations in otolith crenulations or/and an indirect contribution through its effect on otolith growth, which impacts global otolith shape. In addition, some proteoglycan and polysaccharide are present in both the saccular endolymph and the organic matrix (Murayama et al. 2005). Even if their role in otolith

biomineralization is unknown, variability in their quantity and composition in prey could also impact otolith shape in the same way as proteins. In this study, the location of variations in otolith shape related to food composition were identified from reconstructed shapes. Although these reconstructions were "caricatures" predicted from a statistical model limited to individuals from the eastern English Channel and to our observations in terms of individual-state, they highlighted the large number of otolith shape areas impacted by food composition suggesting the importance of understanding otolith biomineralization 3D processes. Otolith shape variation could be also explained by variations of spatial distribution of precursors spatialization due to some physical constraints on the otosac which would impact the otolith shape. However, the current lack of knowledge regarding such processes prevents from evaluating the likelihood of this hypothesis.

Absence of food quantity influence on otolith shape

No significant relationship between food quantity and otolith shape was detected in this study. This result contrasts with several works who showed experimentally that food quantity impacted otolith shape both indirectly via variation in otolith growth creating variation in "global" otolith shape and directly on otolith crenations (Gagliano & McCormick 2004, Cardinale et al. 2004, Hüssy 2008). We could raise the assumption that in the present study, the quantity of ingested food did not differ sufficiently between individuals in order to observe a significant influence on otolith shape. Likewise, Hüssy et al. (2004) did not observed any effect of food quantity on otolith opacity and on the ratio between water-soluble and the water- insoluble proteins in the organic matrix whereas, under more severe food restriction for a longer period, Høie et al (2008) observed that more translucent otolith material was deposited. Such apparent discrepancies are well reconcile under the light of interactions between temperature and food effects (either synergetic or antagonistic) on otolith

opacity (Fablet et al. 2011). Moreover, here food quantity was measured as the sum of the weights of all prey items found in an individual's stomach. However, prey items in stomach contents are digested at varying degrees according to individuals, which can introduce a bias in the estimation of inter-individual differences in ingested food quantity (Gannon 1976).

Limitations of the study

The imprecision of food quantity measure highlights a potential, more general, limitation of stomach contents analysis that only provides a snapshot of fishes' diet. In this study, fishes were sampled over only a month (October) only. The interpretation of the results thus relies on the assumption that observed inter-individual differences in diet may be consistent but may need a sufficiently long period of time to be related to inter-individual otolith shape differences. It should be noted here that the assumption is concerned with the representativity of inter-individual differences, i.e. individuals' specialization, and not of individuals' diet itself. In other words, the assumption is that diet difference at a given time gives an index of dietary specialization even though individuals' diet may vary through time. To our knowledge, such an assumption has never been directly confirmed nor invalidated in fish given that no longitudinal study on fish diet, i.e. with repeated observations of prey selectivity or stomach content on the same individuals, was performed for testing. Although the possibility that this hypothesis does not hold cannot be totally ruled out, several arguments can be brought in its support. There is ample literature on the importance and prevalence of individual diet specialization (see reviews in Bolnick et al. 2003; Araújo et al. 2011). This has been notably observed on long-term trophic specialization in freshwater and marine vertebrates (e.g. Bearhop et al. 2006, Newsome et al. 2009, Hückstädt et al. 2011, Rosenblatt et al. 2015) including fish (e.g. Beaudoin et al. 1999, Svanbäck & Persson 2004, Matich et al. 2011) which support this assumption based on isotopic data. Consistent inter-individual differences in several behavioural traits that may affect diet have also been documented in

fish (see review in Mittelbach et al. 2014) such as habitat use and movements (e.g. Matich & Heithaus 2015) or boldness (e.g. Ward et al. 2004, Harcourt et al. 2009). A more technical argument is that the presence of multiple prey items per stomach ensures that cross-sectional samples of individuals' diet are relevant to estimate individual diet specialization (Araújo et al. 2011). It should also be noted that, despite its limitations, stomach content analysis is the only way to obtain an indication of ingested food quantity in natural condition. In contrast, carbon stable isotope ratios could provide a temporally-integrated view of individuals' diet composition and account for seasonal changes in diet, but without allowing the quantification of the amount of food ingested. Additionally, the precise identification of the consumed prey items from carbon stable isotope ratios when using the so-called mixing models requires knowledge of the isotopic ratios of all potential preys and of the isotopic fractionation between preys and consumers (Post 2002, Fry 2007). Still, variation of carbon stable isotope ratio (in muscles and/or otolith) across individuals could be used to describe individual diet composition changes linked to otolith shape variability. Such analysis would complement the results obtained in this study. Moreover, individuals' diet could be represented by an energy content value based on energetic prey categories determined with appropriate literature. As for taxonomic composition, energetic composition of stomach contents will only provide a snapshot of the energy content of individuals' diet. One way to obtain a temporally-integrated measure of individuals' diet energetic content would be to use somatic growth back-calculation based on otolith macrostructure associated with an energy allocation model in order to determine individuals' energy acquisition rate (Brunel et al. 2012). This method could be efficient to disentangle the direct and the indirect (through otolith growth) influences of diet on otolith shape.

Likewise, the environmental variables i.e. temperature, depth, salinity, longitude and latitude, represent a snapshot of the habitat experienced by individuals as they were measured

at the same sampling site and were averaged over a single month. Similarly to stomach contents, their use in the analyses relies on the assumption that they are representative of inter-individual differences of the environment experienced over a sufficiently long-time period to be related to otolith shape. Such an assumption may seem unlikely considering fish mobility which could explain why the environmental matrix was not significantly related to otolith shape for the majority of the studied species (Figure 4). In order to have a wide temporally-integrated view of the environment experienced by individuals, otolith chemistry analyses such as the oxygen isotopic ratios variation as an index for temperature (Kalish 1991) or the Sr/Ca ratio as an index for salinity (Secor 1992) could be used in future studies. It should be noted that fishes considered in this study have all been sampled in October representing only a single season. It would be interesting to consider the implication of seasonality on the relationship between diet and environment on the one hand and otolith shape on the other.

In summary, an intra-population relationship between diet and otolith shape was detected for several roundfish and flatfish species sampled in the English Channel. Detailed analyses revealed that both main and secondary prey categories partially explain otolith shape variability and that this influenced both the otolith's global shape and some finer details. Finally, the contribution of diet relative composition to otolith shape variation was much higher than that of ingested food quantity represented by the weight of prey items. Gagliano and McCormick (2004) had suggested that otolith shape could be used to discriminate fine scale events, such as the magnitude and periodicity of feeding in wild fish populations, in addition to the discrimination of stocks and populations based on coarser aspects such as life-history differences. The present study shows that diet composition is also a source of otolith shape variability through direct and/or indirect (via otolith growth) ways. This introduces a

novel potential interpretation of three classically known effects on otolith shape. First, otolith shape variation across age and size is generally assigned to ontogenetic changes in metabolism and physiology (Campana & Casselman 1993, Mérigot et al. 2007). Ontogenetic changes in diet composition could also contribute directly to otolith shape variation, thereby acting as a confounding factor (Morat et al. 2012, Vignon 2012). Likewise, sexual dimorphism in otolith shape is generally attributed to physiological differences between sexes. However, sexual dimorphism in diet composition, especially at the time of mating, has been documented in several fish species (Casselman & Schulte-Hostedde 2004, Tsuboi et al. 2011) and could thus also explain otolith shape dimorphism. Finally, environmental abiotic factors, such as temperature and salinity, are also known to influence otolith shape variation (Lombarte & Lleonart 1993) and spatial variation in otolith shape are often interpreted as resulting from habitat differentiation (Morat et al. 2012). However, such variation in abiotic factors is generally related to differences in prey categories available to individuals, such that geographical variation in diet composition could also generate geographical variation in otolith shape (Vignon 2012).

Annex I. Individual variables, factors (A : age, L_T : total length, Se : sex, M : maturity) and environmental variables (T : temperature, Sa : salinity, D : depth, $L_o \times L_a$: interaction of the longitude and latitude) acting on otolith shape according to the model and the species studied. For each species, effects kept in the model selected by AIC-based bidirectional elimination are indicated by the *F* statistic (while respecting effect marginality, type 2 tests) with numerator degrees of freedom as exponent and denominator degrees of freedom as index together with the corresponding *P*-value indicated by the following symbols: <5 %*, <1 %, <0.1 %***.**

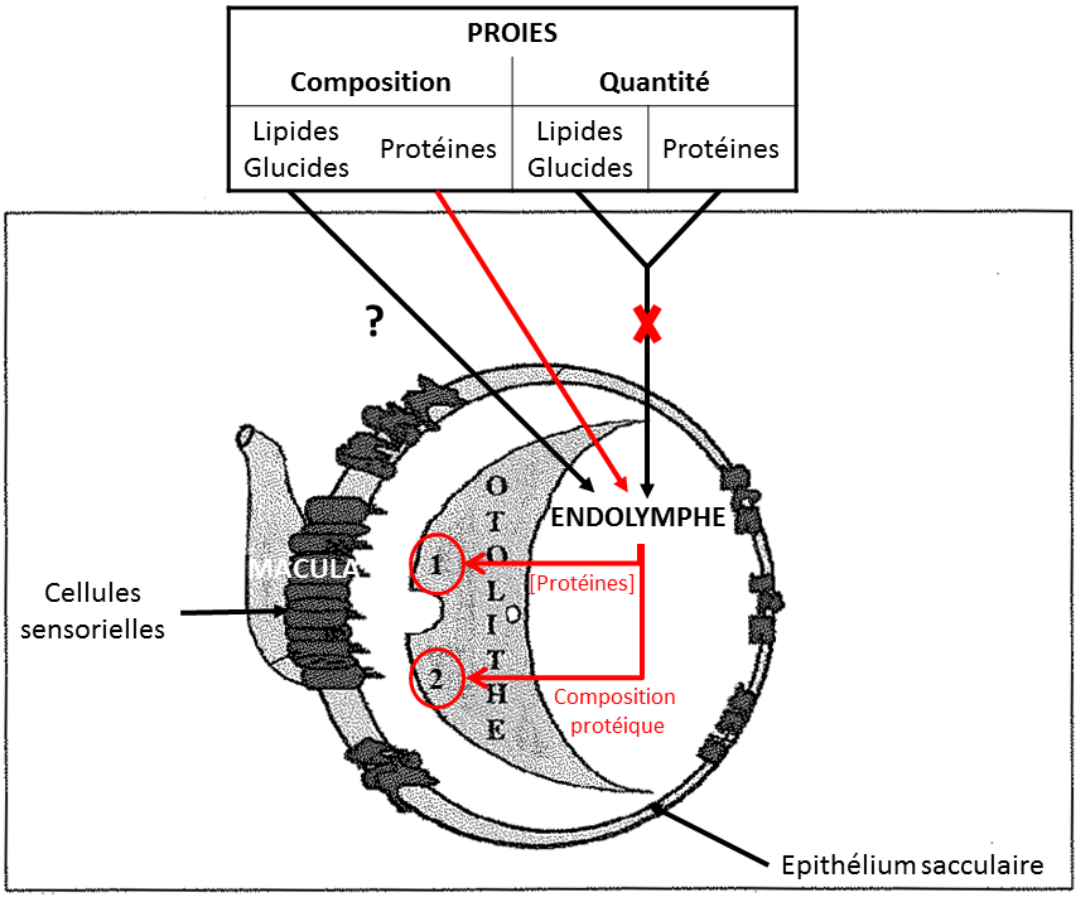
Species	A	L_T	Se	M	T	Sa	D	$L_o \times L_a$
Influence of diet								
Striped red mullet		$2.98\,^{1}_{33}$ *	$4.46\,^{2}_{33}$ ***					
Tub gurnard	$2.64\,^{3}_{18}$ *		$5.67\,^{1}_{18}$					$0.16\,^{1}_{18}$
Red gurnard					$3.02\,^{1}_{25}$ *			3.10^{1}_{25} *
European plaice	$2.11\,^{6}_{26}$ *		$3.00\,^{2}_{26}$ *	$2.40\,^{1}_{26}$	$3.56\,^{1}_{26}$ *	$4.29\,^{1}_{26}$ **		
Common sole	$2.30\,^{5}_{19}$ *	$2.87\,^{1}_{19}$ *	$2.45\,^{2}_{19}$ *					
Influence of prey categories								
Striped red mullet		$4.41\,^{1}_{36}$ *	$4.41\,^{2}_{36}$ ***					
Tub gurnard								
Red gurnard	$1.30\,^{3}_{16}$	$0.17\,^{1}_{16}$		$2.54\,^{1}_{16}$	$0.73\,^{1}_{16}$			$0.53\,^{1}_{16}$
European plaice	$2.65\,^{6}_{31}$ **						$2.44\,^{1}_{31}$	
Common sole	$1.62\,^{5}_{26}$	$3.13\,^{1}_{26}$ *			$3.15\,^{1}_{26}$ *			

Annex 1 (continued)

Food composition *vs* food quantity								
Striped red mullet		$3.80\,{}^{1}_{32}$ **	$4.38\,{}^{2}_{32}$ ***					
Tub gurnard								
Red gurnard				$1.98\,{}^{1}_{23}$	$3.73\,{}^{1}_{23}$ *			$3.64{}^{1}_{23}$ *
European plaice	$2.02\,{}^{6}_{25}$ *		$3.01\,{}^{2}_{25}$ *	$2.58\,{}^{1}_{25}$	$3.34\,{}^{1}_{25}$ *	$4.25\,{}^{1}_{25}$ **		
Common sole		$3.87\,{}^{1}_{22}$ *			$2.13\,{}^{1}_{22}$			$0.64\,{}^{1}_{22}$

Schéma conceptuel des hypothèses soulevées dans le chapitre 2

Hypothèses soulevées pouvant expliquer la variabilité morphologique et donc les différences de biominéralisation des otolithes selon le régime alimentaire de l'animal.



1 La quantité totale de protéines dans l'endolymphe sacculaire peut varier en fonction de la quantité de protéines dans les proies consommées. La vitesse de synthèse de la matrice organique et donc de dépôt de $CaCO_3$ serait donc impactée affectant la vitesse de croissance otolithe et donc sa « forme globale ».

2 La composition protéique de la matrice organique, à savoir le ratio entre les protéines solubles et insolubles dans l'eau, peut changer en fonction de la composition des aliments, impactant la structure cristalline (orientation, la morphologie et le polymorphisme) du $CaCO_3$ précipité et, par conséquent, la forme des otolithes. Par conséquent, la composition protéique alimentaire pourrait avoir une contribution directe aux variations de forme de l'otolithe ou / et une contribution indirecte à travers son effet sur la croissance des otolithes, ce qui affecte la forme globale des otolithes.

Chapitre 3
Diet composition influences otolith shape of sea bass (*Dicentrarchus labrax*) to a limited extent due to ontogenetic canalization

- Publication en cours de rédaction

ABSTRACT

Understanding the sources of otolith morphological variation is a prerequisite for using otolith shape as an indicator of fish population or age. Otolith shape results from genetic, ontogenetic and environmental factors that have confounding effects on otolith shape variability. Previous studies have shown that food amount affects otolith structure, opacity and shape, and that diet composition impacts otolith chemical composition. In this study, the potential effect of diet composition on otolith growth and morphogenesis in sea bass (*Dicentrarchus labrax*) was investigated under controlled conditions. More precisely, the impact of a reduced dietary concentration of some essential dietary fatty acids on sagittal otolith ontogenetic trajectories was evaluated at early life stages. Individuals were exposed to dietary treatments during two consecutive periods, at larval and juvenile stages, according to a partial factorial design. Individual otolith ontogenetic trajectories were followed by oxytetracyclin marking of otolith structure and shape described by geometric morphometrics. The effect of the two dietary treatments on body mass growth, otolith growth and otolith morphogenesis was assessed while accounting for the allometry with body mass. A delayed effect of the larval dietary treatment was detected on otolith shape of 7-month-old juveniles and on otolith morphogenesis during the 2 following months. In contrast, dietary treatments had no detectable effect on somatic and otolith growth. This indicates that diet composition can affect otolith morphogenetic trajectories independently from the relationship between somatic and otolith growth. However, effect magnitude was weak, juvenile dietary treatment had no immediate effect on otolith morphogenesis and none of the dietary treatments affected final otolith shape at 9 months old. Together, these results suggest a compensatory response such that otolith shape variation generated by phenotypically plastic responses to larval dietary treatment is compensated for by morphogenetic trajectories converging back to a similar shape during the juvenile phase. This compensatory response could be interpreted as ontogenetic canalization of otolith shape related to its role in audition and equilibration. Further studies on the influence of diet composition on otolith shape should focus on dietary amino acid composition rather than fatty acids as they are more directly involved in the biomineralization process.

Keywords: n-3 highly polyunsaturated fatty acids; eicosapentaenoic acid; docosahexaenoic acid; geometric morphometrics; otolith morphogenesis.

RESUME

La variabilité morphologique des otolithes résulte de l'influence de facteurs endogènes, exogènes et ontogénétiques. De ce fait, il est nécessaire d'identifier ces sources de variation pour utiliser la forme des otolithes comme indicateur des populations de poissons. Des études précédentes se sont intéressées à l'influence de la quantité de nourriture sur la structure, l'opacité et la forme des otolithes.

De plus, la composition alimentaire influence aussi la composition chimique de l'otolithe; c'est pourquoi cette étude s'est intéressée à l'influence de la composition alimentaire en termes de concentrations lipidiques essentiels sur la morphogénèse et la croissance des otolithes chez le bar commun (*Dicentrarchus labrax*) en milieu contrôlé. L'impact d'une réduction de concentration en acide gras polyinsaturés essentiels a été évalué sur les trajectoires ontogénétiques des *sagittae* aux stades larvaire et juvénile. Durant cette expérience, les poissons ont été exposés à un premier traitement alimentaire au stade larvaire puis à un second au stade juvénile suivant un plan expérimental factoriel partiel. Dans le but de suivre individuellement les trajectoires ontogénétiques des otolithes, ces derniers ont été marqués à l'oxytétracycline et la géomorphométrie a été utilisée pour décrire la variabilité de forme. L'effet des deux traitements alimentaires sur la croissance somatique du poisson et sur la croissance de l'otolithe et sa morphogenèse a été évalué en tenant compte de l'allométrie avec la masse du poisson. Un effet retard du traitement alimentaire au stade larvaire a été détecté sur la forme des otolithes des juvéniles âgés de 7 mois ainsi que sur la morphogenèse des otolithes pendant les 2 mois suivants. En revanche, les traitements alimentaires n'ont eu aucun effet significatif sur la croissance somatique et celle de l'otolithe. Ces résultats indiquent que la composition lipidique du régime alimentaire peut affecter directement les trajectoires morphogénétiques des otolithes; indépendamment de la relation entre somatique et la croissance des otolithes. Cependant, l'effet était de faible ampleur, le second traitement alimentaire donné au stade juvénile n'avait pas d'effet significatif immédiat sur la morphogenèse des otolithes et aucun traitement alimentaire n'a affecté la forme finale des otolithes de poissons âgés de 9 mois. Ces résultats suggèrent une réponse compensatoire où la variation de forme des otolithes générés par les réponses de plasticité phénotypique due au traitement alimentaire au stade larvaire est compensée par des trajectoires morphogénétiques convergentes vers une forme similaire au cours de la phase juvénile. Cette réponse compensatoire pourrait être interprétée comme la canalisation ontogénétique de la forme des otolithes liée à son rôle dans l'audition et dans le maintien de l'équilibre de l'animal. L'une des perspectives de ces travaux serait de s'intéresser les influences d'autres compositions alimentaires telle que la composition en acides aminés car ils sont d'avantage impliqués dans les processus de biominéralisation des otolithes.

Mots-clés : acides gras polyinsaturés essentiels, acide eicosapentaénoïque, acide docosahexaénoïque, morphométrie géométrie, morphogénèse de l'otolithe.

Introduction

Teleost fishes have three pairs of calcified structures, otoliths, contained in the otic capsule (or saccule) of their inner ear that are involved in their audition and balance. *Sagittae*, the largest of the three otolith pairs, grow by accretion, i.e. deposition of successive aragonitic calcium carbonate layers on an organic matrix that represents a minor fraction of the otolith's total material (Degens et al. 1969). Although sagittal otoliths grow continuously throughout an individual's lifetime, they are metabolically inert such that any material deposited remains unaltered and cannot be resorbed (Campana & Neilson 1985). Due to these two properties, otoliths are veritable black boxes (Lecomte-Finiger 1999) containing reliable fingerprints that are considered as invaluable source of information for reconstructing a fish's entire life-cycle (Campana & Thorrold 2001). A complex relationship exists between otolith and somatic growth in fishes (Panfili et al. 2002, Allemand et al. 2007). It results in that environmental factors (e.g. temperature) and physiological functions (growth and reproduction) influencing somatic growth also affect otolith growth and its governing processes (Cardinale et al. 2004). Otolith biomineralization is an acellular process during which precursor compounds are either synthesized (organic ones) or transported (ionic ones) by secretory cells and ionocytes, respectively, belonging to the saccular epithelium (Payan et al. 2004). In addition, the spatial distribution of ionocytes and secretory cells in the saccular epithelium is such that it creates some concentration gradients of both ions and organic precursors in the endolymph (the fluid filling the otic capsule in which the otoliths are bathing) that are involved in the otolith biomineralization process (Pisam et al. 1998, Payan et al. 1999, Borelli et al. 2001). Therefore, otolith biomineralization is totally dependent on the endolymph composition and spatial variation in this composition. Moreover, despite the presence of organic matrix in minute amounts, it plays an important role in otolith formation especially on morphogenesis due to the contribution of several proteins involved in the control of carbonate calcium crystal

morphology (Nagasawa 2013). Therefore, otolith biomineralization depends on multi-causal processes due to the interaction of many endogenous and exogenous factors (Allemand et al. 2007) that result in high otolith morphological variability.

Otolith shape is species-specific because it has a genetic component, which at the inter-specific level, allows the determination of phylogenetic relationships (Lombarte & Lleonart 1993) or the identification of prey fish species or remains present in stomach and feces contents (Bowen 2000, Boström et al. 2012). At the intra-specific level, otolith shape variability allows fish stocks and populations discrimination (Bolles & Begg 2000, Mérigot et al. 2007, Stransky, Murta, et al. 2008). However, the use of otolith shape as a discriminating tool, be it at the inter- or intra-specific level, requires the identification and the understanding of confounding factors. It is known that ontogeny assessed through size (Hüssy, 2008), age (Castonguay et al. 1991), sexual maturity (Mérigot et al. 2007), or sex (Castonguay et al. 1991, Bolles & Begg 2000), but also environmental factors, such as water temperature (Cardinale et al. 2004), may impact otolith morphology. These processes or factors affect fish metabolism and physiology, consequently, impacting somatic as well as otolith growth and shape variability. Food amount also affects otolith shape both indirectly through an effect on otolith growth generating variation in its "global" shape and directly on the otoliths' crenulations (Gagliano & McCormick 2004, Cardinale et al. 2004, Hüssy 2008). Other than food amount, diet composition has also an effect on otoliths' composition (Sanchez-Jerez et al. 2002). The concentration of trace elements in otoliths such as barium (Ba), strontium (Sr) (Buckel et al. 2004) and manganese (Mn) (Sanchez-Jerez et al. 2002) can be related to the concentration of the same elements in prey items, which suggests a trophic transfer due to the absence of correlation between trace element concentration in otoliths and water (Thorrold et al. 1997). Moreover, variations in otoliths' carbon stable isotope ratio ($\delta^{13}C$) were observed to correlate with variations in muscular $\delta^{13}C$ across diet treatments (Elsdon et al. 2010). Since

diet composition impacts otolith composition, it is plausible then, to hypothesize that diet composition or food quality may affect otolith shape.

Highly polyunsaturated fatty acids (HUFAs), mainly the *n*-3 essential fatty acids, are a fundamental source of energy for fishes, which cannot synthesize them. Among *n*-3 HUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are involved in growth, development and survival in marine fishes (Sargent et al. 1999). Dietary fats have profound effects on gene expression and lead to changes in metabolism, growth and cell differentiation (Cahu et al. 2003). In coastal environments, food webs are mainly supplied with *n*-3 HUFAs by diatoms that represent an important source of essential lipids for primary consumers such as zooplankton that are then preyed upon by fish larvae and planktivorous fishes (Pahl et al. 2010). However, *n*-3 HUFA contents in diatoms may decrease with some environmental factors affected by global change such as ocean acidification and warming (Gómez & Souissi 2008, Pahl et al. 2010, Bermúdez et al. 2015).

In this study, we investigated experimentally the potential effect of food quality on sagittal otolith shape in sea bass (*Dicentrarchus labrax*). More precisely, we addressed the impact of a reduction in the dietary concentration of some *n*-3 HUFAs, namely EPA and DHA, on individual sagittal otolith growth and morphogenesis reared under controlled conditions at early stages (from eggs to 10 month-old juveniles). An original approach was used to monitor otolith growth and morphogenesis at the individual level based on oxytetracyclin (OTC) marking of the otolith structure at several points in time. The effects of a low HUFA dietary concentration on otolith length and shape changes described by geometric morphometrics were tested while accounting for body size variation. This approach allowed the disentangling of direct and indirect, i.e. through somatic growth, diet composition effect on otolith ontogenetic trajectories.

Materials and Methods

Specimen collection, fish rearing treatments, and otolith marking

200 000 sea bass (*Dicentrarchus labrax*) eggs were purchased from a commercial hatchery (Aquastream, Ploemeur, France). The eggs were placed into a 150 L incubator with water at 15 °C and 35 psu until hatching. Daily post-hatching (dph) larvae were then distributed among twelve 38 L tanks (3 600 larvae per tank). Throughout the experiment, water temperature, salinity and oxygenation were maintained at 20 °C, 35 psu and more than 6 $mg.L^{-1}$, respectively.

Seven dph, individuals were then exposed to various dietary treatments according to an experimental design organized into three phases described below (Figure 1).

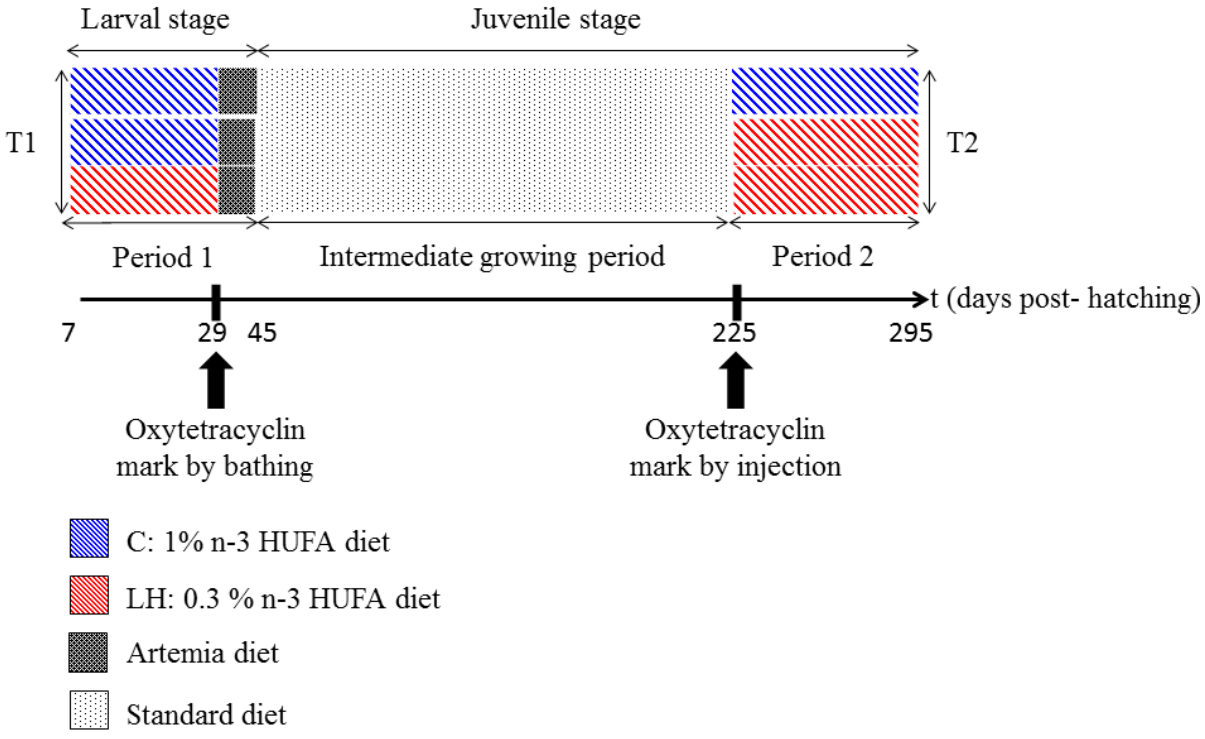


Figure 1: Experimental design for sea basses that were administered dietary treatment T1 during Period 1 (at the larval stage), standard diet during the Intermediate Growing Period, and dietary treatment T2 during Period 2 (at juvenile stage). See text for more details.

Larval stage: first dietary treatment period (Period 1)

Larvae were exposed to two dietary treatments (T1) differing in their *n*-3 HUFA concentration from 7 to 29 dph. Feeds were made at the PFOM Unit, UMR 6539 LEMAR (IFREMER Institute at Plouzané, France) and consisted in either standard (T1=C) or low *n*-3 HUFA (T1=LH) feed pellets. LH feed was especially reduced in DHA and EPA concentrations: together they represented only 0.3 % of feed dry weight (1% for control). Each dietary treatment was administered to 7 replicated 38 L tanks. 53.1% ± 12.4 and 16.0% ± 6.5 of the larvae from treatments C and LH, respectively, survived until the end of the larval stage. From 30 to 44 dph, each experimental group was fed with *Artemia* (Figure 1).

Juvenile stage: intermediate growing period (IG Period)

Following the larval stage (45 dph), replicate tanks were pooled in one raceway tank of 300 L per treatment with approximately 1 200 juveniles per tank. These fishes then underwent an intermediate growing period from 45 to 225 dph during which they were fed with a standard diet containing 1 % EPA+DHA/dry weight (made at INRA-NuMEA, St Pée/Nivelle, France) and maintained at 20 °C.

Juvenile stage: second dietary treatment period (Period 2)

After the IG Period (225 dph), 160 individuals were randomly drawn from each tank, tagged subcutaneously (passive integrated transponder; PIT-tag), weighed (initial weight, $W_{t=225}$) and randomly distributed among two 67 L tanks for juveniles that were fed with C diet during Period 1 and one 67 L tank for individuals previously fed with LH diet. Until 295 dph, fishes were again exposed to two dietary treatments (T2) according to the following scheme. Individuals exposed to the C treatment during Period 1 were either administered with similar C diet again or an LH diet (one tank each). Individuals that experienced the LH treatment during Period 1 were administered the LH diet again (one tank). Therefore, the experimental design was a fractional factorial design between two larval dietary treatments (Period 1) and

two juvenile dietary treatments (Period 2) with individuals experiencing either one of the following "diet history": C-C, C-LH, LH-LH.

Otolith marking

In order to follow otolith morphogenesis, otoliths were marked twice during the whole experiment duration using OTC antibiotics. Initial otolith marking at the end of Period 1 were done by immersing individuals for 4.5 hours in an OTC solution of 300 $mg.L^{-1}$. Secor et al. (1991) had marked 35-45 day-old striped bass (*Morone saxatilis*) by immersion in an OTC solution of 250 $mg.L^{-1}$ for 2h. However, the success of otolith marking was not consistent across individuals. Consequently, we chose to increase the OTC concentration and the exposure time in order to improve marking success. Second, at the end of the IG Period, otoliths were marked by an intraperitoneal injection of OTC at 30 $mg.kg^{-1}$ in each individual fish. The success of marking by immersion is likely lower in juveniles than in larvae due to metabolic differences (Secor et al. 1991), which is why an injection method was preferred at juvenile stage. The dose of OTC administered by injection follows the recommendation of 25-35 mg OTC per kg of wet fish established for sablefish (McFarlane & Beamish 1987). Before the injection, each fish was weighed (g) in order to dilute the OTC dose to be administered (mL) to a volume of physiological serum equal to 1% of its wet weight (initial weight, $W_{t=225}$). At the end of Period 2, each fish was re-weighed (final weight, $W_{t=295}$) and then sacrificed to extract the sagittal otolith pair from its otic cavity.

All experiments were conducted in strict compliance with the Guide for the Care and Use of Laboratory Animals.

Otolith shape analysis and measures

Each unbroken right otolith was cleaned and placed with its proximal face (convex side) against the bottom of a petri box, which was filled with 40 mL of water to increase the intensity of the fluorescent OTC mark. Its distal face (concave side) was observed under a

microscope (ZEISS AXIO) equipped with an UV light source (excitation of 430 nm and emission of 550 nm) and fitted with a digital camera (Axio Cam MRc). Given that the microscope's field of view did not cover the whole otolith, successive images covering the entire area of the otolith were automatically taken at various depths under UV light at ×5 magnification using a motorized stage coupled with ZEISS® ZEN Imaging® 2011 software (www.zeiss.com). An integrated mosaic image of the whole otolith (Figure 2) was created by combining the partial images having the best resolution across depths. Unfortunately, only the otolith mark by OTC injection at the end of the IG Period was visible. Consequently, only the outline of this fluorescent ring could be extracted (initial otolith shape, thereafter) using Adobe® Photoshop® CC software, in addition to the external outline of the otolith (final otolith shape, thereafter). Due to the image quality, the success of marking and low light diffusion, a precise outline of otolith shape at the time of OTC marking could indeed be extracted. After binarization, the outline of the initial and final shape were digitized as pixel coordinates using the tpsDig software version 1.40 (from the TPS package, Rohlf 2006).

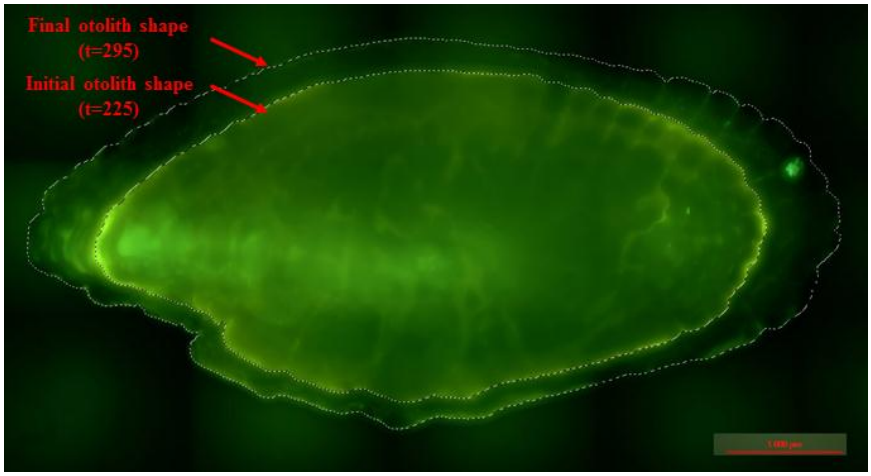


Figure 2: Mosaic image of an otolith of sea bass under UV light with the outline of its fluorescent ring named initial shape and the outline of its final shape.

Elliptical Fourier analysis (Kuhl & Giardina 1982) and (Semi)-landmark-based geometric morphometrics (Bookstein 1997b) are the two main methods for the quantitative description of a shape. The former describes shape by a series of trigonometric functions that

have no direct morphological interpretation whereas the latter represents shape by a series of points distributed along the outline that can be directly interpreted in morphological terms. As the main aim of this study was to describe variation in ontogenetic trajectories due to diet composition influence, it was necessary to follow the changes in morphological characteristics trajectories between the initial and final otolith shapes as well as to compare these between individuals. Consequently, semi-landmark-based geometric morphometrics was used in this study. Landmarks are specific recognizable morphological points that are biologically homologous between individual. However, otoliths have only a few landmarks along their outline which is insufficient to capture the structural information of their shape (Gunz & Mitteroecker 2013). Therefore, semi-landmarks, which are points obtained by geometrical construction relative to landmarks (Bookstein 1997a) were used to describe otolith shape.

Initial and final otolith shapes were described by 1 landmark, defined as the intersection between the major axis (the largest antero-posterior axis) and the otolith outline in the rostrum region, and 59 semi-landmarks equally spaced along the contour starting from the landmark. A Generalized Procrustes Analysis (GPA) was performed to superimpose the landmark and semi-landmarks coordinates by sliding semi-landmarks along the outline so as to minimize Procrustes distance with respect to the average shape taken as a reference. This method adds a step in the classical GPA where semi-landmarks are slid along the outline curve so as to minimize tangential variation to the curve and thus to match as close as possible the position of corresponding points in the reference shape thereby establishing a geometric homology between all samples, consequently removing arbitrary spacing (equally spaced) effects (Adams et al. 2004, Gunz & Mitteroecker 2013). Alternatively, minimization of the bending energy can be used as a criterion for semi-landmarks sliding (Bookstein 1997a). We chose to use Procrustes distance instead because variation in the semi-landmarks

coordinates obtained was much lower than that obtained using bending energy criterion, suggesting better points homologies between individuals with the former method. Additionally, the two criteria are known to yield similar results when shape variation is small (Gunz & Mitteroecker 2013) which was the case in this study (see Results section). The resulting matrix S_t of superimposed (semi-)landmarks coordinates allowed describing initial, $S_{t=225}$, and final, $S_{t=295}$, otolith shapes.

Initial, $O_{L,t=225}$, and final, $O_{L,t=295}$, otolith length were measured on mosaic images. Otolith length $O_{L,t}$ was defined as the largest distance along the otolith antero-posterior axis and its change through time was considered as a proxy of otolith growth in order to dissociate the direct food effect on otolith shape from the indirect food effect through somatic and then otolith growth.

Statistical analysis

Body growth, otolith growth and otolith morphogenesis were analyzed according to the same principle based on two complementary set of analyses (Model 1 and Model 3, Figure 3), but with statistical models that differed in a way where the allometric effect of body mass was included. There is indeed a well-recognized allometric relationship between otolith morphology (shape and length) and individual body mass, but also between body growth rate itself and body mass. Consequently, the fish body mass logarithm at relevant time points was added to all models as a covariate, potentially in interaction with other relevant factors, in order to take into account body size allometry which is the main source of otolith length and shape and body growth rate variation. Log-transformation was then used because of the decreasing influence of body mass as fish grows, typical of allometric relationships.

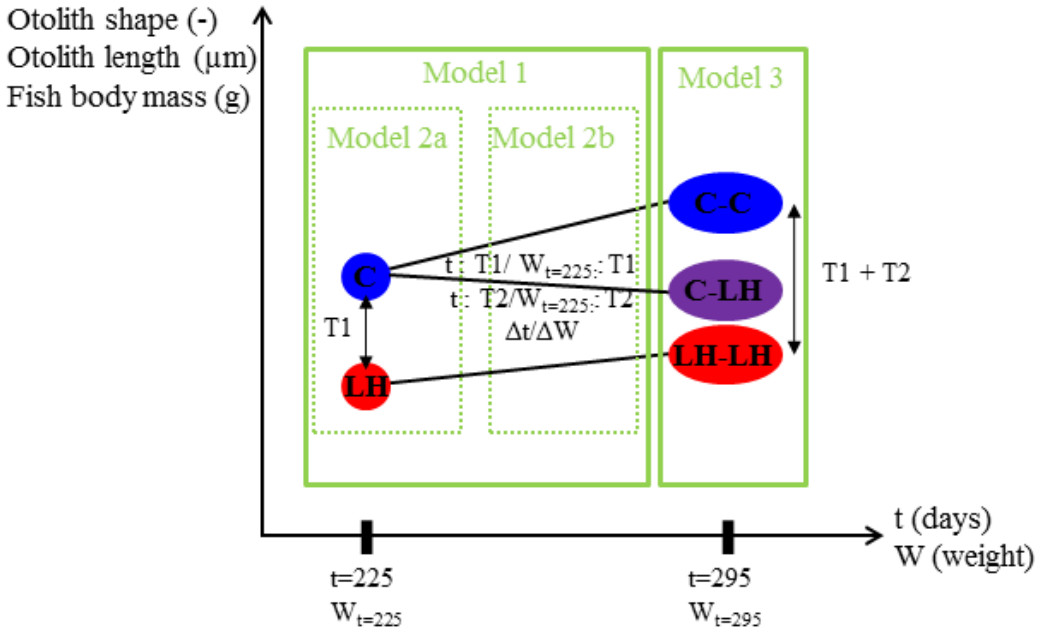


Figure 3: Schematic of the linear (mixed effect) models used to analyze the effect of the two dietary treatments (T1 and T2) on fish body mass, otolith length and shape. ANCOVA-like models were used to analyze the effect of treatment T1 on initial values and of treatment T1 and T2 on ontogenetic changes for body mass, otolith length (Model 1) and otolith shape (Models 2a and 2b). The effect of dietary treatment T1 and T2 on final body mass, otolith length and shape were analyzed by ANOVA-like models (Model 3). See text for more details.

The first set of analyses (Model 1, Figure 3) investigated the effect of the two dietary treatments T1 and T2 on body growth, otolith growth and otolith morphogenesis from the end of the IG Period (t=225) to the end of Period 2 (t=295) using linear mixed effect models in order to account for the longitudinal nature of data, i.e. repeated measures on the same individual at multiple time points. Models were specified according to an ANCOVA-like design with T1 and T2 as fixed factors, an indicator of ontogenetic stage as fixed covariate and individual as a random intercept effect. For body growth, age t in dph was used as an indicator of ontogenetic stage and the allometric effect of initial body mass on growth rate was included in the model. The model was specified as:

$$\log(W_{t,i}) \sim T1 + t + t\times T1 + t\times T2 + \log(W_{t=225,i})\times(t + t\times T1 + t\times T2) + i \quad (1a)$$

where $W_{t,i}$ is body mass of individual i at time t, the fixed factor T1 represents the effect of dietary treatment during Period 1 on the intercept, i.e. on initial weight $W_{t=225}$ at the end of the IG Period (Figure 3), the fixed covariate t is age in dph with the corresponding slope representing temporal change in body mass, i.e. growth rate, during Period 2 (Figure 3),

the fixed interaction t×T1 represents the delayed effect of T1 on growth rate (Figure 3), the fixed interaction t×T2 is the immediate effect of dietary treatment T2 administered during Period 2 on growth rate (Figure 3), the interactions with $\log(W_{t=225,i})$ represent the allometric effect of initial body mass on growth rate and its response to dietary treatments, and the random effect *i* represents individual variation in the intercept. For otolith growth and morphogenesis, individual body mass $W_{t,i}$ was used as an indicator of ontogenetic stage instead of age t thus allowing to account for both temporal change in ontogenetic stage and inter-individual body mass variation at a given time point. The model was specified as

$$Y_{t,i} \sim T1 + \log(W_{t,i}) + \log(W_{t,i})\times T1 + \log(W_{t,i})\times T2 + i \quad (1b)$$

where the response variable $Y_{t,i}$ is either log-transformed otolith length $\log(O_{L,t,i})$ (univariate response) or otolith shape $S_{t,i}$ (multivariate response) of individual i and the various effects are interpreted as for growth rate. Regarding the multivariate response S_t, current algorithms for linear mixed effect models cannot deal with such a large number of dimensions (60 points with x and y coordinates, i.e. 120 dimensions). Consequently, the number of dimensions was reduced by performing a principal component analysis on S_t and selecting a subset of the resulting principal components (PCs) as response matrix according to the broken stick model (Borcard et al. 2011). The broken stick method ensures to keep the significant sources of shape variation and it is recommended to use the 6 first PCs that represents 76.5 % of shape variation in the dataset. These were then used as response variable in Model 1b instead of S_t to analyze otolith morphogenesis.

In order to ensure that the loss of information due to the use of a subset of PCs did not bias our results on otolith morphogenesis, we compared the results of Model 1b with those from analyses that kept all otolith shape variation intact but could not include random effects to account for the longitudinal nature of our data. Two redundancy analyses (RDA) were performed to model the same effects on otolith shape as in Model 1b. The first one (Model 2a,

Figure 3) tested for T1 effect on initial otolith shape $S_{t=225}$ (the intercept in Model 1b, Figure 3) while accounting for the allometric effect of initial body mass $W_{t=225,i}$:

$$S_{t=225,i} \sim T1 + \log(W_{t=225,i}) + \log(W_{t=225,i}) \times T1 \quad (2a)$$

The second one (Model 2b, Figure 3) modeled the individual change in otolith shape $\Delta S_i = S_{t=225,i} - S_{t=295,i}$ during Period 2 (equivalent to the slope with $\log(W_t)$ in Model 1b, Figure 3) as depending on a delayed effect of dietary treatment T1 and an immediate effect of T2 while accounting for the allometric effect of initial $W_{t=225,i}$ and final $W_{t=295,i}$ body mass:

$$\Delta S_i \sim T1 + T2 + \log(W_{t=225,i}) + \log(W_{t=295,i}) + (\log(W_{t=225,i}) + \log(W_{t=295,i})) \times (T1 + T2) \quad (2b)$$

Model 2 was equivalent to Model 1b with the disadvantage of not accounting for individual random effects but with the advantage of keeping all otolith shape variation in the analysis.

The second set of analyses (Model 3, Figure 2) investigated the effect of the two dietary treatments (T1 and T2) on final body mass, otolith length and shape using linear models as Model 1 could not test directly these effects. Models included the allometric effect of initial body mass $W_{t=225,i}$ for final body mass and of final body mass $W_{t=295,i}$ for final otolith length and shape:

$$\log(W_{t=295,i}) \sim T1 + T2 + \log(W_{t=225,i}) + \log(W_{t=225,i}) \times T1 + \log(W_{t=225,i}) \times T2 \quad (3a)$$

$$Y_{t=295,i} \sim T1 + T2 + \log(W_{t=295,i}) + \log(W_{t=295,i}) \times T1 + \log(W_{t=295,i}) \times T2 \quad (3b)$$

where again the response variable $Y_{t,i}$ is either log-transformed otolith length $\log(O_{L,t,i})$ or otolith shape $S_{t,i}$ of individual i. Note that a classical linear model was used for the former whereas a RDA was performed for the latter.

For all models except RDAs, the most parsimonious model was obtained by a bidirectional elimination based on the Akaike Information Criterion. The significance of selected variables at 5 % was tested by likelihood ratio tests between nested models while respecting marginality of the effects (type 2 tests; Fox & Weisberg 2011) that are supposed to

follow a χ^2 distribution under the null hypothesis. For RDAs, the bidirectional elimination and the selected effects significance was based on the p-value determined by permutation tests at a threshold of 5 %. Normality and homoscedasticity of the residuals was checked by visual inspection.

All statistical analyses were performed using the 'vegan' (Oksanen et al. 2013), 'geomorph'(Adams & Otarola-Castillo 2013), 'nlme' (Pinheiro et al. 2015), 'lme4' (Bates et al. 2015), 'Car'(Fox & Weisberg 2011) and 'MASS' (Venables & Ripley 2002) packages in the statistical environment R (R Development Core Team 2013).

Results

Initial body mass $W_{t=225}$ (Model 1a intercept, Figure 4), somatic growth (Model 1a slope, Figure 4) and final body mass $W_{t=295}$ (Model 3, Figure 4) were unaffected by dietary treatments T1 and T2 that were absent from the reduced models (Table I, Model 1a and 3). Both somatic growth and final body mass increased significantly with initial body mass (Table I, Model 1a $\log(W_{t=225,i})\times t$ effect, Model 3 $\log(W_{t=225,i})$ effect) confirming the expected allometric relationship.

Table I: Analysis of the effect of dietary treatments on fish body growth by linear (mixed effect) models. Results are shown for reduced models resulting from bidirectional elimination. For each selected variable, the estimate, its standard error (SE), the χ^2 statistic (for Model 1a) or the F statistic (for Model 3), the associated degrees of freedom (df) and the resulting p-value (P) for a type II test are given. *<0.05; **<0.01, *<0.001**

	Estimate	SE	df	χ^2/F	P	
Model 1a: Initial body mass and growth						
t	0.247	0.204	1	3627.617	<0.001	***
$\log(W_{t=225,i})\times t$	0.147	0.061	1	5.690	0.017	*
Model 3: Final body mass						
$\log(W_{t=225,i})$	0.893	0.061	1/116	211.070	<0.001	***

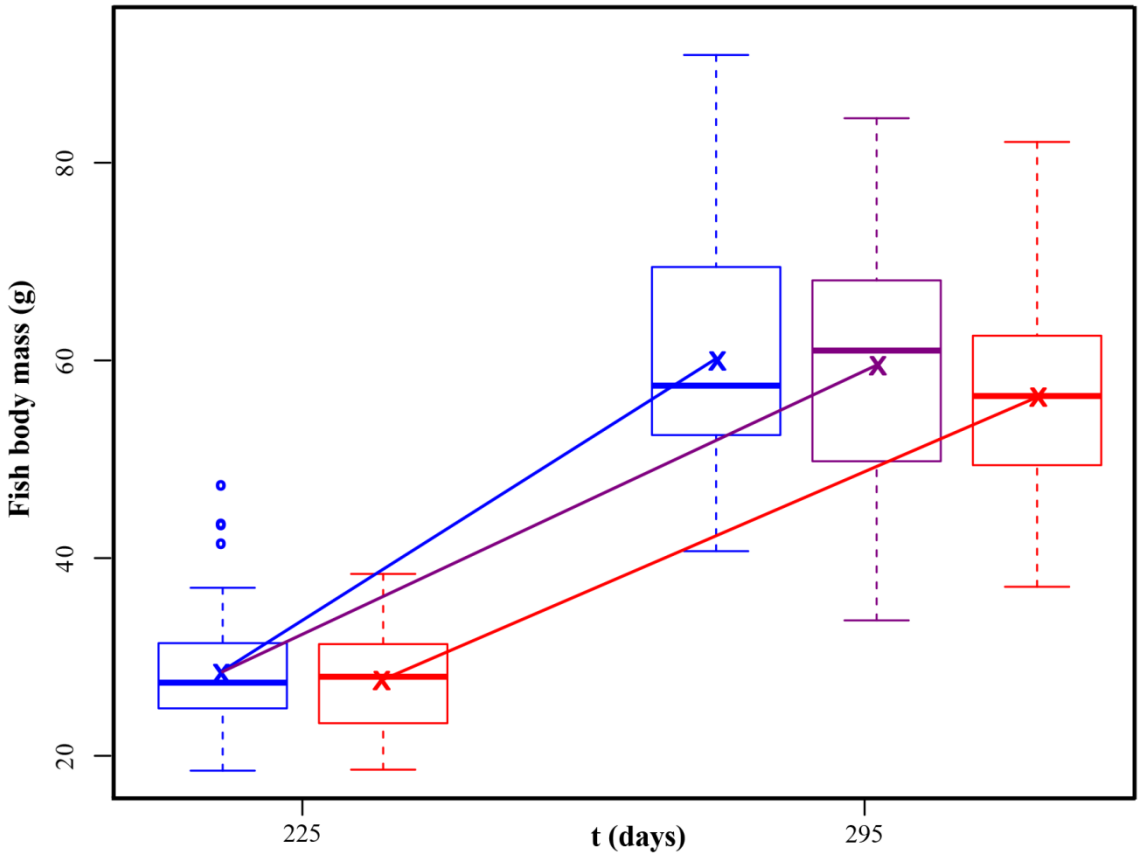


Figure 4: Boxplot of initial body mass $W_{t=225}$ at the end of the IG Period according to dietary treatment T1 (C in blue and LH in red) and of final body mass $W_{t=295}$ at the end of Period 2 according to the combination of dietary treatments T1 and T2 (C-C in blue, C-LH in purple and LH-LH in red). The bottom and top of the boxes are the first and the third quartiles of the data distribution, the horizontal segment and the cross inside the boxes are the median and the mean, respectively, whiskers represent the most extreme data point within 1.5 interquartile range and circles are data points out of this range.

Likewise, dietary treatments T1 and T2 did not affect significantly initial otolith length (Model 1b intercept, Figure 5), or otolith growth with body mass (Model 1b slope, Figure 5) or final otolith length (Model 3, Figure 5) despite the fact that dietary treatment T1 was kept in the reduced model (Table II, Model 1b and 3). Otolith length increased significantly with both temporal change in body mass (Table II, Model 1b $\log(W_{t,i})$ effect) and inter-individual variation in body mass (Table II, Model 3 $\log(W_{t=295,i})$ effect) due to the known allometric effect.

Table II: Analysis of the effect of dietary treatments on otolith growth by linear (mixed effect) models. Results are shown for reduced models issued resulting from bidirectional elimination. For each selected variable, the estimate, its standard error (SE), the χ^2 statistic (for Model 1b) or the F statistic (for Model 3), the associated degrees of freedom (df) and the resulting p-value (P) for a type II test are given. *<0.05; **<0.01, *<0.001**

	Estimate	SE	df	χ^2/F	P	
Model 1b: Initial otolith length and growth						
T1	-0.036	0.020	1	1.083	0.299	
$\log(W_{t,i})$	0.247	0.003	1	12452.642	<0.001	***
$\log(W_{t,i})$×T1	0.008	0.005	1	2.482	0.115	
Model 3: Final otolith length						
T1	-0.185	0.125	1/115	1.191	0.277	
$\log(W_{t=295,i})$	0.196	0.016	1/115	228.012	<0.001	***
$\log(W_{t=295,i})$×T1	0.044	0.031	1/114	2.039	0.156	

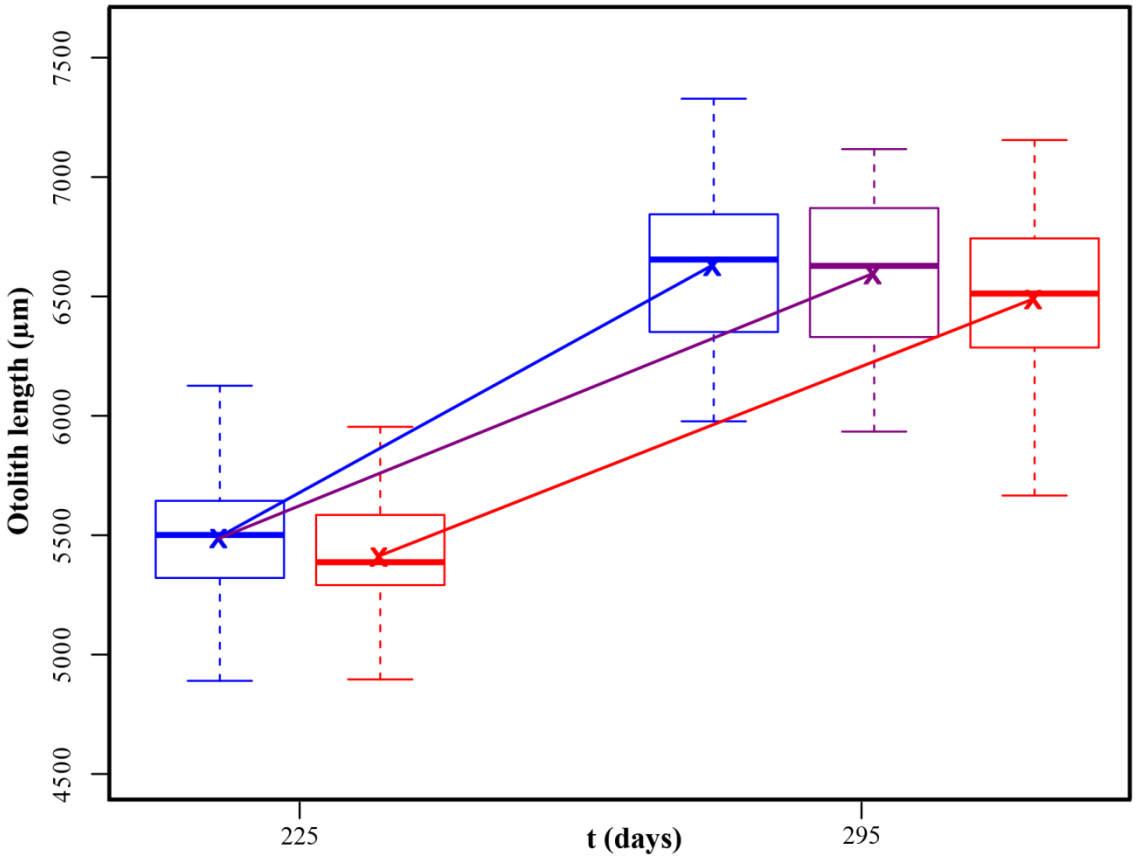


Figure 5: Boxplot of initial otolith length $O_{L,t=225}$ at the end of the IG Period according to dietary treatment T1 (C in blue and LH in red) and of final otolith length $O_{L,t=295}$ at the end of Period 2 according the combination of dietary treatments T1 and T2 (C-C in blue, C-LH in purple and LH-LH in red). Boxplots are designed as in Figure 4.

Unlike somatic and otolith growth, otolith morphogenesis was significantly affected by dietary treatments (Figure 6). More precisely, dietary treatment T1 administered during the larval stage (Period 1, Figure 1) had a significant delayed effect on initial otolith shape

measured at the end of the IG Period (Table III, model 1b, T1 effect). A thin-plate spline deformation grid of initial otolith shape from the LH treatment relative to the C one illustrates that this effect was weak and affected mainly the *excisura major* (the indentation between the rostrum and the antirostrum) and the otolith posterior part (Figure 6, pair A-B). Dietary treatment T1 had also a delayed effect on otolith morphogenesis during Period 2 (Figure 6, pairs A-C, A-D and B-E): the temporal change in otolith shape with body mass (Table III, Model 1b, $\log(W_{t,i})$ effect) varied according to dietary treatment T1 experienced earlier, i.e. during Period 1 (Table III, model 1b, $\log(W_{t,i})$×T1 effect). In contrast, no immediate effect of dietary treatment T2 was detected on otolith shape change during Period 2. These results were supported by those from Models 2a and 2b that admittedly did not account for the correlation between otolith shapes of the same individuals measured at different points in time but kept all otolith shape variation intact. T1, initial body mass $\log(W_{t=225,i})$ and their interaction affected significantly initial otolith shape (Table III, model 2a, T1, $\log(W_{t=225,i})$ and $\log(W_{t=225,i})$×T1 effects) and the interaction between T1 and final body mass $\log(W_{t=225,i})$ impacted significantly otolith shape change during Period 2 (Table III, model 2b, $\log(W_{t=225,i})$×T1 effect) with the effect T1 being marginally significant. Surprisingly, no dietary treatment had a significant effect on final otolith shape at the end of Period 2 (Table III, model 3, Figure 6 pairs F-G and F-H). Only final body mass $\log(W_{t=295,i})$ affected marginally final otolith shape (Table III, model 3, $\log(W_{t=295,i})$ effect).

Table III: Analysis of the effect of dietary treatments on otolith morphogenesis by multivariate linear (mixed effect) models. Results are shown for reduced models resulting from bidirectional elimination. For each selected variable, the χ^2 statistic (for Model 1b) or the F statistic (for Model 2, 3a, 3b), the associated degrees of freedom (df) and the resulting p-value (P) for a type II test are given. Given that these analyses are multivariate, no estimate and associated standard error are given because they are not directly interpretable. *<0.05; **<0.01, *<0.001**

	df	χ^2/F	P	
Model 1b: Initial otolith shape and shape change				
T1	6	16.692	0.010	*
$\log(W_{t,i})$	6	502.871	<0.001	***
$\log(W_{t,i})$×T1	6	19.623	0.003	**
Model 2a: Initial otolith shape				
T1	1/115	2.385	0.028	*
$\log(W_{t=225,i})$	1/115	2.331	0.041	*
$\log(W_{t=225,i})$×T1	1/114	2.523	0.021	*
Model 2b: Otolith shape change				
T1	1/115	1.965	0.094	
$\log(W_{t=295,i})$	1/115	1.430	0.156	
$\log(W_{t=295,i})$×T1	1/114	5.247	0.005	**
Model 3: Final otolith shape				
$\log(W_{t=295,i})$	1/116	1.972	0.071	

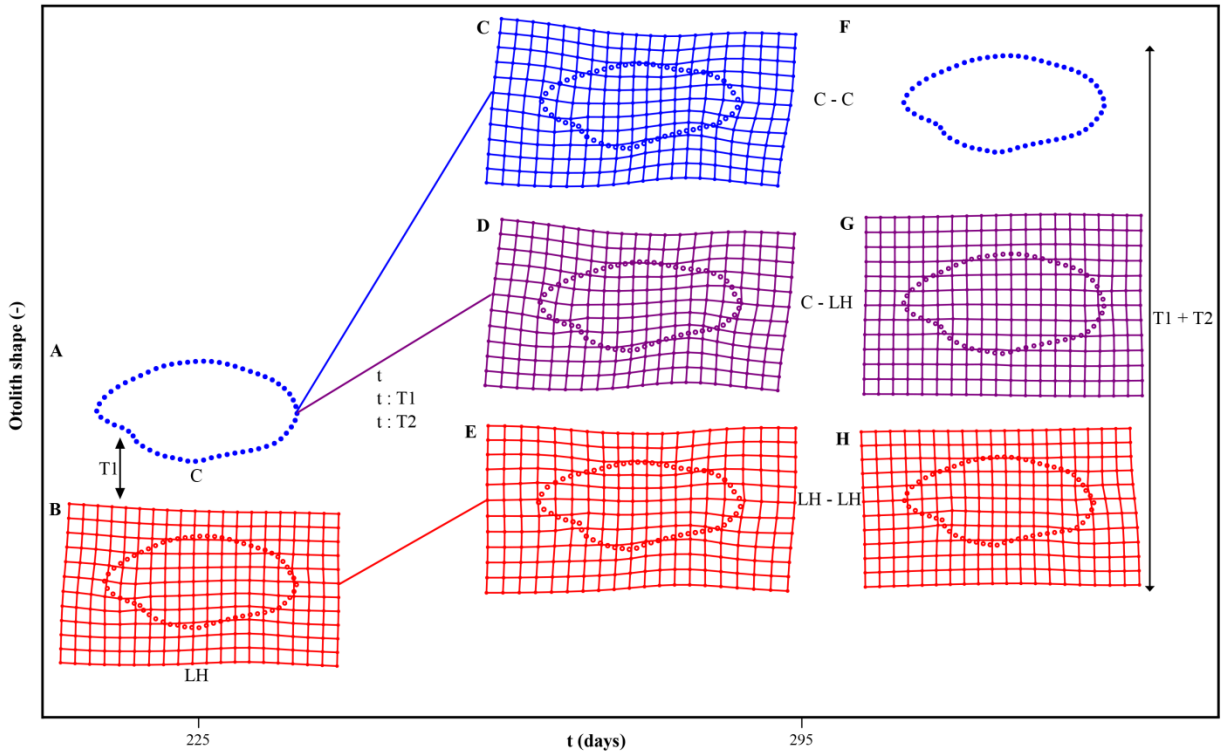


Figure 6: Otolith morphogenesis according to dietary treatments. Mean (semi-)landmark coordinates were calculated to obtain the average otolith shape at the end of the IG Period and of Period 2 according for each combination of dietary treatments. Thin-plate spline (TPS) transformation grids (Bookstein 1997b) are produced to visualize the dietary treatment effect locations with a magnification factor of 4. Transformation grids are produced relative to different reference shapes to help visualize different effects. Treatment T1 effect on initial shape $S_{t=225}$ at the end of the IG Period is illustrated by comparing C diet (A in blue; reference shape) with LH diet (B in red). Treatment T1 and T2 effects on otolith morphogenesis, i.e. changes between initial $S_{t=225}$ and final $S_{t=295}$ otolith shape at the end of Period 2, are illustrated by comparing C diet (A in blue; reference shape) with both C-C (C in blue) and C-LH (D in purple) diet history as well as LH diet (B in red, reference shape) with LH-LH diet history (E in red). Treatment T1 and T2 effects on final otolith shape $S_{t=295}$ are illustrated by comparing C-C diet history (F in blue, reference shape) with C-LH (G in purple) and LH-LH (H in red) diet history.

Discussion

Otolith has the advantage of retaining a permanent record of individual fish life and environmental history without material degradation and reabsorption (Campana & Thorrold 2001). Therefore, otolith shape can be used as a biological indicator, but provided that the sources of shape variation are identified and understood at the intra-population scale. Otolith shape variation results indeed from the impact of many confounding factors acting together such as genetic, individual state (e.g. sex, age) or environmental variability (Vignon & Morat 2010). Some authors have highlighted the relationship between otolith shape and

environmental conditions mediated by the relationship between somatic and otolith growth (Campana & Casselman 1993, Cardinale et al. 2004). The underlying assumption is that environmental factors acts indirectly on otolith shape by essentially altering somatic and otolith growth rates, which in turn affect otolith shape (Hüssy 2008, Vignon & Morat 2010). Some studies have addressed the effect of food quantity on somatic growth and otolith shape (e.g. Gagliano & McCormick 2004), but to our best knowledge no study has investigated the effect of diet composition on otolith shape. This study precisely investigated the potential effect of diet composition in terms of HUFA concentration on otolith growth and morphogenesis while accounting for the effect of somatic growth. The study of otolith morphogenesis relied on a temporal monitoring of otolith shape changes at the individual level (longitudinal data) by OTC marking of otolith structure at 2 points in time: at the end of Period 1 and of the IG Period (Figure 1) where the otolith shape at the end of Period 2 was directly obtained after otolith extraction from the sacrificed animals. Unfortunately, only the OTC mark at the end of the IG period was detectable and, thus, allowed for the investigation of the delayed effect of dietary treatment T1 on otolith shape and length. OTC marking by individual immersion at the end of Period 1 was indeed unsuccessful in this study, which prevented us from analyzing the immediate effect of T1 on otolith shape and length at the larval stage. The appropriate OTC concentration for marking by immersion is highly species-specific and is a compromise between minimizing fish mortality due to OTC exposition and obtaining a detectable fluorescent OTC mark on the otolith (McFarlane & Beamish 1987). In this study, the OTC concentration used was probably not enough to obtain a detectable mark on the otolith structure and/or the immersion time was insufficient. Another possibility could be that the use of the entire otolith, as done in this study, is not appropriate for observing OTC marking at such very young stage. Examining a cross section of the otolith instead could be more relevant to detect the OTC mark. Despite the absence of a usable mark at the end of

Period 1, an effect of dietary treatment T1 on otolith morphogenesis was detected. Therefore, diet composition appears as one biotic environmental factor that can affect otoliths' morphogenetic trajectory.

Diet can affect otolith morphogenesis directly

A delayed effect of the first dietary treatment (standard diet, C, *versus* low HUFA concentration diet, LH) T1 was detected on otolith shape at the end of the IG Period and on otolith morphogenesis during Period 2. In contrast, none of the dietary treatments (T1 and T2) had a significant effect on body and otolith growth. This suggests that environmental variation in diet composition, such as in terms of lipids, can affect otolith morphogenesis directly, i.e. independently from the relationship between somatic and otolith growth. Hüssy et al. (2004) showed in experimental condition that temperature impacted more on the amino acid composition of otoliths' organic matrix with a change in the ratio between water-soluble and -insoluble protein fractions than on body growth in cod (*Gadus morhua*) juveniles. In addition, food ration level has been shown to generate differences in both otoliths' global shape and finer details of their outline crenulations in two tropical fish species (Gagliano & McCormick 2004, Hüssy 2008). Taken together, the results of this study and of previous ones support the assumption that diet, either at food ration or composition level could impact the otolith morphogenesis, both indirectly (through the somatic-otolith growth relationship) and directly. However, given that larvae were pooled at the end of the larvae stage, the effects observed afterwards could also reflect individual tank effects due to the absence of replicates. Moreover, the significant delayed effect of the first dietary treatment T1 on otolith shape at the end of the IG Period and on otolith morphogenesis during Period 2 could be related to differential mortality between individuals characterized by different otolith shapes. In order to test the differential mortality hypothesis, it will be interesting to compare otolith shape from

OTC marked individual (i.e. individuals alive at the end of the experience) and otolith shape from samples taken at each period of the experience and, thus, including individual mortalities during the experiment. Such samples were collected during the experiment.

The absence of diet effect on final otolith shape suggests otolith shape canalization

The delayed effect of the first dietary treatment T1 detected on otolith shape at the end of the IG period confirms that otoliths depend on recent but also past history. This is in line with the fact that growing inertia resulting from past otolith growth affects present otolith growth (Gutiérrez & Morales-Nin 1986), which is responsible for a time-lag between environmental stimuli and otolith growth response (Maillet & Checkley Jr 1991, Morales-Nin 2000b). The additional effect of T1 on otolith morphogenesis during Period 2 corroborates the fact that diet composition can affect otoliths' morphogenetic trajectory at later stages (Figure 7).

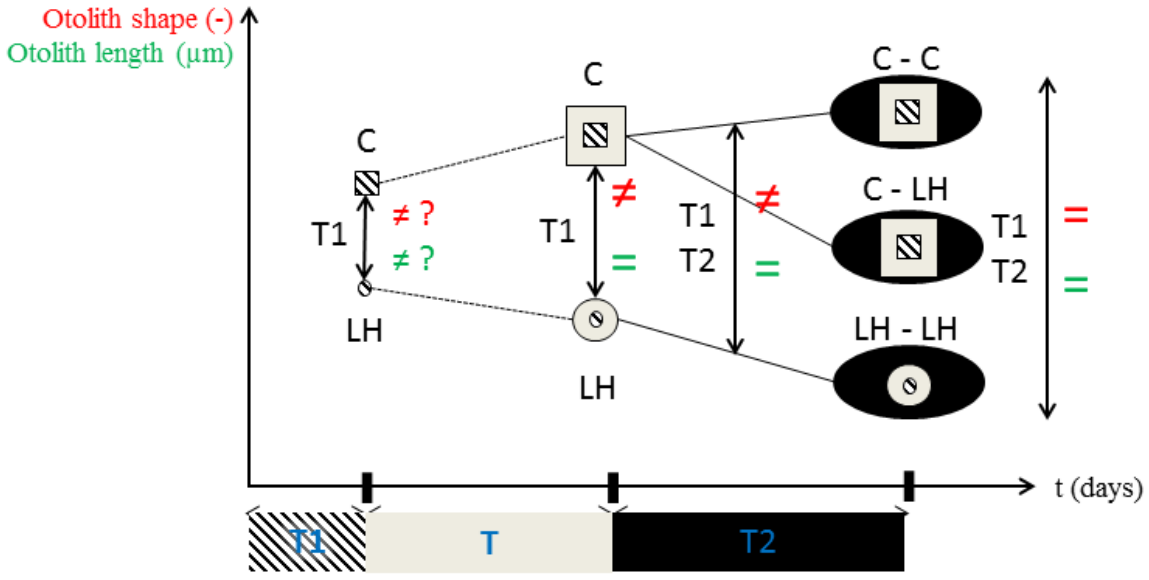


Figure 7: Effect of dietary treatments on otolith morphogenesis (in red) and growth (in green).

Therefore, the ontogenetic growth direction or growth axis of otolith can be modified by environmental condition such as diet composition. A similar result was already observed in

common bluestripe snappers (*Lutjanus kasmira*) during their ontogenetic habitat shift from estuaries to reefs located either in the channel or the outer-reef off French Polynesia. The shift in habitat induces changes in both the magnitude and rate of otolith growth as well as in otolith morphogenetic trajectory that diverge between the two adult environments (Vignon 2012).

However, in the present study, neither an immediate effect of dietary treatment T2 on otolith morphogenesis nor the dietary treatments on the final otolith shape was detected. Some hypotheses may be raised to explain these results. First, the absence of treatment T2 effect could simply be linked to the above-mentioned time-lag between environmental stimuli and otolith response in terms of morphogenesis. The experiment may have been stopped too early to observe any effect. Second, otolith growth or accretion rate is larger at larval stage than at juvenile stage. Therefore, any environmental effect influencing otolith growth direction (and thus its shape), such as that of diet composition, should be easier to detect during larval stage than at juvenile stage due to stronger amplification leading to larger relative effects (Hüssy 2008). This hypothesis may again explain the absence of dietary treatment T2 effects during juvenile stage and the presence x of treatment T1 effect after the larval stage. Our observations are consistent with the finding that otolith growth increments are under stronger environmental influence at immature stage than at adult stage in *Merluccius* species (Lombarte et al. 2003). It, however, contrasts with other studies that found weaker morphological variation at early stages that at later ones (Capoccioni et al. 2011, Vignon 2012).

Despite the delayed effect of treatment T1 on otolith shape at the end of the IG Period and its morphogenetic trajectory during Period 2, no dietary treatment effect was detected on the final otolith shape. This result may suggest a compensatory response such that otolith shape variation generated by phenotypic plasticity in response to diet treatment at larval stage

is compensated for by morphogenetic trajectories converging back to a similar shape during the juvenile phase. This compensatory response could be considered as a sign of ontogenetic canalization of otolith shape. An otolith's shape is implicated in its movements in response to a sound wave to produces additional stimuli that the fish may be able to process for additional cues on the characteristics and direction of the oncoming sound (Krysl et al. 2012). Otolith shape depends mechanistically on the biomineralization process (Borelli et al. 2003) which is under genetic control (Söllner et al. 2003). Genetic control of biomineralization could therefore impose constraints of otolith ontogeny resulting in a canalized shape. This hypothesis is strengthened by the fact that the amplitude of shape variation observed at the end of the IG Period was relatively weak. In conclusion, the fact that otolith shape converged towards similar shape despite diet-induced variability at earlier stage suggests that (i) the degree of otolith shape canalization increases along development (while the degree of plasticity decreases) and (ii) the plastic effect of diet composition at early stages is not large enough compared to later canalization of otolith ontogeny.

Dietary lipids composition and otolith biomineralization

As already mentioned, otolith shape variation resulting from dietary treatment T1 was relatively weak (Figure 5, deformation grids with ×4 magnification). Besides the potential limitation of plastic otolith shape response to diet due to ontogenetic canalization, the absence of interaction between lipids and the otolith biomineralization process could also explain this result. Otoliths are biominerals with a minor organic fraction representing 0.2 to 10 % of their total material (Degens et al. 1969). The organic matrix is composed mainly of proteins and glycoproteins (Borelli et al. 2001, Nagasawa 2013). Therefore, the weak magnitude of the dietary treatment effect could be explained by the fact that HUFA do not play an important role or are not involved in otolith biomineralization. To confirm or infirm this interpretation

versus the canalization hypothesis, and thus progress in the evaluation of the potential effect of food quality on otolith growth and shape, a similar experiment for the same or other fish species could be carried out but with varying concentrations in amino acids such as aspartate and glutamate that are essential for acidic proteins found in the water-soluble fraction of the otolith organic matrix. The latter assume the interaction with calcium and regulate crystal formation (Nagasawa 2013).

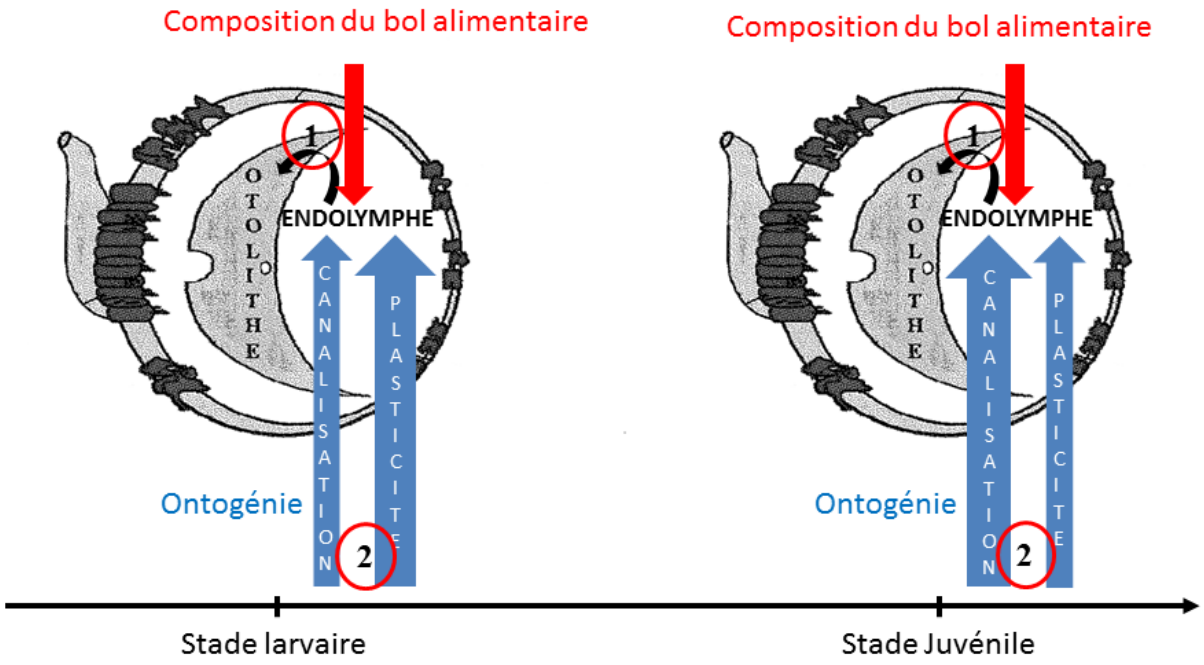
However, HUFA, especially n-3 essential fatty acids such as EPA and DHA, are involved in growth, development and survival in marine fish (Sargent et al. 1999). More particularly, they are essential in the development of the nervous system, especially the cerebellum (Furuita et al. 1998). It was observed that the absence of DHA in the diet of juvenile herrings (*Clupea harrengus*) led to impaired visions (Bell et al. 1995). These previous results raised the question of the potential implication of n-3 HUFA in the good development and functioning of fish inner ears. If this were the case, a reduction in the dietary concentration of these essential lipids could affect the function and/or morphology of fish inner ears and, consequently, otolith biomineralization. For instance, DHA improves stress resistance in milkfish *Chanos chanos* with a decrease of opercular deformities for larvae fed with DHA enriched live preys (Gapasin & Duray 2001). Instead of investigated systematic otolith shape differences between dietary treatments, another method to check is the implication of *n-3* HUFA in the fish audio-vestibular system development and functioning to estimate differences in the degree of otolith shape fluctuating asymmetry (FA) that is often considered as an individual indicator of stress (but see Díaz-Gil et al. 2015). Investigating the existence of a link between dietary n-3 HUFA concentration and the degree of FA would allow confirming or infirming the involvement of n-3 HUFA in developmental and physiological processes related to otolith biomineralization. Another interest of this subject is related to the fact that in coastal ecosystems n-3 HUFA originate mainly from diatoms (Pahl

et al. 2010), which growth rate and n-3 HUFA content generally decrease with some environmental factors affected by global change (e.g. increasing temperature, decreasing salinity) (Gómez & Souissi 2008, Pahl et al. 2010, Chen 2012), although generalization across diatom species proves sometimes difficult (Chen 2012). The identification of a link between dietary n-3 HUFA concentration and otolith FA would then provide a tool for assessing the effect of a global change-induced reduction in n-3 HUFA primary production on fish performances or fitness.

In summary, biotic environmental factor such as n-3 HUFA concentration in diet can affect plastically otolith shape and morphogenesis of sea bass at early stages. The absence of effect on fish somatic and otolith growth suggests a direct effect of food composition on otolith shape and morphogenesis, i.e. an effect independent from the relationship between somatic and otolith growth. However, otolith shape seems under strong canalization effect such that plastically diversified shapes at early stage converged towards similar shape at later stage suggesting that the amplitude of plastic diet composition effect is weak compared to the degree of canalization of otolith ontogeny.

Schéma conceptuel des hypothèses soulevées dans le chapitre 3 :

Hypothèses soulevées pouvant expliquer les différences de morphogénèse et de croissance des otolithes sous des conditions alimentaires différentes en termes de composition du bol alimentaire.



1 La composition alimentaire en termes de concentration en acides lipidiques polyinsaturés essentiels affecte la composition de l'endolymphe et donc engendre de la variabilité de forme.

2 L'importance de la canalisation qui vient contrecarrer la plasticité phénotypique engendrée par la concentration en acides gras polyinsaturés du bol alimentaire.

Discussion générale

La forme des otolithes est un outil très utilisé en halieutique (Campana 1999, Campana & Thorrold 2001) et ce à plusieurs échelles d'organisation biologique : au niveau individuel/intra-populationnelle, inter-populationnelle ou interspécifique (**Introduction**). Cependant, elle résulte de l'influence de multiples facteurs à la fois endogènes (génétiques), exogènes (facteurs environnementaux biotiques et abiotiques) et ontogénétiques qui agissent simultanément et engendrent une grande variabilité morphologique. Une utilisation robuste et non biaisée de la forme des otolithes comme outil nécessite :

1) d'identifier les facteurs qui engendrent sa variabilité ;
2) d'évaluer l'importance relative de leurs effets ;
3) de localiser les effets de ces facteurs au niveau de la forme de l'otolithe ainsi que leurs directions dans le but d'associer une variation morphologique à une propriété biologique ; et
4) d'identifier les relations entre ces facteurs et la biominéralisation des otolithes puisque les variations morphologiques de l'otolithe reflètent une variabilité au niveau des processus aboutissant à la biominéralisation.

Le dernier point rejoint un intérêt plus fondamental qui est la compréhension des processus de biominéralisation et l'identification des facteurs qui la déterminent ainsi que de leurs modes d'action. En ce sens, la variabilité morphologique des otolithes peut apparaître comme un proxy des variations en termes de leur biominéralisation permettant d'identifier, voire d'étudier, les relations entre les facteurs et les processus de biominéralisation des otolithes.

Jusqu'à aujourd'hui, la variabilité de forme des otolithes a été principalement étudiée à l'échelle interspécifique et inter-populationnelle (**Introduction**). Les études à l'échelle intra-populationnelle concernent principalement l'asymétrie fluctuante entre otolithes droit et gauche qui est utilisée comme indicateur de stress ou de condition et donc de bonne santé de

l'individu (Grønkjær & Sand 2003, Gagliano & McCormick 2004). Au-delà de la variabilité intra-individuelle résultant de l'asymétrie fluctuante, Hüssy (2008) a montré de manière expérimentale que la variabilité de la quantité de nourriture est responsable de variations morphologiques interindividuelles des otolithes chez les jeunes morue, affectant leur longueur ainsi que le nombre et la taille des lobes secondaires. Ces observations soulèvent l'importance potentielle de la variation morphologique des otolithes à l'échelle intra-populationnelle. Celle-ci pourrait être noyée ou confondue avec la variabilité observée à l'échelle inter-populationnelle engendrant ainsi un biais dans les analyses reposant sur la forme des otolithes.

C'est pourquoi cette thèse s'est intéressée à la variabilité morphologique des otolithes à l'échelle intra-populationnelle à travers l'étude de deux de ses sources potentielles : l'asymétrie directionnelle et le régime alimentaire, principalement la composition du bol alimentaire. Les conclusions et les perspectives de ces travaux s'articulent autour de 3 axes :

1) Les sources de variabilité de la forme des otolithes étudiées à l'échelle intra-populationnelle.
2) L'importance relative entre les sources de variation morphologique intra-populationnelle : Plasticité versus canalisation et causalité des effets.
3) L'utilisation de la forme des otolithes comme outil naturel biologique.

Les sources de variabilité de la forme des otolithes et leurs effets à l'échelle intra-populationnelle.

Au travers de ces travaux de recherche, deux sources de variabilité morphologique ont été mises en évidence à l'échelle intra-populationnelle. L'asymétrie directionnelle chez les pleuronectiformes et le régime alimentaire de l'individu.

Asymétrie directionnelle : source de variabilité intra-individuelle

Il existe trois types d'asymétrie ou de déviation par rapport à une symétrie parfaite entre les structures paires de l'organisme (Palmer et al. 2010). Elles sont caractérisées par des différences de distributions (moyenne et variance) des variables qui mesurent la différence entre le côté gauche et le côté droit (Figure 1).

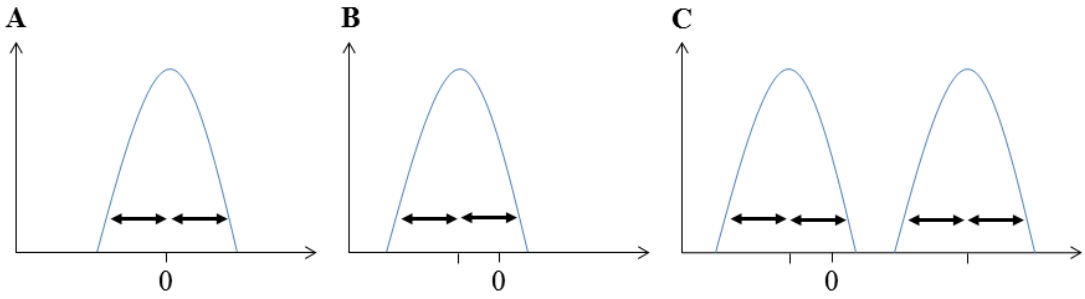


Figure 1: Les 3 différents types d'asymétrie selon Palmer(1994) : l'asymétrie fluctuante (A), la symétrie directionnelle (B) et l'antisymétrie (C). Les flèches noires représentent l'asymétrie fluctuante.

La symétrie directionnelle (Figure 1 B) est définie comme un biais récurrent de la mesure vers l'un des deux côtés, ce qui engendre une distribution dont la moyenne est différente de zéro. L'antisymétrie (Figure 1 C) est également présentée comme une asymétrie récurrente entre les deux côtés mais dont, contrairement à l'asymétrie directionnelle, la direction peut s'inverser aléatoirement selon les individus. Elle est donc caractérisée par une distribution bimodale. Et enfin l'asymétrie fluctuante (Figure 1 A) est la mesure d'une asymétrie aléatoire entre les côtés, sa distribution étant caractérisée par une distribution normale de moyenne 0 et dont la variance représente les déviations par rapport à une symétrie parfaite. Cette dernière est

souvent utilisée comme proxy des performances des individus comme par exemple la fitness. En effet, l'instabilité développementale d'un organisme est supposée se refléter dans son incapacité à ajuster son développement à une forme idéale ; c'est-à-dire une symétrie parfaite entre les structures paires à symétrie bilatérale de l'organisme. Comme cette instabilité développementale n'est pas directement mesurable, un moyen de l'évaluer est donc de mesurer l'asymétrie fluctuante sous l'hypothèse qu'elle reflète bien l'instabilité développementale et qu'elle est corrélée positivement à divers stress génétiques et/ou environnementaux et négativement à la fitness (Palmer 1994, Palmer et al. 2010, Díaz-Gil et al. 2015).

En ce qui concerne la forme des otolithes, l'utilisation de l'asymétrie fluctuante comme indicateur de stress ou de fitness est controversée (Díaz-Gil et al. 2015). Comme l'asymétrie de forme des otolithes résulte potentiellement de la somme des différents types d'asymétrie énoncés précédemment, il est nécessaire dans un premier temps de tester l'existence de chacun d'entre eux et de les isoler afin d'éviter de sous- ou surestimer leurs effets respectifs et donc d'introduire de biais dans l'analyse (Palmer et al. 2010). Le **Chapitre 1** de cette thèse a permis de montrer que, contrairement aux poissons ronds, les pleuronectiformes présentent une asymétrie directionnelle de forme entre les otolithes droit et gauche qu'il convient de prendre en compte dans les études reposant sur l'asymétrie fluctuante. En plus de la chimie (Loher et al. 2008, Kajajian et al. 2014), de la vitesse de croissance et de la masse (Sogard 1991, Fischer & Thompson 2004, Helling et al. 2005), la forme est donc une nouvelle propriété de l'otolithe qui présente une asymétrie directionnelle chez les pleuronectiformes. L'existence d'une asymétrie directionnelle n'exclut pas pour autant la possibilité de conduire des études sur l'asymétrie fluctuante chez ces espèces. Il convient simplement de définir l'asymétrie fluctuante non plus par rapport à une symétrie parfaite entre otolithes droit et gauche, mais par rapport à l'asymétrie directionnelle attendue.

Ceci revient en pratique à mesurer l'asymétrie fluctuante comme une déviation par rapport à la moyenne de la distribution des différences qui dans le cas d'asymétrie directionnelle sera par essence différente de 0. De même, dans le cas de l'antisymétrie, l'asymétrie fluctuante pourra être mesurée comme les déviations mesurées au sein des deux modes par rapport à la moyenne de ces modes (Figure 1). En résumé, toute variation morphologique intra-individuelle des otolithes ayant un caractère systématique peut être prise en compte et retirée lors des études d'asymétrie fluctuante.

Le régime alimentaire : source de variabilité interindividuelle

L'approche *in situ* du **Chapitre 2** a permis de mettre en évidence une corrélation entre le régime alimentaire, décrit à la fois par la quantité et la composition en proies ingérées par l'animal, et la forme des otolithes chez quatre espèces de poissons d'intérêt commercial en Manche Orientale. La composition du bol alimentaire expliquait davantage de variation de forme des otolithes que celle expliquée par la quantité de nourriture ingérée par l'animal. De plus, la variation de forme des otolithes était corrélée à la fois à des catégories de proies principales mais aussi secondaires où des reconstructions de formes ont révélé que la composition alimentaire impactait à la fois la forme globale de l'otolithe mais aussi ces détails tels que la crénulation du bord de l'otolithe et le largueur du renfoncement entre le rostre et l'antirostre (*excisura major).*

Ces résultats étendent les conclusions de Hüssy (2008) qui n'avait étudié que la quantité de nourriture comme source de variabilité de forme et suggèrent que la composition du régime alimentaire peut être une source de variabilité morphologique inter-individuelle de la forme des otolithes.

Les études s'intéressant à la relation entre environnement et otolithe ont souvent suggéré un impact indirect des facteurs environnementaux sur la croissance de l'otolithe et sa

forme au travers de la relation entre croissance somatique et de l'otolithe (Campana & Casselman 1993, Cardinale et al. 2004, Vignon & Morat 2010). En effet, en modifiant la croissance somatique du poisson, l'environnement affecte la croissance de l'otolithe et donc sa forme. De ce fait, l'étude du lien entre la macrostructure de l'otolithe utilisée comme indicateur de croissance somatique au travers de la mesure des distances inter-anneaux et la forme des otolithes peut-être réalisée. Un exemple est celui de la corrélation entre la croissance somatique estimée à partir de la macrostructure et la forme des otolithes pour identifier les stocks de hareng *Clupea harengus* en mer d'Irlande (Burke et al. 2008a). L'approche expérimentale du **Chapitre 3** sur le bar a permis de montrer que les facteurs environnementaux, notamment la composition du bol alimentaire, pouvaient également impacter directement la biominéralisation des otolithes. En effet, selon la composition lipidique de l'alimentation de l'animal en termes de concentration en acide gras polyinsaturés essentiels, des différences de forme et de morphogénèse des otolithes de bar ont été mises en évidence sans pour autant observer de différence significative sur la croissance somatique de l'individu et tout en incluant la relation allométrique avec la masse corporelle des individus.

Les perspectives de ces travaux sont bien entendu de confirmer ces résultats chez d'autres espèces de poissons marins mais également d'étudier l'importance d'autres types de nutriments que les lipides dans l'influence de la composition du régime alimentaire. Dans l'étude du **Chapitre 3**, les acides gras polyinsaturés essentiels ont été choisis parce qu'ils interviennent dans le développement du système nerveux, la croissance et la survie des larves de poissons (Sargent et al. 1999). Mais il est difficile de faire un lien direct entre ces molécules et la biominéralisation des otolithes. En effet, la fraction organique des otolithes est essentiellement composée de protéines et de protéoglycans (Payan et al. 1999, Borelli et al. 2001). Il est donc difficile d'interpréter la corrélation trouvée entre la composition lipidique de l'alimentation et la morphogénèse de l'otolithe à l'échelle des processus de

biominéralisation des otolithes puisque les acides gras polyinsaturés essentiels n'interviennent *a priori* pas dans les processus de constitution de la matrice organique. Ceci pourrait d'ailleurs expliquer au moins en partie la faible amplitude des effets observés dans le **Chapitre 3** même s'ils étaient significatifs. L'étude de l'effet des variations de la composition du régime alimentaire en termes d'acides aminés pourraient être intéressante et ce pour plusieurs raisons. La première est que les acides aminés ingérés participent à la synthèse des protéines qui interviennent dans la précipitation du cristal de l'otolithe. Ainsi une réduction de la concentration des acides aminés dans le bol alimentaire, soit de la quantité totale d'acides aminés ingérés, pourrait engendrer une réduction de la synthèse totale des protéines de l'animal et donc une baisse de la croissance somatique et par conséquent une baisse de croissance de l'otolithe et une modification de sa forme. La seconde raison est que les glycoprotéines qui composent la matrice organique comme par exemple, l'Otolith Matrix Macromolecule-64 (OMM-64) ou l'Otolith Matrix Protein-1 (OMP-1), interviennent dans le polymorphisme du cristal qui se dépose sur l'otolithe − la déposition sur carbonate de calcium pouvant se faire sous forme d'aragonite ou de vatérite −, sa croissance et son orientation. Elles sont donc en grande partie responsables de la forme des cristaux du minéral et donc de la forme de l'otolithe. Ces protéines possèdent une région de fixation du calcium grâce à sa composition riche en glutamate (Hüssy et al. 2004). De ce fait, une variation du régime alimentaire en termes de concentration d'acide glutamique est susceptible d'affecter la croissance de l'otolithe, le polymorphisme des cristaux de carbonate de calcium qui le constitue et donc sa forme.

Importance relative des sources de variation morphologique intra-populationnelle : Plasticité versus canalisation et causalité des effets.

Au-delà de simplement identifier l'asymétrie directionnelle et le régime alimentaire comme sources de variabilité morphologique intra-populationnelle des otolithes, leurs impacts sur la forme des otolithes ont été quantifiés et localisés à travers ces travaux de recherche.

Localisation et quantification des effets

Des reconstructions moyennes ou des prédictions de forme des otolithes ont permis d'identifier les caractéristiques morphologiques des otolithes affectées par les deux facteurs étudiés dans les différents chapitres de cette thèse. L'asymétrie directionnelle observée chez les pleuronectiformes dans le **Chapitre 1** est illustrée principalement par une différence de largueur et de longueur entre les otolithes droit et gauche (Figure 2 A). L'amplitude de cet effet varie entre 2.06 et 17.50 % de différence de recouvrement entre les formes des otolithes droit et gauche et entre 0 et 11.83 % de différence de longueur entre les deux otolithes. En effet, l'otolithe appartenant à l'oreille interne située du côté du flanc aveugle possède toujours l'otolithe le plus large et le plus long que l'espèce considérée soit dextre ou sénestre. Ceci corrobore des résultats antérieurs basés sur la masse de l'otolithe (Sogard 1991, Fischer & Thompson 2004, Helling et al. 2005). En effet, la vitesse de croissance est plus importante pour l'otolithe provenant du côté du flanc aveugle et donc sa longueur plus importante comme observé dans ce chapitre.

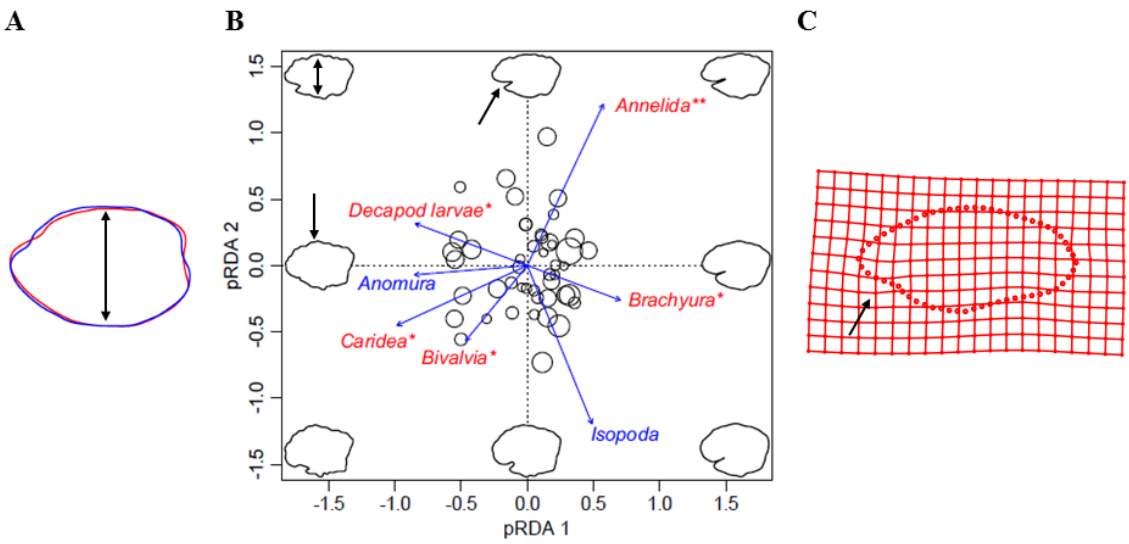


Figure 2: Caractéristiques morphologiques des otolithes affectées (flèches noires) par l'asymétrie directionnelle chez la cardine franche *Lepidorhombus whiffiagonis* (A), par la composition des proies ingérées chez le rouget barbet de roche *Mullus surmuletus* (B) et par la concentration en acides gras polyinsaturés essentiels chez le bar commun *Dicentrarchus labrax* (C).

Dans le **Chapitre 2**, le renfoncement entre le rostre et l'anti-rostre mais aussi l'aspect denté du bord de l'otolithe (Figure 2 B) sont modifiés selon le régime alimentaire des individus. Le régime alimentaire, décrit à la fois par la composition et la quantité de proies ingérées, explique entre 10 et 16 % de la variabilité morphologique des otolithes. Seule la composition du bol alimentaire impacte de manière significative la forme des otolithes et explique entre 12 et 14 % sa variabilité. Le **Chapitre 3** confirme de manière expérimentale l'effet de la composition du bol alimentaire, notamment en termes de concentration en lipides polyinsaturés essentiels, sur la variation de forme des otolithes avec de nouveau une localisation des effets au niveau du renfoncement entre le rostre et l'anti-rostre observé grâce aux grilles de déformation (Figure 2 C).

Ces zones de variation morphologique de l'otolithe s'ajoutent à celles déjà connues. Les facteurs endogènes ont des impacts sur la forme de l'otolithes qui peuvent être soit localisés telle la génétique qui influence la forme du rostre et de l'anti-rostre de l'otolithe (Vignon & Morat 2010) soit globaux telle l'ontogénie qui impacte la forme globale de l'otolithe en provocant son allongement et une forme de plus en plus elliptique au fur et à

mesure du développement (Galley et al. 2006) due au fait que l'axe antéro-postérieur se développe bien plus rapidement que l'axe dorso-ventral. Il en est de même pour les facteurs environnementaux ou exogènes. Ils peuvent impacter la forme globale des otolithes en affectant la vitesse de croissance des otolithes comme chez l'anchois, par exemple, pour lequel les individus dont la croissance est plus lente possèdent des otolithes plus larges que ceux dont la croissance est plus rapide (Pecquerie et al. 2012). Mais ils peuvent affecter également des caractéristiques plus locales comme le nombre de lobes secondaires (Hüssy 2008) ou l'aspect dentelé du bord (Gagliano & McCormick 2004). Cette diversité des caractéristiques morphologiques de l'otolithe soumises à variations, et ce en fonction des facteurs agissant comme source, soulève l'importance de la compréhension des processus de biominéralisation des otolithes, et particulièrement en trois dimensions, ainsi que des mécanismes d'action des différents facteurs sources sur ces processus. Néanmoins, il serait tout d'abord nécessaire de tester la généralité des effets observés à partir d'études sur d'autres espèces de poissons ronds et plats. En ce qui concerne l'asymétrie directionnelle, il serait intéressant de regarder comment le degré d'asymétrie évolue au cours des stades développementaux puisque les figures 2 et 3 suggèrent une augmentation de la valeur de l'asymétrie à la maturité sexuelle chez les poissons plats étudiés. Dans ces travaux, deux méthodes multivariées ont été utilisées pour étudier la forme des otolithes: l'analyse des ellipses de Fourier et la géomorphométrie. L'avantage de l'utilisation des méthodes multivariées est de décrire précisément les variations de forme, particulièrement celles à petite échelle générées par des effets comme les effets environnementaux ou l'effet du régime alimentaire étudié dans cette thèse. D'une part, la méthode des ellipses de Fourier a permis d'étudier de manière très précise la variabilité morphologique des otolithes puisqu'elle est basée sur tout le contour de l'otolithe, permettant ainsi une description et une reconstruction des formes très détaillée tout en évitant un nombre trop important de descripteurs en les

combinant au travers d'une ACP. D'autre part, la morphométrie géométrique a été cruciale pour l'étude de la morphogénèse des otolithes en rendant possible un suivi temporel de points semi-homologues et une visualisation aisée de la localisation des effets grâce à la construction de grilles de déformation. Cependant, d'autres méthodes pourraient être utilisées pour venir corroborer ces résultats et/ou les compléter. Par exemple, la représentation « curvature scale space », qui est basée sur une fonction de courbure du contour de l'otolithe, permettrait de mesurer le taux de changement de la direction du bord et ainsi d'obtenir une cartographie des singularités de courbure de la forme de l'otolithe (Parisi-Baradad et al. 2005). De ce fait, les zones de variations du contour de l'otolithe seraient facilement identifiées et ceci de manière précise. Cela permettrait par la suite de focaliser l'étude de l'effet des facteurs étudiés spécifiquement sur ces zones de variation préalablement identifiées.

L'importance de la canalisation

La canalisation (Waddington 1940) est définie comme la capacité de produire un phénotype unique malgré la variabilité génétique ou environnementale rencontrée au cours de l'ontogenèse (West-Eberhard 2003). Autrement dit, la canalisation est une réduction de la flexibilité ou l'instabilité développementale qui rend le développement d'un phénotype adapté résistant aux perturbations de l'environnement et à la variabilité génétique qui pourraient produire des écarts du phénotype exprimé par rapport à son optimum. La canalisation s'oppose ainsi à la plasticité phénotypique qui est la capacité d'un organisme caractérisé par un génotype donné à exprimer différents phénotypes en fonction de l'environnement qu'il rencontre. La canalisation résulte généralement de l'évolution du système développemental induite par les pressions de sélection naturelle liées au fait que la fonction de certaines caractéristiques phénotypiques dépend de leur stabilité.

L'amplitude faible à modérée des effets intra- et inter-individus observés sur la forme des otolithes dans les différents travaux de cette thèse suggèrent une canalisation de la forme

des otolithes. Le **Chapitre 1** montre que l'asymétrie directionnelle n'excède jamais 18% de différence de forme entre les otolithes droit et gauche quelle que soit l'espèce considérée. Le **Chapitre 2** (Chapitre 2, Figure 5) montre une variabilité de forme liée au régime alimentaire plus importante mais restant dans l'ensemble relativement modérée. Le **chapitre 3** suggère que la composition lipidique du régime alimentaire de l'animal durant le stade larvaire influence certes la forme des otolithes en début de stade juvénile et leur trajectoire de morphogénèse par la suite mais pas leur forme finale mesurée plus tardivement pendant le stade juvénile. La canalisation de la forme des otolithes serait due aux pressions de sélection naturelle liées au bon fonctionnement de l'oreille interne. En effet, l'otolithe possède un rôle essentiel en tant que mécanorécepteur du système audio-vestibulaire de l'animal. Ses changements de position sous divers stimuli (oscillation à des fréquences auditives, gravité, accélérations de l'individu) stimulent les cils apicaux des cellules sensorielles de la *macula* qui transmettent l'information au système nerveux sous forme d'un message électrique traduisant les déformations des cils par l'otolithe. De plus, au-delà de ses changements de position, la forme même des otolithes est impliquée dans les réponses aux stimuli auditifs. L'irrégularité de la forme de l'otolithe permet que sa réponse à une stimulation auditive ne soit pas seulement une simple réponse de va-et-vient. La complexité de sa forme engendrait plutôt un mouvement de balancier (« rocking motion ») qui permettrait la création de stimuli additionnels que le poisson serait capable d'interpréter pour caractériser et localiser la direction du signal sonore reçu (Krysl et al. 2012). Etant la fonction de la forme de l'otolithe, l'hypothèse de canalisation est vraisemblable puisque la forme dépend mécaniquement de la biominéralisation (Borelli et al. 2003) qui est elle-même sous contrôle génétique (Söllner et al. 2003). Le contrôle génétique du processus de biominéralisation est donc susceptible de contraindre la trajectoire ontogénétique de l'otolithe de sorte à canaliser sa forme. Un parallèle entre forme et composition chimique des otolithes peut-être réalisé en termes de

canalisation. Les éléments incorporés dans l'otolithe sont régulés à la fois par l'environnement mais aussi par des facteurs endogènes. À cela s'ajoute l'homéostasie qui est un processus de régulation permettant de maintenir certaines caractéristiques physiologiques, notamment la concentration de certains éléments ou molécules chimiques, à des valeurs optimales pour le bon fonctionnement et la survie de l'organisme. Le passage des éléments entre l'environnement et le plasma sanguin à travers plusieurs barrières physiologiques (branchies, paroi intestinale) est considéré comme un processus des plus complexes (Sturrock et al. 2014) qui altère les concentrations absolues et relatives des éléments (Campana 1999) et contribue à une composition chimique de l'otolithe relativement stable assimilable à de la canalisation.

Les résultats du **Chapitre 3** suggèrent que la canalisation serait de plus en plus importante au fur et à mesure que le développement progresse avec une disparition des effets observés au cours du stade juvénile due à un phénomène de réponse compensatoire. Ceci rejoint l'hypothèse selon laquelle les effets des facteurs environnementaux seraient plus forts et donc plus facilement détectables au cours des premiers stades de vie des poissons. En effet, le taux d'accrétion des otolithes étant plus important aux jeunes stades, les effets environnementaux seraient plus facilement détectables car la moindre variation devrait être rapidement amplifiée (Hüssy 2008). Ces résultats ont été obtenus grâce à un suivi individuel de la morphogénèse des otolithes au moyen de marquages chimique de la structure des otolithes à l'oxytétracycline (Figure 3).

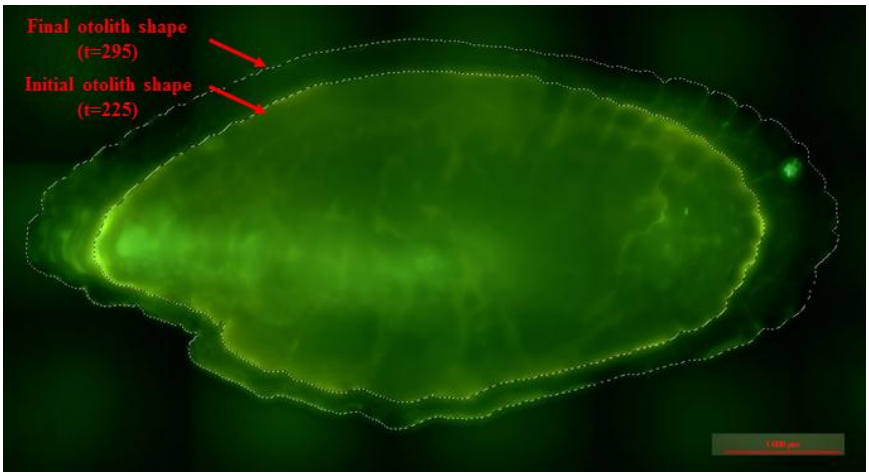


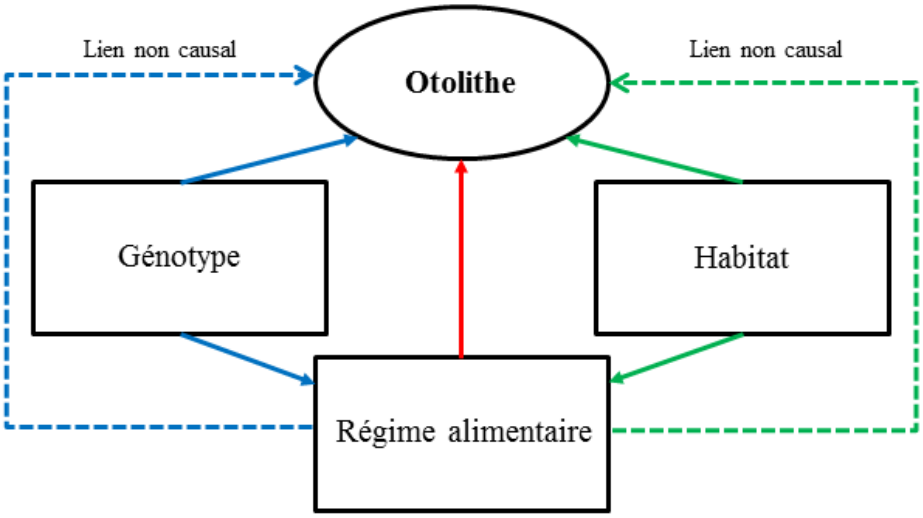
Figure 3 : Image mosaïque d'un otolithe de bar commun marqué à l'oxytétracycline sous lumière UV avec les contours détourés de l'anneau fluorescent et de la finale de l'otolithe.

Ceci a permis notamment de prendre en compte l'effet de la variabilité individuelle de croissance et ses effets via l'allométrie entre masse corporelle et forme des otolithes. Cette approche originale s'est avérée relativement performante pour étudier à l'échelle individuelle l'influence de facteurs environnementaux sur les trajectoires de morphogénèse des otolithes de bar au stade juvénile en milieu contrôlé. Il serait intéressant de confirmer ces résultats dans le futur en utilisant la même approche mais en multipliant les marquages dans le temps afin d'avoir un suivi individuel plus fin de la morphogenèse des otolithes, le **Chapitre 3** n'étant basé que sur 2 points. Confirmer ces résultats sur d'autres espèces marines serait également nécessaires. Ces études pourraient être réalisées en milieu contrôlé mais aussi en milieu naturel pour des espèces relativement territoriales et sédentaires (e.g. poissons de récifs, espèces avec phénomène de « homing ») de manière à assurer leur recapture.

La difficulté de déterminer la causalité des effets dans les études *in situ*

Démêler les effets des différents facteurs qui agissent sur la variabilité de forme des otolithes et en inférer des liens de causalité peut s'avérer être très difficile particulièrement pour les études réalisées *in situ*. En effet, les variables mesurées sur le terrain sont souvent inter-corrélées entres elles, voire avec des variables négligées ou impossibles à mesurer, de

sorte qu'il est difficile de juger de la causalité des variables explicatives sur la variable expliquée à partir de simples modèles statistiques à cause des effets confondants qui en découlent. Ceci est vrai pour l'effet du régime alimentaire sur la forme des otolithes détecté à l'échelle interindividuel par l'étude in situ du **Chapitre 2**. Le lien entre la variabilité interindividuelle de la composition du régime alimentaire et la variation interindividuelle de la forme pourrait en effet être un lien causal direct (Figure 4). Dans ce cas, cela signifierait que le régime alimentaire (composition de la nourriture incorporée par les individus) influence la morphogenèse, et donc la biominéralisation des otolithes. Cependant, ce lien pourrait s'avérer être non causal. Les deux facteurs potentiellement confondants majeurs pourraient être l'habitat et le génotype. Concernant l'habitat, s'il varie entre individus en termes de certains paramètres environnementaux (autres que ceux pris en compte dans les analyses du **Chapitre 2**) et que ces derniers possèdent une influence sur les processus de biominéralisation des otolithes, une variabilité morphologique des otolithes sera observée. De plus, la disponibilité des proies est connue pour varier également en fonction de l'habitat (Estes et al. 2003, Araújo et al. 2011) et entraîner une variation interindividuelle du régime alimentaire. De ce fait, la forme de l'otolithe pourrait co-varier avec le régime alimentaire sans lien de causalité réel parce que ces deux facteurs sont corrélés avec un facteur commun, l'habitat. Concernant le génotype, il est connu qu'il détermine en partie le comportement des individus, et donc potentiellement leur régime alimentaire (Futuyma & Moreno 1988). De même, les processus physiologiques sont également partiellement dépendants du génotype des individus. Une fois de plus, les deux aspects, forme des otolithes et régime alimentaire pourraient ainsi co-varier avec un facteur commun, le génotype, induisant une association sans lien de cause à effet réel.



Le régime alimentaire est directement lié au processus de biominéralisation d'où un lien causal entre régime et forme de l'otolithe.

L'habitat influence à la fois le régime alimentaire des poissons et le processus de biominéralisation des otolithes. Il en résulte un lien non causal entre régime alimentaire et forme de l'otolithe.

Le génotype influence à la fois le régime alimentaire et le processus de biominéralisation des otolithes. Il en résulte également un lien non causal entre régime alimentaire et forme de l'otolithe

Figure 4 : Hypothèses sur l'association entre la variabilité morphologique de l'otolithe et la variabilité du régime alimentaire de l'animal observée *in situ* à l'échelle intra-populationnelle.

En cas de facteurs potentiellement confondants connus et mesurables *in situ*, il est possible d'inclure une mesure de ces derniers dans les analyses, généralement en tant que co-variables, afin de supprimer leurs effets et de réussir à démêler ceux des variables d'intérêt. Le **Chapitre 2** a utilisé cette approche pour supprimer l'effet potentiellement confondant de facteurs environnementaux liés l'habitat sur la forme des otolithes et réussir à extraire l'effet du régime alimentaire. Dans cette étude, les facteurs environnementaux considérés étaient la température, la salinité et la profondeur et ont été mesurés au point et durant la période de capture de chaque individu. La limitation de cette approche provient non pas du principe même mais de la mesure de ces co-variables. Les données utilisées sont uniquement révélatrices d'un instant donné et pas forcément des conditions environnementales rencontrées par l'animal à plus long terme. Il en est de même pour les contenus stomacaux qui

révèlent le régime alimentaire de l'animal à un instant donné mais pas obligatoirement sur le long terme. L'interprétation des résultats du **Chapitre 2** repose donc sur l'hypothèse que le régime alimentaire et les conditions environnementales observés pour un individu à un instant donné sont représentatives de son régime et des conditions rencontrées à plus long terme ou, plus précisément, que les différences interindividuelles observées à un instant donné sont représentatives des différences à plus long terme. Même si cette hypothèse est supportée indirectement par la littérature sur l'importance et la prévalence de la spécialisation écologique et/ou du régime alimentaire à l'échelle individuelle (voir les revues de Bolnick et al. 2003, Araújo et al. 2011), notamment chez les poissons (e.g. Beaudoin et al. 1999, Svanbäck & Persson 2004), elle n'en reste pas moins difficile à prouver puisqu'il semble qu'aucune étude longitudinale n'existe sur le sujet chez les poissons.

Dans un tel cas de figure, l'expérimentation est la seule approche permettant de confirmer des liens de causalités potentiels identifiés à partir d'études *in situ*. C'est pourquoi, dans le but de valider la corrélation entre la composition du régime alimentaire et la forme des otolithes observée dans le **Chapitre 2**, une expérience en milieu contrôlé a été menée chez le bar dans le **Chapitre 3** qui a permis de montrer l'effet d'une réduction de la concentration des lipides polyinsaturés essentiels sur la morphogénèse des otolithes qui cependant reste de faible amplitude. L'expérimentation est une approche prometteuse et nécessaire pour identifier les relations de causalité entre les variabilités des facteurs endogènes et/ou exogène et la variation de forme des otolithes. Á travers les travaux de cette thèse, seul un facteur à fait l'objet d'une étude expérimentale. Pour mieux comprendre les effets multiples du milieu naturel, une approche multifactorielle avec un design expérimental suivant un plan factoriel constituerait une perspective de travail intéressante pour évaluer à la fois l'effet individuel de chacun des facteurs (importance relative, localisation sur la forme de l'otolithe…) mais aussi leurs effets conjoints, ce qui permettrait de révéler les effets synergiques, voire même

amplificateurs, ou au contraire antagonistes. De plus, il serait nécessaire de tester la généralité des effets observés grâce à des études expérimentales réalisées sur d'autres espèces de poissons ronds que le bar commun étudié dans cette thèse, ainsi que chez des espèces de poissons plats.

L'utilisation de la forme des otolithes comme outil dans l'identification des populations.

L'identification des stocks de poissons est une composante importante dans la gestion durable des stocks des ressources halieutiques (Begg et al. 1999, Cadrin et al. 2005). Le cas le plus simple serait celui où le stock est associé à une population au sens biologique. Cette dernière est définie comme un ensemble d'individus d'une même espèce vivant dans un écosystème donné et possédant des caractères communs transmissibles par hérédité (Daget & Le Guen 1975). Dans ce cas, le stock correspond à la partie exploitable de la population associée. Dans la pratique cette association n'est pas toujours aussi simple puisque le stock est une unité de gestion souvent définie sur la base d'unités géographiques administratives, et non sur des bases biologiques, et peut donc regrouper plusieurs populations ou à l'inverse n'être qu'une fraction de la partie exploitable d'une population (Laurec & Le Guen 1981). Dans le cas le plus simple, l'utilisation de la forme l'otolithe comme outil pour discriminer les populations (ou/et stocks) d'une espèce repose sur un principe simple : grouper les individus de sorte à minimiser le ratio de la variabilité morphologique des otolithes intra-groupes sur la variabilité intergroupes. Ainsi, les populations identifiées sur la base des variations morphologiques des otolithes sont généralement associées à des localisations géographiques avec des conditions environnementales (chimie, physique, biocénose) propres.

Afin d'utiliser la forme des otolithes comme un indicateur de population robuste, il est donc nécessaire de démêler ses variations associées aux différentes échelles d'organisation biologiques pertinentes, autrement dit, de démêler la variabilité observée à l'échelle intra-populationnelle de celle rencontrée à l'échelle inter-populationnelle. En effet, la variabilité à l'échelle intra-populationnelle pourrait être confondue avec la variabilité inter-populationnelle. Ceci pourrait engendrer un biais dans l'identification des populations du fait d'une interprétation écologique erronée des groupes identifiés qui repose bien souvent sur l'hypothèse que la variabilité morphologique intra-population est négligeable par rapport à la variabilité inter-population. Vignon (2015) a notamment montré que l'effet des facteurs environnementaux locaux contribue autant à la variation morphologique des otolithes que les patrons d'hétérogénéité environnementale à large échelle et ce quel que soit le niveau de l'échelle d'observation. Si la variation interindividuelle ou intra-populationnelle est plus importante que celle inter-populationnelle et qu'elle est confondue avec cette dernière, alors l'identification des populations basée sur de la variabilité morphologique des otolithes pourrait être biaisée selon deux scénarios assez schématiques (Figure 5).

Le premier scénario correspond à celui où la variabilité morphologique des otolithes va permettre d'identifier plus de populations qu'il n'en existe réellement, car la variabilité à l'échelle intra-populationnelle est forte (Figure 5, partie gauche). C'est le cas par exemple d'une population panmictique unique dont les individus relativement sédentaires (e.g. poissons récifaux, anadromes ou catadromes, territoriraux) sont dispersés sur une aire géographique regroupant trois habitats différents caractérisés par des facteurs environnementaux propres en dehors des périodes de production. Du fait de l'influence environnementale, trois morphotypes d'otolithe vont être trouvés dans cette population par ailleurs homogène génétiquement entraînant l'identification de 3 stocks distincts sur la base de la variation morphologique des otolithes. Dans notre cas, l'effet du régime alimentaire, qui

dépend lui-même en partie de l'habitat, a été mis en évidence comme une source de variation de forme des otolithes (**Chapitres 2 et 3**) mais d'autres facteurs environnementaux, comme par exemple la température ou la salinité, pourraient aussi être impliqués, d'autant que ces différents facteurs co-varient généralement entre habitats. Une perspective de recherche future serait de quantifier les effets respectifs de chacun des facteurs environnementaux à l'échelle intra-populationnelle en plus de déterminer la localisation sur la forme de leurs effets respectifs et conjoints comme décrit dans la partie précédente.

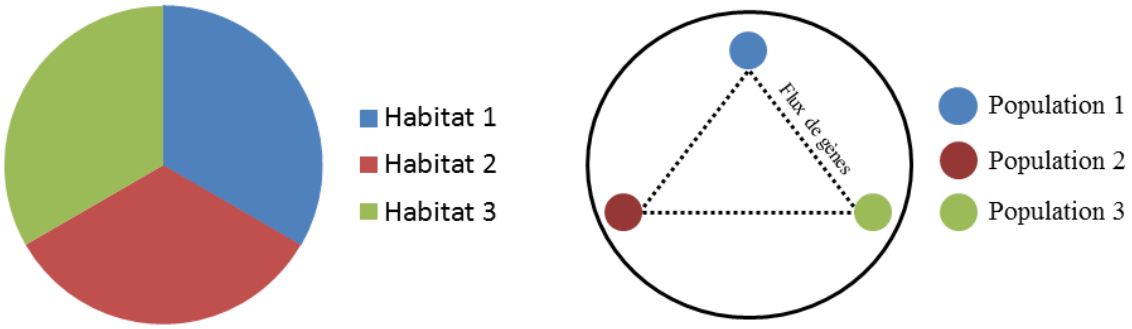


Figure 5 : Illustration de deux cas particuliers où la variabilité phénotypique des otolithes à l'échelle intra-populationnelle est plus élevée que celle inter-populationnelle : Une seule population avec 3 habitats différents (à gauche) et une métapopulation dont les habitats sont similaires (à droite).

Le second scénario est celui où les variations de forme des otolithes vont permettre d'identifier moins de stocks qu'il n'y en existe parce que la variabilité inter-populationnelle est faible et sera potentiellement masquée par la variabilité intra-populationnelle (Figure 5, partie droite). Ceci pourrait se produire, par exemple, dans le cas d'une métapopulation formée de trois populations isolées géographiquement et démographiquement mais non génétiquement différenciées à cause de flux de gènes suffisants ou d'une isolation récente. En cas d'habitats similaires, aucune source de variation inter-populationnelle n'affectera la forme des otolithes de sorte qu'une seule population sera identifiée au lieu de trois. Une caractéristique commune de ces deux scénarios est que la variabilité environnementale

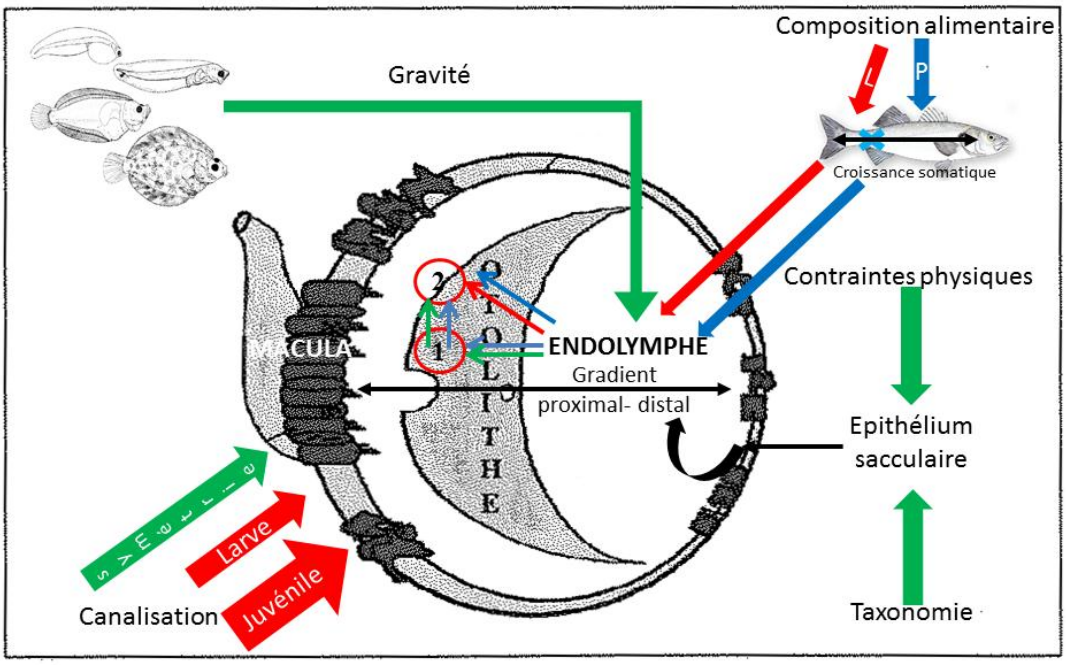
responsable de la variation morphologique des otolithes est plus importante à échelle spatiale locale qu'à large échelle.

Ces deux scénarios représentent bien entendu des cas de figure extrême et c'est le ratio entre la variabilité morphologique des otolithes aux échelles intra- et inter-populationnelle et aux échelles spatiales locale et globale qui gouverne le succès de l'identification des stocks. C'est une des raisons pour lesquelles l'identification de stocks nécessite une approche holistique, c'est-à-dire basée sur l'utilisation de différents outils complémentaires dont la combinaison des résultats permet d'augmenter la probabilité de distinguer correctement les stocks (Begg & Waldman 1999). La morphologie des otolithes est l'un de ces outils mais sa microchimie et sa structure peuvent être aussi utilisées. D'autres outils ne provenant pas de otolithes et qui permettent de couvrir d'autres aspects de la biologie des poissons peuvent également être utilisés comme par exemple les marqueurs génétiques, les ratios isotopiques de certains éléments issus de divers organes (McMahon et al. 2010), ou les marques électroniques.

Conclusions

Cette thèse a mis en évidence le rôle de l'asymétrie directionnelle et du régime alimentaire *in situ* et en milieu contrôlé, particulièrement sa composition, comme sources de variabilité morphologique des otolithes à l'échelle intra-populationnelle (Figure 6). Au-delà de la significativité des effets, leur quantification a permis de montrer l'importance de la canalisation de la forme des otolithes résultant *a priori* du rôle de mécanorécepteur de ces dernières dans le fonctionnement du système audio-vestibulaire des poissons. L'asymétrie directionnelle de forme entre les otolithes droit et gauche observée chez les pleuronectiformes était en effet modérée, n'excédant pas 18 % de différence. De même l'approche innovante de suivi individuel de la morphogénèse des otolithes de bar par marquage chimique a permis de

montrer expérimentalement la réduction de la flexibilité développementale de la forme face à une réduction de concentration alimentaire des lipides polyinsaturés essentiels durant le stade juvénile. La réussite de ce suivi encourage fortement à appliquer ce type d'approche chez d'autres espèces de poissons et à une résolution temporelle plus élevée aussi bien en milieu contrôlé que naturel pour les espèces de poissons sédentaires et/ou territoriales. Enfin, l'identification par reconstruction de forme des caractéristiques morphologiques affectées par l'asymétrie directionnelle et le régime alimentaire ont permis de pointer diverses zones de variation de forme des otolithes qui se recoupent avec les effets d'autres déterminants connus, ce qui soulèvent l'importance de la compréhension des processus de biominéralisation des otolithes, notamment en trois dimensions.



1 Croissance de l'otolithe.

2 Forme de l'otolithe.

Figure 6 : Schéma récapitulatif des hypothèses soulevées, dans chacun des chapitres de cette thèse, expliquant la variabilité morphologique des otolithes à l'échelle intra-populationnelle: asymétrie directionnelle (Chapitre 1, en vert), régime alimentaire (Chapitre 2, en bleu, P=protéines), concentration en lipides polyinsaturés essentiels (Chapitre 3, en rouge, L=lipides polyinsaturés essentiels).

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ANNEXE I

Identifying blue whiting (Micromesistius poutassou) stock structure in the Northeast Atlantic by otolith shape analysis

- Publication:

Mahé K., Oudard C., **Mille T**., Keating J., Goncalves P., Worsoe Clausen L., Petursdottir G., Rasmussen H., Meland E., Mullins E., Pinnegar J. K., Hoines A., Trenkel, V., (2015). Identifying blue whiting (*Micromesistius poutassou*) stock structure in the Northeast Atlantic by otolith shape analysis, Canadian Journal of Fisheries and Aquatic Sciences, *in press 2016*.

ABSTRACT

Information on stock identification and spatial stock structure provide a basis for understanding fish population dynamics and improving fisheries management. In this study otolith shape analysis was used to study the stock structure of blue whiting (*Micromesistius poutassou*) in the northeast Atlantic using 1693 samples from mature fish collected between 37° and 75°N and 20°W and 25°E. The results indicated two stock components located north and south of ICES Divisions VIa and VIb (54°5 to 60°5 N, 4° to 11°W). The central area corresponds to the spawning area west of Scotland. Sampling year effects and misclassification in the linear discriminant analysis suggested exchanges between the northern and southern stock components. The results corroborate previous studies indicating a structuring of the blue whiting stock into two components, with some degree of mixing in the central area of distribution.

Keywords: *Micromesistius poutassou,* otolith shape, Fourier descriptors, stock discrimination, northeast Atlantic.

RESUME

L'identification et la connaissance de la structuration spatiale de stocks sont essentielles pour étudier la dynamique des populations de poissons et ainsi gérer les pêcheries. Dans cette étude, la forme des otolithes a été employée pour comprendre la structuration des stocks des merlans bleus (*Micromesistius poutassou*) dans le nord-est de l'Océan Atlantique à partir de 1693 poissons matures échantillonnés entre 37°-75° N et 20°O-25°E. Les résultats ont indiqué deux composantes de stock séparées entre l'ouest de l'Irlande et de l'Ecosse correspondant à la zone de reproduction. Les effets du facteur « année d'échantillonnage » et la mauvaise classification dans l'analyse linéaire discriminante suggère des échanges entre les composants nordique et méridionale. Ces résultats corroborent des études antérieures indiquant une structuration des merlans bleus en deux composantes avec un certain niveau de mélange.

Mots-clés: *Micromesistius poutassou,* forme de l'otolithe, descripteurs de Fourier, discrimination de stock, nord-est de l'Océan atlantique.

Introduction

Blue whiting (*Micromesistius poutassou*) is a pelagic gadoid whose distribution in the northeast Atlantic extends from Spitsbergen in the north to Morocco in the south (Zilanov 1968; Bailey 1982; Heino and Godø 2002; Monstad 2004; ICES 2012). The blue whiting fishery is one of the largest in the north Atlantic (Trenkel et al. 2014). Several European nations have extensively fished on this species, especially since the late 1990s, with annual catches ranging from 100 000 to 2 400 000 tonnes (ICES 2013). The most important spawning areas are located to the west of Scotland and Ireland, in the water column at the shelf breakat depths of 250 to 450 metres (Bailey 1982; Isaev and Seliverstov 1991; Standal 2006). Juveniles and adults make annual migrations in early summer to the main feeding areas, located primarily in the Norwegian Sea in the north and in the Celtic Sea and Bay of Biscay in the south (Bailey 1982; Carrera et al. 2001, Fig. 1).

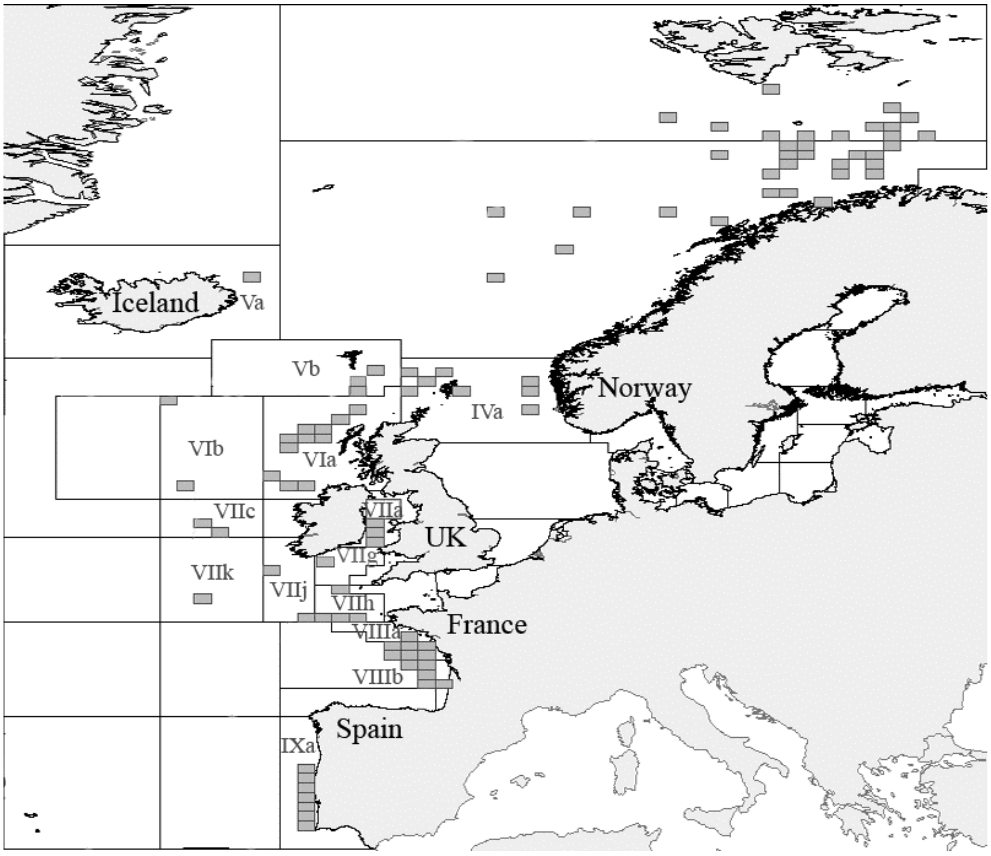

Figure 1: Map of location of blue whiting samples collected between 2006 and 2011. Blue whiting feeding areas are located in the Norwegian Sea (IIa & IIb), the Celtic Sea (VIIg-j) and the Bay of Biscay (VIIIa & b).

Stock identification and information on spatial structure provide a basis for understanding population dynamics and in turn to improve fisheries management. Several techniques can be used for stock identification, including tagging experiments, analysis of spatial variation of genetic or morphometric markers, differentiation of life-history variables, parasites and contaminant concentrations (Pawson and Jennings 1996; Cadrin et al. 2014). Otolith shape analysis is an efficient stock identification tool linked to genetic heterogeneity and the influence of environmental factors on otolith shape (Cadrin and Friedland 1999; Campana and Casselman 1993; Torres et al. 2000; Cardinale et al. 2004; Swan et al. 2006; Vignon and Morat 2010).

Otolith morphology is influenced by biotic and abiotic factors (Cardinale et al. 2004; Capoccioni et al. 2011). Analysis of the outer shape of otoliths has previously been used for stock discrimination of various species (haddock: Begg and Brown 2000; cod: Galley et al. 2006; Petursdottir et al. 2006, Stransky et al. 2008; striped red mullet: Benzinou et al. 2013). Legua et al. (2013) applied otolith shape analysis to identify two distinct stocks of southern blue whiting (*Micromesistius australis*), an Atlantic and a Pacific stock.

Several basic descriptors exist to describe the external contour of otoliths (e.g., coefficient of form, roundness, circularity, rectangularity and ellipticity) in addition to more complex geometric morphometric analyses (Ponton 2006; Ramirez-Perez et al. 2010; Vergara-Solana et al. 2013), wavelet functions (Parisi et al. 2005; Sadighzadeh et al. 2014) and the geodesic method (Benzinou et al. 2013). Of these, the elliptical Fourier analysis currently remains the most widely used and powerful method to describe otolith external shapes (Aguera and Brophy 2011; Capoccioni et al. 2011; Fergusson et al. 2011; Legua et al. 2013; Paul et al. 2013). This method has the advantage of being unaffected by short-term changes in fish condition (Campana and Casselman 1993) due to environmental variations (Campana 1999). Nevertheless, its biological interpretation is more complex than that of

linear morphometric descriptors (Stransky and MacLellan 2005). Some studies have combined the elliptical Fourier analysis and some basic descriptors of otolith contours to help interpretation of results (Campana and Casselman 1993; Begg and Brown 2000; Galley et al. 2006; Merigot et al. 2007; Fergusson et al. 2011; Legua et al. 2013).

For blue whiting, all previous morphological, physiological, genetic, parasite and otolith shape research suggested the existence of at least two stock components in the north-east Atlantic, though the spatial range differed between studies (Karasev 1990; Isaev and Seliverstov 1991; Skogen et al. 1999; Ryan et al. 2005; Brophy and King 2007; Was et al. 2008; Keating et al. 2014). However, these studies focused primarily on the spawning grounds west of Ireland and regions further north, with very few samples from the southern part of the range, i.e. the Bay of Biscay and areas further to the South.

In the current study, analysis of the perimeter and elliptical Fourier descriptors of sagittal otoliths were combined. The otolith shape analysis were performed to discriminate stock components of blue whiting in the north-east Atlantic using individuals sampled over a wide geographic area, from Portuguese waters to Spitsbergen and Iceland.

Materials and methods

Sample collection

Blue whiting samples from ten ICES Divisions between Spitsbergen and the Portuguese waters (Fig. 1) were collected in 2006, 2008, 2010 and 2011 by seven institutes (IFREMER, France; CEFAS, UK (England and Wales); IMR, Norway; DTU, Denmark; MRI, Iceland, AFBI, UK (Ireland) and IPMA, Portugal) during international scientific surveys, on-board fishing vessels and from fish markets. This collection includes all samples used by Keating et al. (2014).

Sex and maturity of the sampled individuals was determined by macroscopic examination of gonadal maturity. Only mature fish were included in this study to minimize the effect of sexual maturity which can change otolith shape (Cardinale et al. 2004). This yielded a total of 1693 mature individuals ranging from 16 to 42 cm total length (mean 27.80, Table 1), corresponding to ages 1 to 13 years. The size for the 878 females ranged from 16 to 42 cm, while the 537 males had sizes from 16 to 35 cm total length. Sagittal otoliths were extracted from all 1693 fishes; all otoliths were weighed.

Otolith shape analysis

Blue whiting otoliths are flat with rounded dorsal and ventral margins. The *rostrum* is large and straight and therefore, the *antirostrum* is indistinct (Fig. 2). Images of the whole left sagittal otoliths were scanned (Epson V750) under reflected light and stored with high resolution (3200 dpi). Image processing was performed using the image analysis system TNPC (Digital processing for calcified structures, version 7, www.tnpc.fr) with the *sulcus acusticus* facing up. Otolith length and width were measured and the contour of each otolith was extracted using the automatic threshold in the TNPC software. To describe otolith contours, Elliptic Fourier Analysis (EFA; Lestrel 1997) was carried out. For each otolith, the first 99 elliptical Fourier harmonics (Hi) were extracted and normalised with respect to the first harmonic using the TNPC software and were, thus, invariant to otolith size and rotation and starting point of the shape measurements (Kuhl and Giardina 1982).

Table 1. Number of blue whiting otolith samples by year, sex (M: males, F: females) and ICES Division. Total length characteristics (mean±S.D.) are given in parentheses.

		Year of sampling				Total
Area (ICES Division)	Sex	2006	2008	2010	2011	
Norwegian Sea (IIa)	F			105 (32.1±2.8)		105 (32.1±2.8)
	M			96 (29.7±1.8)		96 (29.7±1.8)
Spitzbergen and Bear Island (IIb)	F			33 (33.4±2.2)		33 (33.4±2.2)
	M			9 (30.3±1.1)		9 (30.3±1.1)
Northern North Sea (IVa)	F		30 (28.0±1.5)	48 (28.1±5.6)	24 (22.1±3.7)	102 (26.7±4.9)
	M			67 (25.8±5.3)	20 (22.0±3.0)	87 (24.9±5.1)
Iceland Grounds (Va)	F	112 (27.4±2.1)			14 (31.5±1.3)	126 (27.8±2.4)
	M				52 (32.3±1.7)	52 (32.3±1.7)
Faroes Grounds (Vb)	F		26 (27.1±1.9)			26 (27.1±1.9)
	M		12 (26.3±1.0)			12 (26.3±1.0)
West of Scotland (VIa)	F			25 (30.9±1.2)	218 (33.0±2.8)	243 (32.8±2.7)
	M			35 (29.0±0.7)	81 (30.5±2.2)	118 (30±2.0)
Rockall (VIb)	F		30 (29.2±1.2)	15 (31.4±1.7)		45 (30.0±1.7)
	M		31 (26.7±0.7)	25 (29.3±1.3)		31 (27.9±1.6)
Irish Sea (VIIa)	F				16 (18.9±2.3)	16 (18.9±2.3)
	M				24 (17.5±1.4)	24 (17.5±1.4)
Porcupine Bank (VIIc)	F			28 (32.1±1.2)		28 (32.1±1.2)
	M			22 (29.5±0.9)		22 (29.5±0.9)
Celtic Sea (VIIg & h)	F	70 (25.1±4.1)				70 (25.1±4.1)
	M	32 (25.7±3.4)				32 (25.7±3.4)
Southwest of Ireland – West (VIIk)	F			23 (31.5±1.3)		23 (31.5±1.3)
	M			9 (29.7±0.7)		9 (29.7±0.7)
Bay of Biscay (VIIIa&b)	F	34 (22.2±3.5)			118 (24.2±4.4)	152 (23.3±4.3)
	M	12 (21.4±2.5)			118 (21.2±4.5)	113 (21.1±4.5)
Portuguese Waters – East (IXa)	F		24 (25.2±1.8)		16 (20.8±2.7)	40 (23.9±2.9)
	M		33 (22.6±2.4)		16 (23.0±1.5)	49 (22.7±2.2)

To determine the number of harmonics needed to reconstruct the otolith outline, the Fourier Power (PF) was calculated for each individual otolith k as a measure of the amount of contour rebuilt by each harmonic:

$$PF(n_k) = \sum_{HI=1}^{n_k} \frac{A_{HI}^2 + B_{HI}^2 + C_{HI}^2 + D_{HI}^2}{2} \qquad (1)$$

Where A_{HI}, B_{HI}, C_{HI} and D_{HI} are the parameters of the *HI*th harmonic and n_k is the total number of harmonics included. The value of n_k was chosen such that $PF(n_k)$ explains 99.99% of variance in contour coordinates or, in other words, such that shape is reconstructed at 99.99% (Lestrel 2008).

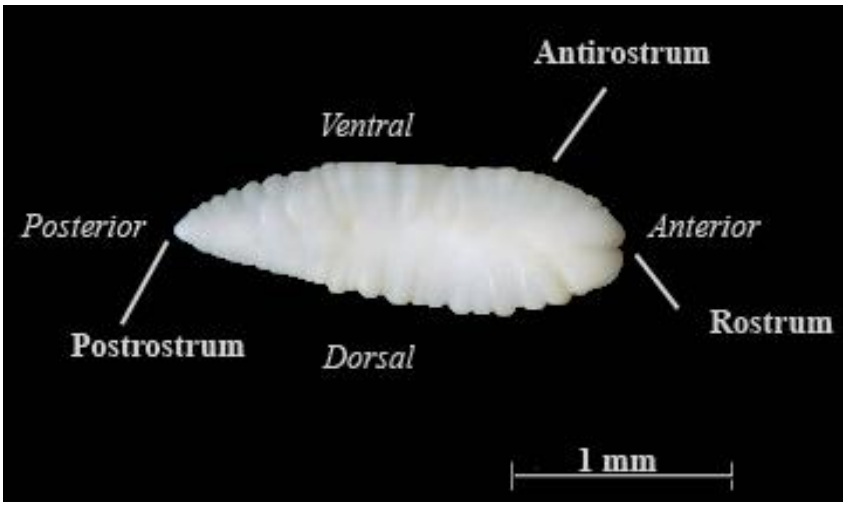

Figure 2: Photograph of a whole blue whiting otolith.

Statistical analyses

Otolith perimeter was used as an integrative variable to study geographic and temporal differences in overall otolith shape. Keating et al. (2014) reported wider otoliths for a given otolith length for individuals sampled in the north-eastern part of the spawning area compared to the southwestern part; the perimeter should reflect such differences. Perimeter was modelled as a function of sex (factor), ICES Division (factor), sampling year (factor) and total length (linear) as well as all two-way interactions with length. For this a linear model with Gaussian error distribution was fitted using stepwise variable selection based on AIC to select

the final model. Total length was centred by subtracting the mean length in the data (27.86 cm) to decouple the estimation of the slope and intercept in the linear model. An analysis of covariance (ANCOVA) was carried out for the final model and a Tukey HSD (honest significant difference) post-hoc test was used for pairwise comparison between intercepts and slopes (for length) of ICES Divisions (α-level=0.05). To visualise geographic differences, the perimeter was predicted for the year 2010 for a total length of 28 cm (mean length in data set) and 38 cm.

To investigate potential explanations for otolith shape differences in more detail, multivariate analyses were carried out. To reduce the number of dimensions and to avoid collinearity between shape descriptors, Principal Components Analysis (PCA) was applied to EFDs (Elliptical Fourier Descriptors) of otolith contours (Rohlf and Archie 1984). Significant principal components (PC) were selected with the 'broken stick method' (Legendre and Legendre 2012). This method assumes that if the total variance (sum of the eigenvalues) is divided randomly among the various components, then the expected distribution of the eigenvalues will follow a broken-stick distribution. The contribution of original variables to the corresponding eigenvectors (PC loadings) were analyzed to understand which shape features had most influence on each PC. Next, two Redundancy analyses (RDA) were carried out. RDA is an extension of multiple regressions to multivariate response data and an extension of principal component analysis (Legendre & Legendre 2012). The first RDA aimed at removing the variance explained by total fish length. In the second RDA, the residuals of the first RDA, referred to as RDA residual matrix, were related to the explanatory variables of interest, i.e. sex, ICES Division and sampling year. A permutation test was used to test the significance of each explanatory variable. The magnitude of multicollinearity between exploratory variables of interest, e.g. total length, sex, year of sampling and ICES

Division was evaluated by calculating variance inflation factors (threshold value for VIF =10; Borcard et al. 2011).

To determine whether otoliths collected in different ICES Divisions could be distinguished based on their contour shapes, stepwise Linear Discriminant Analysis (LDA) was applied to select the discriminant variables among the Fourier harmonics (Rencher and Christensen 2012). To evaluate the resulting discriminant functions, the percentage correct classification of individuals to ICES Divisions was calculated using jack-knifed cross-validation and Wilk's lambda criteria (Klecka 1980).

Finally, a cluster analysis was performed on the normalised Fourier harmonics to group individuals with similar otolith contour shapes. For this, Ward's hierarchical algorithm based on squared Euclidean distances was used. To visualise differences in otolith shape between groups of individual fishes, the average otolith shape of each group was formed by the outline reverse Fourier transform using the first 34 normalised harmonics.

All statistical analyses were performed using the 'lsmeans', 'multcomp', 'Vegan', 'MASS', 'CAR', 'FactoMinR', 'HH' and 'Ellipse' packages in the statistical environment R (R Core Team 2014).

Results

Otolith perimeter increased linearly with total length as expected (Fig. 3a). Linear modeling revealed that sex, ICES Division and year of sampling influenced both the intercept, corresponding to the perimeter for a 27.86 cm individual and the slope of the linear relationship between perimeter and total length (R^2=0.94).

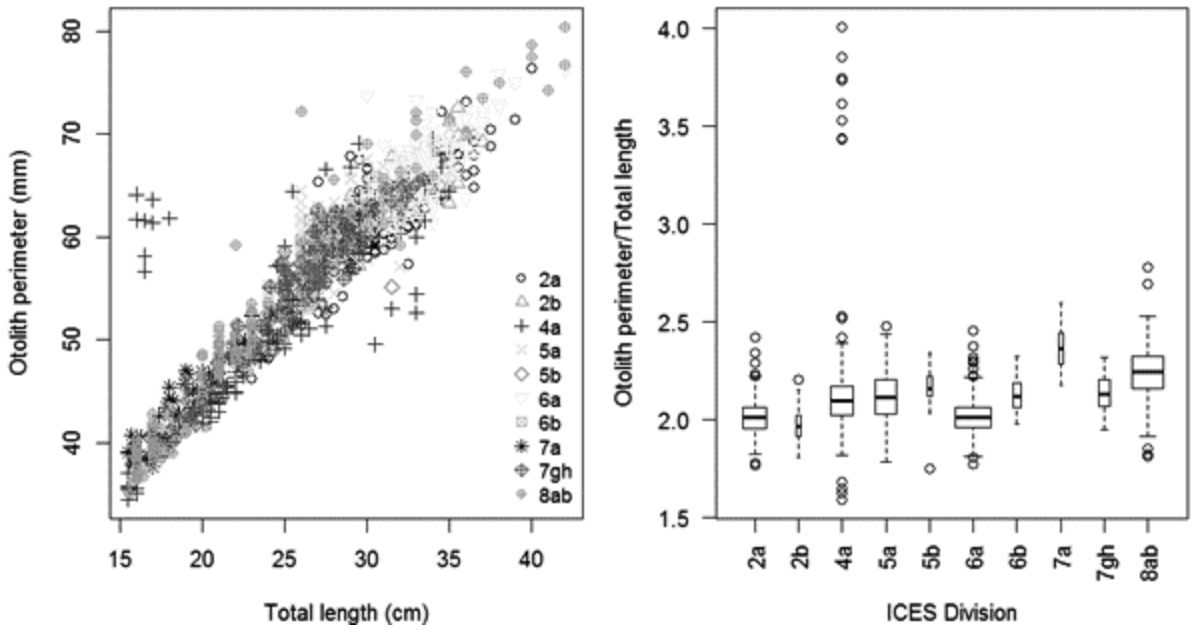

Figure 3 : a) Scatterplot of otolith perimeter as a function of body length and b) predicted perimeter with 95% confidence intervals for best fitting linear model for male and female fish with 28 and 38 cm total length. Northern ICES Divisions are in grey.

The ANCOVA showed that sex was the most important variable for explaining variations in the intercept, with ICES Division and sampling year explaining less (Table 2). In contrast, year was the most important factor for explaining variations in the slope (Table 2). Using a Tukey HSD pairwise comparison test, the intercept for ICES Division IXa was found to be significantly smaller ($p<0.05$) than for all other Divisions. For all other Divisions the predicted perimeter at 28 cm total length corresponding to the intercepts of the linear model decreased more or less continuously from North to South (Fig. 3b). The Tukey HSD test did not identify any ICES Divisions for which the slope was different from all other Divisions. However, slopes also generally decreased from North to South with the exception of Iceland and Faroes Waters (Divisions Va and Vb), leading to a less marked North-South gradient for predicted perimeter at 38 cm (Fig. 3b).

Table 2. Analysis of covariance for otolith perimeter. Interactions between factors are noted by a colon (:) (df -degree of freedom; SS - sum of squares; MS - mean sum of squares).

Factor	df	SS	MS	F value	P value
Length	1	1597.84	1597.84	24121.02	$< 2\ 10^{-16}$
Sex	1	48.13	48.13	726.53	$< 2\ 10^{-16}$
ICES Division	12	120.01	10.00	150.98	$< 2\ 10^{-16}$
Year	3	24.24	8.08	121.97	$< 2\ 10^{-16}$
Length:Year	3	7.46	2.49	37.52	$< 2\ 10^{-16}$
Length:ICES Division	12	13.65	1.14	17.18	$< 2\ 10^{-16}$
Length:Sex	1	0.62	0.62	9.42	0.002176
Residuals	1647	109.1	0.07		

Among the 99 Fourier harmonics extracted to describe otolith contours, the first 34 harmonics explained more than 99.99% of the otolith variation and were thus used for the multivariate analysis. Principal Components Analysis of these first 34 Fourier harmonics (i.e. 156 Coefficients of Fourier, see eq. 1) explained 84.01% of the total variance and the first and the second PC accounted for 36.82% and 14.24% of the total variance respectively. Only the first seven PCs were significant as determined by their eigenvalues exceeding the threshold eigenvalue generated by the broken-stick model (>3.9% of the total variance). In the RDA when the first seven PCs were related to total length, the relationship was found to be significant ($p<0.001$). Therefore in the second RDA, the residuals from the first RDA were used to test the explanatory variables of interest, e.g. sex, year of sampling and ICES Division, as well as second order interactions. All VIF values were smaller than 1.7 providing no evidence for multi-collinearity between exploratory factors. ICES Division ($p<2.10^{-16}$) and the interaction between year and ICES Division ($p<0.05$) both explained differences in otolith shapes (Table 3). Given no sexual dimorphism was found (sex: $p=0.538$; Table 3), males and females were combined in the subsequent LDA and cluster analysis.

Table 3. Summary of Redundancy analysis (RDA) (n = 1693) of blue whiting otolith shapes (df - degree of freedom).

Factor	df	P value
Year	3	$2.2\ 10^{-16}$
ICES Division	12	$2.2\ 10^{-16}$
Sex	1	0.538
Year : ICES Division	36	$2.2\ 10^{-16}$
Year : Sex	3	0.071
ICES division : Sex	12	0.059

The LDA provided discriminant functions for year-ICES Division combinations using the 34 Fourier harmonics and sampling year as explanatory variables. The overall jack-knifed classification success was 28.41% (Table 4). The analysis showed significant differences among groups of blue whiting sampled in different ICES Divisions and years (Wilks'λ = 0.1769; F=24.843; p<0.001). The misclassification percentage for each group was highest in the west of Scotland area (ICES Division VIa) and the Rockall area (ICES Division VIb), where individuals were misclassified to the Norwegian Sea (IIa) (Table 4).

Table 4. Jackknifed correct classification matrix of the linear function discriminant analysis for mature blue whiting (N=1693) between sampling ICES areas and year based on the first normalized 34 harmonics. The percentages in each row represent the classification into the ICES sampling Divisions given in columns (correct classification in grey square), sample sizes are given in parentheses. Overall classification success is 28.41%, Wilks' λ = 0.354.

		IIa	IIb	IVa			Va		Vb	VIa	VIb	VIIa	VIIg-h	VIIIa-b		%correct
		2010	2010	2008	2010	2011	2006	2011	2008	2011	2008	2011	2006	2006	2011	
IIa	2010	49	7	2	9	0	7	3	3	104	5	3	0	2	7	24
IIb	2010	12	6	1	1	0	4	1	0	15	0	0	0	0	2	14
IVa	2008	7	1	2	2	0	5	1	0	9	2	1	0	0	0	7
	2010	18	1	2	19	15	5	1	2	33	3	8	0	4	4	17
	2011	1	0	1	20	9	2	0	1	3	1	1	0	6	9	20
Va	2006	18	4	3	4	2	19	0	10	36	2	4	3	2	5	17
	2011	16	3	1	2	1	2	5	2	31	2	1	0	0	0	8
Vb	2008	9	0	3	0	1	6	1	4	10	3	1	0	0	0	11
VIa	2011	47	6	2	8	2	19	7	2	180	6	4	0	3	13	60
VIb	2008	11	1	2	3	0	4	2	7	17	8	2	1	0	3	13
VIIa	2011	0	1	0	3	2	0	0	1	0	0	2	22	7	2	55
VIIg-h	2006	7	0	0	7	2	3	0	0	12	2	14	3	6	46	14
VIIIa-b	2006	0	0	1	1	4	0	0	0	2	0	12	3	9	24	20
	2011	4	0	1	4	5	7	1	0	21	3	35	9	17	92	42

The misclassification percentages were particularly high from the Norwegian Sea to the West of Scotland in the North and from the Irish Sea and the Porcupine Bank to the Portuguese Waters in the South. When data from different years of sampling were grouped for a given ICES Division in the LDA, the overall jack-knifed classification success increased only by 6% (34%, Wilks' $\lambda = 0.141$; $F=23.402$; $p<0.001$) (Table 5).

Table 5. Jackknifed correct classification matrix of the linear function discriminant analysis for mature blue whiting (N=1693) between sampling ICES areas based on the first normalized 34 harmonics. The percentages in each row represent the classification into the ICES sampling Divisions given in columns (correct classification in grey square), sample sizes are given in parentheses. Overall classification success is 34.44%, Wilks' $\lambda = 0.089$.

	IIa	IIb	IVa	Va	Vb	VIa	VIb	VIIa	VIIc	VIIk	VIIgh	VIIIab	IXa	%correct
IIa	42	1	1	2	0	129	0	0	0	0	1	25	0	21
IIb	13	5	0	3	0	14	0	0	0	0	0	7	0	12
IVa	22	0	29	13	2	95	0	1	0	0	0	24	3	15
Va	12	5	22	33	5	71	3	7	1	0	3	15	1	19
Vb	3	1	8	4	2	19	0	0	0	0	0	1	0	5
VIa	62	0	5	7	2	255	5	3	1	0	2	15	4	71
VIb	2	1	5	6	2	38	31	2	6	0	1	4	3	31
VIIa	1	0	0	0	0	11	0	25	2	0	0	1	0	63
VIIc	0	0	0	3	0	28	7	0	6	0	0	0	6	12
VIIk	0	0	0	1	0	22	4	0	5	0	0	0	0	0
VIIgh	4	0	0	1	1	67	0	4	3	1	1	19	1	1
VIIIab	8	1	4	6	1	124	3	11	8	8	7	76	7	29
IXa	0	0	0	3	0	11	2	0	0	0	0	1	78	82

With the aim of achieving a more reliable classification ICES Divisions were combined. Combining ICES divisions into two areas, 71% of individuals were assigned correctly to the northern (from Norwegian Sea to West of Scotland: ICES Divisions IIa-VIa, N=1009) and the southern areas (from Rockall to the Portuguese Waters: ICES Divisions VIb-IXa, N=684) (Wilks'$\lambda = 0.893$; $F=28.834$; $p<0.001$). The hierarchical cluster analysis performed on the matrix of the 34 Fourier harmonics identified two clusters of fishes (Fig. 4).

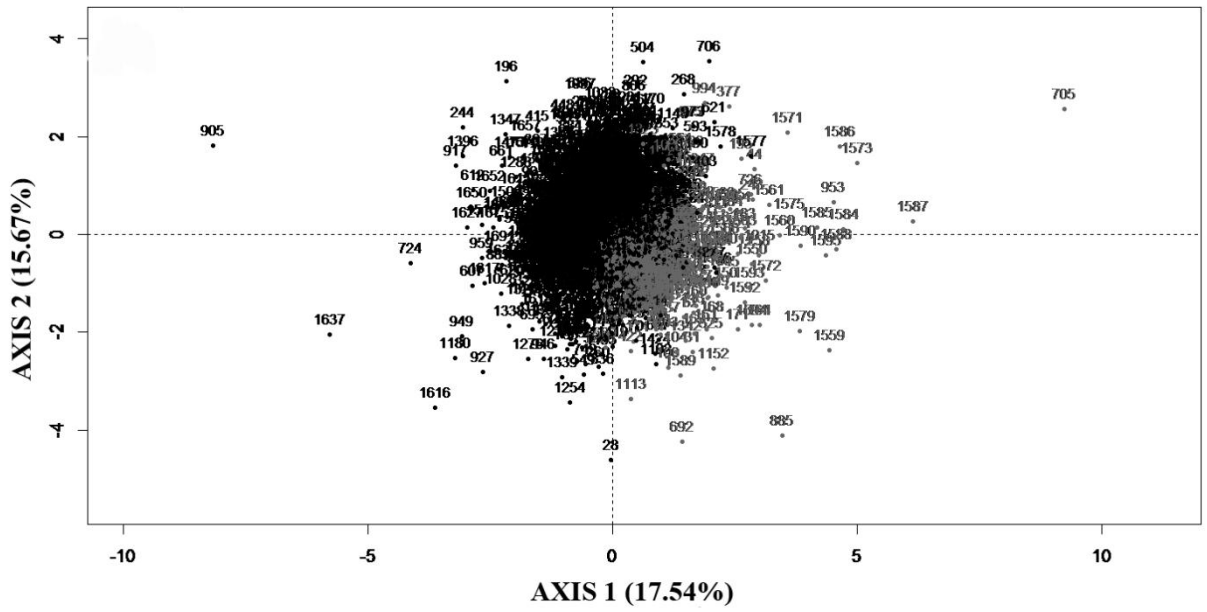

Figure 4: Average individuals from the two clusters identified by hierarchical cluster analysis using Ward's hierarchical algorithm based on the squared Euclidean distances for all blue whiting (N=1693) on the first 2 dimensions. The dots with sample numbers represent individuals.

Cluster 1 (N=573) was exclusively composed of blue whiting sampled from the Norwegian Sea to the West of Scotland (ICES divisions: IIa, IIb, IVa, Va, Vb, VIa and VIb). All fish from the Spitsbergen and Bear Island were found exclusively in cluster 1.Conversely, cluster 2 (N=1120) included individuals sampled in all ICES Divisions and years. For the southern ICES divisions (from Irish Sea to the Portuguese Waters) individuals were found in cluster 2.

Combining the ANCOVA on otolith perimeter which revealed a spatial gradient with the multivariate analyses (LDA and cluster analysis) two geographical areas of blue whiting stock structure were identified:

- Northern zone : from Norwegian Sea to West of Scotland (ICES Divisions IIa-VIa)
- Southern zone : from Rockall to the Portuguese Waters (ICES Divisions VIb-IXa)

To visualise differences in average shapes between clusters, the reconstructed outlines of the mean Fourier Descriptors of the two clusters were plotted as overlay picture (Fig. 5). The main shape differences between clusters occurred in the direction of the small axis of the otoliths, especially in the upper (dorsal) part.

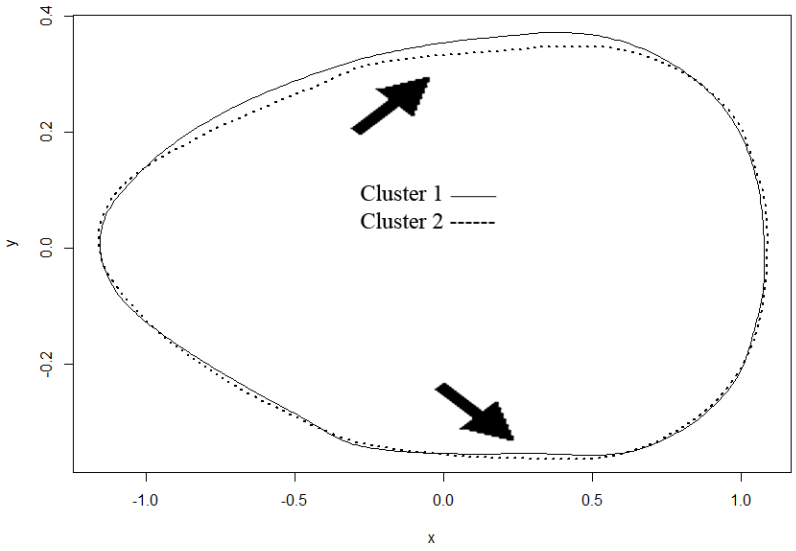

Fig. 5. Mean otolith outline shapes formed with reverse Fourier transform of the outline using the first 34 harmonics showing the overlap and variations between two clusters (arrows show the divergence areas).

Discussion

The use of linear morphometric descriptors was found to be of limited use for *M. poutassou* (Keating et al. 2014), that is why the perimeter was used as a single additional morphometric descriptor which gave results in agreement with the elliptical Fourier descriptors. In addition the descriptors allowed the evaluation of the relative contribution of sampling year and ICES Division to the explanation of inter-individual variability. The number of selected Fourier harmonics (PF=34) was higher than those previously identified for *M. poutassou* (PF=10, Keating et al. 2014) and *M. australis* (PF=14, Legua et al. 2013). A majority of studies using this technique compute the cumulated Fourier power PF (Fourier Power) using EFDs (Elliptical Fourier Descriptors) averaged across the full sample or a part of it, so that the selected harmonics describe the average otolith shape. In this study however, in order to ensure that each individual otolith in the sample was reconstructed with a precision of 99.99%, the PF value was calculated for each individual otolith such that individual shape

was reconstructed at 99.99%. The maximum number of harmonics across individual otoliths was then used to statistically reconstruct each individual otolith in the sample. This value was higher than the cumulated PF using averaged EFDs.

ANCOVA and redundancy analysis were carried out to explore the effects of size (total length of fish), sampling year and sex (only for RDA). Since several authors have provided evidence for allometric otolith growth (Castanguay et al. 1991; Campana and Casselman 1993; Begg et al. 2001; Cardinale et al. 2004; Capoccioni et al., 2011). Moreover, Campana and Casselman (1993) found that sexual maturity can modify the final contour of otoliths. Therefore in our study, only otoliths from mature adults were used. Further, a significant correlation between total length of fish and morphological otolith parameters (perimeter and EDS) was found. To remove this effect the residuals of the first seven PC from the normalised elliptical Fourier descriptors were used.

The effect of sex on otolith perimeter was weak but significant and non-overall shape was found. A sex effect have also been reported on other species, e.g. for herring (*Clupea harengus*, Bird et al. 1986), orange roughy (*Hoplostethus atlanticus*, Gauldie and Jones 2000), cod (*Gadus morhua*, Paul et al. 2013) and southern blue whiting (*Micromesistius australis*, Legua et al. 2013). Combining data from different years has been done before in otolith shape analysis (DeVries et al. 2002; Tracey et al. 2006; Farias et al. 2009; Neves et al. 2011). For example, Friedland and Reddin (1994) and Campana and Casselman (1993) found no year effect for the otolith shape of Atlantic salmon and cod. In contrast, both the ANCOVA for perimeter and the RDA for EFDs of *M. poutassou* in the present study indicated significant sampling year effects. This difference could be explained by migrations for reproduction or feeding. Further, sampling was carried out over several years spread over different months. Consequently, the year effect could be due to true interannual differences, as identified in the previous small scale otolith shape study of *M. poutassou* (Keating et al.

2014), and/or due to seasonal differences such as found for pacific sardine (*Sardinops sagax*) (Felix-Uraga et al. 2004; Vergara-Solana et al. 2013). However, a season effect was not tested in the present analysis due to a lack of sufficient sampling coverage across seasons. In addition, a significant interaction between sampling year and geographical area was found in the RDA, which corroborates the results obtained in other gadoids species (Castonguay et al. 1991; Begg and Brown 2000; Petursdottir et al. 2006).

This large scale study, which identified two broad stock components of blue whiting, is in accordance with previous smaller scale studies which also suggested the existence of several stock components of the blue whiting in the northeast Atlantic (Karasev 1990; Isaev and Selivestof 1991; Skogen et al. 1999; Ryan et al. 2005; Brophy et al. 2007; Brophy and King 2007; Was et al. 2008; ICES, 2012; Keating et al. 2014). Karasev (1990), comparing parasite types and infestation rates, concluded that there were three separate stocks from the north to the south: (1) the Barents Sea, Spitzbergen and Iceland (ICES Divisions IIb, Va), (2) the Norwegian Sea, Faeroes and Hebrides (ICES Divisions IIa, Vb, VIa), and (3) the Porcupine Bank, Celtic Sea, Bay of Biscay and areas near Shetland (ICES Divisions VIIg-h & VIIIa-b). Other studies have investigated the existence of only two subcomponents. Although the genetic variability in blue whiting is low from Gibraltar to Norway (Mork and Gjaever 1995), differentiation has been found between the south, Bay of Biscay and Celtic Sea, and the north, Porcupine bank to Papa bank (Was et al. 2006; 2008). A recent study of blue whiting otolith shapes (Keating et al. 2014), which was focused on ICES Divisions VIa and VIb, identified a geographical stock limit along a line from Porcupine Bank to Rockall Bank (Fig. 1). The location of spawning determines the probability of blue whiting eggs drifting northwards or southwards (Skogen et al. 1999) and define the division line between these two stock components. This division line which varies between years was determined to be approximately at 54.5°N corresponding to the northern edge of Porcupine bank. The Skogen

et al. (1999) study covered the years 1976 to 1995, which was a period of strong subpolar gyre activity hence probably a period with a constrained spawning area (Hátún et al. 2009), resembling the conditions for the data collection of the present study, thus the division line was expected to have been in a similar position.

Brophy and King (2007) based on otolith growth increments; found that juvenile growth rate exhibits a north-south gradient with individuals on the feeding grounds in the Bay of Biscay showing a faster growth than individuals on feeding grounds in the Norwegian Sea, and those from the west of Ireland presenting an intermediate pattern in growth. Further, back calculated juvenile growth rates of spawning individuals sampled on Porcupine Bank were more similar to growth increments of individuals from the Bay of Biscay, while juvenile growth rates for individuals sampled in the most northern part of the spawning area were slower and more similar with the individuals of the northern feeding areas. Such spatial retention of juveniles as suggested by their differential growth rate suggest that individuals from the southern feeding grounds, e.g. the Bay of Biscay might spawn primarily at the southern end of the spawning area while individuals from the feeding areas in the Norwegian Sea spawn at the northern range. Given the division line of drifting eggs, this will ensure some separation between the northern and southern stock components. However, due to interdecadal variations in the strength of the North Atlantic subpolar gyre and more locally interannual variations in currents, together with variations in spawning location and drifting direction of eggs will contribute to the persistence along the time of mixing between the northern and the southern component.

Otolith shapes are influenced by environmental conditions and genetic traits (Farias et al. 2009; Vignon 2012). Environmental conditions regulate the quantity of material deposited during otolith formation (Gauldie and Nelson 1990; Smith 1992; Campana and Casselman 1993; Galley et al. 2006).Thus, for the same environmental conditions, differences in otolith

shapes are known to be driven by genetic differences (L'Abee-Lund and Jensen 1993; Cardinale et al. 2004; Galley et al. 2006) as well as individual growth Vignon and Morat (2010) found that genetic variations only affected the otolith shape locally, mainly in the rostral and antirostrum parts. Conversely, Reichenbacher et al. (2007; 2009), concluded that the posterior and postero-ventral angles of sagittal otoliths differed as a consequence of environmental factors rather than different genetic information. In the present study, variations of otolith shape were observed locally in the dorsal and ventral parts (Fig. 5). Thus both environmental conditions and genetic components could explain these differences. However, genetic differentiation in blue whiting seems to be low from Gibraltar to Norway and there is a high probability of genetic mixing on the spawning grounds (Mork and Gjaever 1995). Therefore, the main factors influencing the observed otolith shape differences of blue whiting could be environmentally driven, such as temperature, salinity, food availability, water depth and oxygen. Temperature gradients have been found to influence the spatial distribution and the growth of blue whiting (Kloppman et al. 2001; Hátún et al. 2009; Payne et al. 2012; Trenkel et al. In Press), and they might also explain, at least partly, the difference in otolith shape, similar to that found for *M. australis* (Legua et al. 2013).

In conclusion, though it has proven difficult to disentangle what drives the observed differences in otolith shape between the northern and southern blue whiting stock components identified in the present study. Otolith shape analysis was proven to be a useful tool to describe the two spatial components of the Northeast Atlantic blue whiting stock.

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ANNEXE II

Otolith shape as a valuable tool to evaluate the stock structure of swordfish (*Xiphias gladius*) in the Indian Ocean

- Publication :

Mahé K., Evano H., **Mille T**. and Bourjea J. (soumis). Otolith shape as a valuable tool to evaluate the stock structure of swordfish (*Xiphias gladius*) in the Indian Ocean. African journal of Marine Science.

- Rapport de contrat :

Bourjea J., Mille T., Evano H., Mahe K. (2015). OTOLITHE –ESPADON : Evaluation de l' Expertise/structure du stock d'espadon de l'océan indien à partir des analyses de forme des otolithes, Rapport final de contrat FEP 39703/DMSOI/2013, 57p.

- Rapport d'expertise :

Mahe K., Evano H., **Mille T**., Bourjea J. (2014). Otolith shape as a valuable tool to evaluate the stock structure of swordfish (*Xiphias gladius*) in the Indian Ocean. DCF - Data Collection Framework (Programme national pour la collecte des données de base : pêche thonière intertropicale française), IOTC–2014–WPB12–12, 12p.

ABSTRACT

Swordfish (*Xiphias gladius*) is an oceanic-pelagic species currently fully exploited by several fisheries in the Indian Ocean, with suspicion of overexploitation in the southwest, but without a clear understanding of the real stock structure within this Ocean. Population structure of the Indian stock was studied in the western Indian Ocean using 395 individual samples collected from 2009 to 2014 stratified in space. Sagittal otoliths of the fish were removed and shape analysis performed on these calcified pieces. Otolith morphometric data and normalized Elliptical Fourier Descriptors (EFDs) were then extracted automatically by the dedicated image-analysis system TNPC. Additionally, a Principal Components Analysis (PCA) on EFDs was realised to remove the co-linearity effect between them. Firstly, the side effect was tested by a Redundancy analysis (RDA) combined to permutation tests on 91 individual samples and showed no significant differences in the outline shape between the right and left otoliths. Consequently, all 395 sagittal otoliths were used to identify stocks among several geographical areas (La Reunion, Mozambique channel, Rodrigues, South Africa, South Malagasy, Sri Lanka and Thailand) within the Indian Ocean. To investigate the effects of sex, year of sampling, lower jaw fork length and geographical area on variations in otolith shape, RDA with permutation tests was realised. These tests demonstrated no significant effects, neither by sex ($p=0.121$), sampled year ($p=0.725$), or lower jaw fork length ($p=0.464$). Only geographical area appeared to be significant ($p<0.05$). Furthermore, Linear Discriminant Analysis (LDA) was performed and overall jacknifed classification success reached 30%. Finally, a clustering analysis has been realised using Ward's hierarchical algorithm, which discriminated 3 different groups. However, each group was composed by some individual samples from all geographical areas. In conclusion, all these results did not show a clear geographical separation, which corroborates the recent genetic analysis at the Indian Ocean scale which identified only a single swordfish stock component in this area.

Keywords: *Xiphias gladius*, otolith shape, Fourier descriptors, stock discrimination, Indian Ocean.

Introduction

Swordfish (*Xiphias gladius,* Linnaeus, 1758) is a highly migratory oceanic species, currently fully exploited by several commercial fisheries in the Indian Ocean as a target species (i.e. European Union longline fleet) or a by-catch product (i.e. Taiwan longline fleet). The spatial structure of large pelagic species is difficult to know due to their cosmopolitan distribution, large population size, high fecundity and ability to easily migrate inter-ocean distances (Nakamura, 1985). Consequently, it is difficult to split the population of these species into stock units which are defined by growth and renewal rates pertinent to geographic areas where fisheries are undertaken (Cadrin *et al.*, 2014). On the basis of the last swordfish stock assessment of this species in the Indian Ocean (IOTC–WPB12, 2014), levels of catches in the whole Indian Ocean for 2009–2013 (average of 26 510 tons) were considered below the estimated maximum sustainable yield (MSY; 39.4 tons (25.0–92.4)). The Scientific Comity of the Indian Ocean Tuna Commission (IOTC; IOTC–SC16, 2013) requested the population structure to be considered as the assessment focused on the southwest Indian Ocean (SWIO) as an independent stock on the basis of the fishery data. Most of the evidence indicated that the resource has been overfished in the past decade, with the current level of catches (7 349 tons in 2013 with an estimated MSY: 9 100 –10 600; IOTC–WPB12, 2014) indicating a stock not subject to overfishing but overfished. Such a result clearly emphasizes the importance of better knowing the spatial structure of this species at the scale of the Indian Ocean, and its relation with adjacent oceans (i.e. Atlantic and Pacific).

Stock identification and spatial structure information provide a basis for understanding fish population dynamics and provides reliable resource assessment for fishery management (Reiss *et al.*, 2009). Several techniques may be used to identify the stock limits, such as tagging experiments, analyses of spatial and temporal variation of genetic or morphometric markers, differentiation of life-history variables, parasites and contaminant concentrations

(Pawson and Jennings, 1996; Cadrin *et al.*, 2014) or parasite composition (Garcia *et al.*, 2011). Lu *et al.* (2006) suggested using few mitochondrial sequences (i.e. female inheritance) from several locations in the Indian Ocean, to establish the existence of genetic differences between swordfish in the north of Madagascar and other northern sampling sites in the Indian Ocean. Moreover, 3 others studies identified some genetic differentiation in the Indian Ocean, including the Western Indian Ocean (Jean *et al.*, 2006; Muths *et al.*, 2009; Bradman *et al.*, 2011) but all showing some inconsistent conclusions on stock structure due to the marker used, no stratification of the sampling in space and time and the lack of information on reproductive behaviour. The reproductive biology of swordfish in the Indian Ocean is partially known. There are three described spawning grounds: the Gulf of Bengal, the Somalia coast where spawning take place after April and the Reunion island where spawning is believed to take place from October to November (Yabe *et al.*, 1959; Palko *et al.*, 1981; Mejuto *et al.*, 2006; Poisson and Fauvel, 2009). Consequently, this could be explained by the difference in conclusion from previous studies investigating the genetic stock structure of swordfish. A recent global study on population genetics using multi-genetic marker approach and spatio-temporal analysis based on 2231 individuals samples suggested there is a single panmictic population (i.e. a single stock) of swordfish in the Indian Ocean (Muths *et al.*, 2013). This study also concluded the need of using other stock discrimination approaches to unravel the real stock structure of this species.

In the present study, swordfish stock structure was investigated using the shape of sagittal otoliths. Otolith morphology is influenced by biotic and abiotic factors (Cardinale *et al.*, 2004; Capoccioni *et al.*, 2011). Analysis of the outline of otoliths has previously been used for stock discrimination (haddock: Begg and Brown, 2000; cod: Galley *et al.*, 2006; Petursdottir *et al.*, 2006, Stransky *et al.*, 2008; striped red mullet: Benzinou *et al.*, 2013). Based on the logistic and sample collection presented in Muths *et al.* (2013), this study

focuses on individual swordfish sampled over a wide geographic area, from South Africa to Thailand, and otolith shape analysis performed to discriminate components of swordfish in the Indian Ocean stock.

Materials and methods

Sample collection

Ethical approval was not required for this study, as all fish were collected as part of routine fishing procedures. Swordfish samples from South Africa to Thailand (Fig. 1; Table 1) were collected from 2009 to 2014 by observers onboard commercial fishing vessels or at landing (with due care collecting the related fishing information).

This study was realized on the samples of the genetic study (Muths *et al.*, 2013) where monthly samples were collected from October 2009 to November 2010 to obtain specimens from both spawning and non-spawning seasons. The specimens from South Africa were collected in December 2013 and March 2014. Information on sample location (exact latitude and longitude or 5° square) was systematically noted and the swordfish were classified in 7 sampling areas in respect of different fishery statistical units. Sex and maturity of the sampled individuals were determined by macroscopic examination of gonads. Only mature fish were included in this study to minimize the effect of sexual maturity which may change otolith shape (Cardinale *et al.*, 2004). Sagittal otoliths were extracted from a total of 395 individuals ranging from 56 to 300 cm lower jaw fork length (mean 155.0±38.5 cm).

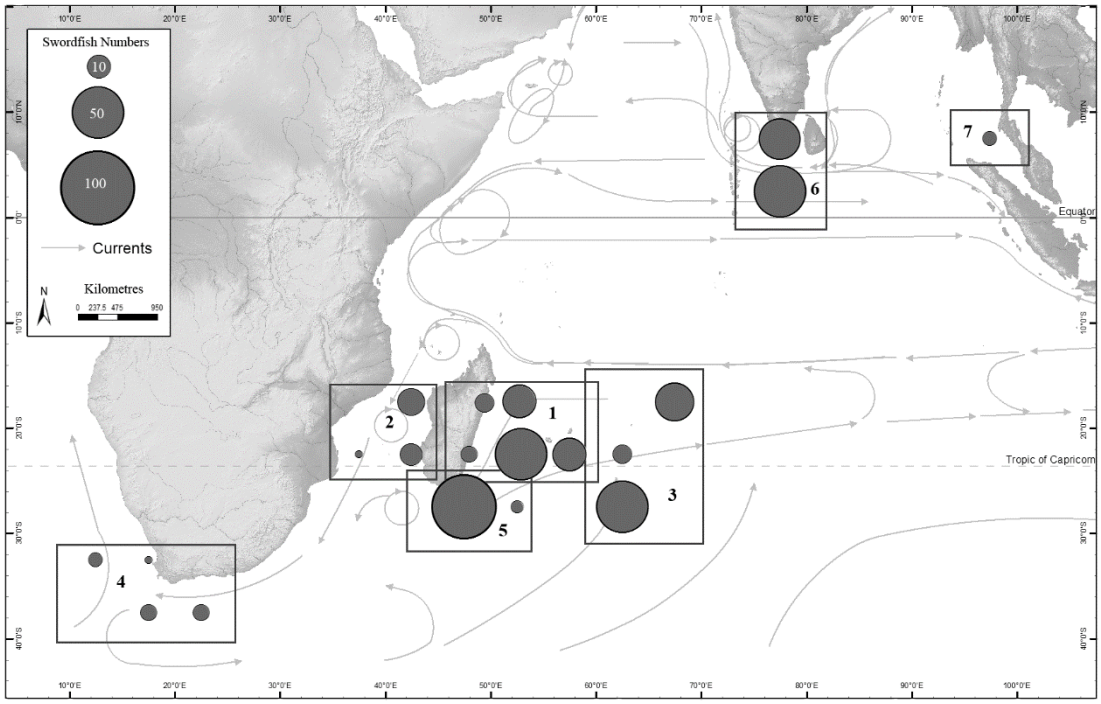

Figure 1: Map of location of swordfish samples collected between 2009 and 2014 in the Indian Ocean. Dark grey rectangle indicates sampling area aggregation (1: La Réunion, 2: Mozambique, 3: Rodrigues, 4: South Africa, 5: South Malagasy, 6: Sri Lanka and 7: Thailand).

Table 1. Number of swordfish otolith samples by year, sex (Male, Female, Other: no identification of sex for gutted fish) and sampling area. Mean lower jaw fork (LJF) length characteristics by sampling area are given.

Sampling area	LJF (cm)	Sex			Sampling year				Total
		Male	Female	Other	2009	2010	2013	2014	
La Réunion	143.0±23.0	38	59	10	61	46			107
Mozambique	135.1±38.6	11	10	13		34			34
Rodrigues	165.2±29.8	29	54	0	39	44			83
South Africa	185.9±36.9			20			5	15	20
South Malagasy	150.4±34.2	21	48	2	62	9			71
Sri Lanka	167.3±57.5	8	20	48	23	53			76
Thailand	98.2±20.3		3	1		4			4
Total	155.0±38.6	107	194	94	185	190	5	15	395

Otolith shape analysis

Swordfish otoliths present a large dorsal area, between the *excisura major* and the *excisura minor*, very distinct from the *antirostrum* (Fig. 2). Images of the whole left and right sagittal otoliths were scanned (Epson V750) under reflected light and stored with high resolution (3 200 dpi). Image processing was performed using the image analysis system TNPC (Digital processing for calcified structures, version 7, www.tnpc.fr) with the *sulcus acusticus* facing up. In order to compare left and right otolith shapes, a mirror image of left otoliths was used.

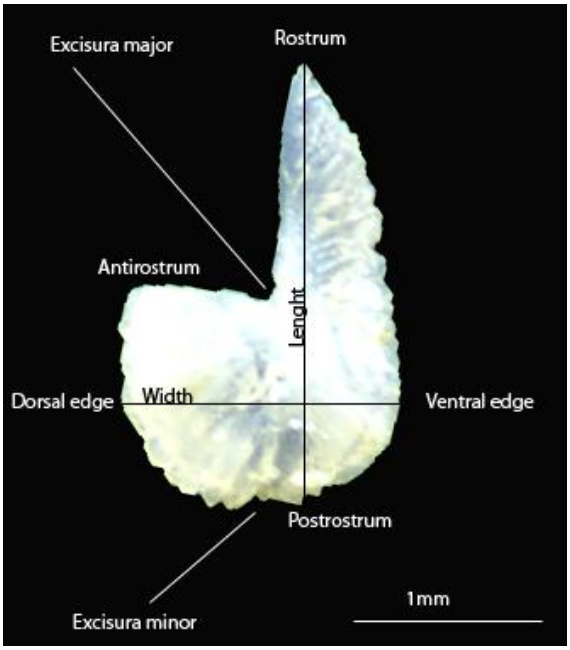

Figure 2. Photograph of a whole swordfish sagittal otolith.

Otolith length and width were measured and the contour of each otolith was extracted using the automatic threshold in the TNPC software (Fig. 2). To describe otolith contours, Elliptic Fourier Analysis (EFA; Lestrel, 2008) was carried out. For each otolith, the first 99 elliptical Fourier harmonics (Hi) were extracted and normalised with respect to the first

harmonic using the TNPC software and were, thus, invariant to otolith size, rotation and starting point of the shape measurements (Kuhl and Giardina, 1982). To determine the number of harmonics required to reconstruct the otolith outline, the Fourier Power (PF) was calculated for each individual otolith *k* as a measure of the amount of contour rebuilt by each harmonic:

$$F(n_k) = \sum_{HI=1}^{n_k} \frac{A_{\mathrm{HI}}^2 + B_{\mathrm{HI}}^2 + C_{\mathrm{HI}}^2 + D_{\mathrm{HI}}^2}{2}$$

Where A_{HI}, B_{HI}, C_{HI} and D_{HI} are the parameters of the *HI*th harmonic and n_k is the total number of harmonics included. The value of n_k was chosen such that $PF(n_k)$ explains 99.99% of variance in contour coordinates or, in other words, such that shape is reconstructed at 99.99% (Lestrel, 2008).

Statistical analyses

To investigate variation sources for otolith shape differences due to factors such as inner ears location (side), lower jaw fork length, sex, sampling year and sampling area, multivariate analyses were carried out. Firstly, Principal Components Analysis (PCA) was applied to selected Elliptical Fourier Descriptors (EFDs) matrix (*EFD*s as columns and individual otolith as lines) of otolith contours (Rohlf and Archie, 1984) and a subset of the resulting principal components were selected as otolith shape descriptors according to the broken stick model (Legendre and Legendre, 2012). This allowed us to decrease the number of variables used to describe otolith shape variability while ensuring that the main sources of shape variation were kept, as well as to avoid co-linearity between shape descriptors (Rohlf & Archie 1984). Secondly, the side effect tested 91 individual samples which have both the left and the right otoliths, by Redundancy analysis (RDA). RDA is an extension of multiple regressions to multivariate response data and an extension of principal component analysis (Legendre and Legendre, 2012), combined with permutation tests (marginal effect, type II;

Fox and Weisberg, 2011) on the selected Principal Components (PC) matrix. To visualise differences in otolith shape between right and left sides, an average otolith shape of each side group was rebuilt based on average EFDs. Thirdly, the lower jaw fork length (cm), sex, sampling year and sampling area effects were tested on 395 individual samples (304 images of right otoliths and 91 mirror images of left otoliths were combined in this study) using a RDA, where the explained matrix was combined with permutation tests on the selected PC matrix and the explanatory matrix was the tested effects and their interactions between them. To test the significance of each explanatory variable, a permutation test (marginal effect, type II) was used (Legendre and Legendre, 2012). Finally, to verify the ontogenic effect on the otolith shape, a linear relationship was used between the ratio otolith length/otolith width and the lower jaw fork length of fish.

To discriminate fish classified in 7 sampled fishery areas based on their otolith shapes, a Linear Discriminant Analysis (LDA) with Jacknifed prediction was applied on the residuals of RDA model to remove the significant effects tested previously on the otolith shape (Rencher and Christensen, 2012). To evaluate the resulting discriminant functions, the percentage correct classification of individuals to sampling area was calculated using jacknife cross-validation and Wilk's lambda criteria (Klecka, 1980). To complete, a cluster analysis (Ward's hierarchical algorithm based on squared Euclidean distances) was performed on the selected EFDs matrix to classify individuals with similar otolith shapes.

All statistical analyses were performed using the 'Vegan' (Oksanen *et al.*, 2013), 'MASS' (Venables and Ripley, 2002), 'CAR'(Fox and Weisberg, 2011), 'FactoMinR' (Lê *et al.*, 2008), 'HH' (Heiberger and Holland, 2004) and 'Ellipse' (Murdoch and Chow, 1996) packages in the statistical environment R (R Core Team, 2014).

Results

Among the 99 Fourier harmonics extracted to describe otolith contours, the first 43 harmonics explained at least 99.99% of the otolith variation and were thus used for the multivariate analysis. The Redundancy analysis (RDA) combined to permutation tests showed no significant difference between the left and right otolith shape (Fig. 3).

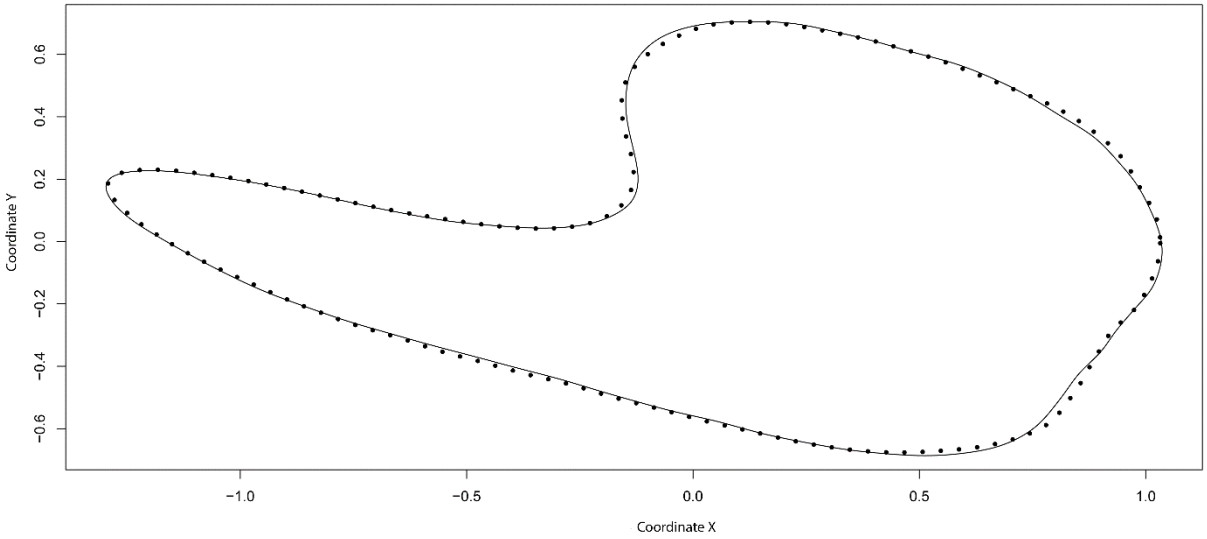

Figure 3. Mean otolith outline shapes formed with reverse Fourier transform of the outline using the first 43 harmonics showing the overlap and variations between right (dark grey dash line) and left (grey solid line) otolith shape of swordfish from the Indian Ocean (N=91).

As the left and right otoliths of swordfish were comparable, 304 images of right otoliths and 91 mirror images of left otoliths were combined in this study. Principal Components Analysis of these first 43 Fourier harmonics showed that the first and the second PCs accounted for 35.9% and 25.2% of the total variance respectively. Only the first seven PCs were significant as determined by their eigenvalues exceeding the threshold eigenvalue generated by the broken-stick model. The effect of lower jaw fork length ($p=0.464$), sex ($p=0.121$), sampling year ($p=0.725$) and sampling area ($p=0.002$) were tested by a RDA. The relationship between the ratio otolith length/otolith width and the lower jaw fork length of fish proved size effect was not significant ($p>0.05$).

Only sampling area effect was significant ($p<0.05$) and sampling area was used as an explanatory variable in the subsequent LDA. The overall jacknifed classification success was 30% (Table 2).

Table 2. Jackknifed correct classification matrix of the linear function discriminant analysis for mature swordfish (N=395) between sampling areas based on the first normalized 43 harmonics. The percentages in each row represent the classification into the sampling area given in columns (correct classification in grey square). Overall classification success: 30%.

Sampling area	South Africa	South Malagasy	Mozambique	La Réunion	Rodrigues	Sri Lanka	Thailand	%
South Africa	4	3	2	1	9	1	0	20
South Malagasy	5	19	4	20	9	13	1	27
Mozambique	3	2	5	11	9	4	0	15
La Réunion	5	17	16	41	14	13	1	38
Rodrigues	2	12	8	23	28	10	0	34
Sri Lanka	2	12	14	13	12	21	2	28
Thaïlande	0	2	1	0	1	0	0	0

The analysis showed significant differences among groups of swordfish sampled in different areas of the Indian Ocean (Wilks'λ = 0.017; F=1.255; p=0.001). The misclassification percentage for each sampling area was explained by closed and distant areas (Table 2). The hierarchical clustering analysis performed on the matrix of 43 Fourier harmonics identified three clusters of fishes (Fig. 4). All sampling areas were divided in practically the same way (Table 3).

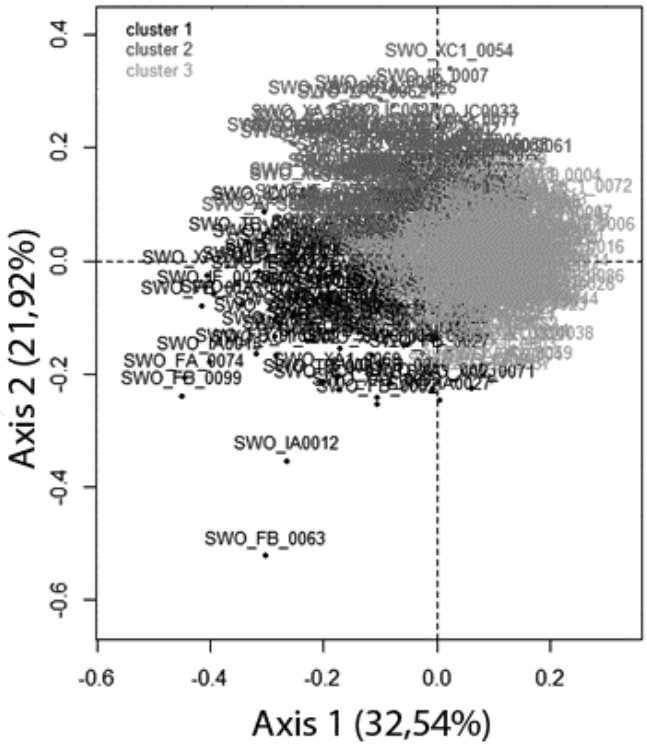

Figure 4: Average individuals from the three clusters identified by hierarchical cluster analysis using Ward's hierarchical algorithm based on the squared Euclidean distances for all swordfish (N=395) on the first 2 dimensions. The dots with sample numbers represent individuals.

Table 3. Classification matrix of the hierarchical clustering on principal components for mature swordfish (N=395) between Sampling areas based on the first normalized 43 harmonics.

Sampling area	Cluster 1	Cluster 2	Cluster 3	Total
South Africa	2	5	13	20
South Malagasy	11	20	40	71
Mozambique	3	13	18	34
La Réunion	20	23	64	107
Rodrigues	12	23	48	83
Sri Lanka	17	17	42	76
Thaïlande	1	1	2	4
Total	66	102	227	395

Discussion

The previous recent genetic study (Muths *et al.*, 2013) identified difference between Atlantic and Indian Ocean swordfish stocks but did not linger on possible sub-divisions in Indian Ocean stock(s). Otolith shape analysis is an another efficient stock identification tool linked to genetic heterogeneity and the influence of environmental factors (Campana and Casselman, 1993; Cadrin and Friedland, 1999; Torres *et al.*, 2000; Cardinale *et al.*, 2004; Swan *et al.*, 2006; Vignon and Morat, 2010). The external contour of otoliths could be described by several techniques such as univariate descriptors (coefficient of form, roundness, circularity…), the geometric morphometric analyses (Ponton, 2006; Ramirez-Perez *et al.*, 2010; Vergara-Solana *et al.*, 2013); wavelet functions (Parisi *et al.*, 2005; Sadighzadeh *et al.*, 2014); growth markers (Benzinou *et al.*, 2013); and the geodesic method (Benzinou *et al.*, 2013). Among these, the elliptical Fourier analysis remains the most widely used and powerful method to describe otolith external shape (Agüera and Brophy, 2011; Capoccioni *et al.*, 2011; Fergusson *et al.*, 2011; Legua *et al.*, 2013; Paul *et al.*, 2013). This method has the advantage to be unaffected by short-term changes in fish condition (Campana and Casselman, 1993) due to environmental variations (Campana, 1999). Nevertheless, its biological interpretation is more complex than one based on linear morphometric descriptors (Stransky and MacLellan, 2005). Some studies have combined the elliptical Fourier analysis and some basic descriptors of otolith contours to help interpretation of results (Campana and Casselman, 1993; Begg and Brown, 2000; Galley *et al.*, 2006; Merigot *et al.*, 2007; Fergusson *et al.*, 2011; Benzinou *et al.*, 2013; Legua *et al.*, 2013). It is worthwhile noting that this approach provided relevant stock structure information on demersal species such as the striped red mullet (*Mullus surmuletus*) in the Northeast European seas (Benzinou *et al.*, 2013).

No study on large migratory and long lived tropical pelagic species has yet been undertaken on the stock identification from the use of otolith shape.

In this study, before analysis of geographical effect on otolith shape, some other known factors affecting shape to disentangle the potential confounding factors were tested (Vignon and Morat, 2010). Firstly, no side effect was observed. Consequently, there was no significant difference between the left and the right otolith shape as for another highly migratory oceanic species, Bluefin tuna (*Thunnus thynnus*) tested by otolith morphological characteristics (Megalofonou, 2006).

Moreover, the sampling used in this study was restricted to adult fish to avoid the sexual maturity effect, which could modify the outline contour of otoliths (Cardinale *et al.*, 2004; Campana and Casselman, 1993). In the present study, the effect of lower jaw fork length, sex and sampling year were not significant. The use of morphological otolith parameters (length and width) allowed us to observe the otolith shape, and conclude that it was comparable during the life of mature swordfish, at least along the size range sampled (56–300 cm). Contrarily, some studies on the Atlantic mackerel (*Scomber scombrus*; Castanguay *et al.*, 1991), Atlantic cod (*Gadus morhua*; Campana and Casselman, 1993, Cardinale *et al.*, 2004, Capoccioni *et al.*, 2011) haddock (*Melanogrammus aeglefinus*; Begg *et al.*, 2001) and common sole (*Solea solea*; Merigot *et al.*, 2007) showed the impact of somatic growth on otolith shape. Sexual dimorphism effect did not significantly affect otolith shape of swordfish as shown in studies of several other species, e.g. Atlantic mackerel (*Scomber scombrus*, Castonguay *et al.*, 1991), haddock (*Melanogramus aeglefinus*, Begg *et al.*, 2000), lake trout (*Salvelinus namaycush*, Simoneau *et al.*, 2000) and Atlantic cod (*Gadus morhua*, Cardinale *et al.*, 2004). Contrarily in others species, e.g. herring (*Clupea harengus*, Bird *et al.*, 1986), orange roughy (*Hoplostethus atlanticus*, Gauldie and Jones, 2000), and southern blue whiting (*Micromesistius australis*, Legua *et al.*, 2013), there was a significant effect of sex on

otolith shape. These authors explained this sexual dimorphism by somatic growth rate differences. However, if female swordfish grow faster and attain larger maximum body size than males, there was no significant effect of somatic growth on the otolith growth (Sun *et al.*, 2002; De Martini *et al.*, 2007). Sampling year had no significant effect for the swordfish, which corroborated others studies on different species, such as Atlantic salmon (*Salmo salar*, Friedland and Reddin, 1994) and Atlantic cod (*Gadus morhua*, Campana and Casselman, 1993).

Finally, a sampling area effect was detected on otolith shape of swordfish a ($p<0.05$). To complete this observation, the linear discriminating analysis indicated significant differences among groups of swordfish sampled in different fishery areas of the Indian Ocean. However, the misclassification percentage of the cross-validation was equal to 70%. Moreover, these specimens were not associated to adjoining sampling areas but all sampling areas regardless the location in the Indian Ocean. In this way, no geographical sub-structures are distinguishable in the Indian Ocean swordfish stock. The cluster analysis corroborated this result with 3 groups composed by all sampling areas. Possible sources of misclassification in otolith shape analysis are individual variability and migration (Campana and Casselman, 1993; Tracey *et al.*, 2006).

Swordfish migration is one of the most complex of the pelagic species and known to be highly variable, with one specimen which travelled 3 053 km during 90 days and 1 other in the same time confined to 21 km from the tagging location (Palko *et al.*, 1981; Sedberry and Loefer, 2001). The Indian Ocean is also divided into two hemispheres involving highly variable marine ecosystems driven by latitudes (Sherman and Hempel, 2008). Consequently, this Ocean is also globally characterized by the westward South Equatorial Current and around 12°S by the hydro-chemical South Tropical Front which separates two large oligotrophic areas: the Indian Monsoon Gyre Province (IMG) in the north and the Indian

South Subtropical Gyre Province (ISSG) (Longhurst, 1998; Schott *et al.*, 2009). However, swordfish is a highly mobile pelagic species with, for example, one swordfish recaptured 6 670 km south-eastward from the point of release in the Indian Ocean (Kadagi *et al.*, 2011). Temperature and oxygen are the main environmental factors which control the migration and distribution of large pelagic fishes (Carey, 1990; Brill, 1994; Prince and Goodyear, 2006; Sund *et al.*, 2006). Swordfish present very high tolerance to variations and extremes of temperature and oxygen and these movements patterns are controlled largely by resource availability and reproduction (Dewar *et al.*, 2011). Consequently, the hydro-chemical South Tropical Front did not seem to be a boundary for this species and it seems that there is significant connectivity between the north and south of the Indian Ocean.

CONCLUSION

Swordfish (*Xiphias gladius*) is a highly migratory oceanic species, currently fully exploited by several commercial and recreational fisheries in the Indian Ocean. This study confirms the hypothesis that otolith shape analysis may assist the implementation of a consistent identification for exploited fish stocks over a large geographical scale. It provides evidence of a single phenotypic stock composed by only one mixing area of swordfish from the southwest Indian Ocean region to the northeast Indian Ocean. This work corroborated results from several genetic markers which concluded the single unique panmictic population for the same geographical area. Future research must investigate otolith shape patterns of swordfish sampled in the south east Atlantic to highlight the connection between Atlantic and Indian Oceans.

Moreover, this type of study could be applied to other pelagic species to improve the management of these resources between the International Commission for the Conservation of Atlantic Tunas and the Indian Ocean Tuna Commission. As already suggested for the

bigeye tuna (*Thunnus obesus*; Durand *et al.*, 2005) and swordfish (Muths *et al.*, 2013), it might be very interesting to investigate the patterns of habitat use of Indian Ocean and Atlantic Ocean of large pelagic fishes in South African waters, with a special focus on the sex-biased dispersal.

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