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Mechanisms underlying the bottom-up control of sardine populations in the Gulf of Lions: insights from experiments and modeling

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Abstract

The Gulf of Lions has faced a sharp drop in the catches of its two main small pelagic exploited species, the sardine *Sardina pilchardus* and the anchovy *Engraulis encrasicolus* since the mid-2000s, despite both population abundances remaining high. This situation has been due to a severe decrease in individual body condition and size as a result of both lower growth and the disappearance of the oldest and largest individuals. While overfishing, predation or disease outbreaks have been refuted to explain this situation, one major hypothesis remained to be investigated. A potential shift in sardine and anchovy diet towards smaller planktonic prey indeed suggested bottom-up control as the main driver of these populations in the Gulf of Lions. The first aim of this thesis was to investigate whether bottom-up processes could explain the changes in sardine growth and condition through changes in both food size and/or quantity and to understand the behavioral and physiological mechanisms involved in this control. The second objective of this PhD thesis was to identify the potential underlying drivers leading to adult overmortality. To do so, we combined an experimental approach on wild sardines maintained in captivity with a modeling approach. Experimentations showed that body condition, growth and storage lipids were significantly impacted by both food size and quantity. Thus, sardines fed on small particles needed to consume twice as much as those feeding on large particles to achieve the same condition and growth. Such results seemed to be linked to higher energy expenditures of sardines while filtering small prey compared to particulate feeding on large prey (sardines being able to shift between two feeding modes according to the prey size). Moreover, our results suggested several adaptations to cope with small food and caloric restriction. The study of the gill raker apparatus involved in the filtration of small prey suggested an increase of the filtration capacity for a given length between 2007-2009 and 2016. Then, sardines fed on small particles exhibited higher mitochondria efficiency and abundance suggesting energy-saving adaptation. Finally, sardines accustomed to feed on small pellets showed lower activity to limit energy expenditure. Nevertheless, all these strategies might incur other costs or may not be enough to compensate the high energy demands of filtration on small prey, as growth and condition remained lower for sardines filtering small prey in all our experiments. Further, sardines fed on large pellets exhibited higher spawning frequency than sardines fed with the same quantity of small ones. The low egg production of these sardines might be explained by a too high body condition of these individuals to observe a change in energy trade-off towards reproduction. For the same reasons, small particle meals did not seem to impact their immunity and stress, leucocyte and cortisol concentrations being similar whatever the feeding treatment. Furthermore, to investigate the hypothesis of adult overmortality, we first studied whether individual could die from starvation and low body reserves. The survival probability sharply decreased when the body condition index became lower than 0.75 and the threshold of 0.72 was identified as the entry in phase III of fasting. While the proportion of sardines reaching such thresholds in the wild remains low, it still increased two-fold in the recent period, reaching about 10% in winter months. A DEB model parameterized using a combination of in-situ and experimental data suggested a lower survival probability for larger fish. Individuals larger than 14 cm, i.e. older than 2-3 years, had a lower than 50 % probability to survive 1 month after the reproduction period. In conclusion, these previous results comforted the two hypotheses of a bottom-up control and an overmortality of adult sardines after reproduction to explain the dynamic and demographic truncation of the sardine population.

Résumé

Le golfe du Lion a été confronté à une forte baisse des captures de ses deux principales espèces exploitées, la sardine Sardina pilchardus et l'anchois Engraulis encrasicolus depuis le milieu des années 2000, malgré des populations abondantes. Cette situation est due à une forte diminution de la condition corporelle et de la taille des individus causée par une croissance plus faible et la disparition des individus les plus âgés. La surpêche, la prédation ou les épidémies ayant été rejetées pour expliquer cette situation, une hypothèse majeure reste à étudier. Un changement du régime alimentaire de ces espèces pour des proies plus petites suggère un contrôle bottom-up comme principal facteur régissant la dynamique de ces populations. Le premier objectif de la thèse était d'étudier si un contrôle bottom-up pouvait expliquer les diminutions de croissance et de condition chez la sardine suite à des modifications de taille et/ou de quantité de nourriture et de comprendre les mécanismes sous-jacents. Le deuxième objectif de cette thèse était d'étudier les facteurs potentiels conduisant à la surmortalité des adultes. Pour cela, nous avons combiné approches expérimentales et modélisation. Les expériences ont montré que la taille et la quantité de nourriture avaient un impact significatif sur la condition, la croissance et le stockage des lipides. Ainsi, les sardines nourries sur de petites proies devaient en consommer deux fois plus que celles nourries sur de grandes proies pour atteindre la même condition et la même croissance. Ces résultats semblent être liés à une dépense énergétique plus élevée des sardines filtrant les petites proies par rapport à une chasse à vue sur de grandes proies. Nos résultats suggèrent plusieurs adaptations pour faire face à des petites proies et à une restriction calorique. L'étude des branchies suggère une augmentation entre 2007-2009 et 2016 de la capacité de filtration des sardines. Ensuite, les sardines nourries avec des petites proies ont montré plus grande efficacité et abondance en mitochondrie, suggérant une adaptation permettant des économies d'énergie. Enfin, les sardines habituées à se nourrir sur de petites proies ont réduit leur activité pour limiter les dépenses énergétiques. Néanmoins, toutes ces stratégies peuvent engendrer des surcoûts ou ne pas suffire à compenser les besoins énergétiques élevés imposée par la filtration, la croissance et la condition des sardines filtrant les petites proies étant restées plus faibles au cours de toutes nos expériences. En outre, les sardines nourries avec de grosses proies présentaient une fréquence de ponte plus élevée que les sardines nourries en même quantité mais sur des petites proies. La faible production d'œufs de ces sardines pourrait s'expliquer par une condition trop élevée pour engendrer un changement de compromis énergétique. Pour les mêmes raisons, les petites proies ne semblent pas avoir d'impact sur leur immunité et leur stress, les concentrations en leucocytes et en cortisol étant similaires quel que soit le traitement utilisé. L'étude de l'hypothèse de surmortalité adulte a permis de montrer que la probabilité de survie chute fortement quand la condition devient inférieure à 0,75 et que le seuil de 0,72 correspond à l'entrée en phase III du jeûne. Alors que la proportion de sardines atteignant de tels seuils dans la nature reste faible, elle a récemment doublé, pour atteindre environ 10% en hiver. Un modèle DEB paramétré à l'aide de données in situ et expérimentales a mis en évidence une plus faible probabilité de survie des individus les plus grands. Ainsi, ceux de plus de 14 cm, c-à-d âgés de plus de 2-3 ans, ont une probabilité inférieure à 50% de survivre un mois après la période de reproduction. En conclusion, ces résultats confortent les hypothèses d'un contrôle bottom-up et d'une surmortalité des sardines adultes après la reproduction pour expliquer la dynamique et la troncature démographique de la population de sardines.

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Synthèse en français

1. Introduction

Les poissons petits pélagiques sont des éléments clés des écosystèmes marins, régulant la dynamique des populations de niveaux trophiques inférieurs et supérieurs, en particulier dans les systèmes d'upwelling marins hautement productifs (Brochier et al., 2011; Cury et al., 2000; Frederiksen et al., 2006; Taylor et al., 2008). Les petits pélagiques représentent également 25% des débarquements dans le monde (en tonnes), principalement par le biais de l'anchois, la sardinelle, la sardine, le maquereau et le hareng (FAO, 2018). Les fluctuations des populations de petits pélagiques peuvent donc avoir des conséquences écologiques, économiques et sociales importantes, et de ce fait la compréhension des mécanismes sous-jacents est extrêmement importante. La dynamique des populations de ces espèces peut être fortement impactée par les fluctuations naturelles de l'environnement (contrôle bottom-up) et de mortalité (contrôle top-down)(Checkley et al., 2017). Tout d'abord, l'importance cruciale de la production et de la disponibilité planctonique sur la dynamique de recrutement des petits pélagiques est connue depuis le travail précurseur de Hjort (1914), et est au cœur des hypothèses de « match-mismatch » et d' « ocean triad » (Bakun, 1996; Cushing, 1990). D'autre part, la (sur)pêche a également été identifiée comme un facteur clé dans l'effondrement de plusieurs stocks, souvent en conjonction avec les fluctuations environnementales (Toresen and Ostvedt, 2000), qui peuvent être exacerbés par l'actuel changement climatique (Brochier et al., 2013; Shannon et al., 2009). Néanmoins, l'importance relative du contrôle bottom-up par rapport au contrôle top-down reste compliquée à évaluer car ils sont souvent entremêlés (Hunt and McKinnell, 2006; Rouyer et al., 2014).

Le golfe du Lion est l'une des zones les plus productives de la mer Méditerranée en raison des upwellings côtiers induits par le vent, la circulation méso-échelle et les apports d'eau douce du Rhône (Millot, 1990; Petrenko et al., 2005). Jusqu'au milieu des années 2000, la sardine (*Sardina pilchardus*) et l'anchois (*Engraulis encrasicolus*) pouvaient représenter jusqu'à 50% du total des débarquements annuels (environ 15000 tonnes) dans le golfe du Lion (Bănaru et al., 2013). Les débarquements de sardines et d'anchois ont cependant fortement diminué depuis 2008, atteignant les niveaux les plus bas enregistrés depuis 150

ans (environ 3000 tonnes), bien que l'abondance de la population soit restée élevée (GFCM, 2017; Saraux et al., 2019; Van Beveren et al., 2016a). La baisse des débarquements de poissons petits pélagiques est généralement due à l'échec du recrutement et/ou de la surpêche (Schwartzlose et al., 1999). Dans le golfe du Lion, cependant, la chute spectaculaire des débarquements a été causée par une diminution importante de la taille et de la condition des individus (poids moyen ± SE: 24,3 ± 0,3 g entre 1993 et 2007 et 11,4 ± 0,2 g entre 2008 et 2018), ce qui a rendu les sardines et les anchois moins attractif pour la pêche (Saraux et al., 2019; Van Beveren et al., 2016a, 2014). La diminution de la taille des sardines est le résultat combiné d'une croissance plus faible et de la disparition, de la population, des individus les plus anciens et donc les plus grands (Brosset et al., 2015; Saraux et al., 2019; Van Beveren et al., 2014). Cette situation est inhabituelle car elle ne découle pas d'un échec du recrutement et/ou de la surpêche. En effet, la pression de pêche et la sélectivité ont été (et sont encore) très faibles et ne peuvent donc pas expliquer la disparition des plus grands individus (GFCM, 2017). Le recrutement est quant à lui resté stable et a même augmenté au cours de la dernière décennie (Saraux et al., 2019). Des études récentes ont également montré que ni l'émigration, ni des contrôles top-down (prédateurs naturels) ou des maladies pouvaient expliquer cette situation (Queiros et al., 2018; Saraux et al., 2019; Van Beveren et al., 2017, 2016b). La principale hypothèse restante est donc le contrôle bottom-up, possiblement lié à la quantité et la qualité de la production de plancton dans le golfe du Lion (Brosset et al., 2015; Saraux et al., 2019; Van Beveren et al., 2014).

2. Objectifs

Le premier objectif de cette thèse était d'examiner l'hypothèse d'un contrôle bottom-up sur les populations de sardines causé à la fois par la taille et la quantité de proies planctoniques mais aussi de mieux comprendre ses mécanismes. Pour ce faire, nous avons développé une approche expérimentale innovante sur les sardines, en étudiant les effets possibles de la nourriture à travers des niveaux d'intégration successifs: au niveau de l'organisme (par ex. la croissance, la condition corporelle, la consommation d'oxygène), de l'organe (par ex. l'appareil branchial, l'intestin), cellulaire (par ex. l'efficacité mitochondriale) et moléculaire (par ex. le cortisol, la balance oxydative). Le deuxième objectif était d'examiner les processus pouvant expliquer une surmortalité des adultes. Tout d'abord, nous avons utilisé une

approche expérimentale pour essayer de relier la condition corporelle d'un individu avec son état physiologique et sa probabilité de survie. Ensuite, nous avons utilisé une approche par modélisation en utilisant le modèle DEB (Dynamic Energy Budget) pour étudier la survie des individus après la reproduction.

Dans un premier temps, pour déterminer si les changements observés dans la nature pouvaient découler d'un changement de la taille et de la quantité de proies, nous avons d'abord étudié les conséquences de différentes tailles et quantités d'aliments sur des paramètres morphologiques (condition corporelle, croissance) et physiologiques (lipides et protéines musculaires, balance oxydative dans le sang). Ensuite, nous avons étudié les mécanismes qui pourraient expliquer les effets de la taille et de la quantité de nourriture sur la croissance et la condition corporelle observées dans la nature en mettant l'accent sur le bilan énergétique associé à l'alimentation. Pour ce faire, nous avons étudié d'une part comment la sardine pouvait maximiser l'extraction d'énergie de son environnement en analysant son appareil branchial et de l'autre côté, les coûts énergétiques associés aux deux comportements alimentaires au moyen d'analyses de respirométrie. Dans la troisième partie, nous avons examiné d'autres traits de vie qui pourraient être influencés par la taille et la quantité de nourriture, ce qui pourrait en partie compenser ou aggraver la situation. Pour cela, nous avons d'abord étudié l'impact de la taille et de la quantité de nourriture sur la capacité de transformation du substrat en énergie au travers de l'étude de l'efficacité de conversion des mitochondries, le moteur énergétique du corps. Nous avons ensuite étudié les effets de la taille des proies sur la reproduction des sardines en s'intéressant à la production d'œufs. Enfin, nous avons étudié l'effet de l'alimentation sur deux paramètres exprimant l'état de santé d'un individu: l'immunité et le niveau de stress. Dans la quatrième partie, nous avons examiné ce qui pourrait conduire à la surmortalité des sardines. Ainsi, nous avons utilisé une approche expérimentale de jeûne pour identifier les seuils de condition corporelle susceptibles de conduire des individus à la mort dans le milieu naturel. Pour ce faire, nous avons suivi l'évolution de la masse corporelle au cours du temps afin d'identifier l'entrée en phase 3 du jeûne (phase critique), ce qui correspond à la mobilisation des protéines pour assurer la survie de l'individu. Dans la dernière partie, nous avons étudié l'hypothèse d'une surmortalité adulte se produisant après la reproduction comme suggérée par Brosset et al. (2016b). Pour cela, nous avons utilisé le modèle DEB qui décrit les flux d'énergie au sein des individus (depuis les apports jusqu'aux utilisations telles que la

croissance ou la reproduction) au cours de son cycle de vie, en prenant en compte des variables forçantes telles que la température et l'alimentation. Nous avons paramétré ce modèle en utilisant à la fois des données expérimentales et provenant du milieu. Nous avons également simulé l'évolution d'une population de sardines au fil du temps en fonction de scénarios environnementaux et en s'intéressant à leur survie.

3. Résultats

3.1. L'hypothèse de bottom-up reste-t-elle encore valable?

En utilisant une approche expérimentale, nous avons d'abord montré que la taille et la quantité de nourriture n'avaient aucun effet sur les lipides structuraux ni sur les protéines, mais avaient un impact significatif sur la croissance, la condition corporelle et les lipides de stockage des sardines. En effet, les sardines nourries avec de petites particules devaient en consommer deux fois plus (en biomasse) que celles qui se nourrissaient sur de grosses particules afin d'obtenir la même condition corporelle et la même croissance. Les taux de croissance mensuels obtenu en captivité sur les sardines nourries avec des traitements intermédiaires (traitement 2 : petite taille en grande quantité; traitement 3: grande taille en petite quantité) étaient proches des taux de croissance observés dans le milieu après 2008 (1,2, 1,0 et 1,5 mm/mois pour les traitements 2 et 3 et dans la nature - voir Matériel supplémentaire de Queiros et al, 2019). Ces résultats ont renforcé le fait qu'une diminution de la taille et/ou de la quantité de proies dans le golfe du Lion restait une hypothèse fiable pour expliquer la dynamique des populations de sardines.

3.2. Effets de taille de nourriture comme conséquence des coûts énergétiques de la filtration

Une plus grande capacité de recherche et de collecte de nourriture dans leur environnement pourrait avantager ces individus, en particulier s'ils vivent en banc comme c'est le cas des petits pélagiques. Pour obtenir la même condition corporelle et la même croissance, les sardines nourries avec de petits granulés devaient en consommer deux fois plus que les sardines nourries avec des plus gros. Sachant que la qualité des aliments et les conditions d'élevage étaient similaires, comment peut-on expliquer le désavantage apparent de se nourrir de petites proies ? Les deux hypothèses possibles et non exclusives reposent sur le

fait que la nourriture était acquise différemment en raison de différences au niveau de l'appareil branchial ou bien que l'énergie dépensée pour la recherche de nourriture dépendait de la taille de la proie. Pour attraper une proie, les sardines peuvent adopter deux stratégies d'alimentation différentes, principalement en fonction de la taille de la proie (Garrido et al., 2007). Dans nos résultats, les besoins en énergie de la nage aérobie utilisée lors de la filtration semblaient plus importants que la chasse à vue anaérobie utilisée pour se nourrir sur des grosses proies.

3.3. Comment expliquer la surmortalité de la sardine?

Bien que nous ayons montré qu'une diminution de la taille ou de la quantité de nourriture était suffisante pour induire une croissance et une condition corporelle plus faibles que celles observées dans le milieu, nous devions néanmoins expliquer l'augmentation de la mortalité adulte provoquant un déséquilibre de la structure en âge de la population en faveur des plus jeunes (Van Beveren et al., 2014). Premièrement, nous avons examiné si les poissons pouvaient mourir de faim et à cause de faibles réserves énergétiques. En utilisant une approche expérimentale, nous avons montré que la survie commençait à diminuer fortement lorsque l'indice de la condition corporelle devenait inférieur à 0,75, la survie atteignant 50% pour une condition corporelle de 0,65. En outre, la perte spécifique de masse corporelle a augmenté environ une semaine avant la mort des individus, au même moment que la condition corporelle tombait en dessous de 0,72 et cela était lié à l'entrée dans la phase III du jeûne. De tels résultats peuvent représenter le potentiel maximum des sardines pour faire face au manque de nourriture car elles étaient moins stressées au cours de l'expérience (pas de prédation, pas de pathogènes) et les seuils trouvés ici pourraient donc être plus élevés dans la nature. Néanmoins, nos résultats ont indiqué que la proportion de sardines dans la nature tombant en dessous de cette condition seuil (0,72) avait doublé au cours de la période récente par rapport aux années précédentes et avait atteint ses niveaux les plus élevés au cours des mois de janvier et février. Cela semble confirmer une probabilité plus élevée de surmortalité chez l'adulte à la fin de la reproduction. En combinant des données expérimentales et in situ, nous avons d'abord paramétré le modèle DEB pour la sardine de Méditerranée. Les simulations basées sur la population in situ ont montré que les individus de 14 cm avaient une probabilité de 20% de survivre un mois après la période de reproduction, mais que cette probabilité chutait jusqu'à 8% après un an. Cette étude a mis

en évidence une mortalité significative des individus les plus importants après la reproduction, correspondant aux individus âgés de plus de 3 ans. Ce travail semble renforcer l'hypothèse de la surmortalité des adultes, même si plusieurs améliorations doivent encore être apportées.

3.4. Effets de la taille des aliments sur d'autres traits

Les traitements alimentaires composés de petites particules semblent n'avoir qu'un faible impact sur l'immunité et le stress des sardines. En effet, les concentrations en leucocytes et en cortisol étaient similaires entre les traitements alimentaires (à la fois grandes et petites tailles). Sachant que les sardines n'avaient pas à faire face à des challenges (pas de prédation et environnement aseptique) et que l'indice de condition corporelle des sardines nourries avec des petites particules tout au long de l'expérience est proche de 1 (état général moyen chez les populations sauvages), les niveaux de leucocytes et de cortisol trouvés au cours de cette étude pourraient correspondre à des concentrations de standard, alors que les variances de ces niveaux traduiraient la variabilité interindividuelle.

Par ailleurs, nous avons aussi étudié l'effet de la taille des aliments sur la reproduction et nous avons découvert que la taille des aliments a un effet significatif sur le nombre d'événements de ponte, ce qui pourrait s'expliquer par une fréquence de ponte plus élevée liée à des individus plus grands. De plus, la relation entre l'indice de condition corporelle et l'investissement dans la reproduction pourrait ne pas être linéaire, mais plutôt supposée être en U. Ainsi, l'investissement dans la reproduction pourrait augmenter lorsque la condition corporelle diminuerait en dessous d'un seuil. Ici, les sardines nourries avec des petites particules avaient une condition corporelle autour de 1, ce qui était peut-être trop élevé pour provoquer un changement de compromis énergétique entre reproduction et survie.

3.5. Les sardines peuvent-elles s'adapter à la restriction calorique ou à des proies plus petites?

Les sardines de la mer Méditerranée sont connues pour être moins bonnes filtreuses que les sardines de zones plus productives (par exemple en Atlantique, voir Costalago et al., 2015). Ici, nous avons étudié si les sardines pouvaient s'adapter à des proies plus petites en augmentant la capacité de filtration de leur appareil branchial. En utilisant des sardines

capturées dans la nature, puis maintenues en captivité pendant 7 mois avec différents traitements alimentaires, nous avons constaté que la longueur de l'arc branchial et la densité de branchiospines étaient significativement corrélées à la longueur du poisson, mais non corrélés à l'abondance et à la longueur des branchiospines. La taille et la quantité de nourriture n'ont pas induit de plasticité dans la structure des branchies (longueur, densité ou abondance) des sardines adultes après 7 mois. Cependant, nous avons constaté une augmentation de la densité des branchiospines pour une longueur de poisson donnée entre 2007-2009 et 2016. Ces modifications peuvent être dues à de la plasticité, à de la sélection naturelle ou à de l'épigénétique, mais cela nécessite des analyses plus approfondies. Une autre façon de faire face à la restriction calorique serait d'adapter ses dépenses énergétiques, soit en modifiant l'efficacité des mitochondries, soit en diminuant son activité. Nos résultats indiquent que la petite taille des aliments semble entraîner une augmentation de l'abondance et de l'efficacité des mitochondries, ce qui suggère que les sardines faisant face à une restriction calorique ont adopté une stratégie d'économie d'énergie pour produire de l'ATP. En outre, la diminution du taux de consommation d'oxygène de base pour les sardines dont l'état corporel est faible soutient la stratégie d'économie d'énergie pour les sardines faisant face à une restriction alimentaire. De même, les sardines nourries avec de petites proies en petite quantité pendant 7 mois affichaient une consommation en oxygène plus faible au niveau du groupe pendant le jeûne, probablement en raison d'une activité plus faible. Une telle diminution semble permettre aux sardines de maintenir une période de jeûne plus longue et d'observer une diminution plus faible de leur condition corporelle. Ces résultats ont montré une réponse plastique des sardines à la privation de nourriture, réduisant leur mortalité. Néanmoins, ces stratégies pourraient entraîner d'autres coûts ou ne suffiraient peut-être pas à compenser les fortes demandes en énergie liées filtration sur des petites proies.

4. Discussion

4.1. Expérimentation sur les sardines

Parmi toutes les expérimentations s'intéressant aux poissons, le poisson zèbre (*Dario rerio*) est sans aucun doute l'espèce la plus utilisée, notamment en raison de similitudes partagées avec l'homme (Kalueff et al., 2014). Les expériences sur les espèces de poissons marins sont

généralement utilisées pour améliorer l'aquaculture ou des études de génétiques, mais rarement pour des problèmes d'écologie appliquée, bien que les expérimentations constituent l'une des approches les plus utiles pour étudier les mécanismes de contrôle des populations (Hunt and McKinnell, 2006). Les études in situ restent complexes et coûteuses sur les poissons marins et celles consacrées aux petits poissons pélagiques ont principalement porté sur des questions spécifiques, telles que les estimations de la taille des stocks. Au cours de ma thèse, nous avons développé une approche expérimentale originale pour examiner les impacts de la taille et de la quantité de nourriture sur la situation des sardines et pour vérifier l'hypothèse de la surmortalité des adultes. Une telle approche expérimentale reste rare en raison de la difficulté de manipuler ces espèces. Bien que des expériences sur les larves de sardines soient fréquemment effectuées, les études expérimentales portant sur des adultes sont peu utilisées. Elles ont principalement servi à étudier les aspects de la captivité ou de la manipulation au cours de l'expérimentation (Bandarra et al., 2018; Marçalo et al., 2008; Peleteiro et al., 2004), des effets des procédures ou des dispositifs de pêche (Goetz et al., 2015; Marçalo et al., 2013, 2010) et le comportement alimentaire (Garrido et al., 2007). De plus, une première expérience de 1 an a été menée sur des sardines adultes mais qui étaient nettement plus grandes et plus lourdes (20,2 cm et 72,2 g, Bandarra et al., 2018), tandis qu'une plus longue étude (1,5 ans) a débuté avec des œufs collectés dans la nature et n'a abouti qu'à une seule sardine vivante après 18 mois (Iglesias and Fuentes, 2014). Enfin, nous avons prouvé que des manipulations et des expériences de longue durée sur les sardines, en particulier sur de petits individus, pourraient être menées à l'avenir.

De plus, l'expérimentation animale doit être conforme aux exigences réglementaires nationales en matière de bien-être animal. À cette fin, la règle des Trois R (Reduce, Refine, Replace - soit Réduire, Améliorer, Remplacer) vise à réduire le nombre d'animaux utilisés, à affiner les procédures expérimentales afin de limiter les souffrances des animaux et à remplacer les animaux par des alternatives non animales lorsque cela est possible (Ibrahim, 2006). En plus d'être particulièrement attentifs aux conditions d'élevage, nous avons également porté une attention particulière au cours de cette thèse au devenir des sardines à la fin des expériences. En tant que telles, les sardines étaient soit données à des aquariums locaux lorsque des sacrifices n'étaient pas nécessaires, soit optimisées afin de recueillir le maximum d'échantillons (muscles pour les protéines et les lipides, sang pour la balance

oxydative, écailles pour le cortisol, les gonades et le cœur pour les télomères, mais aussi à certaines occasions, les branchies, le rein pour l'immunité et l'intestin pour de l'histologie et pour le microbiome). Néanmoins, une telle approche n'aurait pas été possible sans la collaboration de plusieurs experts de différents domaines (mitochondries, immunité, par exemple), ce qui m'a permis d'étudier les effets de l'alimentation sur plusieurs traits d'histoire de vie de la sardine (reproduction, croissance et maintenance) et à plusieurs échelles (organisme, organe, cellule et composant), certaines étant encore à l'étude.

Enfin, le paramétrage de modèles tels que le modèle DEB nécessite généralement beaucoup de données pour trouver le meilleur jeu de paramètres. Les données d'expérimentation représentent les deux tiers des données utilisées lors du processus de paramétrage du modèle DEB, ce qui nous permet de tester (numériquement) certaines hypothèses qui ne pourraient pas être testées autrement (comme par exemple les effets combinés de la nourriture et de la température). Bien que les expériences prennent beaucoup de temps et nécessitent beaucoup de personnel/équipement (par exemple, une étude sur plusieurs années successives, plusieurs températures), elles sont complémentaires de l'approche de modélisation et sont au cœur de toutes les études menées au cours de cette thèse.

5. Limites

Comme pour toutes les expériences, nous avons essayé d'être aussi proches que possible de l'environnement naturel (par ex. pour l'eau et la nourriture) afin d'obtenir le comportement le plus naturel possible des sardines. Par conséquent, les bassins expérimentaux étaient alimentés en eau pompée directement en mer et suivaient des régimes naturels de photo et thermo-période. Le nombre et la densité de poissons par bassin ont également permis la formation de banc. Ensuite, aucune des deux possibilités de fournir des proies vivantes aux sardines (capture dans la nature ou production) ne semblait adéquate pour nos expériences. Outre les difficultés logistiques inhérentes, il aurait été impossible de normaliser les repas en termes de taille et de quantité d'aliments plusieurs fois par jour, tous les jours pendant plusieurs mois. A l'inverse, les granulés d'aquaculture offraient la possibilité de contrôler avec précision les intrants alimentaires. Ne disposant d'aucune information sur la composition des proies à l'état sauvage avant et après la crise, nous avons décidé d'étudier uniquement les effets de la taille et de la quantité de nourriture sur les traits de vie de la

sardine et ainsi sélectionner des granulés d'aquaculture de deux tailles différentes (0,1 et 1,2 mm) et de même qualité en termes de teneur lipidiques et protéiques. Bien que nous soyons conscients de la limitation des aliments inertes par rapport aux proies vivantes, nous nous sommes assurés d'un comportement alimentaire normal après chaque période d'acclimatation. Malgré toutes les dispositions mises en place, les expériences menées au cours de cette thèse ont pu parfois échouer pour plusieurs raisons. Deux exemples sont présentés ci-après. Tout d'abord, malgré nos efforts pour imiter l'environnement naturel, nos connaissances sur la reproduction des sardines sont limitées. Nous avons décidé d'étudier l'effet de la taille de la nourriture sur la reproduction des sardines et donc les pontes n'ont pas été induites artificiellement. Les premières pontes issues des sardines de l'expérience n°2 se sont produites lorsque la température de l'eau est descendue en dessous de 14°C et se sont terminées lorsque la température a dépassé 15-16°C. Cependant, les sardines de l'expérience n°1 (Chapitre 1) n'ont pas pondu en hiver, même les individus nourris avec de grosses particules alors qu'ils avaient une condition corporelle élevée (environ 1,2) et soumis aux mêmes conditions environnementales (les bassins étant alimentés avec la même eau et suivant la même photopériode). Même si les stratégies « income breeding » et « capital breeding » peuvent refléter des points extrêmes dans un continuum de stratégies (Stearns, 1989), les sardines sont connus pour être des « capital breeders » dans le Golfe du Lion. Ainsi, l'absence de ponte chez de tels individus peut être due à de faibles niveaux de stockage d'énergie avant l'expérience (l'expérience a commencé à la mi-novembre, c'est-à-dire environ 3 semaines avant que la température ne descende en dessous de 14°C). Pour y remédier, l'expérience n°3 visait à déterminer si la taille des aliments avant ou pendant la période de reproduction avait une influence sur la reproduction des sardines, c'est-à-dire pour étudier ce gradient capital/income. Cependant, un seul événement de reproduction avec une très faible quantité d'œufs a été observé au cours de l'expérience n°3, tandis que les sardines de l'expérience n°2 se sont reproduites 39 fois au même moment avec la même qualité d'eau. L'absence de reproduction pourrait être due à des conditions d'élevage, les sardines de l'expérience n°2 étaient dans des cuves plus grandes que les sardines des expériences n°1 et n°3 (3 m^3 et 300 L pour les expériences n°1 et n°3, respectivement). Il est intéressant de noter que les sardines de l'expérience n°3 avaient des gonades matures (observées lorsqu'elles ont été sacrifiées en mars). L'absence de ponte dans de petits bassins peut également mettre en avant l'importance de la hauteur

de la colonne d'eau pour la reproduction des petits pélagiques. En effet, le comportement de reproduction des petits pélagiques pourrait être similaire à celui d'autres espèces de poissons libérant leurs œufs dans la colonne d'eau, tels que les mérous (Rowell et al. 2019, Mourier et al. 2019) ou les poissons perroquets (Luckhurst 2011). Pour élucider les comportements de reproduction des sardines, un sonar à courte portée pourrait être utilisé pour identifier le comportement des poissons pendant la reproduction qui se déroule la nuit. Enfin, le fait que les sardines ne se soient pas reproduites peut également résulter d'une incompréhension éthologique de la reproduction des petits pélagiques, telle que le comportement, la taille du banc, etc. Malgré l'absence de reproduction au cours de l'expérience n°3, les échantillons prélevés en mars seront utilisés pour évaluer le coût physiologique de la croissance ou de la reproduction à l'aide de télomères et de la balance oxydative (Bauch et al., 2013; Geiger et al., 2012). Les paramètres morphologiques fournis au cours de cette expérience seront également utilisés pour étudier une compétition potentielle entre individus.

Outre l'étude de reproduction, l'étape finale de l'expérience n ° 3 aurait dû être utilisée pour étudier les différences de consommation d'oxygène des sardines se nourrissant sur des petits granulés par rapport à de gros granulés avec plus de réplicats et un système « start and stop » comparée à l'étude de respirométrie présentée précédemment. Au cours de cette expérience, les sardines ont été réparties dans 8 bassins en fonction de leur indice de condition corporelle (HC et LC, respectivement conditions élevées et faibles), soit 4 bassins pour HC et 4 bassins pour LC. Les sardines ont été nourries avec du gros granulés pendant les 2 premières semaines et avec du petits granulés pendant les 3 semaines suivantes (y compris une semaine pour l'acclimatation à ce traitement alimentaire). Les sardines étaient nourries deux fois par jour et l'alimentation de chaque rangée, constituée d'un bassin de chaque condition, était espacée toutes les 20 minutes afin d'être synchronisée avec le système de respirométrie à flux « start and stop ». Cependant, l'alimentation de la première rangée a entraîné l'agitation des sardines dans tous les autres réservoirs, même si elles n'étaient pas alimentées. Ainsi, la consommation d'oxygène a augmenté avant le début du repas dans 6 des 8 bassins, rendant ces données inutilisables. De plus, les sardines en bon état (c'est-à-dire nourries avec de gros granulés pendant 9 mois) ne semblaient pas s'intéresser aux petits granulés, probablement parce que leur bon état leur permettait d'attendre une meilleure nourriture; de sorte que la consommation d'oxygène n'a pas

augmenté pendant leurs repas. Bien que cette expérience devait être indispensable pour mieux comprendre les mécanismes impliqués dans la croissance et la condition plus faible des sardines nourries avec des petits granulés, il n'a pas été possible de la refaire pendant la période de ma thèse, en raison de plusieurs problèmes logistiques. Néanmoins, un nouveau protocole a été conçu pour cette expérience et pourrait être réalisée dans un proche avenir (voir perspectives).

Outre les limites susmentionnées de l'approche expérimentale, cette étude soulève également certaines limites dans l'exercice de modélisation. L'approche DEB traite de la répartition des flux d'énergie depuis la nourriture jusqu'à son utilisation pour la maintenance, la croissance et la reproduction. Pour paramétrer le modèle DEB, les «meilleures» données correspondent aux bilans énergétiques sur plusieurs tailles de poisson et à plusieurs niveaux de nourriture (Lika et al., 2011). Cependant, ces données n'étant pas disponibles pour notre espèce, nous avons donc utilisé des observations indirectes liées aux flux d'énergie pour estimer les paramètres tels que la croissance. Bien que le paramétrage soit satisfaisant, les erreurs relatives entre certaines observations et prévisions sont relativement élevées et le temps de survie des individus à jeun estimé pendant les simulations était inférieur à l'estimation trouvée lors de l'expérience de jeun. En outre, la prédiction de l'indice gonadosomatique était plutôt basse et pourrait être due à la faible quantité de données disponibles sur la reproduction de la sardine dans la littérature. Ces résultats pourraient également être expliqués par la longue et difficile étape du paramétrage du modèle DEB. Dans notre étude, nous avons en outre ajouté un compartiment au modèle DEB pour prendre en compte la reproduction de la sardine en multiple lots successifs, mais plusieurs hypothèses de modélisation ont dû être formulées en raison du peu d'informations disponibles sur la reproduction comme par exemple le facteur environnemental qui déclenche la reproduction. Enfin, l'exploration de l'espace des paramètres est contrainte par des ensembles de paramètres «réalistes» en fonction d'autres espèces similaires et certains paramètres doivent être corrigés. Néanmoins, deux ensembles de paramètres réalistes pourraient bien corréler aux données mais conduire à une incompréhension du processus physiologique sous-jacent. Ainsi, un faible niveau de nourriture contrebalancé par un κ élevé (allocation au soma) pourrait conduire à la même croissance que la combinaison d'un niveau de nourriture élevé et d'un faible κ. Pour limiter le nombre de paramètres réalistes potentiels, des données supplémentaires (par exemple sur la reproduction ou le contenu

énergétique) seraient nécessaires. Dans cette étude, nous avons considéré que tous les individus partageaient les mêmes paramètres et nous n'avons pas intégré de variabilité interindividuelle, en particulier pendant le processus de simulation. De même, les paramètres environnementaux (température et aliments) dérivés des résultats des modèles 3D hydrodynamiques et biogéochimiques ayant une résolution journalière, mais seules les moyennes mensuelles pour l'ensemble du golfe du Lion et pour toute la hauteur de la colonne d'eau ont été utilisées. De plus, aucune distribution verticale ou spatiale de la sardine (distribution principalement côtière, Saraux et al. 2014) n'a été prise en compte alors que cette dernière pourrait avoir des effets physiologiques notamment dus à la température (par ex. la stratification en hiver) ou à des ressources alimentaires différentes. Néanmoins, les perspectives développées dans la discussion devraient réduire ou permettre d'explorer certaines des limites potentielles susmentionnées.

6. Conclusion

En conclusion, les différentes expériences ont permis de mettre en évidence à la fois l'importance de la taille et de la quantité de nourriture sur la condition corporelle et la croissance des individus. Ces résultats semblent être liés à des dépenses énergétiques plus importantes lorsque les sardines filtrent pour s'alimenter sur des proies de petites tailles que lorsqu'elles chassent les plus grosses. Malgré des adaptations pour faire face à des petites proies ou à une restriction alimentaire (amélioration de la capacité de filtration, meilleur couplage énergétique des mitochondries, réduction de l'activité), ces mesures ne semblent pas contrebalancer les dépenses nécessaires à la filtration. De plus, la reproduction semble être impactée négativement par la taille de nourriture à l'inverse de l'immunité et du stress. Néanmoins l'absence d'effet pourrait être expliquée par une condition corporelle des sardines nourries sur des petites proies trop élevée pour indure une modification de l'allocation énergétique. L'étude de la surmortalité adulte a permis de définir des seuils à partir desquels la probabilité de survie des sardines diminuait drastiquement. Les simulations qui ont suivi ont permis de montrer une plus faible probabilité de survie des individus les plus grands (ceux de plus de 3 ans) qui pourrait ainsi renforcer l'idée d'une surmortalité post-reproduction

Introduction

1. The small pelagics

'*The term 'small pelagic fishes' refers to a diverse group of mainly planktivorous fishes that share the same habitat, the surface layers of the water column, usually above the continental shelf and in waters not exceeding 200 m in depth*' (Dalzell, 1993). They constitute schools made by individuals sharing the same size and body form and consist of one or more species depending on their relative abundance (Bakun and Cury, 1999). Small pelagics are also opportunistic strategists, i.e. with a small size, early maturation and short life (King and McFarlane, 2003). They are at the heart of energetic transfers from lower trophic levels (plankton) to upper ones such as marine mammals, birds and large pelagic fishes (Cury et al., 2000; Pikitch et al., 2014). Thus, they exert major controls on the entire marine food web through either top-down control on the plankton communities or bottom-up control on their predators or wasp-waist control when both top-down and bottom-up controls occur simultaneously (Cury et al., 2000). Simultaneously, these fishes have huge commercial significance as they represent one quarter of worldwide landings (in tons), predominantly through anchovy, sardinella, sardine, mackerel and herring (FAO, 2018). They support important fisheries all over the world and the economy of many countries depend on the small pelagic fisheries (Alheit et al., 2009; Fréon et al., 2005). Therefore, fluctuations in populations of small pelagics can have critical ecological, economic and social consequences as observed in Peru in the early 1970s with the famous collapse of the Peruvian anchovy (Alheit et al., 2009; Allison et al., 2009; Schwartzlose et al., 1999).

Small pelagics are known to widely fluctuate over time complicating the management of their populations (Bakun, 1996). Such strong fluctuations have been recorded over using scale deposition rates, showing that they are not the sole result of exploitation (Guiñez et al., 2014; Valdés et al., 2008). Indeed, their population dynamics are strongly impacted by natural environmental fluctuations (Alheit et al., 2009; Checkley et al., 2017). The crucial significance of plankton production and availability for recruitment dynamics of small pelagics has been known since seminal work by Hjort (1914), and is central to the '*match-mismatch*' and '*ocean triad*' hypotheses (Bakun, 1996; Cushing, 1990). The '*match-mismatch*' hypothesis refers to the need to have a synchrony between plankton availability

and larvae production (Cushing, 1990), while the '*ocean triad*' theory is based on 3 processes (enrichment, concentration and retention) to explain how physical environment could influence the survival of early life stages of pelagic populations (Bakun, 1996). As a consequence, any variable that impacts plankton production may be a potential driver of the small pelagic population dynamic either small-scale (e.g. temperature, wind) or large-scale fluctuations. Large scale fluctuations induced by the North Atlantic Oscillations have involved in several ecological modifications such as dynamic, abundance or spatial distribution of populations (Ottersen et al., 2001; Stenseth et al., 2002). To the same extent, large scale fluctuations of El Niño-Southern Oscillation in the Pacific Ocean are known to negatively impact small pelagic populations such as Peruvian anchovy populations (Barber and Chavez, 1983). However, small pelagics could enable to cope with bad environmental conditions using small scale structure (e.g. anchovy population during El Niño 1997–98 in Bertrand et al., 2004).

Furthermore, worldwide landings (excluding aquaculture) rose from 20 million tons in 1950 to almost 80 million tons in the 80s (Sinclair et al., 2002). Nowadays, most of worldwide marine fish stocks are fully exploited (60%) or overexploited (35%) with large geographic disparities (FAO, 2018). (Over)fishing has also been identified as a key factor in the collapse of several stocks in the world, often synergistically with environmental fluctuations (Essington et al., 2015; Toresen and Ostvedt, 2000).The famous collapse of the Peruvian anchovy in the 1970s could have resulted from both El Niño event and overfishing (Pauly et al., 2002; Stenseth et al., 2002). Similarly, the collapse of the Norwegian herring to the state of commercial extinction in the late 1960s seemed to be due to a combination of unfavorable climatic conditions and overexploitation of adult and juvenile herrings (Engelhard and Heino, 2004). In addition to the direct impact on targeted species, overfishing may also alter pelagic communities and may induce drastic changes in trophic webs through the modification of matter and energy fluxes (e.g. in Bearzi et al., 2006; Cury et al., 2000; Gómez-Campos et al., 2011; Jackson et al., 2001).

Small pelagic fish have a worldwide distribution and are especially well studied in the large EBUS (Eastern Boundary Upwelling Systems) owing to their higher productivity. Even if landings are lower compared to EBUS, small pelagics are also very important for fisheries in other non-upwelling system such as in the Mediterranean basin where small pelagics represent 38% of the total catch (Lacoue-Labarthe et al., 2016). The Mediterranean Sea is

considered as the largest and deepest enclosed sea on Earth and connected to both the Atlantic and Indian Oceans (Coll et al., 2010). The basin is known to be mainly oligotrophic but some coastal enriched spots are induced by environmental conditions and human sewage (see Figure 1c in Coll et al., 2010). Furthermore, the Mediterranean Sea is a marine biodiversity hot spot with a mean of 6.3% of worldwide species inhabiting only 0.82% (in surface area) and 0.32% (in volume) of the world ocean (Bianchi and Morri, 2000; Coll et al., 2010). However, climate change and human impact such as pollution, invasive species and overfishing are important threats for biodiversity especially in the highly populated Mediterranean region (Bianchi and Morri, 2000; Costello et al., 2010). As a result, the Mediterranean Sea has showed an alarming decrease of their main exploited fish stocks since 1990 (Vasilakopoulos et al., 2014).

2. An unusual situation in the Gulf of Lions

The Gulf of Lions is located in the northwestern Mediterranean Sea (Figure 1) with a bathymetry between 0 and 2,500 m and covering about 15,000 km2 (Mellon-Duval et al., 2017). The Gulf of Lions is one of the most productive areas in the Mediterranean Sea mainly due to the dominant forcing drivers such as the strong northwestern (tramontane) and northern (mistral) winds, the western Mediterranean mesoscale circulation and the freshwater input from the Rhone River (Millot, 1990; Petrenko et al., 2005). It represents an important feeding area for fish, birds and mammals, for both resident and migratory populations (Bănaru et al., 2013).

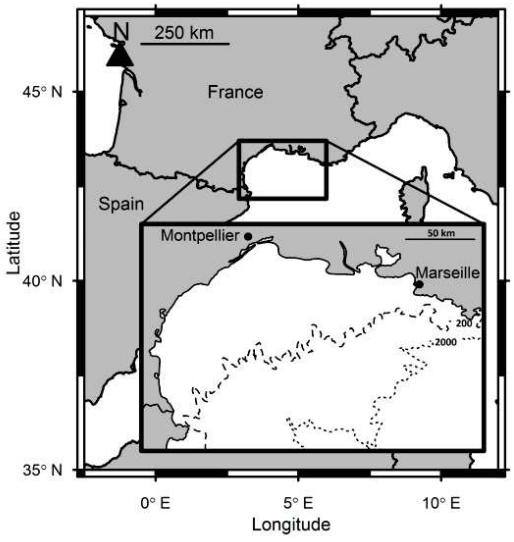

Figure 1: Map of the Gulf of Lions, showing 200 and 2000 m bathymetry

Among the most targeted species, small pelagics as sardines (*Sardina pilchardus*) and anchovies (*Engraulis encrasicolus*) represented about 50% of the total annual landings in the Gulf of Lions until the 2000s (Figures 2A et 2B). In most upwelling systems, when sardines and anchovies occur sympatrically, they fluctuate asynchronously, i.e. alternation between sardine-based ecosystem and anchovy-based ecosystem (Alheit et al., 2009; Schwartzlose et al., 1999). However, landings of small pelagics in the Gulf of Lions have reached their lowest levels recorded in 150 years for both anchovies and sardines at the same time (Van Beveren et al., 2016a) (Figure 2C). More surprisingly, this important decrease in landings occurred despite abundance levels being high (GFCM, 2017b; Van Beveren et al., 2014). Rather, this situation has been explained by a decrease in individual size and weight since 2008 of both sardine and anchovy (sardine being more impacted than anchovy, Figure 2D and 2E), making them economically unfavorable owing to the absence of a market for small individuals (Saraux et al., 2019). The decrease in size resulted from the combination of a lower growth and the disappearance of the oldest and largest individuals (Saraux et al., 2019; Van Beveren et al., 2014). Further, the mean weight has also decreased owing to a weaker body condition of fish observed since 2008 (Figure 2E and 2F)(Van Beveren et al., 2014). In summary, fish are smaller as they grow less and are younger and for a given length, they also appear to be leaner.

Surprisingly, the changes observed in the Gulf of Lions were not due to a lower recruitment (it has remained high since 2008) (Saraux et al., 2019) which is usually highlighted to be responsible of the decline in landings of small pelagics (Schwartzlose et al., 1999). Thus, this unusual situation was primarily investigated by the EcoPelGol project (Ecosystèmes Pélagiques du Golfe du Lion) whose aim was to study the main causes of the changes in small pelagic fish populations in the Gulf of Lions (Saraux et al., 2019). To do so, they investigated several potential sources of this issue (Figure 3): (1) emigration of oldest individuals, (2) top-down controls (i.e. by fisheries or natural predators such as tunas and dolphins), (3) bottom-up controls (i.e. by environmentally-driven food modification), (4) epizootic events and (5) energy allocation trade-offs (Saraux et al., 2019).

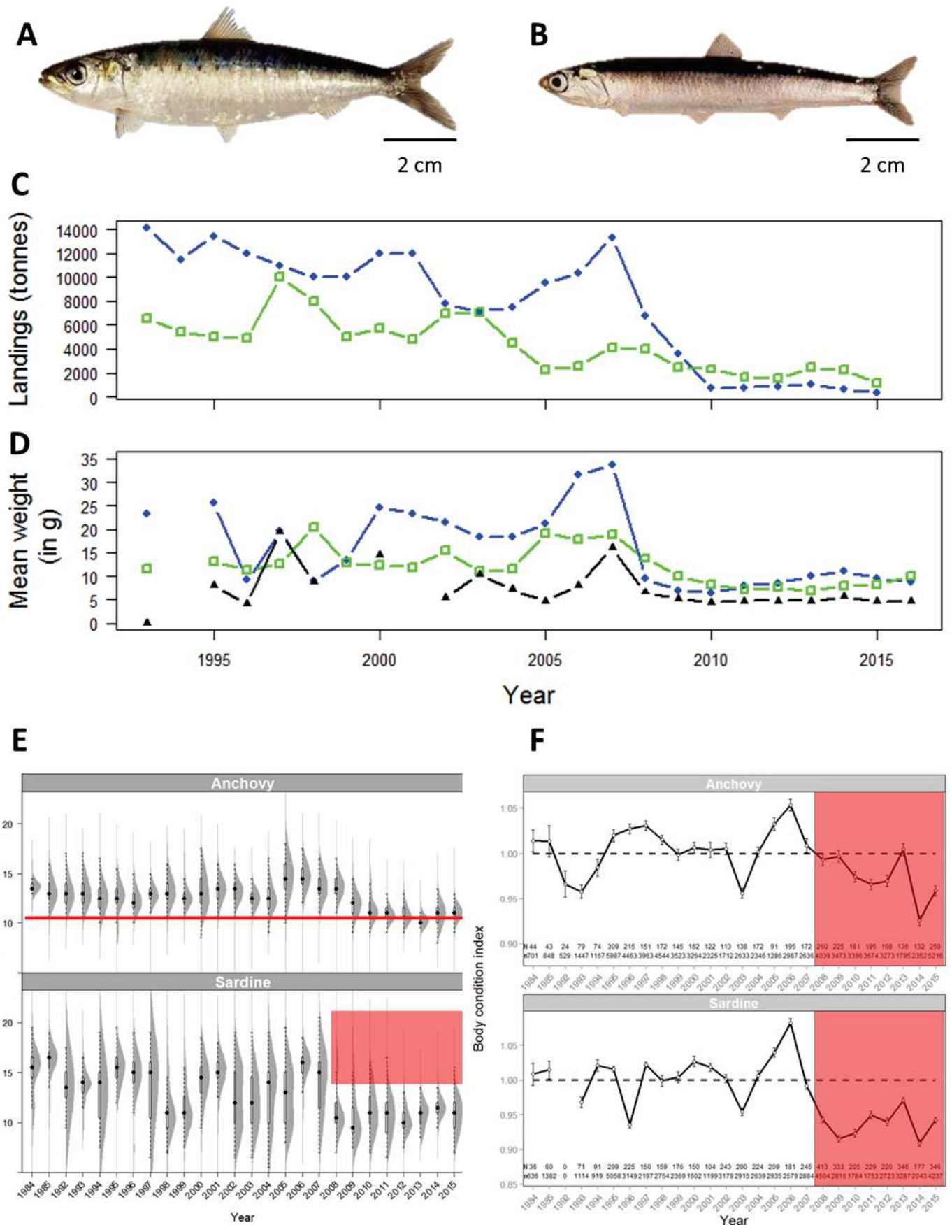

Figure 2: Pictures of the sardine *Sardina pilchardus* (A) and the anchovy *Engraulis encrasicolus* (B). Landings (C), mean weight (D), size distribution (E) and body condition index (F) of sardine (blue full circles) and anchovies (green open squares) [black full triangles correspond to sprat *Sprattus sprattus* in plots (C) and (D)]. Adapted from Saraux et al. 2019

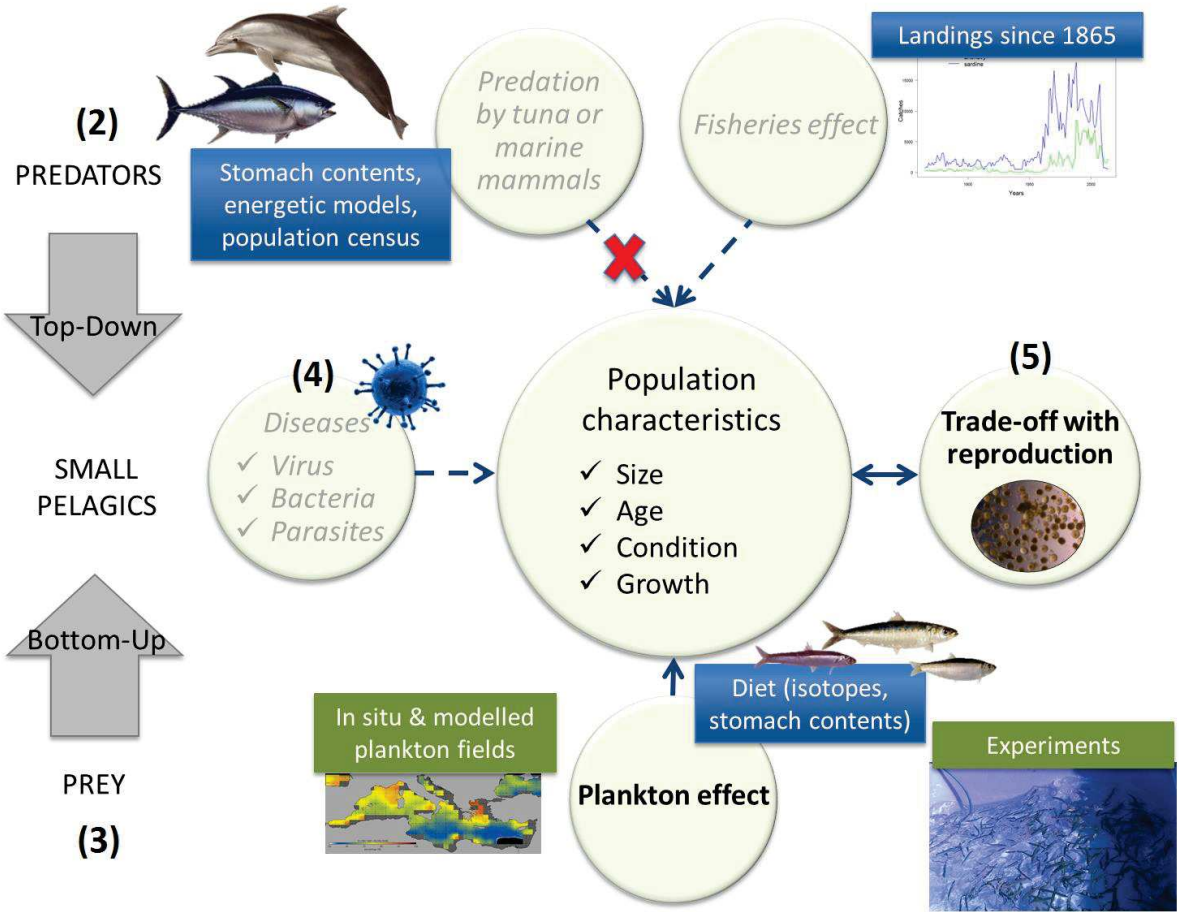

Figure 3: Schematics of the main drivers of small pelagic population dynamics from Saraux et al. (2019). Significant drivers appear in bold, while less important ones appear in grey and in italics. Green items were perspectives for future work

Two hypotheses may explain the disappearance of the oldest individuals from the Gulf of Lions: emigration and/or overmortality of those individuals. Owing to the direction of the powerful North Current (i.e. to the west) and the presence of a continental shelf, if small pelagics had emigrated they should have done so towards Spanish Waters. However, size distributions of both French and Spanish sardines and anchovies were similar between 2002 and 2013 and the disappearance of the largest individuals happened simultaneously in both areas (Saraux et al., 2019). Thus, the hypothesis of an emigration to Spain was not retained to explain the disappearance and adult overmortality appeared the most probable hypothesis. Nonetheless, this result showed that the unusual situation observed in the Gulf of Lions seemed to act at a larger scale in the NW Mediterranean Sea.

The first investigation was led on top-down controls: fishing and (natural) predation pressure. On the one hand, overfishing is known to significantly impact populations and

could modify their structure in favor of small individuals and earlier maturation (Daan et al., 2005; Law, 2000). The mean fishing harvest rates was lower than 15% for both sardine and anchovy, so much lower than the reference level of 40% (Figure 4)(Saraux et al., 2019). Interestingly, this threshold was crossed only once in 1997 and was followed by peak in sardine biomass, suggesting little effect of fishing on these populations (Saraux et al., 2019). Further, the fishing pressure in the Gulf of Lions has remained very low in the last 10 years (< 1 %) and has reached the lowest levels in 150 years, but no sign of recovery has been observed in small pelagic populations (GFCM, 2017b; Saraux et al., 2019; Van Beveren et al., 2016a). Due to low harvest rates and the absence of time concordance between small pelagic decrease in biomass and fishing pressure, overfishing was not considered as a primary source of the observed changes in the Gulf of Lions.

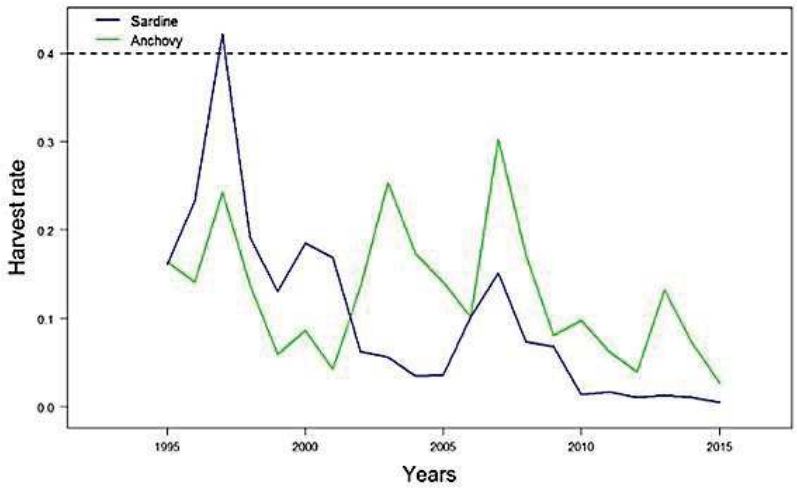

Figure 4: Harvest rates of sardine (in blue) and anchovy (in green) as estimated by the total landings divided by the biomass of the stock assessed by acoustics. Dashed lines indicates the safe level of harvest rates. Plot from Saraux et al. 2019.

On the other hand, the predation pressure (ratio consumed/available) exerted by top predators could be higher than fishing mortality in several areas, increasing population fluctuations (Cury et al., 2000; Jacobsen and Essington, 2018) and so might remove an important part of adults from the population. Due to its recovery in the Gulf of Lions and its diet mainly based on sardines and anchovies (which accounted for more than 80% of its diet in mass and abundance), the Atlantic bluefin tuna (*Thunnus thynnus*) constitutes one of the main predators of these species (Bauer et al., 2015; Van Beveren et al., 2017). To study the predation pressure of the bluefin tuna, Van Beveren et al. (2017) developed an innovative

approach combining several estimations: (1) population abundance derived from aerial survey (Bauer et al., 2015), (2) population structure estimated from landings, (3) energetic requirements from DEB model (Dynamic Energy Budget), (4) stomach content analysis and (5) prey abundance in the Gulf of Lions from PELMED survey (described in Saraux et al., 2014). Then, they performed 10,000 iterations to take into account uncertainties due to previous estimations and using appropriate distribution for each parameters (Van Beveren et al., 2017). Finally, the top-down control exerted by tunas was extremely limited as it reached less than 2% of the available stock for both sardine and anchovy, and no prey-size selectivity was found (Van Beveren et al., 2017). Furthermore, marine mammals seem to be important consumers of prey in various ecosystems, especially cetaceans because of their large body sizes and relatively high metabolic rates (Bowen, 1997; Laran et al., 2010). The predation pressure of the two main dolphin species (the bottlenose dolphin *Tursiops truncatus* and the striped dolphin *Stenella coeruleoalba*) was estimated based on the same approach. To do so, we combined several estimations: (1) population abundances from Bauer et al. (2015) and the demographic compositions based on literature, (2) energetic requirements from empirical relationships, (3) stomach content analysis from stranded individuals, (4) prey energetic values from literature and (5) small pelagic abundances from PELMED survey. The dolphin predation pressure was also estimated through 10,000 Monte Carlo simulations in which each input parameter was drawn from a given distribution (e.g. normal, uniform and gamma distributions) independently of each other. Finally, the top-down control exerted by dolphins was extremely limited as it reached less than 0.1% of the available stock for both sardine and anchovy (Queiros et al., 2018 ; Annex 1). Thus, top-down controls by tuna or dolphin could not explain the unusual situation observed in the Gulf of Lions.

Then, epizootic events are known to have caused massive mortalities in small pelagic populations in the past (e.g. Australian pilchard contaminated by herpesvirus, described in Whittington et al. (1997)). To investigate whether mortality could be induced by diseases, a wide range of potential pathogens (parasites, virus and bacteria) were investigated on both fat and lean sardines sampled throughout an entire year. The investigations revealed the presence of microscopic parasites (e.g. trematodes and coccidia) and some occurrences of bacteria (*Tenacibaculum* and *Vibrio*) but no virus (neither herpesvirus nor nodavirus) or macroparasite was detected. Then, no clear damage to tissues or indication of disease was

observed during necropsy and histological examinations (Van Beveren et al., 2016b). Further, no correlation was found between pathogens and the size or condition of the host (Van Beveren et al., 2016b). It is thus unlikely that an epizootic event may explain the adult overmortality.

Conversely, the decrease of both growth and condition may suggest a bottom-up control mediated by changes in planktonic composition and/or abundance. Indeed, a previous study revealed that the body condition of sardine had a positive correlation with WeMO (Western Mediterranean Oscillation) and both mesozooplankton and diatom concentrations (Brosset et al., 2015b). Interestingly, the diet of sardines seemed to have shifted from large size prey (with a high contribution of cladocerans > 1 mm) before 2008 to smaller prey (copepods < 1 mm) in the most recent years (Brosset et al., 2016a). Similarly, large copepods were replaced by smaller ones (< 1 mm) in the anchovy diet after 2008 (Brosset et al., 2016a). Thus, less energetic content of smaller plankton could explain the decrease in the body condition of small pelagics observed in the Gulf of Lions (Barroeta et al., 2017; Evjemo et al., 2003; Zarubin et al., 2014).

Sardine and anchovy are both able to use two strategies for feeding: particulate feeding and filtration. Nevertheless, sardines have a more efficient filtration apparatus than anchovies, owing to higher filtration area and less spaced gill rakers (Collard et al., 2017). A regime shift toward small prey should thus favor or at least be less detrimental for sardines as they are better adapted to filter-feeding on small plankton. Indeed, sardines are known to dominate upwelling systems when prey size falls (Chavez et al., 2003; Schwartzlose et al., 1999; van der Lingen, 1994). However, in the Gulf of Lions, sardines appeared to be more affected by the regime shift than anchovies (Van Beveren et al., 2014). This result might highlight differences between the feeding strategy of sardines in EBUS vs. other areas (Saraux et al., 2019). For instance, the Mediterranean sardines appeared to have lower numbers and densities of gill rakers (for a given body length) than their Atlantic relatives (Costalago et al., 2015). This observation may reflect a response of the feeding strategy of sardine to the high plankton densities in Atlantic (compared to the Mediterranean). Additionally, interspecific competition was suspected to explain the alternation between sardine and anchovies in the western North Pacific (Nakayama et al., 2018). Here, the increase of the trophic niche overlap between sardine, anchovy and sprat *Sprattus sprattus* (a small pelagic fish with an

increasing population) may act synergistically with the reduction of prey size in particular if resource partitioning becomes lower (Brosset et al., 2016a).

3. Underlying mechanisms

The decrease of the small pelagic body condition observed in the Gulf of Lions seemed to be generalized to the whole Mediterranean Sea, except in the Aegean Sea (Brosset et al., 2017). The sardine population is actually considered as 'ecologically unbalanced' in the Gulf of Lions, but the underlying mechanisms remained unknown (GFCM, 2017). The aim of this thesis was to clear up the mechanisms governing the bottom-up control of sardines and the processes implemented by individuals to cope with this energy limitation. As all mechanisms require energy, energy plays a key role through the entire life of individuals. Energy flow is usually studied because it allows to make links between small scale processes (individuals) to large scale patterns (population) (Ghedini et al., 2017). The available energy for an individual corresponds to the assimilated part of the ingested food (through the digestive apparatus) minus the energy used for prospection and predation. According to life-history theory, this energy is then allocated between the different life-history traits such as survival, reproduction and growth (Stearns, 1989, 1992). As soon as resources become no longer sufficient to pay all costs, trade-offs become unavoidable and thus several internal processes might be affected by these choices (Zera and Harshman, 2001). To extract as much energy as possible from their environment, sardines are able, as mentioned before, to adopt two feeding behaviors, i.e. filter- or particulate-feeding. They are able to switch easily between these two strategies and the switch seems to be prey size-dependent (Garrido et al., 2007, 2008). The study of the feeding apparatus and the feeding behaviors of sardines should be essential to understand the energy expenditure and assimilation of the two feeding modes as well as whether sardines might have adapted to a potential reduction in size of their prey.
Further, the reproduction represents the main life-history trade-off, opposed to growth or maintenance (Williams, 1966b). When food resources shrink, individuals might consider making extreme choices such as either skipping spawning to maximize their survival or investing all energy in reproduction at the expense of survival to pass on their genes to the next generation (Jørgensen et al., 2006). The overmortality of the oldest sardines in the Gulf of Lions might thus be linked to a high reproductive investment despite their low body

condition (condition is a proxy of the energy reserves, see Lloret et al. (2014) and Brosset et al. (2015b)). Indeed, the increase in gonadosomatic index between 2009 and 2016 and the decrease of the length at first maturity from 12.1 cm to 9.6 cm in the recent years seem to suggest that the energy allocation to reproduction may have been kept constant if not increased (Brosset et al., 2016b; Saraux et al., 2019). Thus, this biased energy allocation might have knock-on effects on survival rates of adults after reproduction due to a lack of energy for their own survival, but it required to be validated (Brosset et al., 2016b; Saraux et al., 2019). Sardines are usually considered as capital breeders, i.e. they store energy in spring and summer that can be mobilized later for the reproduction in autumn and winter (Ganias, 2009). Their energy reserve strongly decreased during winter as reproduction and low food resources occur synergistically (Brosset et al., 2015b). This situation may affect sardine survival if they need to cope with low feeding conditions during and/or after reproduction.

Further, the swimming capacity of sardines depends on their ability to mobilize energy to this activity. Small pelagics are able to modulate their swimming behavior between aerobic endurance (e.g. filtration on small prey) and anaerobic sprint (e.g. to avoid predators or feeding on large prey).

On the first hand, coping with low feeding conditions may influence aerobic swimming capacity of sardines. Indeed, the life in school exhibited by small pelagics supposes higher benefits than disadvantages for individuals (e.g. foraging capacity, predator avoidance, swimming activity) (e.g. in Marras et al., 2015) but also requires enough energy to follow the group over long distance. Also, individuals coping with poor energy reserves might have less energy to explore their environment to find prey, leading to a vicious circle for these individuals. On the other hand, coping with low feeding conditions may reduce the anaerobic swimming capacity of sardines. Thus, their escape capacity may be impacted resulting in a potential increase of predation as well as a decrease of their feeding capacity on large prey in movement.

Furthermore, the lack of energy induced by low feeding conditions might also impact the survival of sardines through other processes such as stress or immunity. The ratio between the reactive oxygen species (ROS) production and the antioxidant defenses controls the oxidative stress balance (Finkel and Holbrook, 2000). This balance is known to play a central role as a mediator in trade-offs between growth, maintenance and reproduction (Kirkwood and Rose, 1991; Metcalfe and Alonso-Alvarez, 2010). Imbalance toward ROS could imply

impairment of physiological function leading to macromolecules damages, precocious ageing, age-related disease or cell death, while imbalance toward antioxidants could interrupt physiological process and decrease immunity defenses (Finkel and Holbrook, 2000; Monaghan et al., 2009). Additionally, the oxidative stress balance could be also impacted by glucocorticoid stress hormones like cortisol but their influence seemed to be dependent on the exposure period (Roussel et al., 2004; Tort, 2011; Wingfield et al., 1998). Coping with cortisol may derive energy resources from other activities leading to trade-offs with foraging (Wingfield et al., 1998), growth (Midwood et al., 2014), survival (e.g. predator avoidance, immune response) (Harris and Bird, 2000; Midwood et al., 2014; Tort, 2011; Wendelaar Bonga, 1997; Wingfield et al., 1998) or reproduction (Tort, 2011). Thus, the increase of stress hormones is known to be implicated in the senescence and the post-reproduction death of salmonids (Dickhoff, 1989; Ziuganov, 2005). A prolonged exposition to very low food level could be assimilated to starvation. Similarly to birds and mammals, starvation in fish was divided into 3 phases: (I) a short period with the glycogen consumption, (II) a long period with the mobilization of lipids and (III) a protein degradation period (Bar, 2014; Lignot and LeMaho, 2012). The beginning of the phase III seems to be correlated with the increase of cortisol levels in most fish species even if several exception are recorded (Bar and Volkoff, 2012). Similarly, starvation may have negative consequences on immune functions, although such effects seem to differ between species; starvation has no or positive effect on immune responses in pacu (Gimbo et al., 2015), European eel (Caruso et al., 2010) and Chinese Sturgeon (Feng et al., 2011) but decreases the ability to cope with infection in Atlantic salmon (Martin et al., 2010) and Jade perch (Luo et al., 2013). Thus, stress and immunity might act synergistically with the lack of energy to explain the adult overmortality and both require investigations.

The critical situation of the sardine was made at a population scale and it remained almost impossible to investigate on small pelagics in the wild (see e.g. in Peleteiro et al. (2004)). Experimentation turns out to be one of the most useful approach for studying population dynamics (Hunt and McKinnell, 2006). Even so, experimentation on marine fish species are often performed in a context of aquaculture and fundamental biology, more rarely in applied and fisheries ecology whereas physiological and behavioral information could be complementary to these purposes (Horodysky et al., 2015; Jorgensen et al., 2012; Mckenzie et al., 2016; Ward et al., 2016). Thus, to understand the underlying behavioral and

ecophysiological mechanisms which lead sardine populations to crisis, we used an experimental approach which was completed by modeling.

4. Thesis outlines

For this project, we decided to work on sardine owing to their stronger response to the regime shift. This thesis was developed into 5 parts to answer to 5 questions (Figure 5)

- Chapter 1: Does food size matter?

  To investigate whether the changes that we observed in the wild might derive from a change in prey size and quantity, we first studied the consequences of feeding on different food sizes and quantities on morphological (body condition, growth) and physiological parameters (muscle constituents and blood oxidative balance).

- Chapter 2: Which mechanisms could explain food effects on growth and body condition?

  Secondly, we studied the mechanisms which could explain the food size and quantity effects on the growth and the body condition observed in the wild by focusing on the energy balance associated with feeding. To do so, we investigated on one side how sardine could maximize the extraction of energy from their environment through an analysis of their gill raker apparatus and, on the other side, the energetic costs associated with the two feeding behaviors using respirometry analyses.

- Chapter 3: Which other life history traits could be impacted by food? Could they act as amplifying factors?

  In the third part, we examined other life history traits that could be impacted by food size and quantity, which could either partly compensate or worsen the situation. First, we studied the impact of food size and quantity on the capacity to transform substrate into energy through the study of the conversion efficiency of the mitochondria, the energetic engine of the body. Then, we investigated effects of prey size on the reproduction of sardines using egg production. Finally, we studied the

effect of food looking at two parameters expressing the individual health state: immunity and stress level.

- Chapter 4: Can sardine starve to death?

  Further, we investigated what could drive the overmortality of sardines. First, we used a fasting experimental approach to identify body condition thresholds which would lead individuals to death in the wild (Chapter 4). To do so, we followed body mass changes over time to identify the critical entry in phase 3 of fasting, which corresponds to the mobilization of proteins to survival.

- Chapter 5: Can adult overmortality follow reproduction?

  In the last part, we studied the hypothesis of an adult overmortality after reproduction as suggested by Brosset et al. (2016b). To that end, we used the Dynamic Energy Budget (DEB) which described the individual energy flows (from intakes to uses as growth or reproduction) during its life cycle accounting for external variables such as temperature and food. We parameterized this model using both experimental and in-situ data. And, we simulated the evolution of a sardine cohort over time depending on environmental scenarios monitoring its survival.

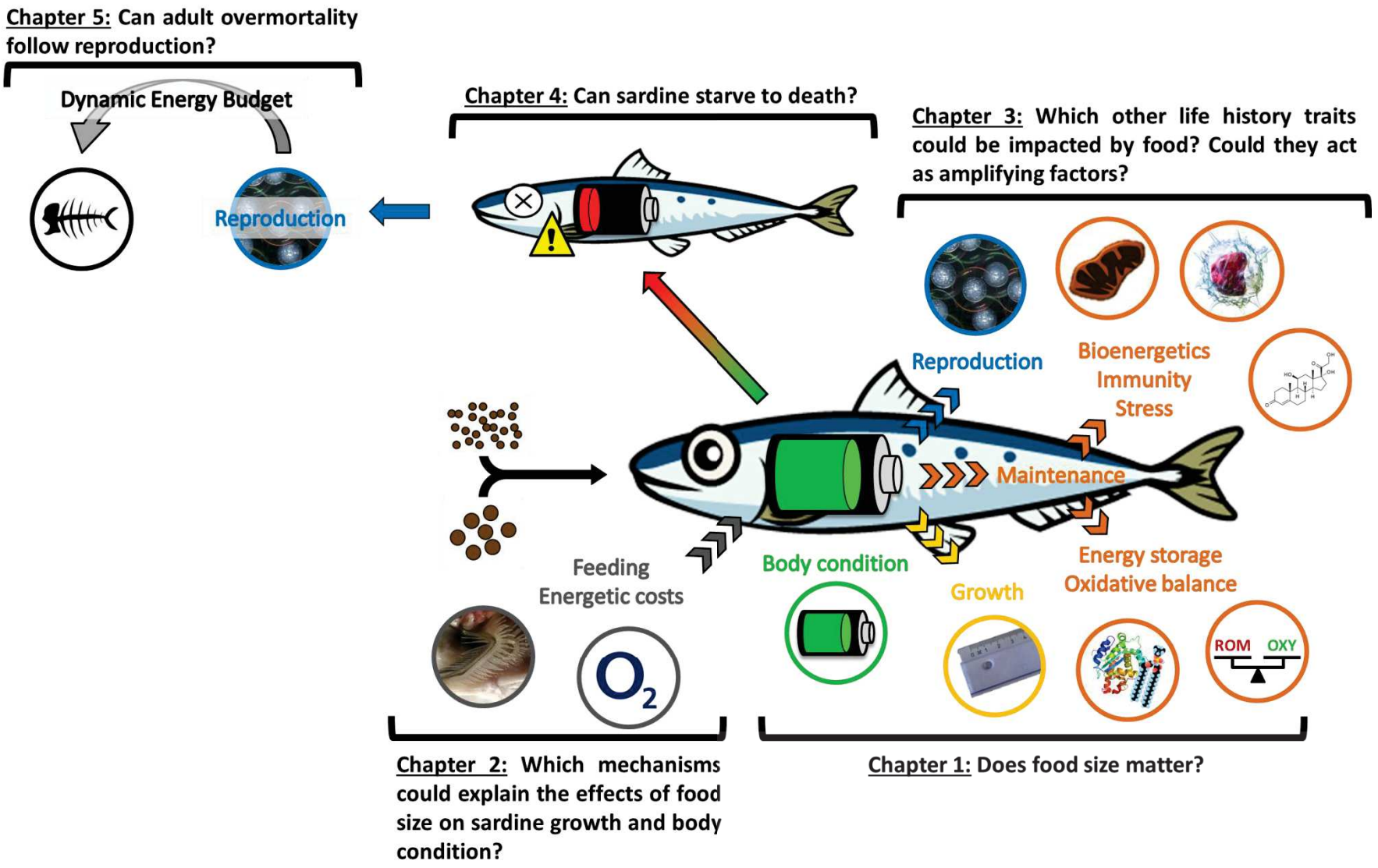

Figure 5: Conceptual framework of this PhD.

Chapter 1: Does food size matter?

Food in the Sea: size also matters for pelagic fish

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Food in the Sea: Size Also Matters for Pelagic Fish

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1. Abstract

Small pelagic fish are key components of marine ecosystems and fisheries worldwide. Despite the absence of recruitment failure and overfishing, pelagic fisheries have been in crisis for a decade in the Western Mediterranean Sea because of a marked decline in sardine size and condition. This situation most probably results from bottom-up control and changes in the plankton community toward smaller plankton. To understand such an unusual phenomenon, we developed an original and innovative experimental approach investigating the mechanisms induced by a reduction in the quantity and size of sardine prey. While experimentations offer the unique opportunity to integrate behavior and ecophysiology in understanding key demographic processes, they remain rarely used in fisheries science, even more so on small pelagics due to the notorious difficulty to handle them. The results revealed that food size (without any modification of its energy content) is as important as food quantity for body condition, growth and reserve lipids: sardines that fed on small particles had to consume twice as much as those feeding on large particles to achieve the same condition and growth. Such a strong impact of food size (based on 100 vs. 1200 mm pellets) was unexpected and may reflect a different energy cost or gain of two feeding behaviors, filter-feeding vs. particulate-feeding, which would have to be tested in further study. As increasing temperature favors planktonic chains of smaller size, climate change might actually accelerate and amplify such phenomenon and thus strongly affect fisheries.

2. Introduction

Small pelagic fish are a key component of marine ecosystems, modulating population dynamics of both lower and upper trophic levels, especially in highly productive marine upwelling systems (Brochier et al., 2011; Cury et al., 2000; Frederiksen et al., 2006; Taylor et al., 2008). Small pelagics also represent 25% of worldwide landings (in tons), predominantly through anchovy, sardinella, sardine, mackerel and herring (FAO, 2018). Fluctuations in populations of small pelagics can, therefore, have critical ecological, economic and social consequences, such that understanding their underlying mechanisms is extremely important. The population dynamics of these species could be strongly impacted by natural environmental fluctuations (bottom-up control) and mortality (top-down control) (Checkley et al., 2017). On the one hand, the crucial significance of plankton production and availability for recruitment dynamics of small pelagics has been known since seminal work by Hjort (1914), and is central to the "match-mismatch" and "ocean triad" hypotheses (Bakun, 1996; Cushing, 1990). On the other hand, (over)fishing has also been identified as a key factor in the collapse of several stocks, often in conjunction with environmental fluctuations (Toresen and Ostvedt, 2000), which may be exacerbated by ongoing global change (Brochier et al., 2013; Shannon et al., 2009). Nevertheless, the relative importance of bottom-up *versus* top-down controls remains complicated to assess since they are often entangled (Hunt and McKinnell, 2006; Rouyer et al., 2014).

The Gulf of Lions is one of the most productive areas of the Mediterranean Sea due to wind-driven coastal upwelling, the western Mediterranean mesoscale circulation and fresh water inputs from the Rhone River (Millot, 1990; Petrenko et al., 2005). Until the mid-2000s, sardine (*Sardina pilchardus*) and anchovy (*Engraulis encrasicolus*) represented up to 50% of the total annual landings (around 15,000 tons) in the Gulf of Lions (Bănaru et al., 2013). Landings of sardines and anchovies have, however, decreased sharply since 2008, reaching the lowest levels recorded in 150 years (around 3,000 tons), although population abundance remains high (GFCM, 2017b; Saraux et al., 2019; Van Beveren et al., 2016a). Declines in landings of small pelagic fishes are typically a result of recruitment failure and/or overfishing (Schwartzlose et al., 1999). In the Gulf of Lions, however, the dramatic drop in catches has been due to a severe decrease in individual body size and condition (mean weight ± SE: 24.3 ± 0.3 g between 1993 and 2007 and 11.4 ± 0.2 g between 2008 and 2018), which has rendered the sardines and

anchovies unattractive for fisheries (Saraux et al., 2019; Van Beveren et al., 2014, 2016a). The decrease in size of sardines is the combined outcome of lower growth and the disappearance, from the population, of the oldest and largest individuals (Saraux et al., 2019; Van Beveren et al., 2014). This situation was not due to recruitment failure and/or overfishing, which is, as mentioned above, unusual. The fishing pressure and selectivity were (and still are) very low and could not explain the disappearance of the large individuals (GFCM, 2017b). Sardine recruitment remained stable and even increased during the last decade in this area (Saraux et al., 2019). Recent studies further showed that this was neither due to emigration, top-down controls (natural predators) or diseases (Queiros et al., 2018; Saraux et al., 2019; Van Beveren et al., 2016b, 2017). The major remaining hypothesis is therefore bottom-up control, linked in some way to the quantity and quality of plankton production in the Gulf of Lions (Brosset et al., 2015b; Saraux et al., 2019; Van Beveren et al., 2014).

Sardines are both filter and particulate feeders, depending on the size of their prey and switch easily between these feeding modes (Garrido et al., 2007, 2008). In the Gulf of Lions, sardines feed on a size range of plankton ranging from 0.1 mm to 1.4 mm in length (Le Bourg et al., 2015). Interestingly, the diet of sardines has shifted from large size prey (with a high contribution of cladocerans > 1 mm) before 2008 to smaller prey (copepods < 1 mm suspected to be less nutritious) in the most recent years (Barroeta et al., 2017; Brosset et al., 2016a; Evjemo et al., 2003; Zarubin et al., 2014). Thus, bottom-up control might explain the poor condition of sardines, with knock-on effects on survival rates of adults after reproduction (Brosset et al., 2016a). This remains, however, to be validated (Saraux et al., 2019).

In this study, we developed an experimental approach to investigate the potential mechanisms for this bottom-up control on sardines. To investigate whether the changes we observed in the wild might derive from a change in prey, we studied the long-term consequences of feeding on different food sizes and quantities on individual body condition and growth through a 7-month experiment on captive adult sardines. While long-term experiments in captivity are rarely performed to inform on fisheries science, especially with difficult-to-maintain small pelagics, we believe that this is an important step to test for hypotheses and better understand the behavioural or ecophysiological mechanisms involved in natural processes. We coupled our experiment with laboratory analyses of physiological parameters, such as muscle constituents (lipids and proteins) and blood oxidative balance. This latter provides insight into levels of metabolism and plays a central role in ageing processes by acting as a mediator in trade-offs

between growth, maintenance and reproduction (Finkel and Holbrook, 2000; Kirkwood and Rose, 1991; Metcalfe and Alonso-Alvarez, 2010).

3. Material and methods

3.1. From capture to maintenance in experimental tanks

Sardines were captured in October 2016 (2 trips on the 10th and 18th of October) by a commercial purse-seiner operating from Sète (South of France). Fishing and acclimation procedures are detailed in Supporting Information.

3.2. Experimental design

449 sardines were distributed homogeneously into 8 experimental tanks of 300 L each (56-57 sardines per tank), so that both the mean and the range in length and weight were comparable among tanks and treatments (Figure 6; Table 1). Prior to transfer, sardines were anesthetized (benzocaine at 140 ppm), total body length and total wet weight recorded to the nearest 0.1 mm and the nearest 0.01 g respectively. A tiny RFID tag (Biolog-id, Bernay, France, 0.03g i.e. <0.2% of sardine lowest body mass) was implanted in the dorsal muscle using a specific injector and allowed individual identification. This procedure caused less than 1% mortality and did not affect their behavior. Tanks were supplied with water pumped directly from the sea and filtered through sand filter (30-40 µm). The photoperiod was adjusted each week to follow the natural cycle and sea water temperature was not controlled except to maintain a minimum of 10°C or a maximum of 25°C (Figure S1). After 10 days of acclimation to these new tanks (sardines fed with the same pellet mix as the one used during acclimation but at 0.3% of the biomass), the experiment started on November 14, 2016 (t_0) and continued until June 15, 2017 (reproduction period between November and March) (Figure 7, Experiment n°1). Biometries were performed every four weeks, with all sardines measured individually (tag read, total body length and body weight recorded). There was very rarely any mortality following the biometries. Relative body condition of each sardine was calculated with the Le Cren index K_n as estimated by Brosset et al. (2015b):

$$K_n = \frac{WW}{0.00607 \times TL^{3.057}} \quad [1]$$

where WW the wet weight in g and TL is the total length in cm.

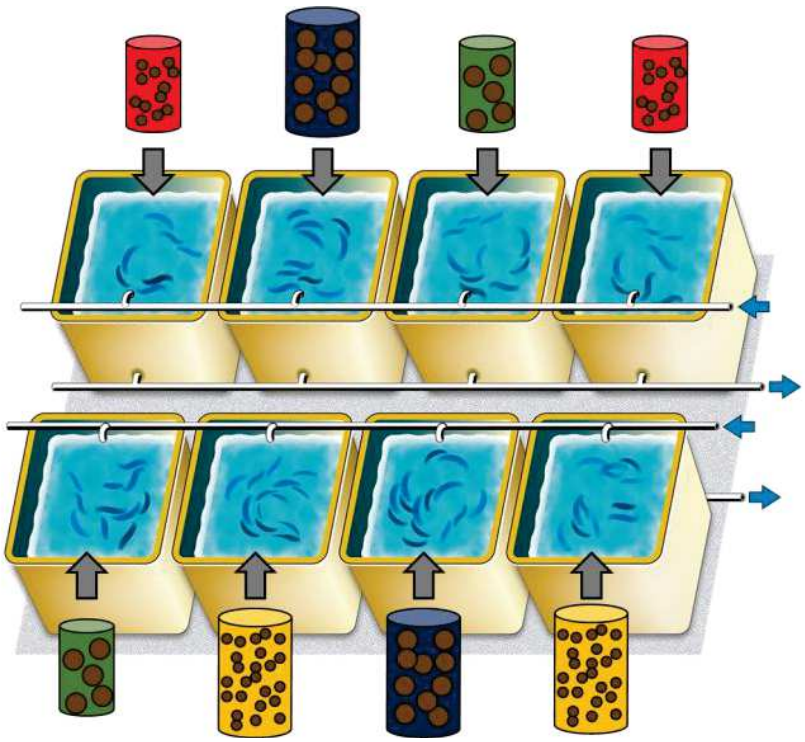

Figure 6: Experimental design to study the impact of food treatment, comprising the 8 tanks and their feeding treatment (red: pellet size of 0.1mm and pellet quantity of 0.3%; yellow: 0.1mm and 0.6%; green: 1.2mm and 0.3% and blue: 1.2mm and 0.6%). Blue arrows represented input and output sea water.

Table 1: Number of individuals, mean length (± SD) and length range (95% CI), mean weight (± SD) and weight range (95% CI), sex ratio (in %) of males (M), females (F) or unidentified (Un.), age class ratio (in %) for each tank and each treatment. Sex ratios and age class ratios are based on samples of March and June (sample size is given by n).

Treatments	Tank	Individuals	Length (mm)	Weight (mm)	Sex (n=231)			Age (n=227)				
					Mature		Immature					
					M	F	Un.	<1	1	2	3	4
1 	1	56	119 ± 9 [98 ; 151]	14.0 ± 3.5 [7.2 ; 27.9]	32%	57%	11%	15%	31%	46%	4%	4%
	2	56	120 ± 8 [98 ; 142]	13.9 ± 2.9 [7.2 ; 21.0]	33%	58%	8%	7%	7%	87%	0%	0%
2 	1	56	119 ± 9 [96 ; 135]	14.0 ± 3.4 [6.7 ; 24.5]	29%	68%	4%	0%	36%	56%	8%	0%
	2	56	120 ± 9 [97 ; 143]	14.3 ± 3.3 [7.7 ; 20.5]	38%	56%	6%	4%	8%	79%	8%	0%
3 	1	56	118 ± 9 [95 ; 143]	14.0 ± 3.3 [6.7 ; 20.1]	36%	52%	12%	4%	21%	71%	4%	0%
	2	56	120 ± 8 [104 ; 144]	14.2 ± 2.9 [7.6 ; 20.5]	41%	53%	6%	3%	3%	84%	6%	3%
4 	1	57	119 ± 9 [97 ; 138]	14.4 ± 3.4 [7.0 ; 23.1]	37%	60%	3%	13%	13%	74%	0%	0%
	2	56	120 ± 8 [101 ; 138]	14.2 ± 3.1 [8.1 ; 22.8]	47%	44%	9%	3%	9%	84%	3%	0%

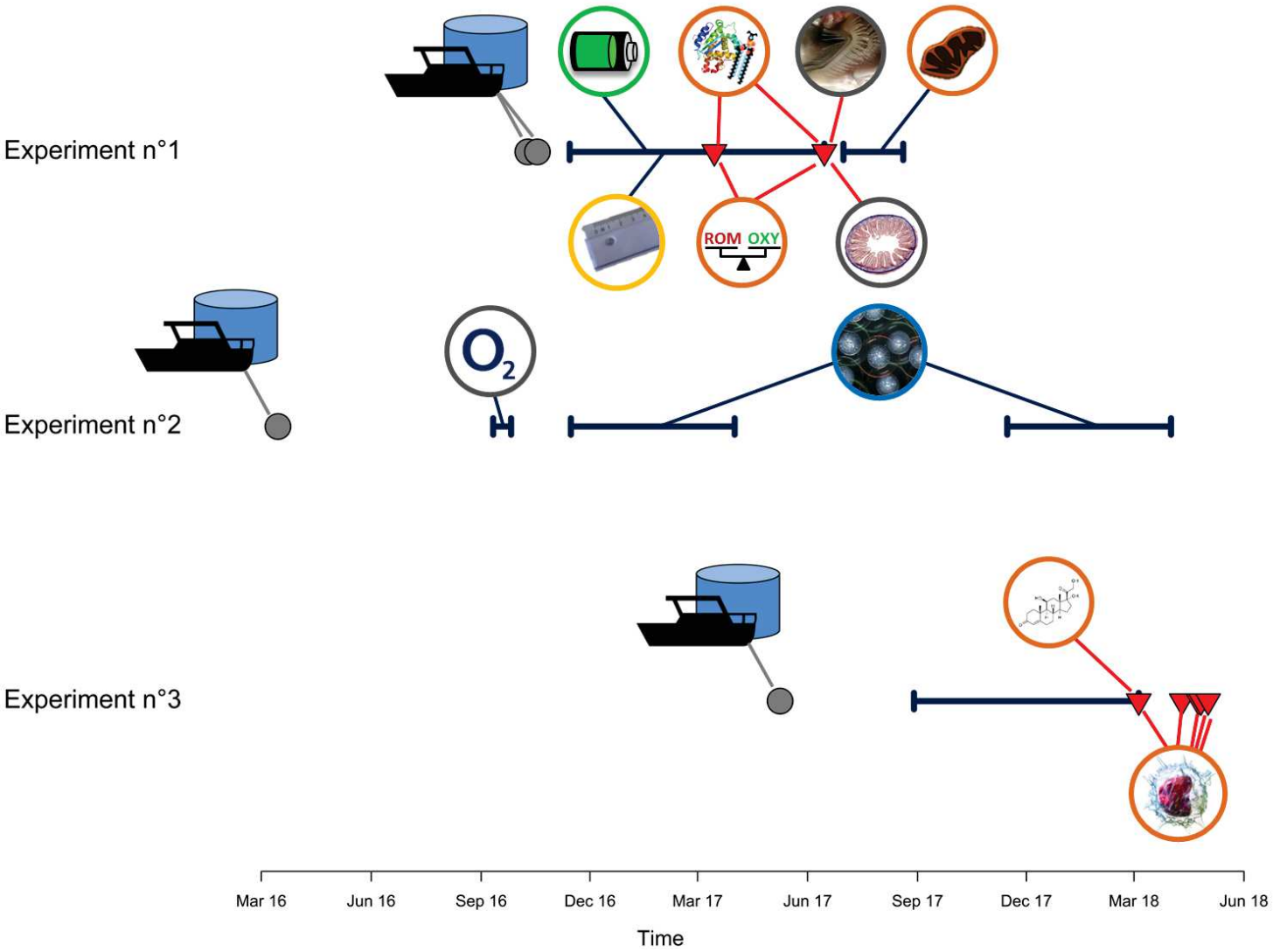

Figure 7: Conceptual framework of the 3 experiments developed during the thesis. The fishing and the beginning of the rearing (acclimatization) are symbolized by grey circles. Each experiment part is represented by blue segments. The sampling events occurring during experiments are depicted by red triangles. Each parameter studied was derived from either a continuous monitoring (represented by blue lines) or from samples (represented by red lines).

3.3. Feeding conditions

For feeding conditions to be as realistic as possible, pellet quantities were selected to mimic weight gains measured in the wild under contrasting conditions (see Supporting Information, Figure S2 and Movie S1). That is, we estimated daily weight gain in the two different periods of good (2005-2006) and bad conditions (after 2010) defined by Van Beveren et al. (2014), using data on weight and age of ~24,000 individuals compiled by Brosset et al. (2015b). Assuming constant growth throughout the year, daily weight gain was estimated between two consecutive ages by:

$$\text{daily weight gain} = \sqrt[365]{\frac{\mu_{age+1}}{\mu_{age}}} \quad [2]$$

where μ_{age} is the mean weight of sardines at a specific age. We found daily weight gain in the wild corresponded to 0.2% and 0.1% of their weight under good and bad conditions, respectively. Using preliminary tests in tanks, these daily weight gains corresponded to daily feeding rates of 0.6% and 0.3% of the total biomass of fish, respectively.

Stomach content analyses indicated that Gulf of Lions sardines were feeding on larger prey during the period of good conditions and smaller ones during poor conditions (Brosset et al., 2016a; Le Bourg et al., 2015), so we also tested for the impact of food size. Ideally, experiments should have been conducted on a continuous range of pellets size, e.g. 100, 300, 600, 900 and 1200 µm, which was, unfortunately, infeasible for several logistic and cost reasons, so that a choice had to be made. Our goal was then to compare two contrasting food sizes within the natural range of sardine diet, which elicited two different feeding modes (i.e. filtering versus catching). In the Gulf of Lions, prey sizes exhibited a bimodal distribution: between 100 and 600 µm and around 1200 µm (Le Bourg et al., 2015). Further, prey smaller than 200 µm could represent until 88% of the numerical contribution of prey to their diet (Nikolioudakis et al., 2012). Finally, sardines are known to switch feeding behaviors depending on prey size; filter-feeding for small prey but particulate-feeding for larger prey (Garrido et al., 2007). The change in their feeding behavior was observed under 300 µm in the Mediterranean Sea (pers. comm.). According to the range of prey size in their diet (Le Bourg et al., 2015), the effects of food size were tested using two contrasting pellet sizes: 0.1

mm (actually ranging between 80 and 250 µm) and 1.2 mm (ranging between 900 and 1500 µm) to avoid any overlap in prey size distributions while ensuring a shift in feeding modes (i.e. filtering versus catching).

The combination of two food sizes (0.1 mm and 1.2 mm) and two quantities (0.3% and 0.6% of the total fish mass in tanks) resulted in four treatments with two replicas each (Figure 6): (i) small size and small quantity (0.1 mm and 0.3%, represented in red in the following – treatment 1), (ii) small size and large quantity (0.1 mm and 0.6%, in yellow – treatment 2), (iii) large size and small quantity (1.2 mm and 0.3%, in green – treatment 3), (iv) large size and large quantity (1.2 mm and 0.6%, in blue – treatment 4). Both sizes of pellet had similar proximate compositions, being 62% and 57% of proteins and 14% and 12% of lipids, for 0.1 mm and 1.2 mm pellets, respectively (lipid class contents are also given in the Supporting Information, Table S1). Sardines were fed four times a day, at the same time, to limit non-ingested food. The total biomass of each tank was estimated weekly (based on linear mass gain between monthly biometries) to re-adjust food intake to account for mass gain.

3.4. Estimation of food loss

To quantify loss of non-ingested food, we performed an additional 2-week experiment described in the Supporting Information (Figure S3).

3.5. Biochemical analyses

The physiological consequences of the diets were further investigated by measuring both lipid and protein content of dorsal epaxial muscle and blood oxidative stress status (d-ROMs and OXY).

Samplings were done at two different times: (i) on the 15th of March 2017 at the mid-experiment (15 sardines per tank) and (ii) on the 15th of June 2017 at the end of the experiment (depending on survival rate, but at least 20 sardines per treatment). Sampled sardines were selected by random draws according to normal distributions fitted on body mass within each tank and anesthetized by benzocaine at 140 ppm. Blood was collected from the caudal vein with a 26G needle into a heparinized syringe and transferred to 1.5 mL Eppendorf tubes. Blood samples were centrifuged at 3000 g for 10 min at 4°C to separate plasma, which was collected and stored at -80°C until further analyses. Sardines were then sacrificed by a lethal dose of benzocaine at 1000 ppm. Sex and maturity were determined by

gonad observation and sagittal otolith pairs were removed from their otic cavity and read to estimate age (ICES, 2011). This showed that sex, maturity and ages were comparable among tanks and treatments (Table 1). Portions of dorsal epaxial muscle were removed, frozen in liquid nitrogen and stored at -80°C for later lipid and protein content analyses.

3.5.1. Muscle energy resources

The energy status was first examined by analyzing lipid and protein content of dorsal muscle. The content of each lipid class was measured in muscle as described by Brosset et al. (2015a). Structural lipids were assumed to comprise phospholipids (PL), sterols (ST), acetone-mobile polar lipids (AMPL), and alcohols (ALC) whereas reserve lipids comprised triacylglycerols (TAG), diacylglycerols (DAG as precursors of TAG) and free fatty acids (FFA) (Lloret et al., 2014; Moltschaniwskyj and Johnston, 2006; Tocher, 2003; Zhol et al., 1995). Proportions of free fatty acids (FFA) were checked to ensure that lipids had not been degraded during sample conservation.

Portions of the same samples were lyophilized and grinded using a ball mill (MM400, Retsch GmbH, Germany). 10 mg of dry muscle powder was then immersed in 1.5 mL of a 10% SDS (Sigma Aldrich, France), 1.5% Protease inhibitor Cocktail (cOmplete, Sigma Aldrich, France), miliQ water solution (lysis solution adapted from Campus et al. (2010)) and subjected to four cycles of 15 min in a ultrasonic bath (300 Ultrasonik, Ney Company, USA) alternatively with 3 minutes vortex. Extracts were then clarified for 10 min at 3000 g at 4°C, and protein content of 25 µL of the upper liquid fraction was quantified by the BCA method (Pierce, Thermo Fisher Scientific, France). Intra- and inter-plate protein variations (based on the same sample repeated over plates) were 6.4% and 16.7% respectively.

Sample sizes associated with these tests varied depending on the amount of muscle in each tissue sample and are indicated in each figure.

3.5.2. Oxidative stress balance

Reactive oxygen metabolites (d-ROMs) and plasma antioxidant defense (OXY) were estimated in plasma using the d-ROMs test and the OXY Adsorbent test, respectively (Diacron International©, Grosseto, Italy) in accordance with Costantini and Dell'Omo (2006). Experimental protocols were modified such as (i) each well of 96-wells microplate was filled with 8 µL of serum for the d-ROMs estimation; (ii) 4 µL of serum was diluted 1:100 for OXY

estimation and (iii) samples were duplicated when possible (depending on available plasma). After incubation, absorbance was measured at 555 nm by microplate automatic reader. The oxidant ability (d-ROMs) was expressed in mg of H_2O_2 equivalent L^{-1} and the antioxidant defense capacity (OXY) in µmol HClO mL^{-1}. Intra- and inter-plate variations were 4.5 % and 3.1 % respectively for d-ROMs estimations, and 9.4% and 7.7% respectively for OXY estimations. Sample sizes associated with these tests varied due to plasma quantity and some sample loss due to hemolysis and are indicated in all figures.

3.6. Data analyses

Mixed-effect models were applied to test for the impact of the different treatments on body condition and length. As body condition index and length distributions on November 2016 were approximated by normal distributions, we built a series of linear mixed-effect models where body condition or length were dependent on two fixed effects: time (months) and food treatment, as well as their interaction. Because of variability among individuals and/or tanks within each treatment, we also introduced a random individual intercept effect (to take into account variations in individual condition/length), a random individual slope effect (to take into account variations among individual slopes in condition/length through time) and a random tank intercept effect (to take into account variations between tanks). We applied the model procedure recommended by Zuur et al. (2009) and the selection of the final model was done using the AIC criterion (Burnham and Anderson, 2002). Body composition and oxidative stress differences between treatments (for the same month) were investigated using parametric (one-way ANOVA) or non-parametric (Kruskal-Wallis) tests and associated post-hoc (Tukey or Dunn test), depending on residual normality and homoscedasticity. Finally, a Principal Component Analysis (PCA) was performed using the individual sardines as objects and terminal body condition index, total length, lipid and protein contents, d-ROMs and OXY as descriptors to summarize all the information and describe the relationships between descriptors.

All statistical analyses were performed in R (R Core Team, 2018) using the FactoMineR (Lê et al., 2008), the FSA (Ogle, 2018), the nlme (Pinheiro et al., 2018), the lsmeans (Lenth, 2016) and the factoextra (Kassambara and Mundt, 2017) packages.

4. Results

Among the 449 individuals distributed within the 8 tanks, length and weight initially varied between 95 and 151 mm and between 6.7 and 27.9 g, respectively (Figure 6; Table 1). Females were in general more abundant (56% F, 37% M and 7% unknown), while age 2 was the dominant age (Table 1).

Estimation of the non-ingested food was low to very low in all experiments; the mean (± SD) food loss was 0.3% (± 0.6%) and 1.2% (±1.1%) for the 1.2mm and 0.1mm pellets, respectively.

4.1. Body condition and total length

Treatment 4 led to the highest body condition and total length at the end of experimental design, whereas treatment 1 led to the lowest ones (Figure 8). Body condition and total length exhibited similar dynamics through time in treatments 2 and 3, and were intermediate compared to treatments 1 and 4 (Figure 8).

The best linear mixed-effect model (Figure S4) explaining both body condition and total length, as selected based on the AIC criterion, both included fixed effects (dates, treatments and their interaction) as well as the random slope and intercept effects for individuals. The random tank effect was never retained in any selection, suggesting no difference between the 2 tanks of the same treatment for body condition and length over time. The diagnostic plots of the final models were satisfactory and residuals were mostly normally distributed (see Supporting Information, Figures S5 and S6). Treatment effects were significant in the body condition model ($p \leq 0.05$) but not in the total length model (p-value > 0.05, see Supporting Information, Tables S2 and S3). Slope of body condition and total length over time exhibited significant differences between all treatments except for intermediate treatments 2 and 3. Body condition of all treatments decreased over time, except treatment 4 (large quantity of large pellets) at +0.01 $month^{-1}$. The steepest decrease occurred in treatment 1, at -0.04 $month^{-1}$ (Table S2). Sardines from treatment 4 grew at a rate of 2.5 mm $month^{-1}$, twice as fast as treatments 2 and 3 (1.2 and 1.0 mm $month^{-1}$, respectively) and 5 times faster than treatment 1 (0.5 mm $month^{-1}$) (Table S3).

For the same food quantity (in terms of mass), sardines that fed on large pellet size showed higher body condition and grew twice as much as those feeding on small pellets (Figure 8;

Tables S2 and S3). Similarly, for the same pellet size, sardines that fed on large quantity exhibited higher body condition and grew 2.5 times higher than those feeding on low quantity (Figure 8; Tables S2 and S3). Furthermore, body condition and growth of sardines that fed on large quantity of small pellets were similar to those that fed on low quantity of large pellets.

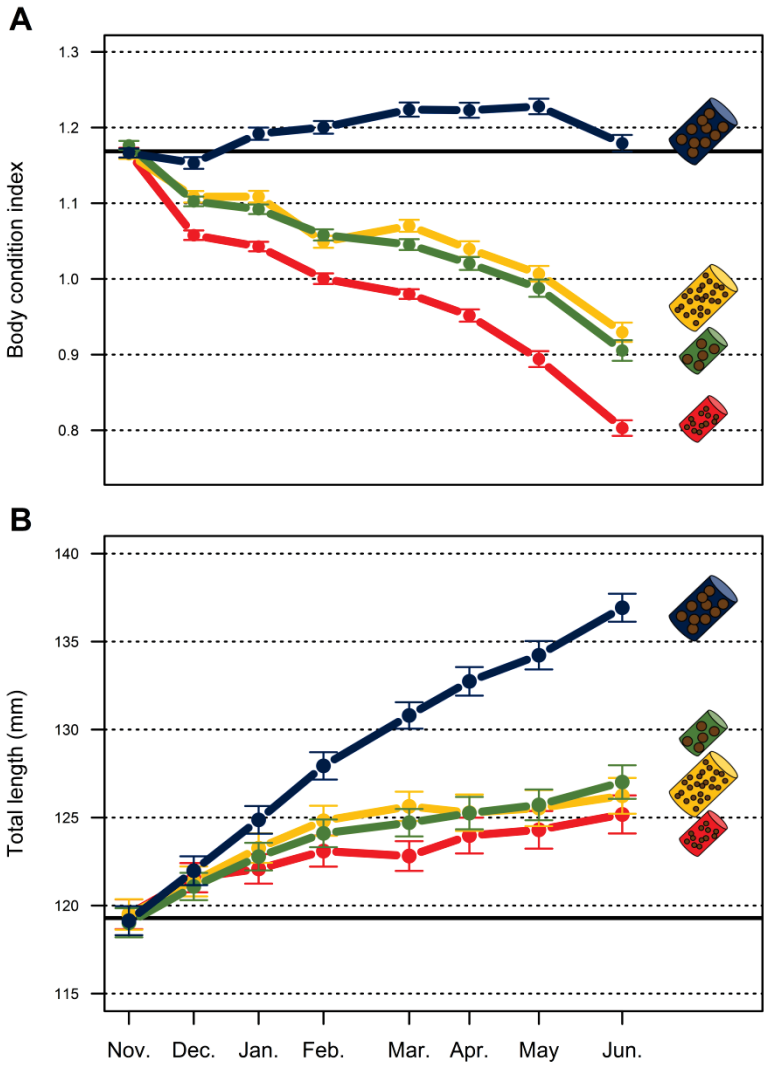

Figure 8: Time series of the mean (± se) body condition (A) and total length (B) of all sardines in each feeding treatment: red: pellet size of 0.1mm and pellet quantity of 0.3%; yellow: 0.1mm and 0.6%; green: 1.2mm and 0.3% and blue: 1.2mm and 0.6%. Black lines represent the mean body condition and total length at the beginning of the experiments.

4.2. Muscle constituents

Reserve lipids for treatment 4 were 2 to 5 times higher than those of the three other treatments, which did not differ significantly (p-value > 0.05) in both March and June (Figure 9A; see Supporting Information, Table S4). By contrast, structural lipids were similar among treatments in March and June (p-value > 0.05, Figure 9B; see Supporting Information, Table

S4), Note, however, that the medians exhibited a positive trend in March according to size and quantity of food (Figure 9B). Protein content exhibited no significant difference between treatments in either March or June (p-value > 0.05), but showed the same pattern as structural lipids in March (Figure 9C; see Supporting Information, Table S4).

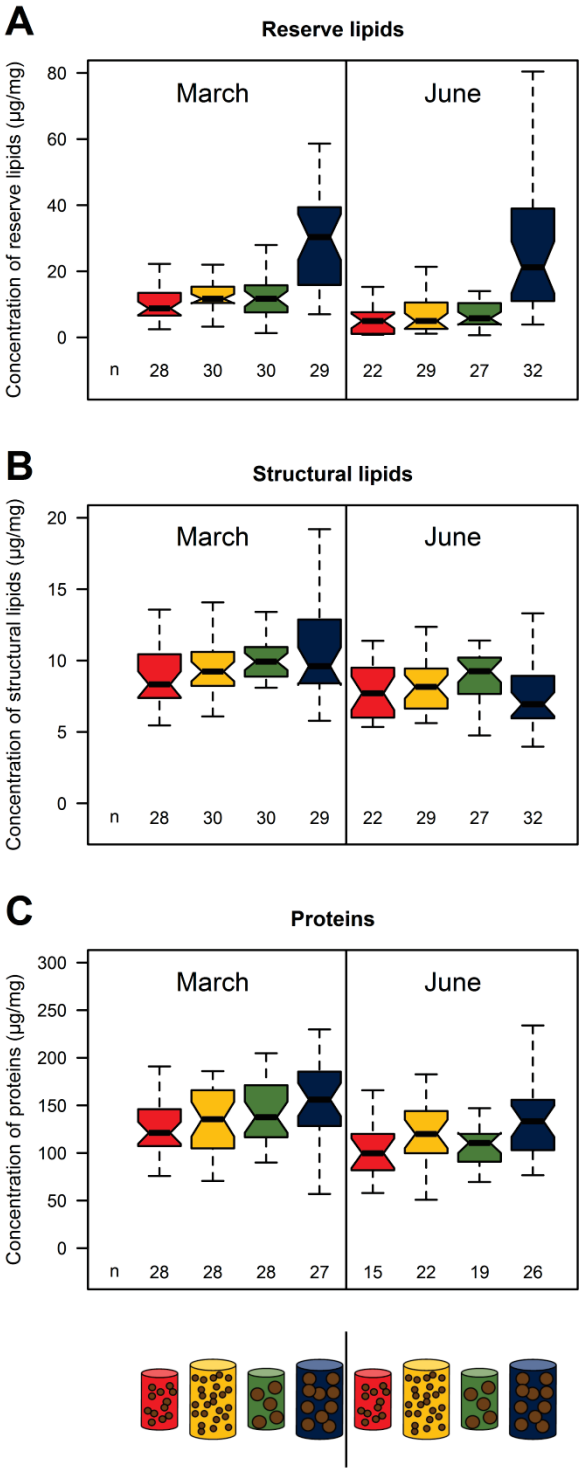

Figure 9: Reserve lipids (A), structural lipids (B), and proteins (C) of sardine muscle from the four feeding treatments (red: pellet size of 0.1mm and pellet quantity 0.3%; yellow: 0.1mm and 0.6%; green: 1.2mm and 0.3% and blue: 1.2mm and 0.6%) in March and June 2017. Concentrations are given relative to muscle wet weight. Sample size for each treatment is given by n below the boxes. Boxplots are presented without outliers for clarity purposes.

4.3. Oxidative stress balance

In March, there were no significant differences in d-ROMs and OXY among treatments (p-value > 0.05, Figure 10; see Supporting Information, Table S4). In June, there were increasing levels of d-ROMs and OXY with food size and quantity: treatment 1 exhibited the lowest values, treatments 2 and 3 intermediate ones and treatment 4 the highest (Figure 10). Only d-ROMs concentrations were significantly different between treatments 1 and 4 (medians: 20.5 and 61.5 mg of H_2O_2 equivalent L^{-1} for treatments 1 and 4, respectively) (Figure 10; see Supporting Information, Table S4).

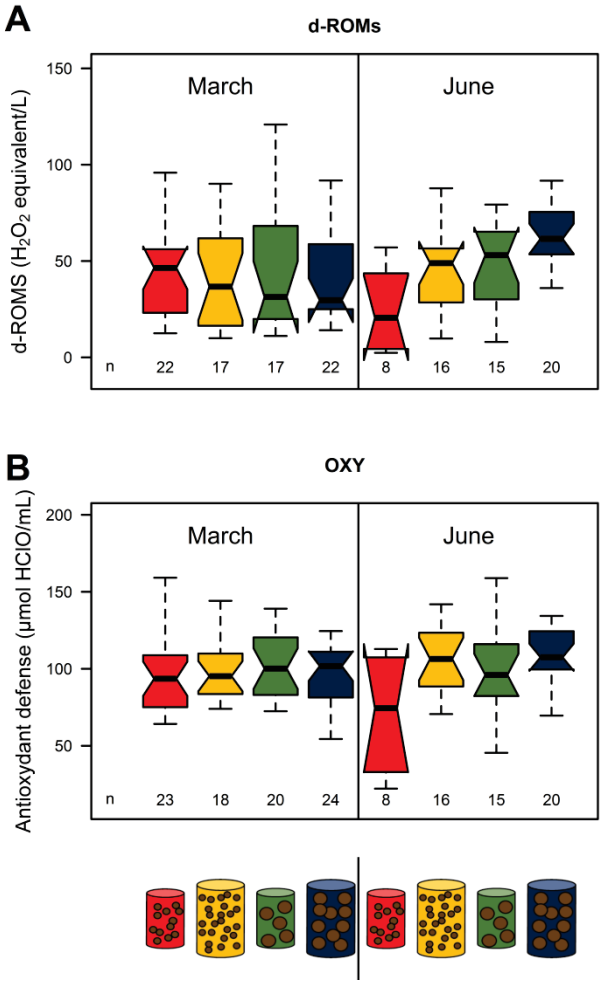

Figure 10: d-ROMs (A) and OXY (B) of the four feeding treatments (red: pellet size of 0.1mm and pellet quantity 0.3%; yellow: 0.1mm and 0.6%; green: 1.2mm and 0.3% and blue: 1.2mm and 0.6%) in March and June 2017. Sample size for each treatment is given by n below the boxes. Boxplots are presented without outliers for clarity purposes.

4.4. Variable covariation

The first 2 components of the PCA explained 48.5% of the total variance observed. The main contributing variables to the first axis were body condition, reserve lipids and total length, whereas d-ROMs and OXY were the two main contributors to the second axis. We super-imposed the treatment for each individual and the projection of the barycenters showed a clear separation between treatment 4 and the three other treatments, especially on the first axis (Figure 11). While the PCA therefore confirms all the above results, it also revealed rather marked individual variation within each treatment, especially treatment 1.

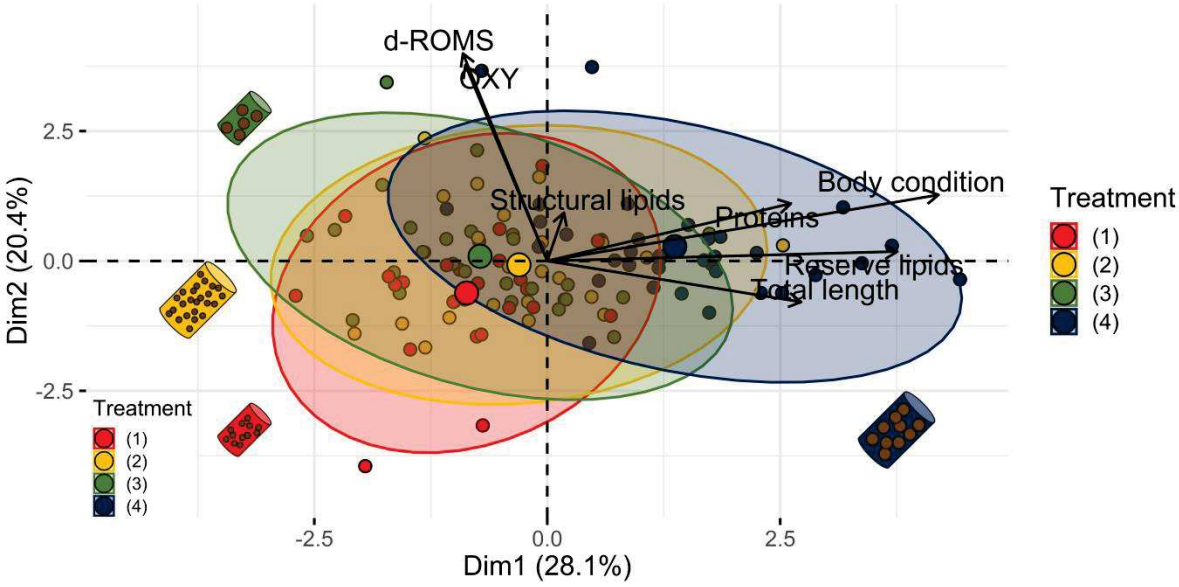

Figure 11: Biplot of the PCA built using body condition, total length, reserve and structural lipids and proteins contents, d-ROMs and OXY as explanatory variables. Ellipses correspond to 95% confidence level for each treatment. The large circles represent the barycenter of the individuals for a given treatment.

5. Discussion

Experimentation on marine fish species are often performed in a context of aquaculture and fundamental biology, but more rarely in applied and fisheries ecology. Nonetheless, controlled experimentation is one of the most useful approach for studying population dynamics (Hunt and McKinnell, 2006). Here, we developed a novel experimental approach on sardines, a species that is notoriously difficult to maintain and handle. We performed a long-lasting experiment (7 months) on a large number of adult sardines (449 individuals) to

validate a specific bottom-up control hypothesis: does food size significantly affect the growth, condition and physiological status of sardines?

5.1. Size and quantity effects

Sardines use particulate feeding on large prey but rely on filter-feeding to capture small prey (Garrido et al., 2008). Both sardine and anchovy are able to switch between the two feeding modes, but the well-developed gill raker apparatus of sardines (larger and less spaced gill rakers than anchovies (*Engraulis encrasicolus*) and denticles on their gill rakers) provides for higher filtration capacity than anchovies (Collard et al., 2017; Rykaczewski, 2009). As a result, in upwelling systems the communities of small pelagics are often dominated by sardines when prey size falls (Chavez et al., 2003; Schwartzlose et al., 1999; van der Lingen, 1994). In light of this, the reduction of planktonic prey sizes in the Gulf of Lions (Brosset et al., 2016a) was expected to be less detrimental for sardines than anchovies. Surprisingly, sardine populations are in a more alarming situation than anchovies, with more striking declines in body condition, growth and adult survival (see Van Beveren et al. (2014)). Our experimental results confirmed the detrimental effect of small prey for sardines of the Mediterranean Sea. To achieve the same growth and body condition, sardines fed on small pellets had to eat twice as much as those feeding on large pellets. Additionally, sardines had to eat large quantity of large pellets in order to store reserve lipids. Knowing that the amount of food ingested was similar (i.e. there was no loss of food in the experiments), how could size have such a marked effect on growth, condition and reserve lipids? It is possible that food is assimilated differently and sardines gain less energy from the smaller particles. Retention efficiency of Atlantic sardines would be estimated at around 70% for 0.1 mm pellets in the filtration mode (using equation in Garrido et al. (2007)) against 100% for large pellets in particulate mode. This could be even worse in Mediterranean sardines, which appear to have lower numbers and densities of gill rakers (for a given body length) than their Atlantic relatives (Costalago et al., 2015) suggesting they might be less adapted to catch small prey. However, an ongoing study of the density of the gill rakers indicated that the filtering efficiency of sardines in the Gulf of Lions may actually be higher than those of the Atlantic sardines nowadays owing to higher gill raker density (unpublished data). Thus, current Mediterranean sardines are likely to catch small prey such as 100 µm prey, even if they correspond to the lower range of sardine prey sizes. It is also possible that they derive the

same energy but spend more, because the filter-feeding mode has high energetic costs. This would have to be investigated directly but information from the literature and our own observations provide support for some speculation. In this study, the two food sizes elicited a marked and visible change in sardine feeding behavior, from particulate feeding (1.2mm) to filter feeding (0.1 mm). Feeding duration seemed to be about 10 times longer for filtration than particulate feeding (up to 20 min for filtration against 2 min for particulate). The energy requirements of sustained aerobic swimming during filter-feeding might be significantly greater than those of rapid bouts of swimming to capture particles (Costalago and Palomera, 2014). Metabolic costs of the filter-feeding mode were higher than those of particulate feeding for the Cape anchovy *Engraulis capensis* (James and Probyn, 1989), but the reverse was observed for the pilchard *Sardinops sagax* (van der Lingen, 1995). In the absence of coherent conclusions on other small pelagic species, we can only hypothesize for Mediterranean sardines that filter feeding may provide lower net gain (so constraining energy allocation to growth and reserves, as was observed in the present study). Nonetheless, this hypothesis remains to be validated by oxygen consumption experiments.

An alternative hypothesis to explain changes in body condition and growth is associated to different energetic and nutritional needs depending on maturity processes and/or engagement in gametogenesis. However, 93% of the fish in our experiment were mature discarding maturity processes as an explanation. Further, our experiment lasted 7 months encompassing both the reproduction period and the period following reproduction and the differences in growth and condition between treatments were observed and of similar importance in both periods, indicating that our results were not biased by potential differences in nutritional needs due to maturity or reproduction.

Structural lipids, proteins and the blood oxidative balance (d-ROMs and OXY) were less or not affected by the feeding treatments compared to body condition, growth and reserve lipids. The fact that structural lipid and protein contents remained steady across treatments suggests that diet provided enough energy for individuals to maintain the basal energetic costs of their structure. The increase in d-ROM levels for treatments 2, 3 and 4 between March and June suggests that individuals might pay short-term costs when displaying higher growth rates (Monaghan et al., 2009). Similar patterns have been shown in species facing dietary restriction during early development and displaying important compensatory growth later in life when food abundance was retrieved (Metcalfe and Monaghan, 2001). OXY levels

followed similar trends to a lesser extent suggesting sardines from treatment 4 were not completely able to buffer d-ROM increase leading to oxidative stress (imbalance towards d-ROMs). On the other hand, treatment 1 displayed lower level of both d-ROMs and OXY, suggesting that sardines might cope with 'poor feeding conditions' by lowering their metabolic rates to limit their living costs (O'Connor et al., 2000). Furthermore, starvation is suggested to have negative consequences on immune functions, although, such effects seem to differ between species; starvation has no or positive effect on immune responses in pacu (Gimbo et al., 2015), European eel (Caruso et al., 2010) and Chinese Sturgeon (Feng et al., 2011) but decreases the ability to cope with infection in Atlantic salmon (Martin et al., 2010) and Jade perch (Luo et al., 2013). Whether such relationship is found in sardines facing 'poor feeding condition' remains to be explored, but it raises concern on the possible additive effect of long-term fasting on individual survival through immune functions impairment.

Sardines are known to be primarily capital breeders (Ganias, 2009; McBride et al., 2015); they store energy during summer and use reserves to invest in breeding during winter. The fact that energetic reserves were so impacted by both food size and quantity may be a cause of significant mortality of the oldest individuals after reproduction: a low reserve storage at the beginning of the reproductive period would impose a trade-off between survival and reproduction, in favor for the latter in oldest sardines (Brosset et al., 2015b; Saraux et al., 2019). Furthermore, seasonal fluctuations of plankton (Costalago et al., 2012; Nikolioudakis et al., 2012) could act synergistically to these declines and punctually reduce even more sardine condition as observed during the winter reproduction period in the Gulf of Lions (Brosset et al., 2015b). Such a difference in reproductive seasonality with income breeder anchovies might help explaining why sardine condition and size have decreased even more than anchovy's.

Finally, the PCA while confirming previous results with treatment 4 easily distinguishable from all others also showed a high variability within each treatment. Such interindividual variability might result from differences in coping abilities under adverse conditions or from competition. For a given quantity of food, the number of pellets was much lower when using large pellets than small ones, thus generating a potential source of competition. As such, it is interesting to note that particulate-feeding on 1.2 mm pellets did not trigger higher competition as the CV was minimal for fish fed of large pellets in large quantities (treatment 4 – blue) both in terms of body condition and length and fish fed with large pellets in small

quantities (treatment 3 – green) displayed a lower CV than fish fed on small particles for length and a similar one for body condition (see Figure S7). Rather, the increase in length or condition variability seemed linked to an increase in interindividual differences when facing adverse conditions. The PCA first two components were less affected by sex or age (Figures S8 and S9). Furthermore, the inter-individual variability observed in March in all physiological variables was similar to the one observed in June, when fish were out of the reproduction season and their maturity levels all equal to 1 (e.g. in Figures 9 and 10), suggesting that their reproductive status was not the source of interindividual variability. Interindividual variability might thus translate differences in other endogenous variables, often viewed as "individual quality" despite the ambiguity of this term (Wilson and Nussey, 2010).

5.2. Amplifying factors and their consequences

Although we have demonstrated that smaller prey size (as of 0.1mm) can act as a key factor in limiting sardine growth, this raises the question of why planktonic prey sizes are falling in the Gulf of Lions. The reduction in prey size might result from a change in community, with implications that extend beyond size to include nutritional quality (Barroeta et al., 2017; Zarubin et al., 2014). In the present study, the composition of both sizes of aquaculture pellets was similar but a reduction in energetic value of smaller prey would presumably exacerbate the effects we have revealed. Oceanographic modifications, induced by global warming, coupled with pollution, are two potential candidates that may explain reduced prey sizes for sardines. Up to 77% of total marine pollution is from terrestrial sources (Williams, 1996) and contaminants such as pesticides, fertilizers, heavy metals and synthetic organic chemicals are all known to affect plankton communities, reducing their diversity and causing irreversible changes in marine ecosystems (Pérez et al., 2011; Shahidul Islam and Tanaka, 2004; Smith et al., 2018). For example, the insecticide Malathion and the herbicide Roundup® significantly decreased copepod abundance, but favored smaller and lower energetic zooplankton (Geyer et al., 2016; Smith et al., 2018). Even in low concentrations, mixture of pesticides had similar effects (Relyea, 2009). Global warming enhances stratification of the water column in the Mediterranean (Coma et al., 2009), which seems to favor population dynamics of small phytoplankton and zooplankton (van der Lingen et al., 2006). The combination of pollution and global change might, therefore, result in a long-

lasting domination of small plankton in the Mediterranean Sea, which could be extremely detrimental to sardine populations in the Gulf of Lions, in terms of fish growth and population demography. More research and more high-resolution observations on the plankton compartment of the Gulf of Lions are needed to assess potential changes in species composition that have affected energy flows through the pelagic community. If this is confirmed, then oceanographic, atmospheric, hydrological parameters and pollutant concentration should be further analyzed to identify the origins of this potential ecosystem regime shift.

Finally, the consequences of such a change in small pelagic populations might have further important repercussions for fisheries and entire ecosystem function. As stated earlier, there has been a major decline in sardine landings in the French Mediterranean fisheries. Fisheries have therefore turned towards other stocks, such as the European hake (*Merluccius merluccius*), which has been overexploited for decades (GFCM, 2017a). Top predators such as large tunas, sharks, marine mammals and birds could suffer from a potential long-term collapse of small pelagic populations (Shannon et al., 2009). In summary, long-term changes in the marine pelagic ecosystem of the Gulf of Lions may lead to critical economic, societal and ecological situations. Sardines may display plasticity or adaptation to cope with smaller prey but this remains to be investigated.

Experimentation on small pelagics is rarely exploited to study ecosystem dynamics, but essential information on trophic controls can be derived from such studies. Our study provides evidence that food size matters as much as food quantity for sardines, which might have repercussions on the entire ecosystem. As increasing temperature favors planktonic chains of smaller size (van der Lingen et al., 2006), climate change might actually accelerate and amplify such phenomenon and thus strongly affect fisheries worldwide.

To sum up the Chapter 1, the take-home messages are:

- Experimentations on sardines remain rarely used whereas they offer the unique opportunity to integrate behavior and ecophysiology in understanding key demographic processes such as trophic controls.

- Food size is as important as food quantity for body condition, growth and reserve lipids whereas structural lipids, proteins and the blood oxidative balance were less or not impacted by the feeding treatments.
- Such results may reflect a different energy cost of two feeding behaviors, i.e. filter-feeding vs. particulate-feeding.

Chapter 2: Which mechanisms could explain the effects of food size on sardine growth and body condition?

1. Introduction

In large upwelling ecosystems, sardines and anchovies usually fluctuate asynchronously (Alheit et al., 2009; Schwartzlose et al., 1999). These out-of-phase fluctuations seem to be linked with a shift in the plankton size, sardines dominating when plankton becomes smaller (van der Lingen et al., 2006). Indeed, sardines have a well-developed gill raker apparatus and they are more efficient to catch smaller prey than anchovy (Collard et al., 2017). Surprisingly, we observed a reversed situation in the Gulf of Lions, where sardines seemed to be more affected than anchovies by a potential reduction of prey size (Van Beveren et al., 2014). Moreover, in the previous experiment (Chapter 1), we showed that with a similar amount of ingested food (i.e. no food loss), sardines fed on small pellets exhibited significant lower growth and body condition index than sardines fed on larger ones. Two possible non-exclusive explanations come to mind, (1) either food is collected differently leading to a lesser energy gain from the smallest particles and/or (2) sardines derive the same energy but spend more to acquire it (i.e. filter-feeding leads to extra energy expenditures compared to particulate-feeding).

To investigate the first hypothesis, we decided to focus our study on the structure of the feeding apparatus of sardines. Mediterranean sardines are known to feed on a large range of prey sizes from small phytoplanktonic prey (~1-100 µm) up to large copepods (> 2 mm) (Le Bourg et al., 2015; Nikolioudakis et al., 2012). Sardines are able to easily switch between filter- and particulate-feeding strategies according to prey size: they filter-feed on small prey and adopt particulate feeding on the larger ones (Garrido et al., 2007). Nonetheless, the sardine efficiency to retain small prey seems to be lower for the Mediterranean sardines as their gill raker density was lower than their Atlantic relatives and these dissimilarities might be explained by differences in plankton productivity between the two areas (Costalago et al., 2015). Thus, this result might explain why sardines may suffer from small prey condition in the Gulf of Lions. During preliminary trials (prior to this thesis), several pellet sizes (0.1, 0.3, 0.8 mm) have been tested to find the size threshold of the switch between the two feeding

modes. This threshold seemed to be around 300 µm for the Mediterranean sardines compared to 700 µm for their Atlantic relatives (Garrido et al., 2007). The feeding apparatus of filter-feeders is divided in two parts: (i) the respiratory system composed by the gill filaments located at the posterior part of the gill arch and (2) the feeding structure including a series of gill rakers, each one covered by a succession of small denticles (or branchiospinules) which improve the retention capacity of sardines (Rykaczewski, 2009).

The second hypothesis was based on the energy spent by the fish while feeding. First of all, sardines seemed to take more time when they filter-fed small particles than when they particulate-fed larger ones (pers. comm.). Also, energy requirements of aerobic swimming during the filtration may be higher than those required for series of bursts of acceleration (Costalago and Palomera, 2014), although this prediction remains debatable as metabolic costs of both feeding strategies seemed to vary according to small pelagic species.: Filter-feeding mode exhibited higher energy requirements than those of particulate feeding for the Cape anchovy *Engraulis capensis* (James and Probyn, 1989), but the reverse was observed for the pilchard *Sardinops sagax* (van der Lingen, 1995). The main explanation of these differences could be the smaller size of Cape anchovy compared to pilchards (van der Lingen, 1995).The hypothesis of higher energy costs of filter feeding for Mediterranean sardines thus remained to be tested for. Metabolic costs are usually measured by respirometry, as rates of oxygen uptake (Killen et al., 2011; Steffensen, 1989).

To test for the two previous hypotheses, we used an experimental approach on wild sardines maintained in captivity. To investigate how sardines could cope with small prey size, we studied the effects of different food sizes and quantities on several parameters involved in the particle catch such as the gill raker density or the denticles. Finally, we studied the oxygen consumption of sardines fed with different food sizes recorded all day long to estimate the energy requirements of both feeding behaviors.

2. Material and Methods

2.1. Gill apparatus

2.1.1. Gill sampling

On the 15th of June 2017, at the end of the previous experiment (Chapter 1), 10 sardines were randomly sampled and sacrificed within each of the four treatments (Figure 7, Experiment n°1). Their gills were removed and stored in the Bouin's solution (cell fixative). After fixation, samples were washed and stored in 70% alcohol. The most external branchial arc was removed from each sardine (on the left or right side depending on its state). Each branchial arc was photographed using binocular microscope (STEMI 305, Zeiss). The length of the gill arch and the length of the gill rakers (located on the ceratobranchial part, Figure 12) were estimated using the software ImageJ (Schneider et al., 2012). Also, the density of the gill rakers was estimated on the ceratobranchial part of the branchial arc and their abundance equaled the gill raker density multiplied by the branchial arc length. Further, preliminary investigations of the series of denticles found along the gill rakers were performed on 6 and 8 individuals from the two extreme treatments (treatments 1 (small quantity of small particles) and 4 (large quantity of large particles), respectively (Figure 13). To do so, the branchial arcs were dehydrated using successive bath at 70%, 90%, 95% and 100% alcohol. Then, samples were dried by a solution of hexamethyldisilazane and were metalized with platinum. The density of the denticles was estimated on the photographs taken by a Scanning Electron Microscopy (SEM) under conventional mode and also using ImageJ software (Schneider et al., 2012).

2.1.2. Data analysis

To study feeding treatment and potential allometric effects on the gill parameters (i.e. branchial arc length, gill raker density), we performed a covariance analysis (ANCOVA) - with a preliminary check on the homogeneity of variances with the Levene's test - where each gill parameter was dependent on treatment and fish length, as well as their interaction. Then, we investigated feeding treatment and allometric effects on the gill raker abundance thanks to a generalized linear model, using Poisson distribution. Further, we built a series of linear mixed effect models for the gill raker length with two fixed effects (fish length and feeding

treatments), as well as their interaction. We added the individual id as a random intercept to take into account the variability of the gill raker lengths among individuals. Finally, we also built a mixed-effect model to study the treatment effect on the denticles, with the treatment as a fixed variable and the individual as random intercept. The selection of the mixed-effect models was done using the AIC criterion (Burnham and Anderson, 2002). The normality of the residuals of each model was then analyzed through a Shapiro-Wilk's test. All data analyses were performed in R (R Core Team, 2018), using the car (Fox and Weisberg, 2018) and the nlme (Pinheiro et al., 2018) packages.

Figure 12: Picture of the first branchial arch of an adult sardine with the branchial arch (orange line) and one gill raker (blue line).

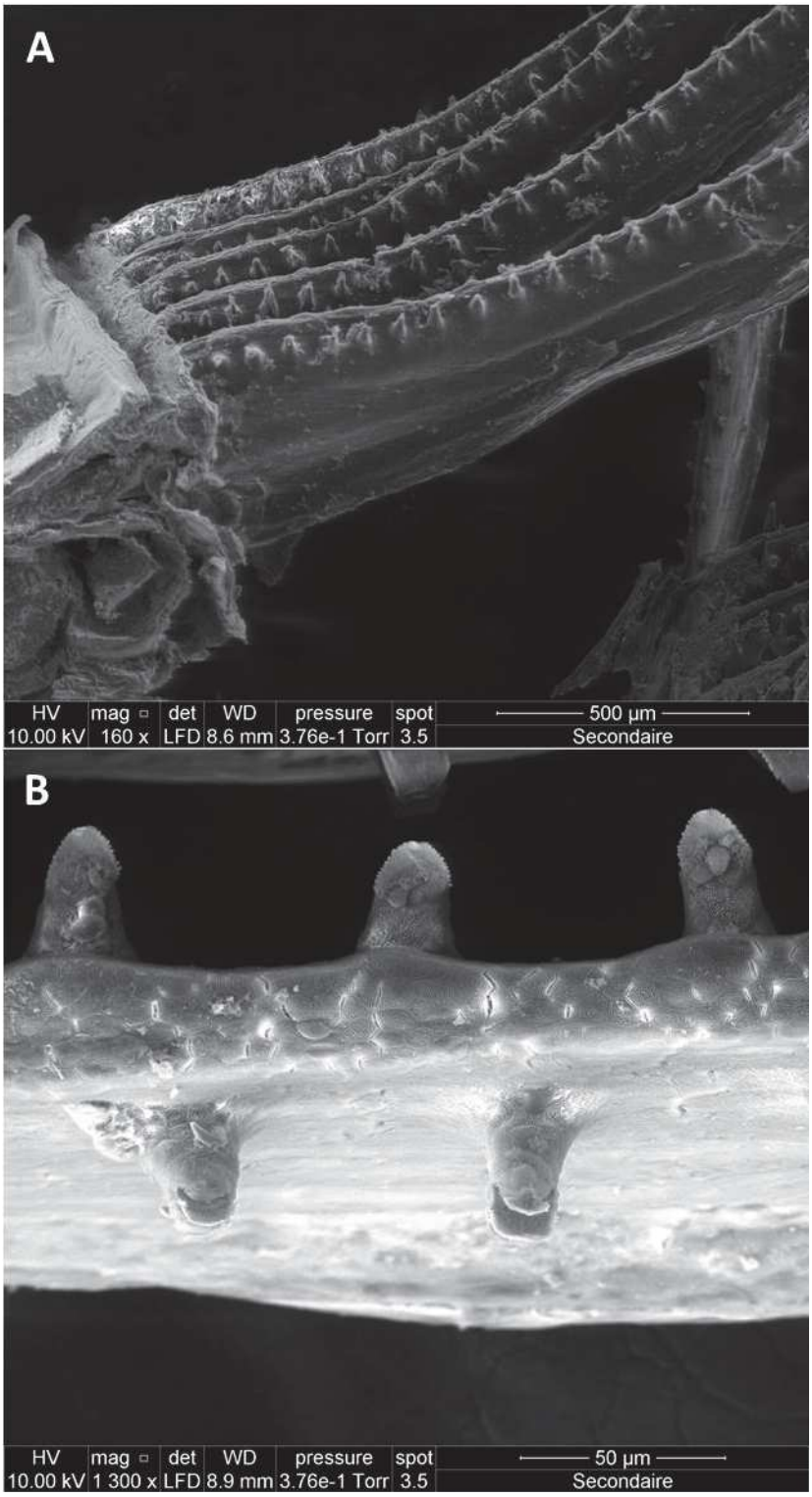

Figure 13: Pictures of the gill raker structure (A) and two series of denticles on the same gill raker (B).

2.2. Energetic costs

The preliminary investigations of sardine energetic costs of feeding were made on a captive sardine population fished on March 2016 (Figure 7, Experiment n°2). Sardines were fished and acclimated following the same procedure described in Queiros et al. (2019). Sardines were then held in a 3 m^3 tank supplied with a flow of aerated seawater, following natural temperature and photoperiod. Before the experiment, sardines were fed with a mix of pellet sizes (0.1, 0.3 and 0.8 mm) at a rate equaled to 2% of the total fish biomass each day, so they were acclimated to both small and large pellets (i.e. filtration vs. particulate feeding).

To estimate the effects of food size on sardine oxygen consumption while feeding, sardines were fed twice a day (at 2% of the total fish biomass) with 0.1 mm pellets during the first week (between September 11 and September 16) and with 1.2 mm pellets during the second week (between September 17 and September 23). Note that the adaptation from 0.1 to 1.2 mm pellets was almost immediate. The oxygen consumption was monitored from 7:30 to 17:30 during 2 consecutive days for each pellet size: on September 13 and 14 and on September 20 and 21 for 0.1 and 1.2 mm pellets, respectively. Results were compared to the basal oxygen consumption of unfed sardines measured on September 26. Every 30 minutes, oxygen concentration, oxygen saturation, temperature and salinity of outflows of two tanks were recorded (i.e. the experiment tank with sardines and the control ones without sardine). The oxygen consumption (in mg/h/kg) was estimated by comparison with a tank without sardines:

$$Consumption\ O_2 = \frac{[O_2]_{control} - [O_2]_{sardines}}{Biomass} \times Q \qquad [3]$$

with $[O_2]$ in mg/L, the water flow rate Q in m^3/h, and the total fish biomass in kg.

Temperature is known to regulate (positively or negatively) the metabolism of fish and thus their oxygen consumption. To take into account temperature differences between the experiments (mean ± SD: 19.1 ± 0.4, 22.0 ± 0.2 and 18.9 ± 0.3 for not fed fish, fish fed on 0.1 mm and fish fed on 1.2 mm, respectively), we corrected oxygen consumption (cO_2) to obtain the same mean oxygen consumption during the night for the 3 feeding conditions (i.e. large pellets, small pellets and unfed).

The corrected oxygen consumption of each feeding condition was compared by the area under the curve using the DescTools package in R (Andri Signorell and mult. al., 2019). This study was a preliminary investigation and another experiment was later designed to strengthen this experiment. However, some problems were encountered in this second experiment (more details in the General Discussion) and we present here only results of the preliminary investigation.

3. Results and Discussion

3.1. Is food collected differently while filter-feeding, leading to a lesser energy gain from the smallest particles?

The total length of fish varied from 113 to 154 mm and individuals from treatment (4) were longer than all other treatments as previously described in Chapter 1. Among the 40 samples, 1 outlier from treatment (1) was removed from all the following analyses (Figure 14).

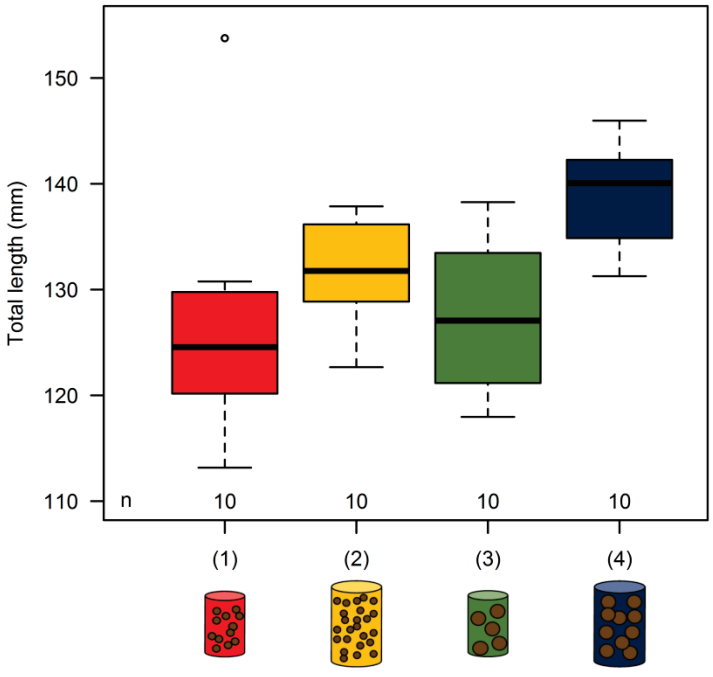

Figure 14: Boxplot of the sardine total length used in the morphological analysis of the gill apparatus for the four feeding treatments (red: pellet size of 0.1 mm and pellet quantity 0.3%; yellow: 0.1 mm and 0.6%; green: 1.2 mm and 0.3% and blue: 1.2 mm and 0.6%). Sample size for each treatment is given by n below the boxes. Outlier was represented by a circle.

The length of branchial arc ranged from 18.2 mm for the smallest individual analysed to 23.2 mm for the largest one (Figure 15A). A linear model analysis testing for the effect of fish length, feeding treatment and their interaction on the branchial arc length revealed no significant effect of food or the interaction between food and fish length (p-value = 0.16 and p-value = 0.08, respectively), but a positive effect of fish length (p-value < 0.001). The equation of the fitted curve was given by:

$$\mathrm{L_{branchial\ arc}} = 9.2\ (\pm\ 3.2) + 0.09\ (\pm\ 0.02) \times \mathrm{TL} \quad [4]$$

with the branchial arc ($L_{branchial\ arc}$) and fish total length (TL) in mm. The standard error of the parameter estimations were given in brackets.

Further, the gill raker density varied between 3.6 and 5.0 gill rakers per mm (Figure 15B). The gill raker density was negatively correlated with fish total length (p-value < 0.001) but no correlation was obtained for the two other variables (p-value = 0.12 and p-value = 0.45 for the treatment and the interaction treatment-fish length, respectively). The equation of the fitted model was given by:

$$d_{gill\ raker} = 7.1\ (\pm\ 0.6) - 0.02\ (\pm\ 0.00) \times \mathrm{TL} \quad [5]$$

with the gill raker density ($d_{gill\ raker}$) in abundance per mm, the fish total length (TL) in mm and the standard error of the parameter estimations were given in brackets.

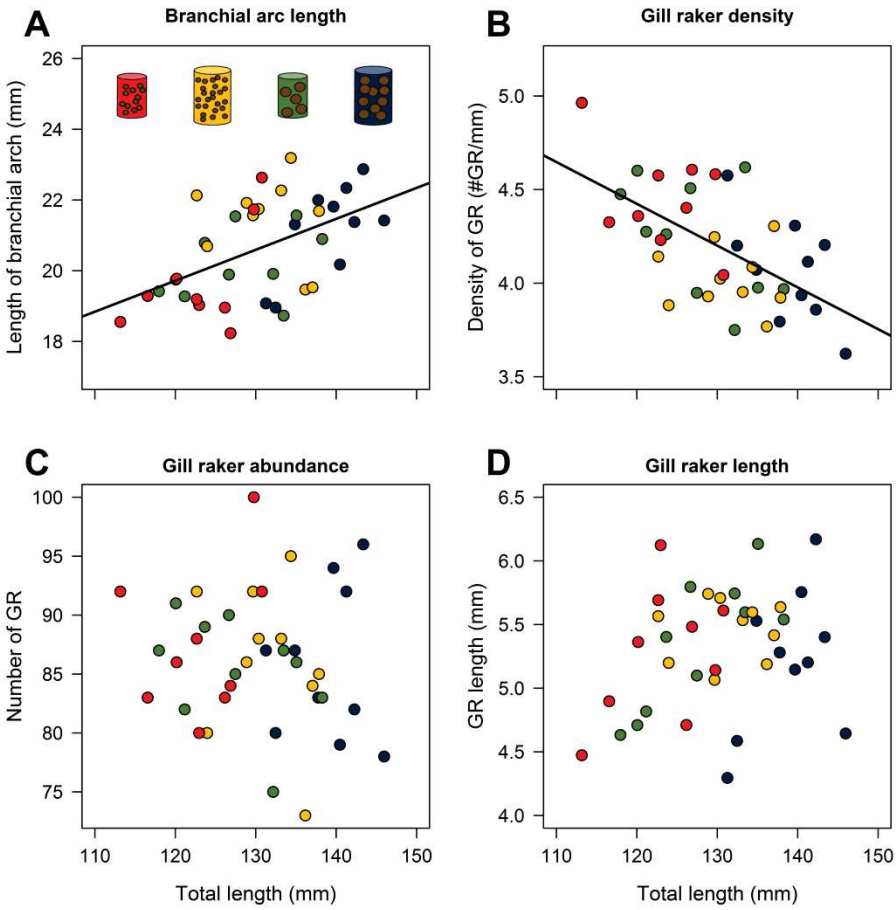

Figure 15: Relationships between the total length of sardine and the branchial arc length (A), the gill raker density (B), abundance (C) and length (D). Colors corresponded to each feeding treatment (red: pellet size of 0.1 mm and pellet quantity of 0.3%; yellow: 0.1 mm and 0.6%; green: 1.2 mm and 0.3% and blue: 1.2 mm and 0.6%). Black lines represented significant relationship (p-value < 0.05) between the morphological structure and the total length of sardine.

Further, similar ranges of the branchial arc length were found between this study and the results obtained on the Atlantic and the Mediterranean sardines (Costalago et al., 2015). The length of the branchial arch and the gill raker density increased and decreased respectively, according to the fish length similarly to their Atlantic and Pacific relatives and to a study made in 2007-2009 in the Gulf of Lions (Costalago et al., 2015; van der Lingen et al., 2009). The gill raker density of sardines from the Gulf of Lions seemed to have changed in almost 10 years. To compare the gill raker density from the study made in 2007-2009 in the Gulf of Lions and our study, we built two linear models with the gill raker density as explicated variable and both fish length, the study period (before vs. now) and their interaction as explicative variables. The comparison of these models made by a covariation analysis revealed that there was no significant difference between the slopes (p-value = 0.89) but a significant difference of their intercepts (p-value < 0.001). This result suggested that for a

given length, the density was higher, and thus the capacity of sardines to retain small prey may have improved during this period (Figure 16).

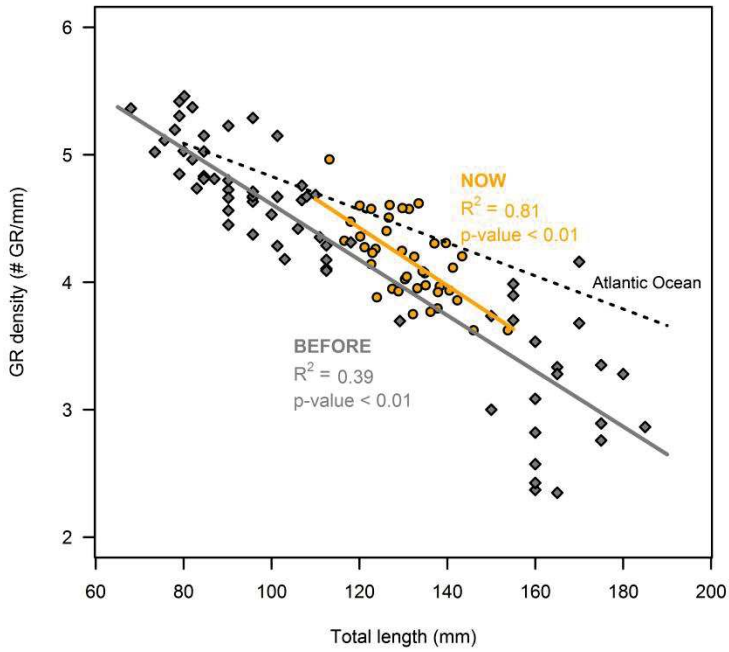

Figure 16: Comparison of the relationship between the gill raker density and the total length for sardines in the Gulf of Lions between 2007-2009 (Costalago et al. (2015): grey solid line) and now (orange solid line). The relationship between the gill raker density and the total length for sardines in the Atlantic Ocean is represented by a black dashed line (Costalago et al., 2015)**. The r-squared ajusted and the p-value of the linear models are given.**

How could morphometric modification of the gill raker density between the two periods be explained? Three distinct but not mutually exclusive hypotheses come to mind, either (1) phenotypic plasticity in response to their environment and/or (2) microevolution due to selection by their environment and/or (3) epigenetic mechanisms. Phenotypic plasticity could be defined as the ability of an individual to exhibit different phenotypes from the same genotype in response to different environmental conditions (DeWitt et al., 1998; Gienapp et al., 2007; Nussey et al., 2007). Microevolution refers to processes implied in the phenotypic diversification within populations for a given species (e.g. mutations, natural selection and genetic drift) (Arnold et al., 2001; Gienapp et al., 2008; Visser, 2008). Epigenetic events could be defined as heritable changes in gene expression that result from a reversible modification of the DNA (e.g. methilation or remodelling chromatin structure) without modification in the DNA sequence which could be induced by environmental factors (Bird, 2007; Bossdorf et al., 2007; Jaenisch and Bird, 2003; Jirtle and Skinner, 2007). In this study, the exposition of adult sardines to 4 feeding treatments (2 sizes and 2 quantities) during 7-months did not influence

the gill raker density of adult sardines. Indeed, the treatment variable was not retained in the linear model built on the gill raker density (note that the treatment was never retained in any of the models), suggesting that this phenomenon could not result from phenotypic plasticity of adult sardines to their environment over a few months. Instead, it could be due to either longer-term plasticity (maybe several months/years) or be induced earlier in life, when gill rakers are in development and become functional after metamorphosis (Rykaczewski, 2009). Moreover, natural selection might also be an explanation of the higher gill raker density of sardine in this study. Indeed, individuals with a higher gill raker density may have a better energy net gain compared to indivudals exhibiting lower density, especially in a context of a decrease in food size in the wild (as suggested to occur in the Gulf of Lions).

Additionally, the gill raker abundance ranged between 73 and 100 gill rakers (Figure 15C). Although comparison with the gill raker abundance found in previous study led on the Atlantic and Mediterranean sardines was not possible because they estimated the gill raker abundances only on the ceratohypobranchial arch, our results seemed to be consistent (ranged between 50 to 70 gill rakers in Costalago et al. (2015)). The analysis of the generalized linear model (Poisson distribution) on this abundance exhibited no significant effect of feeding treatments (p-value = 0.73), fish length (p-value = 0.97) and their interaction (p-value = 0.42). Thus, fish length did not affect the gill raker abundance. These results were consistent with results found on three anchovy species from the southeastern and northeastern Atlantic and from Pacific for the same range of fish length (Rykaczewski, 2009; van der Lingen et al., 2009). However, they went against significant positive relationships found between the gill raker abundance and the fish length for sardines from large upwelling ecosystems (Rykaczewski, 2009; van der Lingen et al., 2009) and from a previous period in the Mediterannean Sea (Costalago et al., 2015). The lack of significant relationship might be explained by the reduced range of fish length used in our study (between 110 and 150 mm) compared to the 50-200 mm range used in other studies. Additionally, the gill raker length was comprised between 4.0 and 6.8 mm. The best linear mixed-effect model explaining the gill raker length was the null model (based on the AIC criterion), suggesting that neither feeding treatment nor fish length nor their interaction have significant effect on the gill raker length (Figure 15D). Conversely to both South West African sardine and anchovy for which the fish length act significantly on the gill raker

abundance, no fish length effect has been found in our study (van der Lingen et al., 2006). Finally, the denticle density on the gill raker varied from 10.9 to 16.7 denticles per mm of gill raker (Figure 17). The best mixed-effect model selected by AIC criterion was the null model, meaning that no treatment effect on the denticles density was observed. The diagnostic plots of the linear models (branchial arc length and gill raker density), general linear models with Poisson distribution (gill raker abundance) and mixed-effect models (gill raker length and denticle density) were mostly satisfactory and residuals were normally distributed (Figures S11).

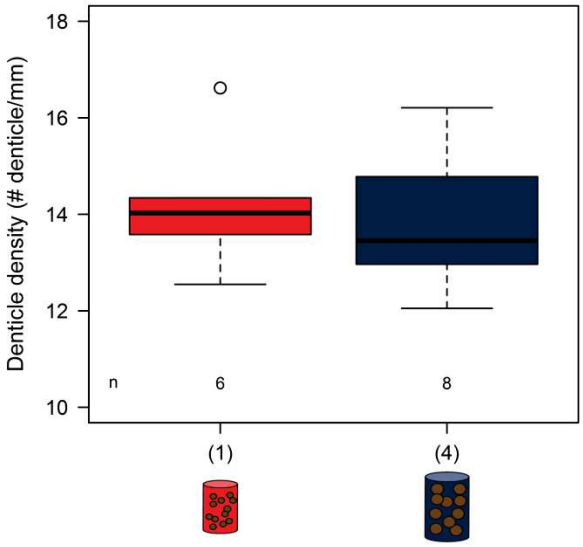

Figure 17: Boxplot of the denticle density (median of each individual) for the treatments (1) (in red: pellet size of 0.1 mm and pellet quantity 0.3%) and (4) (blue: 1.2 mm and 0.6%). Sample size for each treatment is given by n below the boxes.

3.2. Is energy expenditure higher when filter-feeding than particulate feeding?

Thanks to an increase of their gill raker density, sardines may be able to extract more energy from their environment at the moment than they used to 10 years ago. However, the situation of sardines in the wild did not change with time suggesting that this modification might not be enough to cope with the decrease in food size. Thus, could filtration be an energy-consuming feeding mode compared to particulate feeding? To test for this hypothesis, metabolic costs were measured by respirometry using the oxygen consumption on the same fish with only 1 week between the two measurements (length and condition were thus similar between experiments). First, the oxygen consumption exhibited similar fluctuations along the day within each treatment (Figure 18). The two feeding periods were

followed by a significant increase of the oxygen consumption in tanks. This upsurge looked higher for sardines fed with 0.1 mm pellets than for sardines fed with 1.2 mm pellets (Figure 18). Also, with the same amount of food (i.e. 2% of the total fish biomass), the daily oxygen consumption – presented as the ratio of the areas under curve of fed fish and not fed fish – exhibited a mean (± SD) increase of 67.4 (± 0.1) and 89.8 (± 8.4) % for the 1.2 mm (in blue) and 0.1 mm pellets (in red) respectively, compared to sardines not fed (in grey) (Figure 18). Although the food quantity was the same, sardines spent more energy to feed on small particles, resulting in a lower energy net gain. As suggested by Costalago and Palomera (2014), the energy requirements of sustained aerobic swimming during filter-feeding seemed to be significantly greater than particulate-feeding. This phenomenon might act synergistically with the potential longer duration of the filtration compared to particulate feeding but this hypothesis remained to be investigated (see Chapter Discussion). Conversely to the pilchard *Sardinops sagax* for which the filtration required less energy (van der Lingen, 1995), the hypothesis for Mediterranean sardines that filter feeding may provide lower energy net gain seems to be validated by the oxygen consumption experiments. However, oxygen concentration was estimated here in outflowing water which allowed only average estimation of the oxygen consumption owing to delay between the consumption and its estimation and supposing that water was well homogenized. Further, we could not rule out that the difference obtained between treatments was related to the difference in temperature. Another experiment was proposed in the General Discussion to strengthen this preliminary result.

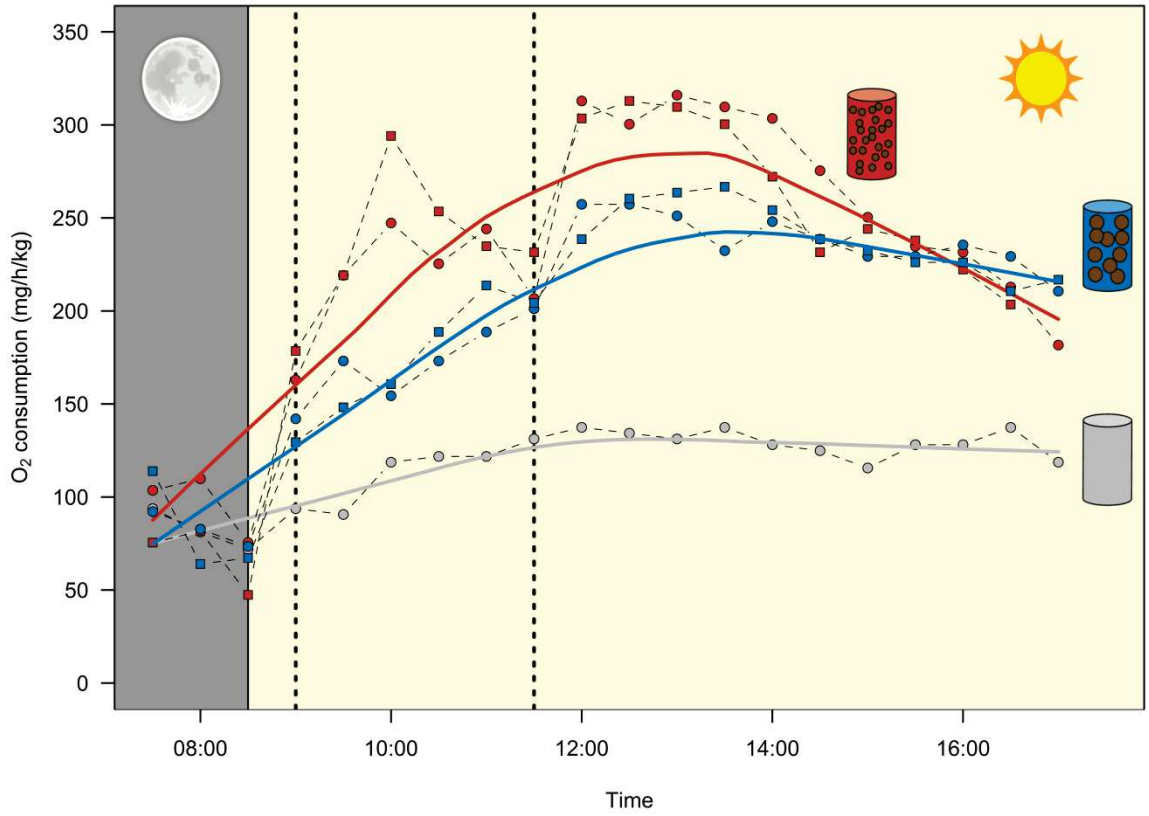

Figure 18: Evolution of the oxygen consumption over time for each feeding treatment (grey: fasting; red: pellet size of 0.1 mm and pellet quantity of 2% and blue: 1.2 mm and 2%). Colored lines represented smooth curve of the oxygen consumption for each treatment. Vertical black solid line represents when lights were turned on. Vertical dotted black lines correspond to the two meal times.

To sum up the Chapter 2, the take-home messages are:

- The morphological structure of the gill apparatus appeared to have changed in 10 years, in particular through the increase of the gill raker density.
- The daily energetic requirements of filtration seemed be higher than those of particulate feeding but this result remains to be confirmed by a more robust estimation.
- Although sardines may have a better retention capacity, it may not be enough to counterbalance the energy expenditures of the filtration on small prey.

Chapter 3: Which other life history traits could be impacted by food? Could they act as amplifying factors?

1. Introduction

Our experimental study presented in Chapter 1 supported the bottom-up control hypothesis showing that both food size and quantity had significant effects on body condition and growth, as well as on lipid storage and oxidative stress balance (Queiros et al., 2019). Such results highlighted energy trade-offs between growth and maintenance as individuals coping with food restrictions managed to pay some maintenance costs (e.g. no effect on protein content and structural lipids) at the expense of lower body condition, growth or lipid storage. In this context, other life history traits could be impacted by food size and quantity either directly or indirectly through a modification of the energy available (i.e. change in body condition). Such effects might worsen or partly compensate the situation described in Chapter 1 and observed in the Gulf of Lions. In this chapter, we investigated the effect of food size and quantity on other life-history traits, from the transformation of resources into usable energy to the utilisation of this energy and the resulting life-history trade-offs. Further, we investigated whether sardines could partly mitigate these effects by potential adaptations induced by phenotypic plasticity, microselection or epigenetic mechanisms, similarly to the modification observed for the gill raker apparatus (Chapter 2).

First, the oxidative stress balance (ratio between the reactive oxygen species (ROS) production and the antioxidant defenses controls) is known to play a central role in energy trade-offs between growth, maintenance and reproduction (Finkel and Holbrook, 2000; Kirkwood and Rose, 1991; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009). A previous study suggested that sardine fed on small pellets may have reduced their metabolic rates to cope with poor feeding conditions (Chapter 1). Simultaneously, filtration on small prey seemed to be more energy demanding than the particulate feeding (Chapter 2). Both results suggested than sardines fed on small pellets may have better mitochondrial conversion efficiency as they required more energy but exhibited lower ROS concentration (Chapter 1) but this remained to be investigated. Thus, to better understand the

physiological implication of food restrictions on the metabolism, we studied the effect of both food size and quantity through an integrative study of the mitochondria.

Further, reproduction represents the main life-history trade-off, opposed to growth or maintenance (Williams, 1966b). Thus, reproduction success could be modulated by individual body condition. Indeed, low energy reserves create energy trade-offs that could lead to extreme choices such as skipping reproduction to ensure survival (Jørgensen et al., 2006; Rideout and Tomkiewicz, 2011). Sardines are multiple spawners and are usually considered as capital breeders, i.e. they store energy in spring and summer that can be mobilized later for the reproduction in winter, between December and March in the Gulf of Lions (Brosset et al., 2016b), when food availability is limiting (Ganias, 2009; Ganias et al., 2007). After 2008, sardine condition was lower, but energy allocated to the reproduction has increased and sardines have maintained high reproductive investments (Brosset et al., 2016b). Such a high investment in reproduction could impact the survival and we therefore investigated the effect of food size on the reproduction of sardines.

Additionally, during their entire life individuals may cope with several pathogens from virus to macro parasites and thus require defenses. These defenses could represent a high energy demand for individuals, leading to again energy trade-offs between reproduction and survival (Alonso-Alvarez et al., 2004; Deerenberg et al., 1997). These trade-offs may explain extreme situations such as suppression of immunity under stress or malnutrition (Alonso-Alvarez et al., 2004; Hanssen et al., 2004). A reduction of energy allocated to immunity may endanger survival of sardines if they have to cope with pathogens. Therefore, we investigated the food size and quantity effects on the immune defenses of sardines.

Finally, stress could be defined as a situation in which individuals have to cope with a challenge (with real or symbolic danger for its integrity) which they need to overcome or compensate (e.g. physiological responses)(Tort, 2011). Cortisol is a glucocorticoid hormone released into blood after activation of the hypothalamic-pituitary-interrenal (HPI-) axis in response of a stressor, which is widely used to assess stress condition and health of individuals and populations (Aerts et al., 2015; Costantini et al., 2011; Sadoul and Geffroy, 2019). Energy required to cope with stress induced by cortisol could be derived from other essential functions such as reproduction or growth. As a consequence, cortisol and more generally glucocorticoids, have a large range of effects on individuals such as foraging capacity (Wingfield et al., 1998), growth (Midwood et al., 2014; Mommsen et al., 1999;

Sadoul and Vijayan, 2016), immune response (Harris and Bird, 2000; Tort, 2011; Wendelaar Bonga, 1997), reproduction (Mommsen et al., 1999; Tort, 2011) or oxidative stress (Tort, 2011). As cortisol may act as an amplifying factor due to the high implication on several mechanisms (both positive and negative effects, see Schreck & Tort (2016)), we studied the effects of food size and quantity on its concentration and whether its concentration might be used as an integrative response to energy trade-offs.

2. Material and Methods

2.1. Bioenergetics

To investigate the hypothesis that filtration strategy may induce an unbalanced energetic budget, we decided to study the mitochondrial activity. To do so, 46 sardines (i.e. 11 or 12 sardines per treatment) from the experiment described in Chapter 1 were sampled (Figure 7, Experiment n°1 – 4 feeding treatments: 2 sizes (0.1 and 1.2 mm) and 2 quantities (0.3 and 0.6%)).

2.2. Mitochondrial isolation and respiration

Mitochondrial populations were isolated from the red muscle using an ice-cold isolation buffer and concentrated by successive centrifugations. The oxygen consumption and ATP synthesis rates were examined at 20°C in a respiratory buffer. The oxygen consumption was measured with a Clark oxygen electrode and the ATP production was indirectly assayed by spectrophotometry monitoring the production of NADH. The basal oxygen consumption was recorded without the addition of ADP. The maximal oxygen consumption was evaluated with an addition of ADP (500 µM). Also, the non-mitochondrial ATP synthesis activity was taken into account even if it remained low (from 5 to 9% of total ATP production) (Material and Methods are detailed in Thoral et al. *in prep* Annex 2).

2.3. Data analysis

The effects of the food size and quantity on the basal and maximal oxygen consumption were compared using a parametric two-way ANOVA or the non-parametric Scheirer Ray Hare test when normality or homoscedasticity were not validated (tested with Shapiro and Levene tests, respectively). Pairwise comparisons were then made with Tukey comparison (if

ANOVA) or the Dunn's test (if Scheirer Ray Hare test). Further, the relation between the rates of ATP synthesis and oxygen consumption was assessed by a linear model. And the relationship between the basal oxygen consumption and the individual body condition index as well as potential threshold were investigated by a piece-wise linear regression with the 'segmented' package (Muggeo, 2019).

2.4. Reproduction

2.4.1. Sardine population

The effects of food size on the reproduction were investigated on a captive population of sardines during two consecutive years (Figure 7, Experiment n°2). Sardines were fished and acclimated on March 2016 following the approach described in Queiros et al. (2019). Fish were split in two groups: the first group was fed with 0.1 mm pellets whereas the second one was fed with 1.2 mm pellets. The experiment was held in 4 tanks of 3 m^3, two for each group/treatment, during the first year. In the second year, fish of a given group were gathered in a single tank due to a lower total number of fish. The feeding rate equaled 0.6% of the fish biomass in tank during all the experiment but for the summer 2018, when it was increased to 1% to account for high temperatures at this period. Tanks were supplied with seawater pumped directly from the sea and temperature was not controlled and followed natural variations except to remain between a minimum of 10°C and a maximum of 25°C. The photoperiod was weekly adjusted to follow natural cycle. Biometries were performed every month (spaced out during summer months) with the recording of individual (RFID tag) length and weight. Body condition was then estimated according to Le Cren's index (Brosset et al, 2015). To identify sex ratio in the sardine population, we used two identification methods, (1) either through abdominal compression (stripping) or using cannula during biometry (inactive males could not be identified with this method) or (2) direct gonad observation on dead fish.

2.4.2. Spawning and egg parameters

The egg collection system was composed of an egg collector made with a 70-µm mesh inside a larger container full of water to make sure that eggs were maintained in water all the time. After spawning, the sardine eggs are floating and therefore were collected through surface

overflows during the night (Miranda et al., 1990). Each morning, egg collectors were inspected and when present, eggs were collected and put in a 4 L beaker. Given that some floating eggs collected during the night became not viable and sank (probably unfertilized egg), floating and sinking eggs were split. The volume of both categories of eggs was recorded and summed to obtain the total egg volume. The concentration (egg abundance/mL) was estimated by counting under the binocular two replicas of 0.5 mL of floating eggs and converted into viable egg abundance.

Further, quality of the spawn was assessed by egg size, although this approach is still debated (Kjørsvik et al., 1990; Riveiro et al., 2004) while yolk was used as a proxy of maternal investment (Lubzens et al., 2017). To investigate food size effect on the egg and yolk sizes, a subsample of floating eggs was photographed by a camera attached to a binocular microscope and both egg and yolk diameters were estimated on photographs with ImageJ software (Schneider et al., 2012). Note that the spawning of sardine was not artificially induced.

2.4.3. Data analyses

Because the amount of spawning events (and thus eggs) was strongly unbalanced between treatments (see Results), we used a jackknife approach to compare egg quality (egg diameter and yolk diameter) between treatments. This also helped to limit a potential bias due to the non-independence between eggs (we do not know which female spawned for a given spawning event). For each of the 10,000 jackknife resamplings, we randomly draw eggs to have the same sample size between each sample. Egg and vitellus diameters differences between treatments (for the same year) and between years (for the same treatment) were then investigated using the non-parametric Wilcoxon test and results are presented as the proportion of the 10,000 resamplings which were significantly different. The effects of treatments and years on the proportion of both floating eggs and vitellus were assessed using generalized linear model with a binomial link. Statistical analyses were performed in R (R Core Team, 2018).

2.5. Immunity

2.5.1. Sardine population and feeding treatments

Sardines were captured on May 9, 2017 and acclimated following the procedures detailed in Queiros et al. (2019) (Figure 7, Experiment n°3). Sardines were then held in outdoor 4.5 m3 tanks and they were fed with aquaculture pellets (mix of pellet sizes: 0.1 mm, 0.3 mm and 0.8 mm at food rates between 1 and 2%) during 3 months. The experimentation started on August 29, 2017 and ended on April 26, 2018. First, around 1,000 sardines were divided into 2 groups and moved into 2 indoor tanks of 3 m^3 each (Figure 19, part A). Prior to transfer, sardines were anesthetized with benzocaïne (140 ppm), their total body length and total wet weight were recorded (to the nearest 0.1 mm and the nearest 0.01 g). A tiny RFID tag (Biolog-id, Bernay, France, 0.03 g i.e., <0.2% of sardine lowest body mass) was implanted in the dorsal muscle to allow individual identification. On October 25, sardines were transferred into 300 L tanks (4 tanks per treatment; Figure 19, part A). Tanks were supplied with water pumped directly from the sea and filtered through sand filter. Also, the photoperiod was adjusted each week to follow the natural cycle and sea water temperature was not controlled except to maintain a minimum of 10°C and a maximum of 25°C. Biometries were made every 4 weeks (tag read, total length and body weight recorded individually) and the body condition index of each sardine was estimated using the Le Cren's index (Brosset et al., 2015a). During the first phase of the experiment, the first group was fed in large quantity of small pellets, while the second one was fed in large quantity of large pellets (similar sizes and quantity as in Chapter 1; Queiros et al. 2019). On November 28, the feeding treatments were inversed for half of the tanks during the winter period (Figure 19), leading to four feeding treatments: small pellets all along – treatment (1), small pellets then large pellets – treatment (2), large pellets then small pellets – treatment (3) and large pellets all along – treatment (4), each composed of two tanks.

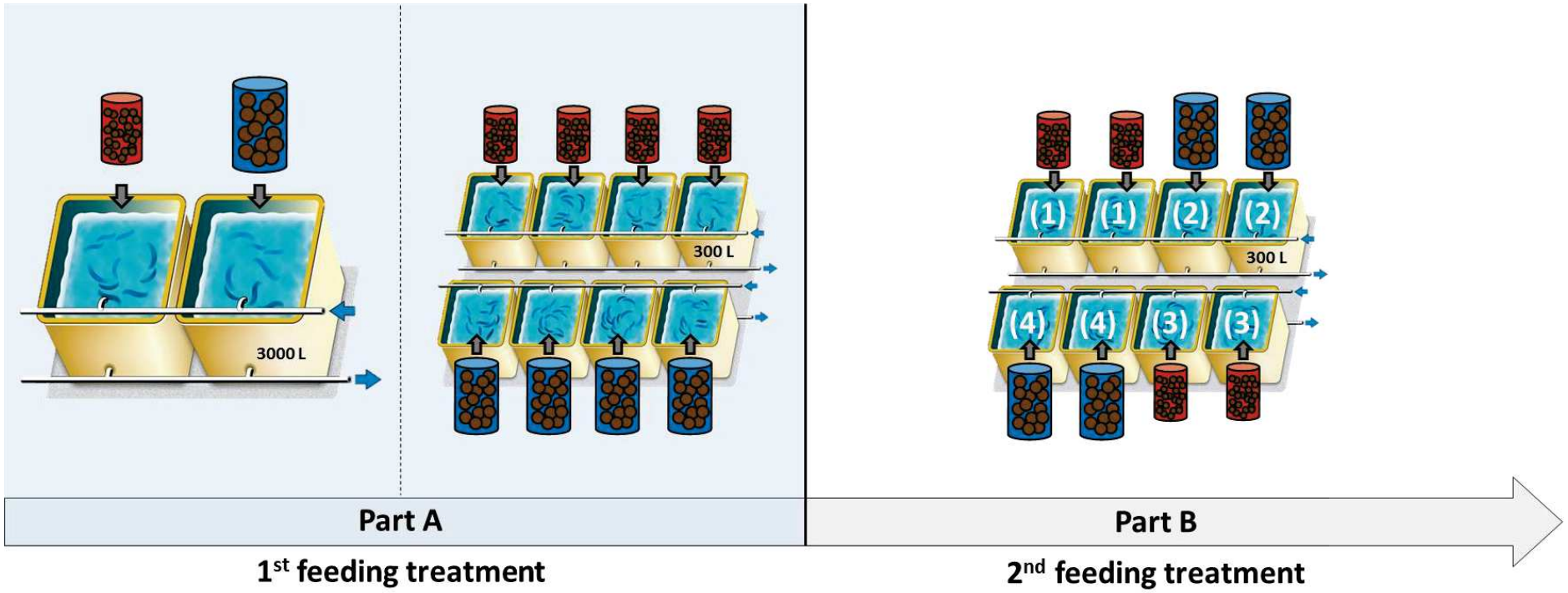

Figure 19: Experimental design used in the experiment n°3, comprising of 2 tanks of 3m^3 and then 8 tanks of 300L. Feeding treatments (red: pellet size of 0.1mm and blue: 1.2mm) are indicated for each of the tank.

2.5.2. Cell collection and preparation

Between 12 and 15 individuals were sampled 4 times during April (3^{th}, 10^{th}, 24^{th} and 26^{th}). At each sampling date, fish were first anaesthetized by benzocaine at 140 ppm and their individual total body length and total wet weight were recorded before blood and head kidney collection. Blood was obtained by caudal vein puncture with heparinized syringe and immediately transferred in L15 medium enriched with 2% fetal bovine serum. Sardines were then sacrificed by a lethal dose of benzocaine at 1000 ppm. After a ventral incision, the head kidney was collected. Single-cell suspension was generated by HK aspiration using a 1-mL syringe filled with heparin and enriched L15 medium followed by forcing the tissues through a 40-µm cell strainer (Falcon) with a plunger from a 1-mL syringe. Sex and maturity were determined by gonad observation.

Leucocytes were isolated using a Ficoll-Paque PLUS gradient media (d=1.077 g/mL, GE Healthcare). Cell suspensions were spun 25 min at 400g to remove erythrocytes and debris and the leucocyte rich interphase was collected, washed in PBS and resuspended in L15 medium. Single-cell suspensions was conserved at 17°C into L15 medium enriched with 0.4% fetal bovine serum overnight until flow cytometry analysis.

2.5.3. Flow cytometry analysis

Leucocytes cells were washed and resuspended in PBS, and were passed through a filter with a 40-µm pore size. Quantification of dead cells was determined by adding propidium iodide (Sigma) at a final concentration of 1 µg/ml. Leucocyte suspensions (1.5×10^6 cells/mL) were resuspended in 1-ml L15 and incubated for 30 min at 17°C with 2'7'-dichlorofluorescin diacetate (DCFH-DA), which is a stable non-fluorescent molecule which is hydrolyzed to DCFH by cytosolic enzymes, then by the action of H_2O_2 (produced in leucocytes), DCFH is oxidised to the fluorescent dichlorofluorescein (DCF). This procedure was helpful to discriminate the different cellular populations. Leucocytes that exhibited positive fluorescence to DCF are noted DCF+ in the following, otherwise DCF-. Flow cytometry analysis and sorting were based on forward scatter, side scatter and DCF fluorescence with a FACSCalibur flow cytometer (BD Biosciences). For each sample, between 10,000 and 20,000 individual cells were recorded. Data were analyzed using FlowJo software Version 8.7. To further describe the gated population, blood and head kidney leucocytes isolated from 4

individuals were sorted using a FACSAria I (BD Biosciences). Four populations per sample were sorted using forward scatter, side scatter and DCF fluorescence.

2.5.4. Cytology analysis

Cytospin preparations were made with ficoll-isolated blood and head kidney cells (4 individuals) and sorted cells. Cells were cytocentrifuged at 300 rpm for 3 min onto glass slides in a Cytospin3 cytocentrifuge (Shandon). Blood smears and cytospin preparations were processed through May-Grünwald and Giemsa stains (Sigma) for cell identification and morphological analyses. Cytospins were imaged using a microscope (LEICA). Images were obtained using a Megaview-III camera (Olympus America) and processed using LAS X.

2.5.5. Data analyses

The differences of body condition, total length and leucocytes (granulocytes, lymphocytes, monocytes and precursors) densities between treatments were investigated using parametric (one-way ANOVA) or non-parametric (Kruskal-Wallis) tests and associated post-hoc (Tukey or Dunn test), depending on residual normality and homoscedasticity. Further, a Principal Component Analysis (PCA) was performed using the individual sardines as objects and body condition index, white corpuscle rates (granulocytes, lymphocytes, monocytes and precursors) and the interval time (duration between the first sampling date and other sampling date of the individual) as descriptors to summarize all the information and investigate the relationships between descriptors. The effect of sex was also investigated using a PCA. All statistical analyses were performed in R (R Core Team, 2018) using the FactoMineR (Lê et al., 2008), the FSA (Ogle, 2018) and the factoextra (Kassambara and Mundt, 2017) packages.

2.6. Stress

2.6.1. Sampling preparation

To investigate the potential stress induced by the food size and quantity, we studied the cortisol deposited in scales, a more integrative tissue than blood (Aert et al 2015). Sampling was made on 120 sardines from the third experiment (Figure 7, Experiment n°3), which were sacrificed on the 3rd March 2018 (i.e. 30 per treatment) by lethal dose of benzocaïne (1,000

ppm). Length and weight were recorded and the body condition was calculated using Le Cren equation (Brosset et al. 2015). Scales of each sardine were collected on the full body and stored in Eppendorf tubes at – 80°C until further analyses. The chronic stress was assessed measuring the cortisol levels in scales using adapted methodological approach from Carbajal et al. (2018). To remove mucus from the scales, 150 to 200 mg of scales were transferred into glass vial of 12 mL with an addition of 3 mL of isopropanol. Vials were vorteed during 2.5 min and the supernatant was removed. This step was repeated 3 times. Residual solvent traces were evaporated under nitrogen flux and samples were frozen at -80°C before to be placed into a lyophilisator during at least 48h. Lyophilized scales were transferred into 2 mL safe lock Eppendorf with a ceramic milling ball. Samples were grinded using a ball mill (MM400, Retsch GmbH, Germany) and 30 mg of dried scales were transferred to 2 mL glass vial. After, 1.5 mL of methanol was added and vials were vortexed for 30 sec. Vials were incubated during 18h at 30°C with slight agitation. After centrifugation at 9500g during 10 min, 1 mL of the supernatant was pipetted and transferred into a new 2 mL glass vial. The supernatant was evaporated under nitrogen flux and reconstituted with 0.2 mL of EIA buffer provided by the Cortisol assay kit (Neogen Corporation Europe, Ayr, UK). Cortisol concentrations were determined in 50 µL of extracted cortisol by using a competitive EIA kits (Neogen® Corporation Europe, Ayr, UK). Samples were run in duplicates and averaged. Only samples with a coefficient of variations (CV) lower than 20% were kept for statistical analyses. Intra-plate cortisol variation was equal to 7.5%.

2.6.2. Data analyses

The cortisol concentration in scales was compared between treatments, using ANOVA or Kruskal-Wallis test (and post-hoc test: Tukey or Dunn test, respectively) depending on the normality and homoscedasticity. Hypothesis of normality and equality of variances were checked with a Shapiro test on the residuals and a Levene test, respectively. The relationship between growth rate and cortisol was assessed using generalized linear model, using gamma distribution owing to overdispersed positive data.

3. Results and Discussion

3.1. Does the filtration strategy induce an unbalanced energetic budget?

To study the effect of food size and quantity on the mitochondrial activity, we studied the relationship between the oxygen consumption and the ATP synthesis rates. Food size had a significant effect on the basal oxygen consumption (both p-value < 0.01) (Figure 20). The mean ± SD basal oxygen consumption rate was lower for fish that fed on small pellets (9.0 ± 3.7 and 13.3 ± 4.3 nmol O/min/mg protein for the small and large food size, respectively). Similarly, only food size had a significant effect on the maximal oxygen consumption rate (p-value < 0.001) with higher values for sardines that fed on large food size (mean ± SD of 44.8 ± 27.2 and 70.3 ± 25.3 nmol O/min/mg protein for the small and large food size, respectively). Then, the slopes of the fitted relationships between the ATP synthesis rate and the oxygen consumption did not differ significantly from each other (p-value > 0.05, Figure 20).

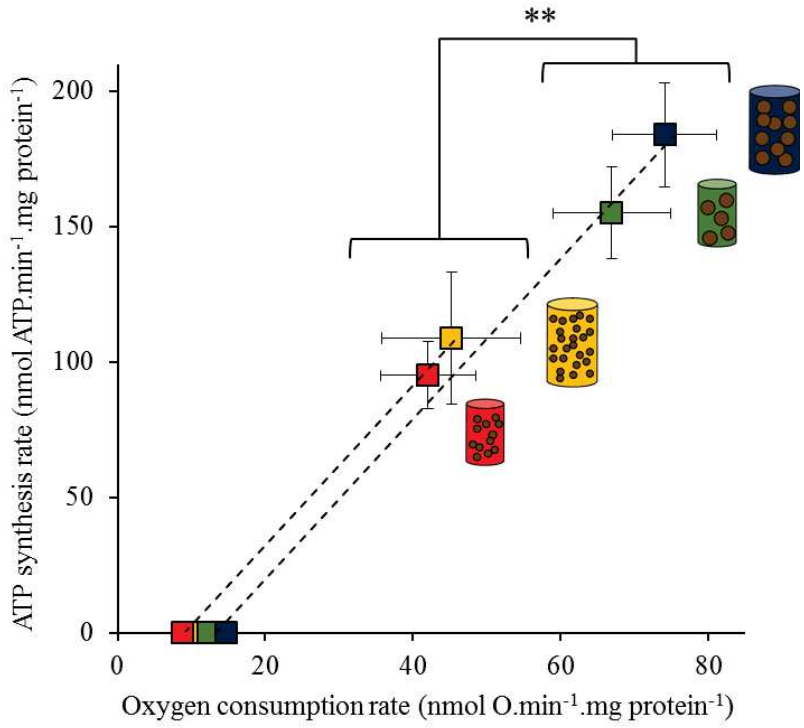

Figure 20: Effects of food size and quantity on mitochondrial oxidative phosphorylation efficiency at the mitochondria. The two trends drawn are those of the "large size" (ATP = 2.96 * Oxygen – 39.21) and "small size" (ATP = 2.95 * Oxygen – 26.70) groups. The values shown are means ± SE (the error bars of the basal oxygen consumption are small enough that they are hidden by the points). ** represents the significant difference between large and small food size treatments (p-value < 0.01). Figure adapted from Thoral et al. in prep – Annex 2.

The oxygen consumed in the basal non-phosphorylating state is used to counteract proton leakage across the mitochondrial inner membrane (Roussel et al., 2004). This basal oxygen consumption is thus an index of maximal energy wastage of mitochondrial metabolism. Thus, these results indicated that the mitochondrial activity of fish that fed on small food size were more efficient than fish fed on larger ones, so consuming less oxygen to produce a given amount of ATP, whereas sardines that fed on large pellets exhibited a more powerful mitochondria. This process was already observed in animals coping with caloric restriction (Bourguignon et al., 2017; Roussel et al., 2018). Thus, when fish are fed with small food size, individuals seem to develop an energy-saving metabolism that could be related to an increase of their mitochondria efficiency and abundance (see Table 2 in Thoral et al. *in prep* - Annex 2). This assumption was also supported by the relationship between the body condition index and the basal mitochondrial respiration. Indeed, the piecewise analysis showed a significant correlation between basal oxygen consumption rate and the body condition index below a condition threshold of 1.07 found for this experiment (p-value < 0.001 and R^2 = 0.45) but no relation after it (p-value > 0.05, Figure 21). Such a decrease in the basal oxygen consumption rate would reflect lower energy dissipation and so a higher coupling mitochondria (i.e. more efficient) occurring in sardines who exhibit a lower condition than the average population over time. Such results were known to occur in muscle of animals suffering caloric restriction (Bourguignon et al., 2017; Roussel et al., 2018). The increase of the efficiency of the mitochondria had been previously also associated to an increase of the food intake in *Salmo trutta* (Salin et al., 2016). However, despite the development of bioenergetics compensation mechanisms to counteract food restriction, such as higher mitochondrial coupling efficiency or the increase of the mitochondrial content in muscle, it may not be sufficient to compensate high energy requirements of the filtration as suggested by the lower body condition and growth exhibited by sardines fed with small pellets.

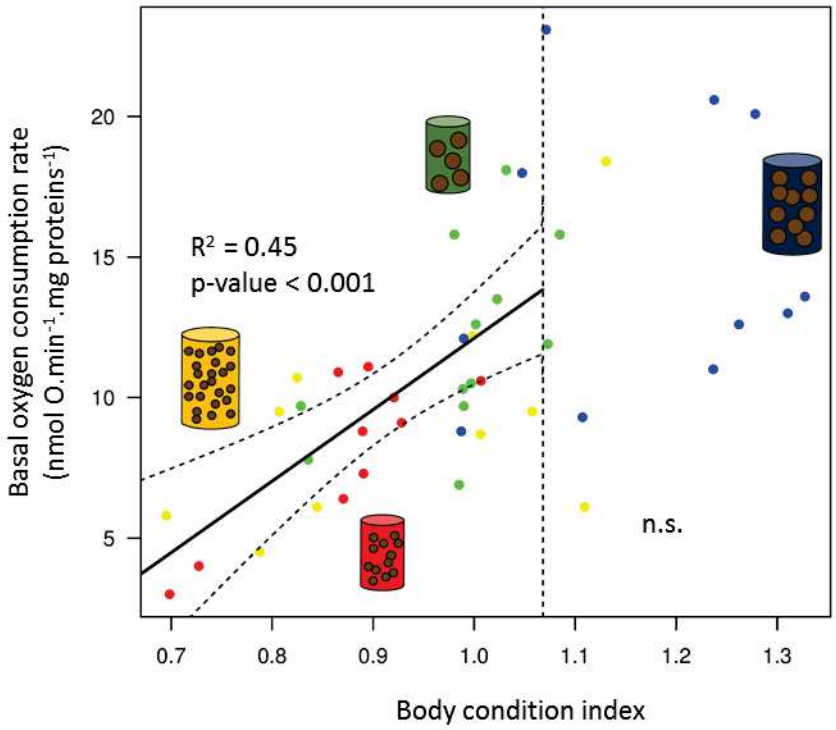

Figure 21: Basal oxygen consumption at the mitochondrial level as a function of the body condition of sardines. A linear model shows that there is a correlation between basal mitochondrial respiration and body condition until the body condition threshold of 1.07. Above this threshold, there is no longer a correlation between these two parameters.

3.2. Is reproduction affected by food size?

Body condition at the start of the experiment was very high (around 1.5) due to the period of acclimation where they were fed ad-libitum to ensure the highest survival possible. As a consequence, body condition decreased in both treatments at the start of the experiment. Nevertheless, body condition of sardines fed on large pellets stabilized after two or three months and then oscillated around 1.4 all along the experiment, while it decreased for almost a year in sardines fed on small pellets before leveling of around a plateau of 1.0. Further, the total length of sardines increased in both feeding treatments but at a much faster pace for sardine fed on large pellets. Thus, sardines fed with large pellets exhibited the highest condition and total length all along the experiment compared to sardines fed on small pellets (Figure 22).

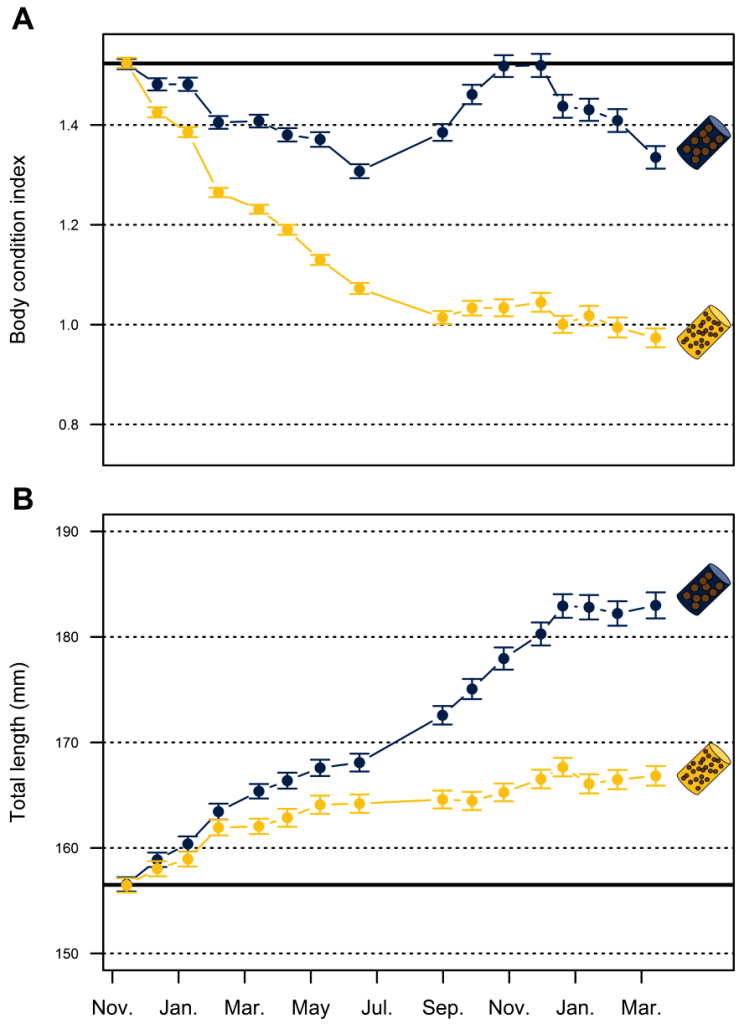

Figure 22: Time series of the mean (± se) body condition (A) and total length (B) of all sardines in each feeding treatment: yellow: pellet size of 0.1mm and pellet quantity of 0.6% and blue: 1.2mm and 0.6%. Black lines represent the mean body condition and total length at the beginning of the experiments.

Reproduction events mainly occurred between December and March when water temperature was between 12 and 15°C Figure 23A. The number of reproductive events was much higher in sardines that fed on large pellets than for sardines fed with small ones (35 vs. 4 events for the first year and 39 vs. 0 for the second). Because the number of females varied between years due to mortality or sacrifice (Figure 23B), we examined the number of reproduction events per female to compare between years and treatments. The number of reproduction events per female was much higher for sardines that fed on large pellets than sardines that fed on smaller ones for both periods: 0.40 and 0.04 events per female in 2016-2017, respectively and 0.63 and 0 events per female in 2017-2018, respectively. While the number of females decreased between the two periods (Figure 23B), the number of

reproduction events per female increased by 50% in 2017-2018 compared to 2016-2017 for sardines feeding on large pellet treatment.

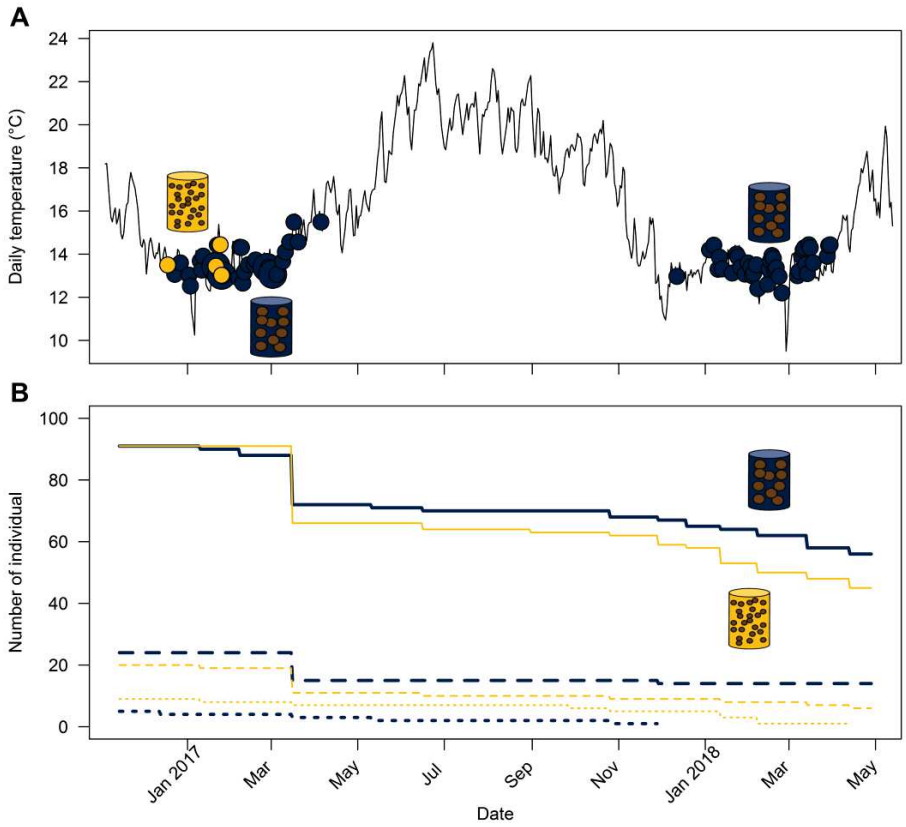

Figure 23: Time series of the sea water temperature (°C), reproduction events (A) and number of female (solid line), male (dashed line) and undetermined individual (dotted line) (B) for each feeding treatments (yellow: pellet size of 0.1 mm and pellet quantity of 0.6%; blue: 1.2 mm and 0.6%). Large circle (A) represents two reproduction events in the same day, i.e. one in each tank during the first year.

Further, the total egg volume per spawning event did not differ significantly between the two treatments for the period 2016-2017 (p-value = 0.13, medians: 0.24 and 0.30 mL for small and large pellet treatments, respectively Figure 24A). However, this volume was significantly higher for sardines fed on large pellets in 2016-2017 than sardines in 2017-2018 (p-value < 0.001, medians: 0.30 and 0.18 mL for 2016-2017 and 2017-2018, respectively, Figure 24A). Similarly, the floating egg volume did not differ significantly between feeding treatments in 2016-2017 (p-value = 0.07) but floating egg volume was significantly higher in 2016-2017 than in 2017-2018 for large pellet treatment (p-value < 0.05, medians: 0.23 and 0.13 for 2016-2017 and 2017-2018, respectively, Figure 24B). Also, the floating egg ratio did not differ significantly between treatments in 2016-2017 or between years for sardines fed on large pellets (p-value > 0.05, Figure 24C). Note that the variability in the ratio of floating

eggs was very large in sardines that fed on small particles (0% to 100% of floating eggs) compared to the ratio in sardines of the other treatment (50% to 100% of floating eggs).

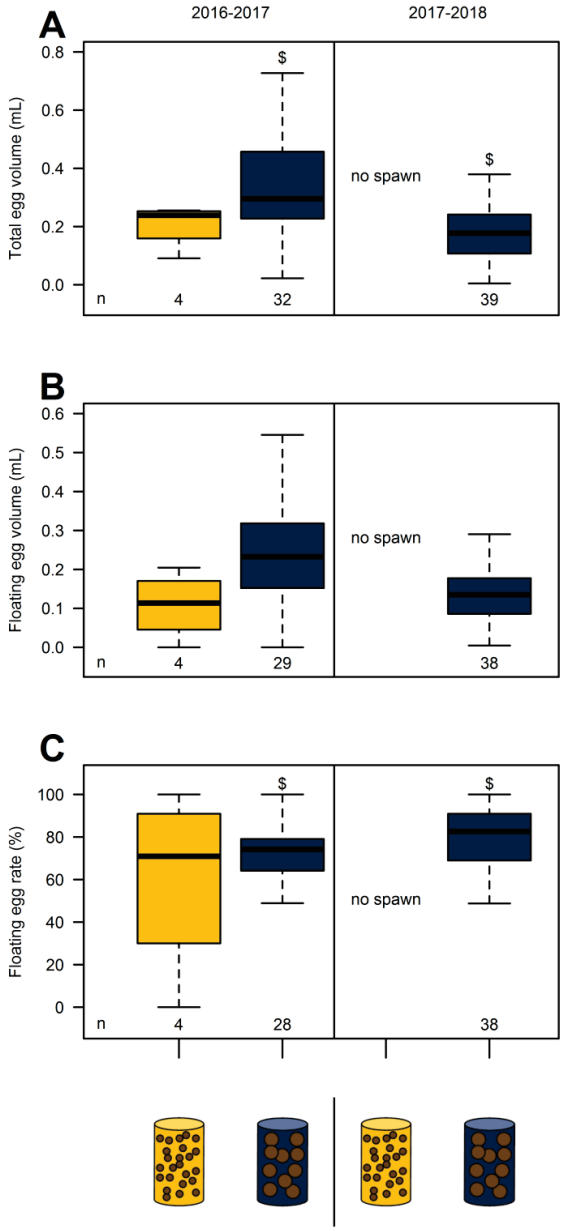

Figure 24: Boxplot of the total (A), floating (B) egg volumes per egg-laying and floating egg rate (C) for each feeding treatment (yellow: pellet size of 0.1mm and pellet quantity of 0.6% and blue: 1.2mm and 0.6%) in 2016-2017 and in 2017-2018. $ indicates a significant difference between groups (p-value < 0.05). The number of observations was indicated by n. Boxplots are presented without outliers for clarity purposes.

Additionally, using the Jacknife resampling procedure, we found that the egg diameter was significantly higher for sardines that fed on small pellets compared to sardines that fed on larger ones (97.5% of resamplings with p-value < 0.05, medians: 1.82 and 1.77 mm for small and large pellet treatments in 2016-2017, respectively, Figure 25A). However, no significant difference was found between years for the large pellet treatment (44.1% of resamplings

with p-value < 0.05, medians: 1.74 and 1.76 mm in 2016-2017 and 2017-2018, respectively, Figure 25A). Similarly, the vitellus diameter was significantly larger for sardines fed with small pellets compare to sardines fed with large pellets in 2016-2017 (99.9% of resamplings with p-value < 0.05, medians: 1.06 and 1.03 mm for small and large pellet treatments, respectively) while no difference was observed between the two periods for the large pellet treatment (4.2% of resamplings with p-value < 0.05, medians: 1.03 mm for both periods, Figure 25B). Vitellus ratio did not differ significantly between the two treatments in 2016-2017 or between years for the large pellet treatment (11.7 and 34.6% of resamplings with p-value < 0.05, Figure 25C).

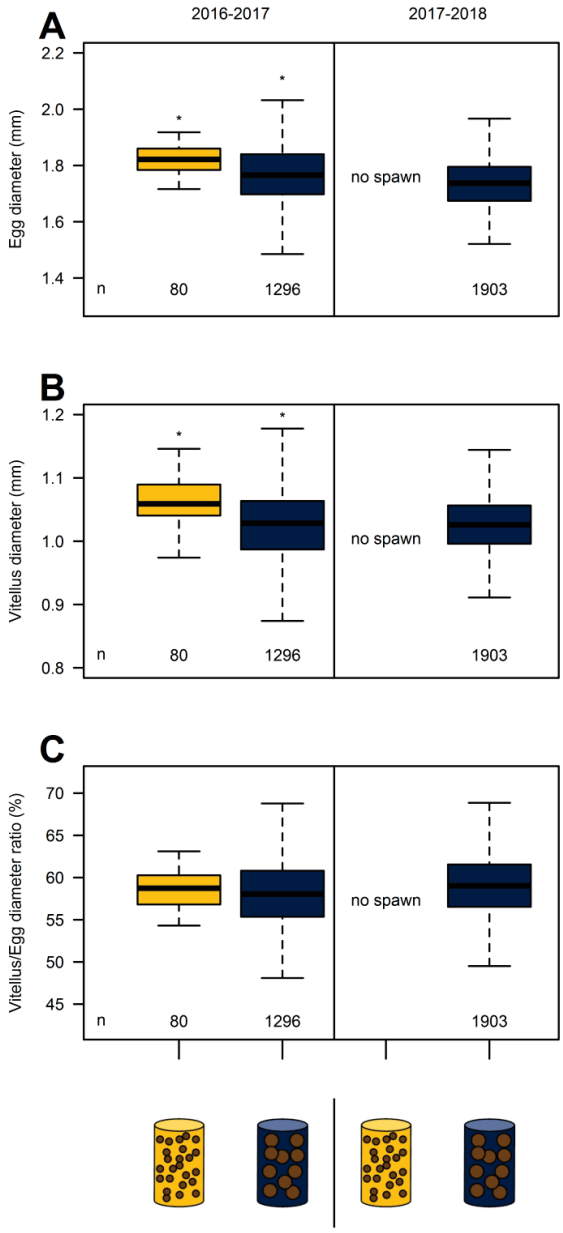

Figure 25: Boxplot of the egg (A), vitellus (B) diameters and the vitellus ratio (C) for each feeding treatment (yellow: pellet size of 0.1mm and pellet quantity of 0.6% and blue: 1.2mm and 0.6%) in 2016-2017 and in 2017-2018. * indicates a significant difference between groups (p-value < 0.05). The total number of observations was indicated by n. Boxplots are presented without outliers for clarity purposes.

These results may suggest that food size impact the reproduction of sardines, first through the number of reproduction events with low or no reproduction event for sardines fed on small pellets. The lack of reproduction events for the small pellet treatment during the second period might be due to either not enough energy to allocate to reproduction (sardines exhibiting lower body condition in 2017-2018 than in 2016-2017) or a low male ratio (< 15%, less than 10 males) leading to an impairment of the reproduction success. However, the number of reproduction events per female was higher in the second year probably due to decrease of the time between two spawning events as expected for larger individuals (Ganias et al., 2007). On top of this higher spawning frequency, larger individuals were expected to have higher batch fecundity (Brosset et al., 2016b). Surprisingly, the total egg volume was significantly higher for sardine fed on large pellets in 2016-2017 compared to 2017-2018. Because we are unable to observe which females actively spawn, we cannot rule out the possibility of a higher egg volume to be linked to more than one female spawning. Further, despite larger total length, the total egg volume produced by sardines fed on large pellets did not differ significantly with that of sardines fed on small pellets in 2016-2017 despite a higher median (Figure 24). Similarly, despite the median volume of floating eggs per batch being twice higher in sardines fed on large pellets compared to sardines fed on small ones in 2016-2017, no statistical difference was highlighted. The fact that both treatments did not differ statistically might probably be due to a too small number of spawning events (n=4 for sardines fed on small pellets). Moreover, the large variability in the ratio of floating eggs for sardines fed on small pellets might have made the situation even worse, as only floating eggs can engender larvae. To counterbalance the low production of floating eggs, sardines might maximize the potential survival of the larvae thanks to higher energy reserves in produced eggs despite their lower body condition (proxy of energy storage). Indeed, larger eggs and yolks might imply precocious hatching and larger larvae at hatching and might increase the survival time if larvae have to cope with food restriction (Pepin et al., 1997; Reznick et al., 1996; Riveiro et al., 2004). Here, despite a decrease in their body condition, sardines fed with small pellets produced larger eggs with a larger vitellus. Surprisingly, this result went against a previous negative relationship found between egg size and body condition index (Brosset et al., 2016b). Nonetheless, this result and all previous interpretations needed to be taken with caution, especially owing to the low number of spawning events and low egg production by sardines fed on small pellets.

In summary, food size had an important effect on the number of spawning events and the volume of eggs produced. While we could expect sardines to maintain their reproduction investment regardless of their body condition (Brosset et al., 2016b), we found different results. However, it has to be pointed that body condition levels were quite different from those observed in (Brosset et al., 2016b). According to reproduction/maintenance trade-offs, one could expect non-linear relationships between body condition and reproductive output (see Figure 26 for a conceptual framework). In particular, when body condition decreases, fish face energetic constraints and might neglect reproduction (even stop it in some cases) in order to allocate more to soma and favor survival and future breeding prospects. Nevertheless, when body condition becomes too small and survival might get impaired, a shift of allocation towards reproduction might arise in the form of terminal investment. Although this is purely conceptual, the fact that adult survival in the wild has been very low since 2008, while it is maintained at high levels in our tanks even in the small pellet treatment might support this hypothesis.

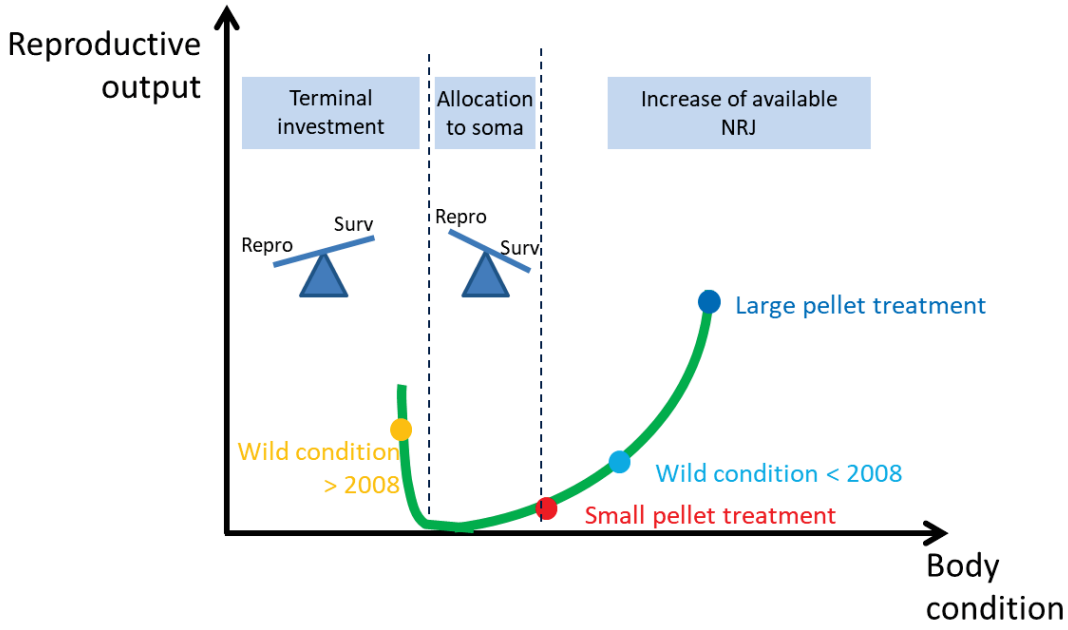

Figure 26: Conceptual framework of the reproductive investment of sardines as a function of their body condition index

The difference in results between previous in situ research (Brosset et al., 2016b) and this experiment might thus derive from the difference in body condition range. By comparing only two points here, it is not possible to examine such potential non-linear relationship and we would need to study the reproduction of sardines under a wider range of body condition

(especially investigate reproduction in captivity of really low condition sardine) to investigate this conceptual framework. Finally, our knowledge on the reproduction of sardines and especially the triggers of spawning events remained limited (see Discussion).

3.3. Does immunity suffer from food restriction?

Immunity was investigated in sardines of four different treatments, one where sardines were fed on small pellets all along, one where sardines were fed on large pellets all along and two cross-treatments where they were fed small then large or large then small pellets. These four treatments resulted in differences in terms of morphometric characteristics of sardines (Figure 27). First, body condition exhibited significant differences between all treatments except for the cross-treatments (2) and (3) (Figure 27). Total length differed significantly between treatments except for sardines fed on large pellets all along and fed on large pellets then small pellets (treatments (3) and (4)), and small pellets all along led to the lowest total length at the end of the experiment (treatment (1), Figure 27).

Further, cell characteristics of leucocytes (e.g. cell and nucleus size, vesicles, granularity) were close to their mammalian counterparts and similar to other teleost species (Stachura and Traver, 2016). Cell identification was thus performed according to a proposed model of adult sardine hematopoietic differentiation, that we developed based on these other species (Figure 28). The major cells isolated by size and granularity and their response to DCF were summarized in Figure 29. Only results on the head kidney are presented here, as blood analyses are still under way.

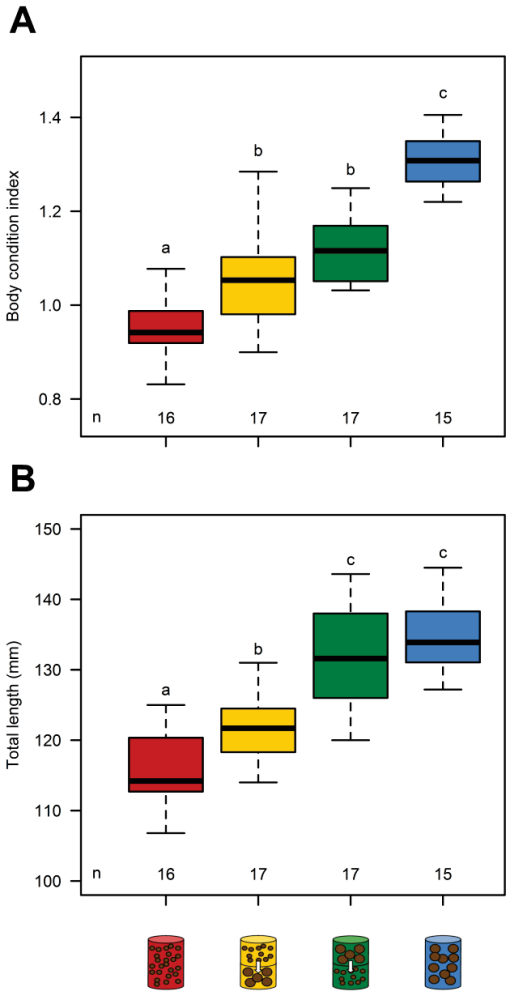

Figure 27: Boxplot of body condition (A) and total length of sampled individuals for each feeding treatment (red: small pellets all along, yellow: small pellets then large pellets; green: large pellets then small pellets and blue: large pellets all along). Boxplot with different superscript letters are significantly different (p-value < 0.05). The total number of observations was indicated by n. Boxplots are presented without outliers for clarity purposes.

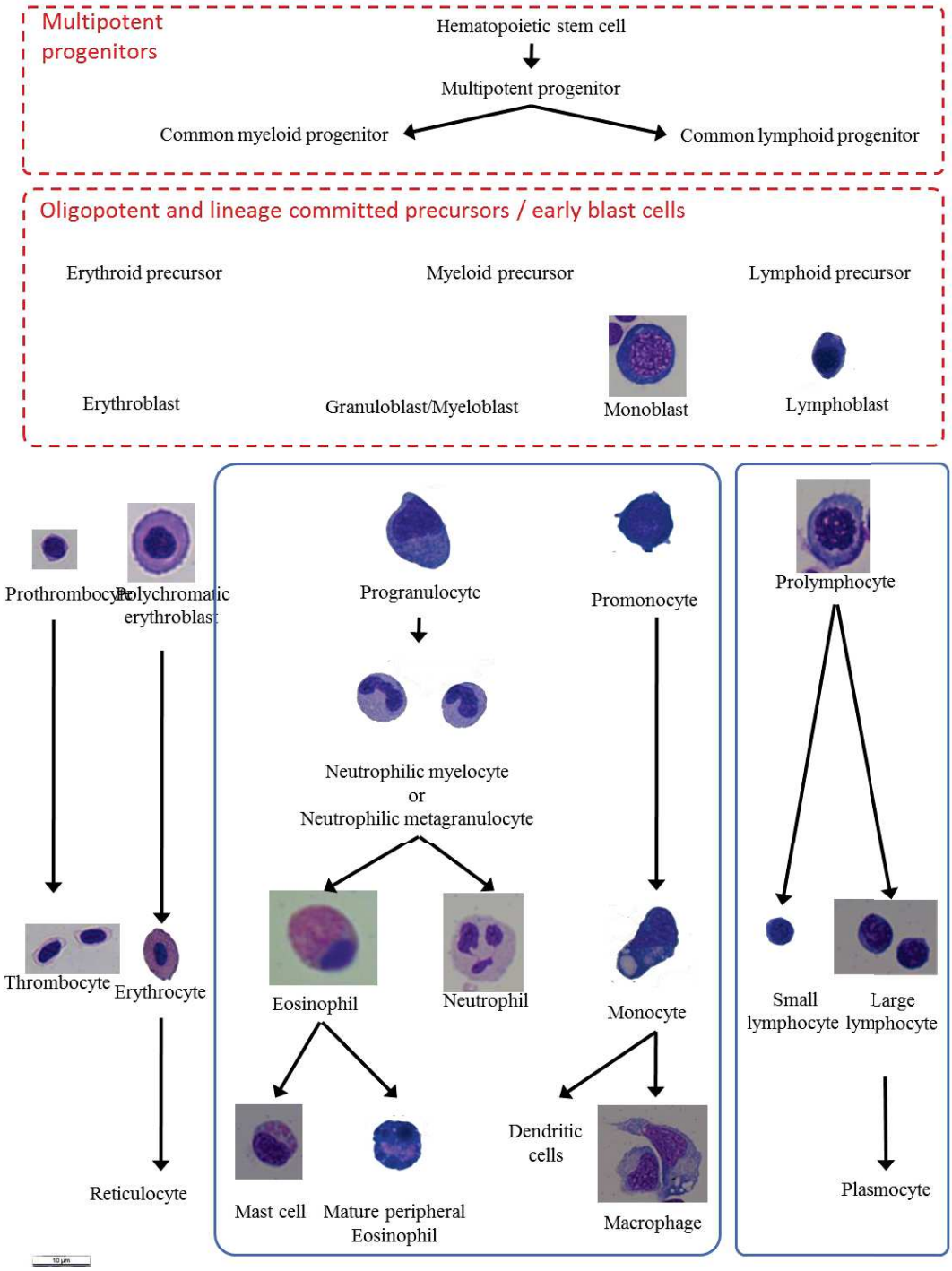

Figure 28: Ficoll-isolated, cytospun, and May-Grümwald/Giemsa stained immune cells from the sardine head kidney and blood and their proposed upstream progenitors. Proposed lineage relationships are based on those demonstrated in zebra fish (Stachura and Traver, 2016). Multipotent and lineage restricted progenitors likely reside in the kidney marrow, but their existence has never been experimentally proved due to a paucity of in vitro assays.

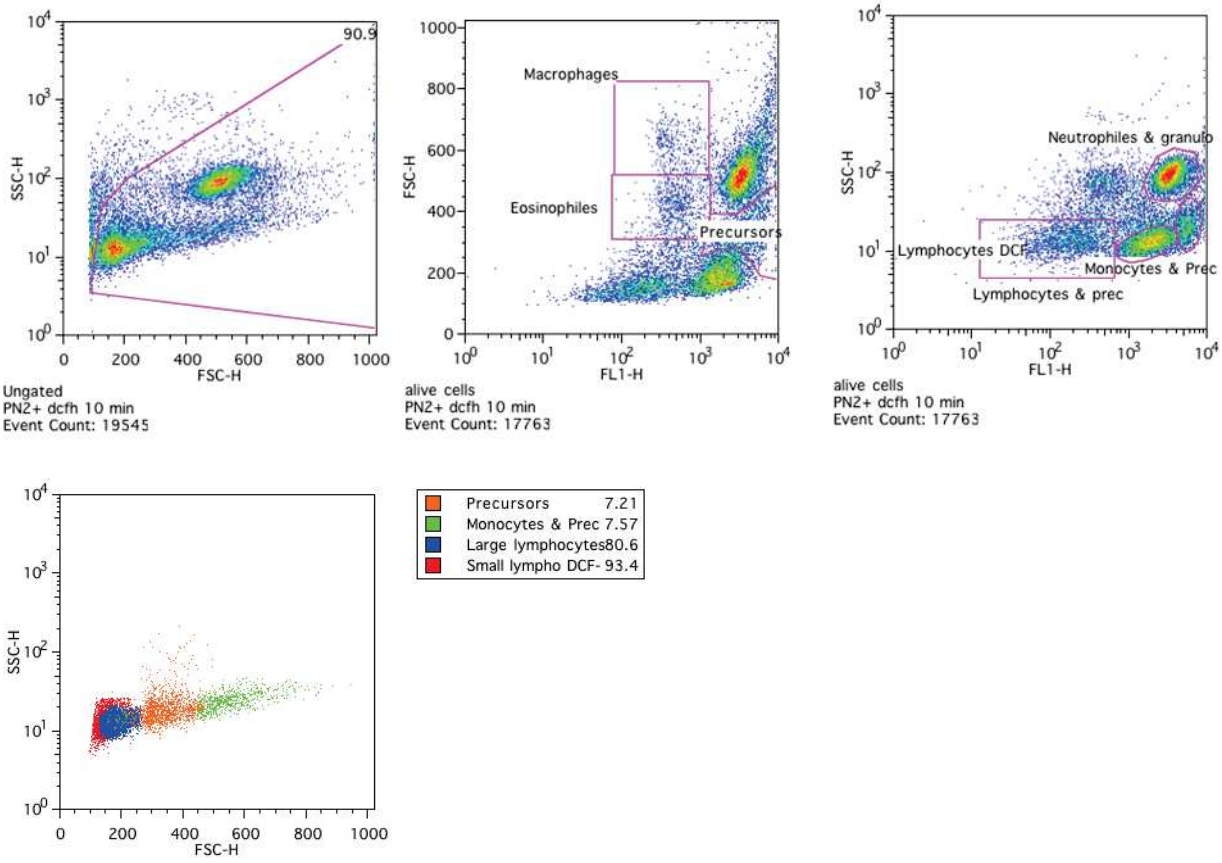

Figure 29: Example of dot plots obtained for head kidney sample using FACS Calibur and cell sorter FACS Aria

Leucocytes are mainly represented by lymphocytes (both DCF+ and DCF-), then by granulocytes, monocytes and precursors (mean ± SD frequency equaled to 44.1 ± 12.8, 28.9 ± 12.5, 11.9 ± 4.6 and 11.0 ± 4.8 %, respectively). The granulocytes (both DCF+ and DCF-) and the precursors did not differ significantly between treatments (Figures 30A, B and F). Conversely, some inter-treatment differences were highlighted in both lymphocytes DCF+ and DCF- and monocytes, mostly through a higher proportion of lymphocytes DCF- and a lower one of lymphocytes DCF + and monocytes in sardines fed with large pellets all along (i.e. treatment 4) compared to the others (see stats on Figures 30 C,D and E).

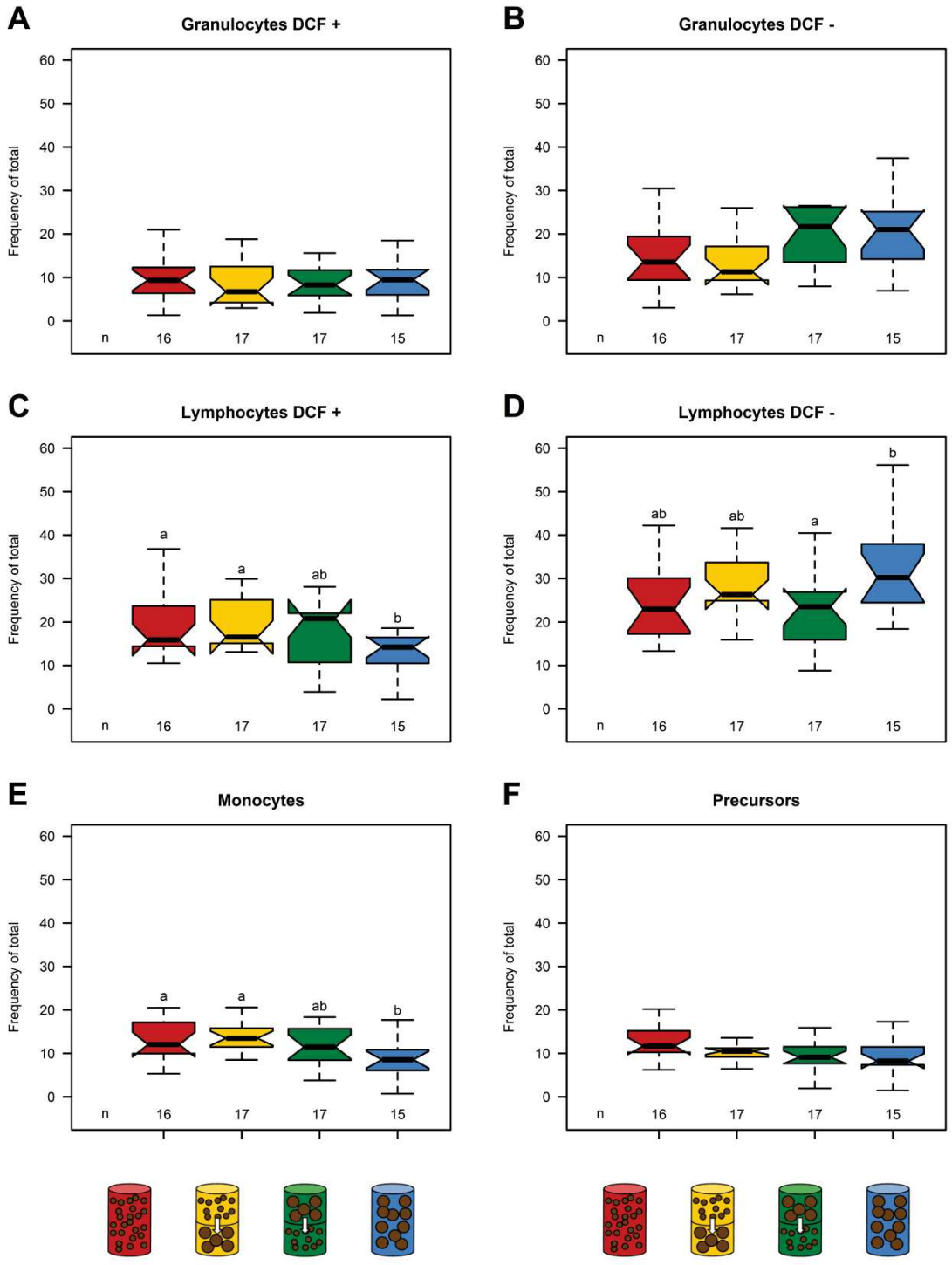

Figure 30: Boxplot of the frequency of granulocytes DCF+ (A) and DCF- (B), lymphocytes DCF+ (C) and DCF- (D), monocytes (E) and precursors (F) for each feeding treatment (red: pellet size of 0.1 mm throughout the experiment; yellow: 0.1 mm then 1.2 mm; green: 1.2 mm then 0.1 mm and blue: only 1.2 mm). Boxplot with different superscript letters are significantly different (p-value < 0.05). The number of observations was indicated by n.

To synthetize all this information, we then investigated all frequencies at the same time as well as morphological parameters and sampling time, using a Principal Component Analysis (PCA). The first 2 components of the PCA explained 52.4% of the total variance observed

(Figure 31). The main contributing variables to the first axis were body condition, monocytes and precursors, whereas the second axis tended to oppose lymphocytes to granulocytes. Note that the DCF + versus DCF- were also distinguished along the first axis for both lymphocytes and granulocytes, indicating that an increase in DCF+ cells might be correlated with a decrease in DCF-. The PCA also indicated a negative correlation between body condition and the frequencies of monocytes and precursors, although no significant linear relationship was found between these variables (p-values > 0.05). Finally, superimposing the treatment for each individual and the projection of the barycenters, only a slight trend along the first axis was observed, indicating a lower body condition but higher frequencies of monocytes and precursors in fish fed with small pellets (Figure 31). Note that the sex had no effect on the leucocytes frequencies as the projection of the barycenters coincided with the origin of the PCA and individual points were distributed over the entire projection space for both sexes (Figure 32).

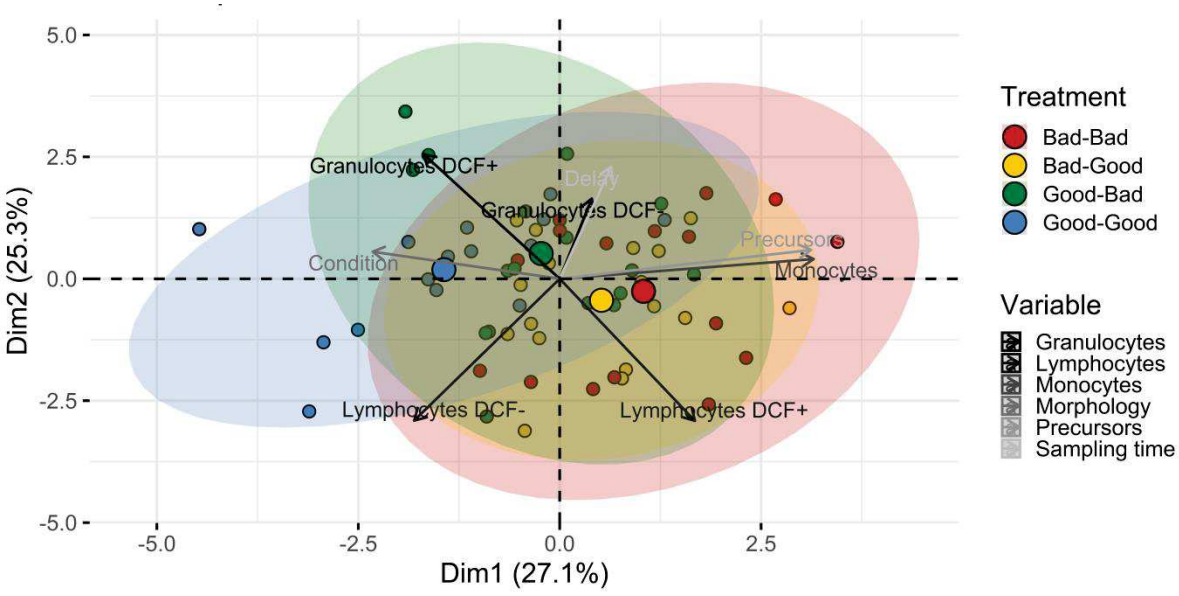

Figure 31: Biplot of the PCA built using body condition, leucocytes (lymphocytes, granulocytes, monocytes and precursors) and the delay between sampling dates as explanatory variables. Ellipses correspond to 95% confidence level for each treatment. The large circles represent the barycenter of the individuals for a given treatment.

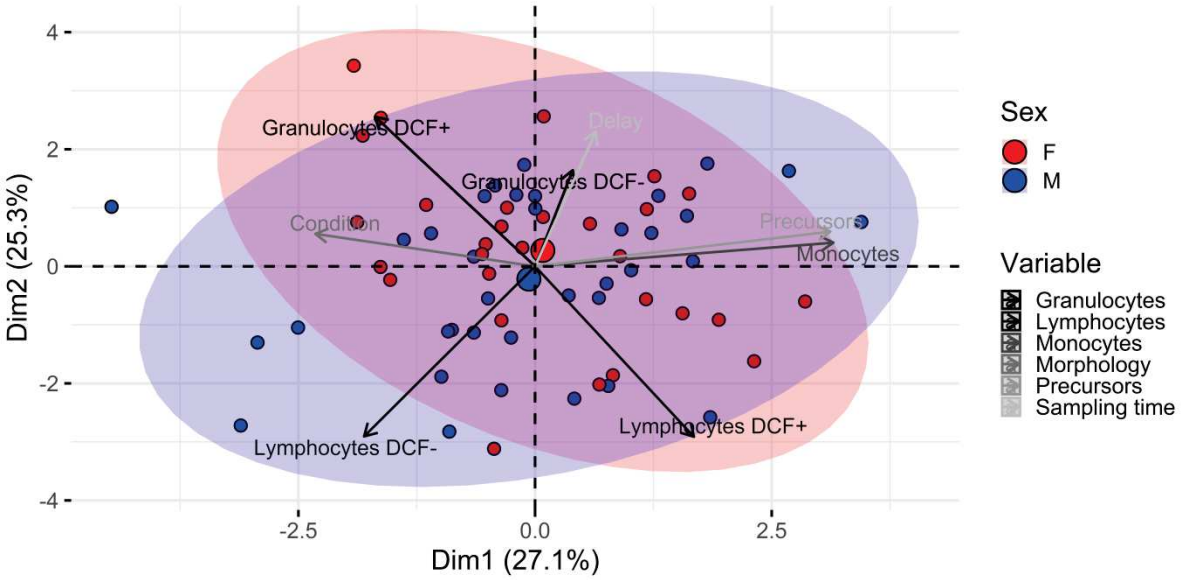

Figure 32: Biplot of the PCA built using body condition, leucocytes (lymphocytes, granulocytes, monocytes and precursors) and the delay as explanatory variables. Ellipses correspond to 95% confidence level for sex (femelle in red and male in blue). The large circles represent the barycenter of the individuals for a given sex.

All these results suggested a slight (but relatively low) impact of the feeding treatment on leucocytes proportions. Inter-treatment differences between lymphocytes frequencies might result from either different cell maturity levels or different populations of lymphocytes (e.g. lymphocytes T or B). The identification of the lymphocyte populations was not possible because the specific antibodies of sardine lymphocytes are unknown. These populations are involved in the specific immunity while monocytes and neutrophils (i.e. granulocytes DCF+) are non-specific phagocytic cells implied in defense against infections and bacteria similarly to eosinophils (granulocytes DCF-), which are also implied in the inflammation process (Davis et al., 2008; Nakanishi et al., 2018). Finally, the relatively low impact of the feeding treatment might result from the fact that leucocyte frequencies found here reflect the basal level of the immune defense of sardines. Indeed, the fact that sardines were reared in controlled environment with tanks supplied with water pumped from the sea but treated using UV-filters ensured the absence of pathogens. The absence of a relationship between the body condition and a potential immunosuppression may result from the fact that the lowest body condition found here remained relatively too high to impact the immune defense (mean ± SD: 0.98 ± 0.10 for sardines fed on small pellets all along). To go one step further, it could be interesting to investigate how these feeding treatments may impact the immune defense of sardines coping with pathogen challenge.

3.4. Does stress vary according to feeding condition?

Cortisol concentration was twice higher for sardines fed on large pellets all along (median: 47.7 pg/mg) than sardines fed on small pellets all along (median: 19.4 pg/mg), although the cortisol concentration in scale did not differ significantly between treatments (p-value > 0.07, Figure 33). Surprisingly, the cortisol concentration in sardines fed on small pellets was lower whereas their body condition reached the lowest values (mean body condition ± SD: 0.93 ± 0.17). Similarly to results found on immunity, the absence of significant effect on the cortisol concentration by feeding treatments (especially treatment (1)) may be explained by a relatively too high body condition index to induce a chronic stress associated with caloric restriction.

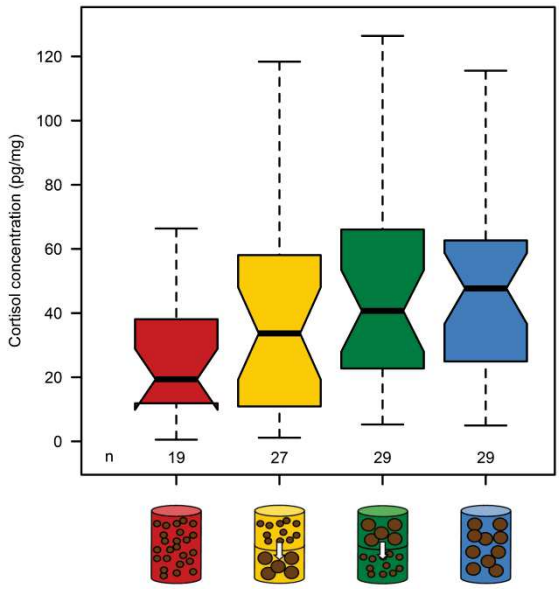

Figure 33: Boxplot of the cortisol concentration in scale for each feeding treatment (red: pellet size of 0.1 mm all along; yellow: 0.1 mm then 1.2 mm; green: 1.2 mm then 0.1 mm and blue: only 1.2 mm). The number of observations was indicated by n.

When fish cope with acute stress, cortisol is known to be negatively correlated with growth rates (McCormick et al. 1998, see review of Mommsen et al. 1999). However, low or intermediate cortisol levels have positive effects on the metabolic rate of individuals (Costantini et al., 2011) and may favor growth. Here, the generalized linear model analysis revealed a significant positive relationship between growth and cortisol levels (p-value < 0.05, Figure 34), supporting the fact that these cortisol concentrations may be too low to generate significant stress conditions for sardines and conversely, that they favor the growth

of individuals fed on large pellets. Yet, the model explained only 7.4% of the variance and those conclusions should be taken with much care (Figure 34).

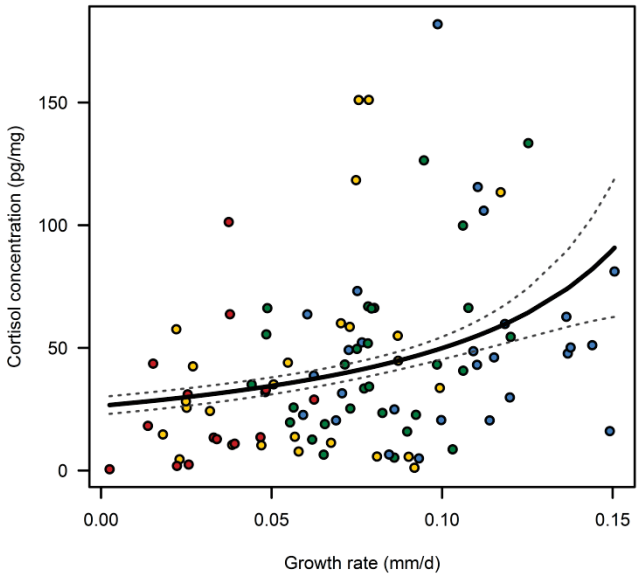

Figure 34: Plot of the cortisol concentration according to individual growth for each feeding treatment (red: pellet size of 0.1 mm throughout the experiment; yellow: 0.1 mm then 1.2 mm; green: 1.2 mm then 0.1 mm and blue: only 1.2 mm). The fit and standard errors of the gamma generalized linear model fitted on data are presented by solid black and dotted grey lines, respectively.

To sum up the Chapter 3, the take-home messages are:

- Sardines displayed plasticity in their mitochondrial activity after a few months of different food treatments. Nevertheless, despite the development of bioenergetics compensatory mechanisms to counteract food restriction (higher mitochondrial efficiency and higher mitochondrial content), these adaptations may not be sufficient to compensate high energy requirements of filtration.
- Reproduction was strongly impacted by the food size with a significant higher number of reproduction events for sardines fed on large pellets in both years of the experiment. While this seems in contradiction with previous results in the wild, it as to be noted that the range of sardine body condition was different in the experiment. Although this would need further investigation, this might result from non-linear relationships between reproduction and individual reserves. Conversely, sardines fed on small pellets produced larger eggs with higher energy reserves, which might increase the potential survival of the larvae. Nevertheless, these results need to be

taken with caution due to the low amount of data (especially for sardine fed on small pellets) and also due to the scarce knowledge on the sardine reproduction.

- Both immunity and cortisol seemed to be not impacted by the feeding treatments and the levels found in this chapter could reflect basal levels in sardines. The absence of significant effect may be explained by a relatively high body condition and thus the absence of significant challenge to cope with.

Chapter 4: Can sardine starve to death?

Can fish starve to death?

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1. Introduction

Survival is one of the main vital rates driving population dynamics, but it is difficult to assess. That is, this either requires longitudinal monitoring of individuals by capture, mark and recapture, or the ability to find dead individuals if there are extreme events such as massive die-offs (Begon et al., 1996). In the particular case of aquatic ecosystems, dead fishes are very rarely found, either sinking to the bottom or being consumed by other animals (but see Griffiths & Kirkwood, 1995). As a consequence, estimation of natural mortality in fishes is often limited to predation mortality and modelled either as a constant or as a function of size (to account both for predation pressure and ageing; Gislason, Daan, Rice, & Pope, 2010). Yet, as in any other species mortality must have multiple sources, such as epidemics or starvation. Understanding fluctuations in natural mortality is of course essential when trying to model population dynamics but is also extremely important for management of exploited species, which is based on stock assessment models that compare fishing mortality (F) to natural mortality (M) (Haddon, 2010; Hilborn and Walters, 1992). A recent study on cod found, for instance, that up to 40% differences in spawning stock biomass (SSB), F and recruitment (R) emerged when comparing stock assessments with a constant M versus a variable M linked to body condition (Casini et al., 2016). This question is crucial in fisheries science, as past studies have showed that natural mortality can significantly vary according to size and growth (Gislason et al., 2010), density dependent processes (Fromentin et al., 2001) or changes in environmental factors (Pershing et al., 2015). Furthermore, the difficulty of observing the vast and remote underwater realm often precludes assessment of whether massive die-offs occur and, if so, what their main drivers might be. There are a few exceptions, such as when dead fishes wash up along shorelines, enabling researchers to investigate the cause of deaths (e.g. discover the occurrence of pathogens; Whittington, Crockford, Jordan, & Jones, 2008).

According to life-history strategy, life-history traits (such as survival) result from the trade-off in energy allocation between growth, maintenance and reproduction (Stearns, 1976; Williams, 1966a). Species have evolved life-history strategies to maximise their population growth and stability across time (Stearns, 1992), but these may not always be optimal, especially if there are sudden unexpected environmental changes. While massive die-offs often occur in response to an abrupt change and extreme conditions (see for instance the

recent effect of the so-called 'blob' heat waves on seabird mortality in the Pacific; Jones et al., 2018), less extreme conditions may modify the energy balance of individuals, due to an increase in energy demands and expenditure (e.g. an increase in temperature and metabolism, maturation of gonads, etc.) or to a decrease in energy resources (e.g. change in prey availability). In both cases energy allocation could be modified, affecting the main vital rates and potentially threatening survival. In marine fishes, we are aware of only one study that has attempted to relate mortality events to starvation (Dutil and Lambert, 2011). Death from starvation might, however, remain largely unnoticed and overlooked in the marine environment. In the present context of accelerating global change, marine systems are under profound pressure from multiple stressors (temperature, ocean chemistry, connectivity, etc.; Harley et al., 2006), leading to changes in population abundance, distribution and phenology, and ultimately to alterations in community structure and diversity (Doney et al., 2012; Pershing et al., 2015). Ocean productivity has been shown to be reduced by climate change (Hoegh-Guldberg and Bruno, 2010). This can affect primary productivity with effects on plankton (Hays et al., 2005) that might propagate through the ecosystem, generating food-limited resources at various trophic levels. It is, therefore, important to investigate the impact of reduced food resources on fishes.

When facing a new selection pressure, such as a change in their environment, animals can either move towards a more favourable area, adapt to the new conditions through micro-evolution mediated by genetic changes and natural selection, or adapt their phenotypic reaction norm to their new environment (Davis et al., 2005). Phenotypic plasticity is where a single genotype expresses different phenotypes depending upon the environment (Gienapp et al., 2008; Nussey et al., 2007; Visser, 2008). Such plasticity can either be fixed by exposure to particular environmental conditions during development or can be reset cyclically, for example labile traits linked to spawning periods (Nussey et al., 2007). Phenotypic plasticity in maturation or growth has also been documented as a response of marine fish to exploitation (Jørgensen et al., 2007). While it is unlikely that adaptive phenotypic plasticity could compensate for extreme climatic events and prevent massive die-offs (see Pershing et al., 2015), it may play an important role in compensating slower or more predictable changes (Levins, 1968). This latter would include regime shifts in food resources, where plasticity in the short term would provide the potential for species to adapt in the medium to long term.

Here, taking sardines as a case study, we use a combination of lab experiments and in situ data to investigate the significance of death by starvation, and whether fish species can adapt to low food resources in order to decrease the risk of mortality. Sardines are small pelagic fish with a worldwide distribution that are known for the profound fluctuations in their populations and their importance as fishery resources (Pikitch et al., 2012). Alternation of boom and bust periods, with major changes in abundance, has been observed in various different upwelling ecosystems (in particular the Eastern-Boundary Upwelling Systems). This is thought to be firstly the result of high and low recruitment rates due to variation in environmental conditions (Checkley et al., 2009; Field et al., 2009; Gushing and Dickson, 1977; Schwartzlose et al., 1999), but overfishing can modify the dynamics and accelerate the decline of those populations and generate their collapse (Essington et al., 2015; Lindegren et al., 2013; Toresen and Ostvedt, 2000). Sardines are interesting because they exhibit a marked flexibility in feeding behaviour, which is often a prerequisite for phenotypic plasticity, by being able to filter-feed when prey are small but abundant and particulate-feed when prey are larger (Garrido et al., 2007, 2008).

In the North-Western Mediterranean Sea, sardines have historically been a very important fishery. Over the last decade, however, sardine biomass has decreased due to a sharp decline in individual size and weight, while abundance has increased and recruitment is high (see Saraux et al., 2019; Van Beveren et al., 2014 for more details). The decrease in size is mainly related to higher natural mortality of older individuals, but this cannot be accounted for by overfishing, predation pressure or pathogens (Queiros et al., 2018; Van Beveren et al., 2016b, 2016a, 2017). Therefore, a bottom-up control of the sardine population, linked to a shift in diet, has been proposed as a mechanism underlying lower growth and body condition (Brosset et al., 2016a; Saraux et al., 2019), possibly coupled with a change in energy allocation towards reproduction (Brosset et al., 2016b). Nevertheless, whether sardines might die from low body condition and starvation remains an open question.

To better understand the link between sardine energy reserves and mortality, we performed a fasting experiment on wild sardines maintained in captivity. This enabled us to estimate mortality probabilities and sardine physiological states at different body conditions. As for birds and mammals, fish usually undergo 3 different phases of fasting (Bar, 2014) where: phase I is usually characterized by a rapid decrease in body mass, the use of glycogen reserves and the progressive use of lipids; phase II involves a relatively extended period

where body mass loss is relatively low and constant, and lipid reserves are the main energy source, and phase III is when reserves are exhausted so the main energy substrate is proteins and rates of body mass loss increase again (Bar, 2014; Cherel et al., 1987, 1991; Cherel and Le Maho, 1985; Le Maho et al., 1981). Using specific body mass loss, namely the rate at which body mass decreases $\left(\frac{1}{m}\frac{dm}{dt}\right)$, as well as respiratory metabolism as indices, we investigated when sardines entered the critical phase III of fasting, to relate this to their body condition. Further, in order to estimate whether phenotypic plasticity played a role in affecting the relationship of body condition to mortality, we used sardines that had been maintained on four different feeding treatments for seven months, one mimicking conditions in the wild before 2008, one mimicking the current period after 2008, and two intermediate conditions (see below for more details).

2. Methods

All procedures were in accordance with the French and the EU legislation regarding animal experimentation (APAFIS, Permissions N° 7097-2016093008412692 and N° 10622-2017071711101242).

2.1. Sardine capture and acclimation

Sardines were captured in October 2016 by a dedicated commercial purse seiner off Frontignan (Hérault, South of France) and transported to the IFREMER research station at Palavas-les-Flots. They were held in quarantine tanks until confirmation of an absence of pathogens, notably nodavirus. During quarantine the sardines were weaned from live food (*Artemia nauplii*) onto commercial aquaculture pellets, as described by Queiros et al. (2019).

2.2. Experimental design

449 sardines were distributed into 8 experimental 300L tanks, so that both the mean and coefficient of variance in length and weight were similar between tanks. Upon transfer, sardines were individually marked, under anaesthesia (benzocaine at 140 ppm), using a tiny RFID (Radio Frequency Identification) tag (Biolog-id, Bernay, France, 0.03g, i.e. <0.2% of sardine lowest body mass) implanted in the dorsal muscle with a specific injector. This procedure caused less than 1% mortality and did not affect their swimming behaviour. After

10 days of acclimation to the new tanks, a 7-month experiment started, with details reported in (Queiros et al., 2019). Briefly, four feeding treatments were applied, comprising two different sizes of pellets (0.1mm and 1.2mm) at to two different quantities (0.3% and 0.6% of the biomass), with 2 tanks per treatment. That is, i) treatment LP-LQ: large particles in large quantities, ii) LP-SQ: large particles in small quantities, iii) SP-LQ: small particles in large quantities and iv) SP-SQ: small particles in small quantities. All other variables (e.g. water quality, temperature, photoperiod) were the same among tanks. Sardines were individually measured (length and weight) once a month and their tissues sampled on two occasions (at mid-experiment and at the end). This enabled us to assess the effect of food size and quantity on sardine growth, condition and physiological state (see Queiros et al., 2019 and Chapter 3). For these variables, we found that sardines from the LP-SQ and SP-LQ treatments exhibited very similar results (Queiros et al., 2019).

At the end of this experiment (June 15th, 2017), sardines were sampled randomly from the four feeding treatments and assigned to 8 smaller tanks (50L), to start the fasting respirometry experiment. About 150g of sardines were placed per tank but, because of differences in mean body mass of sardines from the four treatments, the number of individuals varied from 8 to 16 among tanks. Sardines were left to acclimate for 12 days in these new tanks before the experiment started.

One day prior to the experiment, sardines were measured again (length and weight) and body condition estimated as the Le Cren index (see Brosset et al., 2015). Body condition of sardines varied depending on their initial feeding treatment (Figure S10). Sardines fed LP-LQ had higher initial body condition than those fed LP-SQ or SP-LQ (Bonferroni-adjusted Wilcoxon tests, $P< 0.001$), these latter two did not differ ($P=0.87$). These three treatments all exhibited significantly higher body condition than sardines fed SP-SQ ($P< 0.001$). Therefore, we only consider three initial treatments in this experiment: (i) good initial feeding condition (sardines fed on LP-LQ), (ii) intermediate initial feeding condition (sardines fed on SP-LQ and LP-SQ) and (iii) poor initial feeding condition (sardines fed on SP-SQ). Unfortunately, due to a problem in the air system in two tanks, sardines died during one night (1 tank from the LP-LQ and 1 tank from the LP-SQ), the experiment was finally run in 6 tanks (see table 1).

Tanks were supplied with water pumped directly from the sea and filtered through sand (30–40 µm). The photoperiod was adjusted each week to follow the prevailing natural cycle and sea water temperature was not controlled, except to not exceed 25°C.

Biometries were performed once a week with all sardines measured individually (tag read, length and body mass recorded) under anaesthesia (benzocaine at 140 ppm). Body mass and total length were assumed to change linearly in between two biometries such that, when needed, daily values were estimated through interpolation. Tanks were checked at least three times a day for mortality and dead or moribund fish (on the flank, no attempt to escape) were removed on these occasions and immediately measured and weighed. At each biometry, the number and biomass of fish per tank were checked. Whenever the number of fish decreased below 5 in one of the tanks, fish of this tank were transferred to the other tank of the same treatment.

2.3. Respirometry

The rearing tanks were custom-designed to measure metabolic rate as O_2 uptake by automated stop-flow respirometry (Steffensen, 1989), as previously described in (McKenzie et al., 2007, 2012). Briefly, each tank was fitted with a central vertical PVC pipe that was perforated around the base. It housed a submersible pump that drew in water from the perforations and delivered it out through a flexible tube fixed to the outer wall of the tank, so constantly mixing the tank water. For 45 min of every hour, fresh aerated water was pumped from a large biofiltered reservoir (Vol. approx. 100 l) into the central PVC pipe of each tank, to maintain dissolved O_2 levels close to air saturation in the water holding the sardines; the water returned to the reservoir through a standpipe overflow. The pump in the reservoir was controlled by an electrical timer, and was turned off for 15 min of each hour, at which point the water level settled at the overflow to provide a constant volume, but the water continued to be mixed by the pump in the central pipe. Each tank was fitted with an O_2 optode (Pre-Sens sturdy dipping probe, www.presen.de) attached to an O_2 meter (Pre-Sens OXY-10 mini), which used the manufacturers software to record the linear declines in O_2 saturation in each tank, due to consumption by the sardines. Water O_2 saturation never fell below 70% during the 15 min of closed cycle respirometry and was rapidly restored when the tanks received a flow of aerated water from the reservoir. The fact that this flow entered the central pipe meant that the sardines were not aware of the hourly cyclical changes in flow regime.

Oxygen uptake by the fish (MO_2) was then calculated on the stored files using R software and a custom script. The O_2 saturation (in %) was transformed into O_2 concentration based

upon established values of O_2 solubility as a function of temperature and salinity. Temperature was monitored continuously by a probe linked to the O_2 meter, salinity was measured once a day every morning. The slopes of decreasing oxygen concentration over time were estimated through a linear model using an automated R script (see Figure S11); the first and last minute of the measurements were removed before estimating the slopes. Only slopes with an $R^2 > 0.8$ were retained, and measurements collected during fish handling or any intervention on the tanks were removed. The MO_2 was calculated in mg kg^{-1} h^{-1}, from the decline in water O_2 concentration and considering the total volume of water and the total biomass of the fish (McKenzie et al., 2007; Steffensen, 1989). The hourly measures of MO_2 were averaged to provide a measure of metabolic rate for the entire day. Standard metabolic rates represent metabolic costs of maintenance and were estimated as the 10%-quantile of daily measurements per tank for days in which more than 10 measurements were available. The surface of the tank was open to the atmosphere but surface exchange was so limited between air and water that no corrections were applied (McKenzie et al., 2007). A tank respirometer was run in parallel in the system, but without any sardines, to measure background oxygen consumption by the biofiltered water. This did not represent more than 5 % of fish MO_2, therefore no corrections were applied.

2.4. Sardines in the wild

Body conditions of reared sardines (i.e. from the above starvation experiment) were then compared to body condition of wild sardines before and after the sharp decline in their body condition (see below). Wild sardines were sampled from scientific surveys and commercial fisheries in the Gulf of Lions (NW Mediterranean Sea). PELMED (PELagiques en MEDiterranée, doi: 10.18142/19) surveys have been conducted each July since 1993 by the R/V "L'Europe", to assess small pelagic biomass in the Gulf with a combination of acoustics and trawls. A random sample of sardines in each trawl was collected and the morphometric variables of size (to the nearest mm) and body mass (to the nearest 0.1g), as well as maturity stage (by visual assessment according to ICES, 2008), were determined for each fish. Maturity stages were described on a scale from 1 to 6, with increasing development of gonads in stages 2 to 4, spawning period during stage 5 and post-spawning period during stage 6 and a resting period during stage 1. During other months, samples were collected from commercial fisheries and brought back to the lab for analyses. Samples consisted of

one crate of fish taken randomly from a pelagic trawl or a purse seine net before any sorting had occurred. Once in the lab, the same variables were measured as described for the scientific survey. Data for sardines therefore spans from 1971 to 1978 and 1993 to 2018.

Body condition was estimated for all sardines with the Le Cren index (see above). According to previous studies, sardine body condition decreased profoundly in 2008, to remain low since then (Brosset et al., 2015b; Saraux et al., 2019; Van Beveren et al., 2014). As a consequence, data were categorised into two periods: i) the past, being all data collected before 2008, i.e. 6764 sardines, and ii) the present, being all data collected since 2008, i.e. 14,668 sardines.

2.5. Statistical analyses

All statistical analyses were performed with R v.3.5.0 (R Core Team, 2018). Values are given in the text as mean ± SD, and statistical tests were considered significant at $p < 0.05$. When data were not independent from each other due to repetitions within individuals (e.g. body condition over time), a mixed model was used (either linear mixed model or generalized linear mixed model depending on the distribution of the data) with the individual effect set as a random intercept. Number of observations (n) and number of individuals (N) are then reported. Model selection was done according to Akaike's information Criterion (AIC), and when a difference in AIC was smaller than 2, the most parsimonious model was retained (Burnham and Anderson, 1998). When investigating binary variables, such as survival, a binomial distribution was used. Treatments or maturity stages were compared using Wilcoxon tests, as normality was violated. When multiple testing was performed (comparison between treatments, etc.), a Bonferroni correction was used (Legendre and Legendre, 2012). Finally, whenever appropriate, breakpoints in the data were identified using the "segmented" package in R (Muggeo, 2019).

3. Results

3.1. Body condition

The number of fasting days, the treatment and their interaction were all retained in the best model (as selected by AIC, LMM, n = 2090, N = 78) to explain body condition, suggesting that the decrease in body condition throughout the fasting period varied between treatment

(Figure 35). The rate of decrease in body condition was higher in sardines in good initial feeding condition (-0.008 ± 0.000 per day) than in sardines in intermediate or poor condition (-0.006 ± 0.000 per day for both cases).

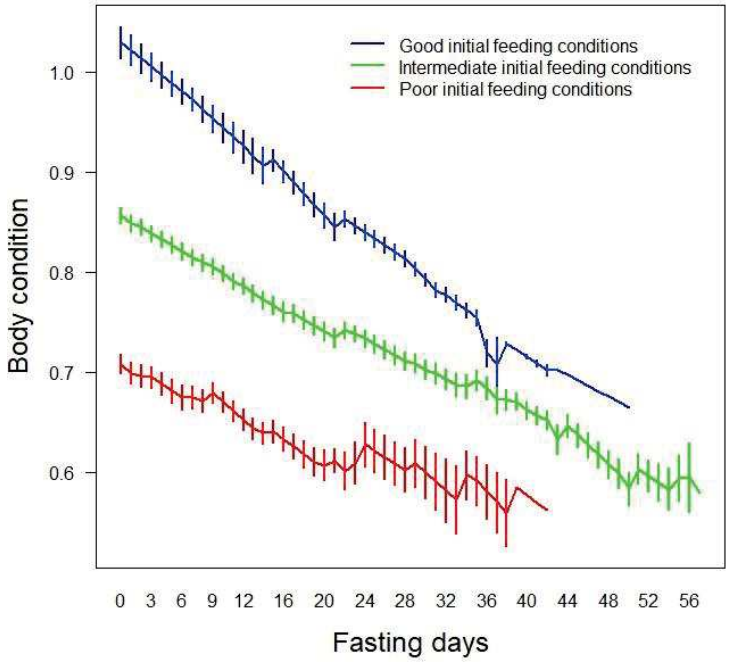


Figure 35: Mean ± SE body condition along the fasting experiment for each of the three initial feeding condition treatments.

3.2. Survival to fasting

During the experiment, sardines died between day 1 and day 57 (Figure S12). The cumulative death rate shows that a quarter of the sardines died after 2 weeks and half after 3 weeks, but it finally reached 90% after 50 days of fasting. The first mortality event occurred at a body condition of 0.84 for sardines in good initial feeding condition and slightly later for sardines in intermediate or poor initial condition (0.77; Figure S13).

The probability to survive 1-week of fasting was affected by body condition and the treatment the sardine originated from (GLMM binomial, n=313, N=78, Figure 36A). That is, it increased with body condition, but was lower in sardines in good initial feeding condition than in sardines in intermediate or poor initial condition (Figure 36A). Looking at all individuals together, the probability to survive 1-week of fasting decreased slightly from a body condition of 0.9 and then very rapidly between 0.75 and 0.6 (reaching 50% at 0.65; Figure 36B).

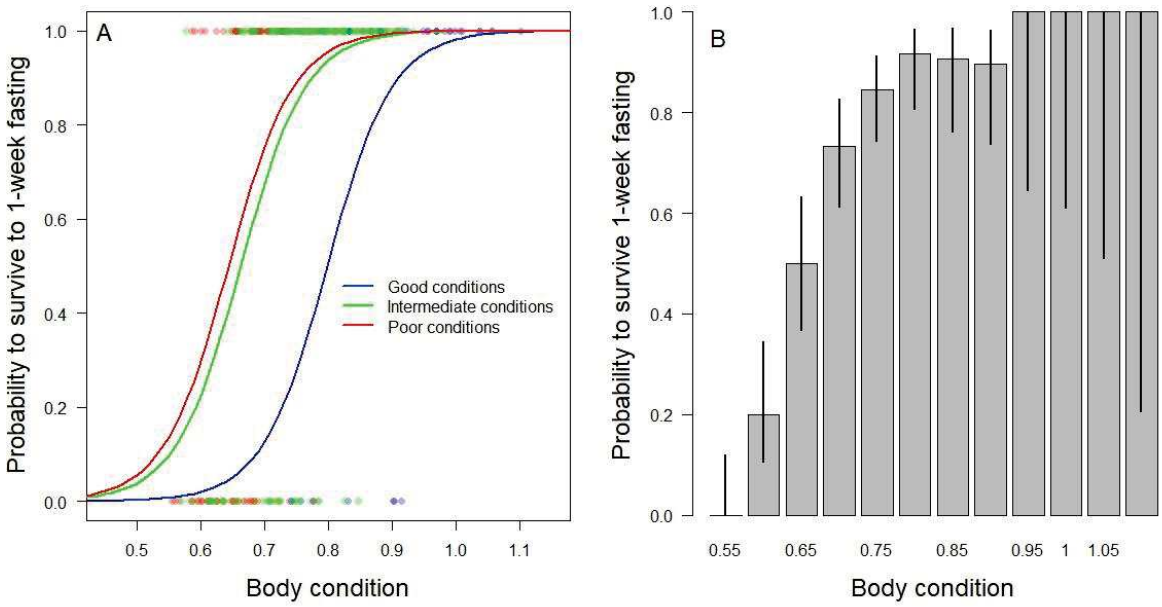


Figure 36: Probability to survive to 1-week fasting according to body condition. A) Empirical data are shown as 0 and 1 survival points, while lines represent the probability to survive predicted by the model according to their initial feeding conditions. B) Mean empirical probability to survive to 1-week fasting according to bins of body condition. The lines represent the 95% confidence interval associated with this empirical probability (according to a Bernouilli distribution).

The mean duration for which sardines were able to sustain fasting was higher for animals in good or intermediate initial condition (31.5 ± 4.3 d and 32.8 ± 2.5 d) compared to those in poor initial condition (16.3 ± 1.8 d). The number of days sardines survived fasting was best explained by their initial body condition and treatment group, but the interaction was not retained in the best model. The higher the initial body condition, the higher the number of days sardines survived (LM, *effect* = 142.6 ± 25.4 d, p-value < 0.001, n = 78; Figure 37). When the initial body condition was accounted for in the model, sardines in intermediate or poor initial feeding condition were able to sustain fasting for longer than sardines in good initial condition (LM, Intermediate – Good *effect* = 26.0 ± 6.2 d, p-value < 0.001, Poor – Good *effect* = 30.6 ± 9.3 d, p-value < 0.001).

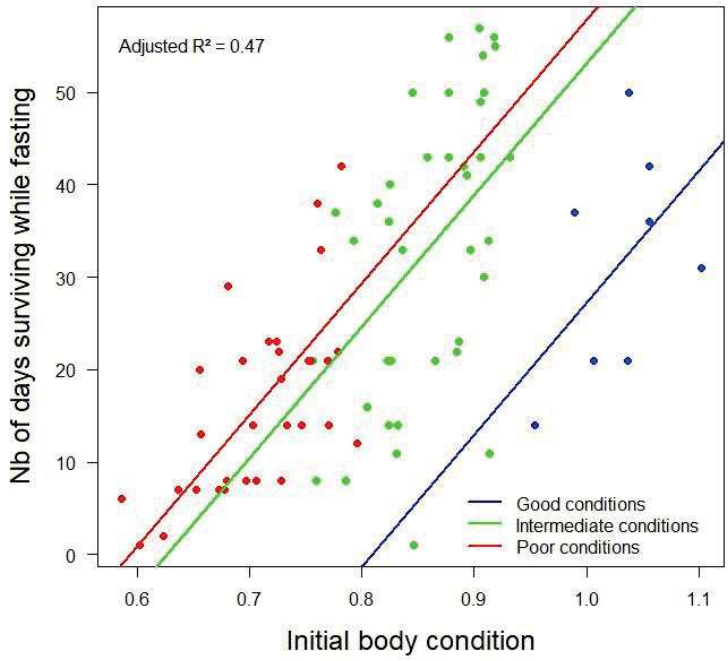


Figure 37: Number of days sardines survived fasting according to their initial body condition at the start of the fasting experiment. Colour indicates the feeding treatment sardines originated from and lines show the prediction from the LM for each of the treatment.

3.3. Specific body mass loss

Specific body mass loss increased sharply between 8 and 9 days before sardines died in all three treatments (Figure S14). During their first period of fasting, body mass loss was fairly constant around 0.79 ± 0.00 % of body mass per day (Figure 38). Eight days before death, this rate started increasing to reach a mean body mass loss of 3.59% ± 0.36% the day before death (Figure 38).

Similarly, a segmented regression model showed that specific body mass loss was relatively low and stable in sardines of body condition higher than 0.72 (0.84 ± 0.02 %), but increased sharply when body condition fell below 0.72 (Figure 39). This was similar among treatments (Figure S15)

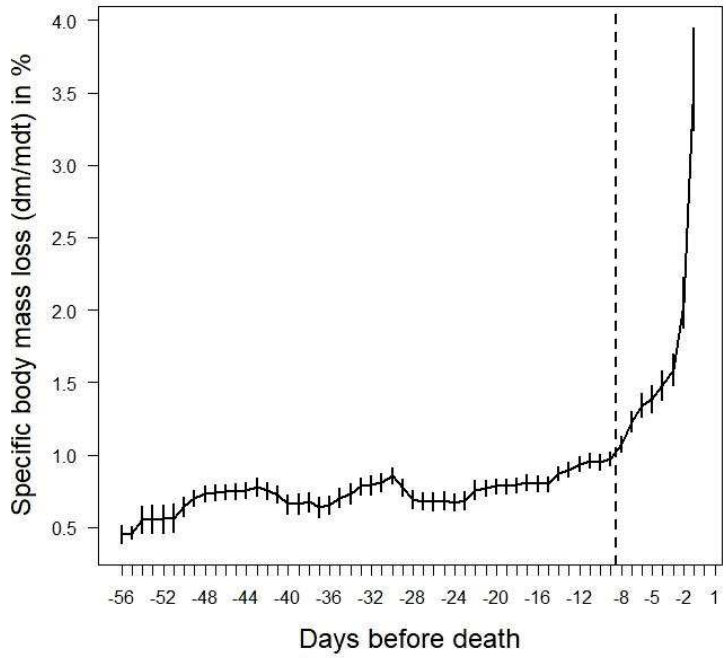


Figure 38: Mean ± SE specific body mass loss (dm/mdt) per day along time. As individuals died at different time in the experiment, the number of days has been estimated relative to death. The specific body mass loss is expressed as %. The vertical dashed line shows a rupture in the slope.

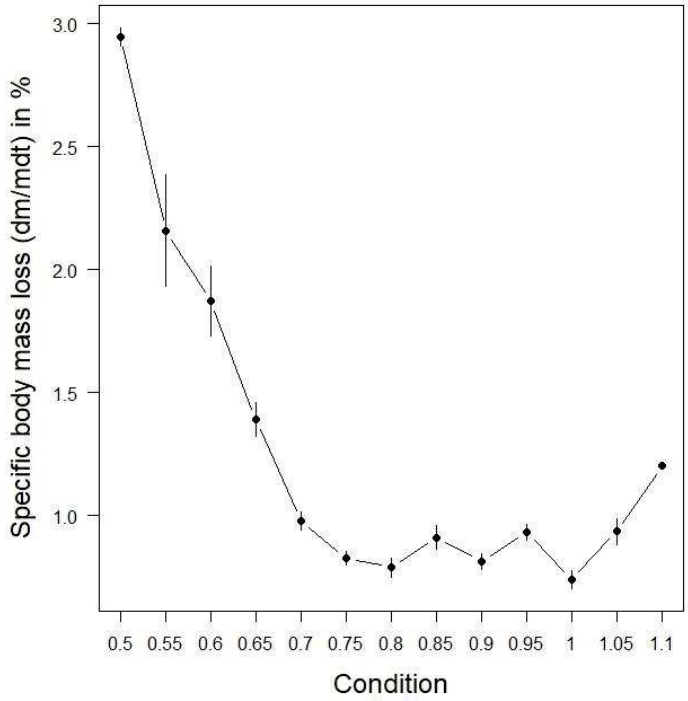


Figure 39: Mean ± SE specific body mass loss (dm/mdt) according to bins of body condition. The specific body mass loss is expressed as %.

3.4. Respiration rates

Focusing on the first 10 days of experiment, metabolic rates varied depending upon the initial feeding condition of the sardines (Figure S16). Notably, sardines in poor condition had a lower metabolic rate than sardines in good or intermediate condition (p-value = 0.019 for both, Bonferroni-corrected Wilcoxon tests). Standard metabolic rates (estimated as the daily 10% quantile and representing mostly maintenance metabolism) did not differ among treatments (p-value > 0.131, Bonferroni-corrected Wilcoxon tests; Figure S16). However, the difference between mean daily respiration rate and daily standard respiration rate was significantly lower in sardines in intermediate or poor initial feeding condition than in those in good initial condition (p-value = 0.003 and p-value < 0.001 respectively, Bonferroni-corrected Wilcoxon tests; Figure S16).

Metabolic rate increased strongly when sardine mean body condition decreased below 0.64 ± 0.01 in a tank (according to segmented linear regressions), while it was constant above this body condition (259.8 ± 41.6 mg $O_2.h^{-1}.kg^{-1}$; Figure 40). Interestingly, the breakpoint was similar in sardines in intermediate or poor initial condition (0.63 ± 0.02 and 0.65 ± 0.01 respectively) but was much higher in sardines in good initial condition (0.79 ± 0.04).

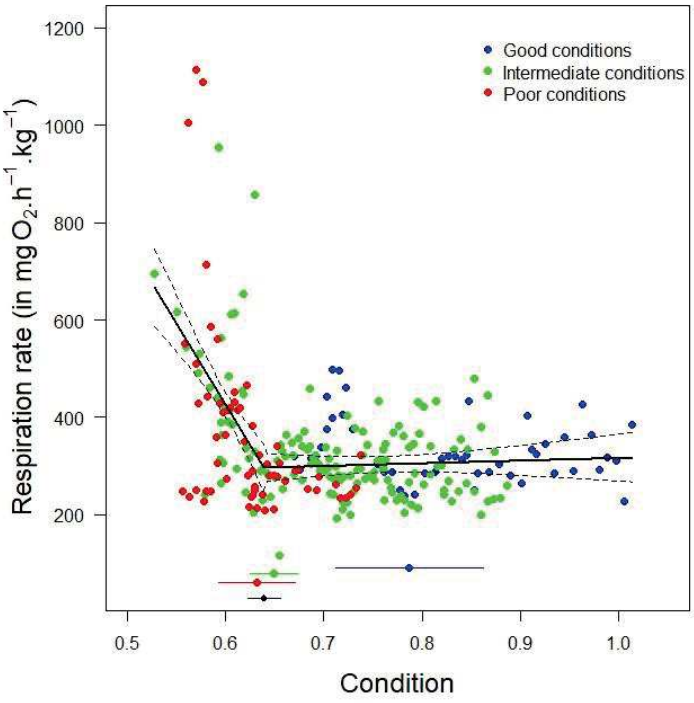


Figure 40: Mean daily metabolic rate (expressed in mg $O_2.h^{-1}.kg^{-1}$) of sardines in a given tank as a function of mean body condition of sardines in that tank that day. Colour indicates the treatment sardines originated from. The segmented regressions are indicated by the black line and the 95% confidence intervals with dashed lines. The breakpoint along with its 95% CI is also indicated at the bottom of the figure in black. Breakpoints and their 95% CI estimated for each treatment are indicated at the bottom of the figure in colour.

3.5. Body condition in the wild

Sardine body condition in the wild was significantly higher in the past (i.e. before 2008) than in the present (1.12 ± 0.00 vs. 0.98 ± 0.00, Wilcoxon test, p-value < 0.001, n = 6764/14668 respectively, Figure S17). Further, body condition varied among months, peaking in spring/summer (as well as in early autumn for the past period only) and reaching its lowest level in December, January and February for both periods (Figure S17). Finally, body condition decreased with maturity stages in both periods (Figure S18, Bonferroni-corrected Wilcoxon tests). While it decreased almost linearly with maturity stages in the present, the contrast appears mainly to be between maturity stages 5 and 6 (i.e. during or post-spawning) and among the first four maturity stages in the past (despite no significant differences in some cases due to very low sample size in some maturity stages).

When comparing against the critical body condition defined in our experiments, only 0.1% of the sardines sampled before 2008 were below the 0.65 threshold that appeared critical for 1-week survival, and only 0.2% of the sardines sampled since 2008. The occurrence of sardines below the second body condition threshold (i.e. 0.72 which corresponds to the entry into phase 3 fasting according to body mass loss, Figure 39) also appeared rather low, although it almost doubled when comparing the present to the past (2.3% vs. 1.2%; Figure 41). The occurrences were not, however, evenly distributed among months, being more probable during winter, especially in the present period, where they reached 6 and 9 % of the population in January and February, respectively (Figure 41).

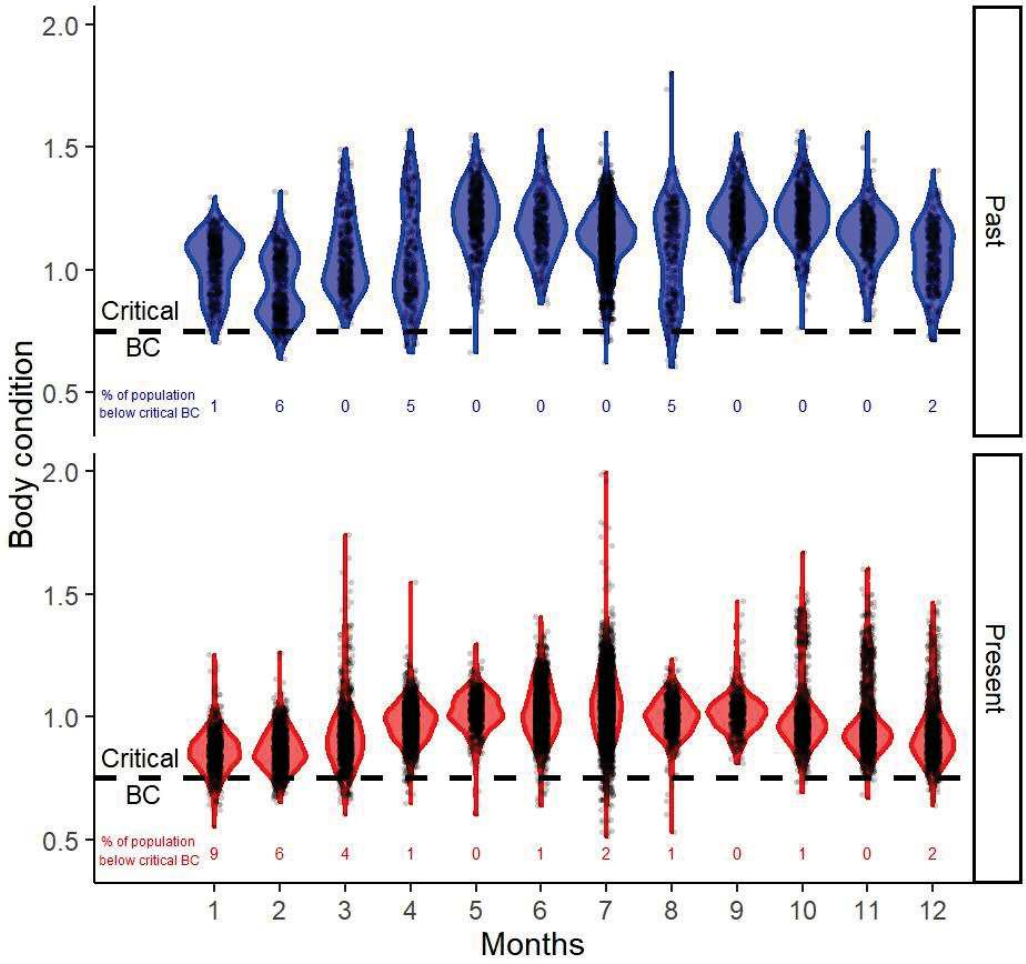


Figure 41: Distribution of body condition of sardines sampled in the wild before (in blue, upper panel) or after 2008 (in red, lower panel) for each month of the year. Horizontal dashed lines indicate the threshold of body condition corresponding to an entry in phase 3 of fasting. The percentage of the population below this critical threshold of body condition each month is indicated at the bottom of each panel.

4. Discussion

Although there has been quite significant research focus on starvation as a cause of mortality in larval and juvenile fishes (Hurst 2007), studies on adult fishes are much rarer (Dutil and Lambert, 2011; Lambert and Dutil, 1997). As mortality is complicated to observe in marine fish populations, we used experiments in tanks to investigate the extent to which wild adult sardines might be at risk of death from starvation. The results revealed that sardines were able to survive fasting for extended periods (up to 56 days under our conditions) and to reach very low body condition before they risked mortality from starvation. It is well known that fishes can survive extended periods of food deprivation although the actual duration varies among species, life stage and water temperature

(McCue, 2010; Navarro and Gutiérrez, 1995; Wang et al., 2006). According to our study, survival in sardines remained high until a Le Cren's body condition of 0.75 (i.e. 25% lower than the global average from measurements on wild populations). When fish condition decreased below this, survival dropped rapidly, to only 50% at a body condition of 0.65.

The experimental approach was able to reveal when the sardines started relying on protein as the main fuel for metabolism. That is, specific body mass loss and mass-specific metabolic rates increased markedly below a certain body condition, enabling us to define a critical body condition that indicated when sardines entered into phase III of fasting. Despite clear thresholds for mean population responses (see for instance Figure 39), there was significant variation among individuals, especially when they were at very low condition. Such inter-individual differences might reveal the importance of individual quality in sardine physiological responses to starvation and survival, despite individual quality being still a debated concept and terminology (Bergeron et al., 2011; Wilson and Nussey, 2010). Individuals within fish species are known to exhibit wide variation in their tolerance of feed deprivation, and this can have both a physiological and behavioural basis (Auer et al., 2016; McKenzie et al., 2014; Norin and Metcalfe, 2019; O'Connor et al., 2000). Thus, further studies are required to elucidate the physiological or behavioural correlates of the individual responses to a fasting challenge in sardines. In the current study, the critical threshold for entry into phase III of fasting was much more accurately defined by the individual indicator of specific body mass loss than by the tank level indicator of group metabolic rate. That is, although individual fish in a given tank derived from the same initial feeding condition, their body condition varied at the beginning of the experiment (Table 1). Further, rates of mass loss and metabolism both sped up during phase III of fasting, leading to death in about 8 days. Fish transitioned from phase II to phase III over a short period, such that a mixture of fish in the different fasting phases were present in a given tank at a given time, contributing to the group's overall metabolic rate. Nonetheless, the tank respirometry clearly indicates that entry into phase III of starvation was associated with a marked increase in metabolic rate by the sardines. It is not known why this occurred; it may have reflected the lower energetic efficiency of using proteins as a main fuel compared to lipids or a desperate increase in activity in search of food (the so-called refeeding signal described in birds and mammals; Groscolas et al., 2000; Koubi et al., 1991; Robin, Boucontet, Chillet, & Groscolas, 1998; Spée et al., 2010), which would obviously have exacerbated the rate of mass loss.

When compared to in situ values observed over the years, the proportion of the population sampled in nature that was below the critical condition of entry in phase-3 fasting was minimal (≤ 2%). Moreover, almost none of the fish sampled in-situ exhibited the low body condition with a 50% probability of surviving a week (i.e. 0.65). Such a result is very similar to that obtained in Atlantic cod (Dutil and Lambert, 2011), but needs to be taken cautiously, as sardines of low body condition might be excluded from schools or might already have died for other reasons linked to a weakened physiological state. Indeed, low body condition can seriously impair fish swimming activity (Faria et al., 2011; Martinez, 2003). As a consequence, starving sardines might not be able to sustain the continuous aerobic swimming required to follow the school and might get isolated. Poor swimming performance, especially burst swimming capacity (Martinez, 2004) would render them prone to predation, also due to the absence of the dilution effect that is gained by being in a school (Lehtonen and Jaatinen, 2016; Rieucau et al., 2014). This raises the important point that our estimates of mortality in tanks represent a maximum potential of sardines to resist fasting and that it is highly likely that mortality would occur much earlier in the wild. Indeed, the fasted sardines were very weak during the experiment, as attested by the increase in death rate during and after biometries (Figure S12). Apart from handling once a week, our fish were subjected to less stress than in nature, with no predation, pathogens, etc.

Nevertheless, the proportion of Gulf of Lions sardines that were below the critical condition of entry in phase III of fasting (i.e. 0.72) was about twice as high in the present period (2.3%), after the changes in population condition and age structure were observed (Saraux et al., 2019; Van Beveren et al., 2014), than in the past (1.2%). Interestingly, when looking at the monthly values, the critical condition of entry in phase III occurred mostly in January/February (reaching 9%), which corresponds to the end of the spawning season for sardines and also to the coldest period. The fact that, among maturity stages, the proportion of body condition below the critical threshold was highest in stage 6, post-spawning, suggests that a depletion of reserves partly due to reproduction remains a valid hypothesis (Brosset et al., 2016b). While we cannot derive mortality estimates directly from this study, the results confirm that mortality might have increased after 2008 and be primarily in winter at the end of reproduction.

Another interesting finding corresponds to the differences observed among our three treatments. Sardines that were in intermediate or poor feeding condition at the outset

displayed a much stronger resistance to fasting than fish that were in good feeding condition. This not only shows that extended caloric restriction promotes subsequent tolerance of fasting (McCue et al., 2017) but, most of all, it clearly demonstrates a plastic response by the fish to their environmental conditions. All the sardines were caught in the wild and our assignment to the different treatments ensured that they were in similar body condition before starting the feeding trials described in experiment (Queiros et al., 2019). Thus, it seems extremely unlikely that the differences in fasting tolerance among the treatments in the current study could derive from genetic selection or early environment exposure. They were more likely a plastic response at the adult stage (McCue et al., 2017). The fact that body condition did not drop as fast in sardines that had been maintained for an extended period in a state of caloric restriction presumably reflects differences in energy expenditure. This is borne out by the tank respirometry, at the start of the experiment (1st 10 days), daily metabolic rates were lower in sardines in poor initial feeding condition than in the other two treatments (Figure S16). It is well established that fishes reduce their metabolic expenditure when exposed to caloric restriction or extended fasting and that the two main strategies are a reduction in spontaneous activity and a reduction in basal metabolism (Auer et al., 2016; McKenzie et al., 2014; Norin and Metcalfe, 2019). These two processes are not mutually exclusive (Auer et al., 2016). While further work is required to establish the relative contributions of behaviour and physiology, in particular video tracking to quantify activity and studies of metabolism from the whole animal down to the cell and mitochondrion (Auer et al., 2016), the tank respirometry provides some preliminary insights. If we assume that the 10%-quantile rate of O_2 uptake each day was an estimate of standard metabolic rate, this indicates that costs of maintenance were similar among treatments. When these daily values were, however, subtracted from daily mean metabolic rate, the difference was significantly higher in the fish in good initial condition, compared to the other two treatments. This difference in metabolic rate, of the fasting fish, might represent in large part costs of spontaneous swimming activity, perhaps exploratory foraging (Auer et al., 2016). This indicates that the fish that had been exposed to caloric restriction had developed an adaptive behavioural plasticity to reduce their energy expenditure. The fact that fish in good initial feeding condition, with no history of caloric restriction, were not able to engage this adaptive response indicates that such behavioural plasticity is not instantaneous. One potential explanation for this is that the responses to caloric conditions are under neuro-

hormonal or endocrine control, and require some weeks or months to be expressed (Secor and Carey, 2016).

To sum up the Chapter 4, the take-home messages are:

- Sardines were highly resistant to fasting when maintained in tanks free of stressors such as predation or pathogens.
- Experimental measurements of specific body mass loss and metabolic rates enabled us to define a critical threshold of body condition, which we could then relate to in situ measurements.
- Despite probably being an underestimation, we showed that the proportion of the wild sardine population that was below such a critical threshold increased in the present and was highest in the winter post-spawning period.
- We showed that, when previously maintained under caloric restriction, sardines display important behavioural plasticity that improved their ability to tolerate fasting, by reducing rates of body mass loss and so increasing their survival.

Chapter 5: Can adult overmortality follow reproduction?

1. Introduction

Small pelagics play a key role in marine ecosystems especially in upwelling ecosystems, transferring energy from lower to upper trophic levels (Cury et al., 2000; Essington et al., 2006). Thus, they operate major controls on the entire marine food web through top-down control on the plankton communities, bottom-up control on their predators or wasp-waist control when both top-down and bottom-up controls occur simultaneously (Cury et al., 2000). Fluctuations in populations of small pelagics can have critical ecological, economic and social consequences due to their huge commercial significance for fisheries (Alheit et al., 2009; FAO, 2018; Fréon et al., 2005).

Small pelagic fish have a worldwide distribution and are especially well studied in the large EBUS (Eastern Boundary Upwelling Systems) owing to their higher productivity (e.g. Barange et al. 2009, Bertrand et al. 2011, van der Sleen et al. 2018). Even if landings are lower compared to EBUS, small pelagics are also very important for fisheries in other non-upwelling systems, such as the Mediterranean basin where small pelagics represent 38% of the total catch (Lacoue-Labarthe et al., 2016). The Gulf of Lions is located in the northwestern Mediterranean Sea and is one of the most productive areas in the Mediterranean Sea. Sardines (*Sardina pilchardus*) and anchovies (*Engraulis encrasicolus*) could represent 50% of the total annual landings in the Gulf of Lions until the 2000s (Demaneche et al., 2009). However, landings of small pelagics have sharply decreased since 2008 in this area reaching their lowest values in 150 years and remaining low thereafter (GFCM, 2017b; Van Beveren et al., 2016a). Simultaneously, the mean weight and length as well as the body condition of small pelagics have dropped in the Gulf of Lions (Van Beveren et al., 2014). The drop in catches was thus not explained by a decrease in abundance, but by the absence of market for smaller and skinnier sardines (Saraux et al., 2019). The decrease in size resulted from the combination of a lower growth and the disappearance of the oldest and the largest individuals from the population, leading to a demographic truncation of the population which is now highly dominated by 0 to 2 years old fish (Brosset et al., 2015b; Saraux et al., 2019; Van Beveren et al., 2014). Surprisingly, these changes in population dynamics were not explained by overfishing or changes in recruitment because the

exploitation rate was rather low and recruitment remained high (Saraux et al., 2019). This unusual situation was neither explained by emigration nor top-down controls (fishing or natural predators) nor diseases (Queiros et al., 2018; Saraux et al., 2019; Van Beveren et al., 2016b, 2017). The most likely hypothesis remains an environmentally-driven change of the plankton production (in terms of quantity and/or quality) to explain the current situation of sardine and (to a lesser extent) anchovy in the Gulf of Lions (Brosset et al., 2015b; Saraux et al., 2019; Van Beveren et al., 2014). A recent experimental study further supported this hypothesis showing that both food size and quantity are crucial for sardine growth and body condition (Queiros et al., 2019). Indeed, individuals that fed on small prey needed to eat twice as much to reach the same length and body condition as sardines that fed on larger ones (Queiros et al., 2019). Furthermore, sardines are known to be mainly capital breeders in the Gulf of Lions (Ganias, 2009; McBride et al., 2015), i.e. they store energy when food resources are high (in spring) and later use these reserves during the reproduction period when food resources are more limited (in winter). Brosset et al. (2016) suggested that sardines maintained high reproductive investments in the recent period (i.e. after 2008) despite their decrease in body condition and reserves. When facing low feeding conditions, individuals need to trade-off their energy allocation between different life-history traits. If investment in reproduction did not change, this implies less energy for other functions, such as maintenance. Because reproduction occurs in winter when food abundance is lower, it could be detrimental for fish if feeding conditions dropped a lot during or after the reproduction period as they may not have enough energy for themselves.

In the present work, we thus aimed to examine if the persistence of reproduction at low body condition would lead to adult overmortality in the sardine population of the Gulf of Lions; a process that would explain the current demographic truncation observed in this population since 2008. To do so, we applied Dynamic Energy Budget (DEB) theory (Kooijman, 2010; Nisbet et al., 2000) to model individual life-history traits and the allocation trade-offs between them. In particular, we examined growth and reproduction, with a focus on the reserves available during and after reproduction to pay maintenance costs. DEB models have been widely used to study small pelagics fish ecological traits (Gatti et al., 2017; Pethybridge et al., 2013), including reproduction (Einarsson et al., 2011; Pecquerie et al., 2009). Yet, reproduction data for multiple-batch spawners are scarce and require dedicated modeling assumptions. Here, we developed an “abj” DEB model (i.e. with acceleration during the

larval stage, (Kooijman, 2014; Kooijman et al., 2011) extended with a gonad compartment to account for the batch reproduction of small pelagics fish and including the use of GSI (gonadosomatic index) data in the estimation procedure. Then, we modeled a sardine population based on in-situ data and we followed individuals over time to investigate their survival according to temperature and food scenarios. Finally, we discuss the use of these models to investigate future environment-driven changes on growth, reproduction and survival of sardines.

2. Material and methods

2.1. Standard DEB model and reproduction module

The standard Dynamic Energy Budget (DEB) model (Kooijman, 2010; Nisbet et al., 2000) aims to describe the individual energy flows from the intakes to the uses as growth or reproduction during its life cycle as a function of temperature and food. The standard DEB model deals with one type of food, one type of reserve and one type of structure and also assumes that shape of structure does not change during growth (isomorphy). Each individual is described by four state variables, i.e. the structural volume V (in cm^3), the reserve energy E (in J), the cumulative energy invested into development E_H (in J) and the reproduction buffer E_R (in J). Energy assimilated from food is stored in reserve energy before being used by the following processes: growth and somatic maintenance of structure, allocation of energy to development (in embryos, larvae and juveniles) or to the reproduction buffer (in adults) and maturity maintenance (Figure 42). Individual starts feeding at birth (mouth opening), allocates energy to maturation until it reaches puberty and then this energy is allocated to the reproduction. The DEB model used in this study was the 'abj' model with type M acceleration (Kooijman, 2014), i.e. we assumed growth acceleration between birth and metamorphosis. In the present work, we added a new compartment to take into account that sardines are multiple-batch spawners and that most of the reproduction data available are gonadosomatic index data. Thus, we added a gonad compartment (E_{Go} in J) as a new state variable (Figure 42) and the energy flux from the reproduction buffer to the gonad is function of the season and the size of the individual, as larger females release larger batches of oocytes (see Supplementary Information – Appendix A). Equations of the DEB model used in this study are given in Table 2.

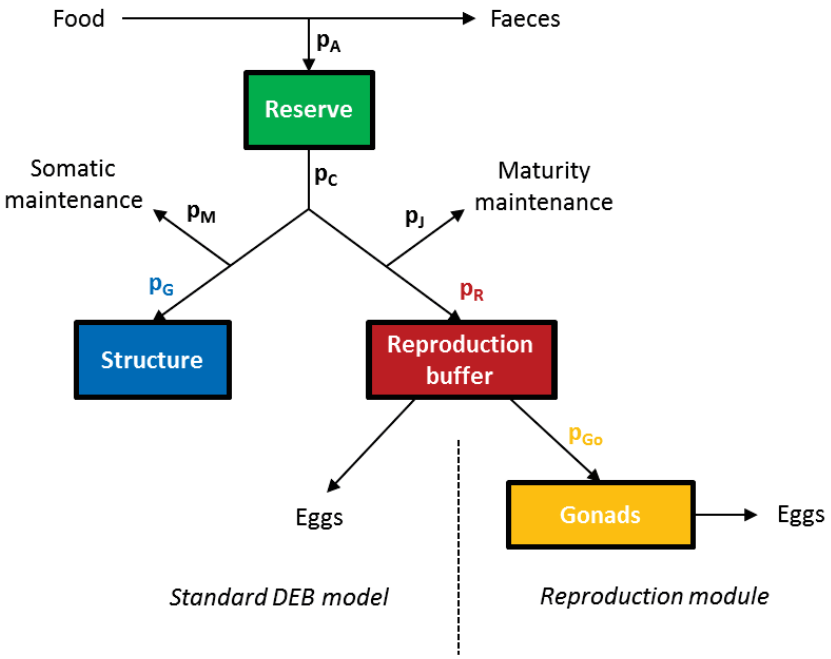


Figure 42: Conceptual framework of the energy flows in the standard DEB model and the specific reproduction module for the Mediterranean sardine.

Table 2: Equations of the DEB model used in this study (see Table 3 for DEB parameter values)

Fluxes / State variables	Formula
Assimilation	$\dot{p}_A = f\{\dot{p}_{Am}\}V^{2/3}$
Mobilization	$\dot{p}_C = E\frac{[E_G]\dot{v}V^{2/3} + \dot{p}_M}{\kappa E + [E_G]V}$
Somatic maintenance	$\dot{p}_M = [\dot{p}_M]V$
Growth	$\dot{p}_G = \kappa\dot{p}_C - \dot{p}_M$
Maturity maintenance	$\dot{p}_J = \dot{k}_J E_H$
Maturity/Reproduction	$\dot{p}_R = (1-\kappa)\dot{p}_C - \dot{p}_J$
Allocation to gonads	$\dot{p}_{Go} = [\dot{p}_{Go}]V$
Reserve energy	$\frac{dE}{dt} = \dot{p}_A - \dot{p}_C$
Structural volume	$\frac{dV}{dt} = \frac{\dot{p}_G}{[E_G]}$
Maturity energy	$\frac{dE_H}{dt} = \dot{p}_R(E_H < E_{Hp})$
Reproduction buffer energy	$\frac{dE_R}{dt} = \dot{p}_R(E_H = E_{Hp})$
Gonad compartment energy	$\frac{dE_{Go}}{dt} = \kappa_R\dot{p}_{Go}$

2.2. Links between state variables and observations

Because state variables are not directly measurable in the field, the calibration and validation of the model requires transformation which link these state variables to observations.

The physical length L (total length that we measured, in cm) was calculated using the structural volume V (cm^3) and the shape coefficient δ such as:

$$L = \frac{V^{1/3}}{\delta} \quad [6]$$

Here, we assumed that shape was different before and after metamorphosis, i.e. larvae (δ_{larvae}) vs. juvenile and adult stages (δ). Further, the wet weight W (g) is the sum of the weight of the structure (W_V), the energy reserve (W_E), the reproduction buffer (W_R) and the gonads (W_{Go}). Assuming that reserve, reproduction buffer and gonads have the same composition (so the same energy content), the total wet weight W (in g) and the gonadosomatic index GSI (in %) are calculated as follows:

$$W = d_V V + \frac{E+E_R+E_{Go}}{\rho_E} \quad [7]$$

$$GSI = \frac{E_{Go}/\rho_E}{W} \times 100 \quad [8]$$

with d_V the density of the structural volume (assumed to be equal to 1 g cm^{-3}) and ρ_E the energy content of 1 g of reserve (J g^{-1}).

The body condition of individuals was also calculated using the Le Cren index as estimated by Brosset et al. (2015):

$$K_n = \frac{W}{0.00607 \times L^{3.057}} \quad [9]$$

with W the wet weight in g and L the total length in cm.

All physiological rates are dependent on body temperature. For a species-specific optimal range of temperatures, we used the following temperature correction function (Kooijman, 2010):

$$k(T) = k(T_{ref}) \times cor_T \quad [10]$$

$$cor_T = \exp\left(\frac{T_A}{T_{ref}} - \frac{T_A}{T}\right) \quad [11]$$

with the absolute temperature T (in K), the chosen reference temperature T_{ref} (here set at 293 K, i.e. 20°C), the Arrhenius temperature parameter T_A (in K), and k a given physiological rate at T and T_{ref}, respectively. In the present work, we did not consider the effect of temperature outside of the optimal range for the Mediterranean sardine population for simplicity's sake. However, these effects are fully documented in (Kooijman, 2010) and at the cost of four extra parameters, would be easily implementable in the model.

2.3. Parameter estimations

To estimate model parameters (Table 3), we used a minimization algorithm with a defined simulation setting. We used the AmP procedure implemented in the Matlab routines of the DEBtool software (Marques et al., 2018). This method aims to minimize the weighted sum of squares deviation between model predictions and observations (Marques et al., 2019). We thus compared model predictions to the following observations from both experiment and in-situ data: ages, length at age, weight at age, gonadosomatic index across time, length across time, weight across time and weight-length relationships (Table 4).

2.4. Specific handling rules to pay maintenance costs

To cover maintenance costs during food limitations (e.g. during winter), we used handling rules for which the modification of energy flows were detailed in Figure 43. First, if somatic maintenance costs could not be paid by energy flow from reserve, they were paid from the reproduction buffer and as soon as this buffer was empty, structure was used to pay maintenance costs (similarly to phase III presented in Chapter 4). The re-assimilation of the vitellogenic oocytes in the gonads (i.e. atresia) is an usual phenomenon observed in sardine. The occurrence and intensity of atresia mostly occur during the regression phase of gonads (Brosset et al., 2016b), so that, we hypothesized that only energy from the reproduction buffer could be used to cope with extra maintenance costs. The maturity maintenance costs being small relatively to the somatic maintenance costs, we also assumed that they were

always covered. More complex assumptions could be implemented in the model, but this would require a dedicated study to explore the different physiological mechanisms that sardine may implement to cope with food limitations (e.g. in Chapter 4) and an extensive sensitivity analysis which was outside the scope of the present work.

Table 3: Parameter values of the sardine DEB model. Rates are given at the reference temperature T1 = 286 K (= 13◦C).

Symbol	Value	Units	Definition	Reference
T_{ref}	293	K	Reference temperature	
T_A	8000	K	Arrhenius temperature	Lika et al., 2011
κ_X	0.8	-	Digestion efficiency of food to reserve	
κ_P	0.1	-	Faecation efficiency of food to faeces	
κ_R	0.95	-	Reproduction efficiency	
$\dot{k}_J$	0.002	d^{-1}	Maturity maintenance rate coefficient	
s_G	0.0001	-	Gompertz stress coefficient	
$\{\dot{p}_{Am}\}$	53.8	J cm^{-2} d^{-1}	Maximum assimilation rate	*Calibration*
$\dot{v}$	0.068	cm d^{-1}	Energy conductance	
κ	0.88	-	Allocation fraction to soma	
$[\dot{p}_M]$	58.61	J d^{-1} cm^{-3}	Volume-specific somatic maintenance	
$[E_G]$	5035	J cm^{-3}	specific cost for structure	
h_a	6.17×10^{-9}	d^{-2}	Weibull aging acceleration	
δ	0.196	-	Shape coefficient after metamorphosis	
δ_{larvae}	0.104	-	Shape coefficient for larvae	
E_H^H	0.033	J	Maturity at hatch	
E_H^B	0.12	J	Maturity at birth	
E_H^J	12.86	J	Maturity at metamorphosis	
E_H^P	8611	J	Maturity at puberty	
f_estim	0.80	-	In-situ scale functional response	
$[\dot{p}_{Go}]$	2.69	J d^{-1} cm^{-3}	Volume-specific allocation rate to gonads	

Table 4: Observations and relative errors between model predictions and observations. The reference pers. comm. refers to experimental data obtained during my thesis.

Data	Value	Units	Reference	Relative error
Age at hatch at 13.5°C	3	d	pers. comm.	0.028
Age at hatch at 12.6°C	3.4	d	pers. comm.	0.005
Age at birth at 13.5°C	9.5	d	pers. comm.	0.218
Age at metamorphosis at 16.5°C	45	d	(Iglesias and Fuentes, 2014)	0.044
Age at puberty at 17°C	365	d	(Brosset et al., 2016b)	0.067
Life span at 17°C	2920	d	Ifremer database	0.023
Yolk diameter	0.10	cm	pers. comm.	0.015
Length at hatch	0.36	cm	pers. comm.	0.038
Length at birth	0.54	cm	pers. comm.	0.253
Length at metamorphosis	1.7	cm	(Garrido et al., 2016)	0.105
Length at puberty	9.6	cm	(Brosset et al., 2016b)	0.226
Ultimate total length	20	cm	Ifremer database	0.158
Egg dry weight	0.039	mg	pers. comm.	0.126
Weight at birth	0.25	mg	(Ré and Meneses, 2008)	0.347
Weight at puberty	6.1	g	Ifremer database	0.083
Ultimate wet weight	57.6	g	Ifremer database	0.244
GSI at several food levels	[1.4 ; 4.9]	%	pers. comm.	[0.086 ; 0.293]
In-situ total length over time	Figure 3A		Ifremer database	0.073
In-situ wet weight over time	Figure 3B		Ifremer database	0.259
Weight-length relationship fraction to soma	Figure 3C		Ifremer database	0.239
GSI over time for two fish lengths	Figure 3D		Ifremer database	[0.432 ; 0.454]
Total length over time at several food levels	Figure 4A		pers. comm.	[0.020 ; 0.435]
Wet weight over time at several food levels	Figure 4B		pers. comm.	[0.005 ; 0.175]
Weight-length relationship at several food level	Figure 4C		pers. comm.	[0.124 ; 0.164]
Total length of larvae over time	Figure 4E		pers. comm.	0.494

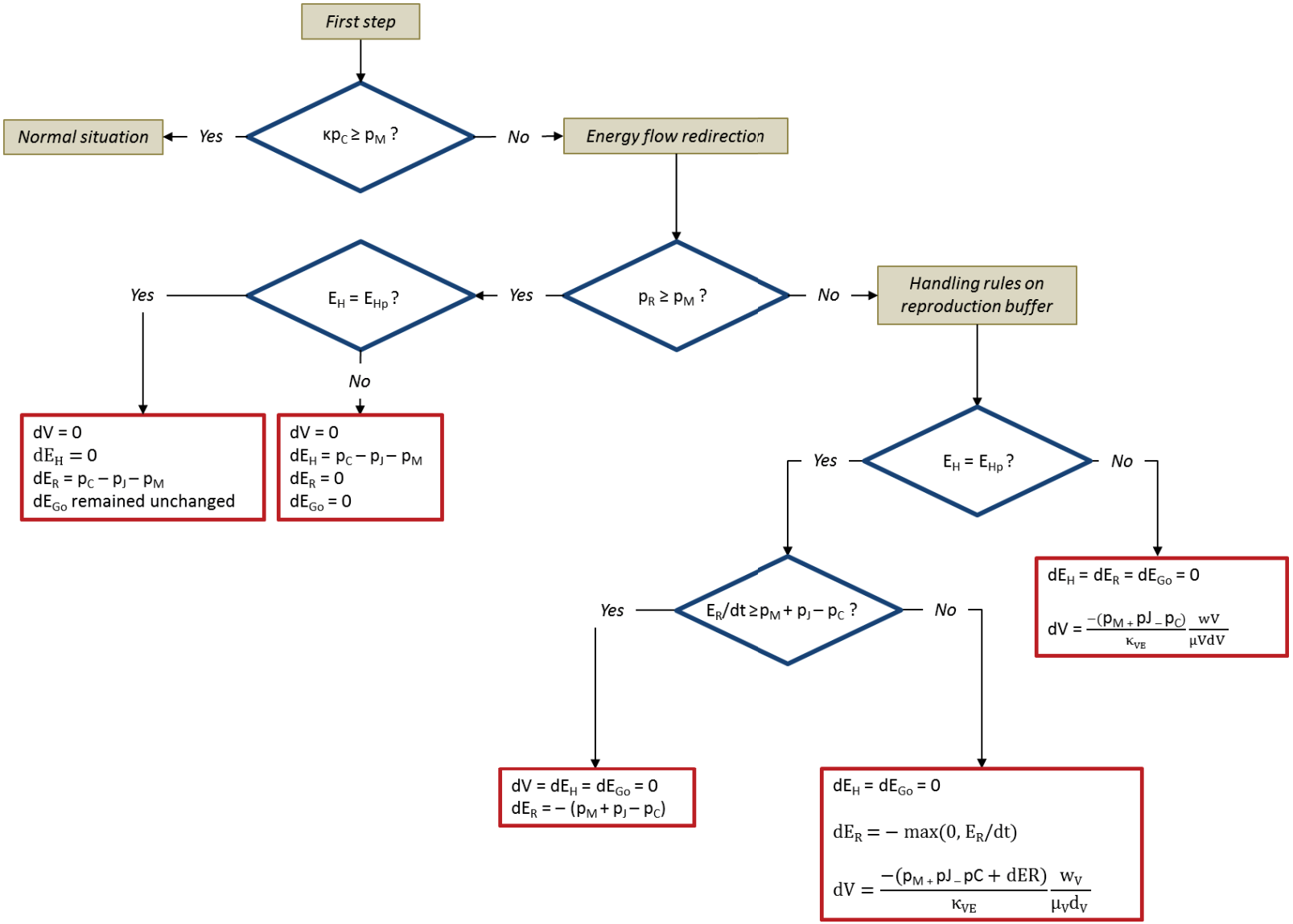


Figure 43: Framework of the specific handling rules to pay maintenance costs

2.5. Simulation

The objective of this simulation was to investigate the hypothesis of the adult sardine overmortality after reproduction. To this end, we used our sardine DEB model with the specific handling rules to pay maintenance costs applied on a simulated sardine population, each individual facing temperature and scaled functional response derived from the wild. To do so, we had to define the environmental conditions (here food and temperature) that would be used for the simulations as well as to specify the initial conditions for the DEB model.

2.5.1. Forcing variables: temperature and food density

The monthly temperature cycle was obtained from the outputs of the 3D hydrodynamical model SYMPHONIE applied to the French continental shelf of the Gulf of Lions (0–200 m isobaths) between 2008 and 2017 (see Marsaleix et al. 2008). The position of sardine in the water column can be managed at a hourly scale with this model but opposing results have been found according to the study area (Giannoulaki et al., 1999; Zwolinski et al., 2007). Thus, for simplicity's sake, we assumed that sardines experienced the monthly median temperature of the Gulf of Lions and temperature was integrated on the full height of the water column.

The plankton cycle was derived from the outputs of the coupled 3D hydrodynamical model SYMPHONIE and the biogeochemical Eco3M-S model (Kessouri et al., 2017) applied to the Gulf of Lions (0-200 isobaths) between 2008 and 2013. We used monthly mean plankton concentrations to build plankton time series. Sardine prey size had a bimodal distribution, between 100 and 600 µm and between 700 and 1400 µm (Le Bourg et al., 2015). But prey smaller than 200 µm may also represent a significant part of their diet (Nikolioudakis et al., 2012). Thus, we summed micro and mesoplantkon (both phyto and zoo) concentrations to build food density time series. Then, to obtain a scaled functional response (= ingested food as a function of food density) of type II (with saturation), we used the equation $f = X/(X + X_K)$ with X the plankton concentration (in mg of C) and X_K the half-saturation coefficient (in mg of C). The half-saturation coefficient was estimated using the constraint that the in-situ scaled functional response found during parameter estimation (f_estim) was equal to the mean of the time series of f_insitu ($=X / (X + X_K)$).

For both temperature and food density, we fitted Fourier series on the mean values of each month to obtain continuous function for the integration of the differential equations and we converted food density in scale functional response time series (Figure S19).

2.5.2. Initial conditions for the sardine population

The starting point of the simulations was defined on March 1st, which corresponds to the end of the spawning season. The simulated sardine population was composed of 10,000 individuals and for each individual, we defined values for each of the five state variables: V, E, E_H, E_R, E_{Go}.

First, we derived length and body condition of the 10,000 individuals from data provided by commercial pelagic trawlers on March between 2008 and 2019 (this preliminary population did not take into account the demographic truncation of the population observed in the wild – lots of large individual – and needed to be refined in further study). After 2008, the length at first maturity was 9.6 cm (Brosset et al., 2016b). As we focused on an adult population and individuals smaller than 9.6 cm represented only 2.3 % of our samples, these individuals were removed from the data. Thus, we assumed that all individuals reached maturity on March 1st and E_H was set to E_H^P. On March 1st, we assumed that the reproduction buffer E_R and the gonads E_{Go} were empty (=0) (see Supplementary Information – Appendix A).

The length and body condition index of in-situ sardines were approximated as normal distributions (Figure S20). The length and body condition index of each simulated individual were then drawn using the multivariate normal distribution approach, which takes into account the variability of each distribution and their correlations (*mvnorm* function (Venables and Ripley, 2002)).

Structural volume was estimated using equation [6] and the individual weight was also estimated using equation [9]. Then, a quantile regression analysis of weight-length relationship of in-situ sardine parameters led to two fitted equations on the 2.5 (W_{min}) and 97.5 quantiles (W_{max}, Figure S21). Finally, for a given length, energy reserve (in J) of each individual could be estimated using the two previous equations as follows:

$$E = \frac{(W - W_{min})}{(W_{max} - W_{min})} \times [E_m] \times V \quad [12]$$

with $[E_m]$ the maximal reserve density (in J. cm^{-3}) and equaled to $\{\dot{p}_{Am}\}/\dot{v}$

2.5.3. Could we explain overmortality of adult sardines after reproduction period?

In Chapter 4, we found that the survival probability sharply decreased when body condition index became lower than 0.75. Here, we stopped the runs of the simulation for each individual when its body condition index decreased below this threshold. Thus, probabilities to survive after 1 month and 1 year after the reproduction period were assessed for each length class (i.e. 0.5-cm classes).

3. Results

3.1. Goodness-of-fit between data and model predictions

The level of completeness of data was equal to 5 on a maximum of 10 (estimation detailed in Lika et al. 2011), which was a relatively good completeness level. The mean relative error (MRE) between model predictions and observations was equal to 17.5% and ranged between 0.5% and 49.4% (Table 4). Relative errors for predicted ages were lower than 5% for all ages (except for age at birth) whereas larval growth and seasonal GSI presented the highest relative errors with values higher than 40% (Figures 44 and 45, Table 4). Growth in length and weight and length-weight relationships showed similar intermediate relative errors, mostly comprised between 10 and 25% (Figures 44 and 45, Table 4).

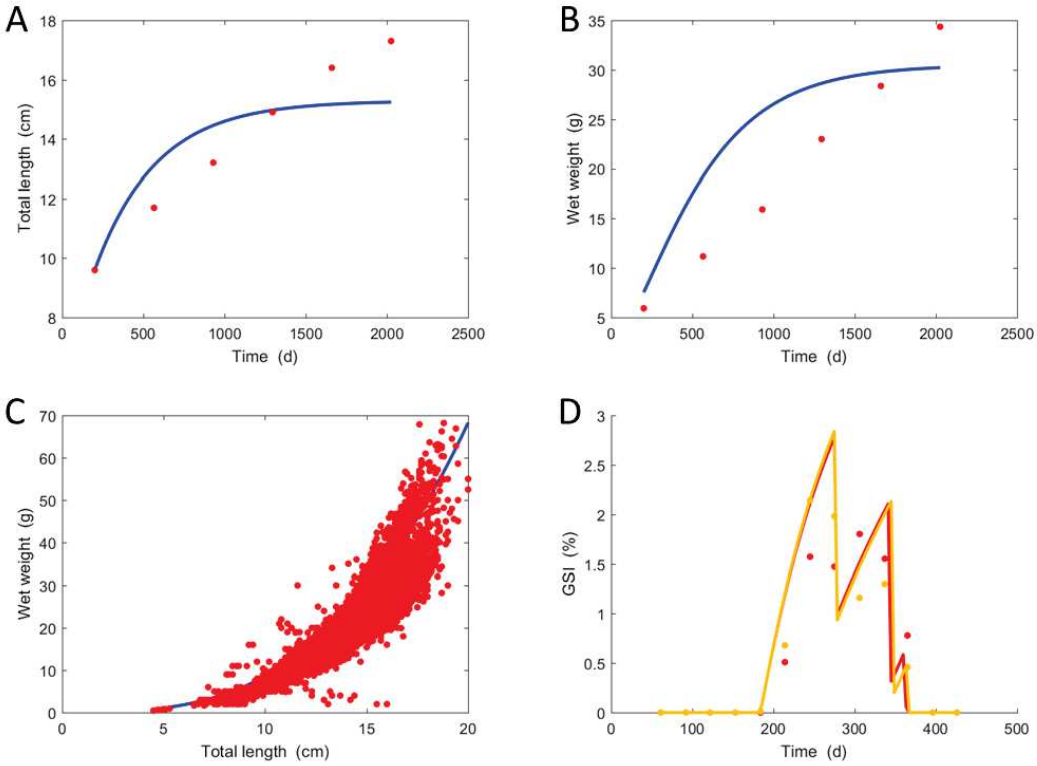


Figure 44: Comparisons of model predictions (continuous lines) to observations (dots) for in-situ total length (A) and wet weight (B) over time, length-weight relationship (C) and gonadosomatic index over time for 13 cm and 16 cm-fish lengths (D) (red and yellow, respectively).

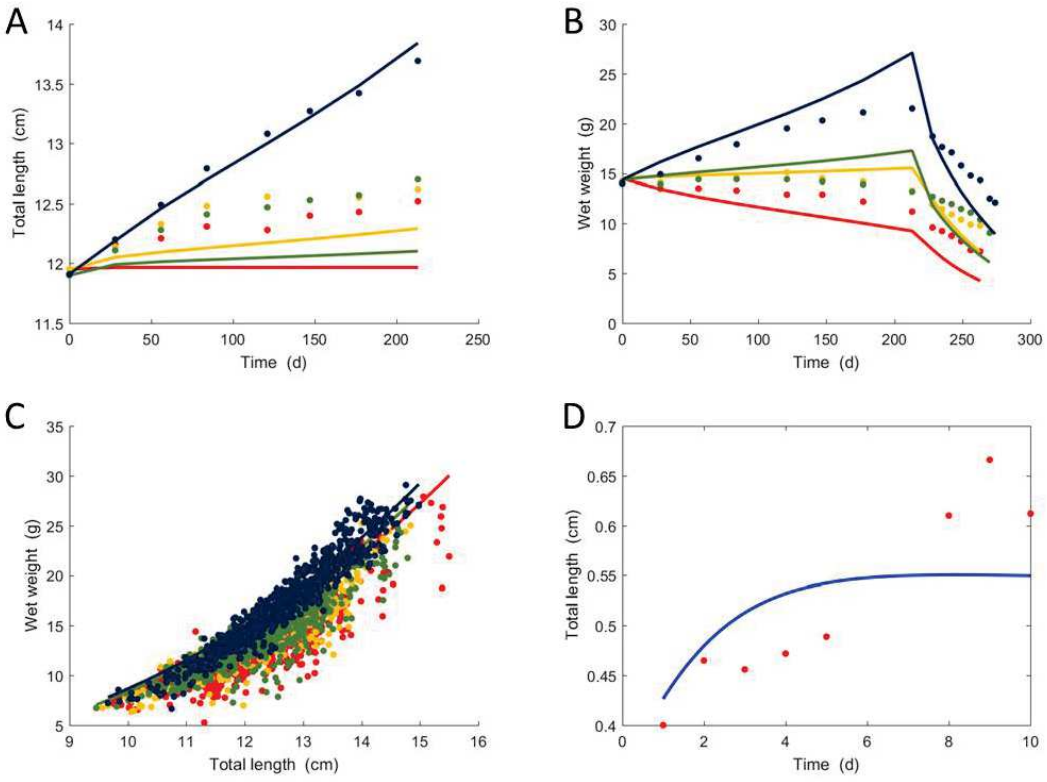


Figure 45: Comparisons of model predictions (continuous lines) to observations (dots) for experimental total length (A) and wet weight (B) over time, length-weight relationship (C) and total length over time since hatch (D)(colors in A, B, C and D corresponded to scale functional level f, red: 0.43; yellow: 0.67; green: 0.64 and blue: 0.90).

3.2. Simulations

Temperature fluctuated between 12 and 23°C with values around 13°C in March, at the end of the spawning season, while the scaled food functional response reached its highest value in March at 0.85 and decreased until values around 0.74 in November (Figure S19). The mean ± SD total length and body condition of simulated sardine population at the beginning of the simulation was equal to 14.0 ± 1.9 cm and 0.88 ± 0.09, respectively and captured well the in-situ population pattern in March (Figure S22).

The survival probabilities decreased significantly with fish length whatever the duration of the post-reproduction period, exhibiting an obvious shift of survival probability between small and large individuals (Figure 46). The probability of survival was higher than 80% for individuals smaller than 12.5 cm and 11.5 cm for 1 month and 1 year after the reproduction period, respectively. Individuals of the 14-14.5 cm size class had a probability of 50% to survive after 1 month but only of 8% after 1 year months. Thus, the survival probability of a given length class decreased significantly with time after the reproduction period, which is particularly obvious when looking at length at which 20% of survival is reached (i.e. > 19 cm after one month against 13.5 cm after a year). Finally, all length classes exhibited mean survival probability higher than 20% after 1 month post-reproduction whereas the survival probability after 1 year fell below this threshold of 20% for fish larger than 13.5 cm.

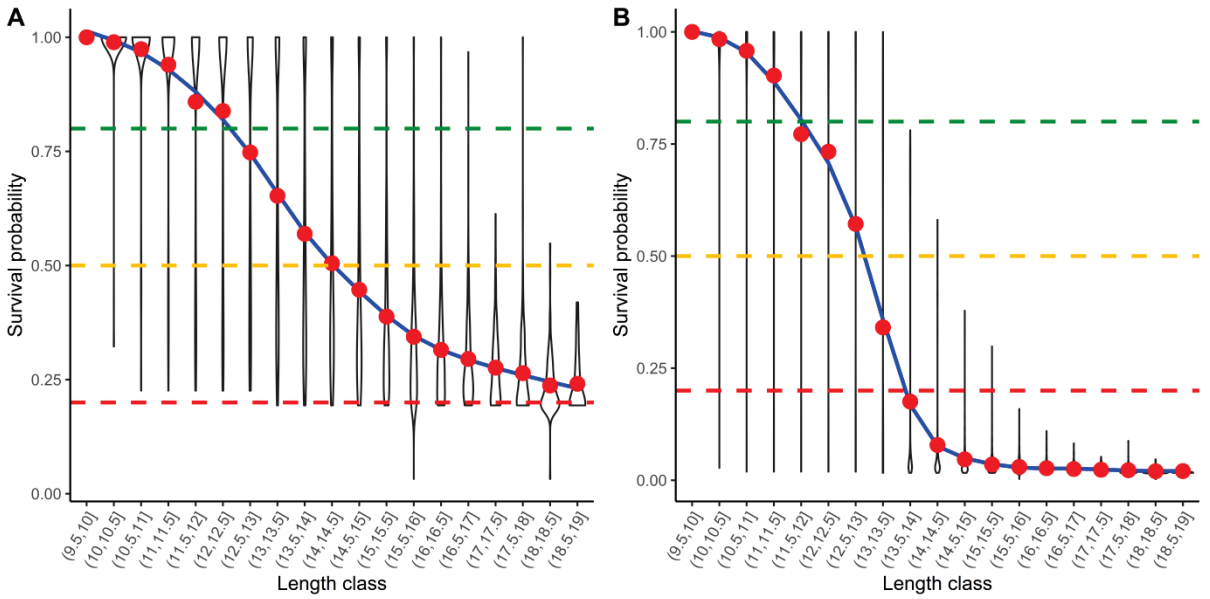


Figure 46: Violin plots of the probability to survive after (A) 1 month and (B) 1 year after the reproduction period for each length class. Red dots represent mean of each length class and fitted survival curves are represented by blue lines. Dashed lines represent 80% (in green), 50% (in yellow) and 20% (in red) of survival probability.

4. Discussion

4.1. Model fit

Here, we investigated the hypothesis of an adult overmortality following the reproduction period using a DEB modeling approach combining both in-situ and experiment data. The DEB model allowed us to model the entire life-cycle of individuals, using data mainly based on lengths and weights. The overall model goodness of fit was relatively good, as the fit mark was close to 10 and similar to results found for other fish species (Lika et al., 2011). Nevertheless, relative high errors were found for the prediction of the total length and wet weight over time. These errors might result either from the parameterization or from the estimation of the individual age. Indeed, age was assessed using otoliths, which are calcium carbonate structures whose annual growth rings give age of fish in year. In other words, the information on age remains very coarse (i.e. annual) and this is likely to affect the goodness of fit our model. More detailed procedures on age reading from otoliths could help us estimate this age in days, but they are very time-consuming and have not been performed here. Otherwise, data on the monitoring of sardines from egg to adult stages would improve the fit of the length and weight over time, similarly to other species for which these variables are well predicted (e.g. European sea bass in Stavrakidis-Zachou et al. 2019). Yet, sardine individuals cannot be monitored repeatedly in the wild preventing us to access such kind of data in situ. As we studied adult sardines during the simulation, thus data from the adult stage could be weighted during the parameterization to improve these fits (being aware that fit on larvae would decrease). Moreover, the length-weight relationships were rather well predicted with relative error below 25%, considering the substantial amount of data. Similarly, the food intakes were found to be ranged between 0.28 % and 0.44 % of the fish biomass during the parameter estimation and these results were consistent with the estimation of the food intakes by sardine in the wild, i.e. 0.3% of the fish biomass actually (Queiros et al., 2019). Among the Clupeidae family, the DEB parameters were estimated for 23 species (including *Sardina pilchardus*) gathered in the AddmyPet collection[1]. Our study allowed a new estimation of the DEB parameters of the Mediterranean sardine, using a large range of data, combining both experimental and in-situ observations. Regarding parameters,

[1] https://www.bio.vu.nl/thb/deb/deblab/add_my_pet/species_list.html

the specific cost for structure [E_G] was consistent with values found for other clupeids (5222 ± 16 (mean ± SD) and 5035 J cm^{-3} in the AmP collection and this study, respectively). Similarly, the parameter κ (allocation fraction to soma) found during the parameterization (0.88) was rather close to values found for other clupeids (0.81 ± 0.19, AddmyPet collection), but higher than 0.67 found in sardines of the Atlantic Ocean by Gatti et al. (2017). This difference between the Mediterranean Sea and the Atlantic Ocean may be relied on either on the estimation process - Gatti et al. (2017) using anchovy model to build sardine model whereas we built directly the sardine model - leading to two distinct parameters sets and/or on ecological differences between sardines from the two areas. If differences resulted from ecological purposes, thus this result might suggest lower investment in reproduction by the Mediterranean sardines than their Atlantic relatives. However, even if Altantic sardines exhibited higher gonadosomatic index compared to their Mediterranean relative (around 15% and 5% in The Atlantic and in the Mediterranean Sea, respectively), such results could derive either from lower investment in reproduction and/or lower food density in the wild. Indeed, the Mediterranean Sea is known to be an oligotrophic sea compared to the coastal northeast Atlantic Ocean. Thus, the comparison between the Mediterranean and Atlantic sardines require further analyses (e.g. parameterization made simultaneously) and also the identification of which parameters could explain the differences between these populations (e.g. food or physiological parameters).

4.2. The reproduction compartment

We added a new compartment to the DEB model to consider multiple-batch spawning strategy of sardines. The continuous monitoring of the sardine reproduction remained almost impossible due to the fragility of small individuals (e.g. for stripping). Therefore, reproduction data are scarce and modeling assumptions had to be made. For instance, the beginning and end of the reproduction period were fixed during the parameterization, but temperature could also be used as an external trigger to start batch preparation. Indeed, the first spawning events observed during the experiments occurred after the decrease of the temperature below 14°C and the reproduction ended after its increase until around 15°C (Chapter 3). Also, the reproduction period and the batch fecundity might depend on the fish length, with e.g. larger the fish, longer the period. Moreover, the parameterization modeled only 3 reproduction events (Figure 44D) whereas between 5 to 11 events could be expected

in theory if we would use the-batch periods reported by Ganias et al. (2003). However, the total energy transferred to the reproduction buffer could support around 15 reproduction events (based on the batch fecundity and the energy content of eggs), but without taking into account the energy required for the development of the reproduction material. The lack of reproduction event might be explained by the allocation to the gonads $\dot{p}_{Go}$ from September to March, as we assumed that this energy allocation flow was constant over time. Thus, we could also assume to have allocation flow depending on the moment of the year, e.g. low value between September and November following by high value during the reproduction period (December to March). Finally, this study provides a first attempt to estimate the DEB parameters for the Mediterranean sardine. Although this work has been rather time-consuming, it remains preliminary and it requires a new estimation of the parameters before publication.

4.3. Which individuals better cope with the post-reproduction period?

The simulations seemed to suggest that the probability to survive decrease conversely with the individual total length. Individuals of 14.5 cm had a probability of 50% to survive 1 month after reproduction, but this probability dropped down to 8% after 1 year. Based on in-situ length at age estimations, these individuals were between 2 and 3 years old. Thus, these results tend to support the hypothesis of an adult overmortality after reproduction, as suggested by Brosset et al. (2016), which would thus explain the adult disappearance observed in the wild since 2008.

Surprisingly, simulation predicted high mortality levels after the reproduction whereas food condition corresponded to the highest values of the time-series (Figure S19). To investigate the capacity of the model to generate mortality, we performed similar simulation including the same sardine population (10,000 individuals) coping with the same temperature, but with scale functional response equal to zero (i.e. fasting). Coping with these conditions, the median survival time varied between 9 and 17 days for all length classes (Figure 47), whereas the survival time during fasting for sardines of similar condition mainly varied between 10 and 50 days in our experiment (Chapter 4). Thus, energy requirements of the somatic maintenance could be too high and may explain the high mortality observed despite high food levels. Finally, the scale functional response fitted for the simulation required the estimation of the half-saturation coefficient, itself depending on the estimation of the scale

functional response found during the parameterization. Thus, higher half-saturation coefficient might lead to limiting food condition for fish, but this requires further sensitivity analyses.

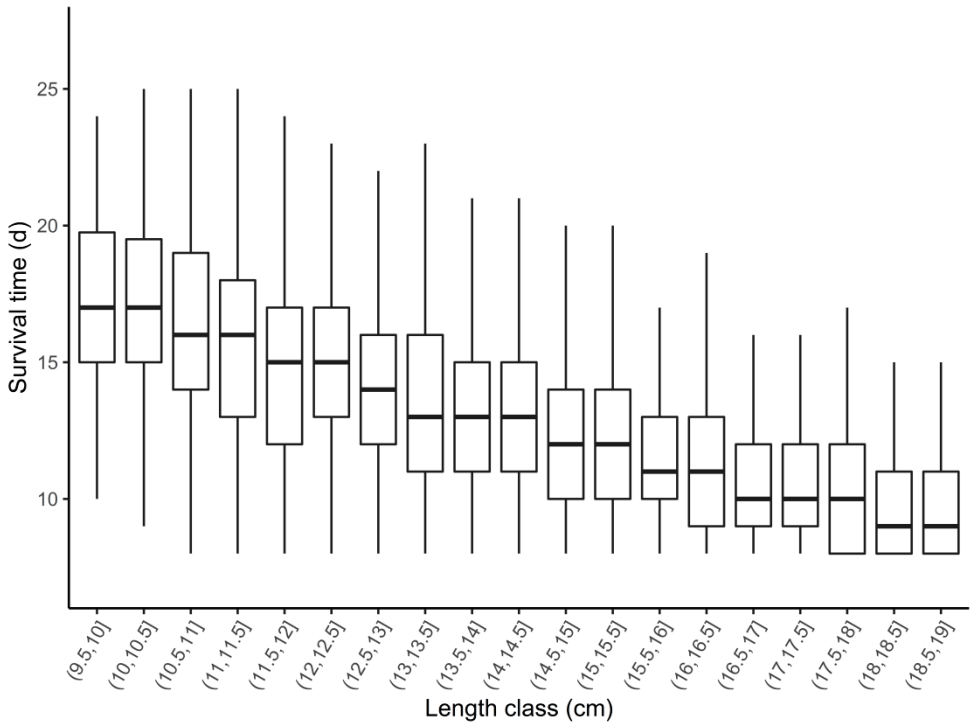


Figure 47: Boxplot of the survival time during fasting (f=0) for each length class. Boxplots are presented without outliers for clarity purposes.

4.4. Further investigations

On top of the methodological improvements already discussed above on the parameterization or the gonad compartment and reproductive assumptions, further work should focus on the simulations to explore more scenarios and offer more insights on the sardine issues in the Gulf of Lions.

First, it could be relevant to run similar simulation to investigate whether the survival probability was higher before 2008, according to temperature and food conditions occurring at that time. Indeed, differences between the two periods that may affect growth or body condition of sardines, could result either from changes in the forcing variables (e.g. food, supported by the diet changes observed by stomach content analysis (Brosset et al., 2016a)and/or adaptation in reaction to changes in their environment. A major reduction in length at first maturity was observed between the two periods, i.e. before and during crisis

(12.1 and 9.6 cm during 2002-2008 and after 2009, respectively, Brosset et al., 2016b). Such a shift was usually documented in populations supporting intensive fishing (e.g. cod in Trippel 1995, Olsen et al. 2005 or hake in Mendo & Carrasco 2000) and we put forward that, in the case of the Gulf of Lions sardine, this could also result from changes in environmental modifications. In other words, the decrease of the feeding conditions due to a shift toward smaller and lower energetic plankton communities in the Gulf of Lions would partially be counterbalanced by an earlier the reproduction investment. Similarly, despite the GSI reached their lowest values in 2009, the GSI increased straight after to reach higher values nowadays than before the crisis (Brosset et al., 2016b). Also, the lower weight loss of individuals that have coped with bad feeding conditions during fasting experiment could also be explained by phenotypic plasticity. Finally, it could be relevant to further analyze the DEB parameters of sardines between the two periods and identify which parameters could explain the differences of such food and/or physiological parameters.

It would be also of interest to study the sensitivity of the survival probability according to the food concentrations. In this study, we used the monthly mean values of plankton concentration to build the scale functional response. We could therefore integrate variability in the food time series. In particular we could study changes in the phenology of the planktonic production. Additionally, the time-series of micro and mesoplankton were summed to establish the plankton time-series used during the simulation. Despite both micro and mesoplankton occur in sardine diet, mesozooplankton represents the major part (Brosset et al., 2016a; Le Bourg et al., 2015). Thus it could be relevant for further simulation to investigate the effect of the mesozooplankton time-series on the survival probability of sardines as its concentration in March was among the lowest values of the year (Figure S23). Further, we only investigated the future of sardines after the reproduction periods in this study. Nonetheless, the survival probability could be significantly lower during the reproduction period, especially when food availability (especially mesozooplankton) reached its lowest values (Figure S23). Thus, it would be relevant to investigate the mortality over time and identify the periods for which the mortality is maximal.

Finally, the above limitations also show that if these simulations are of great interest to investigate some specific questions, such as the causes of adult overmortality, this work remains also preliminary and need further exploration before publication.

To sum up the Chapter 5, the take-home messages are:

- The DEB model parameterization for the Mediterranean sardine was relatively good.
- First outcomes of the simulations tend to support the hypothesis of an adult overmortality after the reproduction period.
- Nevertheless, several methodological improvements in the parameterization and further work in the simulations should be made before publication.

Discussion, limits and perspectives

The first objective of this PhD was to examine the hypothesis of a bottom-up control on sardine populations driven by both food size and quantity of their planktonic prey and to better understand the underlying mechanisms. To do so, we developed an innovative experimental approach on sardines, investigating effects of prey through successive integrative levels: organism (e.g. growth, body condition and oxygen consumption), organ (e.g. gill apparatus, intestine), cell (e.g. mitochondria efficiency) and compound (e.g. cortisol, oxidative balance) levels. The second objective was to examine the potential drivers for adult overmortality. First, we used an experimental approach to try and link an individual body condition (as assessed in the wild) with its physiological state and survival probability. Second, we used a modeling approach through the DEB model to study individual survival according to given environmental conditions. Information of all chapters is combined below to summarize the potential effects of the bottom-up control induced by prey size and quantity on sardines.

1. Result summary

1.1. Is bottom-up control hypothesis still valid?

The unusual situation observed in the Gulf of Lions (i.e. decrease in stock biomass despite good recruitments) was due to a decrease in the growth and body condition of both sardine and anchovy populations, leading to a drop of these fisheries (Van Beveren et al., 2014). Among the main hypotheses tested for to explain this situation, the bottom-up control driven by the potential reduction of the prey size appeared as the most probable one at the start of my PhD. Others potential mechanisms, such as overfishing, top-down control or epidemic were refuted in a previous project (Saraux et al., 2019). Using an experimental approach, we first showed that both food size and quantity have no effect on structural lipids and proteins, but has a significant impact on the growth, body condition and storage lipids of sardines (Chapter 1). Thus, sardines fed on small particles needed to consume twice as much as those feeding on large particles to achieve the same condition and growth. Monthly growth rates in captivity exhibited by sardines fed on intermediate treatments (i.e.

treatment 2: small pellets in large quantity; treatment 3: large pellets and small quantity) were close to the rate found in the wild after 2008 (1.2, 1.0 and 1.5 mm month-1 for treatments 2 and 3 and in the wild – see Supplementary material from Queiros et al. 2019). These results stressed the key role of prey size, almost as important as prey quantity and strengthened the fact that a decrease of the food size and/or quantity in the Gulf of Lions remained a reliable hypothesis to explain the dynamic of the sardine populations.

1.2. Food size effects as consequences of filtration energy costs

Higher foraging capacity and ability to collect food from their environment might give advantages to individuals, especially if they live in school, such as small pelagic fish. To achieve the same body condition and growth, sardines that fed on small pellets needed to consume twice as much as sardines that fed on larger ones. Knowing that the food quality and rearing conditions were similar, how could we explain the apparent disadvantage to feed on small items? The two possible non-exclusive hypotheses were based on the fact that either food was acquired differently due to gill apparatus differences or the energy spent while foraging depended on prey size. To catch prey, sardines are able to perform two different feeding strategies, mainly depending on prey size (Garrido et al., 2007). In our results, the energy requirements of aerobic swimming during the filtration seemed higher than anaerobic burst during particulate feeding. However, oxygen was continuously estimated in outflowing water which did not allow instantaneous estimation of energy costs of feeding strategy. This is due to the delay between oxygen consumption and its measure (due to transit time in large tank) and the short feeding duration for particulate feeding (around 2 min). This preliminary estimation required to be further investigated by cyclical intermittent stopped-flow respirometry to estimate the energy requirements of both feeding modes (Steffensen, 1989).

1.3. How to explain sardine overmortality?

While we showed that a decrease in food size or quantity was enough to induce lower growth and body condition as observed in the wild, we still had to explain the increase in adult mortality resulting in an unbalanced wild population composed mainly by ages 0 and 1 (Van Beveren et al., 2014). First, we investigated whether fish could die from starvation and low body reserves. Using an experimental approach, we showed that 1-week survival started

to sharply decrease when the body condition index became below 0.75, reaching 50% for a body condition of 0.65. Further, the specific body mass loss increased around one week before sardines died at the same time as body condition dropped below 0.72 and linked to the entry in phase III of fasting. Such results may represent maximum potential of sardines to cope with starvation as they were less stressed during experiment (no predation, no pathogens) than they would be in the wild. Nevertheless, our results indicated that the proportion of sardines in the wild falling below this threshold condition (0.72) doubled in the recent period compared to before and reached their highest levels during the months of January and February. This seems to confirm a higher probability of adult overmortality occurring at the end of reproduction and this was further investigated, using a DEB modelling approach. Using a combination of experimental and in-situ data, we first parameterized the Mediterranean sardines DEB model. Then, simulated populations based on this model showed a decrease of the survival probability after the reproduction period. Individuals of 14 cm had a probability of 20% to survive 1 month after the reproduction period, but this probability dropped to 8% after 1 year and if the length at which 20% of survival is reached is still > 19 cm after one month, it is only at around 13.5 cm after a year. This study highlighted a significant mortality of the largest individuals after the reproduction, corresponding to individuals older than 3 years old, which tends to support the hypothesis of the adult overmortality. However, several improvements in the model are still need to fully endorse the above results.

1.4. Effects of food size on other traits

Small food size treatments seemed to have low impact on the immunity and stress of sardines. Indeed, leucocyte and cortisol concentrations were similar between the 4 feeding treatments. According to the fact that sardines were not challenged (no predation and aseptic environment) and the body condition index of sardines fed with small pellets all along the experiment was close to 1 (i.e. global average body condition from wild populations), leucocyte and cortisol levels found during this study might correspond to their basal concentrations, while the variances in these levels would be due to inter-individual variability.

Second, we studied the effect of food size on reproduction and found an important effect of food size on the amount of reproduction events, which could be explain by a higher

spawning frequency linked with longer individuals in better condition. The relationship between the body condition index and the reproductive investment might be nonlinear (e.g. U-curve), increasing when body condition decreases up to a given threshold (and probably decreasing beyond that level). Here, sardines fed on small pellets (Chapter 3) still had a body condition around 1, which might be too high to observe a change of energy trade-off between reproduction and survival.

1.5. Can sardines adapt to caloric restriction or smaller prey?

Sardines in the Mediterranean Sea were known to be less good at filtration compared to sardines from more productive areas (e.g. Atlantic, see Costalago et al. 2015). Here, we examined whether sardines could adapt to smaller prey by increasing the filtration capacity of their gill apparatus. Using sardines caught in the wild and then maintained in captivity for 7 months with different food treatments, we found that branchial arc length and gill raker density were significantly correlated to the fish length, but not with the gill raker abundance and length. Both food size and quantity did not induce plasticity in the gill raker structure (length, density or abundance) of adult sardines after 7 months. However, we found an increase of the gill raker density for a given fish length from 2007-2009 to 2016. These modifications may be due to either plasticity (over a longer time period), natural selection or epigenetic, but this requires further analyses.

Another way to cope with caloric restriction might be to adapt energy expenditure either through a change in mitochondrial efficiency or through a decreased activity. Our results indicate that small food size seemed to lead to an increase of the mitochondria abundance and efficiency, suggesting that sardines coping with caloric restriction have adopted energy-saving strategy to produce ATP. Nonetheless, sardines that fed on large pellets had more powerful mitochondria. Also, the decrease in the basal oxygen consumption rate for sardines with low body condition supports the energy-saving strategy when food is limited. Similarly, sardines that were fed with small pellets in small quantity for 7 months exhibited lower oxygen consumption while fasting, probably due to a lower activity. Such a decrease seemed to enable sardines used to caloric restriction to sustain longer period of fasting. These results would show a plastic response of sardines to food deprivation.

Nevertheless, these strategies might incur other costs (e.g. more energy efficient mitochondria through a better coupling also suggest higher oxidative stress, lower activity

levels might result in lower chances of finding food in the wild) or may not be enough to compensate high energy demanding of the filtration on small prey size.

2. Experimentation on sardines

2.1. Original approach

Among all experimentations based on fish, the zebrafish (*Dario rerio*) is undoubtedly the most used species especially owing to similarities shared with humans (see Kalueff et al. 2014). Experimentation on marine fish species are usually performed for aquaculture improvements or genetics, but rarely for applied ecology issues, although controlled experimentation is one of the most useful approach for studying population control mechanisms (Hunt and McKinnell, 2006). Studies in the wild remain complicated and costly for marine fish and the ones dedicated to small pelagic fish mostly addressed specific issues, such as stock size estimations. In my PhD, we developed an experimental approach to examine the impacts of food size and quantity on sardine condition and test further the hypothesis of adult overmortality. Such an experimental approach remains scarce due to the difficulty of handling these species. While experimentations on sardine larvae are frequently conducted, adults in experimental studies remained barely used. Mostly, they were used to investigate aspects of captivity or handling during experimentation (Bandarra et al., 2018; Marçalo et al., 2008; Peleteiro et al., 2004), effects of fishing procedures or devices (Goetz et al., 2015; Marçalo et al., 2010, 2013) and feeding behavior (Garrido et al., 2007). Furthermore, the only long-lasting experiments (1 year) on adult sardines was conducted on much larger and heavier sardines (20.2 cm and 72.2 g, Bandarra et al. 2018), while the other long-lasting one (1.5 years) started from eggs collected in the wild and left only one sardine alive after 18 months (Iglesias and Fuentes, 2014). Finally, we proved that handling and long-lasting experiments on sardines, in particular on small individuals, could be conducted in the future.

2.2. Experimentation as the pivotal point of this work

Animal experimentation needs to comply with the national regulatory requirements on animal welfare. For this purpose, the Three R's (Reduce, Refine, Replace) aim to reduce the number of animals used, refine experimental procedures to limit animal suffering and

replace animals with non-animal alternatives when possible (Ibrahim, 2006). On top of being especially careful with rearing conditions, we also gave particular attention during this thesis to the use of sardines at the end of the experiment. As such, sardines were either given to local aquariums when sacrifices were not required or their sacrifice was optimized in order to collect the maximum from the samples (e.g. muscles for protein and lipid, blood for oxidative stress balance, scales for cortisol, gonads and heart for telomeres, but also on some occasions, gills, kidney for immunity and intestine for histology and microbiome). Nonetheless, such an extensive and exhaustive approach would not have possible without the collaborations of several experts from different areas (e.g. mitochondria, immunity), which allowed me to investigate the food effects on several life history traits of sardines (reproduction, growth and maintenance) and at several scales (organism, organ, cell and component), some being still under investigation (Table 5).

Additionally, the parameterization of models such as the DEB model requires in general lots of data to find the best parameter simplex. Data from experimentation represented two thirds of data (both 0-variate and 1-variate data) used during the parameterization process of the DEB model, which allows us to (numerically) test some hypotheses that could not be tested otherwise (e.g. combining food and temperature effects). Although experiments were time-consuming and required a lot of people/equipment (e.g. study on several successive years, several temperatures), they were complementary to the modeling approach and central to all studies performed during this PhD.

Table 5: Summary of the collaborations developed during my PhD thesis and my inputs. The MONALISA Team refers to Claire Saraux, Jean-Marc Fromentin, Eric Gasset, Gilbert Dutto, Luisa Metral, Camille Huiban, Lina Leclerc and Lolita Tibeuf.

Chapter	Study	Leader	Collaborators	My inputs
Chapter 1	Experimentation	MONALISA Team		Development Data acquisition Data analysis
	Lipid and protein content	MONALISA Team		Data analysis
	Oxidative stress balance	MONALISA Team	**Dr. Q. Schull** *MARBEC*	Data acquisition Data analysis
Chapter 2	Gill raker structure	**Dr. JH Lignot** *MARBEC*	MONALISA Team	Data analysis
	Oxygen consumption	MONALISA Team	**Dr. D. McKenzie** *MARBEC*	Data acquisition Data analysis
Chapter 3	Bioenergetics	**Dr. L. Teulier** *LEHNA*	MONALISA Team	Data analysis
	Reproduction	MONALISA Team		Development Data acquisition Data analysis
	Immunity	**Dr. E. Farcy** *MARBEC*	MONALISA Team	Data acquisition Data analysis
	Stress	MONALISA Team	**Dr. Q. Schull** *MARBEC*	Data analysis
Chapter 4	Starvation	MONALISA Team	**Dr. D. McKenzie** *MARBEC*	Data analysis
Chapter 5	Modeling	MONALISA Team	**Dr. L. Pecquerie** *LEMAR*	Development Simulation

3. Limits

As all experimental trials, we tried to be as close as possible to the natural environment (i.e. water parameters and food) and natural behavior of sardines. Therefore, experimental tanks were supplied with water pumped directly at sea and followed natural photo- and thermo-period regimes. The number and density of fish per tank also allowed for the formation of schools. Then, none of the two possible ways to provide live prey to sardines (i.e. capture in the wild or production) appeared adequate for our experiments. On top of the inherent logistic difficulties, it would have been impossible to standardize meals in terms of food size and quantity several times a day, every day during several months. On the contrary, aquaculture pellets provided such a chance to precisely control food input. With no information on the composition of prey in the wild before and after the crisis, we decided to study only the effects of food size and quantity on sardine life-history traits and thus selected aquaculture pellets of two different sizes (0.1 and 1.2 mm) of same quality in terms of lipid and protein contents. Although we are aware of the limitation of inert food compared to live prey, normal feeding behavior on aquaculture was ensured after each acclimation periods. Despite the above caution, experiments performed during this PhD could sometimes fail for several reasons and two examples are presented below.

3.1. Reproduction of sardines

Despite our efforts to mimic natural environment, our knowledge on the reproduction of sardines remained limited. We decided to study the effect of the food size on the reproduction of sardines and thus the spawning was not artificially induced. The first reproduction events of sardine from the experiment n°2 (Chapter3) occurred when water temperature decreased below 14°C and ended when temperature increased until 15-16°C. However, sardines from the experiment n°1 (Chapter 1), even those feeding on large pellets in large quantity and exhibiting high body condition (around 1.2), did not spawn during winter, despite similar environmental conditions (tank being provided with the same water). Even though income and capital breeding may reflect extreme points on a breeding strategy continuum (Stearns, 1989), sardines are known to be capital breeders in the Gulf of Lions. Thus, the absence of reproduction event for such individuals may be due to low energy storage levels prior to the experiment (the experiment having started in mid-November, i.e.

about 3 weeks before the temperature dropped below 14°C). To counteract this, the experiment n°3 (Chapter 3) was designed to study whether food size before or during the reproduction period influenced the reproduction of sardines, i.e. to investigate this capital versus income gradient. However, only one reproduction event with a very low egg amount was observed during the experiment n°3, while sardines from the experiment n°2 spawned 39 times at the same time with the same water quality and photoperiod. The absence of spawning might be due to rearing conditions as sardines from the experiment n°2 were in larger tanks than sardines from the experiments n°1 and n°3 (3 m^3 and 300 L for experiments n°1 and n°3, respectively). Interestingly, sardines in experiment n°3 had matured gonads (as observed when sacrificed in March). The absence of spawning in small tanks may also raise the importance of the height of the water column for the reproduction of small pelagic fish. Indeed, the reproductive behavior of small pelagic fish might be similar to the spawning rush exhibited by other fish species releasing eggs in the water column, such as groupers (Mourier et al., 2019; Rowell et al., 2019) or parrotfish (Luckhurst, 2011). To elucidate the reproductive behaviors of sardines, close range sonar could be used to identify movement of fish during nocturnal reproduction. Finally, the fact that sardines did not reproduce may also result from ethologic misunderstanding of the small pelagic reproduction, such as behavior, school size, etc.

Despite the absence of reproduction during the experiment n°3, samples collected in March will be used in near future to assess the physiological cost of growth or reproduction, using telomeres and oxidative stress balance (Bauch et al., 2013; Geiger et al., 2012). Biometric parameters provided during this experiment will also be used to investigate potential inter-individual competition.

3.2. Respirometry

Apart from the reproduction study, the final step of the experiment n°3 should have been used to investigate the oxygen consumption differences of sardines that fed on small vs. large pellets with more replicates and a stopped-flow system compared to the respirometry study presented in Chapter 2. During this experiment, sardines were distributed into 8 tanks according to their body condition index (high vs. low condition, HC and LC respectively), i.e. 4 tanks for HC and 4 tanks for LC. Sardines were fed with large pellets during the first 2 weeks and fed with small pellets during the next 3 weeks (including 1 week for acclimation to this

feeding treatment). Sardines were fed twice a day and the feeding of each row constituted by 1 tank of each condition, was spaced every 20 min to be synchronous with the stopped-flow respirometry system. However, the feeding of the first row led to the agitation of sardines in all other tanks despite them not being fed. As such, oxygen consumption increased before the meal started in 6 of the 8 tanks, preventing us from using these data. Further, sardines in good condition (i.e. fed with large pellets for 9 months) did not appear interested by small pellets, probably as their good condition enabled them to wait for better food; so that oxygen consumption did not increase during their meals. While this experiment was critical to better understand the mechanisms involved in the lower growth and condition of sardines fed with small pellets, it has not been possible to redo it during the timeframe of my PhD, due to several logistic problems. Nevertheless, a new protocol has been designed for this experiment and it might be conducted in near future (see Perspectives).

3.3. Modeling approach

Beside the above limitations in the experimental approach, this study also raises some limitations in the modeling exercise. The DEB approach deals with the allocation of the energy flows due to food into three key compartments: maintenance, growth and reproduction. To parameterize the DEB model, the 'best' data are energy balance at several body sizes and at several food levels (Lika et al., 2011). However, such data was not available for our species, therefore we used indirect observations linked to the energy flows to estimate the parameters, such as growth. Although the parameterization was satisfactory, the relative errors between some observations and predictions remained relatively high and the survival time of individual coping with starvation found during simulation was lower than estimation found during the starvation experiment. Also, the prediction of the gonadosomatic index was rather low and might be due to low amount of available data on the reproduction of sardine in the literature. Such results could also be explained by the long and difficult stage of the parameterization of the DEB model. In our study, we further added a compartment to the DEB model to take into account the multiple-batch reproduction of sardines, but several modeling assumptions had to be made due to little information on the reproduction such environmental factor which triggers spawning. Finally, the exploration of the parameters space is constrained by 'realistic' parameter sets according to other similar

species and some parameters have to be fixed. Nonetheless, two realistic parameters sets could well fit the data but lead to the misunderstanding of underlying physiological process. Thus, a low food level being counterbalanced by a high κ (allocation to soma) could lead to the same growth that the combination of high food level and low κ. To limit the number of potential realistic parameters, additional data (e.g. on reproduction or energy content) would be necessary.

In this study, we considered that all individuals shared the same parameters and therefore we did not integrate inter-individual variability, especially during the simulation process. Similarly, the environmental parameters (i.e. temperature and food) derived from the outputs of coupled 3D hydrodynamical and biogeochemical models at daily resolution, but we only used monthly averages across the whole Gulf of Lions and the full height of the water column. Additionally, we could neither consider vertical nor spatial distribution of sardine (mainly coastal distribution, Saraux et al. 2014) which might have physiological effects due to temperature (e.g. stratification in winter) of food resources. Nonetheless, the perspectives developed below should reduce or investigate some of the above potential limits.

4. Perspectives

Despite the fact that previous results supported used the hypothesis of the bottom-up control induced by the reduction of food size, some questions still remain.

4.1. Could filtration be detrimental for Mediterranean sardines?

Among the perspectives, the hypothesis of higher energy consumption resulting from filtration seemed to be supported by the respirometry study presented in chapter 2, but this remains to be further validated by additional experiments including more replicates. To do so, I just submitted a short-term project for funding in collaboration with Dr. S. Killen, Dr. Q. Schull and Dr D. McKenzie. Sardines would be fished and acclimated to inert food using the same approach as the one detailed in chapter 1. After 2-3 weeks acclimation and upon confirmation of the absence of NODA virus, sardines would be moved into indoor tanks for the respirometry experiments. Around 2000 sardines should be distributed into 8 experimental tanks of 1,000 L each that will be modified to function as automated

respirometers (McKenzie et al., 2007, 2012). During the first week acclimation to the tank-respirometers, fish will be fed with both 0.1 and 1.2 mm aquaculture pellets two times a day. Further, metabolic rate (i.e. energy use) will be measured indirectly as instantaneous oxygen uptake of fish in the tanks, by cyclical intermittent stopped-flow respirometry (Steffensen, 1989), as described in McKenzie et al. (2007, 2012). Contrary to estimation method used in Chapter 2 (i.e. non-automatic diurnal estimation every 30 min and delay between oxygen consumption and its estimation), this method will allow continuous estimation of the oxygen consumption of sardines during the feeding and digestion as well as identification of potential modification of oxygen consumption during the night. During the first 2 weeks of the experiment, oxygen consumption will be measured on 4 tanks fed with only 0.1 mm pellets and the other 4 tanks fed with only 1.2 mm pellets. During the second period of the experiment feeding treatments will be inverted, after a week acclimation to the new feed size. By using freshly fished sardines instead of sardines at the end of a long-term experiment, we will ensure sardines feeding on every meal and oxygen consumption to be measured each time. Further, sardines will all be fed at the same time, to avoid excitation to occur before feeding events. This new study should allow us to complement our understanding about the food size effects on the Mediterranean sardines.

4.2. Does condition have significant impact on sardine swimming activity?

Such results on the effects of the food size on several life history traits may be exacerbated in the wild. Contrary to the wild, sardines were fed every day with aquaculture pellets and they did not face predation and pathogens lowering the energy required for these expenditures. Also, sardines with low body condition index in the wild (i.e. assimilated to a low energy reserve) may exhibit lower swimming performance, especially swimming endurance (Martinez, 2003, 2004). Thus, poor swimming endurance might render these individuals unable to sustain the continuous aerobic swimming and they might get isolated from the school and its positive effects such as avoiding predation and improving foraging. Thus, the decrease of the body condition might lead to a vicious circle, inducing lower food intake and thus reinforcing lower energy reserve. To study the swimming activity of sardines, we used video recorded at the end of the experiment n°3. Movies have been made with GoPro Hero 5 cameras located above the tanks and set at the linear field of view (no fish eye

view) at 1080p and 30 frames per second. Movies were recorded throughout the day and also during the feeding period from the 28th May to the 2nd July 2018. Individual trajectory (speed, velocity) are currently under analyses with a specific tracker program. Also, the link with global school activity could be investigated using custom designed software (Sadoul et al., 2014). This will enable us to first assess the exact duration of meals depending on food size according to changes in fish speed, but also to estimate the level of activity and speed of fish of different conditions outside of the feeding period.

To go even further, the use of stereoscopic video cameras filming during the respirometry study described above might help estimate the distances swum by fish and their accelerations while feeding in order to link them with the oxygen consumption and the energy expenditure of fish. Finally, the use of external individual marks (e.g. with elastomers in McLean et al. 2018) would help investigating inter-individual competition while feeding.

4.3. Is digestion impacted by food size and quantity?

The energy intake of an individual depends on its capacity to catch prey as well as on its capacity to extract nutrients from its food. Intestine is one of the most important organs in the absorption of nutrients (Rašković et al., 2011; Zaldúa and Naya, 2014). The intestinal epithelium of bony fish is composed by a series of villus, themselves constituted by digestive cells, i.e. enterocytes (including its brush border), which play a key role in the digestion and absorption of nutrient owing to the production of digestive enzymes involved in the breaking down of food (Harpaz and Uni, 1999; Silva et al., 2010). Absorption of nutrients depends on both the enzyme activity and the contact area between the brush border and the nutrients.

Owing to the increase of their surface-to-volume ratio, small particles promote digestive processing by enzymes and may lead to the decrease of the energy expenditures due to digestive activity. This observation was supported by the oxygen consumption presented in Chapter 2, where it was shown that oxygen consumption of sardines that fed on small pellets decreased more strongly than that of sardines that fed on large pellets after the last meal.

Further, the digestive tract is an example of phenotypic plasticity (see review of Zaldúa & Naya 2014). An increase of the functional capacity of the digestive tract could occur when internal energy requirements increase or if the food quality decreases (Liu and Wang, 2007; Naya et al., 2007). To counteract food restriction in the wild, individuals might thus improve

their digestive efficiency to maximize the nutrient extraction from their meals. However, if food quality reaches a minimal threshold or if fish have to cope with starvation, the functional capacity could significantly be reduced (German et al., 2010). Thus, the digestive efficiency of sardine might be impacted by the decrease in food quality and/or size (Zarubin et al., 2014). Such phenomenon might be amplified by the predation pressure which induced relatively shorter gut (Relyea and Auld, 2004). Other studies showed that food restriction could impact intestinal structure (Hossain and Dutta, 1991) and also generate stressful situation for fish in the wild. Such a stress and the associated increase in corticosteroids (e.g. cortisol) could lead to alterations of the intestinal structure (Olsen et al., 2002; Söderholm and Perdue, 2001; Sundh and Sundell, 2015). In particular, it may result in the alteration of the ultrastructure of the enterocyte via the increase of junctional gap between cells (until complete detachment of cell-to-cell contacts in extreme cases) and a decrease of the microflora (in the first part of the intestine), owing to the leaching induced by the activation of the mucus production (Meddings & Swain 2000, Olsen et al. 2002, 2005, but see Olsen et al. 2008 and Rosengren et al. 2018). To better understand the relationships between food and microbiome, some intestines of fish from the experiment n°1 were sampled in March and are currently under analyses. These alterations may expose individuals to infection if pathogens reach to translocate across the damaged intestinal wall and result in additional energy costs to get by. Fed individuals (feeding with aquaculture pellets) exhibited higher resistance to stress (i.e. lower intestine damages) than food deprived ones (stop feeding few days prior experiment), but the damage and its persistence seemed to be also species specific (see Olsen et al. 2005, 2008).

During this PhD, we started to investigate the effects of food size and quantity on the digestion, through a morphological analysis of the intestinal tract (intestine circumference and both villus and enterocyte heights). Our results indicated that food quantity might have only effects on fish that fed on small pellets, but not on fish that fed on large pellets. For fish feeding on small pellets in low quantity, the intestine perimeter was indeed lower. However, there was some sampling bias (e.g. bias cut of the intestine) inducing some observation errors and the sample size was too low to get robust statistical comparisons (n = 4 and 6 for some samples). Therefore we did not present those results in Chapter 3 and this analysis should be completed to investigate whether food size and quantity impact the digestive tract.

Finally, to go one step further in the investigation of the digestion, we could also study the enzymatic activity and the pyloric caeca structure. For the latest, their development begins after metamorphosis, when their number increases until a plateau (around standard length of 12 cm, Costalago & Palomera 2014). They have a similar structure as intestine with a single epithelium in column and secretory cells (Harpaz and Uni, 1999) and seems to be involved in both storage, digestion and absorption of food and water (Buddington and Diamond, 1986, 1987). Pyloric caeca may play a key role during food deprivation as the food retention and digestion was reduced compared to the gastrointestinal tract (Hossain and Dutta, 1991) and might increase the ability of individuals to cope with food deprivation.

4.4. Could we model dynamic of sardine population in the Gulf o Lions?

Among the first perspective, the first one was a new estimation of the DEB parameters for years before 2008. I do think that the prediction of the reproduction and biometric parameters (e.g. length and weight) could be improved giving more weight to data referred to adult stage. Also, we could add gonadosomatic index provided by in-situ observation for other fish length. The new estimation of the DEB parameters will be cross-validated by survival time of starved individual found during the simulation to be as close as possible of values found during the experiment. After the parameterization of the DEB model for the actual period, the next step will be the parameterization of the DEB model for the years before 2008 (i.e. before the small pelagic fish crisis in the Gulf of Lions). As presented in the discussion of the Chapter 5, this step will allowed us to investigate whether the differences between both periods are the result of either environmental modification (e.g. lower food size and/or quantity) and/or adaptation of sardines to new environmental conditions. We could further compare the survival of individuals after the reproduction between both periods.

RFID tag allowed us individual identification and therefore individual data acquisition on several parameter, such as growth (length and weight over time) or reproduction investment (gonadosomatic index). This large amount of data could allow us to parameterize individual DEB model and thus to study the potential impacts of inter-individual competition for food or physiological differences among individuals (Sadoul et al. *in prep*). To do so, we to fit the individual data with a new parameter set based on the average one but allowing the

re-estimation of only one (or more) parameter at a time (e.g. κ and $[E_G]$). Nonetheless, this study required a 'good' DEB parameters set as starting point and also high computing capacity to found best parameters for each individual (e.g. 449 individuals only for the first experiment).

After parameterization, the next challenge would be to integrate this DEB model into population model to understand population responses to environmental changes. To do so, the DEB model could be coupled to an individual based model (IBM model) leading to an IBM model based on DEB theory (DEB-IBM, Martin et al. 2012). The transition to DEB-IBM population model would allow us to take into account parameter variability estimated in the previous part using individual parameter estimation. A population model would allow taking into account past and present modifications of the plankton abundance and quality (i.e. size) and spatial effects (e.g. plankton patches, food density according to the distance to the coast). The use of software platform like NetLogo could be a suitable way to build population model (Martin et al., 2012). We could further investigate the sardine population dynamics integrating other factors, such as the combination of fishing pressure with environmental changes (e.g. in temperature and food).

4.5. Are Mediterranean sardine close to their Atlantic relatives?

An interesting perspective could the comparison between the Mediterranean and the Atlantic sardines. Indeed, both populations are geographically separated facing different environments and we could suppose that the genetic flow is rather low if not null between these individuals. Differences found on the gill raker structure between both areas seems to be in favor of this hypothesis (Costalago et al., 2015). Also, the fact that Atlantic individuals are larger and invest more energy in the reproduction (around 15% and 5% in The Atlantic and in the Mediterranean Sea, respectively) may be the result of either higher food levels and/or physiological processes.

Similarly to my PhD, two complementary approaches could be developed to investigate the differences between the two populations: experimentation and modeling. First, a 'common garden' experiment could be developed allowing us to study the phenotype variation individuals according to the environment. Then, interbreeding between both populations and the study of the phenotype variation to the same varying environment would allow the identification of the genetic contribution to a given phenotype. These studies could be

completed with the comparison between the DEB model of Mediterranean and Atlantic sardines, which is already in development. Such comparison could complete the investigation of potential variations exhibited by both populations.

Supporting Information

From capture to maintenance in experimental tanks

Fishing procedures were optimized following Peleteiro et al. (2004) and preliminary tests. Briefly, sardines were concentrated by tightening the net, coaxed gently into buckets so that they were always immersed in water then transferred immediately into 1 m^3 tanks of oxygenated seawater on board. After boat and lorry transport to the IFREMER experimental station at Palavas-les-Flots (Hérault, France), fish were held in outdoor tanks (4.5m^3) supplied with a flow of aerated local seawater at prevailing temperature and photoperiod. The whole operation, from capture to outdoor tanks, took less than 4 hours. The first 2-3 days following capture constituted the most critical period of acclimation, but this varied quite a lot from one fishing event to another, probably due to the sea temperature when the fishing was carried out (the higher the sea temperature, the higher the mortality in the few days after fishing). During the first 5 days, daily prophylactic baths of oxytetracycline (100 ppm) were administered to prevent bacterial infections from fishing injury and scale loss. Over the first week, sardines were fed both *Artemia nauplii* and aquaculture pellets (mix of pellet sizes: 0.1mm, 0.3mm and 0.8mm), with increasing proportions of pellets and decreasing proportions of *Artemia* throughout the week, concluding with meals exclusively of pellets. Pellets were distributed by automatic feeders throughout the day whereas *Artemia* meals were provided once, in the morning. To maximize survival, food rates were high (between 2% and 6% of biomass), such that body condition increased from a mean (± SD) of 1.0 (± 0.1) at fishing to 1.2 (± 0.1) at the start of experiments. Natural swimming and schooling behavior occurred within a few days of capture. After 2 to 3 weeks acclimation (depending on fishing dates) and upon confirmation of the absence of NODA virus, sardines were moved into indoor tanks for experiments.

Feeding conditions

As in most experimental studies, we tried to be as close as possible to the natural environment (i.e. tanks were supplied with water pumped from the sea, followed natural photo- and thermo-period) to have natural behavior of sardines. The number and density of fish per tank allowed for the formation of schools (Figure S2 and Movie S1). To control accurately the quality, quantity and size of the food along the 7-month of the experiment, the use of living prey would not have been appropriate and we therefore chose to feed sardines with

standardized aquaculture pellets having the same quality in terms of lipid and protein contents. Both pellet sizes shared similar lipid class contents except for the phospholipid class (see Table S1). Moreover, the two pellet sizes corresponded to main prey sizes found in the wild during those two periods (Le Bourg et al., 2015). Although we are aware of the limitation of inert food versus live prey, normal feeding behavior on aquaculture pellets was ensured before the start of the study (Movie S1). Finally, based on the mean age at the beginning of the experimentation (i.e. 1.2 years) and on the von Bertalanffy curves adjusted by Van Beveren et al. (2014), monthly growth rates in captivity were similar to the ones in the wild (around 1.5 mm month^{-1} and between 0.5 and 2.5 mm month^{-1}, in Van Beveren et al. (2014) and this study, respectively).

Estimation of food loss

To quantify loss of non-ingested food, we performed an additional 2-week experiment in a single tank, with a similar density of sardines to the previous experiment, where we estimated the quantity of food that (i) deposited on the bottom of the tank and (ii) left the tank through a bottom grid. To do so, 5 glass Petri dishes were placed on the bottom of the tank before each meal (total tank area = 76.6 x 10^{-2} m^2, bottom grid area = 15.6 x 10^{-2} m^2, dish area = 1.45 x 10^{-2} m^2) and a 70-µm-mesh sieve used to filter outflowing water (Figure S3). The experiment comprised 16 meals (4 meals per day) distributed over 10 days. Fish were fed 1.2mm pellet in large quantity on days 1 and 3, fasted on days 2 and 4. Fish were acclimated to 0.1mm pellet on days 5 and 6, then days 7 and 8 repeated days 1 and 2 but using the smaller pellet in large quantity, with days 9 and 10 repeating days 7 and 8. Petri dishes and sieve were removed for analysis 90 minutes after each meal.

Samples from Petri dishes and the sieve were then filtered through a 0.7-µm dry filter (dried beforehand at 60°C in the autoclave for 24 hours and weighed to the nearest 0.0001g), then filters were rinsed with distilled water to remove salt. After manually removing faeces and scales, filters were dried at 60°C in the autoclave for 24 hours and then weighed, so that mass of matter could be estimated as the difference in filter dry weights. The total mass of collected matter was the sum of matter collected by the sieve and matter collected by the 5 dishes weighted by the ratio of tank surface to dish surface (without the bottom grid area):

$$\text{collected } matter = \sum \text{collected matter}_{\text{dish}} \times \frac{S_{\text{tank}} - S_{\text{bottom grid}}}{\sum S_{\text{dish}}} + \text{collected matter}_{\text{sieve}} \quad [1]$$

Finally, the non-ingested food was estimated as the quantity of collected matter during a meal corrected by the mean quantity of collected matter while fish were fasting and expressed as a fraction of the meal size:

$$\text{non-}ingested\ \text{food} = \frac{\text{collected matter}_{\text{meal}} - \text{collected matter}_{\text{fasting}}}{\text{meal size}} \times 100 \qquad [2]$$

Results

Body condition and total length over time: mixed-effect models

The impact of the different treatments on body condition and total length were tested using linear mixed-effect models with both random slopes and intercepts for both individuals and tanks (to study tank effect among treatments). The model selection process was following recommendations provided by Burnham and Anderson (2002) and Zuur et al. (2009). The selected model is presented here using REML estimation. Selected models and violin plots of body condition and total length distributions are presented in Figure S4. For the body condition index, the validation graphs are presented in Figure S5. Homogeneity, normality and independence were checked through plots of Figure S5 and only 3 individuals are considered as outliers (i.e. <1% of all individuals) (Figure S5B). The results of the selected model are presented in Table S2. Simultaneously, the same process was used for the total length parameter. The validation graphs are presented in Figure S6. Homogeneity, normality and independence were checked through plots of Figure S6. The results of this selected model are presented in Table S3.

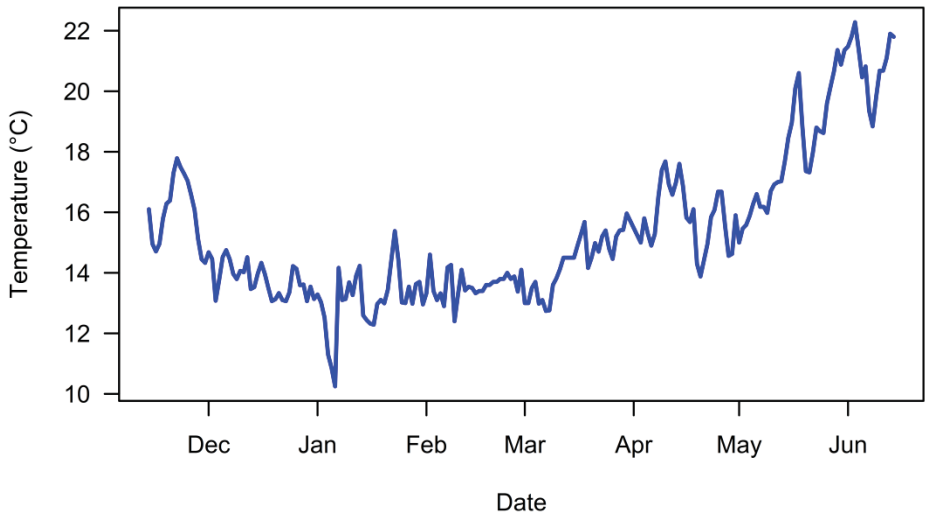


Figure S1: Evolution of the water temperature in tanks throughout the 7-month experiment

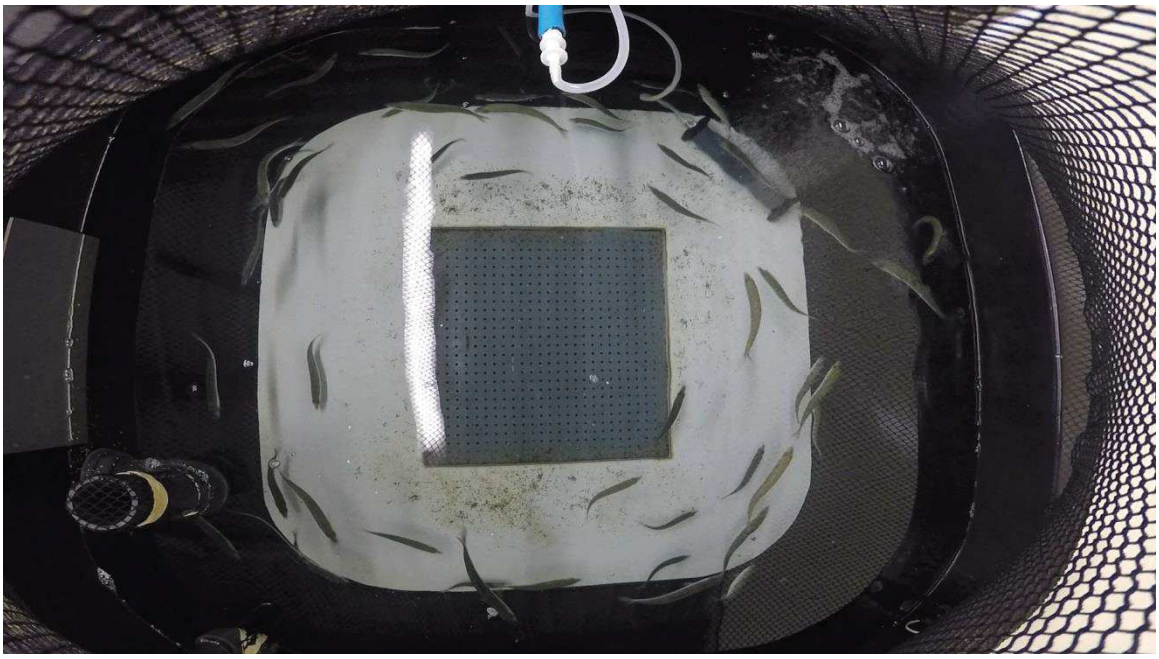


Figure S2: Picture of the swimming and schooling behavior of sardines in the 300 L experimental tank

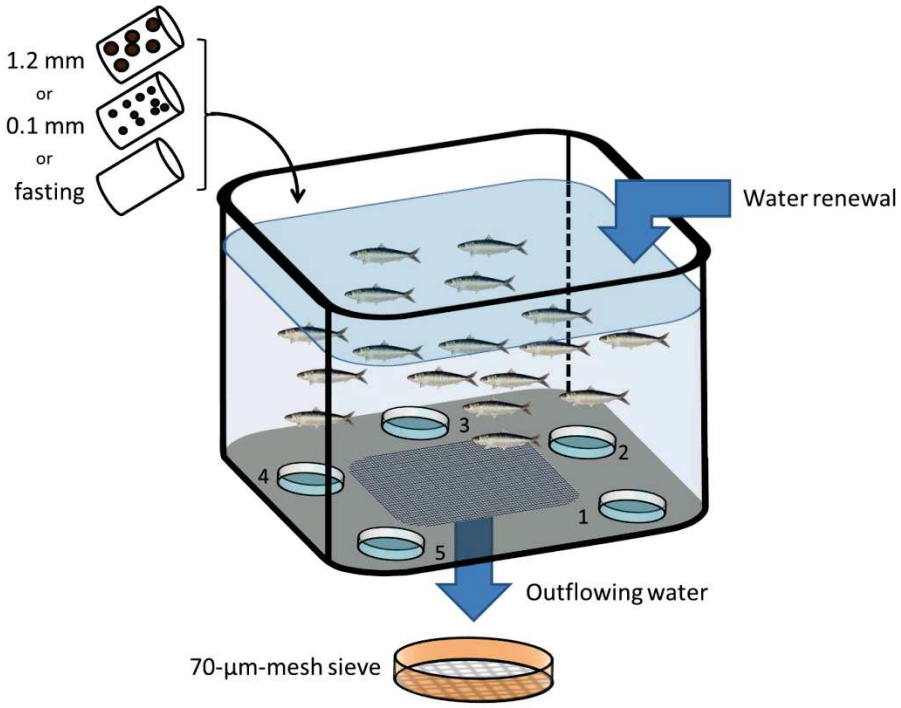


Figure S3: Experimental design to estimate the non-ingested food

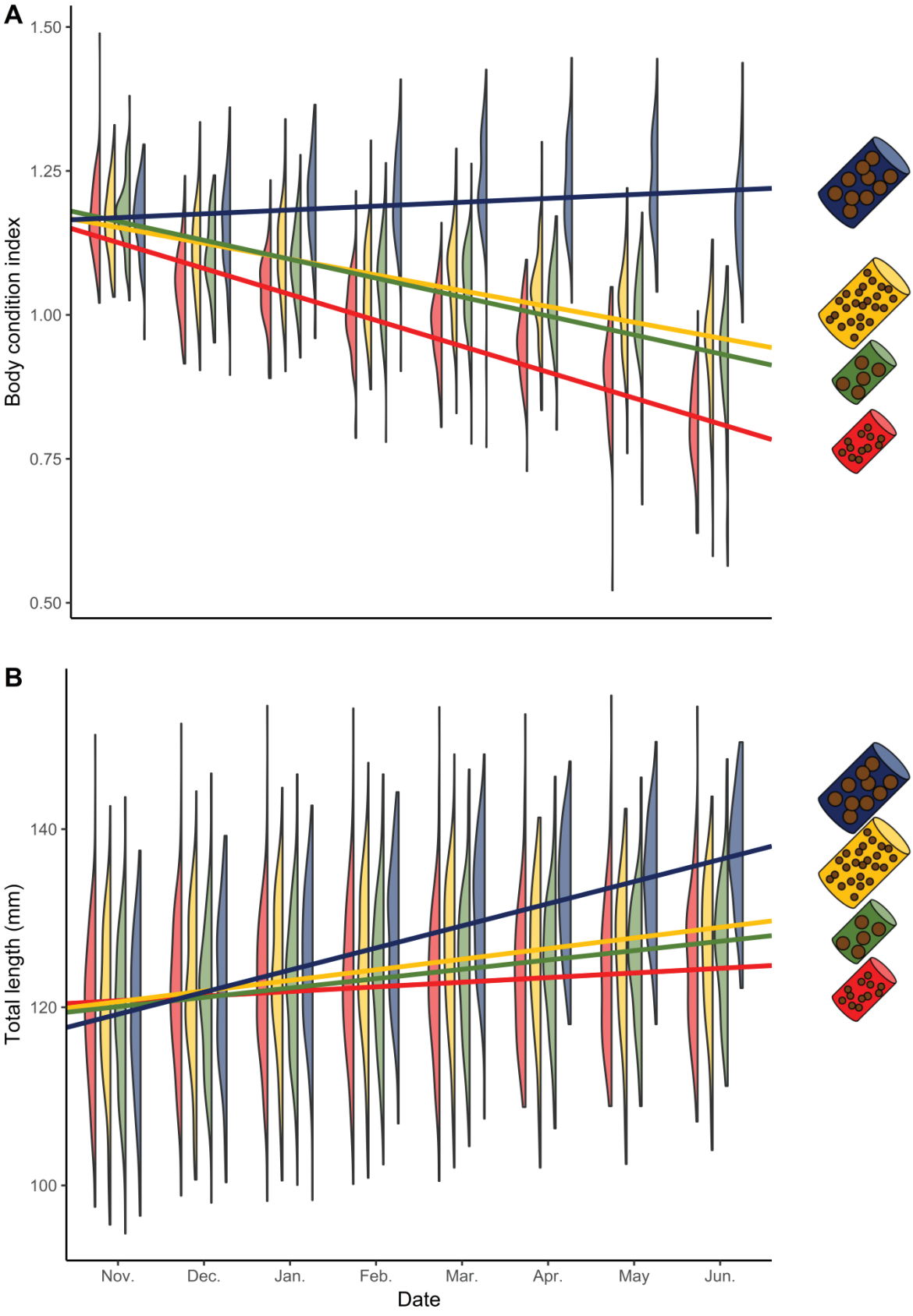


Figure S4: Violin plots of body condition index (A) and total length (B) distributions over time. Selected linear mixed-effect models were added (lines).

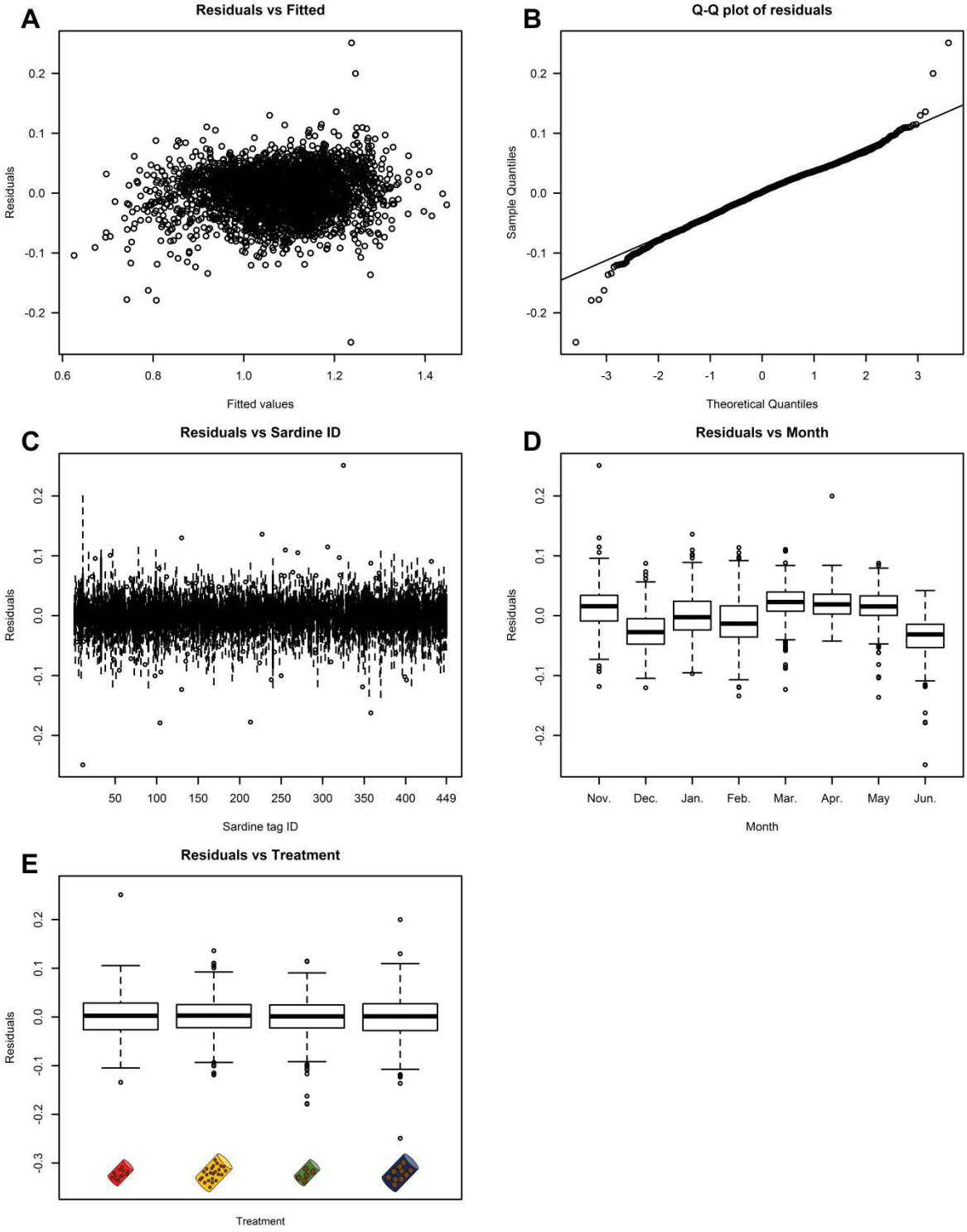


Figure S5: Validation graphs of the selected body condition model. A: Fitted values versus residuals. B: Q-Q plot of the residuals. C: Residuals versus sardine ID. D: Residuals versus time (month). E: Residuals versus treatments

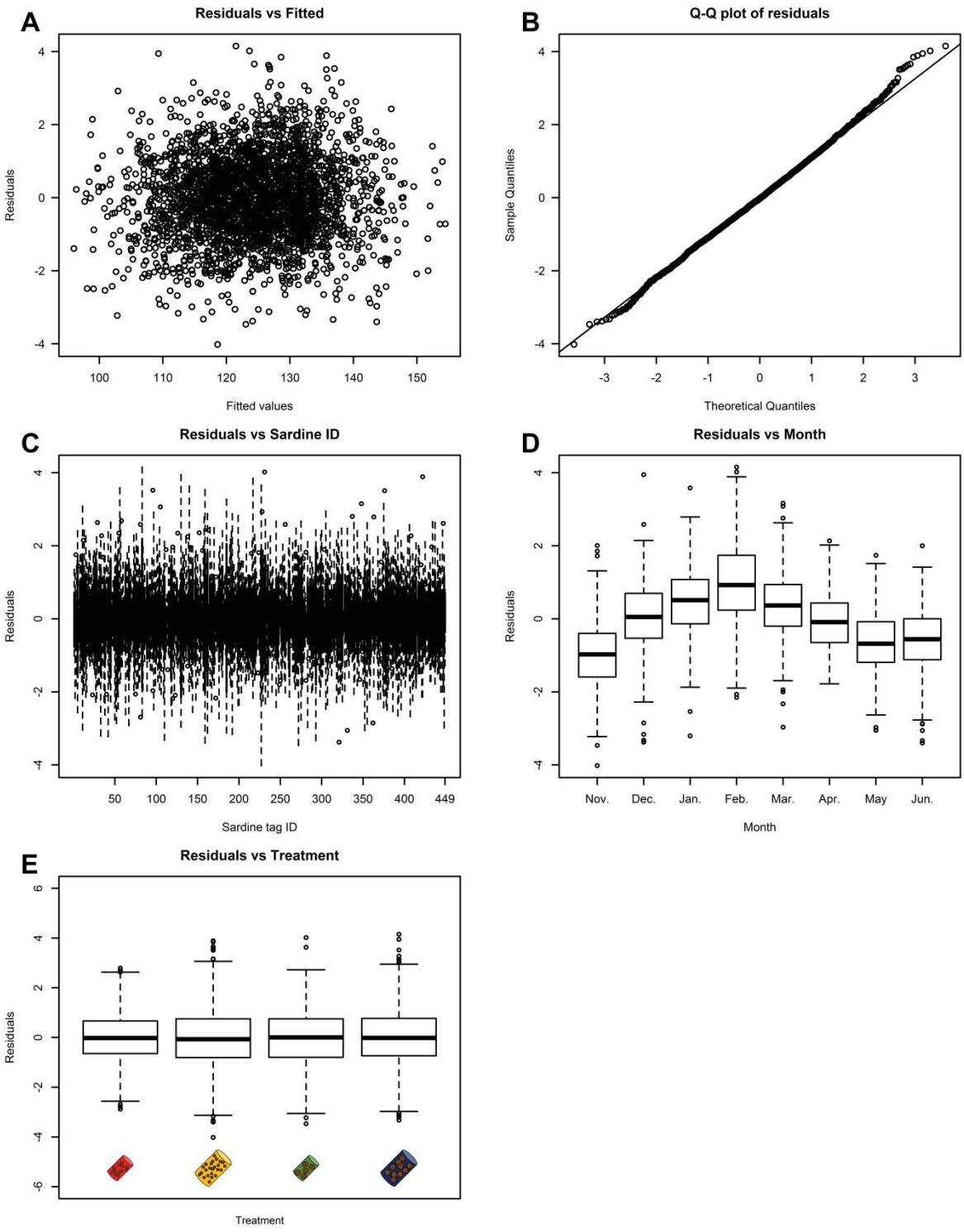


Figure S6: Validation graphs of the selected total length model. A: Fitted values versus residuals. B: Q-Q plot of the residuals. C: Residuals versus sardine ID. D: Residuals versus time (month). E: Residuals versus treatments

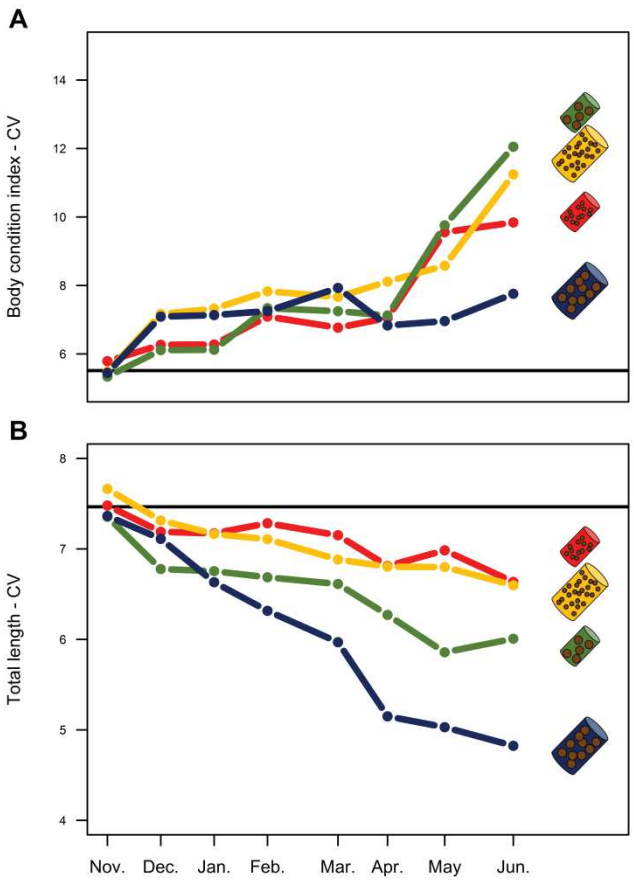


Figure S7: Time series of the CV of the body condition (A) and total length (B) of all sardines in each feeding treatment: red: pellet size of 0.1mm and pellet quantity of 0.3%; green: 0.1mm and 0.6%; yellow: 1.2mm and 0.1% and blue: 1.2mm and 0.6%. Dark line is the mean body condition and total length at the beginning of the experiments.

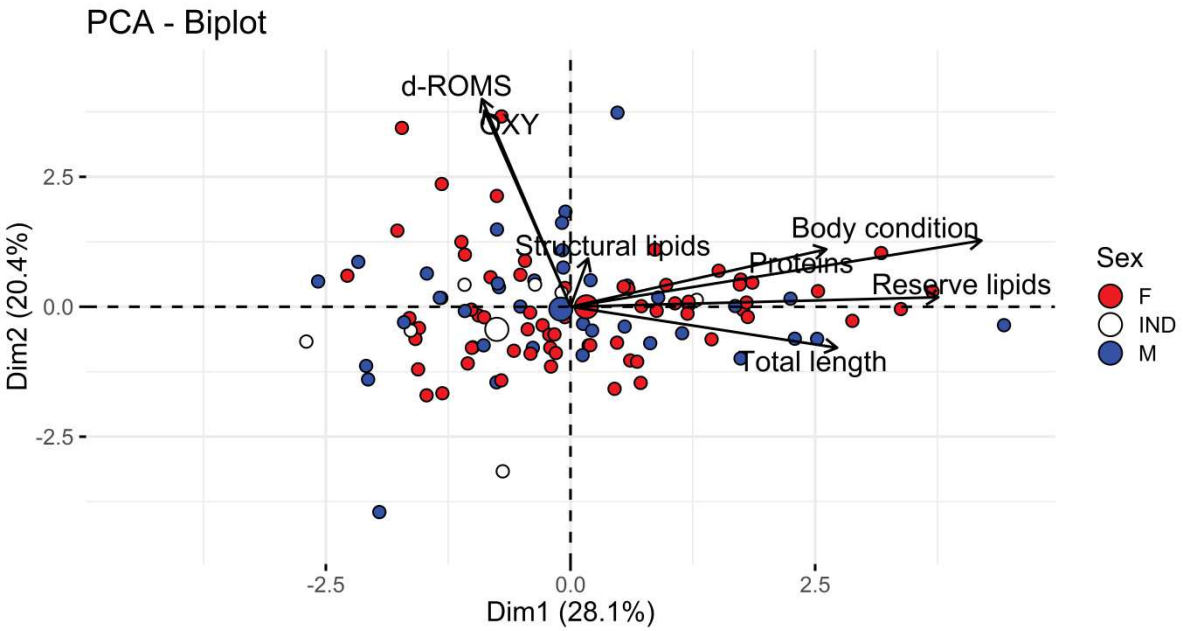


Figure S8: Biplot of the PCA built using body condition, total length, reserve and structural lipids and proteins contents, d-ROMS and OXY as explanatory variables, with grouping by sex. The large circles represent the barycenter of the individuals for a given sex.

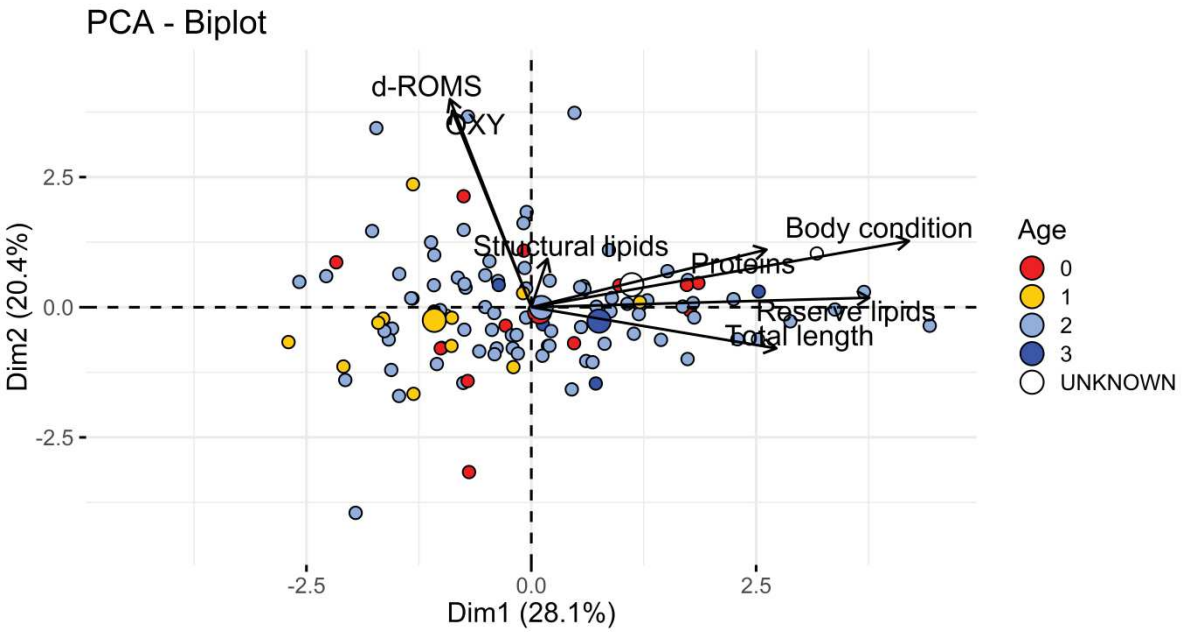


Figure S9: Biplot of the PCA built using body condition, total length, reserve and structural lipids and proteins contents, d-ROMS and OXY as explanatory variables, with grouping by age. The large circles represent the barycenter of the individuals for a given age.

Table S1. Mean ± sd of lipid class contents (µg/mg) of 0.1 and 1.2 mm pellets used in this study: triacylglycerols TAG), free fatty acids (FFA), alcohols (ALC), sterols (ST), acetone-mobile polar lipids (AMPL), diacylglycerols (DAG) and phospholipids (PL).

Pellet size (mm)	TAG	FFA	ALC	ST	AMPL	DAG	PL
0.1	67.1 ± 13.5	4.6 ± 4.3	3.5 ± 2.7	7.0 ± 5.0	12.0 ± 6.6	2.7 ± 2.5	55.7 ± 13.7
1.2	69.0 ± 24.6	3.6 ± 4.1	2.9 ± 2.6	5.7 ± 4.9	10.4 ± 11.3	2.1 ± 2.4	24.6 ± 14.3

Table S2. Results of the selected mixed effect model of body condition over time. Estimations of the predictors of all other fixed effects were based on the estimations of treatment 1. For instance, the intercept of treatment 4 (BLUE) was +1.18 (i.e. 1.14+0.04) and the slope was +0.01 (i.e. -0.04+0.05).

Random effects:

	Standard deviation	*Correlation*
(Intercept)	0.06	(Intercept)
Date	0.01	-0.17

Fixed effects:

Predictors	*Estimates*	*95% CI*	*df*	*p-value*
(Intercept)	1.14	1.12 – 1.15	2565	**<0.001**
Date	-0.04	-0.05 – -0.04	2565	**<0.001**
Treatment 2 (YELLOW)	0.02	-0.00 – 0.04	445	0.050
Treatment 3 (GREEN)	0.02	0.01 – 0.04	445	**0.012**
Treatment 4 (BLUE)	0.04	0.02 – 0.05	445	**<0.001**
Date:Treatment 2 (YELLOW)	0.02	0.01 – 0.02	2565	**<0.001**
Date:Treatment 3 (GREEN)	0.01	0.01 – 0.02	2565	**<0.001**
Date:Treatment 4 (BLUE)	0.05	0.05 – 0.06	2565	**<0.001**
Observations	3018			

Table S3. Results of the selected mixed effect model of total length over time. Estimations of the predictors of all other fixed effects were based on the estimations of treatment 1. For instance, the intercept of treatment 4 (BLUE) was 119.87 mm (i.e. 120.59-0.72) and the slope was +2.48 (i.e. 0.52+1.96).

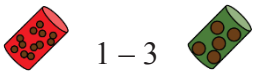
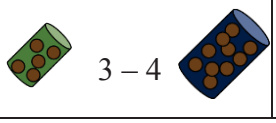
Random effects:

	Standard deviation	*Correlation*
(Intercept)	8.84	(Intercept)
Date	0.61	-0.35

Fixed effects:

Predictors	*Estimates*	*95% CI*	*df*	*p-value*
(Intercept)	120.59	118.95 – 122.24	2565	**<0.001**
Date	0.52	0.39 – 0.65	2565	**<0.001**
Treatment 2 (YELLOW)	-0.06	-2.39 – 2.27	445	0.959
Treatment 3 (GREEN)	-0.44	-2.77 – 1.90	445	0.713
Treatment 4 (BLUE)	-0.72	-3.05 – 1.61	445	0.543
Date:Treatment 2 (YELLOW)	0.67	0.49 – 0.85	2565	**<0.001**
Date:Treatment 3 (GREEN)	0.53	0.35 – 0.71	2565	**<0.001**
Date:Treatment 4 (BLUE)	1.96	1.78 – 2.14	2565	**<0.001**
Observations	3018			

Table S4. p-values of multiple pairwise comparisons between the four treatments for reserve and structural lipids, proteins, d-ROMS and OXY in March and June.

Treatments	Reserve lipids		Structural lipids		Proteins		d-ROMS		OXY	
	March	June	March	June	March	June	March	June	March	June
 1 – 2	0.462	0.741	0.702	0.979	0.684	0.481	1.000	0.150	1.000	0.219
 1 – 3	0.471	0.545	0.068	0.374	0.782	0.768	1.000	0.137	1.000	0.550
 2 – 3	0.808	0.964	0.513	0.710	0.945	0.545	1.000	0.773	1.000	0.837
 1 – 4	**<0.001**	**<0.001**	0.051	0.642	0.129	0.053	1.000	**0.001**	1.000	0.061
 2 – 4	**<0.001**	**<0.001**	0.567	0.825	0.290	0.513	1.000	0.093	0.867	0.546
 3 – 4	**<0.001**	**<0.001**	0.850	0.085	0.946	0.067	0.868	0.180	1.000	0.596

Movies S1: Feeding behavior of sardines fed with aquaculture pellets after acclimation

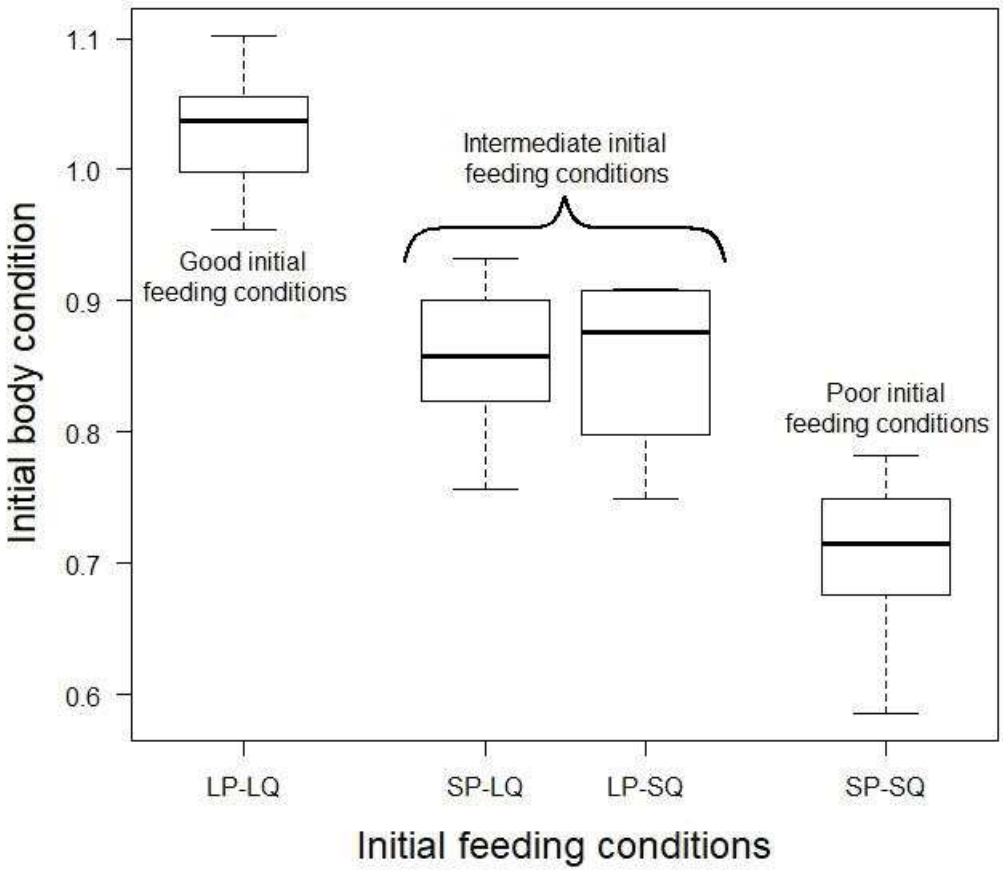


Figure S10: Body condition at the start of the fasting experiment according to the feeding treatment they experienced before. LP and SP stand for large and small particles respectively, while LQ and SQ stand for large and small quantities respectively.

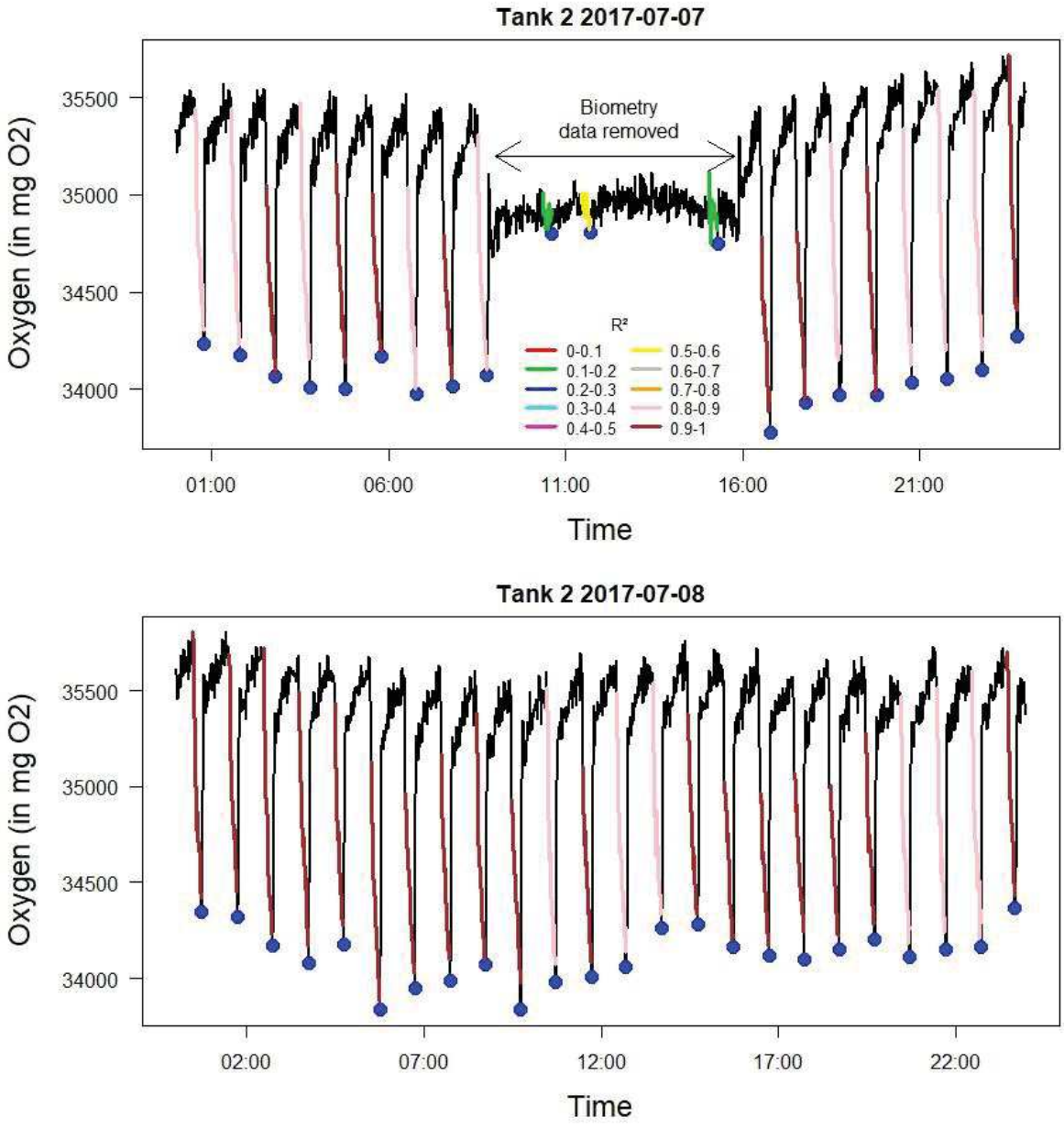


Figure S11: Dissolved oxygen in tank 2 during two days (2017-07-07 and 2017-07-08) as an example of respiration rate estimation. Cycles, during which oxygen consumption are calculated, are indicated in colour depending on the r-square of the linear regression. On the first day, a period was removed as fish were handled during that time for biometry, tanks cleaned, etc.

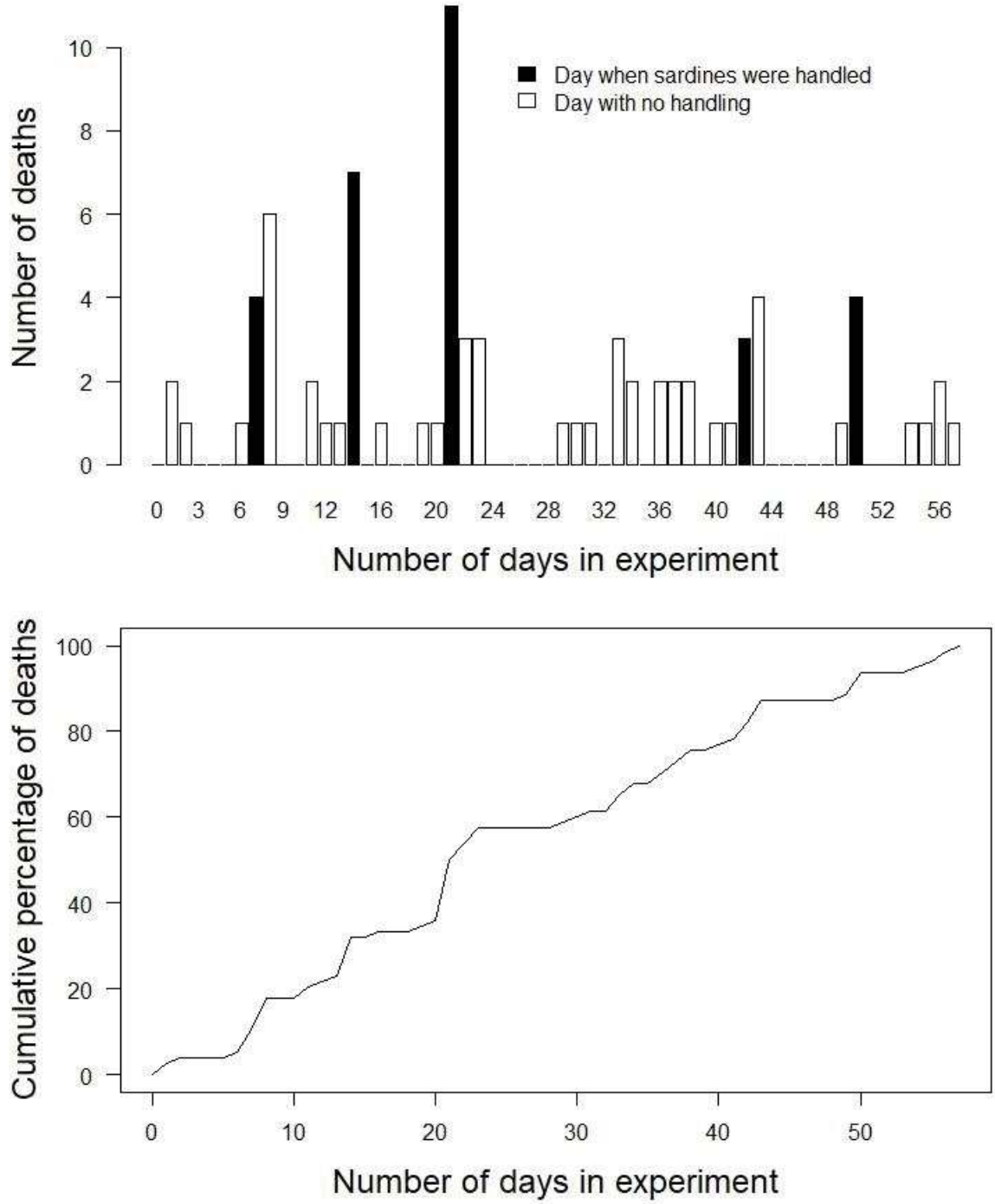


Figure S12: Number of daily sardine deaths along the fasting experiment. Days where sardines were handled are shown in black bars, while days with no handling appear as white bars.

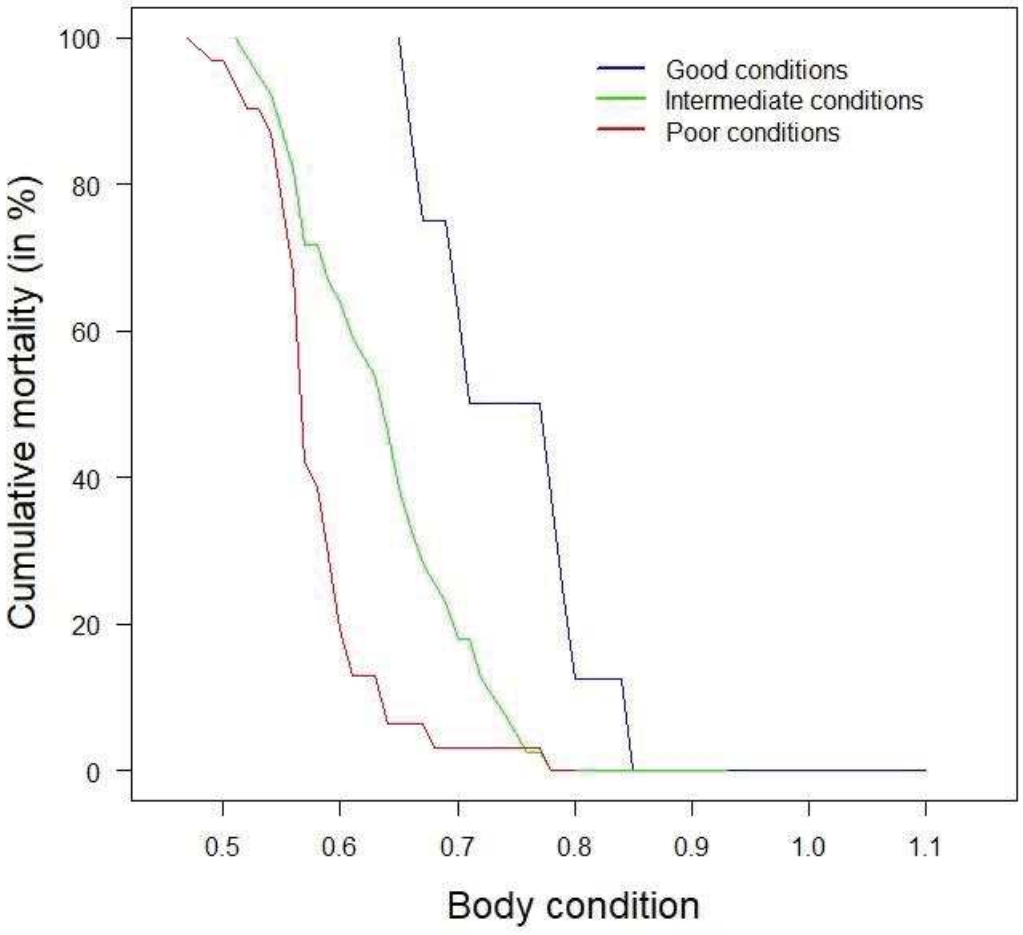


Figure S13: Cumulative mortality of sardines (in %) originating from each of the three initial feeding conditions (as indicated by colours) according to body condition.

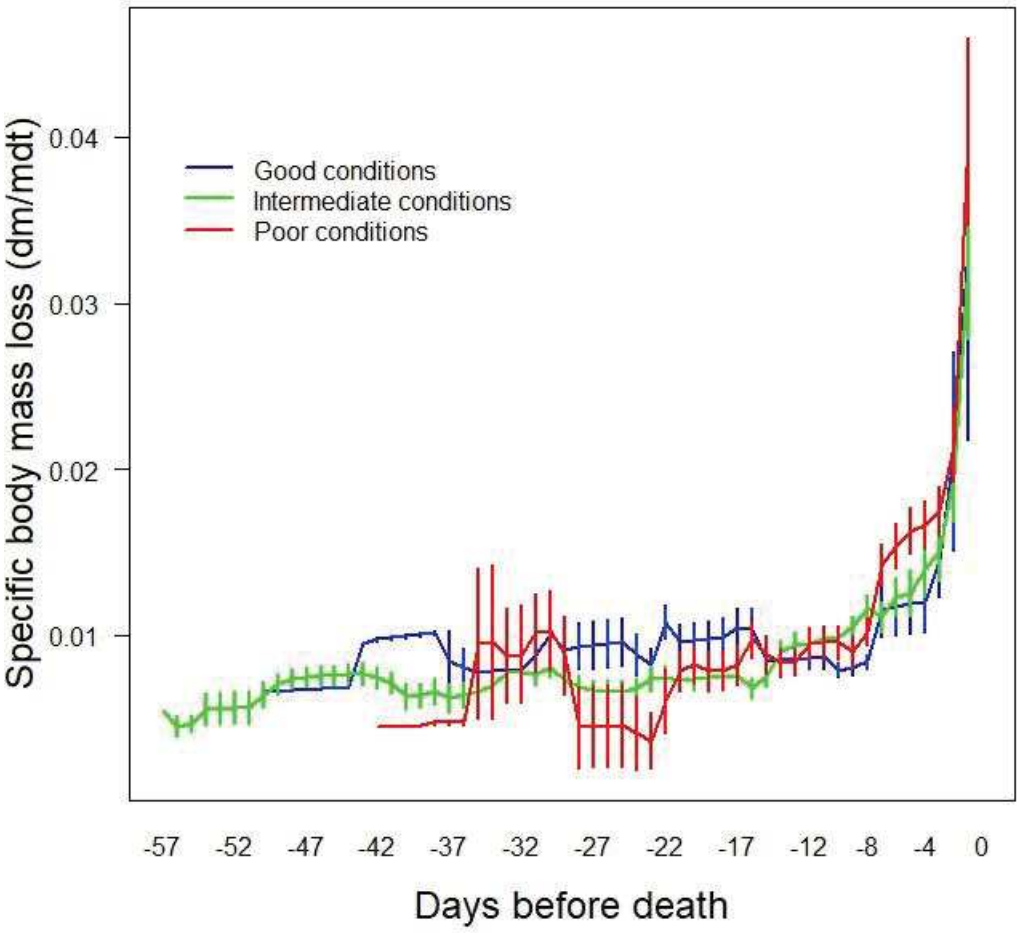


Figure S14: Mean ± SE specific body mass loss (dm/mdt) per day along time according to each initial feeding treatment. Colours indicate the initial feeding treatment sardines originated from. As individuals died at different time in the experiment, the number of days has been estimated relative to death. The specific body mass loss is expressed as %. The vertical dashed line shows a rupture in the slope of all three treatments.

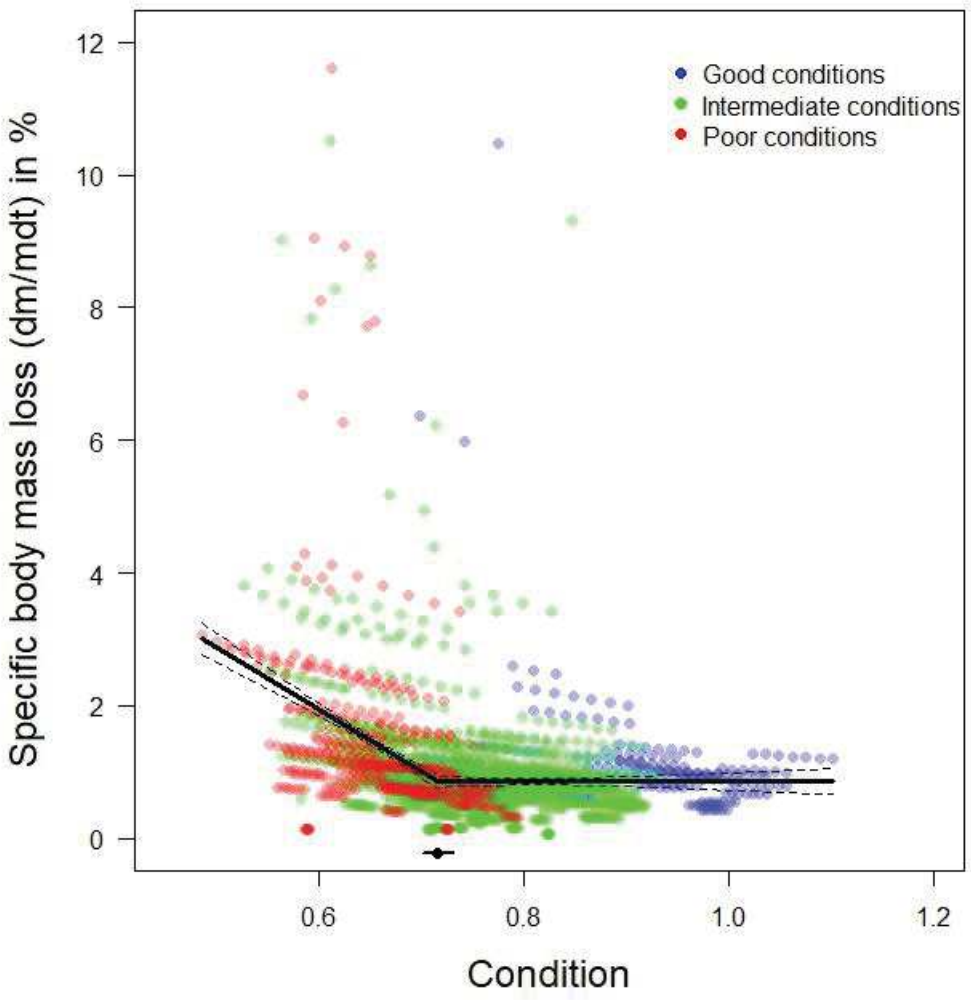


Figure S15: Specific body mass loss (dm/mdt) expressed as % according to body condition. Colour indicates the treatment sardines originated from. The segmented regressions are indicated by the black line and the 95% confidence intervals with dashed lines. The breakpoint along with its 95% CI is also indicated at the bottom of the figure.

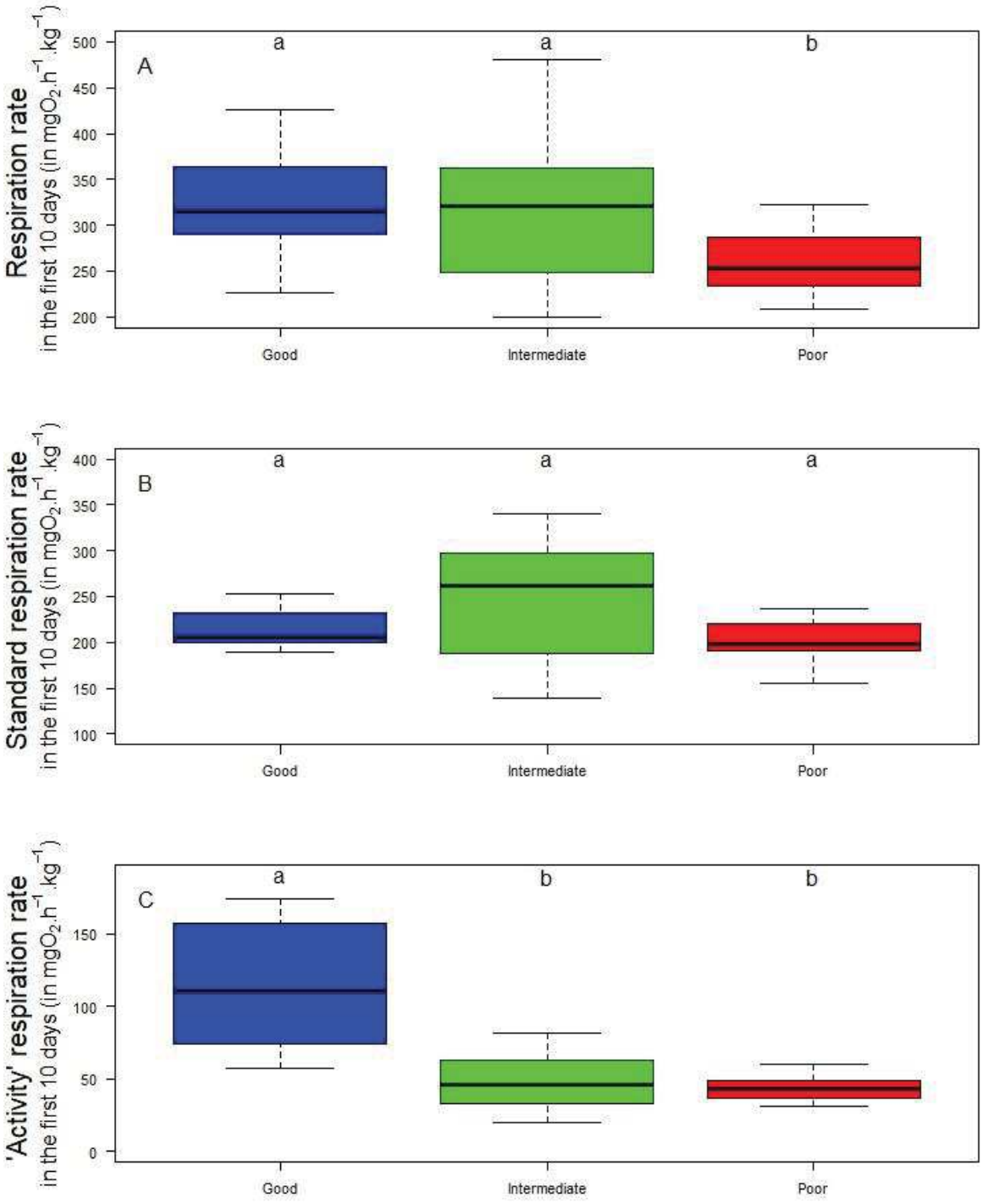


Figure S16: Respiration rates (in mg O2.h-1.kg-1) during the first 10 days of the experiment according to the feeding conditions sardines encountered before the start of the experiment. A) mean daily respiration rate, B) standard respiration rate (as estimated by the 10% quantile and representing mostly maintenance metabolism), C) 'Activity' related respiration rate (as estimated by the difference between the daily mean and minimum

values). Boxes sharing common letters are not significantly different from each other according to Bonferroni-corrected Wilcoxon tests.

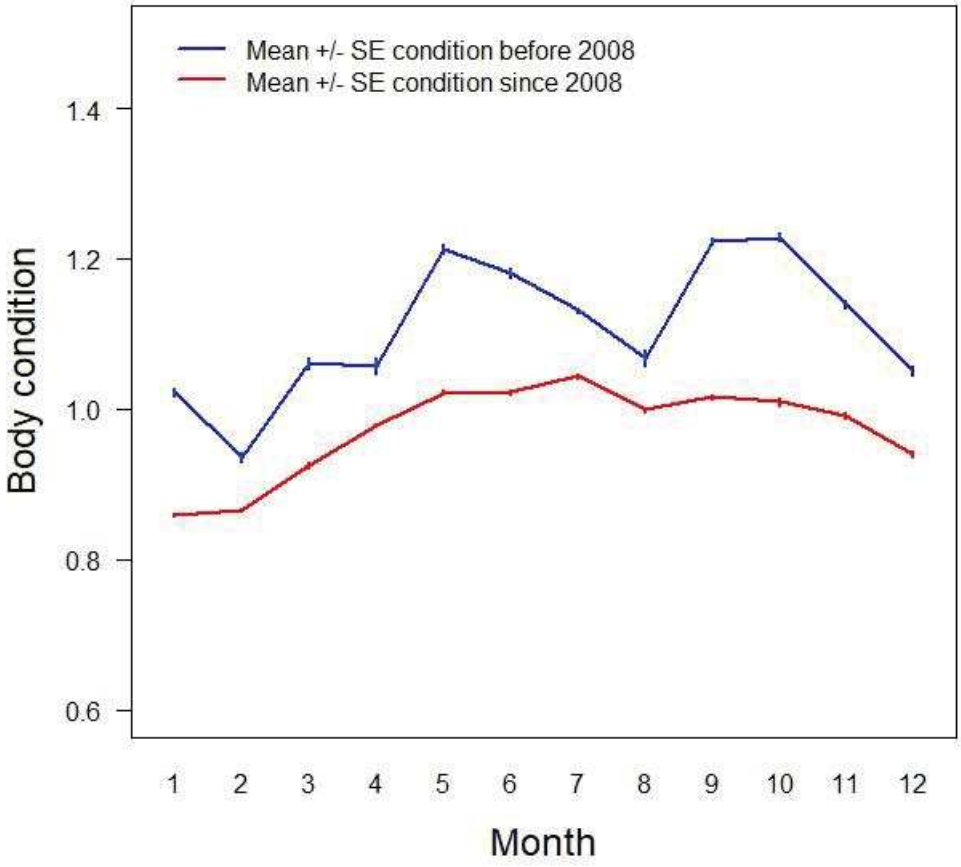


Figure S17: Mean ± SE body condition of sardines sampled in the wild before (in blue) or after (in red) 2008 for each month of the year.

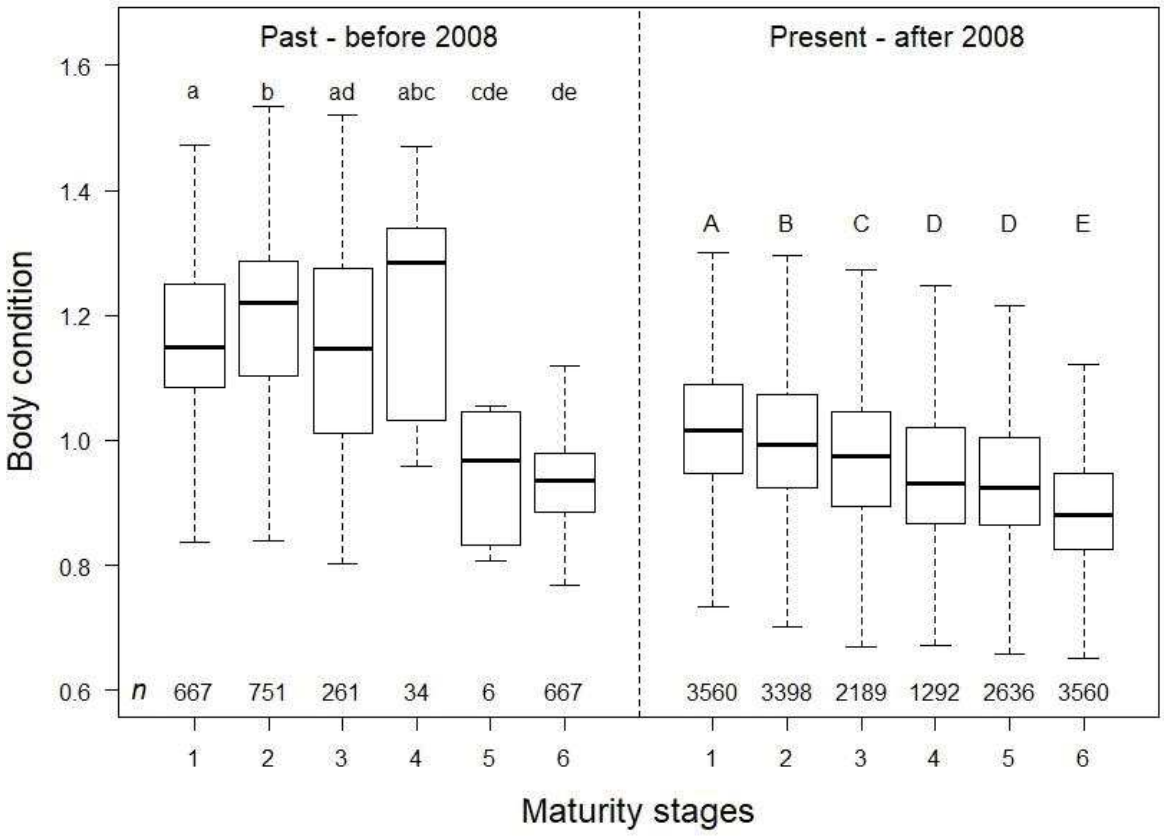


Figure S18: Body condition of sardines sampled in the wild before or after 2008 depending on maturity stages. n indicates the sample size in each category. Boxes sharing common letters are not significantly different from each other according to Bonferroni-corrected Wilcoxon tests. Maturity stage 1 corresponds to sexual rest, stages 2 to 4 to increasing development of the gonads, 5 to active spawning and 6 to post spawning.

Chapter 5

Appendix A: Gonad compartment

Most of spawning events occurred between December and February (Chapter 3) and the in-situ gonadosomatic index also decreased at this period (Brosset et al., 2016b). Without solid information on the reproduction period of sardines, we assumed that the reproduction buffer E_R and the gonads E_{Go} were equal to zero on March 1st. For the same reasons, the energy allocation $\dot{p}_{Go}$ from the reproduction buffer to gonads was assumed to start on September and ended on March and was defined such as:

$$\dot{p}_{Go} = \begin{cases} [\dot{p}_{Go}]V & \text{if allocation period} \\ 0 & \text{otherwise} \end{cases} \quad [A.1]$$

with the volume-specific allocation rate to gonads $[\dot{p}_{Go}]$ (in J d^{-1} cm^{-3}), the temperature correction factor cor_T and the structural length L (in cm). Note that the allocation to gonads also depends on the available energy in the reproduction buffer E_R:

$$\dot{p}_{Go} = \begin{cases} \dot{p}_{Go} & \text{if } E_R > \dot{p}_{Go}dt \\ 0 & \text{otherwise} \end{cases} \quad [A.2]$$

with dt the time step.

Then, we supposed that the energy allocation from the reserve E to the reproduction buffer E_R does not require any chemical transformation. However, the transformation of the reproduction buffer into gonads has an efficiency of κ_R:

$$\frac{dE_R}{dt} = (1-\kappa)\dot{p}_C - k_J E_{Hp} - \dot{p}_{Go} \quad [A.3]$$

$$\frac{dE_{Go}}{dt} = \kappa_R \dot{p}_{Go} \quad [A.4]$$

Further, the reproduction period was fixed from December 1st to March 1st (based on reproduction event observed during experimentations and in-situ GSI (see Brosset et al. 2016). If the energy content in the gonads is higher than the energy requirements for one

batch, we have a spawning event. Energy requirements of one batch E_{batch} (in J) depended on the total fish length and was estimated using equation found by Brosset et al. (2016):

$$E_{batch} = E_0(561.6 \times L - 5455) \quad [A.5]$$

with the physical total length L (in cm) and E_0 the energy content of 1 egg (in J) .

This equation was available for fish between 11 and 15 cm. We assumed the same relation for larger fish but E_{batch} = max(E_{batch}, 0) for smaller ones. Further, the between-batches period was also size dependent: 17 days for individuals smaller than 13cm, 12.25 days for those between 13 and 16 cm and 7.81 days for individuals larger than 16 cm (Ganias et al., 2003). The reproduction period ended on March 1st and spawning ceased until the next reproduction period.

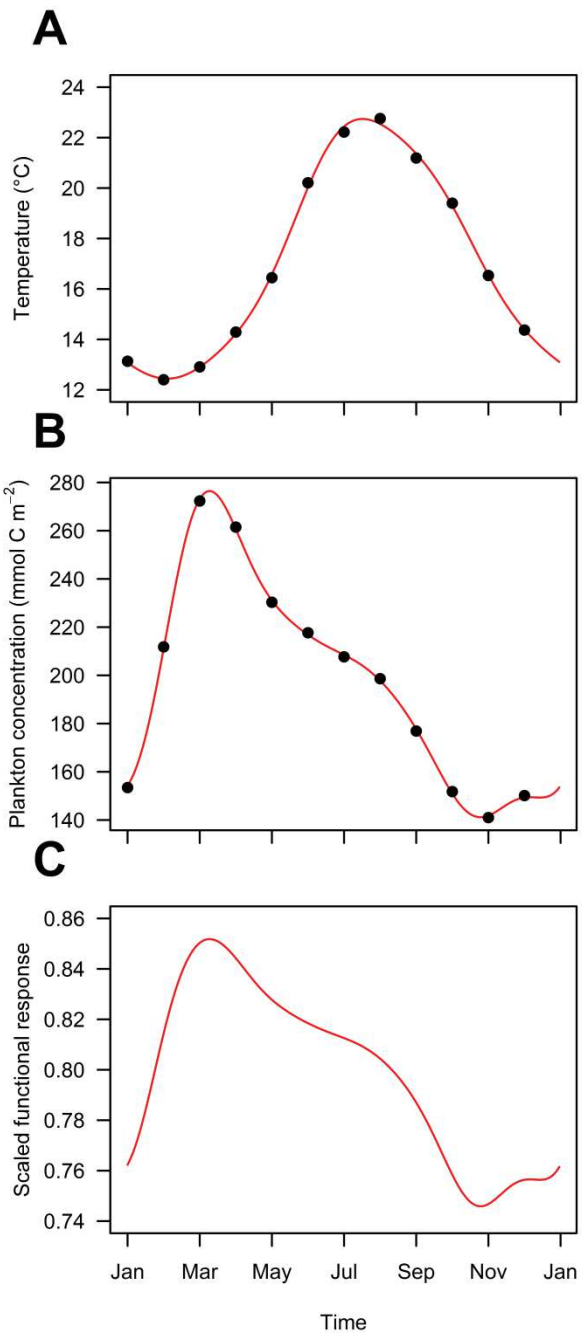


Figure S19: Observation data (black dots) and fitted Fourier series on the median values (red lines) of temperature (A), plankton concentration (B) and scale functional response f (C) in the Gulf of Lions.

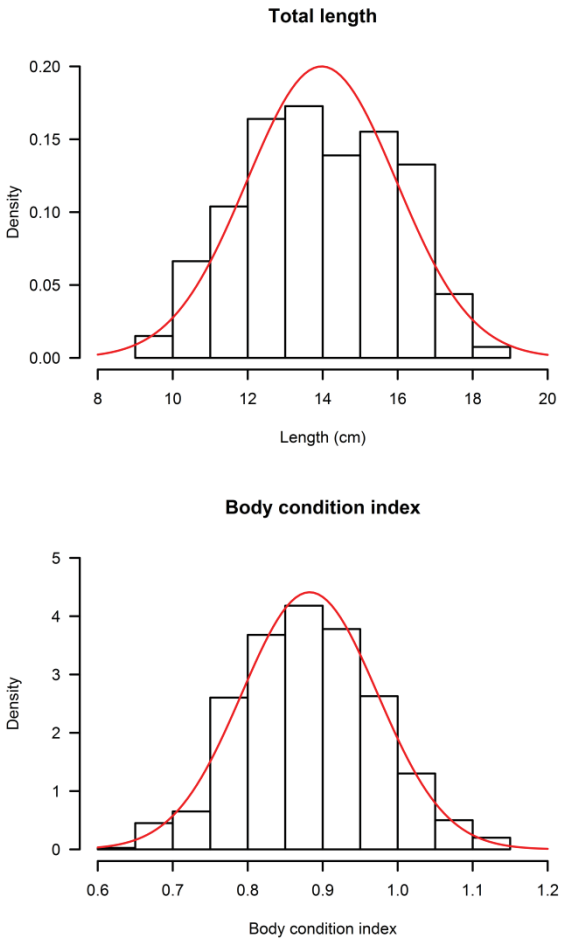


Figure S20: Distribution of total length and body condition index of in-situ sardine population. Red lines represent the normal distribution fit on observations.

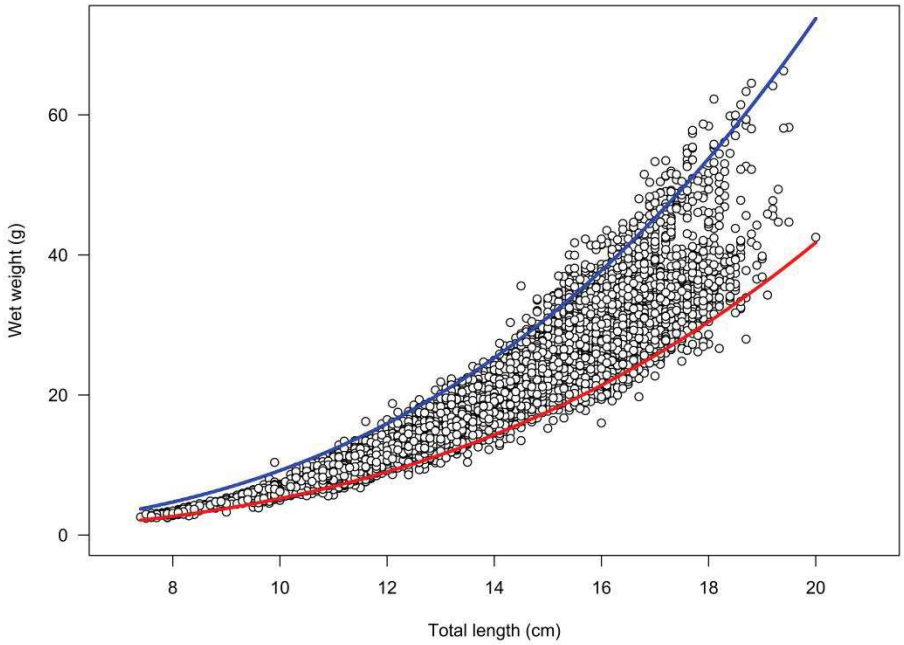


Figure S21: Weight (without gonad) and total length relationship of sardines fished on March between 2008 and 2019. The two quantile regression curves drawn were fitted on the 97.5 (in blue: $W = (0.21 \times L)^3$) and 2.5 quantiles (in red: $W = (0.17 \times L)^3$).

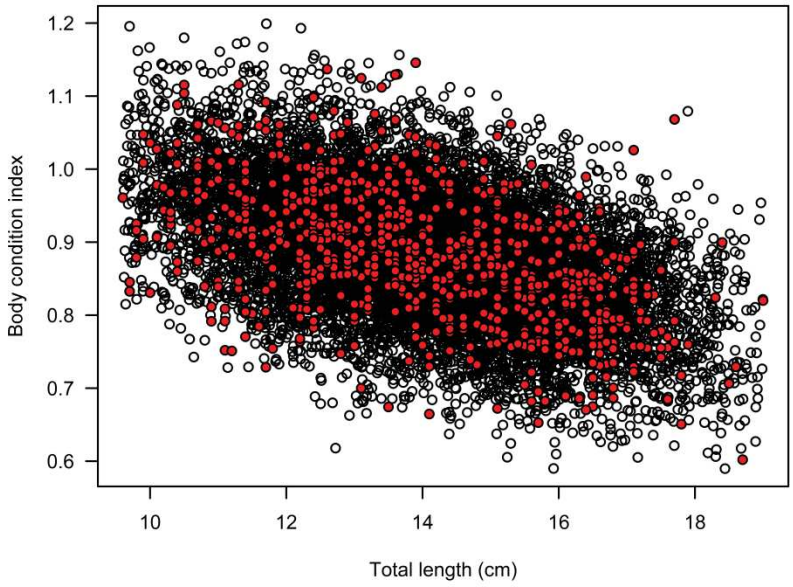


Figure S22: Length and body condition index of in-situ population samples in March (in red) and the 10,000 modeled sardines using in the simulation (in black)

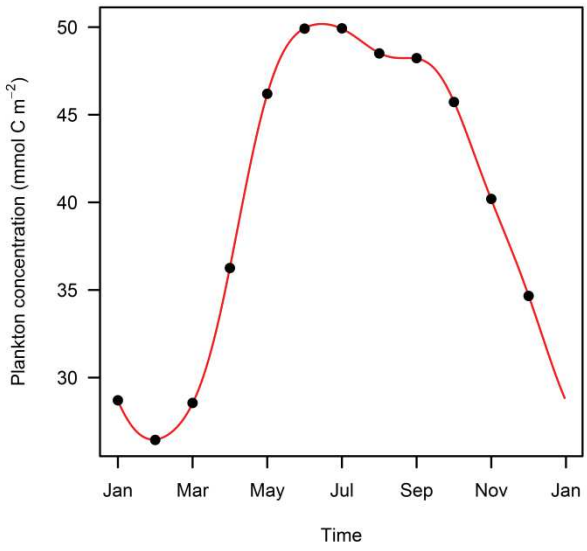


Figure S23: Fitted Fourier series on the median values (red lines) of mesozooplankton concentration in the Gulf of Lions.

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Annex 1

Dolphin predation pressure on pelagic and demersal fish in the northwestern Mediterranean Sea

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Dolphin predation pressure on pelagic and demersal fish in the northwestern Mediterranean Sea

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ABSTRACT: Sardine *Sardina pilchardus*, anchovy *Engraulis encrasicolus* and European hake *Merluccius merluccius* represent a significant part of the commercial landings in the Gulf of Lions (northwestern Mediterranean Sea). However, their stocks have shown severe declines during the last decades due to fishing pressure and/or environmental changes. The aim of this study was to estimate the current predation pressure of bottlenose dolphins *Tursiops truncatus* and striped dolphins *Stenella coeruleoalba* — which are abundant in the area — on sardine, anchovy and hake. To do so, we developed an original approach based on several data sets and models (aerial surveys, stomach contents, allometric and stock assessment models) and Monte Carlo simulations to incorporate various sources of uncertainty due to data limitations. Despite the uncertainties, the results showed that dolphin predation pressure on sardine and anchovy was extremely low in the Gulf of Lions (all simulations <0.5% of the available stock), indicating little impact of dolphins on those populations. However, significant predation pressure on hake (median value: 23%) was detected, a value which might have doubled in the last 30 yr because of hake overfishing. Overexploitation has thus reinforced the natural mortality of hake due to dolphin predation, but this predation pressure remains 2 to 3 times lower than that exerted by fisheries.

KEY WORDS: Predation pressure · Top-down effect · Bottlenose dolphin · Striped dolphin · European hake · Sardine · Anchovy · Gulf of Lions

INTRODUCTION

Although scientists have often assumed that marine ecosystems are mostly controlled by bottom-up processes (Aebischer et al. 1990, Frederiksen et al. 2006), the reverse effect, i.e. top-down control, or even trophic cascades may nonetheless exist (Ainley et al. 2006, Österblom et al. 2006). Other studies have proposed that control in marine food chains is dynamic and that it can alternate between bottom-up and top-down controls (Litzow & Ciannelli 2007, Cury et al. 2008). In particular, marine mammals play a considerable role within marine ecosystems as their abundances and distributions could impact the structure and functioning of those ecosystems (Roman et al. 2014, Kiszka et al. 2015). Although they might forage on a large variety of prey, from phytoplankton to fish or other marine mammals (Bowen 1997, Astruc 2005), they are usually considered as top predators and often feed on prey that are also exploited by fisheries (Kaschner et al. 2004, Lockyer 2007). Even where marine mammals are thought to mostly consume non-exploited prey species, local competitions with fisheries could appear as a result of 'regional and temporal aggregations of marine mammals in highly productive areas which are likely to coincide with high density fishing areas' (Kaschner et al. 2004, p. 57). Consequently, interactions between fisheries

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and marine mammals and even trophic competition in some cases might occur (Bearzi 2002, Pusineri 2005). These frequent interactions between marine mammals and different types of fisheries have double stakes, as they may induce mortality and serious injury for marine mammals but also serious consequences for fishermen due to depredation within the catch (Bearzi 2002, Werner et al. 2015). In particular, incidental mortality of marine mammals due to bycatch represents an issue for dolphin conservation (Brotons et al. 2008, Read 2008, Reeves et al. 2013). On the other hand, depredation (when dolphins take bait or hooked target fish) is an economic concern both worldwide (Read 2005, Snape et al. 2018) and in the Mediterranean Sea, where it affects mostly immobile fishing gear such as trammel nets and gillnets (Bearzi 2002, Gnone et al. 2011, Pardalou & Tsikliras 2018) but also longlines (Bearzi 2002), purse seines (Reeves et al. 2001), trawl nets (Reeves et al. 2001) and fish farms (López 2006). Finally, overfishing of dolphin prey species is also known to impact dolphin populations, along with other human activities such as pollution, habitat degradation or loss, tourism and climate change (Coll et al. 2010, Gonzalvo et al. 2014, Pennino et al. 2017).

Most of the fish stocks in the world are either fully exploited (61%) or overexploited (28%) (Sinclair et al. 2002, FAO 2014). This worrying situation is even more acute in the Mediterranean Sea, where an alarming decrease of the main exploited populations has been observed since 1990 (Vasilakopoulos et al. 2014). In those detrimental conditions, the energetic needs for top predators might not always be fulfilled (Bearzi et al. 2006, Österblom et al. 2006, Cury et al. 2011) but also, any additional pressure might affect the stock even more. In particular, natural predation of marine mammals could have additional or synergetic effects on fish stocks. Although the striped dolphin *Stenella coeruleoalba* — the most abundant dolphin in the Mediterranean Sea — feeds mostly on non-commercial prey, it can sometimes also exploit highly valuable commercial resources, such as European hake *Merluccius merluccius* and small pelagics in the western Mediterranean Sea (Bearzi 2002, Gómez-Campos et al. 2011, Aznar et al. 2017). Additionally, the bottlenose dolphin *Tursiops truncatus* mostly resides on the continental shelf during summer (Laran et al. 2017), where most of the Mediterranean fisheries operate, and feeds preferentially on demersal prey such as the European hake, resulting in potentially high interactions with several local fisheries (Bearzi 2002, Kaschner et al. 2004, Gonzalvo et al. 2014).

In the Gulf of Lions, sardines *Sardina pilchardus* and anchovies *Engraulis encrasicolus* have high economic interest, and, until the mid-2000s, their landings represented up to 50% of total annual landings (Bănaru et al. 2013). European hake is also a significant part of the commercial landings in this area and has a high economic value (Mellon-Duval et al. 2017). However, stocks of European hake and small pelagics (both sardines and anchovies) have shown important declines during the last decades (GFCM 2017a,b). The decline in European hake stock is a clear and well-documented case of overfishing due to overcapacity (GFCM 2017a), while environmental changes appear as the main drivers in the decline in condition and size of small pelagic fish in the Gulf of Lions (Van Beveren et al. 2014, Saraux et al. 2018). In those already poor conditions for small pelagics and hake, natural predation of top predators could have more of an impact. The predation pressure of Atlantic bluefin tuna *Thunnus thynnus* on both sardines and anchovies in the Gulf of Lions has recently been studied and shown to be low (<2%; Van Beveren et al. 2017) but no study has focused on marine mammals in this area. Yet marine mammals seem to be important consumers of prey in various ecosystems, especially cetaceans because of their large body sizes and relatively high metabolic rates (Bowen 1997, Laran et al. 2010). While 3 studies reported prey consumption by dolphins in French marine areas (the Bay of Biscay; Pusineri 2005, Spitz et al. 2018 and the Ligurian Sea; Laran et al. 2010), no study has estimated the predation pressure this could exert on the prey (i.e. prey consumption related to the actual amount of prey biomass). The estimation of predation pressure indeed requires a large and diversified amount of information both on prey and predators (Essington et al. 2001), which is difficult to obtain for marine species because of practical constraints in observing animals (Van Beveren et al. 2017). Bottlenose and striped dolphins are the 2 main dolphin species in the Gulf of Lions (Laran et al. 2017). Their diets are principally composed of fish and cephalopods. European hake is the main prey of bottlenose dolphins and also appears in the striped dolphin diet, while small pelagics, especially sardines, are present in the diet of both dolphin species (Astruc 2005, Gómez-Campos et al. 2011).

In this study, we aimed to assess, for the first time, the predation pressure exerted by the 2 dolphin species (bottlenose and striped dolphins) on the main exploited fish (European hake, sardine and anchovy) in the Gulf of Lions. To do so, we used and adapted an original approach previously developed by Van

Beveren et al. (2017), which combines several data sources on prey and predators (aerial surveys, stomach contents or individual energetic values) and modeling approaches (energetic, stock assessment and statistical models). To take into account the numerous data limitations and estimate the uncertainty associated with our estimations, we further developed a simulation framework, similar to the approach recently used by Spitz et al. (2018).

MATERIALS AND METHODS

Gulf of Lions

The Gulf of Lions is located in the northwestern Mediterranean Sea (Fig. 1) with a bathymetry between 0 and 2500 m and covering about 15 000 km^2 (Mellon-Duval et al. 2017). The dominant forcing drivers in the area are the strong northwestern (tramontane) and northern (mistral) winds, the western Mediterranean mesoscale circulation and the freshwater input from the Rhone River (Millot 1990, Petrenko et al. 2005). The Gulf of Lions represents an important feeding area for fish, birds and mammals, for both resident and migratory populations (Bănaru et al. 2013).

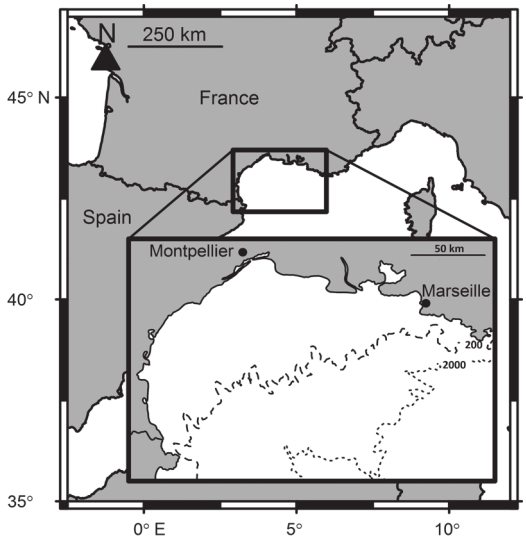

Fig. 1. Gulf of Lions, showing 200 and 2000 m bathymetry

Dolphin predation pressure

To estimate dolphin predation pressure, 5 different processes must be taken into account: 3 regarding the predators (their abundance, diet and energetic demands) and 2 regarding their prey (their energetic values and stock biomass; Fig. 2). Dolphin predation pressure on a given prey was calculated as follows:

$$\text{Predation pressure} = \frac{\sum_{i=1}^{\text{nb}_{\text{dolphin}}} \left(\text{DEE}_i \times \frac{\%W_{i,k}}{\sum_{j=1}^{\text{nb}_{\text{prey}}} \%W_{i,j} \times E_j} \times \frac{1}{\alpha_i} \right) \times 365}{\text{stock}_k} \quad (1)$$

where nb is the number of dolphins or prey, i represents a given dolphin in the population, DEE_i is its daily energy expenditure depending on its mass and

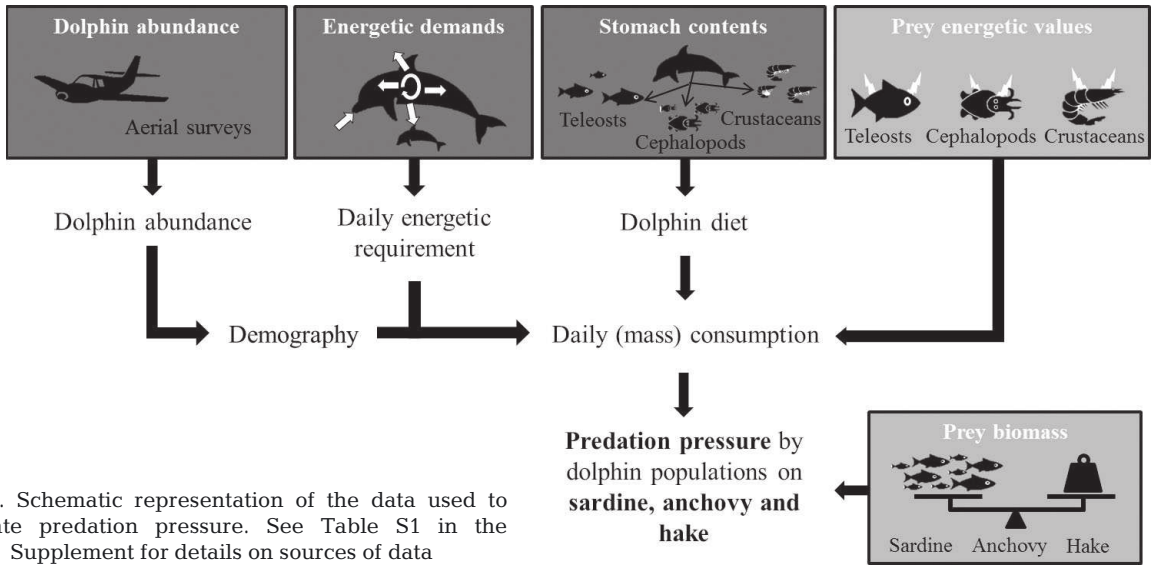

Fig. 2. Schematic representation of the data used to estimate predation pressure. See Table S1 in the Supplement for details on sources of data

reproductive status, $\%W_{i,j}$ is the percentage of biomass of prey j in its diet, E_j is the energy content of prey j, α_i is the assimilation efficiency and stock_k is the stock biomass of prey k.

The estimation of predation pressure thus implies several steps, especially to estimate the dolphin population and demography or their daily consumption. As the estimations of the parameters related to these processes of interest exhibit substantial uncertainty (e.g. unknown energetic values for some prey species in the Gulf of Lions) and variability (e.g. interseasonal and interannual variability in dolphin abundances), a simulation framework was developed, similar to the method employed by Spitz et al. (2018), in which prey consumption and energy requirements were estimated using Monte Carlo simulations. In our simulation framework, each simulation was divided into 3 main parts ('Dolphin population and demography', 'Daily consumption' and 'Predation pressure'), corresponding to 12 successive steps presented in details in the next sections (see Fig. 3 and Table S1 in the Supplement at www.int-res.com/articles/suppl/m603p013_supp.pdf). Predation pressure was estimated through 10 000 Monte Carlo simulations (Manly 2006), in which each input parameter was drawn from a given distribution (e.g. normal, uniform and gamma distributions) independently of each other. The predation pressure results are presented as 95% confidence intervals (CI) to remove outliers.

Dolphin population and demography (Part 1)

Annual dolphin abundances in the Gulf of Lions (95% CI) were estimated from Ifremer aerial surveys from 2000–2003 and 2009–2012 using the line transect approach (Bauer et al. 2015). Following Bauer et al. (2015), and taking into account uncertainties and year-to-year variations, dolphin abundances were estimated first using uniform distribution from aerial survey years (for the choice of the year) and then a draw following a log-normal distribution fitted on density and 95% CI of the chosen year (Fig. 3, Part 1, Step 1). As the aerial surveys did not enable us to discriminate between bottlenose and striped dolphins; see Bauer et al. 2015), we assumed that the dolphin abundance estimated in this study represented the sum of both bottlenose and striped dolphin populations. The striped dolphin is the most abundant species in the northwestern Mediterranean Sea (around 90% of dolphins), with larger group sizes than bottlenose dolphins (Gannier 2005, Gómez De Segura et al. 2008, Laran et al.

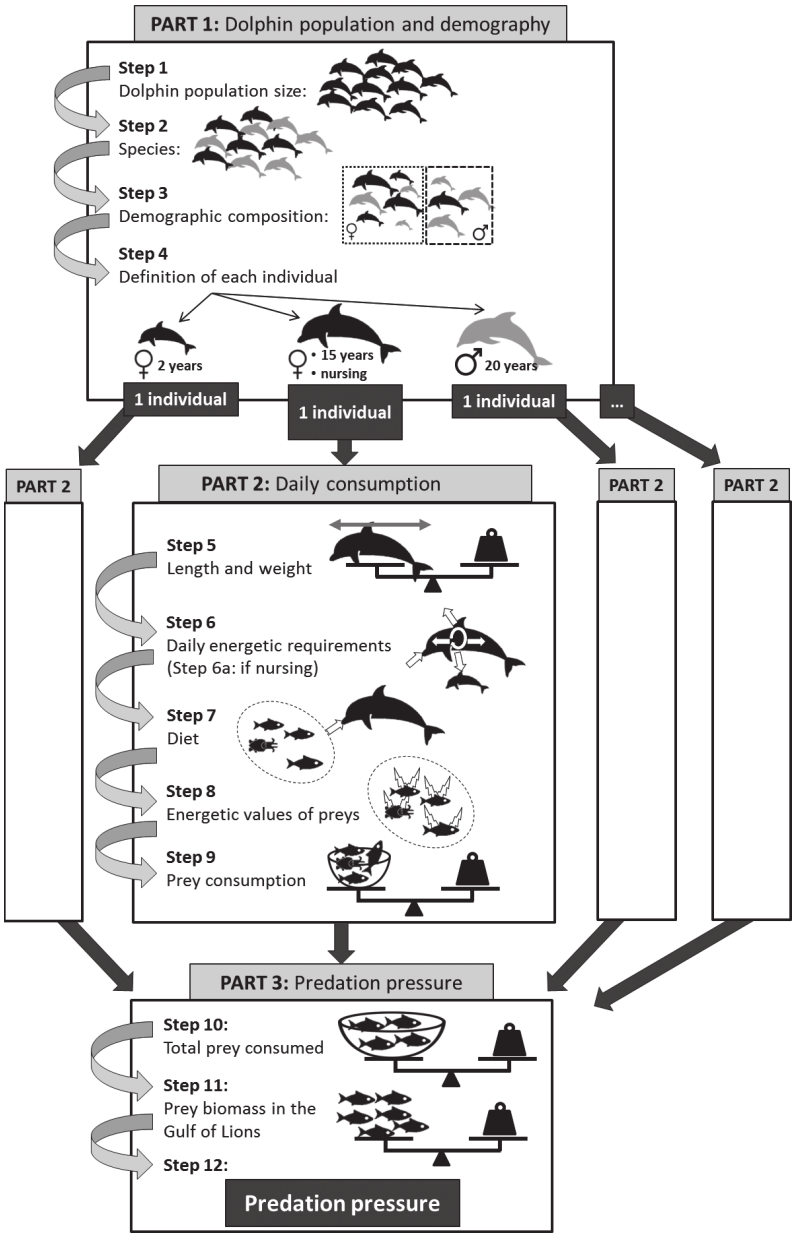

Fig. 3. Schematic representation of the 12 steps (3 parts) of the simulation framework used to estimate predation pressure

2017). However, bottlenose dolphin groups have also been observed in the Gulf of Lions and they could represent a substantial proportion of the dolphin population during summer (Laran et al. 2017, C. Saraux unpubl. data from PELMED surveys). To take this variability into account, a uniform draw was done between 10 and 90% to estimate striped dolphin percentage into the dolphin population for each simulation (Fig. 3, Part 1, Step 2).

Because energy expenditure varies with developmental and reproductive stage as well as body mass (Lockyer 2007), the abundance of dolphins was separated into 3 demographic groups: calves (age 0 to 2; Calzada et al. 1996, Reeves & Read 2003), juveniles (age 2 to 9; Mattson et al. 2006) and adults (age 9 to 35; maximal value observed in Stolen et al. 2002). According to previous studies, dolphin populations are generally composed of 6 to 27% calves, 15 to 30% juveniles and from 43 to 79% mature adults (Wells & Scott 1990, Calzada et al. 1994, Mattson et al. 2006). Sex ratio does not vary much between the 2 species, with 47 to 62% and 43 to 64% of males for bottlenose and striped dolphins, respectively (McFee & Hopkins-Murphy 2002, Stolen et al. 2002, Centro Studi Cetacei 2012). Uniform draws were performed on the above stage-class proportions and sex-ratio values, so that each individual was attributed a sex and stage class (Fig. 3, Part 1, Step 3). Finally, the age of each individual was estimated drawing from a uniform distribution on the age range of its stage class, while a Bernoulli distribution, B(p) (with p equal to the ratio of the number of calves over the number of mature females) was used to assess whether a mature female was nursing. At the end of this first part, each individual of the simulated population was described in terms of species, sex, age and reproductive status (i.e. for mature females whether they were nursing or not) (Fig. 3, Part 1, Step 4).

Daily consumption (Part 2)

Daily energy requirement

Empirical relationships have been commonly employed to quantify requirements of dolphins, baleens or seals, with all of them based on allometry and relationships with body mass (e.g. Sigurjónsson & Víkingsson 1997, Barlow et al. 2008, Laran et al. 2010). Therefore, dolphin age had to be converted into body mass using a combination of age–length and length–mass empirical relationships (summarized in Tables S2 & S3). Due to a lack of information for striped dolphins, but assuming good correlation between the 2 species, length–mass equations of bottlenose dolphins were used here to estimate mass of striped dolphins (Fig. 3, Part 2, Step 5). The existing length–mass relationships were derived using data from stranding and capture–release projects carried out in the Atlantic Ocean (Table S3); however, data on stranded dolphins in this area are consistent with those relationships (see Fig. S1), although striped dolphins in the Atlantic Ocean appear to be bigger and heavier than those in the Mediterranean Sea (Di-Méglio et al. 1996).

The 3 empirical relationships most frequently used to estimate energy requirements of marine mammals (e.g. Barlow et al. 2008, Laran et al. 2010) were applied to estimate dolphin energy requirements:

$$R = \frac{1}{\alpha} \times \beta \times 293.1 \times M_{\text{dolphin}}^{0.75} \quad (2)$$

(Kleiber 1975),

$$R = \frac{1}{\alpha} \times 690.38 \times M_{\text{dolphin}}^{0.783} \quad (3)$$

(Sigurjónsson & Víkingsson 1997),

$$R = \frac{1}{\alpha} \times 2529 \times M_{\text{dolphin}}^{0.524} \quad (4)$$

(Boyd 2002),

where R is daily energy requirement, α is assimilation efficiency [0.6;0.9], β is the active metabolism factor and M_{dolphin} is dolphin body mass.

Eq. (2) results from an adaptation of Kleiber's equation for basal metabolic rate which has been developed for homeotherms and used on terrestrial mammals (Kleiber 1975, Leaper & Lavigne 2007). This equation assumes that the metabolism of cetaceans is 2 to 3 times (sometimes even 5 times) higher than the basal metabolic rate (Lavigne et al. 1986, Kenney et al. 1997, Pusineri 2005, Rechsteiner et al. 2013). We therefore assumed to draw β from a Gamma distribution, with mean equal to 2.5 and the 99% CI upper value equal to 5 (i.e. shape parameter of 8.33 and scale parameter of 0.30). The assimilation factor, which is usually assumed to vary between 0.7 and 0.8 (Lockyer 1981), could vary with prey condition, size and species (Leaper & Lavigne 2007). To take into account this variability, α was randomly drawn following a uniform distribution between 0.6 and 0.9.

In our simulations, R was estimated using first a uniform draw on the 3 empirical relationships (for the

model choice) and then a uniform and gamma draw on its coefficients (α and β) (Fig. 3, Part 2, Step 6). A sensitivity analysis of energy requirement models was performed reproducing the same framework 3 times, once per equation. Finally, nursing females have additional requirements due to suckling. For that reason, their energetic demand requires an increase estimated to be between 48 and 97% (see Kastelein et al. 2002, 2003). This was taken into account in the simulation using a uniform draw on those additional requirements for nursing females (Fig. 3, Part 2, Step 6a).

Dolphin diet

Dolphin diet was estimated using previously published stomach content data. These data were aggregated, summing abundance, weight and occurrence by prey species (see Tables S4 & S5). Briefly, these data had been acquired from by-catch or stranded dolphins along the Mediterranean coasts. Prey was identified using tough pieces (otoliths and cephalopod beaks) and body length and mass were estimated either directly or indirectly through empirical relationships or by using the mean body mass of the species (see Würtz & Marrale 1993, Astruc 2005). From this data, we calculated $\%W_{i,j}$, the percentage of biomass of prey j (family or species) in the diet of dolphin i.

Aside from the 3 species of interest (i.e. hake, sardine and anchovy), all species contributing to at least 1% of biomass were kept for further analyses.

Prey consumption

Daily prey consumption was estimated using energy requirements and diet (in terms of biomass and energetic values of prey; Fig. 3, from Part 2, Step 7 to 9). To convert prey biomass into energy, energetic values of each prey item had to be known. Instead of assuming a generic energetic value for each prey class (e.g. demersal fish), we used specific values for each of the 27 prey species (see Table S6). When energetic values were not available at the species level (in about half of the cases), we used energetic values available at the lowest phylogenetic level. Further, energetic values can vary between years, seasons and geographic zones (Spitz et al. 2010). To take into account both sources of uncertainties, the energetic value of each prey species was estimated using a normal distribution N(μ,σ) of the mean (μ), in which the variance (σ) depended on the taxonomic level at which information was available (Fig. 3, Part 2, Step 8, Table S6). Then the total daily consumption as well as the daily biomass of each prey species consumed per day were estimated for each dolphin as follows:

$$C_i = \frac{R_i}{\sum_j \left(\%W_{i,j} \times E_j\right)} \quad (5)$$

where C_i (kg d^{-1}) is the total amount of prey ingested daily by dolphin i and R_i is the daily energy requirement of dolphin i. This gives the biomass of prey j consumed per day by dolphin i ($C_{i,j}$) as:

$$C_{i,j} = C_i \times \%W_{i,j} \quad (6)$$

The biomasses of European hakes, sardines and anchovies consumed per day were calculated using Eq. (6) (Fig. 3, Part 2, Step 9). As the diet composition and energy content of the prey were assumed to be constant throughout the year, in the absence of more detailed information on seasonal variations, daily consumptions were summed over all dolphins during 1 yr to estimate annual consumptions (Eq. 1 and Fig. 3, Part 3, Step 10). However, our study integrates temporal changes in these 2 parameters, as the diet and energy content data result from samples collected during all seasons.

Predation pressure (Part 3)

Annual stock biomass of small pelagics was calculated using data collected from scientific acoustic surveys carried out by Ifremer every summer from 1993 to 2016 (PELagiques MEDiterranée [PELMED]; described in Saraux et al. 2014). European hake biomass in the Gulf of Lions was derived from data collected during annual standardized bottom trawl scientific surveys (MEDITS Working Group 2013) and stock assessment modelling (GFCM 2017a). Here, for each Monte Carlo simulation, stock biomasses of hake, sardine and anchovy were estimated by uniform draw between minimal and maximal values of stock biomasses to take into account year-to-year variations and inherent uncertainties in stock assessment procedures (i.e. sardine: 26 054 to 264 024 t, anchovy: 13 654 to 112 018 t, hake: 2755 to 5348 t; GFCM 2017a,b) (Fig. 3, Part 3, Step 11). The predation pressure on each species was finally estimated as the ratio between its biomass consumed by dolphins and its stock biomass in the Gulf of Lions

(Fig. 3, Part 3, Step 12). All simulations were performed using R (R Core Team 2018).

RESULTS

Dolphin population

According to Bauer et al. (2015), and taking into account year-to-year variations, dolphin densities were estimated to be between 0.031 and 0.345 dolphins km^{-2} (medians of minimal and maximal boundaries of 95% CI). Multiplying these densities by the surface of the study area, populations of dolphins were estimated at between 460 and 5160 individuals in the Gulf of Lions.

Daily consumption

Dolphin daily energy requirements

Mean mass was higher for bottlenose than striped dolphins regardless of the developmental stage (e.g. adult mean ± SD mass was estimated at 187 ± 17 and 86 ± 4 kg for bottlenose and striped dolphins, respectively; Table 1). Using allometric relationships, the mass of each dolphin was converted into energy requirements. Mean (±SD) energetic demands of bottlenose dolphins were about 1.6 times higher than those of striped dolphins for a given stage (e.g. 61 700 ± 19 800 and 38 200 ± 13 600 kJ d^{-1} $ind.^{-1}$ for adult bottlenose and striped dolphins, respectively; Table 1). Conversely, mass-specific requirements of bottlenose dolphins were lower than those of striped dolphins, but the difference in energy requirements between stages within the same species was small (between 325 and 335 kJ kg^{-1} and between 422 and 439 kJ kg^{-1} for bottlenose and striped dolphins, respectively; Table 1).

Dolphin diet and energetic values of prey

Diets of both dolphin species were dominated by teleosts in terms of prey abundance (about 89 and 63% for bottlenose and striped dolphin, respectively; Tables S4 & S5). However, the picture is different in terms of biomass. Bottlenose diet was dominated by teleosts (86%; Table S4), while that of the striped dolphin was dominated by cephalopods (66%; Table S5). Besides these 2 dominant groups of prey, crustaceans were also present in dolphin diets but represented less than 5% of the prey in terms of abundance and biomass (Tables S4 & S5).

European hake was the dominant prey for bottlenose dolphins in terms of abundance (32%), biomass (30%), frequency and index of relative importance (IRI) (Fig. 4a, Table S4). Although blue whiting was the most abundant prey in terms of abundance (17%) for the striped dolphin (Fig. 4b, Table S5), squids played a key role in its diet in terms of abundance (32%), biomass (65%) and IRI (56%). The main squid species in terms of biomass were the European flying squid, European squid and reverse jewel squid (Fig. 4b, Table S5). Sardine and anchovy consumptions were low for both dolphin species but sardine could represent up to 5% of the striped dolphin diet in terms of biomass (Fig. 4b, Table S5).

Finally, 27 prey species were kept for the study (%W > 1% together with sardine, anchovy and European hake), representing about 94 and 89% of biomass consumed by bottlenose and striped dolphins, respectively (Fig. 4). Energetic values of sardine and anchovy were higher than that of European hake (10.2 ± 2.9, 7.0 ± 3.0 and 3.9 ± 0.2 kJ g^{-1} respectively; Table S6). In general, fish prey had greater energetic values than cephalopods. All energetic values of prey are summarized in Table S6.

Table 1. Mean (±SD) length, mass, individual energetic requirements per day and per kg mass, daily consumption and mass-specific consumption (daily consumption/mass) for all 3 developmental stages of both dolphin species

Species	Stage	Length (cm)	Mass (kg)	Individual daily energetic requirements (kJ d^{-1} $ind.^{-1}$)	Energetic requirements per kg (kJ kg^{-1})	Daily consumption (kg d^{-1} $ind.^{-1}$)	Mass-specific consumption (%)
Bottlenose dolphin	Adults	252 ± 7	187 ± 17	61700 ± 19800	335 ± 121	11.3 ± 3.7	6.1 ± 2.3
	Juveniles	224 ± 15	133 ± 27	42300 ± 10400	325 ± 72	7.7 ± 2.0	5.9 ± 1.4
	Calves	167 ± 23	57 ± 21	–	–	–	–
Striped dolphin	Adults	196 ± 3	86 ± 4	38200 ± 13600	439 ± 152	9.0 ± 3.3	10.3 ± 3.7
	Juveniles	176 ± 13	64 ± 13	26700 ± 7100	422 ± 106	6.3 ± 1.7	9.9 ± 2.6
	Calves	130 ± 13	26 ± 7	–	–	–	–

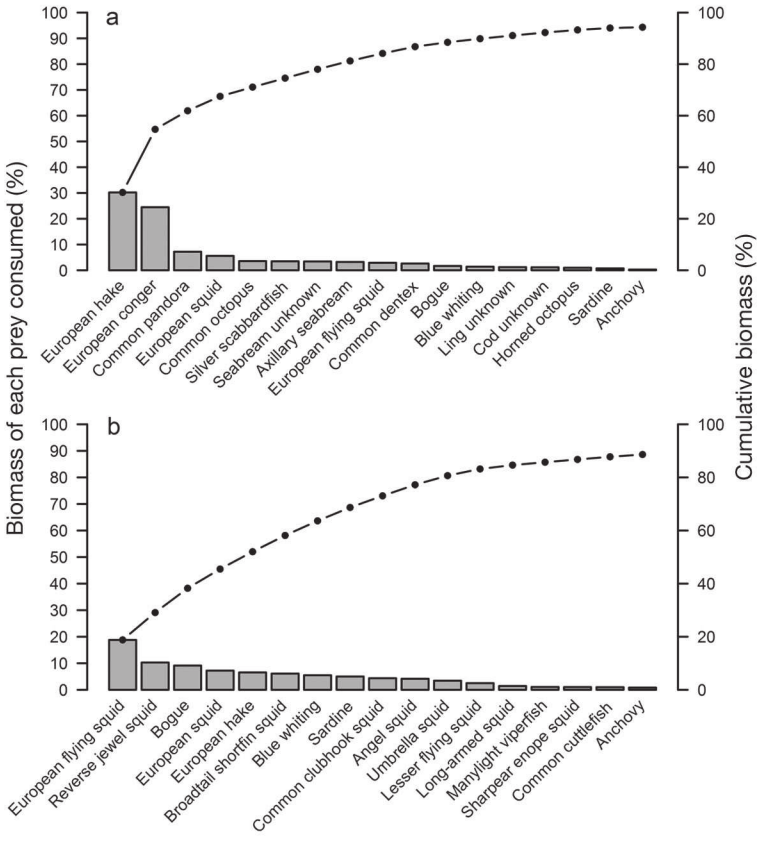

Fig. 4. Percent individual and cumulative biomass of principal prey species consumed by (a) bottlenose and (b) striped dolphins in the northwestern Mediterranean Sea between 1985 and 2012 (see Table 3 for species names)

Dolphin prey consumption

Dolphins consumed 9 ± 4 kg d^{-1} $ind.^{-1}$ of prey, i.e. 8 ± 3% of their own mass per day. This consumption was higher for bottlenose dolphins in terms of kg d^{-1} $ind.^{-1}$ (with 11.3 ± 3.7 kg d^{-1} $ind.^{-1}$) (mean ± SD, for all subsequent values), but higher in terms of mass-specific consumption for striped dolphins (10.3 ± 3.7%, Table 1). Assuming constant daily alimentation throughout the year, the total amount of prey ingested by dolphins in the Gulf of Lions was 6400 ± 6700 t yr^{-1}, which corresponds to 424 ± 444 kg km^{-2} yr^{-1}.

Among the 3 species of interest, dolphins consumed mostly European hake (about 1250 ± 1461 t yr^{-1}), and this consumption was mainly due to the bottlenose dolphin (1065 ± 1353 t yr^{-1}, i.e. 3.1 ± 1.1 kg d^{-1} $ind.^{-1}$; Table 2). Annual consumption of both sardine and anchovy was much lower than hake (203 ± 222 t yr^{-1}) and mostly by striped dolphins (166 ± 198 t yr^{-1}), which mostly consumed sardine (142 ± 169 t yr^{-1}; Table 2). Median values and 95% CI of annual consumption of main prey by bottlenose and striped dolphins are summarized in Table 3.

Dolphin predation pressure

To estimate predation pressure of dolphins in the Gulf of Lions and to take into account the variability of intermediate processes, 10 000 dolphin populations were simulated, corresponding to about 16 million individuals. The median predation pressure of both dolphin species on hake was estimated at around 23% (95% CI: 5–110%; Fig. 5a), while predation pressure estimates on sardine and anchovy were always lower than 0.5% (note that the predation pressure on sardines was twice as high as on anchovies: median of 0.09% [95% CI: <0.1 to 0.5%] and 0.05% [95% CI: <0.1 to 0.2%] for sardine and anchovy, respectively; Fig. 5b,c). Testing the sensitivity of these results to the equations used to estimate daily energetic requirements, we found similar results with all 3 equations: predation pressure estimation on hake (considering each energy relationships separately) led to 21, 24 and 24% for Eqs. (2), (3) & (4) respectively while predation pressure differences were <0.1% for small pelagics (Fig. S2).

Table 2. Mean (±SD) annual and individual daily consumption of European hake, sardine and anchovy by bottlenose and striped dolphins

Species	Annual hake consumption (t yr^{-1})	Individual daily hake consumption (kg d^{-1} $ind.^{-1}$)	Annual sardine consumption (t yr^{-1})	Individual daily sardine consumption (kg d^{-1} $ind.^{-1}$)	Annual anchovy consumption (t yr^{-1})	Individual daily anchovy consumption (kg d^{-1} $ind.^{-1}$)
Bottlenose dolphin	1065 ± 1353	3.1 ± 1.1	26 ± 33	0.1 (<0.1)	11 ± 13	<0.1
Striped dolphin	185 ± 220	0.5 ± 0.2	142 ± 169	0.4 ± 0.2	24 ± 29	0.1 (< 0.1)

Table 3. Median and 95% CI of annual consumption of main prey (in tons) by bottlenose and striped dolphins

Species	Common name	Bottlenose dolphin		Striped dolphin	
		Median	95% CI	Median	95% CI
Merluccius merluccius	European hake	749	112–3693	131	19–649
Conger conger	European conger	607	91–2993	–	–
Pagellus erythrinus	Common pandora	179	27–881	–	–
Loligo vulgaris	European squid	139	21–683	145	21–718
Octopus vulgaris	Common octopus	88	13–436	–	–
Todarodes sagittatus	European flying squid	72	11–357	376	53–1864
Lepidopus caudatus	Silver scabbardfish	87	13–427	–	–
Pagellus sp.	Seabream unknown	85	13–421	–	–
Pagellus acarne	Axillary seabream	80	12–396	–	–
Dentex dentex	Common dentex	65	10–320	–	–
Boops boops	Bogue	42	6–207	183	26–907
Micromesistius poutassou	Blue whiting	34	5–170	110	16–545
Molva sp.	Ling unknown	30	5–149	–	–
Gadidae sp.	Cod unknown	29	4–143	–	–
Eledone cirrhosa	Horned octopus	25	4–123	–	–
Sardina pilchardus	Sardine	18	3–90	100	14–499
Engraulis encrasicholus	Anchovy	7	1–37	17	2–84
Histioteuthis reversa	Reverse jewel squid	–	–	206	29–1021
Illex coindetii	Broadtail shortfin squid	–	–	122	17–606
Onychoteuthis banksii	Common clubhook squid	–	–	88	12–434
Ancistroteuthis lichtensteini	Angel squid	–	–	83	12–413
Histioteuthis bonnellii	Umbrella squid	–	–	69	10–341
Todaropsis eblanae	Lesser flying squid	–	–	50	7–250
Chiroteuthis veranyi	Long-armed squid	–	–	29	4–144
Chauliodus sloanei	Manylight viperfish	–	–	22	3–108
Ancistrocheirus lesueurii	Sharpear enope squid	–	–	21	3–104
Sepia officinalis	Common cuttlefish	–	–	20	3–99

DISCUSSION

Potential sources of variability

This study aimed to estimate the predation pressure of the 2 main dolphin species on commercial fish inhabiting the Gulf of Lions, especially hake, sardine and anchovy. Predation pressure was estimated by combining 5 different processes requiring a large quantity and variety of data on both prey and predators, which probably explains why this work represents, to our knowledge, the first estimation of dolphin predation pressure in the Mediterranean Sea. Nonetheless, some data sources, such as dolphin censuses, displayed high variability, while other data was missing (e.g. prey energetic value at the species level for all species), leading to uncertainties in the predation pressure estimates. To account for such data limitations and quantify the uncertainty around our result, we developed a method based on Monte Carlo simulations in which each parameter was drawn from a given distribution rather than using a mean value.

Sensitivity analyses of similar models have demonstrated that abundance estimates and residency ratios are the most influential parameters in consumption estimations (Smith et al. 2015, Spitz et al. 2018). In our study, dolphin abundance estimation was a process with relatively high variability and uncertainty. Indeed, dolphin abundance varied between 460 and 5160 individuals according to year-to-year variations estimated by Bauer et al. (2015). However, this did not take into account possible seasonal variations, which are suspected to be important but remain difficult to estimate because of a lack of data. Nonetheless, the range of our estimations is close to past estimates of dolphins in the Gulf of Lions. Based on 2 seasons and 1 given year, Laran et al. (2017) estimated striped dolphin abundance to be between 424 and 8300 individuals in winter (95% CI) and bottlenose dolphin abundance from 466 to 3805 individuals in summer (95% CI), while Di-Méglio et al. (2015) estimated bottlenose dolphin abundance over 2 yr as 385 to 1095 individuals (95% CI). Here, the primary source of uncertainty probably arises from the proportion of bottlenose versus striped dolphins that inhabit the Gulf of Lions, which was therefore drawn uniformly using a large range of values, i.e. from 10 to 90% according to different sources

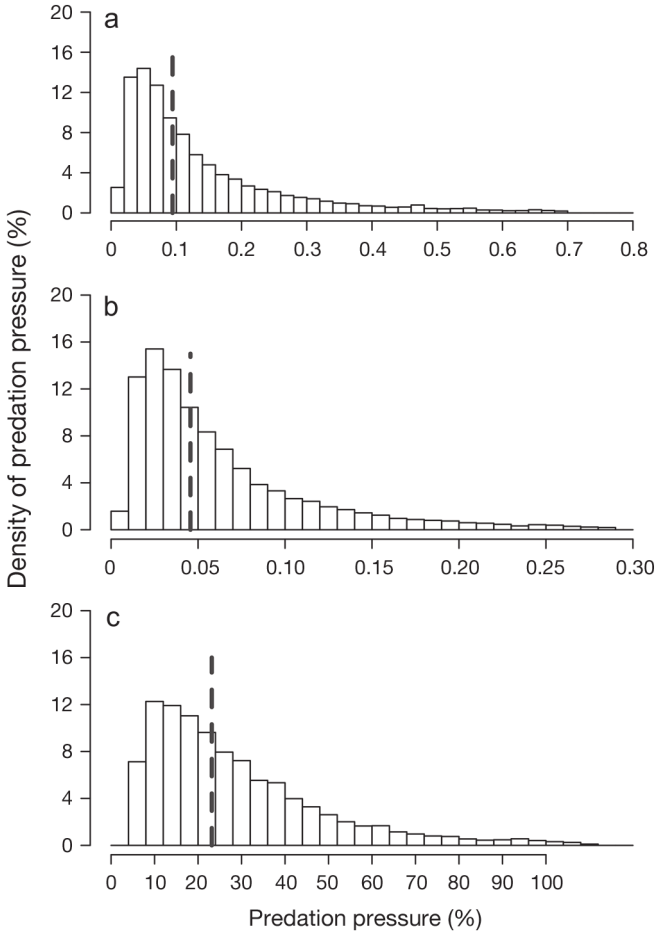

Fig. 5. Dolphin predation pressure (estimated through 10 000 simulations) on (a) sardine, (b) anchovy and (c) European hake. To avoid outliers, 95% CI were plotted. Dashed line = median

(Gannier 2005, Laran et al. 2017, PELMED obs., unpubl. data). As diet differs substantially between the 2 species, this uncertainty propagates into the estimation of hake consumption, which explains the large CI.

After population census, energetic requirements should have the second strongest influence on consumption estimations (Spitz et al. 2018). In our study, we used 3 different energetic models based on the dolphins' body mass, in which a range of values were applied to each model parameter (i.e. assimilation efficiency and active metabolism). Yet the sensitivity analysis on these 3 equations showed that the consequence on the final predation pressure estimate was small for hake (about 1 to 2%) and negligible for both sardine and anchovy (<0.1%), which indicated that the sensitivity of our results did not primarily come from the equations used for dolphin energy requirements, but more clearly from dolphin abundance estimations. Additional energetic requirements of nursing females were not negligible (up to 97%; see Kastelein et al. 2002) and were taken into account in our study.

Finally, the seasonal or interannual variability could not always be explicitly estimated in some parameter values or relationships (e.g. in diet or prey energy content). Still, this variability was integrated by using data and results collected from all seasons.

Consumption estimates and energetic requirements

Our median results are of the same order of magnitude as in other studies. Indeed, our estimates of daily consumption of dolphins are close to estimations found for the Ligurian Sea and the eastern Ionian Sea: between 6.3 and 9.0 kg d^{-1} for the Gulf of Lions (this study), between 2.9 and 6.0 kg d^{-1} in the Ligurian Sea (Laran et al. 2010) and around 5.4 kg d^{-1} for the Ionian Sea (Bearzi et al. 2010). Furthermore, annual consumption removal by dolphins estimated here for the Gulf of Lions stands in between estimates of the other 2 Mediterranean areas: 424 ± 444, 999 and 96 kg km^{-2} yr^{-1} for the Gulf of Lions (this study), the Ligurian Sea (Laran et al. 2010) and the Ionian Sea (Bearzi et al. 2010) respectively. Finally, captive adult bottlenose dolphins exhibit similar daily energetic requirements to those found in this study (up to 330 kJ kg^{-1} of their own mass in captive dolphins at Marineland® of Antibes [M. Oesterwind unpubl. data] compared to 335 ± 121 kJ kg^{-1} calculated in the present study).

Predation pressure on small pelagics

In the Gulf of Lions, bottlenose and striped dolphins mostly showed little interest in sardine and anchovy, which contributed to <1% biomass of their diet, except for sardines in the striped dolphin diet (5% in terms of biomass). Dolphins are known to display a generalist and opportunistic feeding behavior, as with most top predators, and their feeding regime is also area-dependent. While our results are consistent with previous results from the Bay of Biscay (0 and 3% for striped and bottlenose dolphins respectively; Spitz et al. 2006a,b), sardines have appeared as the key prey for striped dolphins (up to 60%) in some neighboring areas (along the eastern

coast of Spain; Gómez-Campos et al. 2011). The relatively low proportion of sardine (and anchovy) in the dolphin diets from the Gulf of Lions might thus be related to the decrease in biomass, mean length and body condition of those prey in this area (Van Beveren et al. 2014). This has already been observed along the Spanish Mediterranean coasts, for instance, where sardines constituted dolphins' main prey in the 1990s before the proportion of sardines in their diet sharply declined in 2007–2008 as a result of sardine overfishing (Gómez-Campos et al. 2011). These low proportions led to low daily consumption of anchovy (0.1 kg d^{-1} $ind.^{-1}$ for both dolphin species) and sardine (about 0.3 kg d^{-1} $ind.^{-1}$ for striped dolphins, and lower for bottlenose). Again, these results are similar to those found in the Bay of Biscay, where the consumption of sardine and anchovy was negligible for both dolphin species (up to 0.2 kg of sardines d^{-1} $ind.^{-1}$ for bottlenose dolphins; Pusineri 2005). Consequently, predation pressure on sardine and anchovy was always very low, i.e. below 0.5% in all simulations (median: 0.09% for sardine and 0.05% for anchovy). Predation pressure of dolphins on both small pelagic species is even lower than that of Atlantic bluefin tuna (0.6 ± 0.2 and 1.9 ± 0.5% for sardines and anchovies respectively; Van Beveren et al. 2017). This predation pressure by dolphins remains significantly lower than the fishing pressure that is estimated (in terms of harvest rate) to be at around 1 and 5% for sardine and anchovy over the last 3 yr respectively (GFCM 2017b). In conclusion, the poor stock status of both sardines and anchovy in the Gulf of Lions (mostly reflected by poor condition and lower growth; see Van Beveren et al. 2014) is unlikely to be due to top-down control by either of the 2 dolphin species. Such a conclusion might also result from the recent absence of common dolphin *Delphinus delphis* in the Gulf of Lions, especially because sardine and anchovy are indeed the main prey of this species regardless of the area considered (Young & Cockcroft 1994, Meynier et al. 2008, Begoña Santos et al. 2014). While the common dolphin might have been expected in the Gulf of Lions due to its preferred habitat (ranging between that of striped dolphin [oceanic habitat] and bottlenose dolphin [coastal habitat]), recent observations are very scarce in the Gulf of Lions, although it seems to have been more common until the middle of the 20th century (Bearzi et al. 2003, Gannier 2017). Reasons for the disappearance of large common dolphin populations in the northwestern Mediterranean basin are still unclear, but may include decrease of prey availability (e.g. due to competition with local fisheries and/or overfishing), bycatch or hunting before their protection by law, environmental changes (e.g. increase of sea water temperature) or contamination by xenobiotics (Bearzi et al. 2003, Gannier 2017).

Predation pressure on hake

In contrast to small pelagics, consumption of hake by dolphins was significant in the Gulf of Lions, mostly due to bottlenose dolphins. This has been documented in other areas, such as the North Atlantic where it was even higher (3 kg d^{-1} $dolphin^{-1}$ in the Gulf of Lions vs. 6 and 8 kg d^{-1} $dolphin^{-1}$ off the Iberian Atlantic coasts and in the Bay of Biscay, respectively; Pusineri 2005, Begoña Santos et al. 2014). Nonetheless, the predation pressure of the bottlenose dolphin on European hake was substantial in the Gulf of Lions, but highly variable among the simulations, ranging from 5 to 110% (95% CI), with a median value at around 23%. This large variability probably originates from uncertainties in dolphin abundance census and proportion of the 2 dolphin species (see above). Predation pressure by dolphins on hake remained, however, lower than fishing pressure, which ranged between 38 and 73% over the last 20 yr (from 1998 to 2017; GFCM 2017a). Similar to the fisheries, bottlenose dolphins mainly target ages 0, 1 and 2 of hakes (93% in abundance; see Astruc 2005), so that fishing pressure and dolphin predation act synergistically on juvenile hakes in the Gulf of Lions, potentially amplifying growth overfishing. Our study brings new, objective (quantified from scientific data) information about marine mammal and fisheries interactions in the Northwestern Mediterranean Sea. These interactions usually occur because of competition for a similar resource, and has become a worldwide concern (Morissette et al. 2012, Snape et al. 2018) — both in terms of conservation, as they result in dolphin mortality due to by-catch (Hall et al. 2000, Bearzi 2002) and in terms of economic losses due to depredation or damage to fishing gear (Bearzi 2002, Hamer et al. 2012), even if they could be overvalued in some cases (Trites et al. 1997, Coll et al. 2007, Gazo et al. 2008). This is true in several parts of the Mediterranean Sea (e.g. Lauriano et al. 2009, Gonzalvo et al. 2014) where interactions with fisheries are increasing (Brotons et al. 2008, Pardalou & Tsikliras 2018). Furthermore, overfishing of hake in the Gulf of Lions has generated a strong decline of this population (see GFCM 2017a), which in turn has

reinforced the natural mortality of hake due to dolphin predation. Indeed, predation pressure of dolphins depends on prey population size. Therefore, it is expected to have been lower in the past, as the hake population was significantly larger. The first hake stock assessments in the Gulf of Lions between 1988 and 1991 (stock already overexploited) estimated hake stock biomass to be between 6041 and 9017 t (Aldebert & Recasens 1996). Based on the same simulation framework and assuming the same dolphin population size and demography, the median predation pressure would indeed decrease to about 12% (95% CI: 3 to 57%). This study shows that the predation pressure of dolphins on hake is substantial in the Gulf of Lions and has been further reinforced by current overexploitation of hake. This might have an important impact, especially on bottlenose dolphin populations in coastal waters (Bearzi et al. 2009, Gonzalvo et al. 2014). Therefore, these interactions should be more carefully considered in the future management plans of the European hake stock in the Gulf of Lions for both the conservation of dolphins and the sustainability of the fisheries.

In conclusion, we used an original approach developed by Van Beveren et al. (2017), but went one step further to account for multiple sources of uncertainties in the estimation of predation pressure. We showed that predation pressure of dolphins on sardine and anchovy in the Gulf of Lions was extremely low, indicating that dolphins probably have had little impact on the population dynamics of those exploited fish. In contrast, the predation pressure of dolphins on hake is substantial in the Gulf of Lions and has been further reinforced by current overexploitation of this population. This situation is even more problematic as both the fishing industry and the dolphins prey on the same resource: hake juveniles; a result that should be considered in future management plans of hake populations.

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Annex 2

Shrinking of fish: bottom-up evidence from bioenergetics

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Shrinking of fish: bottom-up evidence from bioenergetics

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KEY WORDS: aerobic metabolism, body condition, growth, food quality, red muscle, mitochondria

ABSTRACT (250 words)

A significant reduction in the size of ectotherms is being recorded globally and is believed to be a response to global warming. A notable example is the European sardine *Sardina pilchardus* in the Mediterranean Sea, where a decline in the size of individuals has caused a profound drop in total biomass of this important small pelagic species. This is currently hypothesized to be a bottom-up effect, linked to changes in the planktonic communities that the species feeds upon. In particular, sardines change their feeding mode as a function of the size of their prey, using a filtering mode for small items but a direct chase and capture mode for large items. In the current study sardines were reared on artificial feeds, of either small or large size, and in either small or large quantities. After 7 months feeding, the group that received large pellets in large quantities was the only one exhibited a positive specific growth rate and an above-average body condition index. Despite the installation of cellular compensations in sardines that received small pellets with a reduced metabolism and an

increased mitochondrial efficiency, this does not seem sufficient to counteract the reduction in these biometric parameters.

SIGNIFICANCE (120 words)

Global warming could lead to a reduction in the size of plankton in the Mediterranean Sea, which is the main source of sardine food. Interestingly, a reduction in the size of sardines in the Mediterranean Sea has been observed over the past ten years. To evaluate whether a reduction in the size of food could be a trigger of the reduction in the size of sardines, we fed sardines for 7 months on four different diets consisting in small or large pellets in large or small quantities. Results clearly show that the size of food, as well as its quantity, may cause a shrinking of sardines, despite the development of bioenergetics compensations at the cellular level.

INTRODUCTION

Over the last decade, there is growing evidence that aquatic ectotherms are shrinking (Daufresne *et al.,* 2009; Forster *et al.,* 2012), potentially as a consequence of global warming and the temperature-size rule (Atkinson, 1994), This size reduction occurs within and amongst species, throughout the different levels of the fed web, from top predators (Sibert *et al.,* 2006) to primary producers (Sommer *et al.,* 2015). Small pelagic fishes are intermediate but crucial actors in marine food webs (Cury *et al.,* 2000), centrally positioned to link primary producers to top predators (Brosset *et al.,* 2015a ; Checkley *et al.,* 2017; Curry *et al.,* 2000). Throughout the Mediterranean Sea, a small pelagic species that is a major fishery resource, the European sardine *Sardina pilchardus,* has exhibited a drastic reduction in body size and condition index over the last decade, due the disappearance from the population of larger and older individuals (Brosset *et al.,* 2017 Progress in Oceanography. In the Gulf of Lions, for example, the result has been that the species is no longer targeted by fishermen (GFCM 2018)(Van Beveren *et al.,* 2014). Extensive research effort has established that the decline in size and body condition is not due to top down effects such as predation or fishing pressure, nor to pathogens (reviewed in Saraux *et al.,* 2018). Rather, it is now believed that a bottom-up process is at play, linked to a regime shift in the plankton communities that the species feeds upon, towards smaller and less

energy-rich zooplankton (Zarubin *et al.,* 2014; Brosset *et al.,* 2016a). Sardines are opportunistic planktivores (Palomera *et al.,* 2007; Rumolo *et al.,* 2016) that adjust their feeding behaviour to prey size (Costalago *et al.,* 2015), switching from filter feeding on small prey to direct capture for large prey (Garrido *et al.,* 2007). The relative costs of these two feeding modes can differ in small pelagic planktivorous fishes (van der Lingen, 1995) and there is some evidence that filter feeding on small food items may be more energetically expensive than direct capture of large items in *Sardinus pilchardus* (Queiros et al., 2019).

The swimming activity that underlies sardine foraging, whether filtering or particle capture, relies on muscle activity (McKenzie, 2011). In fishes, the swimming muscles represent up to 70% of the body mass and are structurally separated into discrete slow-twitch oxidative 'red' muscle and fast-twitch glycolytic 'white' muscle (Bone 1978; Webb 1998). , represents the major energy consumer in fish. At the cellular level, energy is mainly produced by mitochondria, through the oxidative phosphorylation mechanisms. Mitochondrial oxidative phosphorylation can be more or less efficient, depending on the capacity of oxidizing energy substrates, such as carbohydrates, lipids or proteins, to finally produce ATP. Mitochondrial efficiency is plastic and is known to vary in response to sustained food scarcity or high metabolic energy demand (Salin *et al.,* 2015; Roussel *et al.,* 2018). As the main aerobic tissue used for swimming, fish red muscle represents a prime target to investigate how mitochondrial metabolism can be adjusted in response to the long term energetic status of an animal (Teulier *et al.,* 2019).

Here we aim to assess the proximal consequences, for sardine body composition and red muscle mitochondrial function, of variation in food size and quantity. We sampled animals from four experimental populations, that had been maintained for 7 months on four different diets (Queiros et al. 2019). These diets comprised blab al. They caused significant differences in growth rate, condition, etc…It is known that the amount of food impacts the physiological parameters of fish. Indeed, the growth rate of juvenile cobia *Rachycentron canadum* increases with the daily food intake (Sun *et al.,* 2009) and the body condition of the juvenile olive flounder *Paralichthys olivaceus* changes according to the food ration (Lee *et al.,* 2018). However, the effect of food sizes on fish energy metabolism and

78 body condition has been less investigated, when such question seem even more important in a context
79 of global warming, which induces a size reduction of the plankton (Garzke *et al.,* 2014). In order to
80 measure the impact of food size and quantity on the energy metabolism of sardines, two pellets sizes
81 (0.1mm and 1.2mm) and two quantities (0.3% and 0.6% of the total fish mass in tanks) were combined
82 in resulting 4 different diets: (1) small size and quantity of pellets (SP-SQ), (2) small size and quantity
83 of pellets (LP-LQ). Through a wide integrative point of view linking population dynamics index to
84 mitochondrial bioenergetics, the present integrative study suggests our protocols let us following the
85 hypothesis that filter-feeding mode induces an unbalanced energetic budget, first leading to limit
86 mitochondrial activity and eventually to a shrinking of sardine.

87 **RESULTS**

88 **Evolution of body condition and growth**

89 Diet had a significant effect on sardine body condition (AU, Linear mixed model, Satterthwaite's
90 method, trend of SP-SQ: -0.037 ± 0.003 AU.month^{-1} ; SP-LQ: -0.033 ± 0.003 AU.month^{-1} ; LP-SQ: -
91 0.024 ± 0.003 AU.month^{-1} ; LP-LQ: 0.003 ± 0.003 AU.month^{-1}) but also on their growth (Linear
92 mixed model, Satterthwaite's method, trend of SP-SQ: 0.5 ± 0.2 mm.month^{-1}; SP-LQ: 1.0 ± 0.2
93 mm.month^{-1}; LP-SQ: 1.0 ± 0.2 mm.month^{-1}; LP-LQ: 2.3 ± 0.2 mm.month^{-1}) (**Fig. 2**). There was a
94 significant difference between LP-LQ sardines and the three other groups regarding body condition
95 ($P<0.05$). Indeed, there was a reduction in body condition of LP-LQ, SP-LQ and SP-SQ sardines while
96 this body condition was maintained in LP-LQ sardines all along the 7 months of experiment (**Fig. 2A**).
97 Furthermore, there was also an impact of pellet size on body condition for sardines that received small
98 quantities of pellets, with a significantly higher body condition for LP-SQ sardines compared to SP-
99 SQ sardines ($P<0.05$). Finally, the growth of LP-LQ sardines (**Fig. 2B**) was significantly higher during
100 the experiment compared to the three other groups of sardines ($P<0.05$).

101 **Biometric characteristics**

102 The pellets size and quantity had a significant effect on red muscle mass and fat content (**Table 1**).
103 Sardines which received large pellets in a large quantity (LP-LQ) had higher body condition, body

104 mass and fat content than sardines from other treatments ($P<0.05$), and a higher red muscle mass than
105 sardines that received small pellets ($P<0.05$).
106 The pellets size also had a significant effect on the relative red muscle mass (proportion of the red
107 muscle mass over the entire body mass, "RM/BM" ratio) because sardines that received large pellets
108 had a higher ratio than those that received small pellets ($P<0.05$).

109 **Oxidative metabolism of red muscle**

110 The impact of food was not the same depending on the level of integration (**Table 2**). Indeed, at the
111 mitochondrial level, the FCCP-induced maximal activity of electron transport system (ETS) was
112 significantly lower in sardines fed on small pellets compared to those fed on large pellets ($P<0.01$), but
113 there was no difference in COX activity, a measure of muscle oxidative capacity. At the red muscle
114 fibre level, there was no difference between the four groups ($P>0.05$) but there was an interaction
115 between size and quantity of pellets among the four groups ($P<0.01$) for the maximal ETS activity,
116 and the COX activity of LP-SQ sardines was significantly higher than that of SP-LQ sardines
117 ($P<0.05$). Maximal activities of ETS ($P<0.05$) and COX ($P<0.05$) in LP-LQ sardines were
118 significantly higher than those of SP-LQ sardines. At the fish level, body mass-specific maximal
119 activities of ETS and COX in LP-LQ sardines were significantly higher than in SP-LQ sardines
120 ($P<0.05$). Finally, the mitochondrial content of red muscle of SP-SQ sardines was higher than that of
121 LP-SQ sardines ($P<0.05$).

122 **Mitochondrial oxidative phosphorylation efficiency**

123 **Fig. 3** shows the effect of diet on the maximal rates of ATP synthesis and corresponding oxygen
124 consumption in mitochondria respiring on pyruvate/malate/succinate. Since the relations between ATP
125 synthesis and oxygen consumption has been proven to be linear in isolated mitochondria from
126 different tissues and organisms (Beavis and Lehninger, 1986; Fontaine *et al.*, 1996; Nogueira *et al.*,
127 2002; Teulier *et al.*, 2010; Roussel *et al.*, 2015), we also reported the basal non-phosphorylating
128 oxygen consumption on the graph and draw lines between the basal non-phosphorylating rate and
129 corresponding maximal phosphorylating rate within experimental groups. In accordance with the
130 respiratory parameters reported above, there was a significant effect of the pellets size on maximal

rates of oxygen consumption and ATP synthesis, the highest points to the right in **Fig. 3** ($P<0.001$). Similarly, the basal non-phosphorylating respiration rates measured in the presence of oligomycin (the values on the x-axis) were significantly lower in fish fed on small pellets compared to large pellets groups ($P<0.001$).

The slopes of relationships were the same (small pellets: ATP/O = 3.2 ± 0.2; large pellets: ATP/O = 3.0 ± 0.1 ; $P>0.05$), indicating that the relations between the rates of ATP synthesis and oxygen consumption were parallel between the four experimental groups. Yet, the significant changes in the intercepts with the x-axis indicate that the relations concerning fish fed on small pellets were significantly shifted to the left compared with fishes fed on large pellets. This indicates that muscle mitochondria from fish fed on small particles were more efficient, consuming less oxygen to produce a given amount of ATP compared with fish fed on large particles (**Fig. 3**). Finally, the quantity of pellets had no significant effect on any of these different parameters.

When total oxidative phosphorylation activity per muscle is calculated by taking into account red muscle mass (**Table 1**) and muscle mitochondrial protein content (**Table 2**), the linear relation between the rates of ATP synthesis and oxygen consumption in LP-SQ fish became superimposed to that in small particles fed fishes (**Fig. 3B**). Hence, the maximal rates of ATP synthesis and oxygen consumption per muscle remained significantly higher and the relation significantly shifted to the right in LP-LQ fish compared with each other groups (**Fig. 3B**).

Evolution of the leak respiration in function of body condition

Body condition, which depended on the diet received by sardines (**Table 1**), had a significant effect on basal non-phosphorylating mitochondrial respiration (**Fig. 4)**. Indeed, a segmented regression analysis showed a significant correlation between basal respiration and body condition below a condition of 1.07 ($R^2=0.45$, $P<0.001$), which concerned SP-SQ, SP-LQ and LP-SQ groups, but none above 1.07 (LP-LQ sardines, $P>0.05$). The oxygen consumed in the basal non-phosphorylating state is devoted to counteract proton leakage across the mitochondrial inner membrane. Thus, its activity is an index of maximal energy wastage of mitochondrial metabolism. As such, a decrease in basal respiration rate

would reflect lower dissipation of energy and thus a higher coupling mitochondria, which was occurring in sardines below a body condition of 1.07 (**Fig. 4**).

DISCUSSION

This study showed an effect of food size and quantity on body condition index, growth performance and muscle bioenergetics in sardines. The fundamental finding of the present study was the strong effects of food size on all of the parameters measured. Since small size pellets trigger a filtration mode of feeding in sardines, our results indicate that foraging resources by filtration unable sardines to maintain their body condition and growth performance, despite the development of bioenergetics compensation in muscle.

Sardines fed on small pellets, with low condition and poor growth, had reduced mitochondrial oxidative phosphorylation activity compared with fish fed on large pellets, but they had higher coupling efficiency. Hence filtration behaviour induced by small pellets feeding led to a development of an energy-saving metabolism. More generally, our results indicate that mitochondrial energy-saving metabolism develops in sardines when they go below a body condition threshold of 1.07 (**Fig. 4**). Such energy saving mechanisms, involving less powerful but more efficient mitochondria, are known to develop in muscle of animals suffering caloric restriction (Zangarelli *et al.,* FASEB J 20, 2006; Bourguignon *et al.,* 2017; Roussel *et al.,* 2018).

However, sardines fed on small pellets exhibited a similar red muscle fibres oxidative capacity to the other groups of sardines. Hence, additional compensatory mechanisms have settled down at the level of muscle, which is partly explained by a higher mitochondrial density in red muscle of sardines receiving small pellets (**Table 2**). Increased mitochondrial content have also been previously reported in organisms undergoing caloric restriction, which could be ascribed for a reduction of mitochondrial autophagy after a caloric restriction (Boengler, 2007). Nevertheless, these compensatory mechanisms (i.e. higher coupling efficiency, increased mitochondrial content in muscle) were not sufficient to restore whole muscle ATP production in sardines fed on small pellets (**Fig. 2B**). Interestingly, it has been reported that depressed mitochondrial oxidative phosphorylation capacity may underlay inadequate food intake in *Salmo trutta* (Salin *et al.,* 2016a). All these observations thus suggest that

sardines suffering caloric restriction were unable to increase their energy intake, impairing their ATP homeostasis and so their body condition and growth over time. The present study therefore reinforces the importance of mitochondria in the allocation of resources into performances (Salin *et al.,* 2012, 2015, 2016b; Bourguignon *et al.,* 2017) and supports the hypothesis of filtration mode leading to a mismatch in the energy balance in sardines (Queiros et al., 2019). Filtration behaviour is characterised by a continuous energy consumption over time (van der Lingen, 1995). The present study gives a mechanistic explanation (mitochondrial metabolism failure) supporting the hypothesis that the energy supplied by the filtration did not compensate for this high energy demanding behaviour, resulting in a negative energy balance in fish, at least in sardines.

In contrast, sardines fed with large pellets had a higher mitochondrial capacity, growth performance and body condition than sardines filtering small pellets. Particulate feeding involves a very high but transient energy demand (Garrido *et al.,* 2007), so that the mitochondrial capacity observed could allow sardines to support their peak activity during chasing. However, the growth advantage of having a more powerful mitochondria was blunted in sardines receiving small quantity of food (group LP-SQ). Indeed, this group of sardines exhibited a lower body condition and growth than sardines fed on large pellets in large quantity. Despite powerful mitochondria, a large decrease in whole muscle ATP production due to a lower mitochondrial content may partly explain the poor body condition and impaired growth in this group of fishes (**Fig. 2B**). In turn, it appears that this caloric-restricted group (LP-SQ) has not implemented cellular compensation to counterbalance the low energy intake, since their mitochondria exhibited as high oxidative phosphorylation capacity as fished fed on large pellets in large quantity. All results taking together, the present study therefore suggests that mitochondrial bioenergetics was rather primarily constrained by the mode of foraging (induced by pellet size) than by the quantity of energy received. It remains that sardines fed on large pellets in small quantity had a better body condition and growth performance than fish filtering the same quantity of energy (group SP-SQ). This reinforced the above hypothesis that chasing mode of foraging would provide a better energy balance than filtering mode in sardines.

In conclusion, food size matter. On the whole, the present results show that the size of particles strongly constrains mitochondrial bioenergetics and the capacity of muscle to maintain ATP

212 homeostasis, and in fine the body condition and growth performance of sardines. The present study
213 also suggests that sardines filtering small particules were unable to maintain their body condition and
214 growth despite the development of energy compensatory mechanisms. In a context of global warming
215 and size reduction of the plankton, our data might therefore provide a mechanistic explanation of a
216 shrinking of sardine.

217 **METHODS**

218 All experimental procedures were in accordance with France legislation regarding the protection of
219 animals used for experimentations (APAFIS, permission n°7097-2016093008412692).

220 **Fishing and sardines stabling**

221 Sardines were captured in October 2016 off Sète (South of France). After fishing, sardines were
222 transported to the Ifremer experimental station of Palavas-Les-Flots (Hérault, France). They were first
223 put into 4.5 m^3 outside tanks for acclimatization and quarantine purposes. During that time, sardines
224 were fed ad-libitum, first with a mixture of aquaculture pellets (mix of pellet sizes: 0.1mm, 0.3 mm
225 and 0.8mm) and *Artemia nauplii* for a week and then only with pellets. After having undergone
226 bacteriological and viral analyses, sardines were transferred to indoor tanks in November 2016. More
227 details are given in Queiros *et al.*, in 2019.

228 On transfer, body mass and total size of sardines were recorded, and a RFID tag implanted for
229 individual identification (Biolog-id, Bernay, France). Of note, RFID tag mass was 0.030g,
230 representing 0.3% of the smallest sardine body mass]) implanted for individual identification. Sardines
231 were then distributed into 8 experimental tanks of 300L each, so as to ensure similar mean and
232 standard deviation of the mass distribution in each tank (14.1 ± 3.2g), 56 to 57 sardines were allocated
233 per tank. After 2 weeks of acclimation to these new tank, the experiment started for 7 months.
234 Subsequently, mass and size of sardines were recorded every 4 weeks from November 2016 to June
235 2017 to monitor individual growth and body condition. Body condition of each fish was calculated
236 following the Le Cren index Kn as estimated by Brosset *et al.*, 2015b:

$$K_n = \frac{WW}{0.00607 \times TL^{3.057}}$$

237 where TL is the total length (cm) and WW is the wet weight (g).

238 **Feeding conditions**

239 Two pellet sizes (0.1mm and 1.2mm) and two quantities (0.3% and 0.6% of the total fish mass in
240 tanks) were combined resulting in 4 different diets: (1) small size and quantity of pellets (SP-SQ), (2)
241 small size and large quantity of pellets (SP-LQ), (3) large size and small quantity of pellets (LP-SQ),
242 (4) large size and quantity of pellets (LP-LQ). The two quantities were estimated by preliminary
243 analyses so as to reproduce growth patterns observed in the wild before and after 2007-2008 (REF).
244 The two pellets sizes were selected to elicit two different feeding behaviours (filtration feeding vs.
245 particulate feeding) while being both in the natural range of sardine food size (Le Bourg *et al.*, 2015).
246 Both types of pellets had a similar composition with 62% and 57% of proteins and 14% and 15% of
247 lipids for the pellets of 0.1mm and 1.2mm, respectively. Thus, groups that received the same quantity
248 of pellets received approximately a similar amount of energy (cumulative energy during one month for
249 one sardine per group: LP-LQ = 60.1 ± 0.2 KJ; SP-LQ = 50.4 ± 1.3 KJ; LP-SQ = 24.5 ± 0.7 KJ; SP-
250 SQ = 22.7 KJ). Further, food loss was estimated as a function of the size of pellets received and
251 assumed as insignificant (0.3 ± 0.6% for large pellets and 1.2 ± 1.1% for small pellets, Queiros *et al.,*
252 2019). Sardines were fed 4 times a day, and the biomass of each tank was estimated weekly (based on
253 linear mass gain relationships established through monthly biometrics) to adjust food intakes. More
254 details can be found in Queiros *et al.,* 2019.

255 **Red muscle fibre preparation and oxidative activity**

256 At the end of the experimentation, 6 or 5 sardines were randomly removed from each tank every day
257 over a period of 1 week, i.e. 11 to 12 fishes per treatment, and were sacrificed by a lethal dose of
258 benzocaine (1000ppm). Mass and size of sardines were recorded. The intramuscular fat was measured
259 (MFM-992 fatmeter, more details in Brosset *et al.*, 2015b) and the sex was determined. As the
260 measurements were done in June, the sardines were out of breeding season and therefore at sexual rest
261 (Brosset *et al.,* 2016b).

262 A piece of red muscle was sampled and weighed (5.0 ± 1.5mg) (**Fig. 1**), and its respiration was
263 measured at 20°C using Oxygraph-2K high resolution respirometers (Oroboros® Instruments – WGT
264 Austria) with a suit protocol adapted from Teulier *et al.,* 2019. The total mass of red muscle was

265 measured. Only red muscle oxygen consumption was measured, as it was shown that white muscle
266 respiration is low in sardines (Teulier *et al.,* 2019). Before each session, oxygen electrodes were
267 calibrated with air-saturated respiration buffer Mir05 (110mM sucrose, 0.5mM EGTA, 3mM MgCl2,
268 60mM K-lactobionate, 20mM taurine, 10mM KH2PO4, 1g/L fatty acid-free bovine serum albumin
269 and 20mM Hepes, pH 7.1) and zero oxygen after a sodium dithionite addition. Muscle fibres
270 respiration was measured in MiR05 using a mixture of respiratory substrates (5mM pyruvate/ 2.5 mM
271 malate/ 5 mM succinate). Then the phosphorylating respiration was measured by adding 1mM ADP.
272 The integrity of mitochondria within muscle fibres was systematically tested by adding 10µM
273 cytochrome c. Thereafter, a sequential addition of 1µM p-trifluoromethoxy-carbonyl-cyanide-phenyl
274 hydrazine (FCCP) was performed in order to measure the maximal activity of the electron transport
275 system (ETS). Antimycin A (12.5µM), an inhibitor of complex III, was injected to fully inhibit
276 pyruvate/malate/succinate-supported respiration. Then, 5mM ascorbate and 500mM N,N,N',N'-
277 tetramethyl-p-phenylene-diamine were added and the maximal respiration rate associated with isolated
278 cytochrome c oxidase activity (complex IV of the ETS) was recorded.

279

280 **Mitochondrial isolation, respiration and content**

281 Mixed mitochondrial populations were isolated from red skeletal muscle in an ice-cold isolation buffer
282 containing 100mM sucrose, 50mM KCl, 5mM EDTA, 50mM Tris-base, pH 7.4 at 4°C. Briefly,
283 skeletal muscles were homogenized with a Potter-Elvehiem homogenizer and treated with subtilisin (1
284 mg/g muscle wet mass) for 5 min in an ice bath. The mixture was diluted 1:2, homogenized and then
285 centrifuged at 1000g for 10 min. The supernatant was centrifuged at 9000g for 10 min, and the pellet
286 was re-suspended in 10mL of isolation buffer and centrifuged at 1000g for 10 min to pellet any
287 remaining cell debris contamination. The resulting supernatant was filtered through cheesecloth and
288 centrifuged at 9000g for 10 min. The pellet containing mitochondria was washed once by suspension
289 in the isolation buffer and recentrifugation at 9000g for 10 min. All steps were carried out at 4°C.
290 Protein content of the mitochondrial preparation was assayed in duplicate at 540nm using the biuret
291 method, with bovine serum albumin as a standard. The absorbance of the same volume of

mitochondria was also assayed at 540nm in a solution containing 0.6% Na-K-tartrate and 3% NaOH and subtracted in order to take into account any contamination with pigments absorbing at 540nm.

Mitochondrial oxygen consumption was measured at 20°C in respiratory buffer (120mM KCl, 5mM KH_2PO_4, 1mM EGTA, 2mM $MgCl_2$, 0.3%fatty acid-free bovine serum albumin (w/v), and 3mM HEPES, pH 7.4) using a Clark electrode (Rank Brother Ltd, Cambridge, UK). Mitochondria (0.4-1mg/mL) were energized with a mixture of respiratory substrates containing 5mM pyruvate, 2.5mM malate and 5mM succinate. Phosphorylating respiration activity was initiated by the addition of 1mM ADP. The basal non-phosphorylating rate was obtained by the addition of oligomycin (1µg/mL). The maximal activity of the electron transport system was initiated by the addition of 2µM FCCP. Thereafter, antimycin (10µM) was added to fully inhibit pyruvate/malate/succinate-supported respiration, then ascorbate and TMPD (5mM/0.5mM) were added and the maximal respiration rate associated with isolated cytochrome-c oxidase activity was recorded.

The mitochondrial content of skeletal muscle was estimated from the ratio between the oxygen consumption rates of muscle fibres (expressed per gram of muscle) and mitochondria (expressed per milligram of protein). For each individual, the mitochondrial content was the mean of the two values obtained by using ADP- and FCCP-induced oxygen consumption activities.

Mitochondrial oxidative phosphorylation efficiency

Oxygen consumption and ATP synthesis rates were measured at 20°C in respiratory buffer supplemented with 20mM glucose and 1.5U/mL hexokinase (Teulier *et al.*, 2010; Colinet *et al.*, 2017). Muscle mitochondria were energized with a mixture of substrates (5mM pyruvate, 2.5mM malate, 5mM succinate). ADP (500µM) was added to initiate the mitochondrial ATP synthesis. After recording the phosphorylating respiration rate, four 100 µL samples of mitochondrial suspension were withdrawn from the respiratory chamber every 2 min and immediately quenched in 100µL of ice-cold perchloric acid solution containing 10% $HCLO_4$ and 25mM EDTA. After centrifugation of the denatured protein (15000g for 5 min), supernatants were neutralized with a KOH solution containing 0.2M KOH and 0.3M MOPS. The ATP production was determined from the slope of the linear accumulation of glucose-6-phosphate content over the sampling time interval (6 min). Glucose-6-

phosphate content was assayed spectrophotometrically at 340nm by monitoring the production of NADH in an assay medium (50mM triethanolamine-HCl, 7.5mM $MgCl_2$, 3.75mM EDTA, pH 7.4), supplemented with NAD (0.5mM) and glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (0.5U). The same procedure was performed in the presence of oligomycin (1µg/mL) to measure the level of oligomycin-insensitive ATP synthesis production in our mitochondrial suspension. This value was taken into account to calculate the rate of mitochondrial ATP synthesis that is specific to the mitochondrial ATP synthase activity and associated with mitochondrial oxygen consumption (Teulier *et al.*, 2010; Colinet *et al.,* 2017). In the present study, the non-mitochondrial ATP synthesis activity represented between 5 and 9% of total ATP production, depending on the experimental group (data not shown).

Statistical analysis

Using the software R3.5.1, the statistical significance of observed differences was assessed using Two-way ANOVA or Scheirer Ray Hare test, when normality or homoscedasticity were not validated, to estimate the effects of size and quantity of pellets on sardines for biometric characteristics, muscular respiration and mitochondrial parameters. The effect of diet on these different parameters was also measured in function of sardine's sex using a Two-way ANOVA or Scheirer Ray Hare test. The difference of evolution of body condition and growth between the different groups was analysed by a linear mixed model with the individual as a random effect on the intercept, to account for repeated measurements over the seven months (Queiros *et al.*, 2019). The correlation between body condition and basal respiration was analysed by a segmented regressions model. It is worth nothing that within each experimental group, the gender of sardines had no significant effect on any studied parameters (see Supplemental data).

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FIGURES

Figure 1. Illustrative pictures of skeletal muscle distribution in sardines.

Figure 2. Evolution of body condition (A) and growth (B) of sardines depending on their diet. The data shown are means ± s.e (n=11-12 per group). The blue, green, yellow and red curves correspond to the LP-LQ, LP-SQ, SP-LQ and SP-SQ groups, respectively. "LP-LQ" corresponds to "large pellets – large quantity", "LP-SQ" to "large pellets – small quantity", "SP-LQ" to "small pellets – large quantity" and "SP-SQ" to "small pellets – small quantity". These data were analysed with a linear mixed model.

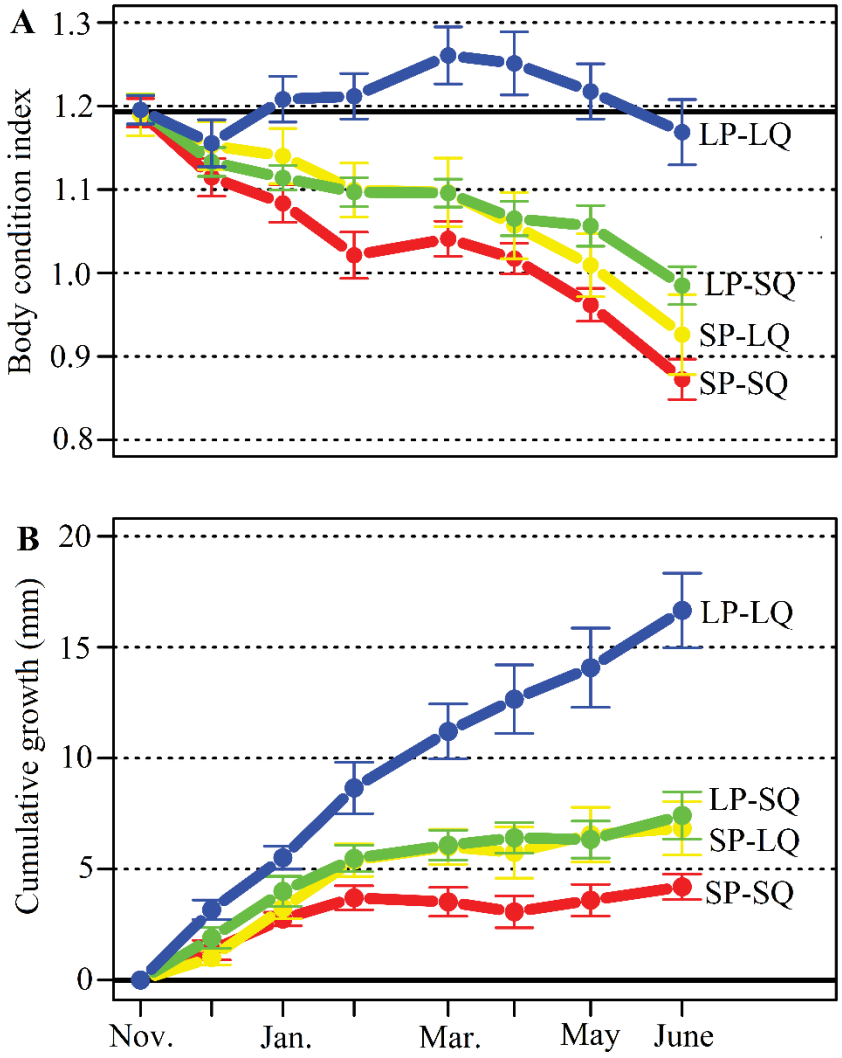


Table 1. Characteristics of sardines used to measure the respiration of red muscle fibres. These data correspond to the measurements made on the day of the sacrifice of sardines. Values are mean ± s.e.m. These data were analysed using Two-ways ANOVA (multiple comparisons test: Tukey test) or using Scheirer Ray Hare test (multiple comparisons test: Dunn test) when normality was not validated. Data with different superscript letters are significantly different at $P<0.05$.

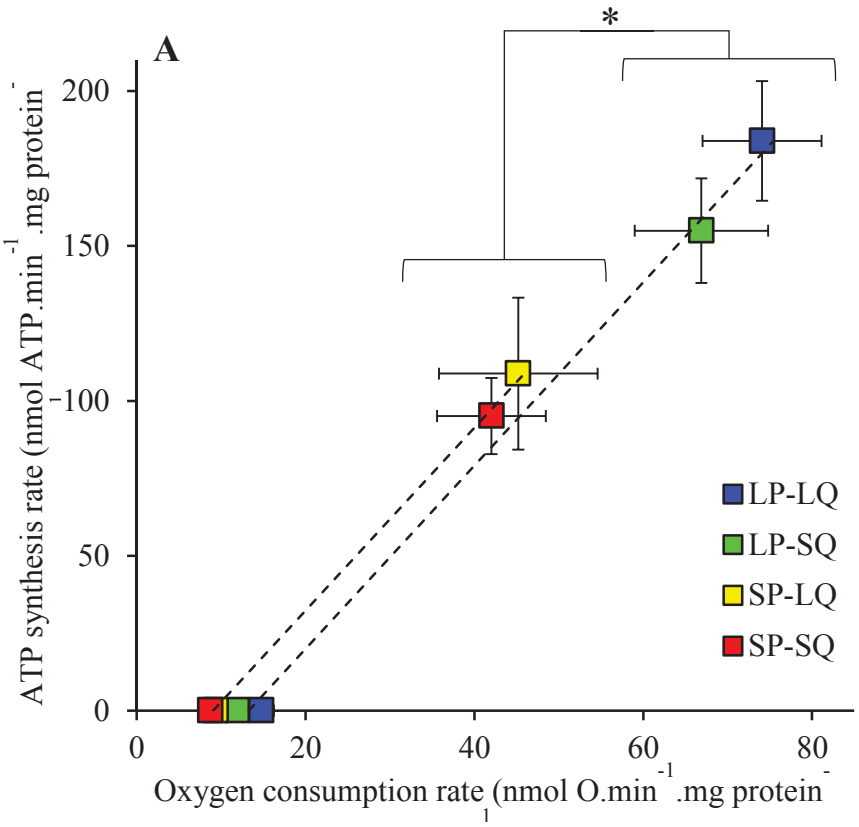
	LP-LQ	LP-SQ	SP-LQ	SP-SQ
n	11	12	11	12
(male/female/indeterminate)	(6/3/2)	(5/6/1)	(3/6/2)	(3/8/1)
Body condition	1.17 ± 0.04^{a}	0.98 ± 0.02^{b}	0.93 ± 0.05^{b}	0.87 ± 0.02^{b}
Body mass BM (g)	19.23 ± 1.38^{a}	13.98 ± 0.62^{b}	14.22 ± 1.19^{b}	12.81 ± 0.73^{b}
Red muscle mass RM (g)	1.59 ± 0.17^{a}	1.18 ± 0.06^{ab}	0.98 ± 0.13^{b}	0.91 ± 0.07^{b}
RM/BM (%)	8.11 ± 0.39^{ab}	8.46 ± 0.20^{b}	6.70 ± 0.45^{c}	7.09 ± 0.30^{ac}
Fat content (%)	13.46 ± 1.40^{a}	8.86 ± 0.76^{b}	8.12 ± 0.81^{b}	6.93 ± 0.37^{b}

Table 2. Effect of diet on uncoupled respiration rate, cytochrome c oxidase (COX) activity and mitochondrial content of red muscle. The uncoupled respiration rate and the cytochrome c oxidase activity are represented at four different level of integration: per gram of fish, on the red muscle level (nmol O_2/s), on the red muscle fibres level (pmol O_2/s^{-1}.mg $muscle^{-1}$) and on the red muscle mitochondria level (nmol O/min^{-1}.mg $proteins^{-1}$)). The mitochondrial content of red muscle is expressed in milligram of proteins per gram of red muscle. These data were analysed using Two-ways ANOVA (multiple comparisons test: Tukey test) or using Scheirer Ray Hare test (multiple comparisons test: Dunn test) when normality was not validated. Letters indicate a significant difference between groups ($P<0.050$).

Oxidative activities		LP-LQ	LP-SQ	SP-LQ	SP-SQ
Mitochondrial level (nmol $O.min^{-1}$.mg $protein^{-1}$)					
FCCP-induced maximal respiration ETS activity		90 ± 9	96 ± 7	59 ± 12	65 ± 12
Cytochrome-c oxidase activity		225 ± 20	237 ± 22	178 ± 23	204± 23
Red muscle fibre level (pmol $O_2.s^{-1}$.mg $muscle^{-1}$)					
FCCP-induced maximal respiration ETS activity		40 ± 6	27 ± 3	24 ± 3	38 ± 6
Cytochrome-c oxidase activity		157 ± 20^{ab}	180 ± 30^{a}	78 ± 8^{b}	141 ± 38^{ab}
Fish level (nmol $O_2.s^{-1}$ or nmol $O_2.s^{-1}.g^{-1}$ fish)					
FCCP-induced maximal respiration ETS activity	Total	73 ± 15^{a}	$35 \pm 7^{a,b}$	29 ± 7^{b}	$34 \pm 7^{a,b}$
	Specific	3.7 ± 0.6^{a}	3.9 ± 1.4^{ab}	1.6 ± 0.2^{b}	2.5 ± 0.5^{ab}
Cytochrome-c oxidase activity	Total	262 ± 48^{a}	216 ± 51^{ab}	85 ± 15^{b}	138 ± 34^{ab}
	Specific	13.2 ± 2.0^{a}	15.2 ± 2.6^{a}	5.1 ± 0.7^{b}	10.4 ± 3.0^{ab}
Mitochondrial content (mg protein.g $muscle^{-1}$)		54.2 ± 6.6^{ab}	34.8 ± 5.4^{a}	56.8 ± 8.5^{ab}	71.1 ± 11.2^{b}

Mitochondrial level (nmol $O.min^{-1}$.mg $protein^{-1}$) / FCCP-induced maximal respiration ETS activity : **LP-SQ-SP-LQ p= 0.0541719**
Red muscle fibre level (pmol $O_2.s^{-1}$.mg $muscle^{-1}$) / FCCP-induced maximal respiration : Size*Quantity p=0.00931, pas de différence entre les groupes

Figure 3. Effects of size and quantity of pellets on mitochondrial oxidative phosphorylation efficiency at the mitochondria level (A) and red muscle level (B). The two trend curves (A) drawn are those of the "large pellets" (LP: y = 2.96x – 39.21) and "small pellets" (SP: y = 2.95x – 26.70) groups. These data were analysed using Two-ways ANOVA (multiple comparisons test: Tukey test) or using Scheirer Ray Hare test (multiple comparisons test: Dunn test) when normality was not validated. The values shown are means ± s.e.m. (the error bars of the basal oxygen consumption are small enough that they are hidden by the points). An asterisk represents a significantly difference between diets composed of different size pellets (difference between large and small pellets) (*P<0.050; **P<0.010).



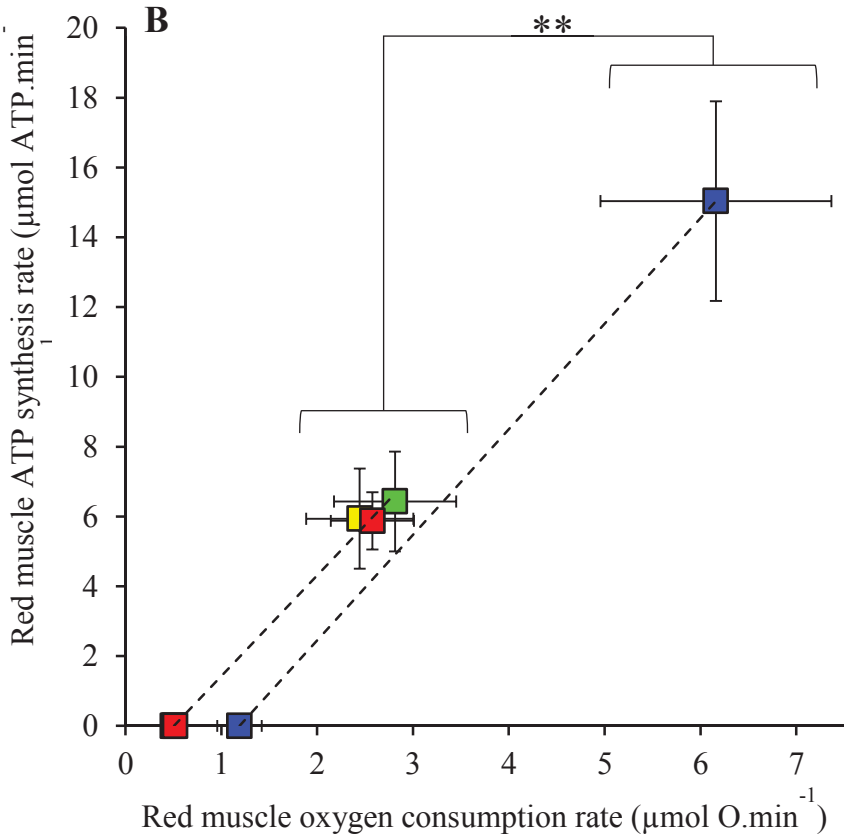
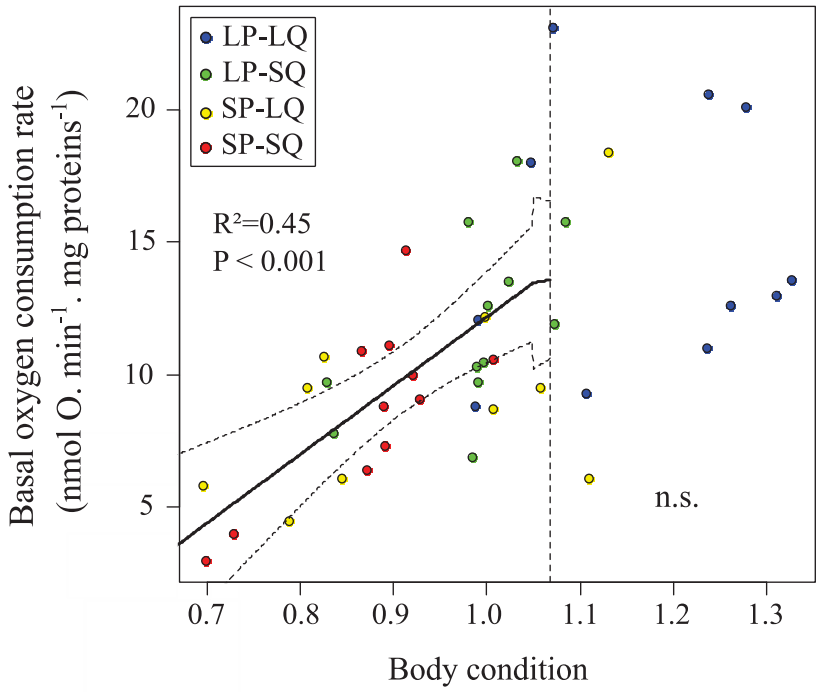


Figure 4. Basal oxygen consumption at the mitochondrial level as a function of the body condition of sardines. A linear model shows that there is a correlation between basal mitochondrial respiration in nmol $O.min^{-1}.mg\ proteins^{-1}$ and body condition when the body condition is less than 1.07 ($P<0.001$). Above this threshold, there is no longer a correlation between these two parameters.



612 **SUPPLEMENTAL DATA**

613 **Table S1. Parameters of Two-ways ANOVA (A) or Scheirer Ray Hare test (SRH) used in the study.** Different effects were tested: sex effect inside the different sardine's
614 groups (interaction between sex effect and treatment effect), the effects of size and quantity of pellets, and the interaction between these two effects.
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Tested variable	Effect	Df	F-value / H-value	Test	P-value
Sardine characteristics					
Body condition	Sex:Treatment	3	0.958	A	0.425
	Size	1	27.283	A	5.450e-06
	Quantity	1	12.723	A	9.360e-04
	Size:Quantity	1	3.717	A	0.061
Body mass (BM)	Sex:Treatment	3	2.772	A	0.058
	Size	1	8.094	SRH	0.004
	Quantity	1	5.851	SRH	0.016
	Size:Quantity	1	1.167	SRH	0.280
Red muscle mass (RM)	Sex:Treatment	3	1.982	A	0.137
	Size	1	15.152	A	3.490e-04
	Quantity	1	4.551	A	0.039
	Size:Quantity	1	2.359	A	0.132
RM/BM	Sex:Treatment	3	0.282	A	0.838

	Size	1	16.515	A	2.070e-04
	Quantity	1	1.190	A	0.281
	Size:Quantity	1	0.002	A	0.962
Fat content	Sex:Treatment	3	0.878	A	0.466
	Size	1	16.099	A	3.250e-04
	Quantity	1	11.111	A	0.002
	Size:Quantity	1	3.993	A	0.054
Mitochondrial content of red muscle	Sex:Treatment	3	1.696	A	0.191
	Size	1	4.691	SRH	0.030
	Quantity	1	0.479	SRH	0.489
	Size:Quantity	1	3.125	SRH	0.077
Mitochondrial respiration					
Maximal respiration rate	Sex:Treatment	3	1.042	A	0.388
	Size	1	9.334	A	0.004
	Quantity	1	0.365	A	0.549
	Size:Quantity	1	1.000e-04	A	0.993
Cytochrome c oxidase activity	Sex:Treatment	3	0.465	A	0.709
	Size	1	3.589	A	0.066

	Quantity	1	0.729	A	0.399
	Size:Quantity	1	0.122	A	0.729
Fibres respiration					
Maximal respiration rate	Sex:Treatment	3	0.481	SRH	0.923
	Size	1	2.822	SRH	0.093
	Quantity	1	7.000e-04	SRH	0.980
	Size:Quantity	1	3.736	SRH	0.053
Cytochrome c oxidase activity	Sex:Treatment	3	0.811	A	0.498
	Size	1	5.934	A	0.020
	Quantity	1	2.541	A	0.119
	Size:Quantity	1	0.585	A	0.449
Muscle respiration					
Maximal respiration rate	Sex:Treatment	3	0.583	SRH	0.900
	Size	1	4.293	SRH	0.038
	Quantity	1	1.098	SRH	0.295
	Size:Quantity	1	4.450	SRH	0.034
Cytochrome c oxidase activity	Sex:Treatment	3	0.076	A	0.972
	Size	1	10.316	A	0.003

	Quantity	1	0.001	A	0.976
	Size:Quantity	1	1.488	A	0.230
Individual respiration					
Maximal respiration rate	Sex:Treatment	3	0.942	SRH	0.815
	Size	1	5.947	SRH	0.015
	Quantity	1	0.062	SRH	0.804
	Size:Quantity	1	3.092	SRH	0.079
Cytochrome c oxidase activity	Sex:Treatment	3	0.121	A	0.947
	Size	1	12.440	A	0.001
	Quantity	1	0.023	A	0.880
	Size:Quantity	1	0.160	A	0.160
Mitochondrial efficiency					
Oxygen consumption rate	Sex:Treatment	3	2.463	A	0.081
	Size	1	9.807	A	0.003
	Quantity	1	0.277	A	0.601
	Size:Quantity	1	0.145	A	0.705
ATP synthesis rate	Sex:Treatment	3	3.414	SRH	0.332
	Size	1	9.866	SRH	0.002

	Quantity	1	0.508	SRH	0.476
	Size:Quantity	1	0.509	SRH	0.476
Basal oxygen consumption rate	Sex:Treatment	3	1.876	A	0.155
	Size	1	11.746	A	0.001
	Quantity	1	2.374	A	0.131
	Size:Quantity	1	0.761	A	0.388
Trend	Sex:Treatment	3	9.447	SRH	0.024
	Size	1	0.902	SRH	0.342
	Quantity	1	0.959	SRH	0.327
	Size:Quantity	1	0.155	SRH	0.693

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