Université du Québec
à Rimouski

Quels sont les compartiments biologiques les plus affectés par l'exposition à une température élevée durant l'ontogénie ? Une étude de cas sur le vers marin *Ophryotrocha labronica*

Mémoire présenté

dans le cadre du programme de maîtrise en gestion de la faune et de ses habitats

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

PAR

© FANNY VERMANDELE

Avril 2019

Composition du jury :

Christian Nozais, président du jury, Université du Québec à Rimouski

Piero Calosi, directeur de recherche, Université du Québec Rimouski

Philippe Archambault, codirecteur de recherche, Université Laval

Réjean Tremblay, examinateur externe, Institut des sciences de la mer de Rimouski

Dépôt initial le 18 décembre 2018 Dépôt final le 30 avril 2019

REMERCIEMENTS

Je tiens, d'abord et avant tout, à remercier profondément mon directeur de maîtrise, Piero Calosi, ainsi que mon codirecteur Philippe Archambault, pour m'avoir guidée à travers cette aventure. Merci d'avoir cru en moi et de m'avoir permis de développer ma propre idée de recherche. Merci, Philippe, de m'avoir ouvert les portes de ton laboratoire aussi tôt dans mon développement académique. C'est en grande partie grâce à toi et à la façon dont tu transmets ta passion que je me suis initialement immergée au monde de la recherche. Piero, merci pour ta confiance, mais surtout pour ta présence tout au long de ce parcours. Ton écoute, ta patience, ton calme et ton soutien, particulièrement suite aux imprévus de la vie, ont fait toute la différence. Merci également pour les incroyables opportunités que tu m'as offertes à travers mon parcours. Elles m'ont permis de grandir scientifiquement.

Merci également à tous les membres de mon laboratoire présents à un moment ou un autre de ma maîtrise, qui ont animé ce parcours et ont aidé, de près ou de loin ce projet: Gloria, Fanny, Ella, Marie-Hélène, Cynthia, Mathilde, Christine, Francis, Juliette, Nicholas, Catherine et Maude. Merci, Gloria et Fanny, pour vos conseils tout au long de la maîtrise. Marie-Hélène, merci pour ta présence, ton écoute et merci d'avoir pris soin de moi... et surtout de mon estomac lors des longues journées d'expérience ! Ella, merci infiniment pour ton support et ton écoute sans faille. La dernière ligne droite de ma maîtrise n'aurait certainement pas été la même sans ta présence. Je veux également remercier mes amies Sara Wing, Anne-Claire Sohet et Audrey Dutilly qui m'ont encouragée tout au long de la maîtrise.

France Dufresne, Astrid Tempestini, Pierre Blier, Pierre De Wit, Alain Caron et Diana Madeira, merci pour votre aide et vos précieux conseils lors des différentes analyses. Merci, France, de m'avoir laissé utiliser ton laboratoire pour les analyses génétiques. Une

immense merci également à Christian Nozais et Réjean Tremblay, membres de mon jury, de prendre le temps de commenter ce projet.

Je tiens également à souligner les organismes subventionnaires suivants, sans qui, le projet n'aurait pas été possible: le Conseil de recherches en sciences naturelles et génie du Canada (CRSNG), le Fonds québécois de la recherche sur la nature et les technologies (FRQNT), l'Académie des Sciences Royale Suédoise pour la bourse KVA, le Groupe de recherche sur les environnements nordiques BORÉAS et le Centre de la Science de la Biodiversité du Québec (CSBQ).

Finalement, je souhaite remercier tous les membres de famille, ici ou en Europe, ainsi que celle de Louis pour vos encouragements constants qui m'ont gardée motivée dans cette aventure. Alice, merci d'être toujours présente pour moi. La confiance que tu portes en moi me donne des ailes. Je veux remercier du fond du cœur mes parents, sans qui rien de tout cela ne serait possible. C'est grâce à vous et à votre audace que je peux aujourd'hui travailler dans le domaine de mes rêves. Merci pour votre soutien, vos encouragements, votre aide, mais surtout merci d'être toujours là lorsque j'en ai besoin. L'accomplissement de ce projet de maîtrise n'aurait pas été le même sans votre présence. Pour terminer, je veux dire un merci tout spécial à mon compagnon Louis qui m'a épaulée tout au long de ce processus. Merci, Louis, pour ton soutien, mais surtout pour ta patience incroyable. Tu m'as gardée motivée dans les moments plus difficiles. Merci de croire en moi et de me soutenir dans mes aspirations. Cela fait toute la différence.

RÉSUMÉ

En mettant en évidence la façon dont les différents compartiments biologiques sont finement ajustés et interconnectés, l'utilisation d'une approche intégrative facilite l'identification des mécanismes sous-jacents aux réponses plastiques des organismes à la température. En améliorant notre compréhension de l'implication des ajustements mitochondriaux et métaboliques dans l'acclimatation des organismes aux températures élevées, cette approche est particulièrement pertinente dans le contexte du réchauffement climatique. Toutefois, les outils nécessaires à son développement sont rarement disponibles pour de nombreux ectothermes marins. L'objectif de ce projet est de développer et appliquer une approche intégrative permettant d'identifier les mécanismes de réponse des ectothermes marins exposés aux températures élevées durant l'ontogénie. En utilisant, l'annélide marin *Ophryotrocha labronica* La Greca et Bacci, ce projet vise à identifier à travers l'ontogénie (1) les ajustements mitochondriaux et métaboliques potentiellement impliqués dans les réponses des organismes aux températures élevées et (2) les conséquences potentielles de ces ajustements sur les traits d'histoire de vie. Pour ce faire, les individus ont été exposés, dès leur naissance, à une température contrôle (24 °C, température moyenne enregistrée au pic de densité de la population naturelle) et une température élevée (30 °C, +2-3 °C par rapport à la température maximale mesurée dans leur habitat). Les traits d'histoire de vie (taille, survie, fertilité et fécondité) étaient mesurés sur des groupes d'individus quotidiennement, du sixième jour d'exposition jusqu'à leur première reproduction. Ces groupes étaient ensuite congelés afin d'analyser l'effet de la température sur l'expression des gènes et des traits physiologiques mitochondriaux et sur les profils métabolomique. Nos résultats montrent que l'exposition des organismes à 30 °C affecte principalement leur profil métabolomique en modifiant les voies et taux métaboliques, mais n'affecte pas fortement les traits d'histoire de vie. Ces résultats confirment que les individus d'*O. labronica* tolèrent la température élevée utilisée dans cette étude (30 °C). Néanmoins, puisque les effets néfastes de l'exposition aux températures élevées peuvent s'accumuler dans le temps et que les ajustements plastiques peuvent changer selon les générations, il serait pertinent d'inclure cette approche intégrative aux expériences multigénérationnelles. Ces études pourraient améliorer notre compréhension des mécanismes qui sous-tendent la réponse des espèces au réchauffement climatique.

Mots clés : Approche intégrative, Réchauffement climatique, Plasticité Phénotypique, Expression génétique, Mitochondries, Registre aérobie, Profils métabolomique, Performance, Annélide marin

ABSTRACT

By highlighting how different biological compartments are finely tuned and interconnected, the use of an integrative approach can facilitate the identification of the mechanisms underpinning organisms' plastic responses to temperature. By improving our understanding of the involvement of mitochondrial and metabolic adjustments in organisms' acclimation to high temperature, this approach is particularly relevant in the current context of global warming. However, the tools necessary for its development are rarely available for numerous marine ectotherms. The objective of this project is to develop and apply an integrative approach enabling to identify response mechanisms of marine ectotherms exposed to high temperature during ontogeny. Using, the marine annelid *Ophryotrocha labronica* La Greca and Bacci, this project aims to identify through ontogeny (1) the mitochondrial and metabolic adjustments potentially involved in organisms' responses to high temperatures, and (2) the potential consequences of these adjustments on life history traits. In order to do this, individuals were exposed from their birth to a control temperature (24 °C, the average temperature recorded at highest population density) and a high temperature (30 °C, +2-3 °C relative to the maximal temperature recorded in their habitat). Life history traits (size, survival, fertility, and fecundity) were measured on groups of individuals daily, starting from the sixth day of exposure to their first reproductive event. These groups were then frozen in order to analyze the temperature effect on mitochondrial gene expression and physiological traits, and on metabolomics profiles. Our results show that organisms' exposure to 30 °C affects mainly the metabolomics profiles in *O. labronica* by altering metabolic pathways and rates, but does not strongly impact life history traits. These results confirm that *O. labronica*'s individuals can tolerate the elevated temperature used in this study (30 °C). However, as detrimental effects caused by an exposition to elevated temperatures can accumulate through time, and as plastic adjustments can change with generations, it would be useful to include this integrative approach within multigenerational experiments. These researches could help us improve our understanding of the mechanisms underpinning species' responses to global warming.

Keywords: Integrative approach, Global warming, Phenotypic plasticity, Gene expression, Mitochondria, Aerobic scope, Metabolomics profiles, Fitness, Marine annelid

TABLE DES MATIÈRES

REMERCIEMENTS vii

RÉSUMÉ ix

ABSTRACT x

TABLE DES MATIÈRES xi

LISTE DES FIGURES xiii

INTRODUCTION GÉNÉRALE 1

CHAPITRE 1 Dans quel compartiment biologique l'impact de l'exposition aux températures élevées est le plus visible durant l'ontogénie 13

RÉSUMÉ EN FRANÇAIS DU PREMIER ARTICLE 13

IN WHICH BIOLOGICAL COMPARTMENTS IS THE IMPACT OF EXPOSURE TO ELEVATED TEMPERATURE BETTER OBSERVED DURING ONTOGENY? 15

ABSTRACT 16

1. INTRODUCTION 17

2. METHODS 22

2.1 Study species 22

2.2 Experimental design and setup 23

2.3 Determination of life history traits 29

2.4 Mitochondrial genome assembly and annotation 29

2.5 Mitochondrial gene expression measurement 31

2.6 Determination of mitochondrial physiological traits 32

2.7 Determination of the metabolomic profile 33

2.8 Data calculation and statistical analyses 34

3. RESULTS 41

3.1 Mitochondrial gene expression 41
3.2 Mitochondrial physiological traits 42
3.3 Metabolomics profiles.....44
3.4 Life-history traits.....51
3. DISCUSSION 54
4.1 Mitochondrial gene expression 57
4.2 Mitochondrial physiological traits 58
4.3 Metabolomics profile 59
4.4 Life history traits.....62
4.5 Conclusion and future perspectives 64
REFERENCES 68
SUPPLEMENTARY TABLES.....82
APPENDICES 88
CONCLUSION GÉNÉRALE.....89
ANNEXE 1 93
RÉFÉRENCES BIBLIOGRAPHIQUES.....118

LISTE DES FIGURES

Figure 1 : Réponses possibles des espèces face aux changements environnementaux 6

Figure 2 : Courbe de performance thermique des organismes illustrant la gamme de tolérance à la température pour différents traits 8

Figure 3 : Représentation du concept d'allocation d'énergie et des coûts de la plasticité phénotypique 10

INTRODUCTION GÉNÉRALE

Qu'ils soient unicellulaires ou multicellulaires, aquatiques ou terrestres, tous les organismes doivent maintenir leur homéostasie pour survivre. Il s'agit de l'un des principes unificateurs en biologie (Moyes et Schulte, 2008; Reece *et al.*, 2012; Woods et Wilson, 2015). Or, ce maintien de l'homéostasie, qui se définit comme étant la préservation d'un équilibre interne en dépit de l'environnement externe (Cannon, 1929; Cooper, 2008; Moyes et Schulte, 2008; Reece *et al.*, 2012; Smith et Smith, 2012), peut représenter un véritable défi pour les organismes (Smith et Smith, 2012; Boyd *et al.*, 2016; Somero *et al.*, 2017) en raison de l'hétérogénéité de l'environnement (Boyd *et al.*, 2016). En conséquence, la distribution des organismes se limite généralement aux régions géographiques présentant des conditions biotiques et abiotiques favorisant le maintien de l'homéostasie et permettant aux organismes de croître et de se reproduire (Taylor, 1984; Brown *et al.*, 1996; Gaston, 2003; Bozinovic *et al.*, 2011; Wiens, 2011; Sunday *et al.*, 2014a). Si les facteurs affectant la distribution des organismes sont nombreux, et résultent d'une interaction complexe entre les traits physiologiques, phénologiques et écologiques (Lee *et al.*, 2009; Bozinovic et Naya, 2015), la compréhension des processus impliqués dans les patrons de distribution des espèces demeure par contre limitée (Gaston, 2009; Diamond, 2018).

Distribution géographique et plasticité physiologique

Parmi les différents processus impliqués dans les patrons de distribution des espèces, les traits physiologiques seraient l'un des facteurs majeurs dictant à la fois les limites et l'étendue de l'aire de distribution des espèces (Gaston, 2009; Bozinovic et Naya, 2015; Boyd *et al.*, 2016). Plus spécifiquement, la tolérance physiologique des organismes à la température expliquerait en partie l'étendue de leur distribution latitudinale,

particulièrement chez les ectothermes marins (Diamond, 2018). En ajustant leur tolérance physiologique, les organismes peuvent en fait s'acclimater à différentes conditions environnementales (Bozinovic *et al.*, 2011; Bozinovic et Naya, 2015), et ce, grâce à leur plasticité phénotypique (Ghalambor *et al.*, 2007; Boyd *et al.*, 2016). Cette plasticité, définie comme étant la capacité d'un génotype à produire plusieurs phénotypes selon l'environnement expérimenté (Bradshaw, 1965; Pigliucci, 2001; West-Eberhard, 2003; Boyd *et al.*, 2016), permet aux organismes d'augmenter ou d'ajuster la gamme de températures qu'ils peuvent tolérer, ce qui influence leur aire de distribution (Calosi *et al.*, 2008; Bozinovic *et al.*, 2011; Bozinovic et Naya, 2015).

Le rôle clé joué par la plasticité phénotypique des organismes dans la détermination des patrons de distribution des espèces fait l'objet de plusieurs hypothèses. Parmi celles-ci, l'Hypothèse de Variabilité Climatique (HVC, Gaston, 2003; Bozinovic *et al.*, 2011) qui postule que l'étendue de la distribution latitudinale des espèces reflèterait leur tolérance thermale (Gaston, 2003; Parmesan, 2006; Sunday *et al.*, 2012). Autrement dit, puisque la variabilité environnementale augmente généralement avec la latitude, les populations et espèces habitant aux plus hautes latitudes possèderaient une plus grande fenêtre de tolérance physiologique (Bozinovic *et al.*, 2011) et seraient plus plastiques (Chown *et al*., 2004; Bozinovic *et al.*, 2011) que leurs congénères des latitudes plus basses (Brown *et al*., 1996). Cette hypothèse s'appliquerait particulièrement aux ectothermes marins (Parmesan, 2006; Sunday *et al*., 2012; Diamond, 2018). Ainsi, considérant à quel point la différence de plasticité phénotypique et donc de tolérance physiologique entre les espèces reflète leur patron de distribution, l'étude des réponses plastiques des organismes en fonction des paramètres environnementaux est primordiale si nous voulons appréhender dans quelle mesure ces réponses contribuent à modifier l'aire de distribution des espèces en cas de changements environnementaux (McNab, 2002; Sunday *et al.*, 2012; Forsman, 2015). Pour ce faire, une approche mécanistique et intégrative est indispensable puisqu'elle constitue une base fondamentale pour comprendre les patrons actuel et futur de distribution des espèces (Chow et al., 2004 ; Bozinovic *et al.*, 2011; Bozinovic et Naya, 2015 ; Diamond, 2018). Or, malgré le nombre grandissant d'études mentionnant l'importance d'utiliser une

approche intégrative (Bartholomew, 1986; Pigliucci, 2003; Pörtner *et al.*, 2006; Wake, 2008; Bozinovic et Naya, 2015), son utilisation en recherche demeure marginale (Strange, 2005; Bozinovic et Naya, 2015).

Vers une approche intégrative

Historiquement, les études portant sur la biologie des organismes avaient tendance à privilégier une approche plutôt réductionniste qui valorisait la déconstruction des organismes en différents compartiments pour les étudier séparément et mieux comprendre leur fonctionnement (Bozinovic et Naya, 2015). Par ailleurs, avec l'avènement des techniques moléculaires et génétiques, les différentes disciplines biologiques telles que l'écologie, la physiologie et l'évolution ont divergé et sont souvent considérées comme des entités indépendantes (Dobzhansky, 1964; Bartholomew, 1986; Strange, 2005; Bozinovic et Naya, 2015). Or, le fait d'étudier les différents traits des organismes sans tenir compte de leurs potentielles interactions, et ce, tout en faisant abstraction des mécanismes évolutifs, peut nuire à notre compréhension des mécanismes de réponse des espèces (Aubin-Horth et Renn, 2009; Martin, 2015). Cela est particulièrement préjudiciable dans le cas de la plasticité phénotypique, puisqu'il s'agit d'une réponse complexe (Forsman, 2015). La modification du phénotype des organismes inclut des ajustements dans de nombreux traits, que ce soit au niveau morphologique, physiologique, comportemental ou encore de l'histoire de vie (Schlichting et Pigliucci, 1998; West-Eberhard, 2003; Schulte *et al.*, 2011). Par ailleurs, il y a des limites à la plasticité phénotypique, puisque les réponses plastiques engendrent des coûts énergétiques importants inhérents au maintien de l'homéostasie cellulaire et des mécanismes régulateurs (Hoffman, 1995; DeWitt *et al.*, 1998; Angilletta, 2009; Godlbod et Calosi, 2013; Martin, 2015; Woods *et al.*, 2015). Ces coûts énergétiques peuvent modifier voire réduire le budget énergétique des espèces, forçant ces dernières à redistribuer l'énergie disponible en priorisant certaines fonctions au détriment des autres, ce qui nuit par le fait même à la valeur sélective des organismes (Stearns, 1989; Roff et Gélinas, 2002; Calosi *et al.*, 2013; Chevin *et al.*, 2013; Thor et Dupont, 2015). En outre, les

modifications phénotypiques peuvent varier en fonction de l'ontogénie des organismes et entre les générations (Sunday *et al.*, 2014a; Shama *et al.*, 2016; Gibbin *et al*., 2017a; Diamond, 2018; Donelson *et al.*, 2018), ce qui complique encore davantage la compréhension des mécanismes plastiques. À cela s'ajoute le fait que la plasticité phénotypique peut être elle-même adaptative, neutre ou non-adaptative, ce qui peut influencer à quel point les changements phénotypiques favorisent la persistance des espèces dans un nouvel environnement (Ghalambor *et al.*, 2007; Gibbin *et al*., 2017b).

Par conséquent, considérant la complexité des réponses plastiques, il est primordial d'utiliser une approche plus intégrative, dans l'optique de comprendre les mécanismes à l'œuvre (Pigliucci, 2003; Bozinovic et Naya, 2015; Forsman, 2015; Woods *et al.*, 2015. Ce type d'approche utilise un ensemble d'outils et de techniques pour comprendre les processus à l'échelle de l'organisme issus des interactions dynamiques entre les différents niveaux et traits biologiques (Zheng *et al.*, 2014). Pour ce faire, il faut d'une part comprendre la variabilité des réponses plastiques des organismes à différents niveaux d'organisation biologique, soit des gènes aux traits d'histoire de vie. D'autre part, il faut intégrer la réponse entre ces différents niveaux biologiques afin d'évaluer comment ces derniers sont finement ajustés et interconnectés (Bozinovic *et al.*, 2011, Bozinovic et Naya, 2015). La combinaison des outils et modèles statistiques spécifiquement développés pour les données de type « -omiques » aux analyses de réseaux (*network analyses*) permet cette intégration entre les différents niveaux biologiques. Cependant, ces analyses demeurent majoritairement développées pour les organismes modèles, pour lesquels le génome ainsi que les voies biologiques impliquées dans l'expression génique, le métabolisme et la transmission des signaux sont bien connus (Shannon *et al.*, 2003).

Plasticité phénotypique et changements globaux

Le développement d'une telle approche intégrative pour mieux comprendre les réponses plastiques des organismes est particulièrement crucial dans le contexte actuel des

changements globaux (Bozinovic et Naya, 2015; Chown et Gaston, 2008). Les changements dans les paramètres environnementaux engendrés par l'augmentation des activités anthropiques (Parmesan et Yohe, 2003; Calosi *et al.*, 2019) représentent l'un des plus grands défis actuels auxquels les espèces sont confrontées (Bellard *et al.*, 2012). Ces changements menacent non seulement la survie des espèces et la biodiversité, mais également le fonctionnement des écosystèmes (Angilletta, 2009; Somero, 2010; Barnosky *et al.*, 2011; Ceballos *et al.*, 2017). Les conséquences des changements globaux se font d'ailleurs déjà sentir. Les taux d'extinction actuels des espèces indiqueraient que la planète serait maintenant entrée dans l'ère de sa sixième extinction de masse (Ceballos *et al.*, 2015; Ceballos et Ehrlich, 2018) et la migration de plusieurs espèces vers de plus hautes latitudes a déjà été observée (Parmesan et Yohe, 2003; Perry *et al.*, 2005; Diamond, 2018). Les écosystèmes marins risquent d'être particulièrement perturbés dans les prochaines années de par le rôle régulateur du climat des océans. Ils subiront non seulement une augmentation de la température des eaux et des variations de la salinité, mais ils subiront également des phénomènes d'hypoxie et d'acidification, caractérisés respectivement par une diminution de la teneur en oxygène et une diminution du pH de l'eau (IPCC, 2014).

Parmi les changements, l'élévation globale des températures de 2 à 4°C prédite d'ici la fin du siècle (IPCC, 2014) risque de modifier fortement les écosystèmes marins (Munday *et al.*, 2013; Reusch, 2014). En influençant directement la vitesse des réactions biochimiques au sein des organismes (Hochachka et Somero, 1984; Schulte, 2015), la température peut en effet modifier leur physiologie et leur comportement, particulièrement chez les ectothermes (Pörtner *et al.*, 2006; Deutsch *et al.*, 2008; Angilletta, 2009). Par conséquent, pour survivre au réchauffement climatique, les espèces devront migrer, s'acclimater grâce à leur plasticité phénotypique ou encore s'adapter (Figure 1, Deutsch *et al.*, 2008; Hofmann *et al.*, 2010; Sunday *et al.*, 2014b; Calosi *et al.*, 2016; Boyd *et al.*, 2016). Or, considérant la vitesse et l'intensité à laquelle l'élévation des températures survient, seule une fraction des espèces sera en mesure de migrer assez rapidement pour 'suivre' le climat (Chen *et al.*, 2011). Quant aux processus adaptatifs, ils pourraient ne pas être mis en place assez rapidement pour éviter l'extinction puisque ces processus se

réalisent à l'échelle des générations (Etterson et Shaw, 2001; Peck, *et al.*, 2010). La plasticité phénotypique des organismes apparaît donc actuellement comme le principal mécanisme qui permettrait aux espèces de répondre aux effets directs du réchauffement climatique (Peck *et al.*, 2010; Massamba-N'Siala *et al.*, 2012; Calosi *et al.*, 2016), tout en laissant le temps aux processus adaptatifs de se mettre en place, ou en les facilitant (Figure 1; Chevin *et al.*, 2010; Munday *et al.*, 2013; Sunday *et al*. 2014b).

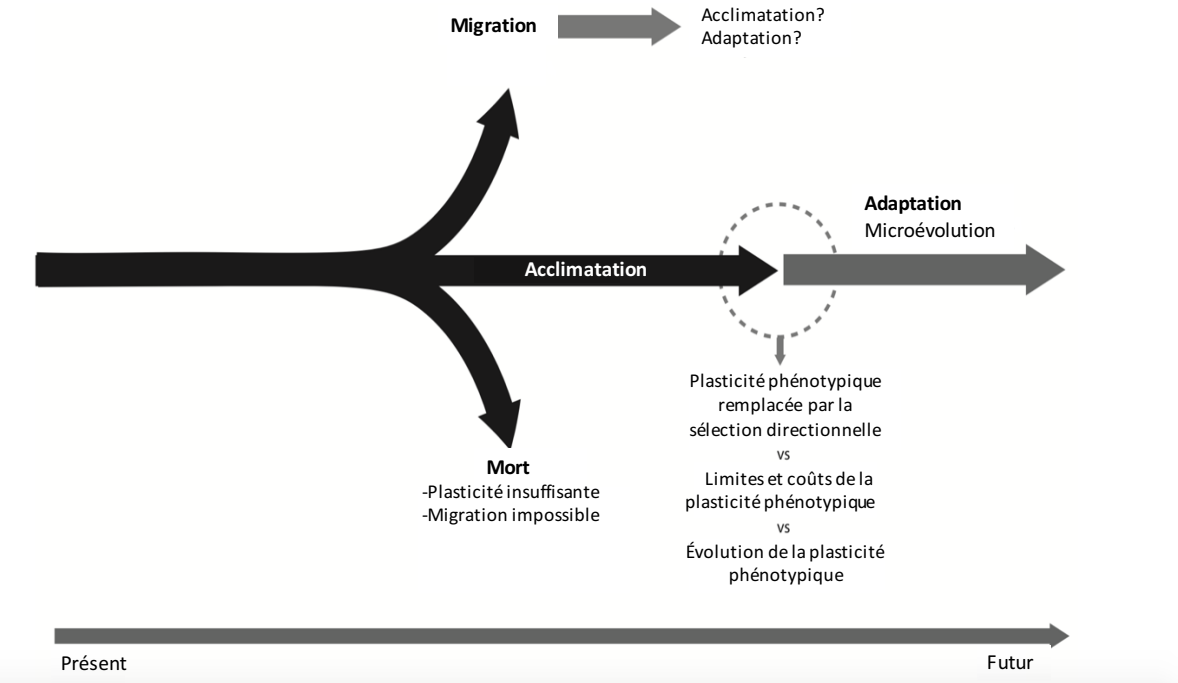


Figure 1 : Réponses possibles des espèces face aux changements environnementaux. Le cercle pointillé indique que la relation entre l'acclimatation et l'adaptation est encore incertaine. Image tirée de Boyd et al. (2016)

Réponses des ectothermes à la température

Un nombre accru d'études tente de comprendre les mécanismes plastiques impliqués dans l'acclimatation des organismes aux températures élevées. La compréhension de ces mécanismes pourrait permettre de mieux mesurer les coûts et conséquences potentiels associés à l'acclimatation thermique des organismes, et ainsi, discerner le lien unissant la plasticité phénotypique et l'adaptation rapide (Schulte *et al.*, 2015; Calosi *et al.*, 2016). Or, la majorité des études se sont uniquement intéressées à l'acclimatation thermique des organismes en se concentrant sur certains traits biologiques seulement, par exemple, les gènes, les protéines, les voies métaboliques ou les traits d'histoire de vie (Pörtner *et al.*, 2006). L'utilisation d'une approche intégrative, telle qu'évoquée précédemment, manque cruellement si nous voulons comprendre la façon dont les réponses des différents niveaux s'intègrent et influencent l'acclimatation des organismes dans le contexte du réchauffement climatique (Pörtner *et al.*, 2006). Pour répondre efficacement à ces interrogations, il est primordial que l'utilisation de cette approche intégrative se base sur les hypothèses et connaissances antérieures concernant l'effet de la température sur les organismes (Schulte, 2015).

De façon générale, l'influence de la température sur la performance des organismes (par exemple leur survie, leur développement et leur reproduction) est représentée par une courbe de performance thermique qui a la forme d'une courbe de cloche : la performance augmente à partir d'une température critique minimale et atteint un plateau à la température optimale. Puis, la performance diminue brusquement jusqu'à la température critique maximale (Figure 2; Huey et Kingsolver, 1989; Angilletta *et al.*, 2002; Angilletta, 2009; Schulte *et al*., 2011; Dell *et al.*, 2013; Schulte, 2015). Cette courbe définit la gamme de température qu'une espèce peut supporter, gamme qui varie en fonction du trait regardé. En effet, la gamme de température permettant à une espèce de se reproduire est généralement moindre que celle dans laquelle une espèce peut survivre (Figure 2; Schulte *et al.*, 2011; Schulte, 2015).

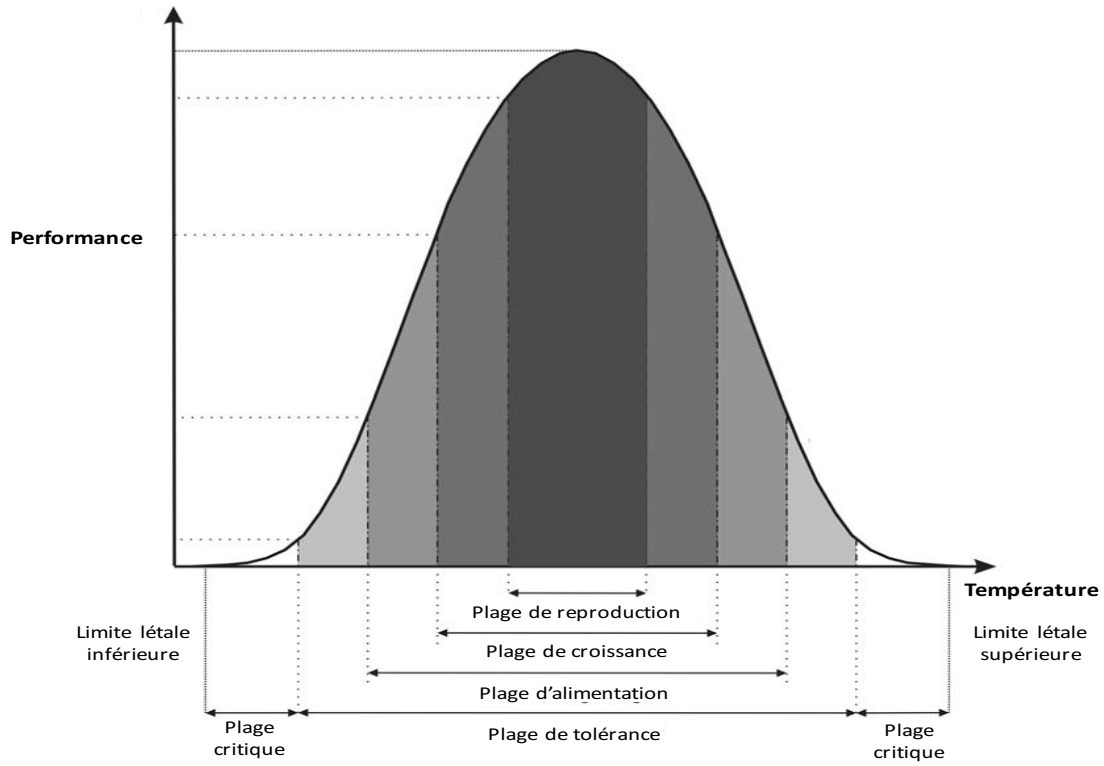


Figure 2 : Courbe de performance thermique des organismes illustrant la gamme de tolérance à la température pour différents traits. La forme et l'étendue des plages de tolérance de ces courbes de performances varient en fonction des stades de vies. Image modifiée de Helaouët et Beaugrand (2009)

La forme de cette courbe serait induite, entre autres, par les limites biochimiques et physiologiques imposées par la température (Angilletta, 2009; Schulte, 2015). Au niveau biochimique, les températures extrêmes limitent la performance des organismes en dénaturant les enzymes et les protéines (Hochachka et Somero, 1984; Angilletta, 2009; Schulte *et al.*, 2011). Néanmoins, la température critique maximale limitant la performance des organismes survient généralement à des températures plus basses que celles attendues si la limite thermique était causée uniquement par la dénaturation des composantes cellulaires. Cela est particulièrement vrai chez les métazoaires (Pörtner, 2001, 2002). Ces observations ont mené à la formulation de l'hypothèse intitulée '*Oxygen and Capacity limited thermal tolerance'* (OCLTT) proposée par Pörtner et collègues (2001, 2002), qui

s'intéresse au registre aérobie des espèces. Ce registre aérobie, défini comme étant la différence entre le taux métabolique maximal (MM) et le taux métabolique de base (MB), représente en fait l'énergie dont dispose un organisme pour effectuer différentes fonctions (Pörtner, 2001; Claireaux et Lefrançois, 2007; Schulte, 2015). Selon cette hypothèse, c'est la réduction du registre aérobie causée par le décalage entre la demande en oxygène et son apport aux tissus qui définirait la limite thermique supérieure des organismes (Pörtner, 2001, 2002, Pörtner et Knust, 2007; Pörtner et Farrell, 2008; *c.f* les discussions amenées dans Clark *et al*., 2013a,b; Jutfelt *et al.*, 2018). Cette diminution du registre aérobie peut non seulement engendrer une transition du métabolisme aérobie vers le métabolisme anaérobie (Pörtner, 2001, 2002, Pörtner et Knust, 2007; Verberk *et al.*, 2013), mais également affecter le budget énergétique des espèces, nuisant par la même occasion à leur performance (Pörtner, 2001, 2002, Pörtner et Knust, 2007).

Ces modifications du budget énergétique peuvent particulièrement influencer les réponses dans les traits d'histoire de vie. Généralement, la règle de la relation température-taille prédit que les organismes atteignent la maturité sexuelle plus tôt et à une plus petite taille lorsqu'exposés à des températures élevées (Atkinson, 1996; Angilletta, 2009). De plus, ces derniers se reproduiraient plus fréquemment (Prevedelli et Simonini, 2001), mais produiraient des œufs de plus petites tailles (Fox et Czesak, 2000; Bownds *et al.*, 2010; Liefting *et al.*, 2010). Or, considérant que l'élévation des températures peut, d'une part, perturber le registre aérobie des espèces (Pörtner 2001, 2002) et d'autre part augmenter les coûts associés aux réponses plastiques (Hoffman, 1995; DeWitt *et al.*, 1998; Angilletta, 2009), des compromis énergétiques entre les différents compartiments biologiques peuvent surgir (Reznick, 1985; Roff, 1992; Angilletta, 2009). En induisant une réallocation de l'énergie entre les différents compartiments biologiques, ce compromis énergétique se fait généralement au détriment de certains traits d'histoire de vie (Figure 3; Massamba-N'Siala, Prevedelli et Simonini, 2014).

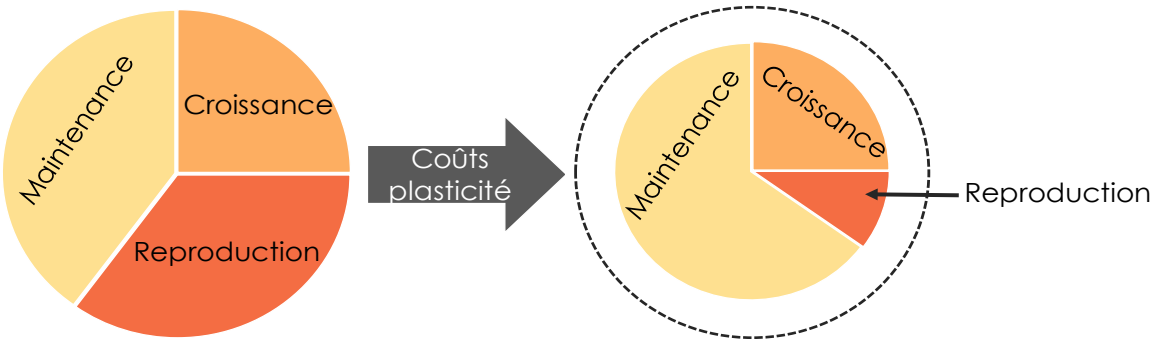


Figure 3 : Représentation du concept d'allocation d'énergie et des coûts de la plasticité phénotypique. Les pointillés illustrent la réduction du budget énergétique des espèces en raison des coûts de la plasticité

Ces observations suggèrent ainsi que la survie des organismes à l'élévation des températures reposerait sur la capacité de ces derniers à maintenir leur registre aérobie, lorsqu'exposés à des températures élevées, et ce, grâce aux ajustements plastiques (Schulte, 2015). À cet égard, les mitochondries pourraient jouer un rôle fondamental dans la détermination de l'acclimatation thermique considérant leur implication fondamentale dans la production d'énergie (Schulte, 2015; Shama *et al.*, 2016). Que ce soit en ajustant l'efficacité ou la densité des mitochondries, la fluidité des membranes ou encore en modifiant les voies métaboliques utilisées, ces réponses plastiques peuvent influencer la quantité d'énergie produite par rapport à la demande en oxygène, influençant de ce fait le registre aérobie des espèces et la température critique maximale qu'une espèce peut supporter (Strobel *et* al., 2013; Chung et Schulte, 2015).

En basant nos connaissances sur l'hypothèse de l'OCLLT et sur le principe de compromis énergétique, l'une des avenues intégratives permettant de comprendre les mécanismes impliqués dans l'acclimatation thermique des organismes impliquerait de 1) s'intéresser aux paramètres influençant l'intégrité ou le fonctionnement des mitochondries, 2) vérifier s'il y a une transition du métabolisme aérobie vers le métabolisme anaérobie, et 3) mesurer les traits d'histoire de vie des organismes afin d'identifier un potentiel compromis énergétique entre la maintenance, la croissance et la reproduction et donc les

conséquences au niveau organismique. Ce sont ces priorités qui ont été retenues en tant qu'objectifs de cette maîtrise.

Objectifs et hypothèses

L'utilisation d'une approche intégrative qui considère l'effet de la température sur différents compartiments biologiques nécessite l'utilisation de nombreux outils qui permettent de mesurer les divers traits d'intérêt génétiques et physiologiques. Ces outils sont généralement disponibles pour des organismes modèles comme *Drosophila melanogaster* (Calosi *et al.*, 2016). Or, dans la majorité des embranchements marins, de tels organismes modèles sont inexistants (Munday *et al.*, 2013; Calosi *et al.*, 2016). L'utilisation d'une approche intégrative pour les organismes marins impose donc préalablement l'identification d'un organisme marin modèle (Krogh, 1929). L'annélide marin *Ophryotrocha labronica* La Greca and Bacci 1962 (Annelida: Dorvilleidae) semble, à cet égard, un bon candidat comme organisme modèle émergent dans les études portant sur la plasticité phénotypique et l'adaptation rapide (Massamba-N'Siala *et al.*, 2014; Rodriguez-Romero *et al.*, 2015; Chakravarti *et al.*, 2016; Gibbin *et al.*, 2017). Il s'élève facilement en laboratoire et possède un temps de génération court, soit de 17 jours environ à 27 °C (Åkesson, 1976; Shain, 2009). Par ailleurs, cette espèce ayant déjà été choisie comme organisme modèle dans les études portant sur les réponses trans et multigénérationnelles face aux changements globaux (Chakravarti *et al.*, 2016; Gibbin *et al.*, 2017), les techniques de mesure liées à sa physiologie et aux traits d'histoire de vie sont déjà développées.

C'est dans ce contexte et cette perspective intégrative, que s'inscrit mon projet de maîtrise, qui s'intéresse aux mécanismes sous-jacents à la plasticité phénotypique dans le contexte du réchauffement climatique chez les ectothermes marins. **Ma maîtrise a pour objectif général d'évaluer l'effet de la température sur les différents compartiments biologiques des organismes à travers l'ontogénie de l'annélide marin *Ophryotrocha***

***labronica*.** En exposant expérimentalement les individus à une température contrôle et une température élevée, mon projet de maîtrise vise à caractériser, à travers l'ontogénie, l'impact de l'exposition des vers à une température élevée sur (1) l'expression des gènes mitochondriaux, (2) l'activité enzymatique mitochondriale, (3) le métabolisme énergétique et (4) les conséquences de ces changements cellulaires et physiologiques sur les traits d'histoire de vie.

En tenant compte des connaissances antérieures relatives à l'impact de la température et du fait que la température élevée utilisée dans cette expérience est à la limite de la plage thermique optimale d'*O. labronica* (Massamba-N'Siala *et al.*, 2012), il est attendu que les organismes augmentent l'efficacité mitochondriale, que ce soit en régulant positivement l'expression des gènes mitochondriaux ou en augmentant l'activité des enzymes mitochondriales, ou modifient les voies métaboliques afin d'augmenter la production d'énergie. Il est également attendu que la température élevée influence négativement la taille et la fécondité des organismes. Finalement, considérant la durée de l'exposition, il est attendu que les organismes maintiennent leur registre aérobie, mais l'augmentation de la demande en énergie durant la maturation sexuelle génèrera un compromis énergétique entre la maintenance cellulaire et les traits d'histoire de vie.

CHAPITRE 1
DANS QUEL COMPARTIMENT BIOLOGIQUE L'IMPACT DE L'EXPOSITION AUX TEMPÉRATURES ÉLEVÉES EST-IL LE PLUS VISIBLE DURANT L'ONTOGÉNIE ?

RÉSUMÉ EN FRANÇAIS DU PREMIER ARTICLE

L'utilisation d'une approche intégrative s'avère particulièrement pertinente pour étudier les mécanismes qui sous-tendent les réponses plastiques des organismes face aux changements de température; cette approche met en évidence la façon dont les compartiments biologiques interagissent et sont finement ajustés de manière à créer une réponse intégrée. L'utilisation d'une telle approche est particulièrement pertinente dans le contexte du réchauffement climatique, puisque les réponses plastiques au niveau des mitochondries et du métabolisme aérobie, pourraient permettre aux espèces de faire face aux effets directs de l'élévation rapide des températures. Cela nécessite néanmoins le développement et l'utilisation d'outils moléculaires et physiologiques qui sont généralement inexistants pour les ectothermes marins, à l'exception de certaines espèces d'intérêt commercial et d'aquaculture, pour lesquelles les outils moléculaires ont été développés récemment. Dans ce contexte, cette étude avait pour objectif de développer et d'utiliser une approche intégrative sur l'annélide marin *Ophryotrocha labronica* La Greca et Bacci 1962 afin de (1) comprendre l'effet de la température élevée sur les réponses mitochondriales et métaboliques et (2) identifier les conséquences sur la croissance, la survie et la reproduction des organismes, et ce, à travers l'ontogénie des individus. Pour ce faire, les individus ont été exposés expérimentalement, dès leur naissance, à une température contrôle (24 °C, température moyenne enregistrée au pic de densité de la population naturelle) et une température élevée (30 °C, augmentation de 2-3 °C par rapport à la température maximale mesurée dans leur habitat). Chaque jour, du sixième jour

d'exposition jusqu'à leur première reproduction, un groupe d'individus était prélevé et congelé afin de mesurer l'effet de la température sur les traits biologiques suivants : expression des gènes mitochondriaux, traits physiologiques mitochondriaux et profils métabolomiques. Les traits d'histoire de vie, soit la taille, la survie ainsi que la fertilité et la fécondité, étaient préalablement mesurés. Nos résultats ont démontré que l'exposition des organismes à la température élevée (30 °C) affecte principalement le profil métabolomique d'*O. labronica* en modifiant les voies et taux métaboliques, mais n'affecte pas fortement sa croissance, sa survie et sa capacité de se reproduire. Ces résultats confirment qu'*O. labronica* est en mesure de tolérer la température élevée utilisée dans cette étude. Néanmoins, sachant que les effets néfastes de l'exposition aux températures élevées peuvent s'accumuler dans le temps et que les réponses plastiques peuvent changer avec les générations selon l'environnement expérimenté par les générations précédentes, il serait intéressant d'inclure cette approche intégrative dans les expériences multigénérationnelles. Ces études pourraient contribuer à améliorer notre compréhension des mécanismes impliqués dans la réponse des organismes au réchauffement climatique.

Mots-clés : Approche intégrative, Réchauffement climatique, Plasticité phénotypique, Expression génétique, Métabolisme

Cet article, intitulé « *In which biological compartments is the impact of exposure to elevated temperature better observed during ontogeny*? » est destiné à être soumis pour publication à la revue Journal of Experimental Biology d'ici l'été 2019. Les coauteurs Gloria Massamba-N'Siala, Philippe Archambault et Piero Calosi ont contribué à l'élaboration de l'étude et à la révision du manuscrit, avec le soutien et les précieux conseils de France Dufresne, Pierre De Wit et Astrid Tempestini pour le développement des analyses génétiques, celui de Pierre Blier pour les analyses enzymatiques mitochondriales et Diana Madeira pour les analyses métabolomiques. Les coauteurs Diana Madeira et Astrid Tempestini ont également contribué à la révision du manuscrit.

IN WHICH BIOLOGICAL COMPARTMENTS IS THE IMPACT OF EXPOSURE TO ELEVATED TEMPERATURE BETTER OBSERVED DURING ONTOGENY?

Vermandele Fanny[1]*, Gloria Massamba-N'Siala[1], Madeira Diana[2], Tempestini Astrid[1], Dufresne France[1], De Wit Pierre[3], Blier Pierre[1], Archambault Philippe[4], Calosi Piero[1a]

[1]Département de Biologie, Chimie et Géographie, Université du Québec à Rimouski, Rimouski, QC, G5L 3A1, Canada

[2]Departamento de Biologia & CESAM & ECOMARE, University of Aveiro, Aveiro, Portugal

[3]Department of Marine Sciences, University of Gothenburg, Strömstad, Sweden

[4]Québec Océan, Takuvik, Département de Biologie, Université Laval, Québec, QC, G1V 0A6, Canada

*Corresponding author: fanny.vermandele@uqar.ca

ABSTRACT

The use of an integrative approach is useful to study the mechanisms underpinning organisms' plastic responses to temperature changes; it highlights how different biological compartments interact to create a unique integrated response. This is especially relevant in the context of global warming, as plastic changes at the mitochondrial and metabolic levels, could enable species to face the direct impact of a rapid temperature increase. However, this approach requires molecular and physiological tools, lacking for marine ectotherms. Our study applied an integrative approach on the marine annelid *Ophryotrocha labronica* La Greca and Bacci 1962 to understand the mitochondrial and metabolic responses to elevated temperature throughout its ontogeny, and the subsequent consequence on organisms' fitness. Hatchlings were exposed to a control (24 °C, average temperature recorded at highest population density) and an elevated temperature (30 °C, +2-3 °C from the maximal temperature recorded in their habitat) until their first reproductive event. Groups were sampled daily to measure the effect of temperature in the following traits of the different biological compartments: mitochondrial gene expression and physiological traits, metabolomics profiles and life-history traits. The elevated temperature affected organisms' metabolomics profiles, principally the metabolic pathways and the metabolic flux, but did not impact individuals' growth, survival and ability to reproduce. This indicates that *O. labronica* is able to tolerate 30 °C. However, as detrimental effects can accumulate through time and generations, future studies combining an integrative approach with a multigenerational exposure will be necessary to improve our mechanistic understanding of species responses to global warming.

Keywords: Integrative approach, Global Warming, Phenotypic plasticity, Gene expression, Mitochondrial physiology, Metabolism

1. INTRODUCTION

> "Because of its focus on organisms, natural history is in a unique position to supply questions and integrating links among disciplines. Studies at lower levels will delineate the machinery of structural units, and the complex systems into which these units have been assembled through evolutionary time will be worked out by research at the intermediate and higher level of biological integration. Biology is indivisible; biologists should be undivided"
>
> Bartholomew (1986, p.329)

The use of an integrative approach within an evolutionary framework has regained interest as a way to tackle organism's complexity and responses to his environment (Bartholomew, 1986; Pigliucci, 2003; Pörtner *et al.*, 2006a; Wake, 2008; Bozinovic and Naya, 2015). However, its use in research remains largely overlooked (Strange, 2005; Bozinovic and Naya, 2015). This integrative vision opposes to a more reductionist approach that largely prevailed historically. According to the latter approach, the best way to study organisms' biology is to deconstruct their constituent parts, such as genes, cells, tissues, and study them in isolation. Moreover, since the advent of molecular and genetic techniques, biological disciplines such as ecology, physiology, and evolution have diverged and somewhat become independent entities (Dobzhansky, 1964; Bartholomew, 1986; Strange 2005; Bozinovic and Naya, 2015). This fragmentation has led to underestimate the implications of physiological mechanisms in ecological processes and the importance of evolutionary process in organism's response to their environment (Bartholomew, 1986; Pörtner *et al.*, 2006a; Bozinovic *et al.*, 2011; Bozinovic and Naya, 2015).

The limitations of the reductionist approach are particularly clear when we try to unravel how phenotypic plasticity contributes to an organism's fitness and helps to define species diversity and distribution (Pigliucci, 2003; Pörtner *et al.*, 2006a; Forsman, 2015; Martin, 2015; Woods *et al.*, 2015). Indeed, phenotypic plasticity, defined as the ability of one genotype to express different phenotypes according to the environment experienced

(Bradshaw, 1965; Pigliucci, 2001; West-Eberhard, 2003; Whitman and Agrawal, 2009; Boyd *et al.*, 2016), involves modifications across multiple compartments and multiple traits in a given organism: including morphological, physiological and life-history traits (Schlichting and Pigliucci, 1998; Schulte *et al*., 2011; Woods *et al.*, 2015). Together, these modifications act as a complex, but integrated unit that responds to changes in the environment to sustain and maximize an organism's fitness (Schlichting, 1989; Woods *et al.*, 2015). The fact that these traits interact with each other, and that their interaction can change across an organism's ontogeny, demonstrates the importance to study plastic responses using a 'whole organism' perspective, rather than focusing on single trait plastic responses (Schlichting, 1989; Pigliucci, 2003; Pigliucci and Preston, 2004; Whitman and Agrawal, 2009; Forsman, 2015). In addition, the 'whole organism' perspective is required to truly identify the costs and limits associated with plastic responses. Indeed, phenotypic plasticity is known to be energetically demanding due to the cost of maintaining cellular homeostasis and regulatory mechanisms (Hoffman, 1995; DeWitt *et al.*, 1998; Angilletta, 2009; Godbold and Calosi, 2013; Martin, 2015; Woods *et al.*, 2015; Jarrold *et al.*, 2019). Consequently, these costs can alter individuals' energy budget, leading to a reallocation of the energy available to specific traits at the expense of others, which can affect organisms' fitness (Stearns, 1989; Roff and Gélinas, 2002; Chevin *et al.*, 2013; Thor and Dupont, 2015; Martin, 2015). Finally, the growing evidences that phenotypic plasticity might facilitate rapid adaptation under certain conditions (Visser, 2008; Pfennig *et al.*, 2010; Chevin *et al.*, 2013; Ghalambor *et al.*, 2015), indicates the need to include an evolutionary perspective view investigating how organisms respond to environmental heterogeneity (Bozinovic *et al.*, 2011; Diamond, 2018). Thereby, the successful identification of the mechanisms, limits, as well as ecological and evolutionary implications of phenotypic plasticity relies on the use of a more integrative approach (Pigliucci, 2003; Forsman, 2015, Bozinovic and Naya, 2015).

The development of an integrated approach is of paramount importance for the investigation of the likely impacts of global change on biological systems (Bozinovic and Naya, 2015). Changes in environmental parameters generated by increasing human

activities (Parmesan and Yohe, 2003; Calosi *et al.*, 2019) represent a challenge without precedents for species in modern times (Bellard *et al.*, 2012). Ultimately, global changes will affect species survival and biodiversity levels locally and globally, as well as ecosystems' functioning (Angilletta, 2009; Somero, 2010; Barnosky *et al.*, 2011). With already evidences of shift in species distribution (Parmesan and Yohe, 2003; Perry *et al.*, 2005; Diamond, 2018), and increasing rate of extinctions (Ceballos *et al.*, 2015; Ceballos and Ehrlich, 2018), numerous studies are investigating the mechanisms underpinning species responses to this changing environment (Somero, 2012; Munday *et al.*, 2013; Reusch, 2014). The increase of 2 to 4 °C in temperature expected by the end of the century (IPCC, 2014) will likely deeply affect marine ecosystems (Munday *et al.*, 2013; Reusch, 2014). By affecting the rate of biochemical reactions (Hochachka and Somero, 1984; Schulte, 2015), the temperature can affect the whole organism's fitness, by inducing changes from genes, to physiology and behavior, particularly in ectotherms (Pörtner *et al.*, 2006a; Angilletta, 2009; Deutsch *et al.*, 2008). Considering the rate at which temperature increase is occurring, phenotypic plasticity, as a way for species to thermally adjust to such change, seems to be the only valuable mechanism to enable species to face the directs impacts of global warming (Peck *et al*., 2010; Calosi *et al.*, 2016), while potentially facilitating the implementation of adaptive processes (Ghalambor *et al.*, 2007; Chevin *et al.*, 2010; Sunday *et al*., 2014; Ghalambor *et al.* 2015; Gibbin *et al.*, 2017a). Within this context, the use of an integrative approach to understand the mechanisms underpinning plastic responses to elevated temperature is crucial if we want to improve and refine our predictions of the biological impacts of global warming (Schulte *et al.*, 2011; Calosi *et al.* 2016).

In this regard, developing an integrative approach with a specific focus on mitochondria and metabolism plastic responses is of primary interest, considering that these plastic adjustments can influence organisms' aerobic scope (Pörtner *et al.*, 2006a): i.e. the differences between the maximum metabolic rate (MMR) and the standard metabolic rate (SMR) (Pörtner, 2001; Claireaux and Lefrançois, 2007; Schulte, 2015). Indeed, according to the oxygen and capacity limited thermal tolerance hypothesis (OCLTT), organisms'

upper thermal limit is set by the reduction in organisms' aerobic scope due to a mismatch between oxygen demand and oxygen supply. This mismatch impairs organisms' performance and usually lead to a transition to anaerobic metabolism (Pörtner, 2001, 2002; Pörtner and Knust, 2007; Pörtner and Farrell, 2008). By affecting organisms' bioenergetics, plastic adjustments in mitochondria, the powerhouse of the cell, are likely to play a key role in organisms' acclimation and adaptation to elevated temperature (Shama *et al.*, 2016). Whether it is by adjusting mitochondrial efficiency or density, or by changing specific metabolic pathways used, these plastic responses could fundamentally change the ratio between oxygen demand and energy production, leading to an increase in organisms' aerobic scope and upper thermal limit (Strobel *et al.*, 2013; Chung and Schulte, 2015). Thus, the use of an integrative approach, looking, throughout an organism' ontogeny, at the mitochondrial and metabolism adjustments in different biological compartments, and the consequences on organisms' fitness, could help shed the light on the mechanisms underpinning plastic responses to elevated temperature (Pörtner *et al.*, 2006a). However, this approach usually requires the use of molecular and physiological tools, which are crucially lacking for the vast majority of marine ectotherms, as model organisms are limited in the marine environment (Munday *et al.*, 2013; Calosi *et al.*, 2016). Molecular tools for marine ectotherms have been only developed very recently and are mainly limited to species of aquaculture and commercial interest.

Within this context, **the objectives of this study are to understand the mitochondrial and metabolic responses, and the subsequent impact on organisms' fitness, in marine ectotherms exposed throughout their ontogeny to elevated temperature.** In order to achieve our aim, we conducted a laboratory control experiment exposing the marine annelid *Ophryotrocha labronica* La Greca and Bacci 1962 (Annelida: Dorvilleidae) to an elevated temperature to characterize, across its ontogeny, the following responses: 1) mitochondrial gene expression, 2) mitochondrial physiological traits and 3) energy metabolism in order to 4) provide a mechanistic understanding of any potential consequences observed in fitness related life-history traits. *O. labronica* is an emerging model in comparative biology (Halanych and Borda, 2009) and eco-evolutionary studies in

the context of global change (Rodriguez-Romero *et al.*, 2015; Calosi *et al.*, 2016; Chakravarti *et al.*, 2016; Gibbin *et al.*, 2017b). With life-history traits and physiological measurements methods already developed (Massamba-N'Siala *et al.*, 2011, 2012; Chakravarti *et al.*, 2016; Gibbin *et al.*, 2017b; Jarrold *et al.*, 2019), this species represents an ideal ectotherm model for integrative studies in marine species as only genetic and some physiological tools needed to be developed.

As the elevated temperature treatment used is at the limit of the thermal range supported by this species (Massamba-N'Siala *et al.*, 2012), it is expected that organisms exposed to the elevated temperature treatment increase mitochondria efficiency, either by up-regulating gene expression or increasing CS and ETS activities, and/or by changing metabolic pathways in order to sustain the higher energy demand associated with elevated temperature. In the reproductive phase of their ontogeny, however, as energy demand is particularly high due to the cost of reproduction and the accumulation of temperature deleterious effect, a drop in aerobic scope leading to the transition to anaerobic metabolism could be expected, impairing life-history traits. By developing an integrative 'cell to whole organism' approach measuring the temperature responses in different biological compartments, throughout ontogeny, and the impact on organisms' fitness, this study could improve our understanding of the mechanisms and costs associated to organisms 'plastic responses to elevated temperature.

2. METHODS

2.1 Study species

Characterized by a small size (3-5 mm in length), *Ophryotrocha labronica* is a gonochoric iteroparous species with direct development, colonising the interstices created by subtidal benthic species of nutrient-rich shallow water environments (Paxton and Åkesson, 2007, 2010; Simonini *et al.*, 2009, 2010; Massamba-N'Siala *et al.*, 2011; Thornhill *et al.*, 2009). Females reproduce semi-continuously, laying tubular egg masses that are externally fertilized by the male. Parental cares are provided until the hatch of the eggs to prevent the accumulation of parasite and ensure a good oxygenation of the egg mass, particularly from the mother, although the father can participate to the parental effort (Paxton and Åkesson, 2007).

The initial population used for this study (ca. 100 worms) was sampled in the harbor of La Spezia (Italy; 44°06'29"N; 9°49'44"E) in October 2015 following the sampling method presented in Massamba-N'Siala *et al.* (2011). The specimens were transferred at the Marine Eco-Evolutionary Physiology (MEEP) laboratory at the Université du Québec à Rimouski (Québec, Canada). They were reared for more than 12 generations at room temperature in glass bowls (ø = 9 cm, depth = 3.7 cm) filled with artificial sea water kept at salinity 35 ± 2 (mean ± SD) and pH 8.0 ± 0.2. During this rearing period, the worm culture experienced minimum and maximum temperatures of 20 and 24 °C respectively, which are within the range of optimal performance of the species (Massamba-N'Siala *et al.*, 2012). Sea water was prepared by dissolving artificial salt (Aquarium Sea Salt Mixture, Instant Ocean, Blacksburg, VA, USA) in distilled water. To ensure a good water quality and prevent the accumulation of excreta and fermentation of food, sea water was partially changed once a week (Rodriguez-Romero *et al.*, 2016). Finally, worms were fed *ad libitum* with minced frozen spinach (Åkesson, 1970).

2.2 Experimental design and setup

To characterise the impact of elevated temperature on the different biological compartments along the worms' ontogeny, we exposed different clutches of *O. labronica* individuals to two temperature treatments: 24 °C (control), which is the average temperature recorded in the period of highest population density in their natural habitat (Prevedelli *et al*. 2005), and 30 °C (elevated), which represents the upper end of the optimal performance range of this species (Massamba-N'Siala *et al.* 2012). This elevated temperature corresponds also to an increase of 2-3 °C relative to the maximal temperature (27.6 °C) currently measured in their natural habitat in the Ligurian sea (Massamba-N'Siala *et al.*, 2011) and thus simulates the temperature they will most likely encounter by the end of the century (IPCC, 2014). Clutches were exposed from the hatch to the first reproductive event and different clutches were collected on a daily basis to characterise the following traits in different biological compartments: mitochondrial gene expression, mitochondrial physiological traits, organismal metabolomics/lipidomics (hereafter metabolomics) profiles and life history traits. Given the differential rates of development existing within each clutch and between temperature (Forster and Hirst, 2012), a fixed exposure time that guaranteed most individuals to reach the first reproductive event was used, specifically 20 and 30 d to the elevated and control temperature respectively (Prevedelli and Simonini, 2001; Massamba-N'Siala *et al*., 2011).

To achieve our aims, 135 pairs of worms (F0) were first created using males and females from our laboratory cultures (Fig 1). Each pair was isolated in a well of a six-wells culture plate (Corning Ltd, Sunderland, UK) kept at the same conditions of origin, and followed daily to record every spawning and hatching event. Hatchlings (F1) of F0 pairs were transferred in the experimental system every time that at least three clutches, with the same hatching date, were available. This was necessary to obtain a sufficient starting number of hatchlings. In more details, groups of approx. 36-45 individuals from a given clutches were randomly assigned to a given plate composed of three wells (10-15 indiv. *per* well). In order to reach the set number of 50 indiv. for each of the three wells (to control for

density), a total of 3-5 clutches were used, with approximatively the same number of hatchlings from each clutch, to control for genetic diversity (Fig 1). In total, up to 4-6 plates were prepared on any given day: half for each temperature treatment (Fig 1).

These three wells of every culture plate were then assigned to an aquarium (see the description of the experimental system) and given collection day (6-30 for 24 °C; 6-20 for 30 °C) using a random number generator, and corresponded to a replicate. Even though clutches were exposed to the two temperature treatments since the hatch, no replicates were collected before the sixth day of exposure considering that younger hatchlings were too small to be detected in any analyses of any biological compartment. Transfers continued until three replicates *per* temperature treatment for each day of exposure were obtained. After each transfer, the plates assigned for the elevated temperature treatment were gradually exposed to 30 °C by increasing temperature by 1 °C h^{-1} from control temperature, using an environmental chamber (MLR-351H, Sanyo Electric Co., Osaka, Japan), before they were placed in the experimental system. As for the plates assigned to control temperature, they were placed directly in the experimental system.

Once the date of collection was reached for a replicate, a number of life-history traits (size, survival, fertility, and fecundity) were measured within each of the three wells (see section 2.3). Then, for each replicate, individuals from the three wells were pooled in a 1.5 mL microcentrifuge tube to reach the targeted number of individuals needed for each biological compartment investigated: 60 indiv. for mitochondrial gene expression, 12 indiv. for mitochondrial physiological traits (CS/ETS) and 9 indiv. for metabolomics profile. Samples were stored at -80 °C until analyses were carried out. This experimental design enabled us to have for each temperature treatment and for every date of collection, three samples for each of the biological compartment (Fig 2).

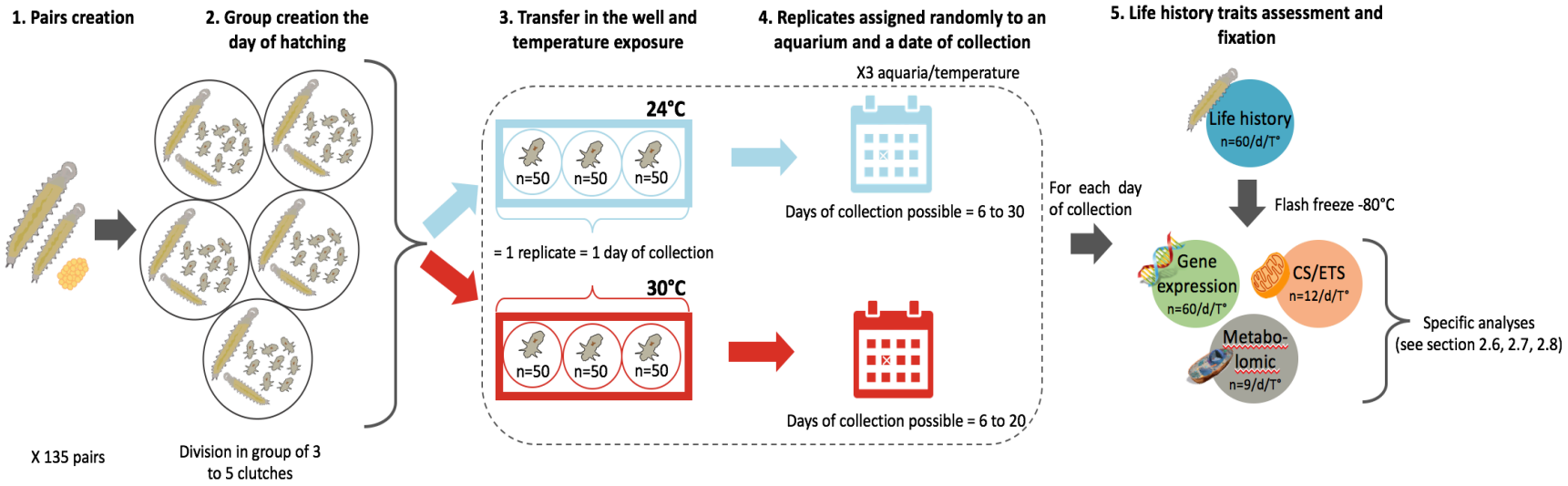


Fig 1. Schematic representation of the experimental design and workflow. The figure represents the workflow completed for every transfer realized in the experiment where F1 hatchlings of the Dorvellid worm *Ophryotrocha labronica* coming from three to five clutches were transferred in the wells of two culture plates, one for each temperature treatments (step 1, 2 and 3). Culture plates were then collected after a certain time for the biological compartment analyses (step 4 and 5). Culture plates in blue and red represent the treatment at 24 and 30 °C respectively.

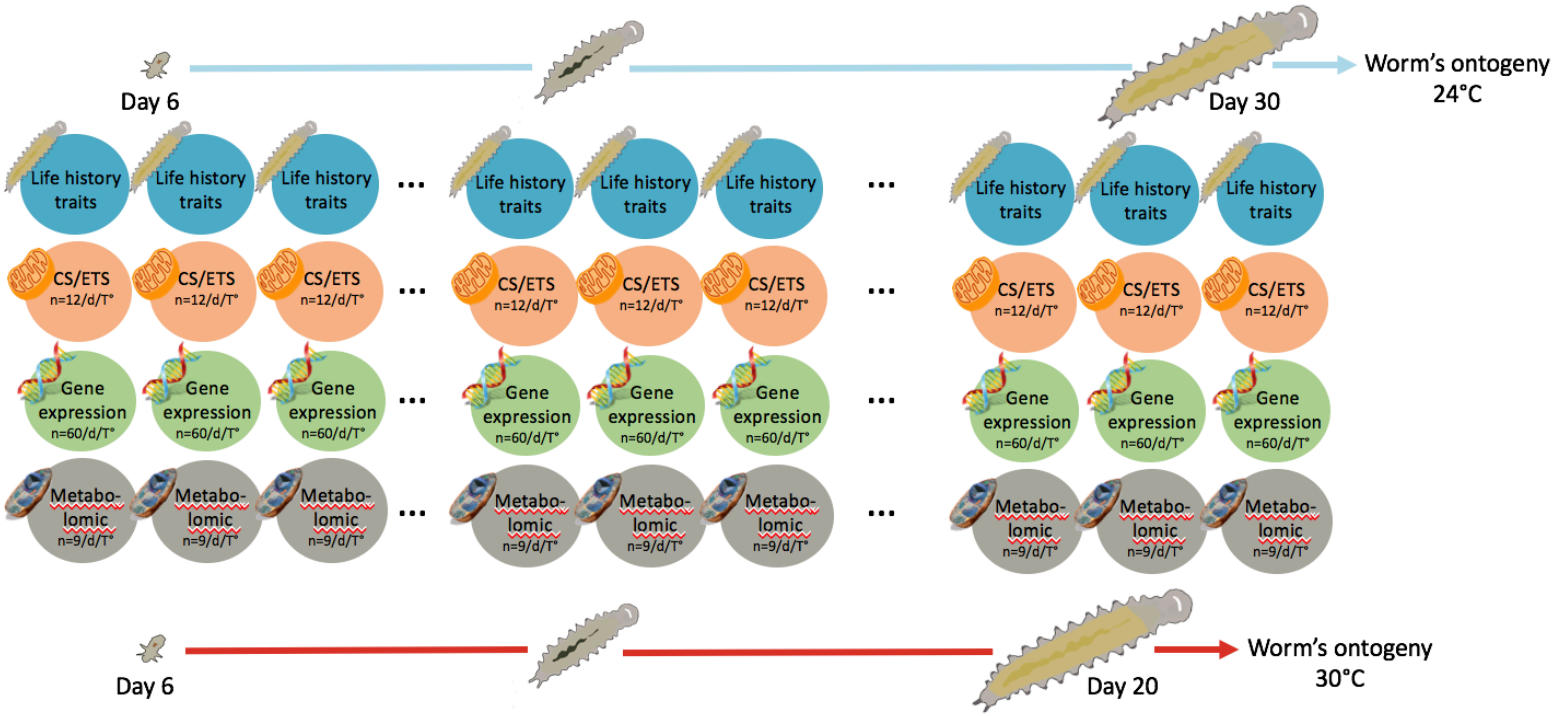


Fig 2. Representation of the samples available for each biological compartment analyses along the worms' ontogeny.

The temperature treatments were applied on the culture plates using a "*bain-marie* experimental system", composed of six deep trays (66 x 47 x 25 cm, vol. 57 L), three *per* temperature treatment. Each tray was half filled with deionized water and heated to either 24 or 30 °C with aquarium heaters (50 W, Eheim Jager, Deizisau, Stuttgar, Germany and 100 W, Hydor, Sacramento, CA, USA respectively), and helped maintaining temperature conditions stable. An aquarium circulation pump (Koralia Nano Evolution 900 Circulation Pump, Hydor) was added in each tray to avoid the formation of thermal gradients (Fig 3), while a Perspex sheet lid was used to minimize thermal fluctuation associated with water evaporation.

An airtight transparent aquarium (37.4 x 24.1 x 14 cm, vol. 9 L) was placed inside each tray (i.e. a total of three aquaria *per* temperature) and used to hold the six-well culture plates (Corning Ltd) hosting the worms (Fig 3). Each aquarium contained one replicate of each day of exposure, for a total of three replicates *per* day of exposure for the 24 and 30 °C treatment. Finally, each aquarium was connected to an air pump (Laguna 4200 LPH, Mansfield, MA, USA) through a Nalgene tube, assuring the constant oxygenation of the sea water in the plates (Table 1). The aquaria were moved between the trays of the same temperature treatment every four days to control for the potential variation in temperature between the trays.

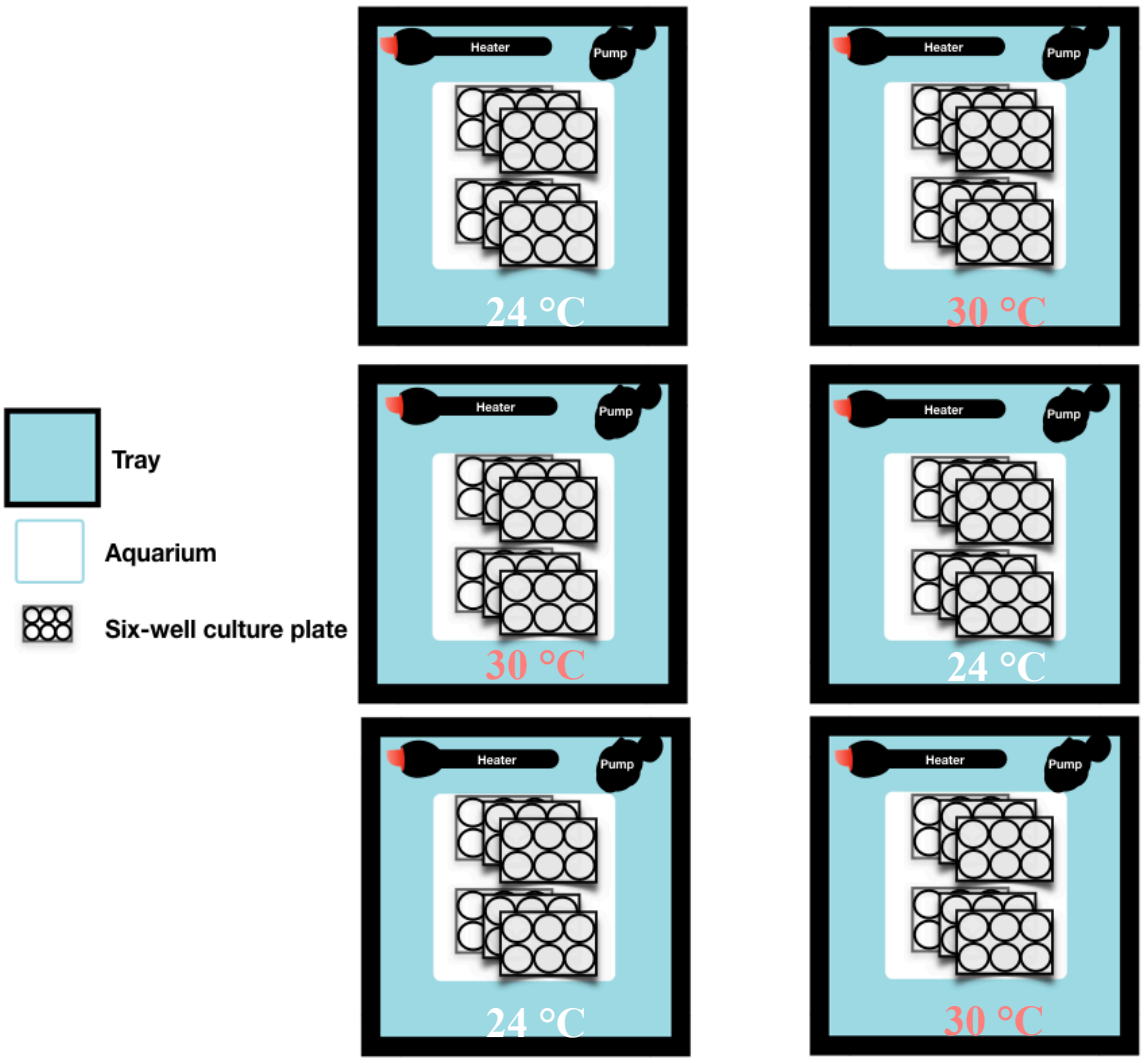


Fig 3. Schematisation of the experimental system and design used. The number of six-well culture plates represented in this figure is used as an example, as the number of plate simultaneously present *per* aquarium varied.

Each culture plate was also covered with a breathable film (Aeraseal, Alpha Laboratories Ltd, Eastleigh, UK) to reduced water evaporation whilst permitting gas exchanges. In addition, the sea water inside each plate was changed every day with new artificial sea water to keep salinity and pH values stable (Table 1). Worms were fed *ad libitum* with minced frozen spinach throughout the experiment. Temperatures and salinity were measured daily in multiples plates of each aquarium. The temperature was measured with a dual input K/J digital thermometer (HH802U, OMEGA, Laval, QC,

Canada, ± 0.1 °C) and salinity with a portable refractometer (DD H2Ocean, MOPS aquarium supplies, Hamilton, ON, Canada ± 0.1). pH of the water was measured every two days with a benchtop pH meter (FE20 FiveEasy^{TM}, Mettler Toledo, Columbus, OH, USA, ± 0.01) and oxygen concentration was checked at least once a week with a portable meter (SevenGo Duo pro^{TM}, Mettler Toledo, Columbus, OH, USA).

Table 1. Physicochemical parameters of the experimental system (mean ± SD) measured in the two treatments.

Treatment	Temperature (°C)	Salinity	pH_{NBS}	Oxygen concentration (%)
Control	24.08 ± 0.40 n = 311	32.75 ± 0.96 n = 65	8.04 ± 0.11 n = 39	96.40 ± 1.77 n = 9
Elevated	29.78 ± 0.82 n = 227	33.92 ± 1.30 n = 107	8.04 ± 0.08 n = 23	99.48 ± 1.84 n = 7

2.3 Determination of life history traits

Body size was determined by counting the number of chætigers of three randomly selected individuals *per* well, for a total of 9 indiv. within each replicate, as a replicate consisted of three wells of every culture plate. The developmental stage, *i.e.* juveniles, adult male or female, of 20 indiv. chosen randomly from each well was also noted to quantified the juvenile : adults ratio. Additionally, the number of individuals remaining in the wells after fixation was counted to calculate the survival rate (%) *per* well with the following equation:

$$Survival = \frac{Number\ of\ individuals\ fixed + number\ of\ individuals\ remaining\ in\ the\ well}{number\ of\ hatchlings\ initially\ transferred\ in\ the\ well} * 100 \quad (1)$$

Finally, fertility and fecundity were measured as the total number of egg masses spawned in each well divided by the number of females in the well, and the number of eggs *per* egg mass, respectively.

2.4 Mitochondrial genome assembly and annotation

In order to obtain the mitochondrial genome of *O. labronica*, next generation sequencing (NGS) was performed with a *de novo* assembly, as no reference mitochondrial genome was available. In more details, a total of 77 indiv. of *O. labronica*

were collected from the cultures and DNA was extracted with E.Z.N.A Tissue DNA kit (Omega Bio-tek, Norcross, GA, USA) following the company protocol. DNA purity and quantity was assessed through spectrophotometry using a NanoVue Plus spectrophotometer (GE Healthcare Life Sciences, Chicago, IL, USA). Then 500 ng of the DNA was sent to the Plateforme d'Analyses Génomiques of the Institut de Biologie Intégrative et des Systèmes (IBIS, Laval University, QC, Canada) where it was fragmented using a covaris M220 (Covaris, Woburn, MA, USA) for 40 s with default setting. The library synthesis was performed with the NEB Next Ultra II (New England Biolabs, Ipswich, MA, USA) on the fragmented DNA previously transferred in PCR tubes, following the company protocol. HT adapters (Illumina, San Diego, CA, USA) were used to barcode the samples. All libraries were quantified and pooled using an equimolar ratio and subjected to an Illumina MiSeq 300 base pair paired-end run (600 cycle, v3 kit).

Raw Illumina reads were trimmed to remove low quality base (Phred score threshold of 20) as well as Illumina adapters using the Trim Galore! tool (v0.4.2, Krueger, 2015) and the quality of the reads was assessed with FastQC (Andrew, 2010). Reads were then assembled using SOAP *de novo* short read genome assembler with default parameters and a custom script to filter the data. (Luo *et al.*, 2012). After the first assembly, all contigs were compared against a database containing the mitochondrial genome of two phylogenetically closest species for which the complete genome was available: *Marphysa sanguinea* (KF733802.1) and *Eurythoe complanata* (KT726962.1) using tBLASTn and Blastx tools from the BLAST+ package available from NCBI (https://www.ncbi.nlm.nih.gov/books/NBK52640/). Contigs from *O. labronica* genome with high similarity (E-value below 1E-4) to this mitochondrial database were annotated with MITOS Web Server, a pipeline specially designed for annotation of mitgenomic sequences (Bernt *et al.*, 2013, http://mitos.bioinf.uni-leipzig.de/index.py). Once annotated, only sequences of the protein-coding genes were kept to design species-specific primers.

2.5 Mitochondrial gene expression measurement

The RT-qPCR analyses were realized on three mitochondrial protein-coding genes: ATP synthase protein 8 (*ATP8*), NADH dehydrogenase subunit 1 (*NAD1*) and cytochrome b (*COB*). Beta-actin (*ACTB*) and the Elongation factor 1 (*ELF1A*) were chosen as housekeeping genes as they are known to have a constant expression regardless of the temperature and the life cycle in many species (Purohit *et al.*, 2016; Wan *et al.*, 2017). However, *ELF1A* was not working and was discarded from the analyses. All primers were designed on the Primer3 software using default parameters (Koressaar and Remm, 2007; Untergasser *et al.*, 2012) and the sequences of the primers are available in Table 2. For the housekeeping gene, we designed the primer from the sequence of *Hydroides elegans* putative cytoplasmic actin mRNA (accession number: FJ823668) available on GeneBank, as it was the closest species to *O. labronica* with a known *ACTB* sequence available.

Table 2. Primer sequences of ATP synthase protein 8 [*ATP8*], NADH dehydrogenase subunit 1 [*NAD1*], cytochrome b [*COB*], designed from the full mitochondrial sequences of *O. labronica*, and of the housekeeping gene Beta-actin [*ACTB*] for the RT-qPCR analyses.

Gene	Primer sequence (5'-3')
ATP8	5'-TGCCACATTTATCTCCTATAACGTG-3'
NAD1	5'-GGGTTTTACTCACTCCGCTATTAAT-3'
COB	5'-TGGAGTAGAGCTAGTAAATTGGGTT-3'
ACTB	5'-ACTGGGACGATATGGAGAAGAT-3'

Total RNA was first extracted from frozen sample assigned for the mitochondrial gene expression (60 indiv. *per* sample) with TRIzol (MilliporeSigma, St. Louis, MO, USA), following to the manufacturer protocol, with a potter pestle to homogenize the sample. RNA was then purified on column using the kit AxyPrep Multisource Total RNA Miniprep (Corning, Corning, NY, USA), following the manufacturer instruction. All centrifugations were conducted at 4 °C. Then the quality (A260/A280) and quantity of RNA were assessed using NanoVue Plus spectrophotometer (GE Healthcare Life Sciences, Chicago, IL, USA). RNA was then converted in cDNA using the High

Capactiy cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA, USA) according to the manufacturer instruction. Each RNA sample was diluted to reach 1 µg for a 20 µL reaction prior to the conversion.

The relative quantification of gene expression of the three targeted and the housekeeping genes was realized on a Light Cycler ® 480 II (Roche, Basel, Switzerland) using a 96 well microplate (LigthCycler®480 Multiwell Plate 96 white, Roche) covered with a sealing foil (LightCycler® 480 Sealing Foil, Roche). The reaction mix was composed of 7.5 µL of 2X SensiFast SYBR® No-ROX Mix (Bioline, Cincinnati, OH, USA), 0.8 µL of each 10 µM primer and 1 µL of cDNA. Nuclease Free Water (Wisent Inc., St Bruno, QC, Canada) was added to the mix to reach a final volume of 15 µL. qPCR was performed with a first denaturation cycle at 95 °C for 2 min and followed by 45 cycles at 95 °C for 5 s, 60 °C for 10 s and 72 °C for 10 s. Each measurement was performed in duplicate for the samples, and in triplicate for the calibration curve. A no template control (NTC) was added for every gene tested in each plate. Finally, cDNA from a pool of 240 worms was added in every plate and served as an internal calibrator between each reaction. The relative expression normalized ratio of the three mitochondrial genes was calculated following the 2-ΔΔCT (Livak and Schmittgen, 2001) using the LightCycle® 480 Software from Roche Life Science.

2.6 Determination of mitochondrial physiological traits

Mitochondrial physiological traits were characterized through the enzymatic activities' measurement of the mitochondrial citrate synthase (CS) and the mitochondrial electron transport system (ETS), which are proxies for mitochondrial density (Rabøl *et al.*, 2006) and mitochondrial capacity (Schmidlin *et al.*, 2015), respectively. Enzymatic measurements were realized using an adapted protocol from Gibbin *et al.* (2017b). Each sample (N = 12 indiv./sample) was first homogenized in a 100 mM phosphate buffer and were then separated in two for CS and ETS measurement respectively. For CS, each sample was mixed with 0.1 mM of 5.5' – dithiobis (2-nitrobenzoic acid) (DTNB), 0.113 mM of acetyl-CoA and 0.15 mM of oxaloacetic acid, while ETS samples were mixed in 0.85mM *ß*-Nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH), 2 mM Iodonitrotetrazolium chloride (INT) and 0.03% Triton™ X-100 (Sigma-Aldrich,

Mississauga, ON, Canada) at pH 8.0. In both cases, enzymatic activity was assessed in triplicate (N = 3) at 27 °C using a UV/VIS microplate spectrophotometer (Perkin Elmer Envision, Foster City, CA, USA). The increase absorbance associated to the reduction of the DNTB (ε_{412} = of 13.6 mL cm^{-1} μmol^{-1}; Thibault *et al.*, 1997) was measured for 10 min at 412 nm for CS activity. For ETS activity, the increase absorbance associated to the reduction of INT (ε_{490} = 15.9 mL cm^{-1} μmol^{-1}; Bergmeyer et al. 1983) was measured at 490 nm for 10 min. In order to normalize the activity by the protein content, protein concentration was assessed using the Bicinchoninic Acid method (BCA) (Smith *et al.*, 1985).

2.7 Determination of the metabolomic profile

In order to characterise the change in metabolites and free fatty acids profiles along the worm's ontogeny for the two temperatures treatments, targeted metabolomics analyses were conducted on specific metabolites associated to the aerobic and anaerobic metabolisms (see Table 1 of Annexe I). The method developed was inspired from Lu *et al.* (2006), originally used for the bacteria *Salmonella enterica,* and was modified to detect metabolites on samples of small size organisms containing high salt content (see *Annexe* I for the full paper describing the method development). Briefly, 250 µL of a cold 8:2 methanol:water-10 mM ammonium carbonate extraction solution was added to each frozen samples containing *O. labronica* individuals. This extraction solution, a novelty of the method developed, enabled to extract metabolites while avoiding the creation of salt abducts when injecting the sample. Samples were homogenized with a potter pestle, centrifuged and the supernatant was then injected in a liquid chromatography system (Accela, Thermo Electron Corporation, San Jose, CA, USA) with a Luna PS C18 guard column (100 mm X 2.1 mm Luna C18; Phenomenex, Torrance, CA, USA) to separate the metabolites. Peaks of targeted metabolites were detected using a high-resolution mass detection (HRMS) performed on an Orbitrap LTQ Discovery mass spectrometer from Thermo system (Thermo Electron Corporation). The absolute quantification of these metabolites (in ng mL^{-1}) was assessed with Xcalibur 2.0 software (Thermo Electron Corporation) using a calibration curve previously created with standards for each targeted metabolite (see Annexe 1 of the '*memoire'* for more details).

2.8 Data calculation and statistical analyses

All analyses were carried on *R software* (version 3.4.2, R Core Team, 2017) with a significant threshold $\alpha = 0.05$, with the exception of metabolomics data which were processed and analyzed on the *Metaboanalyst* software (Chong *et al.*, 2018).

2.8.1 Database preparation for developmental Endpoints and Periods analyses

In order to compare the different biological compartment of *O. labronica* between the two temperatures, but also through the worm's ontogeny, data filtering was first realized on all the dataset. Indeed, as organism's developmental rate is usually shorter at a higher temperature, it creates a gap between the two temperatures: for the same number of days of exposure, individuals exposed at 24 °C were not systematically at the same stage of development than the ones exposed to 30 °C. In this context, comparing the organism's responses to the two temperatures for the same day of exposure would not have allowed us to compare the individuals at a similar developmental stage. As a result, we decided to use an 'Endpoint' and a 'Period' approach that would enable us to compare each biological compartment between the two temperatures on individuals with the same developmental stage.

Endpoints were chosen *a posteriori* using the juvenile : adult ratio, fecundity and fertility life-history traits and were selected for their representation as key developmental stages in the life cycle of *O. labronica* (Fig 4):

- 'Endpoint' 1 (EP #1): First day of the experiment when samples were collected – representing the part of the life cycle where 100 % of the individuals are hatchlings/juveniles;
- 'Endpoint' 2 (EP #2): First day with at least one mature individual in the culture plate – representing mainly the juvenile stage;
- 'Endpoint' 3 (EP #3): First day when there are at least 15 % of adults in the culture plate – representing the stage in the life cycle where spermatogenesis and oocytes maturation has started;
- 'Endpoint' 4 (EP #4): First day when there is a least one egg mass spawned in the culture plate – representing the reproductive stage in the life cycle;

- ‘Endpoint’ 5 (EP #5): First day with at least 60 % of adults in the culture plate – representing the part of the life cycle when the majority of the individuals are adults;
- ‘Endpoint’ 6 (EP #6): Specific day when there is the highest number of egg masses spawned *per* female in the culture plate – representing the stage where the majority of females have spawned one egg mass;

As each of these ‘Endpoints’ was referring to one specific collection day for each aquarium (Table 3), a new database containing only these replicates was created for each biological compartment.

Table 3. Day of collection corresponding to each ‘Endpoint’ (EP) for each aquarium.

Treatment (° C)	Aquarium	EP #1	EP #2	EP #3	EP #4	EP #5	EP #6
24	1	6	8	15	19	25	30
24	2	6	10	16	18	24	30
24	3	6	14	17	25	26	29
30	4	6	14	14	18	19	24
30	5	6	12	12	17	16	17
30	6	6	10	15	15	16	18

In the case of mitochondrial gene expression and mitochondrial physiological traits, analyses were realized, for now, only on the samples corresponding to these ‘Endpoints’ due to technical and financial limitation. As for the life-history and metabolomics analyses, samples for all the days of collection (6-20 for 30 °C and 6-30 for 24 °C) were analyzed, and a ‘Period’ approach, separating the data into three periods of the life cycle (Fig 4), was used for the statistical analysis:

- ‘Period’ 1 (P1): Timeframe when all individuals in the culture plate are juveniles;
- ‘Period’ 2 (P2): Timeframe when individuals in the culture plate are becoming sexually mature (spermatogenesis and oocytes maturation), but where no spawning events have been recorded yet;
- ‘Period 3’ (P3): Timeframe when the majority of the individuals in the culture plates are sexually mature and spawning.

This approach enables to increase the number of replicate *per* 'Period' (more than 10 *per* 'Period' instead of three *per* 'Endpoint', for each temperature), while still comparing between the two temperatures individual within the same stage of development.

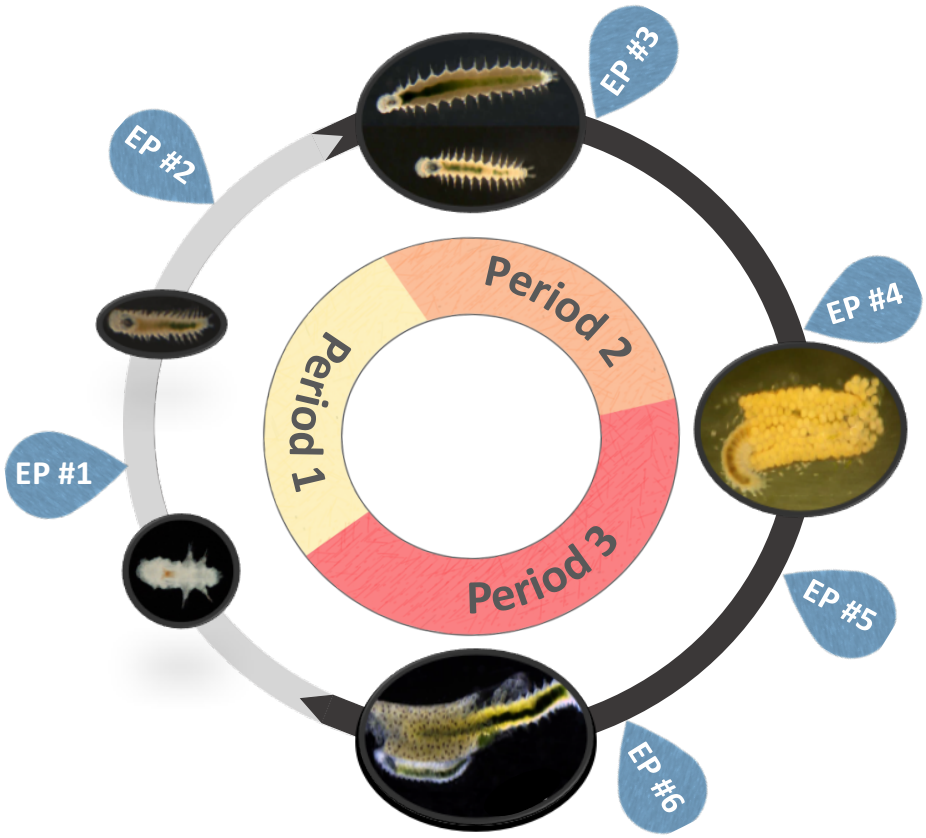


Fig 4. Representation of the developmental 'Endpoints' and 'Periods' along the life cycle of *O. labronica*. Photographs of *Ophryotrocha* used to build the life cycle were taken from Mills *et al.* (2007) for the hatchling, Massamba-N'Siala (2008) for the egg mass spawning and Paxton & Åkesson (2010), for the picture of the juvenile, male and female and the one with the egg mass almost hatched.

A summary of the approach used (developmental 'Endpoint' or 'Period' – hereafter named 'Endpoint' and 'Period') for each statistical analysis is presented in Table 4.

Table 4. Type of approach ('Endpoint' or 'Period') used to statistically analyzed the data on biological parameters measured in different biological compartments in *O. labronica*.

Analysis	**P1**	**P2**	**P3**	**EP #1**	**EP #2**	**EP #3**	**EP #4**	**EP #5**	**EP #6**
ATP8 expression							X	X	X
NAD1 expression							X	X	X
COB expression							X	X	X
CS activity[1]					X	X	X	X	
ETS activity[2]				X	X	X	X	X	
CS : ETS activity					X	X	X	X	
Metabolomics[3]									
Fecundity			X						
Fertility			X						
Size	X	X	X						
Survival	X	X	X						

[1] = mitochondrial citrate synthase, [2] = mitochondrial electron transport system (ETS), [3] = metabolites concentrations.

2.8.2 Mitochondrial gene expression and mitochondrial physiological traits

The effect of elevated temperature on the mitochondrial gene expression and mitochondrial physiological traits along the worms' ontogeny and the interaction was analyzed using a two-way analysis of variance (ANOVA; Type III) with 'Temperature' and 'Endpoints' as factors. For the mitochondrial gene expression, ANOVAs were realized directly on the normalized ratio of gene expression calculated for each sample with the Light Cycler® 480 Software from Roche Life Science. As for the mitochondrial physiological traits, ANOVAs were undertaken on the enzymatic activity normalized by the protein content for the citrate synthase (CS), electron transport system (ETS) and the ratio (CS : ETS). Homogeneity of variance was assessed visually plotting residuals from the model against predicted values and normality of the data was assessed using a Shapiro-Wilk test. In the case of the mitochondrial gene expression, a log10

transformation of the ratio was realized for *ATP8* and *NAD1* as the assumption of homogeneity or normality were not met.

2.8.3 Metabolomics analyses

The metabolomics database containing the concentration of the targeted metabolites for each sample was analyzed using the Metaboanalyst 4.0 web software (Chong *et al.*, 2018). Considering the high amount of missing values in the database, a common issue with LC-MS metabolomics data (Wei *et al,* 2018a), the database was first run through the web tool MetImp 1.2 (Wei *et al.*, 2018a; Wei *et al.*, 2018b), using to workflow proposed by Wei *et al.* (2018a), to correct for missing values. Considering that missing data in our database where due to limit of quantification (LOQ) of the technique, we followed the workflow for the 'Missing not at random (MNAR)' type of missing values. First, the 'modified 80 % rule' was applied to the database, removing metabolites for which missing values existed in more than 20% of the sample in at least one treatment combination. In total, 37 metabolites out of 58 were removed from the database (Table A1 in appendices), leaving only amino acids and free fatty acids in the database. In order to keep more metabolites for the analyses, we tried to increase the threshold of missing values to 40 %, but the gain in metabolites was too low, so we decided to keep the 'modified 80 % rule' instead. Then missing values were replaced using the quantile regression imputation of left-censored data (QRILC), which was the imputation that performed the best for our type of missing data (MNAR) according to Wei *et al*. (2018).

The database was then imported in Metaboanalyst, normalized by the sum and was scaled using the 'auto scaling' option of the software. This scaling permits that all metabolites are considered as equally important and is one of the methods that perform the best in pre-treating the data without changing the biological expectations (Van den Berg *et al.*, 2006). In order to test for the interaction between the factors 'Temperature' and the 'Period', a two-way analysis of variance (ANOVA; Type III) with a False discovery rate correction was realized on the database. As the interaction term was not significant, p-value between 0.4621 and 0.9105 for all metabolites, further statistical analyses were realized on the factors 'Temperature' and 'Period' separately. To explore

the metabolites distribution, two principal component analysis (PCA) were realized, one for the 'Temperature' and one for the 'Period'. In addition, to visually explore the variability in metabolites concentration between treatments, cluster heatmaps with samples and metabolites were realized using the Pearson distance measure and the complete link as a clustering algorithm. Finally, to identify metabolites that are significantly different between the treatments, a Wilcoxon rank-sum test and a Kruskal-Wallis test were realized for the 'Temperature' treatment and the 'Period' respectively, as the assumption of normality was not respected. The choice the use non-parametric test instead of log-transform the data was based on the fact that the log-transformation was helping only removing heteroscedasticity on only a few metabolites (4 out of 21 metabolites) and that log transformation is known to inflate the standard deviation of metabolites in low concentration (Van den Berg *et al.*, 2006). As no *post-hoc* tests were yet available to follow the Kruskal Wallis test on the software, a Dunn's multiple comparison test on the significant metabolites was realized with a Benjamini-Hochberg adjustment.

Finally, in order to understand the functional implication of the metabolites affected by the treatment, two enrichment analysis of the pathway-associated metabolites sets were then realized on the significant metabolites previously identified for the factor 'Temperature' and the factor 'Period' respectively. This analysis enables to assess which potential metabolic and biological pathways were affected. To do so, KEGG and/or HMDB numbers were gathered for the chosen metabolites and imputed in the 'Enrichment analysis' tool of the Metaboanalyst software using the Over-Representation Analysis or Enrichment Analysis (ORA) method. To complement this analysis and visualize the connection between all the metabolites measured, a metabolomics network was created using Cytoscape (version 3.6.1, Shannon *et al.*, 2003) software implemented with the Metscape application (version 3.1.3, Karnovsky *et al.*, 2012). The pathway-based network was build using the Human metabolites database, as it was the only metabolites database available along with the mouse and rat database and either of these choices did not affect the metabolomics network created. The following metabolites were not included in the network as they were not annotated in the database: B-aminoisobytric

acid, Caprylic acid, Tetradecenoic acid, Eicosapentaenoic acid, Palmitoleic acid, Pentadecylic acid and Ginkgolic acid.

2.8.4 Life history traits

Life history traits (fecundity, fertility, size, and survival) were analyzed using linear mixed model analysis (LMM) with 'plate' and 'well' as random effects nested in the factors 'Temperature' and 'Period': to account for the potential variability associated with the experimental system used. For fecundity and fertility, the 'Temperature' was the only fixed factor as reproductive events were only part of the third 'Period'. To account for a potential observer bias, influential data points (outliers) were removed from the database using the Cook's distance with a threshold of 1. Thereafter, LMM were fit by restricted maximum likelihood using the *lmer* function in the *lme4 R package* (version 1.1-18-1, Bates *et al.*, 2015). Homogeneity of variance was assessed visually by plotting residuals from the model against predicted values and normality of the data was assessed using Shapiro-Wilk test. The significance of the fixed factor and the random effects were tested respectively with the Type III ANOVA table with the Satterthwaite's degrees of freedom method and an ANOVA-like table for random effects, both included in the package *lmerTest* (version 3.0-1, Kuznetsova *et al.*, 2017). This approximation was specifically selected considering that it is not overly sensitive to relatively small sample sizes (Luke, 2017). When the factor 'Period' was found significant, multiple comparisons of means were carried out using Tukey contrasts (*multcomp package* version 1.4-8, Hothorn *et al.*, 2008).

When random effects were found not to have a significant effect, they were removed from analyses, which was the case for fertility and size. For the fertility trait, the effect of the fixed factor 'Temperature' was analyzed with a t-test on the $\log_{10}$-transformed data to respect the assumptions. Finally, the influence of 'Temperature' and 'Period' on individuals' size was analyzed through a generalized linear model (GLM) as the assumption of the normal distribution was not met. GLM were fit using the glm function with a *quasi* Poisson distribution, keeping the same structure of fixed factors as the ones used for LMM models. Significativity of the main factors and their interaction

was verified using the Analysis of Deviance Table with a Chi-square distribution and multiple comparisons between every level of the two factors were realized with a Tukey contrast.

3. RESULTS

3.1 Mitochondrial gene expression

The mean of the normalized ratio of expression of ATP synthase protein 8 (*ATP8*), NADH dehydrogenase subunit 1 (*NAD1*) and cytochrome b (*COB*) was relatively stable across the 'Endpoints', thus along the worm's ontogeny, despite the high variation recorded between replicates (Table 5). Moreover, the level of expression for the three genes was not affected by the 'Temperature' and did not differ across 'Endpoints' as indicated by the absence of significant interaction between the terms investigated (see Table 6).

Table 5. Mean (±SE) of the normalized ratio of expression for the three mitochondrial genes *ATP8*, *NAD1* and *COB* according to 'Temperature' and 'Endpoints' in *O. labronica*.

Factors		***ATP8***			***NAD1***			***COB***		
Temperature	Endpoint	n	Mean ±	SE	n	Mean ±	SE	n	Mean ±	SE
24 °C	4	1	0.7488 ±	NA[1]	2	4.7770 ±	4.1470	2	3.2140 ±	2.5111
	5	3	1.3569 ±	0.8780	3	2.1314 ±	0.7692	3	2.8123 ±	0.9856
	6	2	0.4738 ±	0.2092	1	0.4885 ±	NA[1]	2	0.7150 ±	0.0240
30 °C	4	3	0.3376 ±	0.1020	3	3.5588 ±	0.1673	3	6.7400 ±	0.6886
	5	1	0.5416 ±	NA[1]	1	3.8220 ±	NA[1]	1	4.1050 ±	NA[1]
	6	2	0.4077 ±	0.0643	1	0.4121 ±	NA[1]	2	3.2468 ±	2.6862

[1] Data not available as only one sample was analyzed (n = 1).

Table 6. Statistical results of the two-way ANOVA on the effect of 'Temperature', 'Endpoints' and their interaction on the three mitochondrial genes *ATP8*, *NAD1* and *COB* in *O. labronica*.

	ATP8				**NAD1**				**COB**			
	df	Sum Sq	F-value	*P*	df	Sum Sq	F-value	*P*	df	Sum Sq	F-value	*P*
Temperature	1	0.1503	1.4138	0.2793	1	0.0057	0.0186	0.8959	1	14.9200	2.9240	0.1310
Endpoints	2	0.0061	0.0288	0.9717	2	0.4013	0.6601	0.5507	2	7.4770	0.7327	0.5141
Temperature x Endpoints	2	0.0287	0.1351	0.8762	2	0.1792	0.2948	0.7549	2	2.3140	0.2268	0.8027
Residuals	6	0.6378			6	1.8238			7	35.7170		

3.2 Mitochondrial physiological traits

Similarly to mitochondrial gene expression, mean citrate synthase (CS) activity (Fig 5A, Table S1) did not differ between 'Endpoints' and 'Temperatures', nor was the interaction between these terms found to be significant (Table 7). Differently, mean electron transport system (ETS) activity was generally higher than the one measured for mean CS (Fig 5 A, B) for both 'Temperatures' and for all 'Endpoints' (Table S1). However, similarly to mean CS activity, no particular pattern of response was observed (Fig 5B), as indicated by the absence of significant impacts of 'Endpoints', 'Temperatures', and their interaction (Table 7).

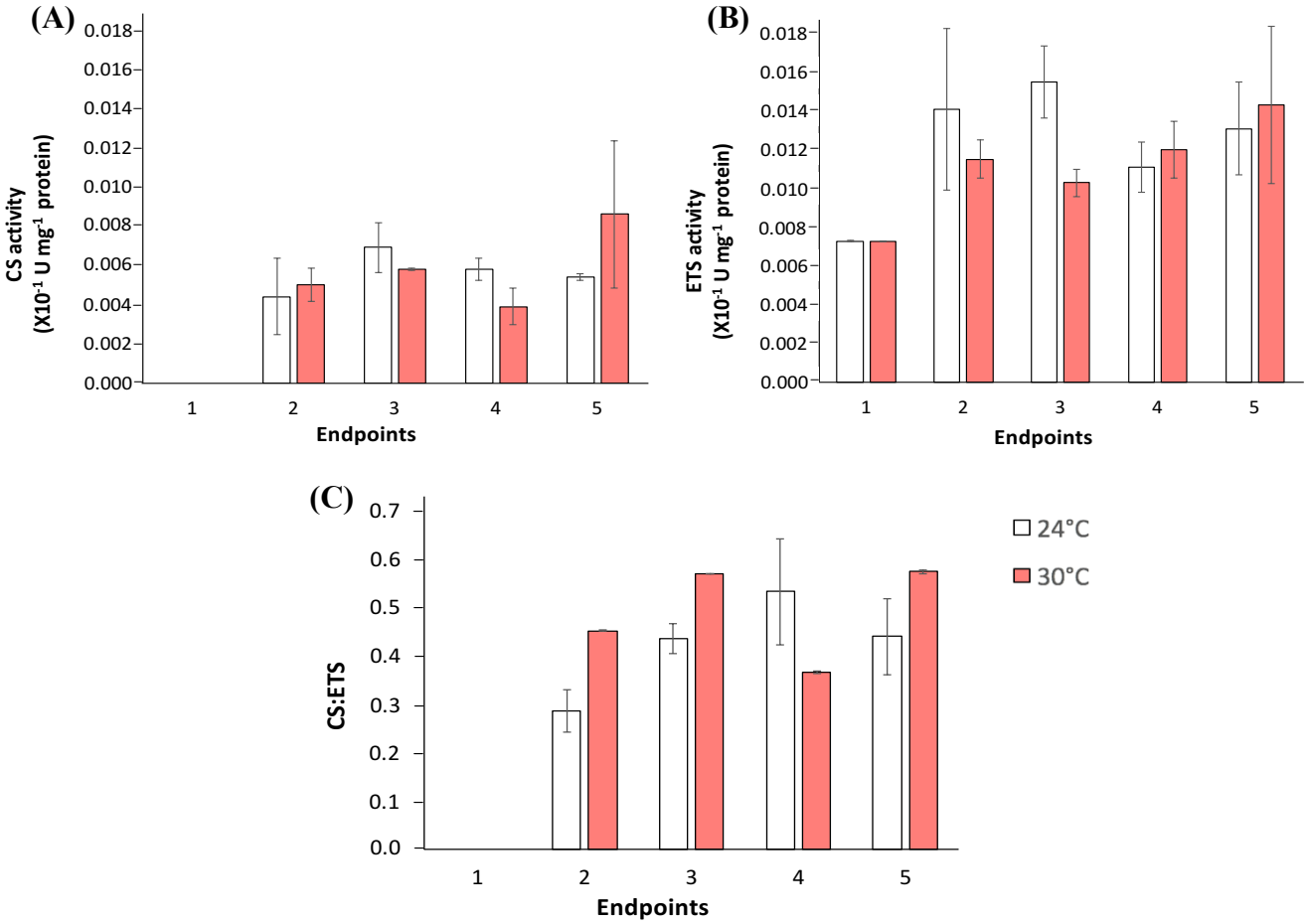


Fig 5. Temperature effect through *O. labronica*'s ontogeny ('Endpoints') on (A) citrate synthase [CS] activity, (B) electron transport system [ETS] activity and (C) the ratio CS : ETS. Errors bars represent mean ± SE with the 24 and 30 °C treatment in white and red respectively.

As for the ratio of CS : ETS (Fig 5), similarly to CS and ETS, there were no significant effect of 'Temperatures' and 'Endpoints' in isolation or combined (Table 7, Table S1).

Table 7. Statistical results of the two-way ANOVA on the effect of 'Temperature', 'Endpoints' and their interaction on citrate synthase [CS], electron transport system [ETS] and the ratio CS : ETS in *O. labronica*.

	CS				ETS				CS:ETS			
	df	Sum Sq	F-value	*P*	df	Sum Sq	F-value	*P*	df	Sum Sq	F-value	*P*
Temperature	1	5.21E-07	0.1000	0.7565	1	0.00	0.0000	0.9966	1	0.0409	2.3583	0.1469
Endpoints	3	9.42E-06	0.6027	0.6239	4	9.32E-05	1.5583	0.2333	3	0.0808	1.5518	0.2452
Temperature x Endpoints	3	1.85E-05	1.1821	0.3521	4	3.81E-05	0.6370	0.6436	3	0.0918	1.7637	0.2002
Residuals	14	7.29E-05			16	2.39E-04			14	0.2430		

3.3 Metabolomics profiles

No clear separation between the metabolomics profile of the worms exposed to 24 and 30 °C (Fig 6A), and between the three 'Periods' investigated was found (Fig 6B).

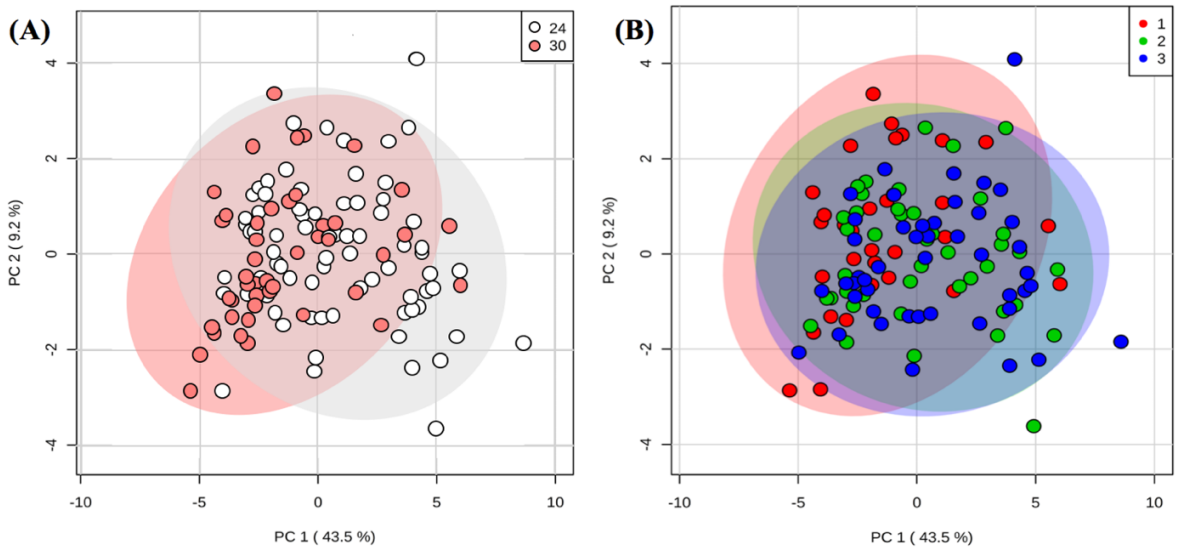


Fig 6. PCA plot representing the variation in metabolites profile in *O. labronica* according to the 'Temperature' of exposure (A) and the 'Periods' (B). Each point corresponds to a sample analysed, with samples of the same color corresponding to the same treatment. Red dots represent the worms exposed to 24 °C and the ones in green to 30 °C for Fig 6A. In case of Fig 6B, the red, green and blue dots correspond to Period 1, Period 2 and Period 3 respectively. 95% confidence intervals are indicated by the shaded area.

In both cases, the two first principal axes explained 52.8 % of the total variation (PC1 = 43.5 % and PC2 = 9.2 %). However, even though samples associated to the 24 °C and the 30 °C treatment are overlapping, there is a clear distinction regarding specific metabolites composition of the 21 metabolites analysed, the difference being driven by the two categories of metabolites: fatty acids and amino acids. Samples at the right side of the PC1 axis are represented by a high concentration of amino acids while samples at the left side of the axis are represented by a higher content of fatty acids (Fig 7A, B).

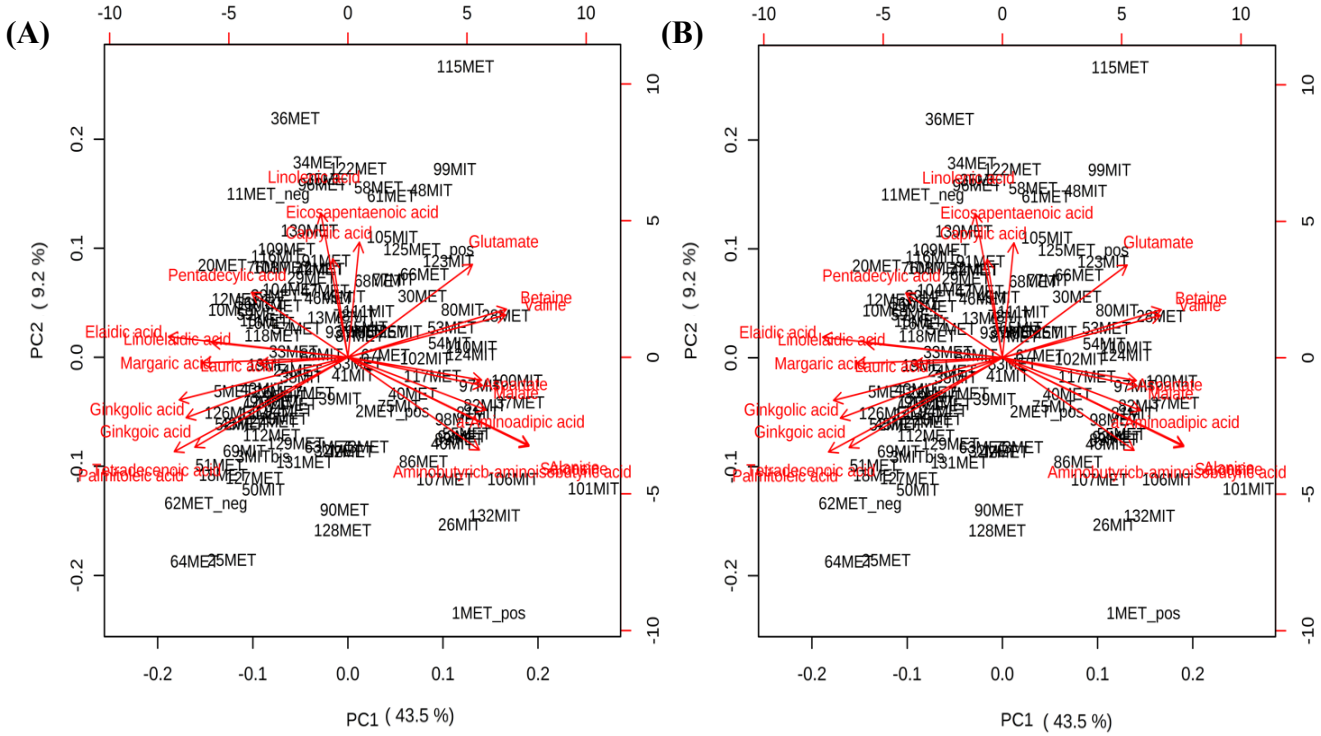


Fig 7. PCA biplot of the metabolites explaining the variation in the samples for (A) the two 'Temperature' treatments, and (B) the three 'Periods'. Samples and metabolites are shown in black and red respectively.

This separation between the amino acids and the lipids according to the 'Temperature' and the 'Period' was confirmed through the cluster heatmaps (Fig 8 A, B), especially in the case of the temperature treatment, where the pattern of responses to elevated temperature was divided in two clusters: one composed in majority of amino acids metabolites and the other one composed with the majority of fatty acids

metabolites. As shown in Fig 8A, these two clusters showed opposite responses to elevated temperature: amino acids concentrations tended to decrease with elevated temperature whereas on the contrary fatty acid concentrations increased with the temperature. This decrease in average concentrations at 30 °C is particularly visible for alanine, sarcosine, betaine and valine amino-acids, while aspartate is the only amino acids which showed only a slight variation in concentration between the two temperatures tested (Fig 8A). Interestingly, the eicosapentaenoic acid was the only fatty acid showing a significant decrease in concentration between 24 and 30 °C, as the concentration of all the other fatty acids increased at 30 °C when compared to 24 °C. However, the magnitude of change of the fatty acids metabolites was more variable than for the amino acids, as the increase in concentration for the linolenic and caprylic acid is small when compared to the one of the linolelaidic, elaidic, palmitoleic and lauric acid (Fig 8A). These high differences in metabolites concentration between the two temperatures treatment were confirmed as 17 out of 21 metabolites were found significantly different between the 24 and 30 °C: see the Table S2 for the statistical result and Fig 8A where significant metabolites are indicated with an asterisk.

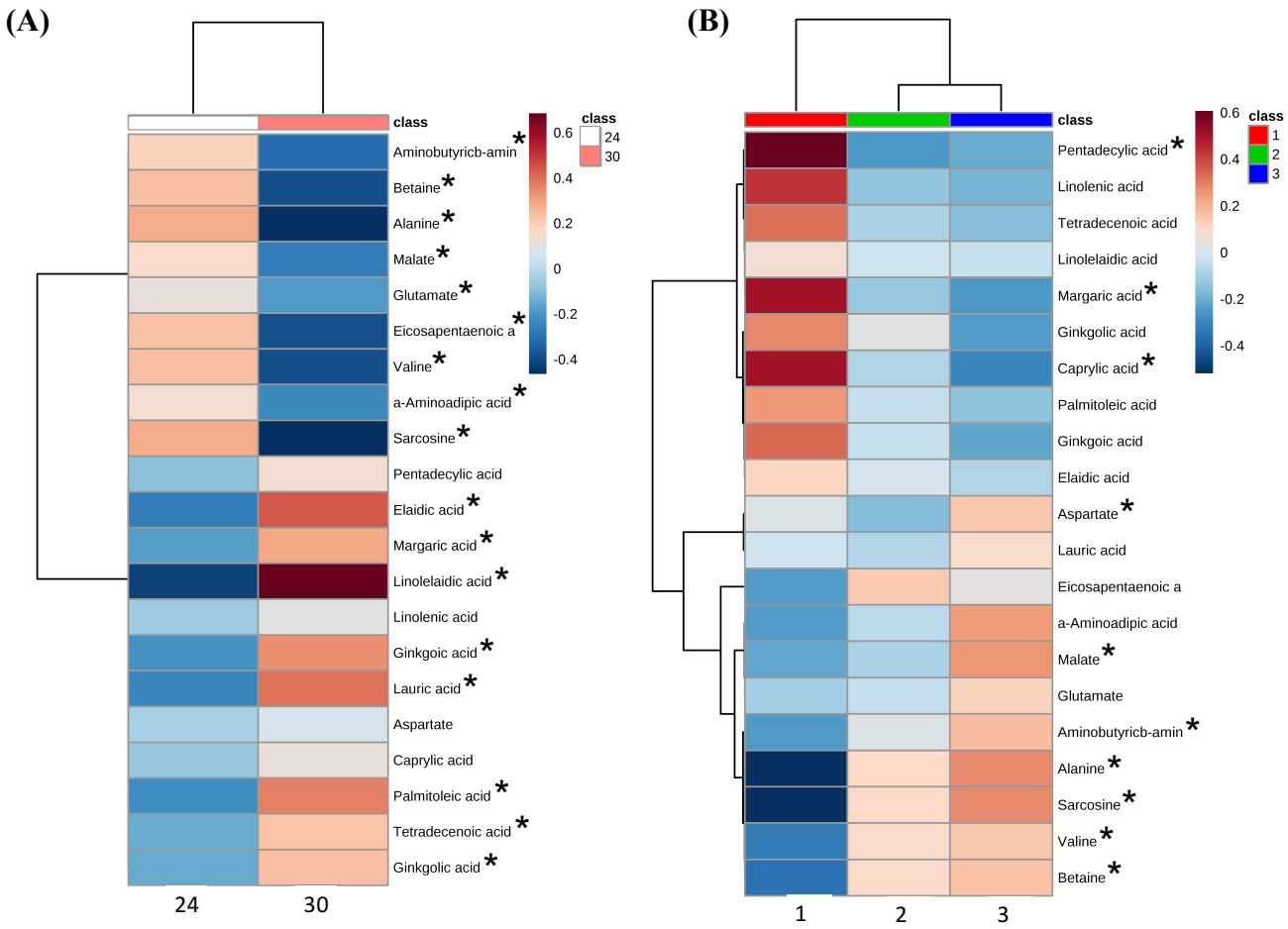


Fig 8. Cluster heatmap of normalized metabolites concentration (A) in individuals of *O. labronica* exposed to 24 and 30 °C and (B) through the three 'Periods'. Each cell denotes the group average metabolite concentration for all the samples of one treatment with each metabolite in a row and treatments in columns. Metabolites for which an increase in concentration in comparison to the average value was recorded are indicated in red while metabolites with a lower concentration than the average are displayed in blue. The brightest of each color represent the magnitude of this difference in concentration relative to the average value. An asterisk indicates metabolites that are significantly different between the treatments (Wilcoxon rank-sum statistical test (q-value < 0.05) in (A) and Kruskal Wallis statistical test (q-value < 0.05) in (B)).

Regarding the variation in metabolites concentration along the three 'Periods' investigated, the cluster heatmap revealed that Period 2 and 3 had more similar metabolomics profiles between each other than to Period 1 (Fig 8B). This difference between Period 1 and the cluster of Period 2 and 3 seems driven particularly by changes in fatty acids concentrations: Period 1 was characterized by a systematically higher fatty acids concentration than the two others Periods, with the exception of the

eicosapentadenoic and lauric acid (Table S3). The concentrations of the pentadecylic, caprylic and margaric acid are particularly high for the first Period whilst they were interestingly amongst the metabolites the least affected by the temperature (Fig 8 A, B). Moreover, similarly to the effect of elevated temperature on metabolites concentration, the amino acids concentrations across the three 'Periods' changed in an opposite fashion than fatty acids. In fact, concentrations of amino acids were generally observed to increase in later 'Periods', particularly for alanine and sarcosine (Fig 8B). The difference observed between Period 1 and the two other Periods was also confirmed, as ten metabolites, in majority amino acids, were significantly different between the three 'Periods' (Table S3). *Post-hoc* analyses highlighted that the first Period was significantly different from the other two in terms of height of the nine metabolites: Aspartate being the only metabolite for which only Period 1 and 3 could be distinguished from each other (Table S4).

Finally, according to the enrichment analysis, the majority of the metabolic pathways potentially affected by the factor 'Temperature' are those associated with amino acids metabolism (Fig 9A). Indeed, all the pathways from 'Glycine and Serine Metabolism' to the 'Glutathione Metabolism' were the ones for which the number of metabolites affected by elevated temperature, were more frequently represented than by chance. Theses pathways had a *p-value* ranging between 0.0135 and 0.0453. Interestingly, the majority of these pathways involved the glutamate and alanine metabolites which were present in a lower concentration in *O. labronica* individuals exposed to 30 °C (Fig 10). No pathways involving fatty acids metabolites were identified to change significantly under elevated temperature or across development, with the exception of the 'Alpha Linolenic Acid and Linoleic Acid Metabolism'.

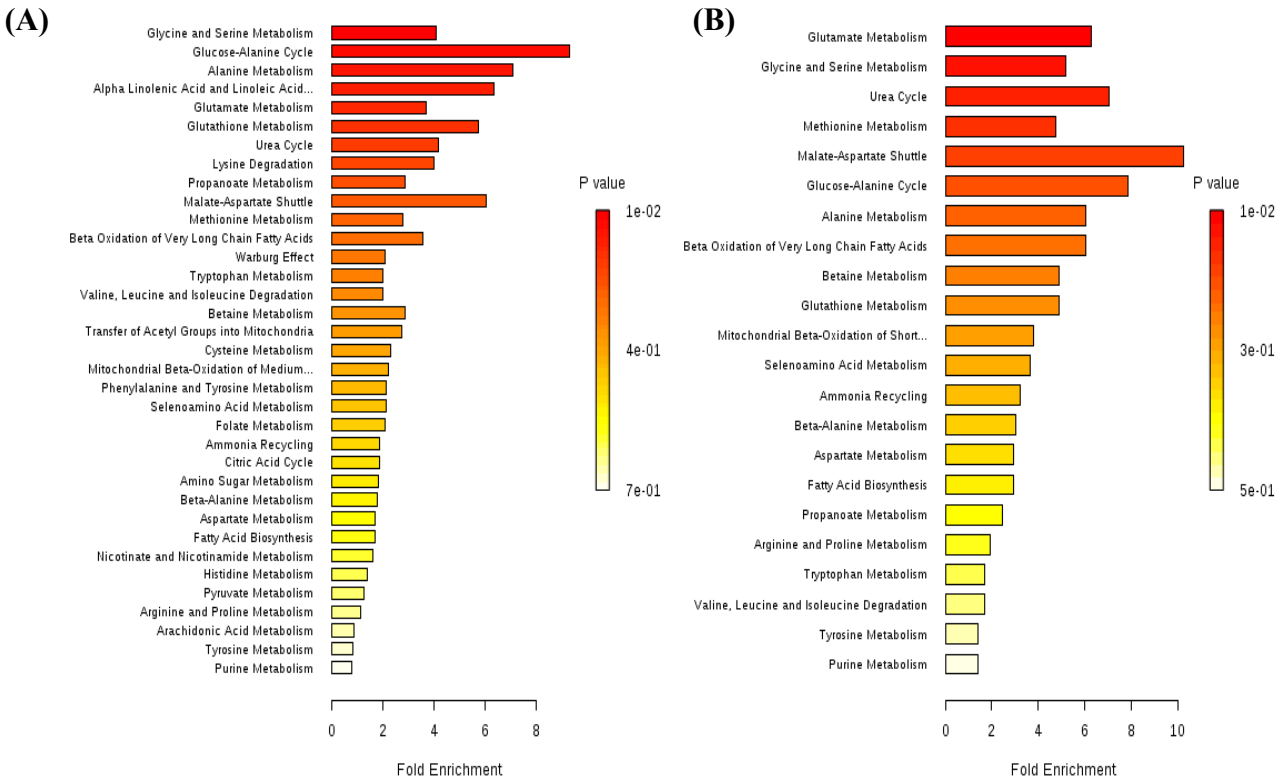


Fig 9. Metabolic pathways potentially (A) affected by the temperature treatment or (B) affected by the worm's ontogeny (Period) in order of decreasing *P* value. The color scale indicates the *P* value, thus, the probability of seeing at least a particular fraction of the metabolites from a certain metabolite set affected by the treatment.

Similarly, the enrichment analysis of the metabolites significantly affected by the factor 'Period' revealed that the potential pathways the most affected (the first three pathways in Fig 9B) where the ones containing at least either the betaine, sarcosine, alanine or aspartic acid metabolites in it, all metabolites that had a lower concentration in the first Period (Fig 10). Theses pathways had a *p-value* ranging between 0.00975 and 0.0303.

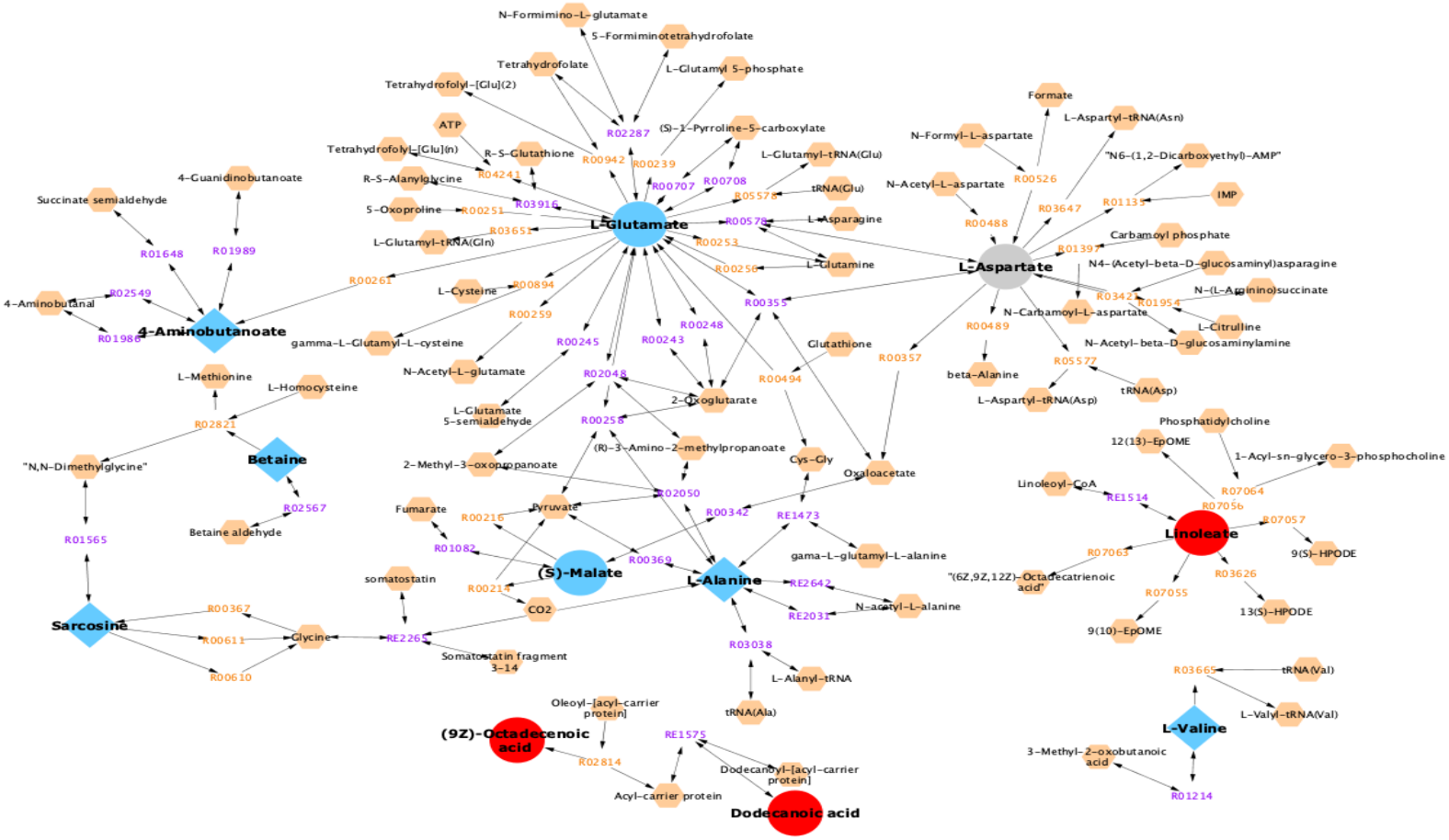


Fig 10. Metabolic network (Cytoscape version 3.6.1 with a Metscape plug-ing version 3.1.3) of all the metabolites analyzed and their associated compounds and reactions. Some lipids are not presented in the metabolic network as they were not available in the software. The color blue indicates metabolites for which the concentration was significantly lower at 30 °C, while metabolites in red indicate the contrary. Finally, the diamond shape corresponds to the metabolites that were significantly different in Period 1.

3.4 Life-history traits

Females exposed to 30 °C showed a significant, but small, decreased in fecundity (Figure 11A; Table 8), as observed by a lower number of eggs *per* egg mass when compared to the ones at 24 °C (Table S5). However, females' fertility (Table S5) was not affected by the temperature (Table 8).

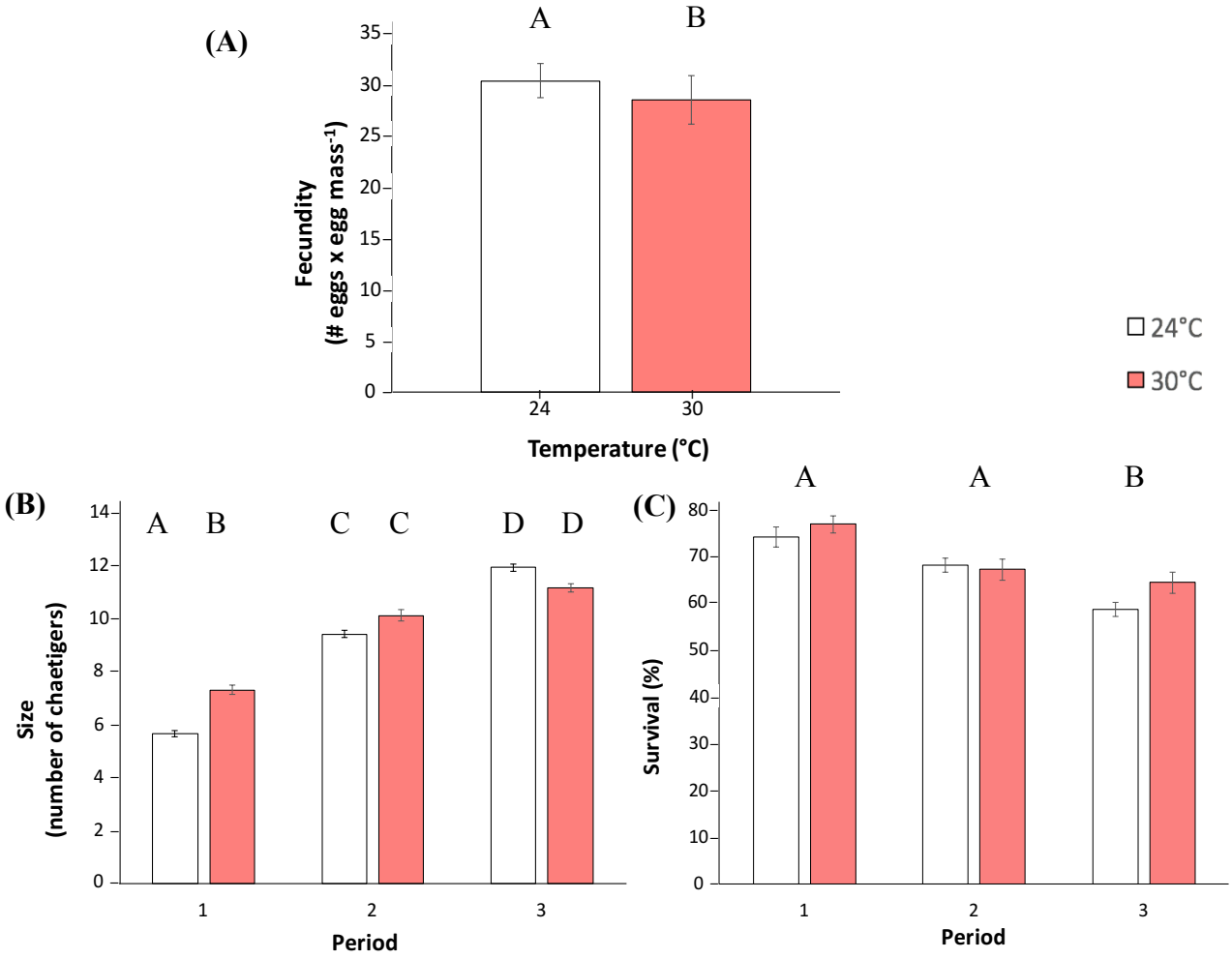


Fig 11. Effect of temperature through *O. labronica*'s ontogeny (Periods) on (A) fecundity, expressed as the number of eggs *per* egg mass, (B) size and (C) individual' survival. Errors bars represent mean ± SE with the 24 and 30 °C treatment in white and red respectively. Capital letters above the bars indicate significant differences ($P < 0.05$) between treatments.

Regarding the size of the individuals (Table S5), the results indicate that individuals' size was differently affected by the factor 'Temperature' across the three 'Periods', as supported by the presence of a significant interaction between the factors 'Temperature' and 'Period' (Table 8). More precisely, organism's size significantly increased across the three 'Periods' and the organisms reared at 30 °C were bigger than the ones reared at 24 °C for the first 'Period' (Table S6). However, no significant differences in the organisms' size were observed between the two temperature treatments for the second and the third 'Period' (Table S6).

Survival rate decreased along the individuals' ontogeny, thus the 'Periods', reaching between 58 and 64 % at 24 and 30 °C respectively for the third 'Period' (Figure 11C; Table S5). Survival rates (Figure 11C) were not affected by elevated temperature but were significantly negatively affected by 'Period' (Table 8). In more detail, survival rates at Period 3 were significantly lower than those reported for Period 1 ($Z = -4.211$, $P < 0.0001$) and Period 2 ($Z = -3.251$, $P = 0.0033$), but no significant difference was observed between Period 1 and 2 ($Z = -1.471$, $P = 0.3035$).

Table 8. Statistical results of the effect of 'Temperature' through *O. labronica's* ontogeny ('Periods') on life history traits. Statistical results for fecundity and survival were obtained through linear mixed models [LMM], while statistical results for the fertility and the size were obtained respectively through an ANOVA and a generalized mixed model [GLM]. Significant statistical differences are indicated in bold.

	Fecundity				**Fertility**			**Size**					**Survival**			
	Num df	Den df	F value	*P*	df	t value	*P*	df	Dev	Resid. df	Resid. Dev	*P*	Num df	DenDF	F value	*P*
Fixed effects																
Temperature	1	24.006	6.8571	**0.0151**	51	-0.17046	0.8653	1	3.93	983	870.15	**0.0476**	1	120.89	0.6613	0.4177
Period	-	-----	-----	-----	--	-----	-----	2	409.73	983	460.42	**<0.001**	2	120.91	15.0395	**<0.0001**
Temperature x Period	-	-----	-----	-----	--	-----	-----	2	22.17	979	438.25	**<0.001**	2	120.91	0.5759	0.5637
Random effects			**Variance**				**Variance**				**Variance**					**Variance**
Well			15.26				-----				-----					26.66
Plate			35.59				-----				-----					114.13
Residuals			42.16				-----				-----					72.04

3. DISCUSSION

Temperature is known to affect virtually all biological compartments, from gene expression and biochemical reactions to cellular functions, metabolism and life-history traits (Hochachka and Somero, 1984; Guderley and St-Pierre, 2002; Angilletta, 2009). Despite the wide impact of temperature on organisms' biology (Hochachka and Somero, 1984; Angilletta, 2009), there is a particular interest in focusing on aerobic metabolism (Schulte, 2015). Firstly, the majority of organisms relies on oxygen for their processes (Schulte, 2015). Secondly, the decrease in organisms' aerobic scope induced by the mismatch between oxygen demand and supply is one of the mechanism potentially setting organisms' upper thermal limit (see the OCLTT hypothesis in Pörtner, 2001, 2002; Pörtner and Knust, 2007; Pörtner and Farrell, 2008; c.f. see discussions in Clark *et al.*, 2013a,b; Jutfelt *et al.*, 2018). The aerobic scope, i.e. the difference between the maximum metabolic rate (MMR) and the standard metabolic rate (SMR), represents, in fact, the energy available that an organism can allocate to different functions (Pörtner, 2001; Claireaux and Lefrançois, 2007; Schulte, 2015). Consequently, the decrease of the aerobic scope could crucially impair organisms' physiological functions and life history traits, such as growth and reproduction (Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Shama *et al.*, 2016), with consequences on organism's fitness and, ultimately, on species' geographical distribution and abundances (Pörtner and Knust, 2007; Schulte, 2015). Therefore, the capacity of an organism to maintain its aerobic scope with increasing temperature, through phenotypic plasticity, will dictate their survival in a warming environment (Schulte, 2015).

Even though no direct measurements of respiration rates were realised in this study to measure the aerobic scope, plastic changes in the biological traits measured here could indirectly change organisms' aerobic scope. At the cellular level, mitochondria, the powerhouse of the cells, are likely to play a key role in organisms' responses to elevated temperature, considering their implications in energy metabolism, biosynthesis and intracellular signaling (Guderley and St-Pierre, 2002; Blier, Lemieux and Pichaud, 2014; Shama *et al.*, 2016). Molecular or cellular changes leading to a modification in the number

of mitochondria's or the efficiency of the mitochondrial respiratory chain can fundamentally change the Phosphate/Oxygen Ratio (P/O ratio), thus the relation between energy production and oxygen demand (Hulbert and Else, 1999; Pörtner, 2002). As a result, this change in the P/O ratio can modify the aerobic scope of an organism and potentially increase their critical upper thermal limit (Pörtner, 2002; Chung and Schulte, 2015). Besides changes at the molecular and cellular levels, changes in metabolism or in energy allocation in life-history traits could also contribute to increase organisms' aerobic scope, as they are intrinsically linked (Schulte, 2015).

Our findings suggest that exposure to elevated temperature affects organisms' higher biological compartment only, thus metabolomics profiles and life history traits (Table 9). As predicted, the elevated temperature seems to have increased the rate of organisms' aerobic metabolism, with consequences on organisms' life history traits. However, no changes relative to the mitochondria functions at the cellular or molecular level are observed. This indicates that changes in energy metabolism levels might have been sufficient to enable worms to face the direct, relatively short-term but across its entire development, impacts of the exposure to elevated temperature, without any evident change in the genes expression and enzymatic activities targeted in this study. Our results regarding *Ophryotrocha labronica* responses to elevated temperature are discussed for each biological compartment measured separately. In addition, the importance of using an integrative approach in future studies looking at organisms' responses to temperature in the context of global warming is discussed.

Table 9. Summary of the direction of response for each of the trait measured in *O. labronica* individuals following the exposure to elevated temperature throughout their ontogeny (represented by the 'Period' or the 'Endpoint' factor). The presence of a statistical interaction between the factor 'Temperature' and either the factor 'Period' or 'Endpoint' is indicated in the second column. The column 24 °C, P1 and EP#1 were used as a reference level to assess the direction of response for the 'Temperature', 'Period' and 'Endpoint' factors respectively. For each trait, red arrows indicate a decline, green arrows, an increase, and the flat line, no changes, in the trait in comparison to the reference level. Striped areas indicate that no information is available.

Traits	Interaction	24 °C	30 °C	P1	P2	P3	EP #1	EP #2	EP #3	EP #4	EP #5	EP #6
ATP8 expression	No	—	—							—	—	—
NAD1 expression	No	—	—							—	—	—
COB expression	No	—	—							—	—	—
CS activity	No	—	—					—	—	—	—	
ETS activity	No	—	—				—	—	—	—	—	
CS : ETS activity	No	—	—					—	—	—	—	
Metabolomics – [Amino acids]	No	—	↓	—	↑	↑						
Metabolomics – [Fatty acid]	No	—	↑	—	↓	↓						
Fecundity		—	↓									
Fertility		—	—									
Size	Yes	—	↑	—	↑	↑						
Survival	No	—	—	—	—	↓						

4.1 Mitochondrial gene expression

Changes in gene expression, i.e. up or down-regulation, following a change in temperature are known to be a fundamental plastic response enabling organisms to face changing environmental conditions (Aubin-Horth and Renn, 2009; Hodgins-Davis and Townsend, 2009; Yampolsky *et al.*, 2014; Shama *et al.*, 2016). Mitochondrial genes encoding the subunits of four of the five complexes of the mitochondrial respiration chain are known to be involved in organisms' acclimation to temperature (Garvin *et al.*, 2015). However, up or down-regulation of mitochondrial gene expression was not observed for the three mitochondrial genes in *O. labronica*, not even through the individuals' ontogeny. Up-regulation of the mitochondrial gene expression could have been expected in the elevated temperature treatment, as this up-regulation could lead to an increase in proteins levels involved in the mitochondrial respiration chains (Shama *et al.*, 2016). This could translate into an increase in ATP production, which could enable organisms to meet higher energy demand (De Wit *et al.*, 2016). This response was particularly expected for individuals exposed to the elevated temperature treatment at the Endpoint 4 of their ontogeny, which corresponds to sexual maturation and reproduction. In addition to having to handle the higher energy demand induced by the increase in temperature, worms have to invest energy both in growth and sexual maturation, two processes both known to be energetically extremely costly (Stearns, 1989; Wieser, 1994; Massamba-N'Siala *et al.*, 2012). On the other hand, down-regulation of these genes, to reduce energetic costs associated to mitochondrial functions, and thus SMR, was expected if the elevated temperature treatment was very close to their upper thermal limit (Shama *et al.*, 2016).

Given that other 10 mitochondrial genes and more than 70 nuclear genes code for subunits in the mitochondrial respiration chain, it is highly possible that changes in gene expression may have occurred for other genes not characterised in our study and thus undetected. Moreover, other elements at the molecular level such as mtDNA and transcription rates can also modulate energy production (De Wit *et al.*, 2016). Therefore, the use of other complementary molecular techniques, including a transcriptomic approach,

would be useful to acquire a more comprehensive understanding of the role of genetic plasticity in organisms' responses to elevated temperature. However, these methods present considerable limits for species such as *O. labronica*, for which we still do not possess a complete genome; the only complete genome available for this species is the mitochondrial genome assembled within the context of this study. The implementation of this approach would thus require a considerable effort well beyond the scope of this study. Our results are in line with the study by Shama *et al.* (2016), looking at transgenerational effects in gene expression in sticklebacks exposed to elevated temperatures. In fact, Shama *et al.* (2016) showed that the immediate environment experienced by the offspring had the lowest impact on genes expressions profiles, in comparison to the environment experienced by their mothers. Thus, changes in gene expression profiles were higher when offspring raised at higher temperature were born from mothers also raised at the same temperature. More specifically, an up-regulation in mitochondria encoded genes was only detected in offspring following a transgenerational exposure to elevated temperature. Offspring exposed solely to the elevated temperature during their development showed instead more changes at the cellular metabolism level (Guderley and St-Pierre, 2002; Shama *et al.*, 2016). Altogether, these results suggest that the timing when exposure to elevated temperature is perceived in an organism' life cycle can fundamentally affect which plastic responses or pathways are set up (Donelson *et al.*, 2018).

4.2 Mitochondrial physiological traits

The timing at which exposure to elevated temperature occurs, and thus when environmental cues are detected by an organism, may also explain our results for the citrate synthase (CS), electron transport system (ETS) activities and their ratio. Citrate synthase and ETS are respectively used as proxies for mitochondrial density (Moyes *et al.*, 1997) and maximum mitochondrial capacity (i.e. metabolic potential) (Schmidlin *et al.*, 2015; Simcic *et al.*, 2015). An increase in both their activities with elevated temperature could indicate an increase in an organism's metabolic potential, and consequently, energy production (Simcic and Brancelj, 2006). An increase in CS and ETS activity has already

been found in *O. labronica* following a transgenerational exposure at 30 °C (but with control temperature at 27 °C) (Chakravarti *et al.*, 2016). However, similarly to our findings, no significant differences in CS and ETS activity and their ratio, for the first generation exposed to the elevated temperature treatment is observed. These findings further suggest that developmental and transgenerational exposure to elevated temperatures trigger different mechanisms of responses in *O. labronica.* In addition, given that the mitochondrial respiration chain is formed by numerous proteins subunits that are closely integrated to form four functional complexes, it is possible that changes in mitochondrial physiological traits may have occurred elsewhere, in the cytochrome *c* oxidase (COX; Complex IV) for instance (Blier *et al.*, 2014). Indeed, this complex is known to be tightly involved in the regulation of mitochondrial respiration (Blier and Lemieux, 2001; Blier *et al.*, 2014).

Another hypothesis explaining our results for the mitochondrial physiological traits is that stress imposed by elevated temperature with the duration of exposure was not sufficiently energetically demanding for the worms to require the adjustment of CS and ETS activities. The fact that CS and ETS activities remain relatively stable throughout their ontogeny could indeed indicate that worms' aerobic scope at 30 °C was sufficient to support organisms' activity in all their biological compartments.

4.3 Metabolomics profile

The idea that the aerobic scope was sufficient to support all organisms' functions at 30 °C is further supported by the fact that no transition from aerobic to anaerobic metabolism has been observed in individuals' metabolomics profiles. Indeed, according to the OCLTT hypothesis, a decrease in the aerobic scope at the upper thermal limit could induce a transition from the aerobic to the anaerobic metabolism (Pörtner, 2006b; Verberk *et al*., 2013). This shift can lead to an accumulation of metabolites associated to the anaerobic metabolism, such as lactate, alanine and acetate, as well as an accumulation of metabolites used as substrate for the Krebs cycle, such as succinate and malate, due to a disruption of the cycle (Lannig *et al.*, 2010; Verberk *et al*., 2013). However, this is not the

case for the *O. labronica* individuals as the concentration of both alanine and malate decreased at 30 °C. As malate is an intermediate compound in the Krebs cycle, its decrease could indicate an increase in the flux of the Krebs cycle to sustain the higher metabolic rate and energy demand (Pörtner, 2001; Gillooly *et al.*, 2001; Schulte, 2015). In addition, the depletion in alanine and other amino acids that can be used as fuel for the Krebs cycle (i.e. glutamate, valine; Hochachka and Somero, 1984) could indicate that *O. labronica* individuals rely mainly on amino acid as a substrate for the aerobic metabolism, rather than on carbohydrates or lipids. This hypothesis is further supported by the enrichment analysis results. All significant pathways identified were associated with the formation of pyruvate for the Krebs cycle. These pathways use, as intermediate compounds, some of the amino acids mentioned before, as well as sarcosine and betaine, which were also found in significantly lower concentration at 30 °C when compared to the control condition (Lehninger, 2005). However, the observed lower concentration of amino acids could also indicate an increase in proteins anabolism, to produce the necessary proteins involved in temperature responses pathways or in cellular maintenance. However, as no proteomics analyses were carried out in this study, we cannot confirm this idea.

Our results show also that increasing temperature leads to an increase in free fatty acid concentration in the cells. In this regard, one of the main known effects of elevated temperature on fatty acid composition is associated with phospholipids composition of the cell membrane (Angilletta, 2009). In response to the increase in membrane fluidity with higher temperature, organisms tend to increase the proportion of saturated fatty acids relative to unsaturated fatty acids to reduce membranes fluidity (Hulbert, 2007; Angilletta, 2009). This change in fatty acid saturation state can reduce membrane leakiness, and thus, limit proton leak across the mitochondrial membrane (Careau *et al*., 2015; Schulte, 2015), and can also influence the activity of important signaling proteins (Török *et al.*, 2014). However, changes in membrane saturation state could not be assessed in this study, as the metabolomics method used here detect free fatty acids, and not fatty acids composing phospholipids. Even if we cannot exclude completely the idea that a certain fraction of

lipids might have undergone hydrolyses during the extraction process, we consider that this fraction is so low that it did not impact our results.

Consequently, the increase in free fatty acid concentration observed in the elevated temperature treatment could correspond to an increase in triglycerides catabolism, which form glycerol and free fatty acids (Hochachka and Somero, 1984). Similarly to the amino acids, free fatty acids can be used as a substrate for aerobic metabolism (Hochachka and Somero, 1984). Their chemical characteristics make them suitable substrate for energy production to sustain high ATP-consuming physiological processes, as they contain more energy *per* unit of mass than carbohydrates or amino acids (Hochachka and Somero, 1984; Weber, 2011). However, they cannot yield ATP quickly (Weber, 2011). Thus, the increase in free fatty acids concentrations detected in *O. labronica* metabolomics profiles could indicate that they are accumulating these metabolites as energy substrates to be used in a near future, in order to support their multiple reproductive events. Indeed, even though our results seem to indicate that *O. labronica* individuals rely on amino acids to produce ATP during the experiment, they could use fatty acids as a substrate in a longer exposure to elevated temperature in order to meet the higher energy demand and still allocate enough energy in reproduction, growth and cellular repairs. In *O. labronica,* the energy demand can be particularly high during the reproductive phase, as they need to invest energy in egg masses production, as well as growth. Moreover, as protein denaturation is a time-dependent process, the longer organisms are exposed to stressful temperature, the more enzyme and proteins denaturation can be important (Schulte, 2015), and translate in higher energy need with time.

Another potential implication of the higher free fatty acids concentrations at 30 °C may be associated with the protection of the cells against the oxidative damage caused by the reactive oxygen species (ROS) (Murphy, 2009; Tomanek, 2015), for which production usually increase with temperature (Abele *et al.*, 2002). In that regard, uncoupling protein UCP2 and UCP3, found in the mitochondrial membrane (Graier *et al.*, 2008) have been recognized for their role of protection against mitochondrial ROS production, although the

mechanisms underpinning this function are still subject of discussion (Nègre-Salvayre *et al.*, 1997; Brand and Esteves, 2005). As they are activated by free fatty acids (Nicholls, 2006; Graier *et al.*, 2008), the increase in concentration of fatty acid at 30 °C could potentially be a plastic response generated to activate the UCP2 and UCP3 uncoupling proteins and hence protect the cells from the deleterious oxidative damage of ROS (Murphy, 2009; Tomanek, 201). As we did not measure the ROS production in this study, however, we cannot confirm this idea. A previous study with *O. labronica* individuals exposed transgenerationally to elevated temperature showed no increase in ROS production in the elevated temperature treatment, which could indicate that they used antioxidant or protection systems such as the UCP2 and UCP3 for protection (Chakravarti *et al.*, 2016).

Finally, the non-significant interaction between the 'Temperature' and the 'Period' factor indicates that temperature affects metabolomics profiles in a similar fashion throughout worms' ontogeny. The relative higher concentration of lipids in the first period in comparison to the second and third period, when individuals are still in the hatchling or juvenile stages, might be associated with the remaining lipid content of their eggs. As they grow, juveniles deplete their lipid reserves while creating a pool of amino acid, a common phenomenon in marine invertebrates used to sustain aerobic metabolism (Hochachka and Somero, 1984).

4.4 Life history traits

Even if our results in individuals' metabolomics profiles seem to indicate an increase in energy metabolism, the associated increase in ATP production did not positively affect all life-history traits, due to potential physiological costs and trade-offs (Jarrold *et al.*, 2019). Trade-offs between life-history traits and physiological systems are frequent in organisms, as each trait competes for the same limited resource, energy, that must be allocated between growth, reproduction, and cellular maintenance (Reznick, 1985; Roff, 1992; Zera and Harshman, 2001). These trade-offs can fundamentally influence organisms' fitness (Massamba-N'Siala *et al.*, 2014). The higher energy requirement for physiological adjustments, maintenance, and reparation in *O. labronica* individuals exposed to 30 °C

could have reduced the available energy for the life history traits (i.e. growth and reproduction), leading to a potential trade-off between these traits. As a result, the individuals seemed to have allocated more energy in their growth, at the expense of reproduction, as individuals exposed at 30 °C are bigger in the first period and of the same size thereafter, but have a lower fecundity (lower number of eggs *per* egg mass).

The fact that individuals exposed to 30 °C reach the same size than the ones exposed to 24 °C for the second and third 'Period' appears not to corroborate to the temperature-size rule which stipulates that individuals reared at higher temperature are smaller when they reach sexual maturity, as temperature increases developmental rates (Atkinson, 1994; Angilletta *et al.*, 2004; Daufresne, 2009). This rule has been supported by an increasing number of studies showing evidence of size reduction in marine and terrestrial ectotherms as a consequence of increasing temperature, both in modern time and in the paleo past (Daufresne, 2009; Gardner *et al.*, 2011; Sheridan and Bickford, 2011; Calosi *et al.*, 2019). Similarly, a decrease in size from 24 to 30 °C in *O. labronica* has also been observed in a previous study (Åkesson, 1976). In our case, individuals reared at 30 °C reached the same size than their counterparts in control condition (24 °C) during their spawning event ('Period' 3), despite the fact that they seem to reach, on average, this developmental stage faster than the worms reared at 24 °C. This observation supports the idea that they allocated more resources to grow in the elevated temperature treatment. Reaching a greater size for reproduction can be advantageous for marine ectotherms' fitness as fecundity generally increases with individuals' size (Angilletta *et al.*, 2004; Arendt, 2010; Massamba-N'Siala *et al.*, 2012). *Ophryotrocha labronica* is no exception, as its fecundity is strongly associated with its number of chaetigers (Massamba-N'Siala *et al.*, 2011). Thus, this could explain why they invested energy in growth to ensure they maintain a good fecundity rate. Nevertheless, this is not the case in this experiment. Even with the same reproduction size than the individuals exposed to 24 °C, they are not able to maintain the same level of fecundity as their counterparts, as they produce fewer eggs *per* egg mass. This result could support our hypothesis of the existence of a trade-off between size and fecundity. By investing more energy to sustain continuous growth, the remaining energy available for

reproduction might not have been sufficient to maintain or even increase their fecundity level in comparison to control condition. However, the decrease in fecundity observed at 30 °C was much lower than the decrease observed in the previous study: 6 % instead of 36 % after a within-generation exposure in Chakravarti *et al.* (2016). The lower fecundity observed may, therefore, be a strategy for *O. labronica* individuals to optimize their reproductive output for later spawning events and not the consequence of a lack of energy to support their reproduction. The fact that the fertility trait remains unchanged between the two temperatures tested tend further to support this idea, or otherwise fertility should have decreased too. In fact, growth continues when organisms reach sexual maturity in this species: *O. labronica* individuals continue to add chaetigers along their spawning events. In addition, the temperature is known to increase the reproduction rate in *O. labronica*, as they spawn more frequently, with a shorter interval between each spawning event (Prevedelli and Simonini, 2001). As a result, they could have invested less energy in their first egg mass, to ensure that they can spawn the next egg mass sooner, at a bigger size.

Finally, survival rate indicates a low level of mortality in the elevated temperature treatment, which confirms that 30 °C is still within the thermal window and the range of full aerobic scope throughout the ontogeny in *O. labronica* (Massamba-N'Siala *et al.*, 2012). As the decrease in the survival rate in the third 'Period' comparatively to the first two 'Period' was similar between the two temperature treatments, we conclude that this decrease in survival rate is the by-product of trade-offs caused by thermal adjustments and costs of reproduction. It also confirms the idea that the increase in energy production through modification in metabolic pathways was sufficient to support organisms' energy needs.

4.5 Conclusion and future perspectives

In light of our results, we can conclude that the elevated temperature affects specifically higher biological compartments (metabolomics profiles and life history traits). However, organisms' responses to increasing temperature are usually complex and include multiple traits that can interact and influence each other (Woods and Wilson, 2015; Woods

et al., 2015). Accordingly, an absence of changes in one trait following an increase in temperature does not necessarily imply that it is not affected by temperature, as changes in other biological compartments could enable organisms to maintain this trait unchanged (Woods and Wilson, 2015). This is why, by enabling to analyse simultaneously different biological traits in different biological compartments through networks analyses, the integrative approach is particularly relevant to study plastic changes. However, development in the statistical tools available for "omics" data and network analyses is needed in order to beneficiate fully from this approach.

In the case of *O. labronica,* exposure to the elevated temperature treatment seems to affect particularly metabolic pathways and metabolism rate, but overall, does not affect individuals' fundamental ability to reproduce, grow and survive after 20 d of exposure. This indicates that individuals of *O. labronica* are able to tolerate and acclimate to this elevated temperature through physiological adjustment of their aerobic metabolism. As plastic adjustments of the metabolism and the metabolic pathways are known for their rapidity (Hochachka and Somero, 1984), it is unsurprising that it is, with the life-history traits, the only biological compartment where differences between the temperatures tested were detected. The fact that the elevated temperature used in this study has already been recorded in the southern part of their natural environment (Massamba-N'Siala *et al.*, 2011), could explain why we did not observe more visible reductions in organisms' fitness on this short-term exposure. However, with the increasing temperature associated with global warming, high temperatures could occur more frequently and on a longer period in their natural environment. Consequently, considering that detrimental effects of temperature can accumulate with the duration of exposure (Schulte, 2015), it would be of great interest to evaluate the effect of elevated temperature on different biological traits of different biological compartments over longer exposition time (e.g. Chakravarti *et al*., 2016, Gibbin *et al.*, 2017b).

More specifically, integrative multigenerational research looking at the effect of temperature and maternal effects could be highly relevant, as many studies, including some

with *O. labronica*, (Massamba-N'Siala *et al.*, 2014; Chakravarti *et al*., 2016; Gibbin *et al.*, 2017b) have demonstrated that the environmental conditions experienced by previous generations can affect future generations' responses to elevated temperature (i.e. transgenerational plasticity, Munday *et al.*, 2013; Chakravarti *et al*., 2016; Gibbin *et al.*, 2017b). However, in *O. labronica* species, these plastic responses differed between the transgenerational and the multigenerational exposure: while transgenerational exposure fully alleviated the negative impacts of the within exposure to elevated temperature in reproductive traits (Chakravarti *et al*., 2016), longer exposure resulted in an important decrease in fecundity, potentially due to the accumulation of harmful effects (Gibbin *et al.*, 2017b). However, few of these multigenerational studies have been conducted on a fine within-generation temporal scale, and with the simultaneous measurement of temperature responses in different biological compartments ranging from genes to the life-history traits, as proposed in this current study. This prevents us from pinpointing the mechanisms involved in long-term plastic responses and the associated costs, leading potentially to erroneous conclusions (Munday *et al.*, 2013). In turn, this can limit our comprehension on how and which mechanisms underpinning multigenerational responses can facilitate rapid adaptation. In this regard, the combination of our integrative approach with a multigenerational one could contribute to a better understanding of the mechanisms underpinning long term plastic responses. In which biological compartments does plastic responses to elevated temperature are most visible? Are they changes in plastic responses across generations of exposure? Are such changes improving species fitness, or are they associated with a high energetic cost that accumulates through time? A multigenerational integrative mechanistic approach will considerably improve our ability to predict the impact of global warming on species' fitness, and to evaluate the ability of marine ectotherms to rapidly adapt. In turns, these findings could be integrated into ecological models and improve our predictions of the fate of biodiversity and ecosystem functioning in a warmer world.

Acknowledgments

The authors would like to thank Francis Beaudet, Juliette Debacker, Marie-Hélène Carignan, Cynthia Thibault and Christine Isabel for their assistance in the experiment, as well as Catherine Marcil for assisting with the culture maintenance and Mathieu Babin for his support and advices on the metabolomics analyses.

Competing interests

The authors declare no competing interest.

Author contributions

F.V., G.M.N., and P.C. designed the experimental design. P.D.W. helped with the genome assembly and the primers design and P.D.W., F.D., A.T. contributed in the development of the mitochondrial gene expression analyses. F.V. and G.M.N performed the research, and F.V. analysed the data with the statistical support of P.C. P.A. and D.M. Finally, F.V. developed the first advanced draft and all graphical support, and all authors contributed to the final manuscript.

Funding

This work was financed by the Discovery Program grant from the Natural Sciences and Engineering Research Council of Canada (NSERC, RGPIN-2015-06500), a grant from the Programme Établissement de nouveaux chercheurs universitaires of the Fonds de Recherche du Québec – Nature et Technologies (No. 199173), a funding from the Fonds Institutionnel de Recherche of the Université du Québec à Rimouski and the Kungl. Vetenskapsakademien grant (KVA) all awarded to PC. FV was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Alexander Graham Bell Canada Graduate Scholarships with a NSERC Michael Smith Foreign Study Supplements, a Fond de Recherche du Québec – Nature and Technologies (FRQNT) Master Scholarship as well as an FRQNT International internship grant (No. 211134).

REFERENCES

Abele, D., Heise, K., Pörtner, H.O. and Puntarulo, S. (2002). Temperature dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria. J. Exp. Biol.* **205**, 1831-1841.

Åkesson, B. (1970). *Ophryotrocha labronica* as test animal for the study of marine pollution. *Helgoland. Wiss. Meer.* **20**, 293-303.

Åkesson, B. (1976). Temperature and life cycle in *Ophryotrocha labronica* (Polychaeta, Dorvilleidae). *Ophelia*. **15**, 37-47.

Andrew, S. (2010). FastQC: a quality control tool for high throughput sequence data. Available online at: *http://www.bioinformatics.babraham.ac.uk/projects/fastqc*.

Angilletta, M.J., Steury, T.D. and Sears, M.W. (2004). Temperature, Growth Rate, and Body Size in Ectotherms: Fitting Pieces of a Life-History Puzzle. *Integr. Comp. Biol.* **44**, 498-509.

Angilletta, M.J. (2009). *Thermal Adaptation. A Theoretical and Empirical Synthesis.* Oxford, UK: Oxford University Press.

Arendt, J.D. (2010). Size-fecundity relationships, growth trajectories, and the temperature-size rule for ectotherms. *Evolution,* **65**, 43-51.

Atkinson D. (1994). Temperature and organism size: A biological law for ectotherms? *Adv. Ecol. Res*. **25**, 1-58.

Aubin-Horth, N. and Renn, S.C. (2009). Genomic reaction norms: using integrative biology to understand molecular mechanisms of phenotypic plasticity. *Mol. Ecol.* **18**, 3763-3780.

Barnosky, A.D., Matzke, N., Tomiya, S., Wogan, G.O.U., Swartz, B., Quental, T.B., Marshall, C., McGuire, J.L., Lindsey, E.L., Maguire, K.C. *et al.* (2011). Has the Earth's sixth mass extinction already arrived? *Nature*. **471**, 51-57.

Bartholomew, G.A. (1986). The Role of Natural History in Contemporary Biology. *BioScience*. **36**, 324-329.

Bates, D., Mächler, M., Bolker, B. and Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* **67**, 1-48.

Bellard, C., Bertelsmeier, C., Leadley, P., Thuiller, W. and Courchamp, F. (2012). Impacts of climate change on the future of biodiversity. *Ecol. Lett.* **15**, 365-377.

Bergmeyer, H. U., Bergmeyer, J. and Grassl, M. (1983). *Methods of enzymatic analysis*. Deerfield beach, USA: Verlag Chemie.

Bernt, M., Donath A., Jühling, F., Externbrink F., Florentz,, C., Fritzsch G., Pütz, J., Middendorf, M. and Stadler, P.F. (2013). MITOS: Improved *de novo* Metazoan Mitochondrial Genome Annotation. *Mol. Phylogenet. Evol.* **69**, 313-319.

Blier, P.U. and Lemieux, H. (2001). The impact of the thermal sensitivity of cytochrome c oxidase on the respiration rate of Arctic charr red muscle mitochondria. *J. Comp. Physiol. B. Biochem. Syst. Environ. Physiol.* **171**, 247-253.

Blier, P.U., Lemieux, H. and Pichaud, N. (2014). Holding our breath in our modern world: will mitochondria keep the pace with climate changes? *Can. J. Zool.* **92**, 591-601.

Boyd, P.W., Cornwall, C.E., Davison, A., Doney, S.C., Fourquez, M., Hurd, C.L., Lima, I.D et McMinn, A. (2016). Biological responses to environmental heterogeneity under future ocean conditions. *Glob. Change. Biol.* **22**, 2633-2650.

Bozinovic, F., Calosi, P. and Spicer, J.I. (2011). Physiological Correlates of Geographic Range in Animals. *Annu. Rev. Ecol. Evol. Syst.* **42**, 155-179.

Bozinovic, F. and Naya, D.E. (2015). Linking physiology, climate, and species distributional ranges. In *Integrative Organismal Biology* (ed. L.B. Martin, C.K. Ghalambor and H.A. Woods), pp. 277-290. New Jersey, USA: Wiley Blackwell.

Bradshaw, A.D. (1965). Evolutionary Significance of Phenotypic Plasticity in Plants. *Adv. Genet.* **13**, 115-155.

Brand, M.D. and Esteves, T.C. (2005). Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. *Cell. Metab.* **2**, 85-93.

Calosi, P., Rastrick, S.P.S., Lombardi, C., Guzman, H.J., Davidson, L., Jahnke, M. Giangrande, A., Hardege, J.D., Schulze, A., Spicer, J.I. and Gambi, M-C. (2013). Adaptation and acclimatization to ocean acidification in marine ectotherms: an *in situ* transplant experiment with polychaetes at a shallow CO_2 vent system. *Phil. Trans. R. Soc. B.* **368**, 20120444.

Calosi, P., De Wit, P., Thor, P. and Dupont, S. (2016). Will life find a way? Evolution of marine species under global change. *Evol. Appl.* **9**, 1035-1042.

Calosi, P., Putnam, H.M., Twitchett, R.J. and Vermandele, F. (2019). Marine Metazoan Modern Mass Extinction: Improving Predictions by Integrating Fossil, Modern and Physiological Data. *Annu. Rev. Mar. Sci.* **11**, 20.1-20.22.

Careau, V., Killer, S.S. and Metcalfe, N.B. (2015). Adding fuel to the "fire of life": energy budgets across levels of variation in ectotherms and endotherms. In *Integrative Organismal Biology* (ed. L.B. Martin, C.K. Ghalambor and H.A. Woods), pp. 219-233. New Jersey, USA: Wiley Blackwell.

Ceballos, G., Ehrlich, P.R., Barnosky, A.D., Garcia, A., Pringle, R.M. and Palmer, T.M. (2015). Accelerated modern human-induced species losses: Entering the sixth mass extinction. *Science*. **1**, e1400253.

Ceballos, G. and Ehrlich, P.R. (2018). The misunderstood sixth mass extinction. *Science*. **360**, 1080-1081.

Chakravarti, L.J., Jarrold, M.D., Gibbin, E.M., Christen, F., Massamba-N'Siala, G., Blier, P.U. and Calosi, P. (2016). Can trans-generational experiments be used to enhance species resilience to ocean warming and acidification? *Evol. Appl.* **9**, 1133-1146.

Chevin, L-M., Lande, R. and Mace, G.M. (2010). Adaptation, Plasticity, and Extinction in a Changing Environment: Towards a Predictive Theory. *PLoS Biol.* **8**, e1000357.

Chevin, L-M., Gallet, R., Gomulkiewicz, R., Holt, R.D. and Fellous, S. (2013). Phenotypic plasticity in evolutionary rescue experiments. *Phil. Trans. R. Soc. B.* **368**, 20120089.

Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G. Wishart, D.S. and Xia, J. (2018). MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucl. Acids. Res.* **46**, 486-494.

Chung, D.J and Schulte, P.M. (2015). Mechanisms and costs of mitochondrial thermal acclimation in a eurythermal killifish (*Fundulus heteroclitus*). *J. Exp. Biol.* **218**, 1621-1631.

Claireaux, G. and Lefrançois, C. (2007). Linking environmental variability and fish performance: integration through the concept of scope for activity. *Phil. Trans. R. Soc. Lond. B. Biol. Sci.* **362**, 2031-2041.

Clark, T.D., Sandblom, E. and Jutfelt, F. (2013a). Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *J. Exp. Biol.* **216**, 2771-2782.

Clark, T.D., Sandblom, E. and Jutfelt, F. (2013b). Response to Farrell and to Pörtner and Giomi. *J. Exp. Biol.* **216**, 4495-4497.

Daufresne M, Lengfellner K and Sommer U. (2009). Global warming benefits the small in aquatic ecosystems. *Proc. Natl. Acad. Sci. U S A*. **106**, 12788-12793.

Deutsch, C.A., Tewksbury, J.J, Huey, R.B., Sheldon, K.S., Ghalambor, C.K., Haak, D.C. and Martin, P.R. (2008). Impacts of climate warming of terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci.* **105**, 6668-6672.

DeWitt, T.J., Sih, A. and Wilson, D.S. 1998. Costs and limits of phenotypic plasticity. *Trends. Ecol. Evol.* **13**, 151-154.

De Wit, P., Dupont, S. and Thor, P. (2016). Selection on oxidative phosphorylation and ribosomal structure as a multigenerational response to ocean acidification in the common copepod *Pseudocalanus acuspes*. *Evol. Appl.* **9**, 1112-1123.

Diamond, S.E. (2018). Contemporary climate-driven range shifts: Putting evolution back on the table. *Funct. Ecol.* **32**, 1652-1665.

Dobzhansky, T. (1964). Biology, Molecular and Organismic. *Am. Zool.* **4**, 443-452.

Donelson, J.M., Salinas, S., Munday, P.L. and Shama, L.N.S. (2018). Transgenerational plasticity and climate change experiments: Where do we go from here? *Global. Change. Biol.* **24**, 13-34.

Etterson, J.R. and Shaw, R.G. (2001). Constraint to Adaptive Evolution in Response to Global Warming. *Science*. **294**, 151-154.

Forsman, A. (2015). Rethinking phenotypic plasticity and its consequences for individuals, populations and species. *Heredity*. **115**, 276-284.

Forster, J. and Hirst, A.G. (2012). The temperature-size rule emerges from ontogenetic differences between growth and development rates. *Funct. Ecol.* **26**, 483-492.

Gardner, J.L, Peters A, Kearney, M.R, Joseph, L and Heinsohn, R. (2011). Declining body size: a third universal response to warming? *Trends. Ecol. Evol*. **26**, 285-291.

Garvin, M.R., Thorgaard, G.H. and Narum, S.R. (2015). Differential Expression of Genes that Control Respiration Contribute to Thermal Adaptation in Redband Trout (*Oncorhynchus mykiss gairdneri*). *Genome. Biol. Evol.* **7**, 1404-1414.

Ghalambor, C.K., McKay, J.K., Carroll, S.P. and Reznick, D.N. (2007). Adaptive *versus* non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Funct. Ecol.* **21**, 394-407.

Ghalambor, C.K., Hoke, K.L., Ruell, E.W., Fischer, E.K., Reznick, D.N. and Hughes, K.A. (2015). Non-adaptive plasticity potentiates rapid adaptive evolution of gene expression in nature. *Nature.* **525**, 372-375.

Gibbin, E.M., Massamba-N'Siala, G., Chakravarti, L.J, Jarrold, M.D. and Calosi, P. (2017a). The evolution of phenotypic plasticity under global change. *Sci. Rep.* **7**, 17253.

Gibbin, E.M., Chakravarti, L.J., Jarrold. M.D., Christen, F., Turpin, V., Massamba-N'Siala, G., Blier P.U., and Calosi, P. (2017b). Can multi-generational exposure to ocean warming and acidification lead to adaptation of life-history and physiology in a marine metazoan? *J. Exp. Biol.* **220**, 551-563.

Gillooly, J.F., Brown, J.H., West, G.B., Savage, V.M. and Charnov, E.L. (2001). Effects of Size and Temperature on Metabolic Rate. *Science.* **293**, 2248-2251.

Godbold, J.A. and Calosi, P. (2013). Ocean acidification and climate change: advances in ecology and evolution. *Phil. Trans. R. Soc. B.* **368**, 20120448.

Graier, W.F., Trenker, M. and Malli, R. (2008). Mitochondrial Ca2+, the secret behind the function of uncoupling proteins 2 and 3? *Cell. Calcium.* **44**, 36-50.

Guderley, H. and St-Pierre, J. (2002). Going with the flow or life in the fast lane: contrasting mitochondrial responses to thermal change. *J. Exp. Biol.* **205**, 2237-2249.

Halanych, K.M. and Borda, E. (2009). Developing Models for Lophotrochozoan and Annelid Biology. In *Annelids in Modern Biology* (ed. D. H. Shain), pp. 1-12. New Jersey, USA: Wiley-Blackwell.

Hochachka, P.W. and Somero, G.N. (1984). *Biochemical Adaptation*. Princeton, USA: Princeton University Press.

Hodgins-Davis, A. and Townsend, J.P. (2009). Evolving gene expression: from G to E to G x E. *Trends. Ecol. Evol.* **24**, 649-658.

Hoffman, A.A. (1995). Acclimation: increasing survival at a cost. *Trends. Ecol. Evol.* **10**, 1-2.

Hothorn, T., Bretz, F. and Westfall, P. (2008). Simultaneous Inference in General Parametric Models. *Biometrical J.* **50**, 346-363.

Hulbert, A.J. and Else, P.L. (1999). Membranes as Possible Pacemakers of Metabolism. *J. theor. Biol.* **199**, 257-274.

Hulbert, A.J. (2007). Membrane fatty acids as pacemakers of animal metabolism. *Lipids*. **42**, 811-819.

IPCC (2014). Climate Change 2014: Synthesis report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.

Jarrold, M.D., Chakravarti, L.J., Gibbin, E.M., Christen, F., Massamba-N'Siala, G., Blier, P.U. and Calosi, P. (2019). Life-history trade-offs and limitations associated with phenotypic adaptation under future ocean warming and elevated salinity. *Phil. Trans. R. Soc. B.* **374**, 20180428.

Jutfelt, F., Norin, T., Ern, R., Overgaard, J., Wang, T., McKenzie, D.J., Lefevre, S., Nilsson, G.E., Metcalfe, N.B., Hickey, A.J.R. *et al.* (2018). Oxygen-and capacity-limited thermal tolerance: blurring ecology and physiology. *J. Exp. Biol.* **221**, jeb169615.

Karnovsky, A., Weymouth, T., Hull, T., Tarcea, V.G., Scardoni, G., Laudanna, C., Sartor, M.A., Stringer, K.A., Jagadish, H.V., Burant, C. *et al.* (2012). Metscape 2 bioinformatics tool for the analysis and visualization of metabolomics and gene expression data. *Bioinformatics*. **28**, 373-380.

Koressaar, T. and Remm, M. (2007). Enhancements and modifications of primer design program Primer3. *Bioinformatics.* **23**, 1289-1291.

Krueger, F. (2015). Trim galore. A wrapper tool around Cutadapt and FastQC to consistently apply quality and adapter trimmer to FastQ files. Available online at: https://github.com/FelixKrueger/TrimGalore.

Kuznetsova, A., Brockhoff, P.B. and Christensen, R.H.B. (2017). lmerTest Package: Tests in Linear Mixed Effects Models. *J. Stat. Softw.* **82**, 1-26.

LaGreca, M. and Bacci, G. (1962). Una nuova specie di Ophryotrocha delle costa tirreniche. *B. Zool.* **29**, 13-23.

Lannig, G., Eilers, S., Pörtner, H.O., Sokolova, I.M. and Christian Bock (2010). Impact of Ocean Acidification on Energy Metabolism of Oyster, *Crassostrea gigas* – Changes in Metbolic Pathways and Thermal Response. *Mar. Drugs*. **8**, 2318-2339.

Lehninger, A.L. (2005). *Lehninger principles of biochemistry*, 4th ed.. New York, USA: W.H. Freeman.

Livak, K.J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* **25**, 402-408.

Lu, W., Kimball, E. and Rabinowitz, J.D. (2006). A high-performance liquid chromatography-tandem mass spectrometry method for quantification of nitrogen-containing intracellular metabolites. *J. Am. Soc. Mass. Spectr*. **17**, 37-50.

Luke, S.G. (2017). Evaluating significance in linear mixed-effects models in R. *Behav. Res. Methods.* **49**, 1494-1502.

Luo, R., Liu, B., Xie, Y., Li, Z., Huang, W., Yuan, J., He, G., Chen, Y., Pan, Q., Liu, Y., *et al*. (2012). SOAPdenovo2: an empirically improved memory-efficiency short-read de novo assembler. *Gigascience*. **1**, 18.

Martin, P.R. (2015). Trade-offs and biological diversity: integrative answers to ecological questions. In *Integrative Organismal Biology* (ed. L.B. Martin, C.K. Ghalambor and H.A. Woods), pp. 291-308. New Jersey, USA: Wiley Blackwell.

Massamba-N'Siala, G. (2008). Microevoluzione e riscaldamento globale: ruolo dei pattern climatici regionali nel differenziamento delle storie vitali di popolazioni del polichete modello *Ophryotrocha labronica* (Polychaeta). PhD thesis, XX cycle, Università di Modena e Reggio Emilia (Italy).

Masamba-N'Siala, G., Simonini, G., Cossu, P., Maltagliati, F., Castelli, A. and Prevedelli, D. (2011). Life-history and demographic spatial variation in Mediterranean populations of the opportunistic polychaete Ophryotrocha labronica (Polychaeta, Dorvilleidae). *Mar. Biol.* **158**, 1523-1535.

Massamba-N'Siala, G., Calosi, P., Bilton, D.T., Prevedelli, D. and Simonini, R. (2012). Life-history and thermal tolerance traits display different thermal plasticities and

relationships with temperature in the marine polychaete *Ophryotrocha labronica* La Greca and Bacci (Dorvillidae). *J. Exp. Mar. Biol. Ecol.* **438**, 109-117.

Massamba-N'Siala, G., Prevedelli, D. and Simonini, R. (2014). Trans-generational plasticity in physiological thermal tolerance is modulated by maternal pre-reproductive environment in the polychaete *Ophryotrocha labronica. J. Exp. Biol.* **217**, 2004-2012.

Mills, S. W., Beaulieu, S.E. and Mullineaux, L.S. (2007). Photographic identification guide to larvae at hydrothermal vents in the eastern Pacific. Available online at: http://www.whoi.edu/vent-larval-id/.

Moyes, C.D., Mathieu-Costello, O.A., Tsuchiya, N., Filburn, C. and Hansford, R.G. (1997). Mitochondrial biogenesis during cellular differentiation. *Am. J. Physiol.* **272**, 1345-1351.

Munday, P.L., Warner, R.R., Monro, K., Pandolfi, J.M. and Marshall, D.J. (2013). Predicting evolutionary responses to climate change in the sea. *Ecol. Lett.* **16**, 1488-1500.

Murphy, M.P. (2009). How mitochondria produce reactive oxygen species. *Biochem. J.* **417**, 1-13.

Nègre-Salvayre, A., Hirtz, C., Carrera, G., Cazenave, R., Troly, M., Salvayre, R., Pénicaud, L. and Casteilla, L. (1997). A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. *FASEB J.* **11**, 809-815.

Nicholls, D.G. (2006). The physiological regulation of uncoupling proteins. *Biochim. Biophys, Acta.* **1757**, 459-466.

Parmesan, C. and Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature.* **421**, 37-42.

Paxton, H. and Åkesson, B. (2007). Redescription of *Ophryotrocha puerilis and O. labronica* (Annelida: Dorvilleidae). *Mar. Biol. Res.* **3**, 3-19.

Paxton, H. and Åkesson, B. (2010). The *Ophryotrocha labronica group* (Annelida: Dorvilleidae) – With the description of seven new species. *Zootaxa*. **2713**, 1-24.

Peck, L.S., Morley, S.A. and Clark, M.S. (2010). Poor acclimation capacities in Antarctic marine ectotherms. *Mar. Biol.* **157**, 2051-2059.

Perry, A.L., Low, P.J., Ellis, J.R. and Reynolds, J.D. (2005). Climate Change and Distribution Shifts in Marine Fishes. *Science*. **308**, 1912-1915.

Pfennig, D.W., Wund, M.A., Snell-Rood, E.C., Cruickshank, T., Schlichting, C.D. and Moczek, A.P. (2010). Phenotypic plasticity's impacts on diversification and speciation. *Trends. Ecol. Evol.* **25**, 459-467.

Pigliucci, M. (2001). *Phenotypic plasticity: beyond nature and nurture*. Maryland, USA: JHU Press.

Pigliucci, M. (2003). Phenotypic integration: studying the ecology and evolution of complex phenotypes. *Ecology Letters*. **6**, 265-272.

Pigliucci, M. and Preston, K. (2004). *Phenotypic Integration: Studying in the Ecology and Evolution of Complex Phenotype*s. Oxford, UK: Oxford University Press.

Pörtner, H. (2001). Climate change and temperature-dependent biogeography: Oxygen limitation of thermal tolerance in animals. *Naturwissenschaften*. **88**, 137-146.

Pörtner, H. (2002). Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **132**, 739-761.

Pörtner, H.O., Bennett, A.E., Bozinovic, F., Clarke, A., Lardies, M.A., Lucassen, M., Pelster, B., Schiemer, F. and Stillman, J.H. (2006a). Trade-Offs in Thermal Adaptation: The Need for a Molecular to Ecological Integration. *Physiol. Biochem. Zool.* **79**, 295-313.

Pörtner, H-O. (2006b). Climate-dependent evolution of Antarctic ectotherms: An integrative analysis. *Deep-Sea Res II*. **53**, 1071-1104.

Pörtner, H.O. and Knust, R. (2007). Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science*. **315**, 95-97.

Pörtner, H.O. and Farrell, A.P. (2008). Ecology. Physiology and climate change. *Science.* **322**, 690-692.

Prevedelli, D. and Simonini, R. (2001). Effect of temperature on demography of *Ophryotrocha labronica* (Polychaeta: Dorvilleidae). *Vie. Milieu*. **54**, 173-180.

Prevedelli, D., Massamba-N'Siala, G. and Simonini R. (2005). The seasonal dynamics of six species of Dorvileidae (Polychaeta) in the harbour of La Spezia (Itlay). *Mar. Ecol.* **26**, 286-293.

Purohit, G.K., Mahanty, A. Mohanty, B.P. and Mohanty, S. (2016). Evaluation of housekeeping genes as references for quantitative real-time PCR analysis of gene expression in the murrel *Channa striatus* under high-temperature stress. *Fish. Physiol. Biochem.* **42**, 125-135.

Rabøl, R., Boushel, R. and Dela, F. (2006). Mitochondrial oxidative function and type 2 diabetes. *Appl. Physiol. Nutr. Metab.* **31**, 675-683.

R Core Team. (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available online at: https://www.R-project.org/.

Reusch, T.B.H. (2014) Climate change in the oceans: evolutionary versus phenotypically plastic responses of marine animals and plants. *Evol. Appl.* **7**, 104-122.

Reznick, D.N. (1985). Costs of Reproduction: An Evaluation of the Empirical Evidence. *Oikos*. **44**, 257-267.

Rodriguez-Romero, A., Jarrold, M.D., Massamba-N'Siala, G., Spicer, J.I. and Calosi, P. (2015). Multi-generational responses of a marine polychaete to a rapid change in seawater *p*CO2. *Evol. Appl.,* **9**, 1082-1095.

Roff, D.A. (1992). *The Evolution of Life Histories*. New York, USA: Chapman & Hal.

Roff, D.A. and Gélinas, M.D. (2002). Phenotypic plasticity and the evolution of trade-offs: the quantitative genetics of resource allocation in the wing dimorphic cricket, *Gryllus firmus*. *J. Evolution. Biol.* **16**, 55-63.

Schlichting, C.D. (1989). Phenotypic integration and environmental change. *BioScience.* **39**, 460-464.

Schlichting, C.D. and Pigliucci, M. (1998). *Phenotypic Evolution: A Reaction Norm Perspective*. Sunderland, USA: Sinauer Associates.

Schmidlin, L., von Fumetti, S. and Nagel, P. (2015). Temperature effects on the feeding and electron transport system (ETS) activity of *Gammarus fossarum*. *Aquat. Ecol.* **49**, 71-80.

Schulte, P.M., Healy, T.M. and Fangue, N.A. (2011). Thermal performance curves, phenotypic plasticity, and the time scales of temperature exposure. *Integr. Comp. Biol.* **51**, 691-702.

Schulte, P.M. (2015). The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *J. Exp. Biol.* **218**, 1856-1866.

Shama, L.N.S., Mark, F.C., Strobel, A., Lokmer, A., John, U. and Wegner, K.M. (2016). Transgenerational effects persist down the maternal line in marine sticklebacks: gene expression matches physiology in a warming ocean. *Evol. Appl.* **9**, 1096-1111.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B. and Ideker, T. (2003). Cytoscape: a software environment for integrated modes of biomolecular interaction networks. *Genome. Res.* **13**, 2498-2504.

Sheridan, J.A and Bickford, D. (2011). Shrinking body size as an ecological response to climate change. *Nat. Clim. Chang*. **1**, 401-406.

Simcic, T. and Brancelj, A. (2006). Effects of pH on electron transport system (ETS) activity and oxygen consumption in *Gammarus fossarum, Asellus aquaticus* and *Niphargus sphagnicolus*. *Freshwater. Biol.* **51**, 686-694.

Simcic, T., Jesensek, D. and Anton, B. (2015). Metabolic Potential, Respiration Rate and Their Relationship in Offspring of Different Sizes of Marble Trout (*Salmo marmoratus Cuvier*). *Turk. J. Fish. Aquat. Sc.* **15**, 39-48.

Simonini, R., Massamba-N'Siala, G., Grandi, V. and Prevedelli, D. (2009). Distribution of the genus *Ophryotrocha* (Polychaeta) in Italy: new records and comments on the biogeography of Mediterranean species. *Vie. Milieu.* **59**, 79-88.

Simonini, R., Grandi, V., Massamba-N'Siala, G., Martino, M.P., Castelli, A. and Prevedelli, D. (2010). Diversity, habitat affinities and diet of *Ophryotrocha* species (Polychaeta, Dorvilleidae) living in Mediterranean harbour habitats. *Vie. Milieu.* **60**, 27-38.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem*. **1**, 76-85.

Somero, G.N. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. *J. Exp. Biol.* **213**, 912-920.

Somero, G.N. (2012). The Physiology of Global Change: Linking Patterns to Mechanisms. *Annu. Rev. Mar. Sci.* **4**, 39-61.

Stearns, S.C. (1989). Trade-Offs in Life-History Evolution. *Funct. Ecol.* **3**, 259-268.

Strange, K. (2005). The end of "naïve reductionism": rise of systems biology or renaissance of physiology? *Am. J. Physiol.* **288**, 968-974.

Strobel, A., Graeve, M., Pörtner, H.O. and Mark, F.C. (2013). Mitochondrial Acclimation Capacities to Ocean Warming and Acidification Are Limited in the Antarctic Nototheniid Fish, *Notothenia rossii* and *Lepidonotothen squamifrons*. *PLoS One.* **8**, e68865.

Sunday, J.M., Calosi, P., Dupont, S., Munday, P.L., Stillman, J.H. and Reush, T.B.H. (2014b). Evolution in an acidifying ocean. *Trends. Ecol. Evol.* **29**, 117-125.

Thibault, M., Blier, P.U. and Guderley, H. (1997). Seasonal variation of muscle metabolic organization in rainbow trout (*Oncorhynchus mykiss*). *Fish. Physiol. Biochem.* **16**,139-155.

Thor, P. and Dupont, S. (2015). Transgenerational effects alleviate severe fecundity loss during ocean acidification in a ubiquitous planktonic copepod. *Glob. Change. Biol.* **21**, 2261-2271.

Thornhill, D.J., Dahlgren, T.G. and Halanych, K. (2009). The evolution and ecology of *Ophyrotrocha* (Dorvilleidae, Eunicida). In *Annelids in Modern Biology* (ed. D. H. Shain), pp. 242-256. New Jersey: Wiley-Blackwell.

Tomanek, L. (2015). Proteomic responses to environmentally induced oxidative stress. *J. Exp. Biol.* **218**, 1867-1879.

Török, Z., Crul, T., Maresca, B., Schütz, G.J., Viana, F., Dindia, L., Piotto, S., Brameshuber, M., Balogh, G., Péter, M. *et al.* (2014). Plasma membranes as heat stress sensors: From lipid-controlled molecular switches to therapeutic applications. *Biochim. Biophys. Acta.* **1838**, 1594-1618.

Untergrasser, A., Cutcutache, I., Koressaar, T., Ye. J., Faircloth, B.C., Remm, M. and Rozen, S.G. (2012). Primer3 – new capabilities and inferfaces. *Nucleic. Acids. Res.* **40**, e115.

Van den Berg, R.A., Hoefsloot, H.C., Westerhuis, J.A., Smilde, A.K. and van der Werf, M.J. (2006). Centering, scaling and transformations: improving the biological information content of metabolomics data. *BMC Genomics*. **7**, 142.

Verberk, W.C.E.P., Sommer, U., Davidson, R.L. and Viant, M.R. (2013). Anaerobic Metabolism at Thermal Extremes: A Metabolomic Test of the Oxygen Limitation Hypothesis in an Aquatic Insect. *Integr. Comp. Biol.* **53**, 609-619.

Visser, M.E. (2008). Keeping up with a warming world; assessing the rate of adaptation to climate change. *Proc. R. Soc. B.* **275**, 649-659.

Wake, M.H. (2008). Integrative Biology: Science for the 21st Century. *BioScience.* **58**, 349-353.

Wan, Q., Chen, S., Shan, Z., Yang, Z., Chen, L., Zhang, C., Yuan, S., Hao, Q., Zhang, X., Qiu, D., Chen, H. and Zhou, X. (2017). Stability evaluation of reference genes for gene expression analysis by RT-qPCR in soybean under different conditions. *PLoS One.* **12**, e0189405.

Weber, J-M. (2011). Metabolic fuels: regulating fluxes to select mix. *J. Exp. Biol.* **214**, 286-294.

Wei, R., Wang, J., Mingming, S., Jia, E., Chen, S., Chen, T. and Ni, Y. (2018a). Missing Value Imputation Approach for Mass Spectrometry-based Metabolomics Data. *Sci. Rep.* **8**, 663.

Wei, R., Wang, J., Jia, E., Chen, T., Ni, Y. and Jia, W. (2018b). GSimp: A Gibbs sampler based left-censored missing value imputation approach for metabolomics studies. *PLoS. Comput. Biol.* **14**, e1005973.

West-Eberhard, M. (2003). *Developmental plasticity and evolution*. New York, USA: Oxford University Press.

Whitman, D.W. and Agrawal, A.A. (2009). What is Phenotypic Plasticity and Why is it Important. In *Phenotypic Plasticity of Insects: Mechanisms and Consequences* (ed. D.W. Whitman, and T.N. Ananthakrishan), pp. 1-63. Enfield, USA: Science Publishers, CRC Press.

Wieser, W. (1994). Cost of growth in cells and organisms: general rules and comparative aspects. *Biol. Rev.* **68**, 1-33.

Woods, H.A., Martin, L.B. and Ghalambor C.K. (2015). Trade-offs and biological diversity: integrative answers to ecological questions. In *Integrative Organismal Biology* (ed. L.B. Martin, C.K. Ghalambor and H.A. Woods), pp. 309-317. New Jersey, USA: Wiley Blackwell.

Woods, H.A. and Wilson, J.K. (2015). An elephant in the fog: unifying concepts of physiological stasis and change. In *Integrative Organismal Biology* (ed. L.B.

Martin, C.K. Ghalambor and H.A. Woods), pp. 119-135. New Jersey, USA: Wiley Blackwell.

Yampolsky, L.Y., Zeng, E., Lopez, J., Williams, P.J., Dick, K.B., Colbourne, J.K. and Pfrender, M.E. (2014). Functional genomics of acclimation and adaptation in response to thermal stress in *Daphnia*. *BMC Genomics*. **15**, 859-870.

Zera, A.J. and Harshman, L.G. (2001). The Physiology of Life History Trade-Offs in Animals. *Ann. Rev. Ecol. Sys.* **32**, 95-126.

SUPPLEMENTARY TABLES

Table S1. Mean (± SE) of the enzymatic activity (10^{-1} U mg^{-1} protein) of citrate synthase [CS], electron transport system [ETS] and the ratio CS : ETS for each Endpoint and between the two temperatures.

Factors		CS (10^{-1} U mg^{-1} protein)				ETS (10^{-1} U mg^{-1} protein)				CS : ETS			
T°	Endpoint	n	Mean	±	SE	n	Mean	±	SE	n	Mean	±	SE
24°C	1	0	NA[1]	±	NA[1]	2	0.0073	±	2.87E-05	0	NA[1]	±	NA[1]
	2	3	0.0044	±	0.0020	3	0.0141	±	0.0042	3	0.2856	±	0.0438
	3	3	0.0069	±	0.0013	3	0.0155	±	0.0018	3	0.4357	±	0.0303
	4	2	0.0058	±	0.0006	2	0.0111	±	0.0013	2	0.5324	±	0.1093
	5	3	0.0054	±	0.0002	3	0.0131	±	0.0024	3	0.4391	±	0.0789
30°C	1	0	NA[1]	±	NA[1]	1	0.0073	±	NA[1]	0	NA[1]	±	NA[1]
	2	3	0.0050	±	0.0009	3	0.0115	±	0.0010	3	0.4508	±	0.1046
	3	3	0.0058	±	5.77E-05	3	0.0103	±	0.0007	3	0.5695	±	0.0300
	4	3	0.0041	±	0.0009	3	0.0120	±	0.0014	3	0.3431	±	0.1193
	5	2	0.0086	±	0.0037	3	0.0143	±	0.0040	2	0.5735	±	0.0639

[1] Data not available as no sample or only one sample was analyzed (n = 0 or 1).

Table S2. Mean (± SE) metabolites concentration (ng mL^{-1}) at 24 and 30 °C with the statistical result (p-value and q-value) of the Wilcoxon rank-sum statistical test. Significant metabolites are indicated in bold.

Metabolites	24 °C				30 °C				*P*	q-value (FDR)
	n	Mean	±	SE	n	Mean	±	SE		
Malate	75	0.164	±	0.136	46	-0.268	±	0.075	**0.0080**	**0.0120**
Glutamate	75	0.114	±	0.104	46	-0.186	±	0.167	**0.0074**	**0.0120**
α-aminoapidic acid	75	0.143	±	0.136	46	-0.233	±	0.081	**0.0371**	**0.0458**
Alanine	75	0.284	±	0.120	46	-0.463	±	0.107	**<0.0001**	**0.0002**
Aspartate	75	-0.035	±	0.076	46	0.057	±	0.206	0.1810	0.2001
Sarcosine	75	0.284	±	0.120	46	-0.464	±	0.107	**<0.0001**	**0.0002**
Valine	75	0.238	±	0.118	46	-0.388	±	0.124	**<0.0001**	**0.0002**
Betaine	75	0.240	±	0.117	46	-0.391	±	0.127	**<0.0001**	**0.0002**
Aminoburtyric-aminoisobutyric acid	75	0.194	±	0.114	46	-0.316	±	0.140	**<0.0001**	**0.0000**
Caprylic acid	75	-0.071	±	0.098	46	0.115	±	0.178	0.3015	0.3166
Lauric acid	75	-0.242	±	0.111	46	0.394	±	0.139	**<0.0001**	**0.0001**
Tetradecenoic acid	75	-0.141	±	0.111	46	0.230	±	0.152	**0.0292**	**0.0383**
Ginkgoic acid	75	-0.209	±	0.112	46	0.341	±	0.142	**0.0013**	**0.0030**
Linolenic acid	75	-0.056	±	0.086	46	0.091	±	0.195	0.4372	0.4372
Eicosapentaenoic acid	75	0.235	±	0.132	46	-0.384	±	0.078	**0.0023**	**0.0044**
Palmitoleic acid	75	-0.226	±	0.103	46	0.368	±	0.158	**0.0019**	**0.0041**
Pentadecylic acid	75	-0.087	±	0.122	46	0.142	±	0.131	0.0556	0.0649
Linolelaidic acid	75	-0.420	±	0.078	46	0.685	±	0.157	**<0.0001**	**0.0000**
Ginkgolic acid	75	-0.147	±	0.113	46	0.239	±	0.146	**0.0251**	**0.0351**
Elaidic acid	75	-0.269	±	0.108	46	0.439	±	0.140	**0.0001**	**0.0003**
Margaric acid	75	-0.180	±	0.111	46	0.293	±	0.147	**0.0033**	**0.0059**

Table S3. Mean (± SE) metabolites concentration (ng mL^{-1}) for the three 'Periods' with the statistical result (χ^2, p-value and q-value) of the Kruskal Wallis statistical test. Significant metabolites are indicated in bold.

Metabolites	**Period 1**				**Period 2**				**Period 3**				$\chi 2$	*P*	q-value
	n	Mean	±	SE	n	Mean	±	SE	n	Mean	±	SE			
Malate	33	-0.218	±	0.105	44	-0.093	±	0.119	44	0.256	±	0.196	**8.103**	**0.0174**	**0.0365**
Glutamate	33	-0.108	±	0.192	44	-0.041	±	0.145	44	0.123	±	0.145	1.835	0.3995	
α-aminoapidic acid	33	-0.244	±	0.107	44	-0.061	±	0.114	44	0.244	±	0.198	6.358	0.0416	
Alanine	33	-0.518	±	0.139	44	0.107	±	0.168	44	0.282	±	0.136	**16.946**	**0.0002**	**0.0022**
Aspartate	33	0.010	±	0.282	44	-0.159	±	0.079	44	0.152	±	0.105	**12.316**	**0.0021**	**0.0074**
Sarcosine	33	-0.514	±	0.139	44	0.105	±	0.168	44	0.280	±	0.137	**16.971**	**0.0002**	**0.0022**
Valine	33	-0.332	±	0.205	44	0.095	±	0.138	44	0.154	±	0.135	**13.382**	**0.0012**	**0.0052**
Betaine	33	-0.356	±	0.198	44	0.101	±	0.139	44	0.166	±	0.138	**13.670**	**0.0011**	**0.0052**
Aminoburtyric-aminoisobutyric acid	33	-0.246	±	0.185	44	0.010	±	0.166	44	0.174	±	0.127	**9.756**	**0.0076**	**0.0200**
Caprylic acid	33	0.510	±	0.250	44	-0.082	±	0.119	44	-0.300	±	0.090	**11.743**	**0.0028**	**0.0085**
Lauric acid	33	-0.019	±	0.144	44	-0.076	±	0.120	44	0.090	±	0.188	0.144	0.9306	
Tetradecenoic acid	33	0.329	±	0.175	44	-0.093	±	0.142	44	-0.154	±	0.152	6.255	0.0438	
Ginkgoic acid	33	0.341	±	0.186	44	-0.035	±	0.136	44	-0.221	±	0.150	6.830	0.0329	
Linolenic acid	33	0.435	±	0.250	44	-0.143	±	0.100	44	-0.183	±	0.116	3.340	0.1883	
Eicosapentaenoic acid	33	-0.244	±	0.117	44	0.148	±	0.175	44	0.035	±	0.154	1.984	0.3708	
Palmitoleic acid	33	0.255	±	0.191	44	-0.043	±	0.144	44	-0.148	±	0.143	2.921	0.2321	
Pentadecylic acid	33	0.607	±	0.226	44	-0.253	±	0.115	44	-0.203	±	0.113	**15.991**	**0.0003**	**0.0024**
Linolelaidic acid	33	0.071	±	0.176	44	-0.017	±	0.154	44	-0.036	±	0.149	0.234	0.8897	
Ginkgolic acid	33	0.283	±	0.203	44	0.030	±	0.146	44	-0.243	±	0.130	4.502	0.1053	
Elaidic acid	33	0.117	±	0.143	44	-0.005	±	0.160	44	-0.083	±	0.161	0.971	0.6153	
Margaric acid	33	0.510	±	0.213	44	-0.129	±	0.132	44	-0.254	±	0.120	**8.503**	**0.0143**	**0.0332**

Table S4. Statistical results of the *post-hoc* Dunn's multiple comparison test with the Benjamini-Hochberg adjustment on the significant metabolites identified after the Kruskal Wallis test. Significant statistical differences are indicated in bold and the group distinction (A, AB, B) are indicated in the second column.

Metabolites		Period 1	Period 2	Period 3
Malate	**Period 1 (A)**	-----	0.3934	**0.0198**
	Period 2 (AB)	0.3934	-----	0.0663
	Period 3 (B)	**0.0198**	0.0663	-----
Alanine	**Period 1 (A)**	-----	**0.0047**	**0.0002**
	Period 2 (B)	**0.0047**	-----	0.2407
	Period 3 (B)	**0.0002**	0.2407	-----
Aspartate	**Period 1 (A)**	-----	0.0859	**0.0014**
	Period 2 (AB)	0.0859	-----	0.0827
	Period 3 (B)	**0.0014**	0.0827	-----
Sarcosine	**Period 1 (A)**	-----	**0.0046**	**0.0002**
	Period 2 (B)	**0.0046**	-----	0.2444
	Period 3 (B)	**0.0002**	0.2444	**-----**
Valine	**Period 1 (A)**	-----	**0.0028**	**0.0024**
	Period 2 (B)	**0.0028**	-----	0.7961
	Period 3 (B)	**0.0024**	0.7961	-----
Betaine	**Period 1 (A)**	-----	**0.0027**	**0.0019**
	Period 2 (B)	**0.0027**	-----	0.7542
	Period 3 (B)	**0.0019**	0.7542	-----
Aminobutyric-aminoisobutyric acid	**Period 1 (A)**	-----	0.0874	**0.0054**
	Period 2 (AB)	0.0874	-----	0.1851
	Period 3 (B)	**0.0054**	0.1851	-----
Caprylic acid	**Period 1 (A)**	-----	**0.0265**	**0.0021**
	Period 2 (B)	**0.0265**	-----	0.2739
	Period 3 (B)	**0.0021**	0.2739	-----
Pentadecylic acid	**Period 1 (A)**	-----	**0.0007**	**0.0011**
	Period 2 (B)	**0.0007**	-----	0.7542
	Period 3 (B)	**0.0011**	0.7542	-----
Margaric acid	**Period 1 (A)**	-----	**0.0353**	**0.0153**
	Period 2 (B)	**0.0353**	-----	0.5616
	Period 3 (B)	**0.0153**	0.5616	-----

Table S5. Mean (± SE) of the life history traits fecundity, fertility, size and survival for the two temperatures.

Factors		Fecundity[1]				Fertility[2]				Size[3]				% Survival			
T°	Period	n	Mean	±	SE	n	Mean	±	SE	n	Mean	±	SE	n	Mean	±	SE
24°C	1	--	------		------	--	------		------	90	5.6444	±	0.1217	51	74.1961	±	2.1680
	2	--	------		------	--	------		------	252	9.4048	±	0.1424	93	68.1505	±	1.5517
	3	56	30.3214	±	1.6129	36	0.2933	±	0.0392	269	11.9182	±	0.1377	93	58.6237	±	1.5420
30°C	1	--	------		------	--	------		------	131	7.2977	±	0.1688	66	76.8485	±	1.8559
	2	--	------		------	--	------		------	90	10.1111	±	0.1983	36	67.1667	±	2.2641
	3	32	28.4688	±	2.3314	18	0.3009	±	0.0492	153	11.1503	±	0.1611	45	64.3556	±	2.2947

[1] Fecundity = number of eggs *per* egg mass

[2] Fertility = number of egg masses spawned in each well *per* number of females in the well

[3] Size = number of chaetiger

Table S6. Statistical results of the multiple comparisons with a Tukey contrast comparing organisms' size between every level of the two factors ('Temperature' and 'Period'). Significant statistical differences are indicated in bold.

Temperature	Period		Temperature	Period	z-value	*P*
30	1	-	24	1	4.679	**<0.0001**
24	2	-	24	1	10.442	**<0.0001**
30	2	-	24	1	10.347	**<0.0001**
24	3	-	24	1	15.651	**<0.0001**
30	3	-	24	1	13.903	**<0.0001**
24	2	-	30	1	6.620	**<0.0001**
30	2	-	30	1	6.757	**<0.0001**
24	3	-	30	1	13.311	**<0.0001**
30	3	-	30	1	11.037	**<0.0001**
30	2	-	24	2	1.284	0.7839
24	3	-	24	2	8.743	**<0.0001**
30	3	-	24	2	6.129	**<0.0001**
24	3	-	30	2	5.357	**<0.0001**
30	3	-	30	2	3.839	**0.0016**
30	3	-	24	3	-1.186	0.8357

APPENDICES

Table A1. Complete list of the 37 metabolites on 58 metabolites analysed selected to be removed from the metabolomics database prior to the statistical analyses due to the high amount of missing values according to the 'modified 80 % rule'.

Metabolites	
Glycerol-3-phosphate	Caproic acid (C6:0)
a-ketoglutarate	Carpic acid (C10:0)
AMP	Undecylic acid (C11:0)
ADP	Tridecylic acid (C13:0)
ATP	Arachidonic acid (C20:4)
NAD	Docosahexanoic acid (C22:6)
NADH	Myristic acid (C14:0)
Pyruvate	Mead acid (C20:3)
Lactate	Palmitic acid (C16:0)
Fumarate	Eicosadienoic acid (C20:2)
Succinate	Stearic acid (C18:0)
Glucose	Eicosenoic acid (C20:1)
Hydroxyproline	Docosadeinoic acid (C22:0)
Serine	Erucic acid (C22:1)
Cysteine	Arachidic acid (C20:0)
Proline	Behenic acid (C22:0)
Methionine	8-oxo-2-dexyguanosine
Tyrosine	8-hydroxyguanosine
Threonine	

CONCLUSION GÉNÉRALE

À travers l'utilisation d'une approche intégrative, mon projet de maîtrise a permis de caractériser la réponse de différents compartiments biologiques à une température élevée durant l'ontogénie des vers marins *Ophryotrocha labronica*. Les résultats ont permis de conclure que l'exposition des organismes à une température élevée affecte les voies et taux métaboliques associés au métabolisme aérobie, sans pour autant affecter grandement la taille, la survie, la fertilité et la fécondité des individus. De plus, aucun changement dans l'expression des trois gènes mitochondriaux et dans l'activité enzymatique mitochondriale n'a été observé. Par ailleurs, ce projet illustre, à travers son développement et son usage, l'intérêt d'une approche intégrative dans les recherches portant sur la réponse des espèces au réchauffement climatique. De fait, cette approche permet, en utilisant un ensemble de techniques sur différents niveaux et traits biologiques, de comprendre comment l'interaction dynamique entre ces traits biologiques influence les processus à l'échelle de l'organisme.

Avancées de l'utilisation d'une approche intégrative pour *Ophryotrocha labronica*

Toutefois, bien que ce type d'approche soit de plus en plus préconisé, son application en recherche se limite généralement aux organismes modèles, pour lesquels nous possédons déjà les outils génétiques et physiologiques (Calosi *et al.*, 2016). Or, ces organismes modèles sont rares dans la vaste majorité des embranchements marins (Munday *et al.*, 2013; Calosi *et al.*, 2016). En ce sens, les méthodes génétiques et métabolomiques développées dans le cadre de cette étude permettent une avancée importante dans la mise en œuvre d'une approche plus intégrative visant à tester des hypothèses spécifiques chez les ectothermes marins. Le développement de ces outils pour l'espèce *O. labronica* s'inscrit dans la lignée d'études précédentes qui ont déjà permis d'optimiser les mesures d'activité enzymatique mitochondriale (Chakravarti *et al.*, 2016; Gibbin *et al.*, 2017a; Jarrold *et al.*,

2019). Ces études ont aussi démontré l'intérêt d'utiliser cette espèce comme organisme modèle émergent pour des recherches multigénérationnelles dans le contexte des changements globaux. La présente étude contribue donc à établir *O. labronica* comme organisme modèle émergent pour des études intégratives portant sur la réponse des espèces aux changements globaux. Par ailleurs, suite aux limitations éprouvées durant les analyses, ce projet permet de cibler les compartiments biologiques pour lesquels les outils et techniques d'analyse doivent encore être développés.

Amélioration des analyses génétiques et métabolomiques

En effet, bien que les méthodes développées offrent de nouvelles avenues de recherche en permettant de relier les patrons d'expression génétiques aux traits physiologiques et d'histoire de vie, ces dernières peuvent être encore optimisées. Dans le cas des analyses génétiques, il serait particulièrement important de tester la stabilité de l'expression du gène de l'actine (*ACTB*) à différentes températures et à divers stades de développement pour le genre *Ophryotrocha*. Si le gène de l'actine a été choisi comme gène de référence dans cette étude puisque son expression n'est perturbée ni par la température, ni par les différents stades de développement chez de nombreux organismes, cela reste à être vérifié pour *Ophryotrocha*. En outre, puisque la séquence de nucléotides est maintenant disponible pour les 13 gènes mitochondriaux (*séquences non publiées*), il serait important de tester différentes amorces pour chaque gène mitochondrial; cela permettrait de sélectionner la meilleure amorce pour chaque gène et, ainsi, améliorer l'efficacité des courbes de calibration des analyses PCR quantitatives.

En ce qui a trait aux analyses métabolomiques, considérant que la méthode développée est modulable, il serait pertinent d'incorporer la détection des lipides membranaires en raison de leur implication potentielle dans l'acclimatation des organismes à la température. Par ailleurs, il serait utile d'optimiser la méthode pour détecter plus fréquemment les métabolites énergétiques clés tels que l'ATP, l'ADP et le NADH, sachant que ces métabolites fournissent des informations clés sur l'état du métabolisme énergétique des organismes. Actuellement, leur détection n'est pas systématique de sorte qu'ils sont

écartés des analyses (voir la table A1 dans l'annexe de l'article). En outre, il serait important d'augmenter la taille des échantillons, particulièrement dans le cas des analyses génétiques et de l'activité enzymatique, afin de tenir compte de la grande variabilité interindividuelle observée dans mon étude. Dans le cas de ces deux compartiments biologiques, d'autres échantillons conservés lors de l'expérience pourront être analysés prochainement afin d'évaluer la réponse de ces compartiments biologiques selon les 'Périodes' plutôt qu'avec l'approche des 'Endpoints'.

Vers des études intégratives multigénérationnelles

Les résultats de cette étude montrent la prépondérance du métabolisme énergétique comme mécanisme de réponse rapide à la température. Il reste maintenant à déterminer si ces réponses sont maintenues à plus long terme: c'est-à-dire sur plusieurs événements de reproduction et sur plusieurs générations. Dans le cadre de telles études, l'ajout de certaines mesures pourrait favoriser notre compréhension de la cascade physiologique impliquée dans les réponses plastiques à la température. L'inclusion d'analyses protéomiques, par exemple, permettrait d'ajouter un niveau de compréhension intermédiaire entre les réponses métaboliques et les conséquences sur les traits d'histoire de vie. Par ailleurs, considérant que cette étude montre l'effet prédominant de la température sur le métabolisme, il serait utile d'incorporer des mesures du taux métabolique pour établir le registre aérobie des espèces et le relier aux profils métabolomiques. En outre, l'intégration des mesures de CTmax pourrait nous permettre de déterminer si les changements au niveau génétique et physiologique permettent aux organismes d'augmenter leur tolérance thermique; cela est particulièrement approprié dans le cadre d'études intégratives multigénérationnelles. L'établissement de lignées isofemelles pour contrôler la diversité génétique serait également une avenue intéressante à considérer dans les expériences futures.

Au final, mon projet de maîtrise concrétise l'usage d'une approche intégrative pour comprendre, avec une échelle temporelle fine, l'effet de la température sur les différents compartiments biologiques. Elle a donné l'occasion d'établir les bases nécessaires à ce genre d'approche en l'appliquant à l'espèce modèle émergente *O. labronica.* Ce faisant, ce

projet offre de nouvelles perspectives de recherche. En particulier, l'intégration de cette approche avec les expériences multigénérationnelles contribuera à une meilleure compréhension des mécanismes et des coûts issus de ces réponses plastiques. L'intégration des réponses plastiques dans les différents compartiments biologiques à travers les générations pourrait également nous aider à identifier les mécanismes facilitant une adaptation rapide. Ces informations sont essentielles si nous voulons améliorer nos prédictions relatives à la capacité des espèces à tolérer les changements environnementaux actuels et déterminer les conséquences sur leur distribution dans le contexte des changements globaux.

ANNEXE 1

A sensitive targeted omics micro-method: a new approach for the characterisation of metabolomic profile in single microscopic marine individuals

Fanny Vermandele[1,†*], Mathieu Babin[2,†], Peter Thor[3], Piero Calosi[1]

[1] Département de Biologie, Chimie et Géographie, Université du Québec à Rimouski, 300 allée des Ursulines, Rimouski, QC G5L 3A1, Canada

[2] Institut des Sciences de la Mer de Rimouski, Université du Québec à Rimouski, 300 allée des Ursulines, Rimouski, QC G5L 3A1, Canada

[3] Norwegian Polar Institute, Fram Centre, 9296 Tromsø, Norway

[†] These authors contributed equally to the manuscript

*** Corresponding Author:** Tel: +1 (418) 723-1986 (ext. 1567)

E-mail address: fanny.vermandele@uqar.ca

ABSTRACT

Background Metabolomics analyses are being increasingly used in environmental studies aiming to characterize how environmental challenges influence the functional status of an organism. However, technical challenges, such as high salt contents in biological matrices and reduced biomass, have limited its use in marine animals.

Objectives We developed a simple and sensitive method to investigate the variation of targeted metabolites in single microscopic marine individuals.

Methods Using stage IV and V copepodites of the Artic copepod *Calanus glacialis,* we developed a targeted approach to carry out analyses on an liquid chromatography-high resolution mass spectrometry (LC-HRMS) platform, using ammonium carbonate to construct a fast "cold-quenching salt-eliminating" extraction procedure. The method's reproducibility and sensitivity, as well as the linearity of standard curves were tested using a homogenate of pooled copepods and a metabolite stock solution. The method was also applied on other marine species (i.e. lobster larvae, juveniles and adults of a marine interstitial annelid) to assess the breadth of its scope of utilisation.

Results and conclusion The method is sufficiently sensitive for application on single microscopic marine animals as the linearity of standard curves, the concentration working range, and the limits of quantification were adequate for this purpose. The use of ammonium carbonate in the extraction solution enabled to extract the samples without the need for dilution. Moreover, the method is applicable to a broad range of small marine organisms, thus being of great interest for the application of targeted -omics approaches on single microscopic individual of marine species.

Keywords: Individual phenotype; Copepods; Marine invertebrates; Global change; Metabolites; LC-HRMS

1 INTRODUCTION

Metabolomics and lipidomics analyses (hereafter called *metabolomics* for simplicity), investigating variation in cells' and tissues' metabolite and lipid composition, are becoming an invaluable tool enabling direct measurement of end-products of metabolic activity (Longnecker *et al.*, 2015; Johnson, Ivanisevic and Siuzdak, 2016). These approaches represent a powerful complement to genomics, transcriptomics and proteomics analyses (Viant, 2007; Goulitquer, Potin and Tonon, 2012; Peng, Li and Peng, 2015). In addition, they represent a powerful mean to provide a mechanistic cellular underpinning for physiological status at the whole-organism level (Viant, 2007; Goulitquer, Potin and Tonon, 2012), even for non-model organisms for which other –omics tools are not yet developed (Viant, 2007). However, the characterization of metabolomics profiles comes with considerable challenges and limitations (Viant, 2007; Miller, 2007; Goulitquer, Potin and Tonon, 2012). In order to improve our understanding of the metabolic pathways involved in the evolution of physiological systems (Goulitquer, Potin and Tonon, 2012) and species-environment interactions across the tree of life (Viant, 2008), these challenges and limitations have to be overcome.

Among all the metabolomics techniques developed, including nuclear magnetic resonance (NMR) and gas chromatography/electron impact-mass spectrometry (GC/EI-MS) (Lafaye *et al.*, 2005), liquid chromatography-mass spectrometry (LC-MS) has been widely used for lipid and metabolite profiling in a wide range of biological samples (Johnson, Ivanisevic and Siuzdak, 2016). Furthermore, the recent introduction of lower cost high-resolution mass spectrometry (HRMS) instruments has fuelled the innovative development of methods for highly sensitive and reproducible targeted and untargeted -omics approaches (Viant and Sommer, 2013; Johnson, Ivanisevic and Siuzdak, 2016; Rochat, 2016). These methods have enabled the application of metabolomics analyses to large scale environmental studies, leading to the development of a new approach in environmental metabolomics (Lankadurai, Nagato and Simpson, 2013). By providing a snapshot of metabolite profiles, metabolomics analyses allow to detect changes in the functional status of an organism under different environmental conditions (Bundy, Davey and Viant, 2009; Peng, Li and Peng, 2015). Such functional status can be correlated to organisms' phenotypes (Bundy, Davey and Viant, 2009; Johnson, Ivanisevic and Siuzdak, 2016). Thus, the application of environmental metabolomics can provide us with important and integrative insights on how organisms respond to environmental challenges (Goulitquer, Potin and Tonon, 2012; Verberk *et al.*, 2013; Putnam, Davidson and Gates, 2016; Calosi *et al*., 2017).

However, whilst many metabolomics methods have been widely used on terrestrial organisms, their application on marine organisms remain relatively scarce (Rochat, 2016; Viant, 2007). This is due in particular to the existence of technical challenges specifically associated with marine organism samples, which tend to be characterized by a high salt content. This characteristic of marine samples creates interference with metabolite detection and identification, affecting mass spectrometry detection by creating salt adducts, which decrease the ability of instruments to detect and identify metabolites (Goulitquer, Potin and Tonon, 2012; Rochat, 2016). In order to offset this problem, most methods turn to solid phase extraction (SPE) (Dettmer, Aronov and Hammock, 2007; Rochat, 2016). However, most unstable or short-life metabolites are lost through this SPE procedure, from extended sample exposure to either heat or acidic conditions. Moreover, polar metabolites cannot be separated from salt using this strategy. That is why, most common metabolomics methods use "cold quenching" as their extraction procedure for reactive metabolites. This slows down or offsets metabolite degradation (Lu, Kimball and Rabinowitz, 2006), thus representing a suitable extraction method for bacteria or plants. However, extraction protocols adapted to marine organism need to eliminate salt content in order to be effective. Fast "cold quenching" salt-eliminating polar conditions are therefore required to properly prepare marine sample for sensitive LC-HRMS analyses.

Another major challenge researchers face in characterizing the metabolomic profile of marine organisms is their reduced biomass, characteristic of the vast majority of marine species particularly during the early development (Vernberg and Vernberg, 2013). This technical limitation has prevented us, to date, from exploring the metabolomic diversity of marine organisms of small or microscopic size, as well as their metabolomic responses to environmental changes (Goulitquer, Potin and Tonon, 2012). However, this characterization of metabolomic profiles in small marine organisms is crucial within the context of the ongoing global ocean change. Thus, the increase in temperature, lowering of seawater pH, and reduction in seawater oxygen and salinity predicted to occur by the end of the century in oceanic and coastal systems (IPCC, 2014) will likely impact marine organisms' survival, development and functions (Brierley and Kingsford, 2009; Byrne and Przeslawski, 2013; Wittmann and Pörtner, 2013). Considering that metabolic changes in response to environmental stress relate closely to changes observed at the whole organismal level (Bartholomew, 1966; Calosi et al, 2017), the implementation of metabolomics approaches to small marine organisms can help shed the light on their physiological abilities. Moreover, whilst the pooling of multiple individuals has been utilised to obtain usable mass for direct infusion HRMS analyses for some marine microscopic species (e.g. Mayor et al. 2015), the undeniable importance of characterizing individual level responses to more accurately predict species sensitivity to the global change (Bennett, 1987; Calosi *et*

al., 2013; Turner *et al.*, 2015) pushes us to improve existing analytical platforms enabling the characterisation of individuals' metabolomic profiles.

In the present study, using copepodites (i.e. copepod larvae) of the ecologically important Arctic marine copepod *Calanus glacialis* (Jaschnov, 1955), we describe a sensitive method for the microscale characterization of the metabolomic profile using a single sample preparation for a single microscopic individual. We describe the development and the validation of the method, which includes the evaluation of the importance of salt elimination in sample preparation, and the matrix effect on spike standard recovery from a multi-individual copepod homogenate. The linear concentration range and limit of quantification (LOQ) of the targeted compounds and the analytical reproducibility were also assessed. The method developed was then applied to larvae and post-larvae stages of the American lobster (*Homarus americanus,* H. Milne Edwards, 1837) as well as adults of a marine interstitial annelid species (*Ophryotrocha japonica*, Paxton and Åkesson, 2010), to test for its versatility in supporting the investigation of individual-level metabolomics responses of marine animals to current and future environmental challenges.

2 METHODS

2.1 Standards, reagents, solvents and solutions

Free fatty-acid standards were obtained by hydrolyzing the FAME 37 standard from Sigma-Aldrich (St. Louis, MO, USA). The amino acid mixed standard was obtained from Phenomenex (Torrance, CA, USA). All other metabolite standards and ammonium carbonate (trace metals-grade, 99.999%) were obtained from Sigma-Aldrich. LCMS-grade acetonitrile (ACN) (OmniSolv, EMD Chemical, Gibbstown, NJ, USA) was obtained from VWR International (West Chester, PA, USA) and Nanopure water (18.0 Ω) was produced with a Barnstead infinity system (Lake Balboa, CA, USA).

The ammonium carbonate solution (50 mM) was prepared by dissolving 4.8 g in 1 L of Nanopure water. Daily extraction solvent solution was prepared by combining 0.4 mL of 50 mM ammonium carbonate solution to 1.6 mL of Nanopure water and 8 mL of methanol for a final 8:2 methanol:water-10 mM ammonium solution.

2.2 Fatty acid and metabolite stock solution

Fatty acid stock solution was prepared by evaporating 250 µL of FAME 37 standard under nitrogen in 1.5 mL PP centrifugal tube. A total of 50 µL of KOH 6.25% (w/v) in Nanopure water was added to the sample and the tube was then heated at 60 °C for 30 min under nitrogen. 950 µL of extraction solvent was then added to eliminate the precipitated

potassium carbonate, forming a white pellet after centrifugation at 8000 rpm. The final supernatant stock solution was transfer to a HPLC amber vial and stored in -80 °C.

Similar, DL-α-glycerophosphate, nicotinamide adenine dinucleotide (NAD), reduce nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADP), reduce nicotinamide adenine dinucleotide phosphate (NADPH), adenosine 5`-triphosphate disodium salt hydrate (ATP), adenosine 5`-diphosphate sodium (ADP), adenosine 5`-monophosphate disodium salt (AMP), fumaric acid, sodium pyruvate, sodium L-lactate, sodium succinate dibasic hexahydrate, D-malic acid and α-ketoglutaric acid disodium salt dehydrate, D-glucose, betaine free base and Nm-phospho-L-arginine were precisely weighed individually directly into clear HPLC vials to make 1 mg mL^{-1} solutions in extraction solvent to create individual metabolite stock solutions.

2.3 Working stock solution and calibration solution

1000 µL of amino acid mixed standard solution, 2000 µL of fatty acid stock solution, 500 µL of glucose and 50 µL of each other individual metabolites stock solutions were pooled into a single solution and diluted to 10 mL with an extraction solvent to produce the working solution. A serial dilution of the working stock solution in the extraction solvent 1:1 was then conducted to prepare 10 working solutions for the calibration curve.

2.4 Specimens collection and preservation

In order to develop the method here presented, copepodites stage IV and V of *Calanus glacialis*, weights 117 ± 27 µgC $indiv.^{-1}$ and 230 ± 43 µgC $indiv.^{-1}$, respectively (mean ± sd) (Thor et al. 2018) were collected from Kongsfjorden and Billefjorden, Svalbard in June 2015 and were brought back to Kings Bay Marine Laboratory (Ny-Ålesund, Svalbard). They were then flash frozen in liquid nitrogen and individually stored at -80 °C in 1.5 and 2 mL PP centrifuge tube. Furthermore, in order to validate the reproducibility and recovery efficiency of the methods, we used adult copepods (*Calanus spp*.) collected in the maritime estuary of Saint Lawrence by vertical tow with a 150 µm net from approximatively 200 m to the surface and stored in batches at -80 °C in 50 ml falcon tubes. As for the determination of the method's scope, larvae and post-larvae of the American lobster (*H. americanus*) were reared in an experimental system at the Department of Fisheries and Oceans Biological at the Saint Andrews Biological Station (New Brunswick, Canada) in July 2016. They were flash frozen individually in liquid nitrogen in 2 mL PP centrifuge tube. Adult worms of the species *Ophryotrocha japonica* were collected in 2010 in the harbour of La Spezia, (Italy), and kept for more than 20 generations in laboratory cultures under control conditions (12:12h L: D photoperiod, temperature of 24

°C, salinity 35 and pH_{NBS} 8.1). Adults were then flash frozen individually in liquid nitrogen and stored at -80 °C in 1.5 mL PP centrifuge tube.

2.5 Sample preparation

2.5.1 Metabolites extraction

Copepodites samples were taken from the -80 °C storage to a liquid nitrogen bath and 250 µL of cold extraction solvent (kept at -80 °C) was added to the sample. The sample was then crushed with a potter pestle (blue pre-sterilize, Axygen, Tewksbury, MA, USA) directly in the tube until a homogenized solution was obtained. Samples were then sonicated (Sonication bath, model Symphony, VWR, West Chester, PA, USA) at room temperature for 3 s. After a controlled centrifugation using a centrifuge (5430R, Eppendorf, Hamburg, Germany) at 10 000 rpm for 3 min at 4 °C, 225 µL of the supernatant was pipette to an amber HPLC vial with glass insert (250 µL, Wheaton, New Jersey, USA) and injected rapidly or stored at -20 °C for no more than 24 h. The schematic representation of the work flow is reported in figure 1.

2.5.2 Copepod homogenate preparation

In order to assess the methods' reproducibility and metabolite recovery, a homogenate of copepods (*Calanus spp.*) was used to mimic the copepod sample. A pool of several copepods was added to 1 mL of Nanopure water and homogenized with a Tissue-Tearor (BioSpecs Products, Bartlesville, OK, USA). Aliquots of 5 µL of the homogenate solution were transferred to several 1.5 mL PP centrifugal tubes and precisely weighed on a microbalance (MX5, Mettler, Columbus, Ohio, USA). Then, in order to assess spike recovery, 10 µL of working stock solution was added to some of the aliquots, corresponding to approximately 10 % of the calibration curve's most concentrated solution.

2.6 LC-HRMS analysis

The liquid chromatography (LC) separation was performed on an Accela from Thermo system (Thermo Electron Corporation, San Jose, CA, USA), equipped with a Luna C5 column and guard from Phenomenex, 150 mm x 2 mm, with the following parameters: autosampler temperature set at 4 °C, column temperature set at 20 °C, injection volume of 25 µL and solvent flow rate of 200 µL min^{-1}. Mobile phase A was composed of ACN:50mM ammonium carbonate water solution (90:10) and mobile phase B was composed of 5 mM ammonium carbonate water solution. Gradient program started at 2 % of phase A over 2 min to 98 % of phase A at 6 min, until the minute 15. The initial condition phase A (2%) was re-established at 17 min and conditioning occurred over 3 min for a total run time of 20 min.

The high-resolution mass detection (HRMS) was performed on an Orbitrap LTQ Discovery mass spectrometer from Thermo system (Thermo Electron Corporation, San Jose, CA, USA), in both positive and negative mode sequentially. Electrospray ionization spray voltage was 5000 V in positive mode and 3200 V in negative mode. Nitrogen was used as sheath gas at 55 arbitrary units with a capillary temperature of 325 °C. Scan range was from 60 to 1500 m/z (mass to charge ratio) for both modes.

2.7 Data processing

HRMS data analyses were performed with Xcalibur 2.0 software (Thermo) with a 10 ppm mass tolerance. Calibration curves were obtained from the area of the working standard solution by extract ion integration. Linear, linear log-log and quadratic log-log relations were used for best fit and some higher calibration points were skimmed to better reflect the sample concentration range for each metabolite.

2.8 Testing for the method scope

Following the development and analytical validation, the method was applied to single individuals of other marine species in order to test the method scope and to apply it to broader range of species. Copepodites of the copepod *Calanus glacialis*, adults of the interstitial annelid *Ophryotrocha japonica* (Paxton and Åkesson, 2010) and stages I to V larvae and postlarvae of the American lobster *Homarus americanus* (H. Milne Edwards, 1837) were tested to determine the method's scope of application regarding sensitivity *vs* sample size. The lobster larva sample preparation had to be modified only in regard to the extraction volume given the size of the individuals: mean weight of 58 ± 2 mg compared, for example, to copepods which had an average estimated weight of 0.68 ± 0.13 mg for stage IV copepodite (Allison Bailey, *pers. communication,* 2017). However, a ratio of 250 µL of extraction solvent to 2 mg of sample was maintained.

2.9 Testing the degradation of the working stock solution

In order to estimate the durability of the working stock solution in terms of metabolite degradation, 225 µL of the working stock solution conserved during nine months in the -20 °C was transferred in an amber HPLC vial with glass insert and was injected directly.

3 RESULTS AND DISCUSSION

3.1 Salts

The development of the method for sample preparation started with the establishment of a "cold quenching" protocol using methanol:water (80:20) as the extraction solution.

During the first method's validation procedure, the recovery of the spiked standard on copepod homogenate was found to be around 50 % for the metabolites of interest, and thus inadequate for our purpose. Close signal investigation supported the calcium and sodium salt adduct interference, a problem already observed in other metabolomics analyses on marine organism samples (Goulitquer, Potin and Tonon, 2012).

In order to validate the influence of sodium (Na) and calcium (Ca) in the sample preparation, sodium chloride (NaCl) was added to a solution. One working stock solution was diluted in methanol:water (80:20), and a second one was diluted in methanol:water (80:20) with 1 % of sodium chloride. Both working stock solutions were diluted using the same dilution factor. The results showed a significant decrease in signal between the working stock solution with and without the NaCl. The decrease in signal did not affect all targeted metabolites by the same factor. For example, AMP, ADP, and NAD signals were found to decrease by 50, 75, and 90 % respectively in both negative and positive detection modes. Therefore, in order to eliminate most Na and Ca during the extraction, ammonium carbonate was added to the extraction solution to precipitate these cations in the form of carbonated compounds at low temperature. This procedure enables us to obtain a significant increase in the metabolite recovery in spiked standard on copepod homogenates for the metabolites of interest. Thus, the addition of ammonium carbonate to the extraction solution seems to be an efficient approach to prepare marine samples for sensitive LC-HRMS analyses, by offsetting the need to dilute the samples prior to the extraction, as it is custom (Shrestha and Vertes, 2009; Goulitquer, Potin and Tonon, 2012; Ghosh *et al.*, 2017). Considering that such dilutions would have decreased our ability to detect metabolites in small organisms, the addition of ammonium carbonate to the extraction solution enables, in one single extraction protocol, elimination of most interfering cations while preserving the sensitivity and sensibility of the method.

3.2 Method performance

Using the calibration curve data, the limit of quantification (LOQ) was determined at the lowest detected concentration with less than 20 % calculation error. The set linear working range was obtained by selecting the LOQ and the highest concentration meeting the same 20 % calculation error criterion. Best fit calibration relations for each metabolite were determined by the highest coefficient of determination (R^2) obtained from one of the following relationships: linear, linear log-log and quadratic log-log. Higher concentration points for the calibration curve for a given metabolite were skimmed *ad hoc* to obtain better linear relationships. In more details and when applicable, calibration points found outside the upper or lower ends of the cloud of points belonging to individuals' concentrations for the given metabolite measured were eliminated from the calibration curve. This displays a

more accurate representation of the biological values obtained and increases the accuracy of metabolite quantification, particularly for metabolites which were found in low concentrations. Table 1 reports the results of the method performance for each of the targeted metabolites.

3.3 Method validation

Reproducibility and standard recovery validation on individual microscopic organisms is a challenging process, and given the biologic base level variability between samples (Miller, 2007), the reproducibility of the analytical method was evaluated on a homogenate of pooled copepods. Due to considerable variation among homogenate sample volumes in a pilot test, it was deemed necessary to weigh the homogenate and normalize the result by mass. Reproducibility could then be assessed by injecting the sample multiple times (table 2). Results shown in Table 2 (% variation section) indicate an average variation of biological material with a 2-20 % range for most compounds.

The same homogenate of pooled copepods was then used as a matrix to determine the standard recovery upon addition. In order to calculate the recovery of added standard (spiked), we used the averaged measured concentration from the reproducibility test results as the base level matrix (copepod) concentration (ng *per* mg of homogenate). Previous tests on copepod samples showed that most detected metabolites were within the first 20 % of the calibration curve's concentration range. In aiming for an average standard addition of half the measured concentration, a value of 10 % of the calibration curve range was used for addition in the metabolite recovery evaluation. The average recovery was calculated on three replicates and the metabolite recovery variation relative to the average was also calculated. The results presented in Table 2 (recovery 10% section) were mostly within the 75-125 % expected range for biological matrix with a 2-20 % base level variation.

The ammonium carbonate buffer used in the sample preparation and mobile phase allowed a slightly basic chromatographic condition. Highly acidic compounds, such as phosphate adenosine derivatives, were detected in both modes with great signal as shown in Table 1. Other ammonium buffer salts were tested and none gained positive signals for phosphate metabolites. Carbonate is believed to act as carbonic acid in the nebulizing process, providing the carbonic acid gaseous phase proton donor ability. Working detection mode could then be chosen according to the linearity, working range and metabolite recovery parameter, which give some flexibility to this method.

3.4 Method scope

The application of the method on the copepod larvae, lobster larvae and juveniles, and interstitial worms adults showed that this method is able to detect many other simple and complex metabolites such as pigments and phospholipids present in those samples, while conserving a high sensitivity of detection for the original targeted compounds (Table 3).

3.5 Working stock solution stability

The detected signal obtained from the working stock solution after nine months of storage at -20 °C was similar to the originally fresh one. This indicates that the working stock solution preparation described above is stable at -20 °C for an extended time period.

4 CONCLUSIONS

The proposed method is simple, sensitive and allows for the investigation of the variation in targeted metabolites present in single microscopic marine individuals. The results showed that the linearity, the concentration working range, and the limit of quantification (LOQ) were adequate for this purpose and that the reproducibility was within the range expected for biological matrices. Moreover, the novelty of using ammonium carbonate to eliminate salt during the extraction process is of great interest in the context of applied -omics to small marine organisms. It enables researchers to quantify, with a good sensitivity, a variety of structurally different metabolites in a single microscopic organism without the need to pool multiple individuals together or to dilute the samples, which would have increased the risk of signal loss. Moreover, the fact that we observed a high sensitivity for many of the targeted metabolites in vastly different sizes of (small) organisms, characterized by different biological structure (incl. as for the juvenile lobsters soft tissues and calcified structure), is a testament that this method is versatile and of great interest for applied -omics on marine organisms. Furthermore, we show that the proposed method can be used on a variety of marine species displaying substantially different sizes, whilst providing useful insights in the metabolic responses of different stages of the life cycle to environmental changes in marine animals.

Considering that only few studies have integrated the characterization of marine organisms' metabolomics profiles to date (Rochat, 2016), the further development of the method presented here could enable us to conduct wider analyses in untargeted -omics. In addition, the method we propose is timely because it allows to quantify, with high sensitivity, many of the most important metabolites associated with the aerobic and anaerobic metabolism in single individuals. The use of an individual-based approach is becoming increasingly important to understand the impact of current and future environmental challenges on marine organisms. Indeed, metabolomics is, among all –omics

approaches, the closest one to measure discrete phenotypes, which more closely relates to both whole-organism phenotypes (Dettmer *et al.*, 2007; Calosi et al. 2017) and measures of fitness (Viant, 2007) than e.g. transcriptomics analysis of RNA expression. It could thus improve our understanding on how changes in metabolites could be correlated to the functional status of a whole organism (Motti, 2012). Hence, the implementation of metabolomics analyses, such as the method proposed here, within integrative studies in marine biology could help to shed light on the physiological cascade and the *fitness* consequences of an organism's response to an environmental change. In the end, these integrative studies could lead to new perspectives in the ecophysiological and evolutionary responses of marine organisms to global change, which are much needed to improve our prediction of the biological impacts of global change and to adopt better conservation strategies.

ACKNOWLEDGEMENTS

The authors would like to thank Pierre Blier for his advices on the metabolites associated to oxidative stress, Nick Beaudreau for the linguistic revision of the manuscript, Jory Cabrol, Fanny Noisette and Cynthia Thibault for providing us respectively the copepods (*Calanus spp.*), lobsters and worms samples. This study was supported by the FRAM High North Research Centre for Climate and the Environment through the Ocean Acidification and Ecosystem Effects in Northern Waters Flagship to PT, as well as by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Program grant, the Programme Établissement de nouveaux chercheurs universitaires of the Fonds de Recherche du Québec – Nature et Technologies (No. 199173) and the Fond Institutionnel de Recherche of the Université du Québec à Rimouski all awarded to PC. FV was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Alexander Graham Bell Canada Graduate Scholarships and a Fond Recherche du Québec – Nature et Technologies Master Scholarship.

AUTHOR CONTRIBUTIONS

MB, PT and PC chose the targeted metabolites on which the method should be developed. MB carried out the method development, helped by FV. MB performed the data analysis. MB wrote the first draft of the manuscript. FV developed the first advanced draft and completed all graphical support. FV, MB, PC all contributed to the first complete draft. All authors contributed to the final manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: Fanny Vermandele, Mathieu Babin, Peter Thor and Piero Calosi declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants performed by any of the authors. To the best of our knowledge, all applicable international, national, and/or institutional guidelines for the care and use of animals, in the present case invertebrates, were followed.

REFERENCES

Bartholomew, G.A. (1966). Interaction of physiology and behavior under natural conditions. **In**: R.I. Bowman, ed. 1966. *The Galápagos: Proceedings of the Symposia of the Galápagos International Scientific Projet.* Berkeley: University of California Press. pp.39-45.

Bennett, A.F. (1987). Chapter 7 – Interindividual variability: an underutilized resource. **In**: M.E. Feder, A.F. Bennett, W.W. Burggren and R.B. Hueym eds. 1987. *New Directions in Ecological Physiology.* New York: Cambridge University Press. pp.147-169.

Brierley, A.S. and Kingsford, M.J. (2009). Impacts of Climate Change on Marine Organisms and Ecosystems. *Current Biology*, 19, 602-614.

Bundy, J.G., Davey, M.P. and Viant, M.R. (2009). Environmental metabolomics: a critical review and future perspectives. *Metabolomics*, 5, 3-21.

Byrne, M. and Przeslawski, R. (2013). Multistressor impacts of warming and acidification of the ocean on marine invertebrates'life histories. *Integrative and Comparative Biology,* 53(4), 582-596.

Calosi, P., Turner, L.M., Hawkins, M., Bertoloni, C., Nightingale, G., Truebano, M. and Spicer, J.I. (2013). Multiple physiological responses to multiple environmental challenges: an individual approach. *Integrative and Comparative Biology,* 53(4), 660-670.

Calosi, P., De Wit, P., Thor, P. and Dupont, S. (2016). Will life find a way? Evolution of marine species under global change. *Evolutionary Applications*, 9(9), 1035-1042.

Calosi, P., Melatunan, S., Turner, L.M., Artioli, Y., Davidson, R.L., Byrne, J., Viant, M.R., Widdicombe, S. and Rundle, S.D. (2017). Regional adaptation defines sensitivity to future ocean acidification. *Nature Communications*, 8, 13994.

Dettmer, K., Aronov, P.A. and Hammock, B.D. (2007). *Mass spectrometry-based metabolomics*, 26(1), 51-78.

Ghosh, S., Tiwari, R., Hemamalini, R. and Khare, S.K. (2017). Chapter 20 – Multi-omic Approaches for Mapping Interactions Among Marine Microbiomes. In *Understanding Host-Microbiome Interactions – An Omics Approach: Omics of Hot-Microbiome Association* (ed. R.P. Singh, R. Kothari, P.G. Koringa and S.P. Singh), pp. 353-365. Springer Nature.

Goulitquer, S., Potin, P. and Tonon, T. (2012). Mass Spectrometry-Based Metabolomics to Elucidate Functions in Marine Organisms and Ecosystems. *Marine Drugs*, 10, 849-880.

IPCC. Climate Change (2014). Synthesis Report. Contribution of Working Groups I, II and II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and LA. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.

Johnson, C.H., Ivanisevic, J. and Siuzdak, G. (2016). Metabolomics: beyond biomarkers and towards mechanisms. *Nature Reviews Molecular Cell Biology*, 17(7), 451-459.

Lafaye, A., Labarre, J., Tabet, J.-C., Ezan, E. and Junot, C. (2005). Liquid Chromatography – Mass Spectrometry and ^{15}N Metabolic Labeling for Quantitative Metabolic Profiling. *Analytical Chemistry*, 77, 2026-2033.

Lankadurai, B.P., Nagato, E.G. and Simpson, M.J. (2013). Environmental metabolomics: an emerging approach to study organisms responses to environmental stressors. *Environmental Reviews,* 21, 180-205.

Longnecker, K., Futrelle, J., Coburn, E., Kido Soule, M.C. and Kujawinski, E.B. (2015). Environmental metabolomics: Databases and tools for data analysis. *Marine Chemistry*, 177, 366-373.

Lu, W., Kimball, E. and Rabinowitz, J.D. (2006). A high-performance liquid chromatography-tandem mass spectrometry method for quantification of nitrogen-containing intracellular metabolites. *Journal of the American Society for Mass Spectrometry*, 17(1), 37-50.

Mayor, D.J., Sommer, U., Cook, K.B. and Viant, M.R. (2015). The metabolic response of marine copepods to environmental warming and ocean acidification in the absence of food. *Scientific Reports,* 5, 13690.

Miller, M.G. (2007). Environmental Metabolomics: A SWOT Analysis (Strenghts, Weaknesses, Opportunities and Threats). *Journal of Proteome Research,* 6, 540-545.

Motti, C. (2012). Environmental Marine Metabolomics: From Whole Organism System Biology to Ecosystem Management. *Journal of Marine Science: Research & Development,* 2, e110.

Peng, B., Li, H. and Peng, X.-X. (2015). Functional metabolomics: from biomarker discovery to metabolome reprogramming. *Protein & Cell,* 6(9), 628-637.

Putnam, H.M., Davidson, J.M. and Gates, R.D. (2016). Ocean acidification influences host DNA methylation and phenotypic plasticity in environmentally susceptible corals. *Evolutionary Applications*. 9(9): 1165-1178.

Rochat, B. (2016). From targeted quantification to untargeted metabolomics: Why LC-high-resolution-MS will become a key instrument in clinical labs. *Trends in Analytical Chemistry,* 84, 151-164.

Shrestha, B. and Vertes, A. (2009). In Situ Metabolic Profiling of Single Cells by Laser Ablation Electrospray Ionization Mass Spectrometry. *Analytical Chemistry*, 81, 8265-8271.

Thor, P., Bailey, A., Dupont, S., Calosi, P., Søreide, J.E., De Wit, P., Gushelli, E., Loubet-Sartrou, L., Deichmann, I., Candee, M., Svensen, C., King, A.L. and Bellerby, R. (2018). Contrasting physiological response to future ocean acidification among Artic copepod population. *Global Change Biology*, 24, e365-e377.

Turner, L.M., Ricevuto, E., Massa-Gallucci, A., Gambi, M.-C. and Calosi, P. (2015). Energy metabolism and cellular homeostasis trade-offs provide the basis for a new type of sensitivity to ocean acidification in a marine polychaete at a high-CO2 vent: adenylate and phosphagen energy pools versus carbonic anhydrase. *The Journal of Experimental Biology*, 218, 2148-2151.

Verberk, W.C.E.P., Sommer, U., Davidson, R.L. and Viant, M.R. (2013). Anaerobic metabolism and thermal extremes: a metabolomic test of oxygen limitation hypothesis in an aquatic insect. *Integrative and Comparative Biology*, 53, 609-619.

Vernberg, F.J. and Vernberg, W.B. (2013). *Functional Adaptation of Marine Organisms*. New York: Academic Press.

Viant, M.R. (2007). Metabolomics of aquatic organisms: the new ‘omics’ on the block. *Marine Ecology Progress Series*, 332, 301-306.

Viant, M.R. (2008). Recent developments in environmental metabolomics. *Molecular BioSystems*, 4, 980-986.

Viant, M.R. and Sommer, U. (2013). Mass spectrometry based environmental metabolomics: A primer and review. *Metabolomics*, 9, 144-158.

Wittman, A.C. and Pörtner, H.O. (2013) Sensitivities of extant animal taxa to ocean acidification. *Nature Climate Change,* 4, 995-1001.

Table 1. Summary of method performance in regard to metabolite detection. Detection mode (MS Mode), detected mass (M/Z), retention time (RT), regression coefficient of the calibration curve (R^2), as well as instrumental limit of quantification (LOQ) and range of detection are provided.

Metabolite	MS Mode	M/Z	RT	R^2	LOQ (ng mL^{-1})	Range (ng mL^{-1})
NADPH	+	746.098+768.081	1.32	0.9950	40.0	40-1275
NADH	+	666.137	1.36	0.9946	20.0	20-630
NADP	+	744.089	1.38	0.9859	150.0	150-600
AMP	+	348.073+370.055	1.48	0.9993	10.0	10-2500
α-glycerophosphate	+	173.022	1.50	0.9953	20.0	20-2500
ATP	+	508.003	1.41	0.9991	10.0	10-2500
Aminoadipic acid	+	162.077	1.51	0.9971	0.5	0.5-80
ADP	+	428.04	1.52	0.9977	10.0	10-2500
Glutamic acid	+	148.06	1.50	0.9988	0.6	0.6-150
NAD	+	332.560+664.116	1.60	0.9975	10.0	10-325
Aspartic acid	+	134.045	1.61	0.9986	0.5	0.5-140
Phospho-L-Arginine	+	255.086	1.72	0.9956	10.0	10-1250
Cystine	+	241.031	1.85	0.9991	1.0	1-250
Hydroxyproline	+	132.066	1.92	0.9980	0.5	0.5-140
Serine	+	106.050	1.91	0.9981	0.75	0.75-100
Threonine	+	120.066	1.91	0.9983	0.5	0.5-120
Aminobutiric acid	+	104.071	1.95	0.9997	0.8	0.75-200
Alanine	+	90.056	1.95	0.9993	0.8	0.75-100
Tyrosine	+	182.082	2.03	0.9963	0.8	0.75-100
Betaine	+	118.086	2.05	0.9998	10.0	10-2500
Methionine	+	150.059	2.06	0.9989	0.5	0.5-150
Proline	+	116.071	2.06	0.9993	0.5	0.5-150
NADPH	-	371.540+382.550+744.084	1.33	0.9953	80.0	80-1250
α-ketoglutaric acid	-	145.014	1.34	0.9919	6.0	6-200
Fumaric aid	-	115.004	1.36	0.9983	10.0	10-625
NADH	-	331.550+664.117	1.37	0.9946	75.0	75-1250
Succinic acid	-	117.019	1.36	0.9971	5.0	5-300
ATP	-	252.490+505.988+527.970	1.40	0.9955	80.0	80-2500
ADP	-	426.022	1.42	0.9945	10.0	10-2500
α-glycerophosphate	-	171.006	1.51	0.9982	20.0	20-2500
Malic acid	-	133.014	1.45	0.9989	10.0	10-1500
AMP	-	346.055	1.48	0.9974	10.0	10-2500
Glutamic acid	-	146.046	1.50	0.9940	1.0	1-150
Lactic acid	-	89.024	1.52	0.9440	50.0	50-1000
Pyruvic acid	-	87.009	1.61	0.9992	75.0	75-2000
C4:0	-	87.045	1.87	0.9554	40.0	40-2600

Glucose	-	179.056+180.066+215.032	1.98	0.9940	100.0	100-26000
C6:0	-	115.076	2.14	0.9990	20.0	20-2600
C8:0	-	143.107	6.58	0.9722	20.0	20-2600
C10:0	-	171.139	6.78	0.9960	20.0	20-5000
C11:0	-	185.154	6.86	0.9989	10.0	10-2500
C12:0	-	199.170	6.88	0.9968	40.0	40-2600
C14:1	-	225.185	6.88	0.9994	10.0	10-2500
C13:0	-	213.185	6.89	0.9962	10.0	10-2500
C15:1	-	239.201	6.97	0.9997	10.0	10-2500
C14:0	-	227.201	7.01	0.9959	40.0	40-5250
C20:5	-	301.217	7.05	0.9963	10.0	10-2500
C18:3	-	277.217	7.05	0.9984	20.0	20-350
C16:1	-	253.217	7.16	0.9878	10.0	10-1250
C15:0	-	241.217	7.17	0.9904	20.0	20-1250
C22:6	-	327.232	7.22	0.9998	10.0	10-2500
C20:4	-	303.232	7.26	0.9909	10.0	10-650
C17:1	-	267.232	7.30	0.9965	40.0	40-625
C18:2	-	279.232	7.34	0.9943	20.0	20-625
C16:0	-	255.232	7.37	0.9568	250.0	250-4000
C20:3	-	305.248	7.47	0.9963	20.0	20-650
C18:1	-	281.248	7.60	0.9800	30.0	30-1000
C17:0	-	269.248	7.65	0.9938	10.0	10-1250
C20:2	-	307.264	7.79	0.9970	10.0	10-650
C18:0	-	283.264	8.05	0.9738	30.0	20-5250
C20:1	-	309.279	8.33	0.9953	10.0	10-2500
C22:2	-	335.295	8.66	0.9972	10.0	10-2500
C20:0	-	311.295	9.48	0.9941	20.0	20-5250
C22:1	-	337.311	9.87	0.9960	10.0	10-2500

Table 2. Estimation of the method reproducibility in terms of percentage of variation and recovery. The percentage of variation represents the standard deviation (in %) calculated from the normalized metabolite concentration of four subsample of copepod homogenate. The normalization was realised with the subsample mass. Method recovery at 10 % represent the mean of three normalized subsample of copepod homogenate that were spiked with the working stock solution at 10 % of the calibration curve and its standard deviation (variation in %). Compounds showing above 125 % recovery with much higher variation on the recovery reproducibility are highlighted in bold. NF indicates metabolites that were *not found.*

Metabolite	**% Variation**	**Recovery 10%**	
	n=4	**n=3**	**% variation**
NADPH	NF	90%	20%
NADH	NF	112%	14%
NADP	NF	110%	33%
AMP	4%	107%	28%
α-glycerophosphate	5%	112%	27%
ATP	NF	93%	4%
Aminoadipic acid	**11%**	**159%**	**27%**
ADP	5%	93%	7%
Glutamatic acid	**7%**	**168%**	**49%**
NAD	NF	100%	12%
Aspartic acid	**2%**	**200%**	**81%**
Phospho-L-Arginine	NF	81%	11%
Cystine	11%	84%	16%
Hydroxyproline	**8%**	**149%**	**28%**
Serine	**8%**	**304%**	**31%**
Threonine	**7%**	**308%**	**24%**
Aminobutiric acid	3%	96%	13%
Alanine	**5%**	**200%**	**22%**
Tyrosine	**12%**	**216%**	**155%**
Betaine	6%	27%	67%
Methionine	**7%**	**173%**	**27%**
Proline	18%	123%	59%
NADPH	NF	108%	14%
α-ketoglutaric acid	13%	113%	16%
Fumaric aid	7%	136%	16%
NADH	NF	98%	11%
Succinic acid	**10%**	**137%**	**19%**
ATP	NF	91%	20%
ADP	12%	74%	14%
α-glycerophosphate	8%	89%	16%
Malic acid	4%	113%	14%
AMP	5%	73%	16%

Glutamic acid	7%	103%	12%
Lactic acid	15%	98%	23%
Pyruvic acid	17%	NF	NF
C4:0	NF	87%	12%
Glucose	8%	91%	12%
C6:0	8%	85%	13%
C8:0	19%	118%	21%
C10:0	2%	101%	15%
C11:0	14%	107%	13%
C12:0	20%	105%	11%
C14:1	NF	111%	7%
C13:0	0%	117%	11%
C15:1	21%	109%	12%
C14:0	17%	115%	6%
C20:5	6%	120%	15%
C18:3	NF	106%	11%
C16:1	8%	109%	12%
C15:0	**35%**	**111%**	**13%**
C22:6	4%	121%	19%
C20:4	14%	104%	14%
C17:1	22%	109%	14%
C18:2	7%	109%	14%
C16:0	**34%**	**173%**	**5%**
C20:3	NF	106%	13%
C18:1	8%	111%	12%
C17:0	**26%**	**108%**	**9%**
C20:2	NF	103%	10%
C18:0	**49%**	**142%**	**16%**
C20:1	6%	110%	14%
C22:2	NF	108%	12%
C20:0	**46%**	**100%**	**4%**
C22:1	5%	102%	16%

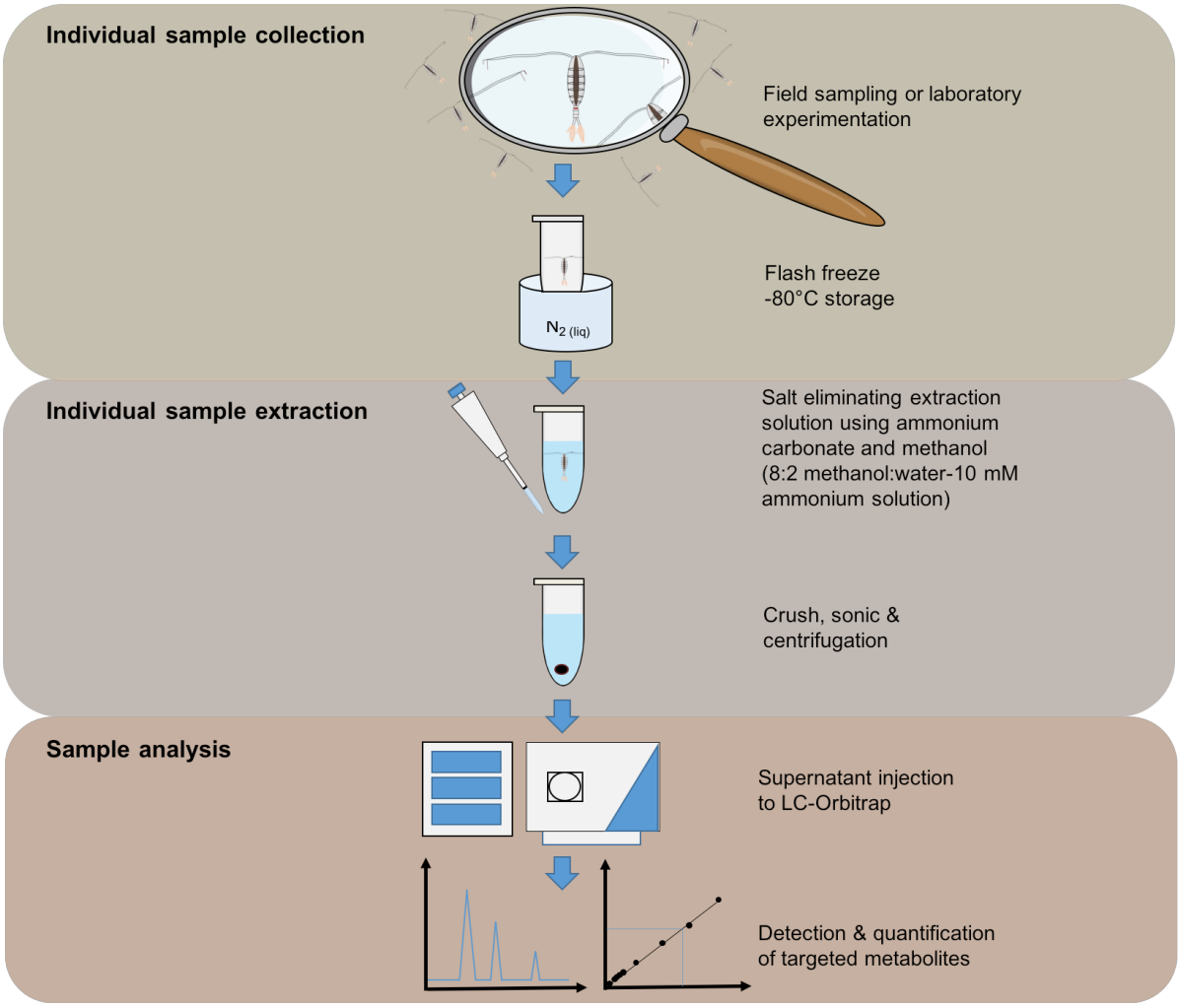
Table 3. Example of the concentration of the targeted metabolites obtained in copepodites stage IV of the copepod (*C. glacialis*), adult annelids (*O. japonica*) and stage III lobster larvae (*H. americanus*), detected with the proposed method. NF indicates metabolites that were *not found*.

Metabolite	***C. glacialis* (ng mL^{-1})**	***O. japonica* (ng mL^{-1})**	***H. americanus* (ng mL^{-1})**
NADPH			NF
NADH	NF	NF	NF
NADP	NF	NF	NF
AMP	432.3	109.1	<LOQ
α-glycerophosphate	NF	NF	5.9
ATP	203.2	24.8	373.7
Aminoadipic acid	0.3	13.7	5.4
ADP	523.1	155.9	0.3
Glutamatic acid	28.2	101.2	888.1
NAD	28.3	NF	153.8
Aspartic acid	69.2	194.2	828.1
Phospho-L-Arginine	38.8	NF	3442.8
Cystine	0.2	NF	<LOQ
Hydroxyproline	0.1	0.2	2.9
Serine	32.2	7.2	39.3
Threonine	11.9	10.0	35.1
Aminobutiric acid	6.4	1.5	3.1
Alanine	220.5		158.8
Tyrosine	2.5	4.7	10.2
Betaine	271.3	33.1	NF
Methionine	1.9	1.5	55.6
Proline	188.5	12.6	
NADPH	NF	NF	NF
α-ketoglutaric acid	3.6	NF	5.3
Fumaric aid	18.6	4.0	141.7
Succinic acid	27.5		67.4
Malic acid		4.9	118.9
Glutamic acid	30.5		
Lactic acid	284.8	<LOQ	NF
Pyruvic acid	49.1	35.0	139.3
C4:0	NF	NF	NF
Glucose	232.9	883.1	1302.4
C6:0	3.0	<LOQ	<LOQ
C8:0	86.7	<LOQ	<LOQ
C10:0	34.6	<LOQ	<LOQ
C11:0	10.4	6.9	<LOQ
C12:0	36.3	80.7	1.5

C14:1	7.2	6.3	NF
C13:0	11.7	27.6	NF
C15:1	NF	NF	NF
C14:0	80.7	<LOQ	NF
C20:5	340.7	<LOQ	35.7
C18:3	NF	16.3	62.4
C16:1	112.7	109.0	49.7
C15:0	NF	<LOQ	NF
C22:6	355.7	NF	NF
C20:4	NF	12.4	22.6
C17:1	NF	NF	NF
C18:2	24.8	20.4	55.9
C16:0	1166.3	3216.7	286.2
C20:3	NF	12.6	NF
C18:1	203.2	104.4	245.0
C17:0	30.7	<LOQ	23.9
C20:2	NF	<LOQ	NF
C18:0	873.2	3228.2	381.1
C20:1	NF	39.7	13.1
C22:2	NF	NF	NF
C20:0	190.7	<LOQ	166.9
C22:1	NF	NF	NF

Figures legend

Fig. 1 Schematic workflow of the proposed method. The figure was created with Microsoft Office PowerPoint 2016 for Mac.



RÉFÉRENCES BIBLIOGRAPHIQUES

Åkesson, B. (1976). Temperature and life cycle in *Ophryotrocha labronica* (Polychaeta, Dorvilleidae). *Ophelia*. **15**, 37-47.

Angilletta, M.J., Niewiarowski, P.H. et Navas, C.A. (2002). The evolution of thermal physiology in ectotherms. *J. Thermal. Biol.* **27**, 249-268.

Angilletta, M.J. (2009). *Thermal Adaptation. A Theoretical and Empirical Synthesis.* Oxford, UK: Oxford University Press.

Atkinson, D. (1996). Ectotherm life history responses to developmental temperature. In *Animals and temperature: phenotypic and evolutionary adaptation* (ed. I.D. Johnston, and A.F. Bennett), pp. 35-69. Cambridge, UK: Cambridge University Pres.

Aubin-Horth, N. et Renn, S.C. (2009). Genomic reaction norms: using integrative biology to understand molecular mechanisms of phenotypic plasticity. *Mol. Ecol.* **18**, 3763-3780.

Barnosky, A.D., Matzke, N., Tomiya, S., Wogan, G.O.U., Swartz, B., Quental, T.B., Marshall, C., McGuire, J.L., Lindsey, E.L., Maguire, K.C. *et al.* (2011). Has the Earth's sixth mass extinction already arrived? *Nature*. **471**, 51-57.

Bartholomew, G.A. (1986). The Role of Natural History in Contemporary Biology. *BioScience*. **36**, 324-329.

Bellard, C., Bertelsmeier, C., Leadley, P., Thuiller, W. et Courchamp, F. (2012). Impacts of climate change on the future of biodiversity. *Ecol. Lett.* **15**, 365-377.

Bownds, C., Wilson, R. et Marshall, D.J. (2010). Why do colder mothers produce larger eggs? An optimality approach. *J. Exp. Biol.* **213**, 3796-3801.

Boyd, P.W., Cornwall, C.E., Davison, A., Doney, S.C., Fourquez, M., Hurd, C.L., Lima, I.D et McMinn, A. (2016). Biological responses to environmental heterogeneity under future ocean conditions. *Glob. Change. Biol.* **22**, 2633-2650.

Bozinovic, F., Calosi, P. et Spicer, J.I. (2011). Physiological Correlates of Geographic Range in Animals. *Annu. Rev. Ecol. Evol. Syst.* **42**, 155-179.

Bozinovic, F. et Naya, D.E. (2015). Linking physiology, climate, and species distributional ranges. In *Integrative Organismal Biology* (ed. L.B. Martin, C.K. Ghalambor et H.A. Woods), pp. 277-290. New Jersey, USA: Wiley Blackwell.

Bradshaw, A.D. (1965). Evolutionary Significance of Phenotypic Plasticity in Plants. *Adv. Genet.* **13**, 115-155.

Brown, J.H., Stevens, G.C. et Kaufman, D.M. (1996). The geographic range: Size, Shape, Boundaries, and Internal structure. *Ann. Rev. Ecol. Syst.* **27**, 597-623.

Calosi, P., Bilton, D.T. et Spicer, J.I. (2008). Thermal tolerance, acclimatory capacity and vulnerability to global climate change. *Biol. Lett.* **4**, 99-102.

Calosi, P., Rastrick, S.P.S., Lombardi, C., Guzman, H.J., Davidson, L., Jahnke, M. Giangrande, A., Hardege, J.D., Schulze, A., Spicer, J.I. et Gambi, M-C. (2013). Adaptation and acclimatization to ocean acidification in marine ectotherms: an *in situ* transplant experiment with polychaetes at a shallow CO_2 vent system. *Phil. Trans. R. Soc. B.* **368**, 20120444.

Calosi, P., De Wit, P., Thor, P. et Dupont, S. (2016). Will life find a way? Evolution of marine species under global change. *Evol. Appl.* **9**, 1035-1042.

Calosi, P., Putnam, H.M., Twitchett, R.J. et Vermandele, F. (2019). Marine Metazoan Modern Mass Extinction: Improving Predictions by Integrating Fossil, Modern and Physiological Data. *Annu. Rev. Mar. Sci.* **11**, 20.1-20.22.

Cannon, W.B. (1929). Organization for physiological homeostasis. *Physiol. Rev.* **9**, 399-431.

Ceballos, G., Ehrlich, P.R., Barnosky, A.D., Garcia, A., Pringle, R.M. et Palmer, T.M. (2015). Accelerated modern human-induced species losses: Entering the sixth mass extinction. *Science*. **1**, e1400253.

Ceballos, G., Ehrlich, P.R. et Dirzo, R. (2017). Biological annihilation via the ongoing sixth mass extinction signaled by vertebrate population losses and declines. *Proc. Natl. Acad. Sci. USA.* **114**, E6089-E6096.

Ceballos, G. et Ehrlich, P.R. (2018). The misunderstood sixth mass extinction. *Science*. **360**, 1080-1081.

Chakravarti, L.J., Jarrold, M.D., Gibbin, E.M., Christen, F., Massamba-N'Siala, G., Blier, P.U. et Calosi, P. (2016). Can trans-generational experiments be used to enhance species resilience to ocean warming and acidification? *Evol. Appl.* **9**, 1133-1146.

Chen, I.C., Hill, J.K., Ohlemüller, R., Roy, D.B. et Thomas, C.D. (2011). Rapid Range Shifts of Species Associated with High Levels of Climate Warming. *Science*. **333**, 1024-1026.

Chevin, L-M., Lande, R. et Mace, G.M. (2010). Adaptation, Plasticity, and Extinction in a Changing Environment: Towards a Predictive Theory. *PLoS Biol.* **8**, e1000357.

Chevin, L-M., Gallet, R., Gomulkiewicz, R., Holt, R.D. et Fellous, S. (2013). Phenotypic plasticity in evolutionary rescue experiments. *Phil. Trans. R. Soc. B.* **368**, 20120089.

Chown, S.L., Gaston, K.J. et Robinson, D. (2004). Macrophysiology: large-scale patterns in physiological traits and their ecological implications. *Funct. Ecol.* **18**, 159-167.

Chown, S.L. et Gaston, K.J. (2008). Macrophysiology for a changing world. *P. Roy. Soc. Lond. B. Bio.* **275**, 1469-1478.

Chung, D.J et Schulte, P.M. (2015). Mechanisms and costs of mitochondrial thermal acclimation in a eurythermal killifish (*Fundulus heteroclitus*). *J. Exp. Biol.* **218**, 1621-1631.

Claireaux, G. et Lefrançois, C. (2007). Linking environmental variability and fish performance: integration through the concept of scope for activity. *Phil. Trans. R. Soc. Lond. B. Biol. Sci.* **362**, 2031-2041.

Clark, T.D., Sandblom, E. et Jutfelt, F. (2013a). Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *J. Exp. Biol.* **216**, 2771-2782.

Clark, T.D., Sandblom, E. et Jutfelt, F. (2013b). Response to Farrell and to Pörtner and Giomi. *J. Exp. Biol.* **216**, 4495-4497.

Cooper, S.J. (2008). From Claude Bernard to Walter Cannon. Emergence of the concept of homeostasis. *Appetite*. **51**, 419-427.

Dell, A.I., Pawar, S. et Savage, V.M. (2013). The thermal dependence of biological traits. *Ecology*. **94**, 1205-1206.

Deutsch, C.A., Tewksbury, J.J, Huey, R.B., Sheldon, K.S., Ghalambor, C.K., Haak, D.C. et Martin, P.R. (2008). Impacts of climate warming of terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci.* **105**, 6668-6672.

DeWitt, T.J., Sih, A. et Wilson, D.S. 1998. Costs and limits of phenotypic plasticity. *Trends. Ecol. Evol.* **13**, 151-154.

Diamond, S.E. (2018). Contemporary climate-driven range shifts: Putting evolution back on the table. *Funct. Ecol.* **32**, 1652-1665.

Dobzhansky, T. (1964). Biology, Molecular and Organismic. *Am. Zool.* **4**, 443-452.

Donelson, J.M., Salinas, S., Munday, P.L. et Shama, L.N.S. (2018). Transgenerational plasticity and climate change experiments: Where do we go from here? *Global. Change. Biol.* **24**, 13-34.

Etterson, J.R. et Shaw, R.G. (2001). Constraint to Adaptive Evolution in Response to Global Warming. *Science*. **294**, 151-154.

Forsman, A. (2015). Rethinking phenotypic plasticity and its consequences for individuals, populations and species. *Heredity*. **115**, 276-284.

Fox, C.W. et Czesak, M.E. (2000). Evolutionary ecology of progeny size in arthropods. *Annu. Rev. Entomol.* **45**, 341-369.

Gaston, K.J. (2003). *The Structure and Dynamics of Geographic Ranges*. Oxford, UK: Oxford University Press.

Gaston, K.J (2009). Geographic range limits: achieving synthesis. *Proc. R. Soc. B.* **276**, 1395-1406.

Ghalambor, C.K., McKay, J.K., Carroll, S.P. et Reznick, D.N. (2007). Adaptive *versus* non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments. *Funct. Ecol.* **21**, 394-407.

Gibbin, E.M., Chakravarti, L.J., Jarrold. M.D., Christen, F., Turpin, V., Massamba-N'Siala, G., Blier P.U., et Calosi, P. (2017a). Can multi-generational exposure to ocean warming and acidification lead to adaptation of life-history and physiology in a marine metazoan? *J. Exp. Biol.* **220**, 551-563.

Gibbin, E.M., Massamba-N'Siala, G., Chakravarti, L.J, Jarrold, M.D. et Calosi, P. (2017b). The evolution of phenotypic plasticity under global change. *Sci. Rep.* **7**, 17253.

Godbold, J.A. et Calosi, P. (2013). Ocean acidification and climate change: advances in ecology and evolution. *Phil. Trans. R. Soc. B*. **368**, 20120448.

Helaouët, P. et Beaugrand, G. (2009). Physiology, Ecological Niches and Species Distribution. *Ecosystems*. **12**, 1235-1245.

Hochachka, P.W. et Somero, G.N. (1984). *Biochemical Adaptation*. Princeton, USA: Princeton University Press.

Hoffman, A.A. (1995). Acclimation: increasing survival at a cost. *Trends. Ecol. Evol.* **10**, 1-2.

Hofmann, G.E, Barry, J.P., Edmunds, P.J., Gates, R.D., Hutchins, D.A., Klinger, T. et Sewell, M.A. (2010). The Effect of Ocean Acidification on Calcifying Organisms in Marine Ecosystems: An Organisms-to-Ecosystem Perspective. *Annu. Rev. Ecol. Evol. Syst.* **41**, 127-147.

Huey, R.B. et Kingsolver, J.G. (1989). Evolution of thermal sensitivity of ectotherm performance. *Trends. Ecol. Evol.* **4**, 131-135.

IPCC (2014). Climate Change 2014: Synthesis report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.

Jarrold, M.D., Chakravarti, L.J., Gibbin, E.M., Christen, F., Massamba-N'Siala, G., Blier, P.U. and Calosi, P. (2019). Life-history trade-offs and limitations associated with phenotypic adaptation under future ocean warming and elevated salinity. *Phil. Trans. R. Soc. B.* **374**, 20180428.

Jutfelt, F., Norin, T., Ern, R., Overgaard, J., Wang, T., McKenzie, D.J., Lefevre, S., Nilsson, G.E., Metcalfe, N.B., Hickey, A.J.R. *et al.* (2018). Oxygen-and capacity-limited thermal tolerance: blurring ecology and physiology. *J. Exp. Biol.* **221**, jeb169615.

Krogh, A. 1929. The progress of physiology. *Am. J. Physiol.*, **90**(2), 243-251.

Lee, J., Janion, C., Marais, E., van Vuuren, B.J. et Chown, S.L. (2009). Physiological tolerances account for range limits and abundance structure in an invasive slug. *Proc. R. Soc. B.* **276**, 1459-1468.

Liefting, M., Weerenbeck, M., van Dooremalen, C. et Ellers, J. (2010). Temperature-induced plasticity in egg size and resistance of eggs to temperature stress in a soil arthropod. *Funct. Ecol.* **24**, 1291-1298.

Martin, P.R. (2015). Trade-offs and biological diversity: integrative answers to ecological questions. In *Integrative Organismal Biology* (ed. L.B. Martin, C.K. Ghalambor et H.A. Woods), pp. 291-308. New Jersey, USA: Wiley Blackwell.

Massamba-N'Siala, G., Calosi, P., Bilton, D.T., Prevedelli, D. et Simonini, R. (2012). Life-history and thermal tolerance traits display different thermal plasticities and relationships with temperature in the marine polychaete *Ophryotrocha labronica* La Greca and Bacci (Dorvillidae). *J. Exp. Mar. Biol. Ecol.* **438**, 109-117.

Massamba-N'Siala, G., Prevedelli, D. et Simonini, R. (2014). Trans-generational plasticity in physiological thermal tolerance is modulated by maternal pre-reproductive environment in the polychaete *Ophryotrocha labronica. J. Exp. Biol.* **217**, 2004-2012.

McNab, B.K. (2002). *The physiological ecology of vertebrates: a view form energetics*. Ithaca, USA: Cornell University Press.

Moyes, C.D et Schulte, P.M. (2008). *Principles of animal physiology*, 2nd ed., San Francisco, US: Pearson Benjamin Cummings.

Munday, P.L., Warner, R.R., Monro, K., Pandolfi, J.M. et Marshall, D.J. (2013). Predicting evolutionary responses to climate change in the sea. *Ecol. Lett.* **16**, 1488-1500.

Parmesan, C. et Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature.* **421**, 37-42.

Parmesan, C. (2006). Ecological and Evolutionary Responses to Recent Climate Change. *Ann. Rev. Ecol. Evol. Syst.* **37**, 637-669.

Peck, L.S., Morley, S.A. et Clark, M.S. (2010). Poor acclimation capacities in Antarctic marine ectotherms. *Mar. Biol.* **157**, 2051-2059.

Perry, A.L., Low, P.J., Ellis, J.R. et Reynolds, J.D. (2005). Climate Change and Distribution Shifts in Marine Fishes. *Science*. **308**, 1912-1915.

Pigliucci, M. (2001). *Phenotypic plasticity: beyond nature and nurture*. Maryland, USA: JHU Press.

Pigliucci, M. (2003). Phenotypic integration: studying the ecology and evolution of complex phenotypes. *Ecology Letters*. **6**, 265-272.

Pörtner, H. (2001). Climate change and temperature-dependent biogeography: Oxygen limitation of thermal tolerance in animals. *Naturwissenschaften*. **88**, 137-146.

Pörtner, H. (2002). Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **132**, 739-761.

Pörtner, H.O., Bennett, A.E., Bozinovic, F., Clarke, A., Lardies, M.A., Lucassen, M., Pelster, B., Schiemer, F. et Stillman, J.H. (2006). Trade-Offs in Thermal Adaptation: The Need for a Molecular to Ecological Integration. *Physiol. Biochem. Zool.* **79**, 295-313.

Pörtner, H.O. et Knust, R. (2007). Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science*. **315**, 95-97.

Pörtner, H.O. et Farrell, A.P. (2008). Ecology. Physiology and climate change. *Science.* **322**, 690-692.

Prevedelli, D. et Simonini, R. (2001). Effect of temperature on demography of *Ophryotrocha labronica* (Polychaeta: Dorvilleidae). *Vie. Milieu*. **54**, 173-180.

Reece, J., Urry, L., Cain, M., Wasserman, S., Minorsky, P. et Jackson, R. (2012). *Campbell Biologie*, 4e ed, Montréal, CAN: Éditions du renouveau pédagogique Inc.

Reusch, T.B.H. (2014) Climate change in the oceans: evolutionary versus phenotypically plastic responses of marine animals and plants. *Evol. Appl.* **7**, 104-122.

Reznick, D.N. (1985). Costs of Reproduction: An Evaluation of the Empirical Evidence. *Oikos*. **44**, 257-267.

Rodriguez-Romero, A., Jarrold, M.D., Massamba-N'Siala, G., Spicer, J.I. et Calosi, P. (2015). Multi-generational responses of a marine polychaete to a rapid change in seawater *p*CO2. *Evol. Appl.,* **9**, 1082-1095.

Roff, D.A. (1992). *The Evolution of Life Histories*. New York, USA: Chapman & Hal.

Roff, D.A. et Gélinas, M.D. (2002). Phenotypic plasticity and the evolution of trade-offs: the quantitative genetics of resource allocation in the wing dimorphic cricket, *Gryllus firmus*. *J. Evolution. Biol.* **16**, 55-63.

Schlichting, C.D. et Pigliucci, M. (1998). *Phenotypic Evolution: A Reaction Norm Perspective*. Sunderland, USA: Sinauer Associates.

Schulte, P.M., Healy, T.M. et Fangue, N.A. (2011). Thermal performance curves, phenotypic plasticity, and the time scales of temperature exposure. *Integr. Comp. Biol.* **51**, 691-702.

Schulte, P.M. (2015). The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *J. Exp. Biol.* **218**, 1856-1866.

Shain, D.H. (2009). *Annelids in Modern Biology*. New-Jersey, USA: John Wiley & Sons.

Shama, L.N.S., Mark, F.C., Strobel, A., Lokmer, A., John, U. et Wegner, K.M. (2016). Transgenerational effects persist down the maternal line in marine sticklebacks: gene expression matches physiology in a warming ocean. *Evol. Appl.* **9**, 1096-1111.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B. et Ideker, T. (2003). Cytoscape: a software environment for integrated modes of biomolecular interaction networks. *Genome. Res.* **13**, 2498-2504.

Smith, T.M. et Smith, R.L. (2012). *Elements of ecology*, 8e ed. San Francisco, USA: Pearson Benjamin Cummings.

Somero, G.N. (2010). The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine 'winners' and 'losers'. *J. Exp. Biol.* **213**, 912-920.

Somero, G.N., Lockwood, B.L. et Tomanek, L. (2017). *Biochemical Adaptation - Response to Environmental Challenges from Lifes' Origins to the Anthropocene*. 1^{e} edition. Sunderland, USA: Sinauer Associates Inc.

Stearns, S.C. (1989). Trade-Offs in Life-History Evolution. *Funct. Ecol.* **3**, 259-268.

Strange, K. (2005). The end of "naïve reductionism": rise of systems biology or renaissance of physiology? *Am. J. Physiol.* **288**, 968-974.

Strobel, A., Graeve, M., Pörtner, H.O. et Mark, F.C. (2013). Mitochondrial Acclimation Capacities to Ocean Warming and Acidification Are Limited in the Antarctic Nototheniid Fish, *Notothenia rossii* and *Lepidonotothen squamifrons*. *PLoS One.* **8**, e68865.

Sunday, J.M, A.E. Bates et Dulvy, N.K. (2012). Thermal tolerance and the global redistribution of animals. *Nat. Clim. Change.* **2**, 686-691.

Sunday, J.M., Bates, A.E., Kearney, M.R., Colwell, R.K., Dulvy, N.K., Longino, J.T., et Huey, R.B. (2014a). Thermal-safety margins and the necessity of thermoregulatory behavior across latitude and elevation. *P. Natl. Acad. Sci-Biol.* **111**, 5610-5615.

Sunday, J.M., Calosi, P., Dupont, S., Munday, P.L., Stillman, J.H. et Reush, T.B.H. (2014b). Evolution in an acidifying ocean. *Trends. Ecol. Evol.* **29**, 117-125.

Taylor, J.A. (1984). *Biogeography: Recent advances and future directions*. 1e edition. New Jersey, USA: Barnes & Noble Books.

Thor, P. et Dupont, S. (2015). Transgenerational effects alleviate severe fecundity loss during ocean acidification in a ubiquitous planktonic copepod. *Glob. Change. Biol.* **21**, 2261-2271.

Verberk, W.C.E.P., Sommer, U., Davidson, R.L. et Viant, M.R. (2013). Anaerobic Metabolism at Thermal Extremes: A Metabolomic Test of the Oxygen Limitation Hypothesis in an Aquatic Insect. *Integr. Comp. Biol.* **53**, 609-619.

Wake, M.H. (2008). Integrative Biology: Science for the 21st Century. *BioScience.* **58**, 349-353.

West-Eberhard, M. (2003). *Developmental plasticity and evolution*. New York, USA: Oxford University Press.

Wiens, J.J. (2011). The niche, biogeography and species interactions. *Phil. Trans. R. Soc. B.* **366**, 2336-2350.

Woods, H.A. et Wilson, J.K. (2015). An elephant in the fog: unifying concepts of physiological stasis and change. In *Integrative Organismal Biology* (ed. L.B. Martin, C.K. Ghalambor et H.A. Woods), pp. 119-135. New Jersey, USA: Wiley Blackwell.

Woods, H.A., Martin, L.B. et Ghalambor, C.K. (2015). Conclusions: the central role of the organism in biology. In *Integrative Organismal Biology* (ed. L.B. Martin,

C.K. Ghalambor et H.A. Woods), pp. 309-317. New Jersey, USA: Wiley Blackwell.

Zheng, H., Jiang, R. et Zhao, Z. (2014). Integrated Approach in Systems Biology. *Comput. Math. Methods. Med.* **2014**, 656473.