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**Nutritional constraints on growth and metabolic rate of  
an ectotherm consumers (*Daphnia magna*) in a context  
of global change: Experimental approach by  
microcalorimetry**

Thomas Ruiz

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**Université Clermont Auvergne**

**Thèse de Doctorat**

Spécialité : Gestion de l'environnement

Présentée par :

**Thomas RUIZ**

Pour obtenir le grade de

**DOCTEUR de l'Université Clermont Auvergne**

**Contraintes nutritionnelles sur  
le taux métabolique et la croissance d'un  
consommateur ectotherme (*Daphnia magna*)  
dans le contexte des changements globaux :  
Approche expérimentale par microcalorimétrie.**

Soutenance : 13 Octobre 2020

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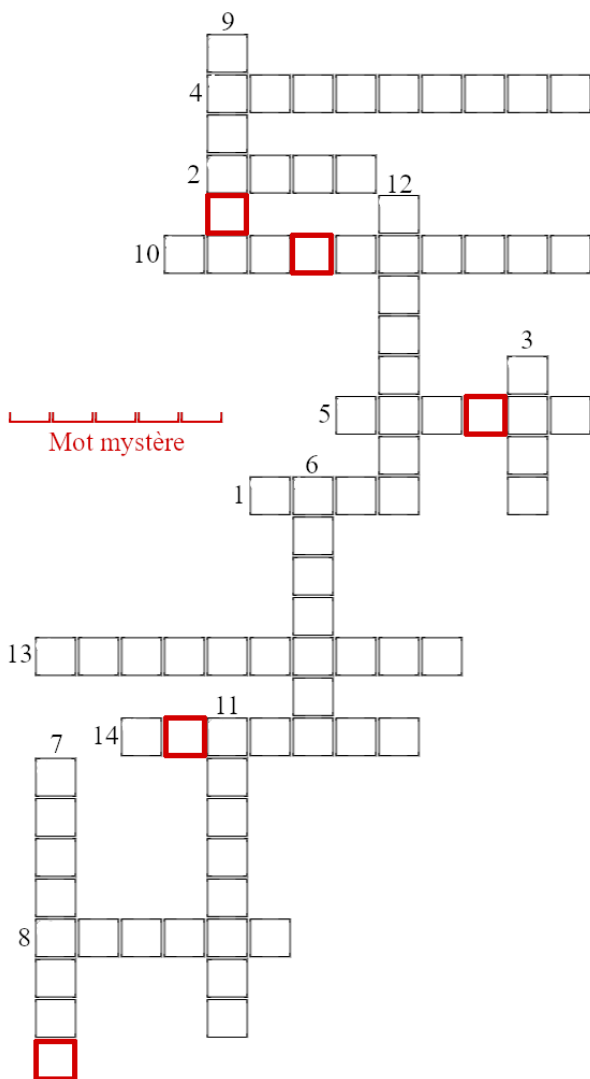
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*“Ecological sciences are a patchwork of approaches, perspectives and theories. They are necessarily so because of the sheer complexity of ecological systems.”*

Robert Sterner 2015

## Résumé :

Dans un contexte de changement global, anticiper les altérations de la structure et du fonctionnement des écosystèmes aquatiques est un enjeu écologique majeur nécessaire à la gestion de ces derniers. Cependant, la fragmentation de l'écologie en une multitude de sous-disciplines est aujourd'hui vue comme un obstacle à toutes prédictions, créant une volonté grandissante d'unifier les différents cadres théoriques de l'écologie. Dans ce contexte, l'importance d'harmoniser l'écologie stœchiométrique et l'écologie métabolique pour affiner nos capacités à prédire la structure et le fonctionnement écosystémique est largement reconnue (Allen and Gillooly, 2009; Hillebrand et al., 2009; Ott et al., 2014). A ce titre, ce travail de thèse s'est principalement consacré à établir les liens entre qualité nutritionnelle et taux métabolique des consommateurs afin de comprendre comment leurs interactions pouvaient affecter la croissance des organismes. L'objectif a également été de comprendre comment des facteurs environnementaux associés au changement global (réchauffement, salinisation) pouvaient affecter cette notion de qualité nutritionnelle ou interagir avec elle par l'intermédiaire du métabolisme de l'organisme.

A cet égard, le volet expérimental de cette thèse s'est focalisé sur le cladocère *Daphnia magna*, organisme clé des réseaux trophiques aquatiques, particulièrement sensible à la qualité de ses ressources alimentaires. Nous avons, dans un premier temps, cherché à déterminer comment l'élévation des températures pouvait moduler la notion de qualité nutritionnelle pour le consommateur. Dans un second temps, la mise au point d'une méthode basée sur la microcalorimétrie a permis d'établir les liens existants entre qualité nutritionnelle et taux métabolique du consommateur. Finalement, nous avons exploré les interactions entre qualité nutritionnelle, taux métabolique et stress osmotique sur les traits de vie de *Daphnia* afin de comprendre comment un facteur environnemental (ici la salinité) pouvait affecter ou être affecté par les liens qualité-métabolisme.

Nos résultats ont, tout d'abord, démontré la forte dépendance thermique des besoins nutritionnels stœchiométriques chez *Daphnia* ainsi que les potentielles conséquences en résultant sur le découplage producteur-consommateur dans un contexte de changement global. Nous avons ensuite démontré les conséquences de ce découplage (i.e. de la qualité nutritionnelle) sur le taux métabolique au repos (RMR) des consommateurs. Nous avons mis en évidence une élévation systématique du RMR lors de diminutions de la qualité nutritionnelle. Ce surcout énergétique se répercutant *in fine* sur la croissance des consommateurs, la mesure du RMR semble ici permettre de quantifier le coût énergétique des contraintes nutritionnelles (stœchiométriques mais aussi biochimiques). Finalement, nous avons pu mettre en évidence les interactions entre qualité nutritionnelle et tolérance à la salinité ainsi que leurs répercussions au niveau métabolique. De façon générale, cette thèse a démontré le rôle central du RMR dans la réponse des organismes face aux stress environnementaux. Ainsi, la mesure du RMR pourrait constituer un indicateur essentiel permettant de prédire la structure et le fonctionnement des écosystèmes dans un contexte de changement global.

## **Abréviations**

AGPI: Acide gras polyinsaturé

ANCOVA: Analysis of covariance

ANOVA: Analysis of variance

BPC: Biomass production cost

C: Carbone

DHA: Docosahexaenoic acid

DNA: Deoxyribonucleic acid

EPA: Eicosapentaenoic acid

GAM: Generalized additive model

GRH: Growth rate hypothesis

HUFA: Highly unsaturated fatty acid

MTE: Metabolic theory of ecology

N: Nitrogen

P: Phosphorus

POPC: 1-palmitoyl-2-oleoyl-phosphatidylcholin

POPG: 1-palmitoyl-2-oleoyl-phosphatidylglycerol

PUFA: Polyunsaturated fatty acid

RMR: Resting metabolic rate

SDA: Specific dynamic action

TER: Threshold elemental ratio

TMT: Threshold minimizing temperature

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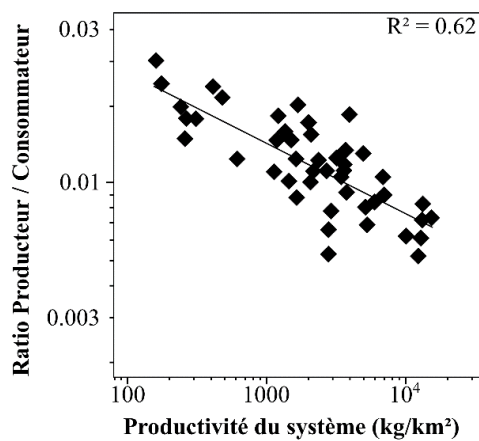
# **Chapitre 1 : Introduction générale**

## **1.1 Introduction :**

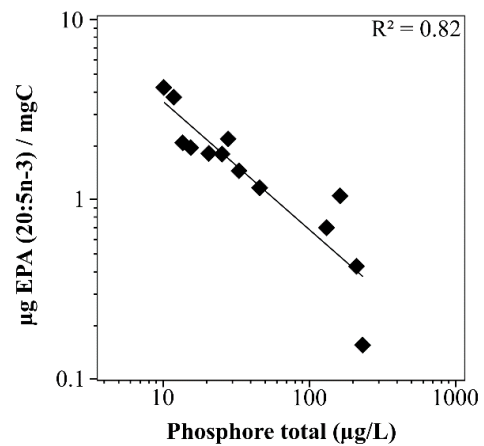
Anticiper les altérations de la structure et du fonctionnement écosystémique en réponse aux stress environnementaux et anthropiques est une préoccupation majeure des écologues mais aussi des gestionnaires. A ce titre, prédire de la production secondaire au sein de ces écosystèmes pourrait constituer un proxy intéressant permettant d'anticiper de telles altérations (Dolbeth et al., 2012). En effet, la production secondaire constitue un marqueur intégratif du développement des populations (croissance, reproduction), de leurs interactions biotiques (prédation, compétition, etc.) ainsi que de leur réponse aux conditions environnementales (Huryn and Wallace, 2000; von Schiller et al., 2017). Cependant, prédire cette production secondaire s'avère souvent problématique, en particulier du fait de la variabilité des efficacités de transferts de matière et d'énergie à l'interface producteur-consommateur (Brett and Goldman, 1997) pouvant engendrer un découplage, parfois important, entre production primaire et production secondaire (Davis et al., 2010).

Néanmoins, une méta-analyse récente (Hatton et al., 2015) remet en question ce découplage producteur-consommateur en démontrant, à large échelle, une forte corrélation entre biomasse des producteurs et biomasse des consommateurs. Plus précisément, la relation avancée par ces auteurs démontre que, si l'efficacité des transferts tend à diminuer dans les milieux plus productifs, la production secondaire reste largement dépendante de la productivité primaire et semble donc prévisible. On constate, malgré tout, dans cette étude la présence d'une variabilité résiduelle importante. Bien qu'elle n'altère pas cette relation à large échelle, elle pourrait brouiller les prédictions à une échelle plus fine. C'est pourtant à ce niveau plus spécifique que prédire la production secondaire est essentiel afin d'orienter l'action des gestionnaires. En milieu aquatique particulièrement, la gestion des populations piscicoles ne peut se faire qu'au cas par cas à l'échelle de chaque masse d'eau spécifiquement et requiert

donc des capacités prédictives à échelle locale (Soranno et al., 2011). A cette échelle, nombre d'études ont exploré la qualité nutritionnelle des producteurs primaires pour les consommateurs herbivores afin de déterminer l'efficacité des transferts entre ces deux niveaux trophiques (Elser et al., 2000; Müller-Navarra, 2008; Müller-Navarra et al., 2000). L'une d'entre elle apparaît ici particulièrement notable (Müller-Navarra et al. 2004) car elle démontre une diminution de la disponibilité en acides gras polyinsaturés (AGPI), notamment en EPA (20:5n-3) qui constitue un déterminant important de la qualité nutritionnelle (Müller-Navarra et al., 2000), avec l'enrichissement du milieu en phosphore. Cet enrichissement en P étant généralement associé à des phénomènes d'eutrophisation et de forte productivité phytoplanctonique (Conley et al., 2009), cette étude semble démontrer une diminution de la qualité nutritionnelle, et donc de l'efficacité des transferts, dans les milieux fortement productifs, une conclusion étonnamment similaire à celle d'Hatton et al. (2015) (**Figure 1**).



*D'après Hatton et al. (2015)*



*D'après Müller-Navarra et al. (2004)*

**Figure 1 :** Comparaison des résultats de Hatton et al (2015) et Müller-Navarra et al. (2004) détaillant l'efficacité des transferts en fonction de la productivité du système.

Une telle concordance entre ces études suggère l'existence de liens entre qualité nutritionnelle des producteurs et modification de ratio producteur/consommateur observé à large échelle, corroborant ainsi les conclusions de Cebrian et al (2009) selon lesquelles la qualité nutritionnelle des producteurs primaires constituerait un déterminant majeur de la structure écosystémique.

Ainsi, prédire la production secondaire et la structuration écosystémique requiert, en premier lieu, de définir clairement la qualité nutritionnelle des producteurs. Cependant, malgré plusieurs décennies de recherches consacrées à cette qualité nutritionnelle (Ahlgren et al., 1990; Bordalo et al., 2018; Brett et al., 2000; Guo et al., 2016; Sterner, 1993), celle-ci reste complexe à définir (Persson et al., 2007). En effet, les déterminants de cette qualité sont nombreux et couvrent une large gamme de composés spécifiques (Peltomaa et al., 2017) incluant notamment, mais non exclusivement, nutriments (N, P - (Elser et al., 2000; Sterner, 1993), acides gras insaturés ((Isanta Navarro et al., 2019; Müller-Navarra et al., 2000), stéroïdes (Martin-Creuzburg and Merkel, 2016; Von Elert et al., 2003), acides aminés (Fink et al., 2011), ou encore macronutriments (Raubenheimer et al., 2005), chacun affectant indépendamment ou de concert différents traits de vie de l'organisme comme sa croissance, sa survie, son succès reproducteur, etc. (**Table 1**).

De plus, il a été démontré que le contexte environnemental pouvait, dans certains cas, modifier les besoins nutritionnels des consommateurs, modulant ainsi de façon relative la qualité nutritionnelle de leurs ressources. A ce titre, il est notamment reconnu qu'une diminution des températures tend à augmenter les besoins en AGPI des consommateurs pour assurer la fluidité de leurs membranes (Masclaux et al., 2009). D'un point de vue stœchiométrique, les besoins élémentaires des consommateurs (C, P) semblent également être affectés par la température, bien que le sujet soit encore à l'origine de vigoureux débats

(Boersma et al., 2016a, 2016b; Winder et al., 2016). Ainsi, du fait de la complexité que revêt la qualité nutritionnelle mais aussi de sa variabilité en réponse au contexte environnemental, il semble impossible de formaliser cette qualité sous une unique valeur permettant de la quantifier de façon directe.

		Déterminant de qualité	Publications
Trait de vie observé	Taux de Croissance	P	(Boersma, 2000; Brett et al., 2000; Elser et al., 2000; Persson et al., 2011; Plath and Boersma, 2001; Urabe et al., 1997)
		N	(Cole et al., 2002; Groeger et al., 1991)
		AGPI indifférenciés	(Martin-Creuzburg and von Elert, 2009; Park et al., 2002)
		18:3n-3	(Sundbom and Vrede, 1997)
		20:5n-3	(Ahlgren et al., 1990; Gladyshev et al., 2008; Müller-Navarra et al., 2004, 2000; Sundbom and Vrede, 1997)
		22:6n-3	(Ahlgren et al., 1990; Müller-Navarra et al., 2004)
		Stérols	(Martin-Creuzburg and Merkel, 2016; Martin-Creuzburg and von Elert, 2009; Von Elert et al., 2003)
		A.Aminés (Arg / Hist)	(Fink et al., 2011)
		Chlorophylle	(Cole et al., 2002)
	Taux de Fécondité	N	(Groeger et al., 1991)
		AGPI indifférenciés	(Martin-Creuzburg and von Elert, 2009)
		18:3n-3	(Sundbom and Vrede, 1997)
		20:5n-3	(Müller-Navarra et al., 2004, 2000; Sundbom and Vrede, 1997)
		22:6n-3	(Müller-Navarra et al., 2004)
		A.Aminés (Arg / Hist)	(Fink et al., 2011)
		Protéine	(Koch et al., 2009)
		Sterols	(Martin-Creuzburg and von Elert, 2009)
	Durée de vie	P	
		N	(Cole et al., 2002; Groeger et al., 1991)
		18:3n-3	(Sundbom and Vrede, 1997)
20:5n-3		(Sundbom and Vrede, 1997)	
Chlorophylle		(Cole et al., 2002)	

**Table 1 :** Liste non exhaustive de publications portant sur la qualité nutritionnelle en milieu aquatique en fonction des traits de vie et déterminants de qualité observés.

L'impossibilité à quantifier cette qualité nutritionnelle a très largement empêché sa prise en compte dans nombre de modèles prédisant la production secondaire, se focalisant plus généralement sur des approches métaboliques (Álvarez and Nicieza, 2005; Brown et al., 2004; West et al., 2001). De manière générale, ces modèles métaboliques se basent sur l'étude des flux d'énergie au sein des écosystèmes en exploitant plus particulièrement les relations allométriques liant taux métabolique et masse de l'organisme afin de prédire la production de chaque niveau trophique (Brown et al., 2004). Si ces approches constituent une base solide en vue de prédire la production secondaire, leur incapacité à intégrer le contexte nutritionnel des consommateurs peut engendrer d'importants biais faussant inévitablement leurs prédictions. Dans ce contexte il apparaît essentiel d'intégrer la notion de qualité nutritionnelle à ces approches métaboliques afin de prédire efficacement la production secondaire, non seulement à large échelle, mais aussi à un niveau plus spécifique afin de faciliter l'action des gestionnaires. Une telle intégration, dont l'importance a déjà été suggérée au cours de la dernière décennie (Allen and Gillooly, 2009; Hillebrand et al., 2009; Moorthi et al., 2016; Ott et al., 2014), pourrait permettre, à terme, de mieux anticiper les réponses des populations et/ou communautés soumises à une pression anthropique grandissante.

## **1.2 Organisation du mémoire de thèse :**

Ce travail de thèse a pour objectif d'explorer les interactions existantes entre qualité nutritionnelle et taux métabolique des consommateurs ainsi que leurs conséquences sur la croissance dans un contexte de changement global.

Le chapitre 2 de ce manuscrit s'est attelé à définir si et comment le contexte environnemental pouvait moduler la qualité nutritionnelle d'une ressource pour les consommateurs. Plus spécifiquement, il a visé à déterminer la dépendance thermique des besoins nutritionnels de *Daphnia magna*, d'un point de vue stœchiométrique (C : P).

Les chapitres suivants ont cherché à comprendre les interactions entre qualité nutritionnelle et taux métabolique des consommateurs. Dans un premier temps le chapitre 3 s'est focalisé sur une contrainte nutritionnelle stœchiométrique avec pour objectif principal de mettre au point une méthode de mesure du taux métabolique exploitant la microcalorimétrie. Le chapitre 4, quant à lui, a permis d'élargir le gradient de nourriture considéré afin de couvrir une large gamme de contraintes nutritionnelles incluant à la fois les approches stœchiométriques (C, P) mais aussi biochimiques (stérols, AGPI). Ce chapitre visait aussi à déterminer les conséquences de ce lien qualité-taux métabolique sur la croissance des consommateurs ainsi que les processus physiologiques impliqués dans ces relations.

Le chapitre 5 a eu pour but d'étudier les conséquences d'un stress anthropique (ici la salinisation) sur les relations masse-métabolisme-croissance définies précédemment. De cette façon, ce chapitre visait à comprendre comment les interactions potentielles entre salinité et qualité nutritionnelle pouvaient se traduire au niveau métabolique et quelles pouvaient en être les conséquences pour la persistance et la croissance des organismes.

Enfin, le dernier chapitre de ce manuscrit (chapitre 6) viendra conclure ce travail de thèse et ouvrir un certain nombre de perspectives établissant des axes essentiels qui permettraient d'approfondir les travaux présentés ici.



## **Chapitre 2 : La dépendance thermique des besoins nutritionnels stœchiométriques, une réponse en U pour Unifier des visions divergentes**

## **U-shaped response Unifies views on temperature dependency of stoichiometric requirements**

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### **2.1 Abstract:**

Temperature and nutrient availability, which are major drivers of consumer performance, are dramatically affected by global change. To date, there is no consensus on whether warming increases or decreases consumer needs for dietary carbon (C) relatively to phosphorus (P), thus hindering predictions of secondary production responses to global change. Here, we investigate how the dietary C:P ratio optimizing consumer growth ( $TER_{C:P}$ : Threshold Elemental Ratio) changes along temperature gradients by combining a temperature dependent  $TER_{C,P}$  model with growth experiments on *Daphnia magna*. Both lines of evidence show that the  $TER_{C,P}$  response to temperature is U-shaped. This shape indicates that consumer nutrient requirements can both increase or decrease with increasing temperature, thus reconciling previous contradictory observations into a common framework. This unified framework improves our capacity to forecast the combined effects of nutrient cycle and climatic alterations on invertebrate production.

## **2.2 Introduction:**

In the last decades, temperature of lake surface water increased on average by  $0.34^{\circ}\text{C}\cdot\text{decade}^{-1}$  (O'Reilly et al., 2015) a trend which may worsen regarding the speed at which global change occurs (Rogelj et al., 2012). In the same time, urbanization and agricultural practices lead to significant alterations of elemental cycles (especially C, N and P) and nutrient availability in the biosphere (Falkowski et al., 2000). These two factors are important drivers of ectotherm herbivore physiology, temperature regulating the rate of all biological process occurring in the organism (Angilletta, 2006) and nutrient uptake fueling physiological needs for growth or reproduction (Elser et al., 2003). The independent effects of temperature and nutrient availability on organisms has long been studied separately, however, in natural environments submitted to global change, these two factors change in parallel, affecting consumer performance interactively (Kingsolver et al., 2006). Hence, it is essential to consider any temperature change within its nutritional context to understand ongoing and future changes in invertebrates' populations and their interactions with other trophic levels.

Several studies have explored the thermal sensitivity of consumer nutritional requirements, particularly focusing on C:N:P stoichiometry (Cross et al., 2015) and two competing predictions emerge from this literature. The first emphasizes the temperature sensitivity of individual metabolic rate (Brown et al., 2004) arguing that a higher metabolic activity resulting from higher temperature should increase the energetic requirements of the organism. As energy results from the oxidation of C-rich compounds, an increased temperature should therefore increase consumer C needs. This hypothesis is supported by experimental evidence both for terrestrial (Lee et al., 2015) or aquatic (Boersma et al., 2016a; Malzahn et al., 2016) consumers which tend to select for C-rich diets at higher temperatures. However, other authors claim that higher temperature also accelerates growth (Frazier et al., 2006).

According to the growth rate hypothesis ((Elser et al., 2003), an increased growth rate should raise nutrient demand (N and P) in order to sustain increasing protein synthesis and DNA replication. This hypothesis is also supported by empirical evidence showing an increasing nutrient demand at higher temperatures both in terrestrial (Lemoine et al., 2013) or aquatic (Persson et al., 2011b; Wojewodzic et al., 2011) consumers. To date, these apparently contradictory results remain a conundrum and the evolution of consumer stoichiometric requirements under warming scenarios a subject of vivid debate (Boersma et al., 2016; Winder et al., 2016). A recent opinion paper by Schmitz and Rosenblatt (2017) however, contains an appealing solution to the above controversy. The authors propose four theoretical models for the interactive effects between temperature and predation stress on consumer stoichiometric requirements. One of their models predicts that both C and nutrient demand can increase with warming and that previous contradictory results could simply reflect different regions of the same underlying U-shaped response

The models in Schmitz and Rosenblatt (2017) are based on the threshold elemental ratio (*TER*), a well-established framework defining how consumer biomass production responds to the elemental composition of food (Frost et al., 2006). The *TER* is the ratio at which elements should be present in diet in order to maximize consumer growth and where the limitation of growth switches from one element to another. The most commonly studied *TER* are those relating carbon (C) to phosphorus (P) or nitrogen (N) and they reflect the switching point from an energy-limited (i.e. C-limited) growth to a nutrient-limited growth. Deviations of food stoichiometry below or above the *TER* induce a dietary (C- or nutrient-) limitation for consumers (Frost et al., 2006) and lead to a unimodal response of growth to food stoichiometry also known as the “stoichiometric knife-edge” (Elser et al., 2016; Zhou and Declerck, 2019). Besides the effects on consumer performance, the *TER* can explain consumer-driven P and N

recycling in ecosystems (Cherif and Loreau, 2013), population dynamics (Elser et al., 2000; Frost et al., 2006) and species interactions (Boersma et al., 2016a). The main innovation in Schmitz and Rosenblatt (2017) is that certain physiological processes underlying the *TER* such as mass specific ingestion and respiration rate do not simply rise exponentially as usually assumed (Anderson et al., 2017a) but rather exhibit a unimodal response to temperature (Kibby, 1971; McMahon, 1969; Yurista, 1999). Under this assumption, the *TER* of a consumer can exhibit a U-shaped response to temperature, an exciting prediction that remains to be tested experimentally.

The aim of our study is two-fold. First, we extend the theoretical work of Schmitz and Rosenblatt (2017) and thoroughly explore the conditions under which the U-shaped response of the *TER* emerges. Departing from a model parametrization based on the freshwater cladoceran *Daphnia magna*, we analyze the robustness of the U-shape to variations in the thermal reaction norms of the physiological traits underlying the *TER*. Second, we experimentally determine how the *TER* of a *Daphnia magna* clone changes with temperature by measuring its somatic growth responses under a factorial combination of temperatures and dietary stoichiometry.

## **2.3 Material and Method:**

### 2.3.1 Model equations and analysis:

The  $TER_{C:nutrient}$  can be expressed as the product of nutrient use efficiency and the consumer's elemental composition:

$$TER_{C:nutrient} = \frac{AE_{nutrient}}{GGEc} * Q_{\frac{c}{nutrient}} \quad eq.1$$

Where  $Q_{C:nutrient}$  is the body composition of organisms,  $AE_{nutrient}$  is the assimilation efficiency of a specific nutrient (N or P) and  $GGE_C$  the gross growth efficiency of C.  $GGE_C$  refers to the proportion of ingested C used for growth, formulated as:

$$GGE_C = \frac{I_C * AE_C - R_C}{I_C} \quad eq.2$$

Where  $I_C$  is the C ingestion rate,  $AE_C$  the C assimilation efficiency and  $R_C$  the C respired.

In a comparable approach to Schmitz et Rosenblatt (2017), we define the thermal reaction norms for each process underlying the  $TER$ . The thermal response of daphnia ingestion rate has been largely documented (Kibby, 1971; McMahon, 1969; Yurista, 1999) and is known to present a temperature optimum below and above which process rate decreases generating a hump-shaped thermal performance curve (TPC) (Angilletta, 2006). We modelled  $I_C$  using a modified Gaussian as:

$$I_C = I_{CMIN} + \Delta I_{CMAX} * e^{-b(T-T_{OPT})^2} \quad eq.3$$

where  $\Delta I_{CMAX}$  is the difference between minimum ( $I_{CMIN}$ ) and maximum ingestion rate.  $T$  is temperature,  $T_{OPT}$  the optimal temperature and  $b$  a coefficient determining the decrease rate around  $T_{OPT}$ . Here, we estimated the shape of C ingestion TPC parameters ( $b$ ,  $I_{CMIN}/I_{CMAX}$  ratio) by fitting  $eq.3$  to the measurements of Yurista et al. (1999) on the arctic cladoceran *Daphnia middendorffiana*. However, to adjust the TPC to our temperate *D.magna* we replaced the thermal optimum ( $T_{OPT}$ ) to 22°C, the acclimation temperature of our clones (**Figure 2**). We also adjusted the maximum ingestion rate ( $I_{CMIN} + \Delta I_{CMAX}$ ) to the measurement of Urabe & Watanabe (1991) on temperate *Daphnia* species fed on *Chlamydomonas reinhardtii*, which is also in accordance with Darchambeau et al., (2003). However, as data on thermal dependence of  $I_C$  are scarce for *Daphnia magna* (McMahon, 1969) we tested how the  $TER_{C:P}$  thermal

reaction is influenced by curvature of the  $I_C$  hump-shaped response by varying  $b$  in the sensitivity analysis (**Figure 2.A**).

In Schmitz and Rosenblatt (2017), respiration is modelled as a hump-shaped thermal reaction norm. While this is valid, experimental evidence indicates that within the temperature range we used in our study, the metabolic rate of *D.magna* (i.e. respiration) rises exponentially. Hence, unlike Schmitz and Rosenblatt (2017) we modeled  $R_c$  as:

$$R_c = ae^{dT} \quad \text{eq.4}$$

Where  $d$  is the scaling exponent and  $a$  the proportionality constant. We measured *D.magna* metabolic rate (see method below) to confirm the exponential rise. Fitting our data to **eq.4** results in a scaling parameter  $d = 0.035$  (**Figure.S1**). We also tested how the predicted  $TER_{C:P}$  reaction norm is affected by variations in the scaling exponent  $d$  ranging from 0.01 to 0.07 (**Figure 2.B**).

Carbon and nutrient assimilation efficiency ( $AE$ ) are typically assumed constant across temperatures in zooplankton  $TER$  models (Anderson et al., 2017b; Frost et al., 2006) and experimental evidence seems to support this assumption (Yurista, 1999). Using previously reported values for *Daphnia* as reference we modelled  $AE_C$  and  $AE_P$  constant with temperature ( $AE_C \sim 0.5$  (Urabe and Watanabe, 1991; Yurista, 1999) and  $AE_P \sim 0.8$  (Anderson and Hessen, 2005; Frost et al., 2006)). As levels of  $AE_C$  and  $AE_P$  reported in literature do not constitute a clear consensus, we tested how the  $TER_{C:P}$  thermal reaction norm responds to increases and decreases of  $AE_C$  and  $AE_P$  levels around these reported values (**Figure 2.C-D**). See Supplementary material for a model with  $AE$  thermal reaction norms (**Figure S2**).

We analysed the elemental content of our *Daphnia* clone (see method below) and showed no significant differences in body C:P ratio (**Figure S3**) across dietary and temperature

treatments, with an average value of 95. Such homeostatic regulation is not uncommon in daphnids fed stoichiometrically imbalanced. However, some studies on other *D.magna* clones and copepods suggested that body C:P can either increase (McFeeters and Frost, 2011) or decrease (Mathews et al., 2018) with increasing temperatures. To test all these scenarios (constant, increasing, decreasing  $Q_{C:P}$ ), we define  $Q_{C:P}$  as:

$$Q_{C:P}(T) = CP_0 + (r * (T - T_{MIN})) \quad eq.5$$

Where  $CP_0$  and  $r$  respectively define the intercept and slope of the  $Q_{C:P}$  versus temperature curve and  $T_{MIN}$  the minimal temperature observed (**Figure 2.E**). By setting  $r$  to 0,  $Q_{C:P}$  becomes a constant ( $Q_{C:P}=CP_0$ ) as it is the case for our *D.magna* clone.

To test the robustness of our predictions, we calculated the *TER* thermal reaction norm (from Eq.1), for 1000 random combinations of parameter values (parameters  $b$ ,  $d$ ,  $AE_C$ ,  $AE_P$ ,  $r$ ) sampled from uniform distributions bound within the biologically relevant ranges defined in **Table 2**. For each obtained *TER* reaction norm we calculated the average slope (at 0.5 °C intervals) below and above optimal temperature (set at 22°C) to describe the shape of  $TER_{C:P}$  thermal reaction. Only slopes above 5% were considered significant. When both slopes are positive or negative or when one of them is zero, we considered the  $TER_{C:P}$  thermal reaction as monotonous. A U-shape (soft or strong) was assigned when the slope is initially negative and then positive. A strong-U  $TER_{C:P}$  slope was assigned when both slopes are above 10%. (**Figure 3**)



C Ingestion		Equation: $I_c(T) = ICmin + \Delta ICmax * e^{-b(T-T_{OPT})}$
$I_{CMIN}$	<b>0.25</b> <sup>1-2</sup> ( $\mu\text{gC}\cdot\text{day}^{-1}\cdot\mu\text{gDW}^{-0.48}$ )	
$\Delta I_{CMAX}$	<b>0.85</b> <sup>1-2</sup> ( $\mu\text{gC}\cdot\text{day}^{-1}\cdot\mu\text{gDW}^{-0.48}$ )	
$b$	(0.05, 0.4) ; <b>0.2</b> <sup>1</sup> Unitless	
$T_{OPT}$	<b>22</b> (°C)	
Assimilation efficiency C		Constant
$AE_C$	(0.3 ; 0.7) <b>0.5</b> <sup>3-4</sup> Unitless	
Assimilation efficiency P		Constant
$AE_P$	(0.6 ; 1) <b>0.8</b> <sup>3-4</sup> Unitless	
C respiration		Equation: $R_c = ae^{dT}$
$d$	(0.01-0.07) <b>0.035</b> this study Unitless	
$a$	<b>0.0269</b> this study ( $\mu\text{gC}\cdot\mu\text{gDW}\cdot\text{day}^{-1}$ )	
Consumer body C:P		Equation: $Q_{C:P} = CP_0 + (r * (T - T_{min}))$
$CP_0$	<b>95</b> this study Unitless	
$r$	(-4,4) ; <b>0</b> this study Unitless	
$T_{MIN}$	<b>18</b> (°C)	

**Table 2:** Modelled thermal reaction norms of the physiological processes underlying the TER model in eq. 1 and associated parameters. Tested parameter ranges (see Figure 2) are given in parentheses, values in bold are the values used for *Daphnia magna*. <sup>1</sup>(Yurista, 1999); <sup>2</sup>(Urabe and Watanabe, 1991); <sup>3</sup>(Anderson and Hessen, 2005); <sup>4</sup>(Frost et al., 2006)

### 2.3.2 *Daphnia* maintenance:

We tested our model predictions using a clonal line of *Daphnia magna* to exclude genotypic response variations. The stock cultures of daphnids were kept in Volvic water© at 22°C on a 14:10h day:night cycle and fed with *Chlamydomonas reinhardtii* (C:P 250 at 3mgC.L<sup>-1</sup>) for more than 3 generations.

### 2.3.3 Preparation of food suspensions:

*Chlamydomonas reinhardtii* was used as food in all experiments. We used 3 freeze-dried stock cultures with respective molar C:P ratio of 95 (high P), 331 (intermediate P) and 1014 (low P) previously prepared (*See* supplementary material for details). Food suspensions were prepared daily by mixing freeze-dried algae stocks diluted in Volvic© water (**Table.S1**). Despite a slight reduction in individual performance, freeze-dried algae were shown to be adequate food for *D.magna* for several generations (Naylor et al., 1993). The P content of algae has no significant influence on their fatty acids composition (**Table.S2**).and the rehydration of freeze-dried algae had no effect on their elemental composition.

### 2.3.4 Experimental procedure:

We used a gradient experimental design in order to capture the non-linearity of *D.magna* growth rate response to diet stoichiometry and temperature (Kreyling et al., 2018). Daphnids were grown on a full factorial combination of 6 stoichiometric treatments (with C:P ratios of 95-172-250-331-553-1014, obtained by mixing the freeze-dried stock cultures) at 6 different temperatures (18-20-22-24-26 and 28°C). For technical reasons however, growth experiments were performed independently for each temperature using the same clonal line and a strictly identical protocol. At the beginning of the experiments, 8h old neonates obtained from the 3<sup>rd</sup> or 4<sup>th</sup> clutch were randomly distributed in 2 replicate glass jars (250mL, 12 ind/jar) per dietary treatments. The culture medium composed of Volvic© water with *ad-libitum* food

(3mgC.L<sup>-1</sup>) was renewed daily to maintain food quantity above the saturating level during all the experiment. Individuals were raised until they reached maturity, which took from 5 to 9 days depending on temperature. They were then pooled by jar, dried 48h at 60°C, weighed (Sartorius-ME36S; Germany; precision:0.001mg) and stored until elemental analysis. The average growth rate in each jar was calculated as follows:

$$\mathbf{Growth\ rate} = \frac{\ln(W_t) - \ln(W_0)}{t} \quad \mathbf{eq.6}$$

where  $W_t$  is the average weight ( $\mu\text{g}$ ) at age of maturity  $t$  (days) and  $W_0$  the average weight ( $\mu\text{g}$ ) of neonates. The growth rate thus calculated refers to the juvenile growth rate which has been shown to represent the best proxy for population increase rate (Lampert and Trubetskova, 1996). After measuring the mean weight, pools of dried daphnids were divided into subgroups to measure their carbon and phosphorus content. In parallel, a subset of mature daphnids from the C:P 250 treatment was used to estimate their metabolic rate. As variation of metabolic rate (a.k.a. respiratory rate) with diet is not included in *TER* model, we performed this measurement on a single dietary treatment which is the diet that daphnids used to consume. The mass-specific metabolic rates obtained by microcalorimetry (Ruiz et al. 2018) were plotted against temperature to determine the temperature dependence of metabolism (**Figure S2**).

### 2.3.5 Elemental and biochemical analyses:

Elemental composition of algae and daphnids were performed in a similar way. Carbon was determined using a CHN analyser (NA-2100-Protein, ThermoQuest-CE Instruments, Italy) and phosphorus content was estimated using a colorimetric determination based on potassium persulfate method (Ruiz et al., 2018). Fatty acid of algae were extracted using chloroform/methanol method and then converted into fatty acid methyl esters by acid-catalysed

transesterification (Denoux et al., 2017). The fatty acid methyl esters were analysed by gas chromatography (6850-Network-GC, Agilent-technologies, USA).

### 2.3.6 Data analyses:

To model the response surface of *D.magna* juvenile growth rate to experimental gradients of temperature and dietary C:P we fitted a Generalized Additive Model (GAM) to the experimental growth rate data. The model of growth rate was constructed as follows:

$$GR_{T,C:P} = f(x_T) + f(x_{C:P}) + ti(x_T, x_{C:P}) \text{ eq.7}$$

where  $f(x_T)$  and  $f(x_{C:P})$  are the smooth terms for temperature and diet stoichiometry whereas  $ti(x_T, x_{C:P})$  is the tensor interaction smooth term. The model was fitted to the data using the mgcv R package (Wood, 2001) and "Restricted Maximum Likelihood" REML method.

To assess the shape of the  $TER_{C:P}$  thermal reaction norm we first estimated the  $TER_{C:P}$  at each experimental temperature separately. For each temperature, we fitted the following modified Gaussian function to the growth rate versus C:P data:

$$GR_T = a + b * e^{-E * \frac{x_{C:P} - c}{d^2}} \text{ eq.8}$$

where  $x_{C:P}$  is the resources C:P ratio,  $a$  the minimum growth rate,  $b$  is the height of the curve,  $c$  is the estimated  $TER_{C:P}$  (i.e. curve's optimum),  $d$  the curve breadth, and  $E$  a scaling parameter. The various parameters including  $c$  ( $TER_{C:P}$ ) were estimated by non-linear least squares regression. The confidence intervals around the  $TER_{C:P}$  were estimated by nonparametric bootstrapping (Efron and Tibshirani, 1986). At each temperature the dataset was resampled 1000 times and the non-linear regression procedure and  $TER_{C:P}$  estimation reiterated. The obtained estimates are strongly robust against the number of bootstrap iterations (**Table.S3**)

and were used to calculate the mean  $TER_{C:P}$  and the 95% confidence intervals ( $CI_{95\%}$ ). A significant difference between  $TER_{C:P}$  estimates at each temperature was concluded in the absence of overlap between their  $CI_{95\%}$ .

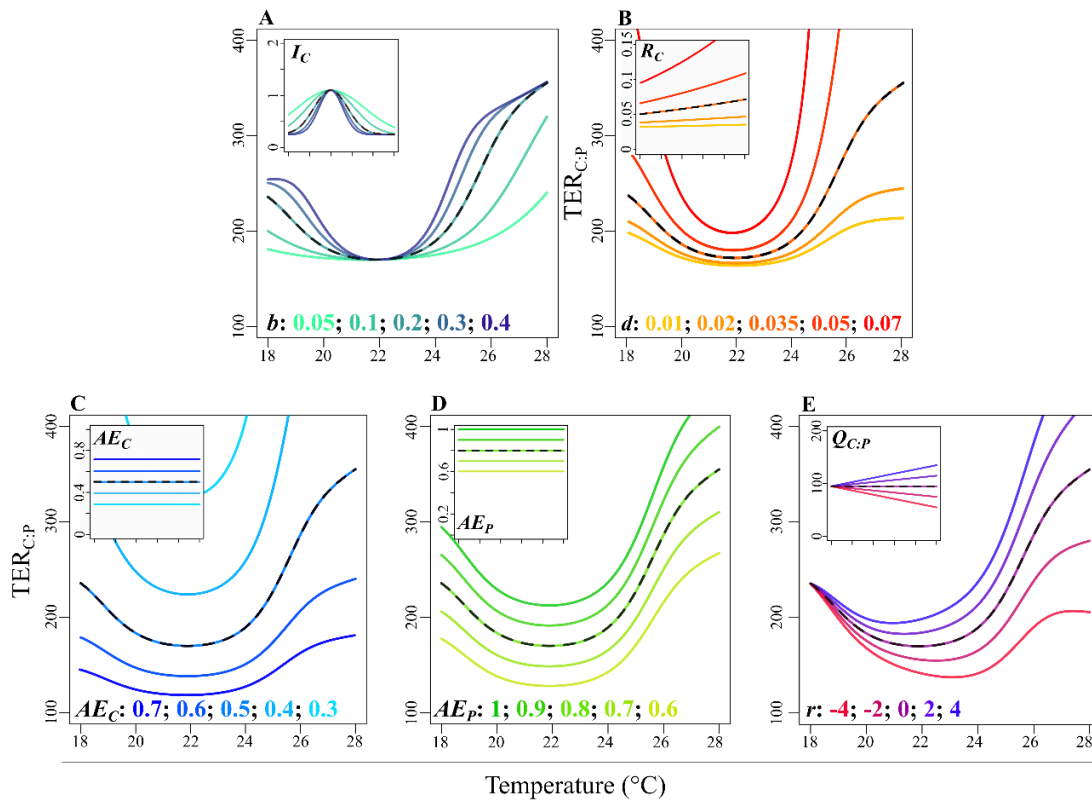
Finally, to determine whether the  $TER_{C:P}$  reaction norm has a true U-shape we used the Two-lines test (Simonsohn, 2018) on the bootstrapped data. Briefly, the two-line test is used to statistically confirm or infirm a U-shape in data series. The test consists of estimating two regression lines, for “low” and “high” values of  $x$  separated at a specific breakpoint. The test confirms a U-shape if the two lines have opposite and individually significant slopes. All analyses were performed using the software R v.3.6.1 (R-Core Team 2019) with the alpha error set at 0.05.

## **2.4 Results:**

### 2.4.1 Model:

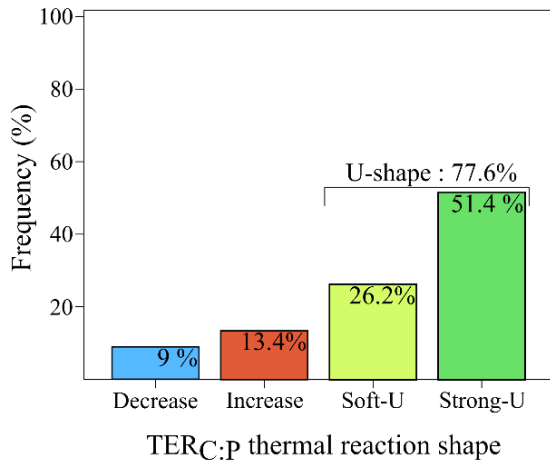
When parametrised for *Daphnia* (**Table.2**), the model predicts that the  $TER_{C:P}$  thermal reaction norm is U-shaped, decreasing between 18 and 22°C and then increasing up to 28°C (**Figure 2**, *dashed line*). The  $TER_{C:P}$  is thus minimized at 22°C which constitutes the threshold minimizing temperature (TMT). Among the model parameters,  $b$ , which controls the concavity of the C ingestion rate thermal reaction norm ( $I_c$ ) has a strong effect on the convexity of  $TER_{C:P}$  thermal response (**Figure 2.A**). Similarly, the temperature scaling exponent of respiration ( $R_c$ ),  $d$ , has a strong effect on the  $TER_{C:P}$  (**Figure 2.B**). The lowest  $d$  value used here (0.01), which corresponds to a  $Q_{10}=1.2$  for  $R_c$ , yields a nearly flat  $TER_{C:P}$  reaction. Increasing the temperature scaling of  $R_c$  up to a  $Q_{10}=2$  (parameter  $d=0.07$ ) strongly increases the convexity of the  $TER_{C:P}$  thermal reaction norm and shifts the minimal value of  $TER_{C:P}$  towards higher C:P ratios. The level of C assimilation efficiency ( $AE_C$ ) also constrains both the level and the convexity of the  $TER_{C:P}$  thermal reaction norm. Reducing  $AE_C$  from 0.7 to 0.3 strongly increases the level and

convexity of the reaction norm (**Figure 2.C**). The assimilation efficiency of P ( $AE_P$ ) also modulates the thermal reaction of  $TER_{C:P}$  in a lesser extent. Increasing  $AE_P$  values from 0.6 to 1 only shifts  $TER_{C:P}$  thermal reaction norm to higher C:P values without altering its convexity (**Figure 2.D**). The scaling of consumer body composition ( $Q_{C:P}$ ) with temperature (defined by parameter  $r$ ) only affects  $TER_{C:P}$  at higher temperatures. Increasing  $Q_{C:P}$  with temperature (parameter  $r=4$ ) leads to a rapid increase of  $TER_{C:P}$  at higher temperatures. Oppositely, decreasing  $Q_{C:P}$  at high temperature ( $r=-4$ ) tends to limit  $TER_{C:P}$  to lower values, slightly reducing the convexity of its thermal response without flattening the U-shape (**Figure 2.E**).



**Figure 2:** Effect of parameter values on the thermal reaction norm of TER. Each panel is associated with the variation of one specific parameter constraining: (A) The concavity (parameter  $b$ ) of the carbon ingestion rate reaction norm  $I_C$ , (B) the temperature scaling (parameter  $d$ ) of carbon respiration  $R_C$ , (C) the level of carbon assimilation efficiency  $AE_C$ , (D) the level of phosphorus assimilation efficiency  $AE_P$  and (E) the slope (parameter  $r$ ) of consumer's body C:P response to temperature. Each parameter is varied individually while the others are kept constant at the values for *D.magna* (see Table 2). Upper left inserts present the temperature responses of physiological process associated with parameter variations. The dashed black line is the model prediction for *D.magna*. See table 2 for details on the selected parameter ranges.

The sensitivity analyses of the model shows that across a large number ( $n=1000$ ) of random parameter combinations (selected within biologically relevant ranges, *see Table.2*), the U-shape  $TER_{C:P}$  thermal reaction norm is predominant with a frequency of occurrence of 77.6% (**Figure 3**).

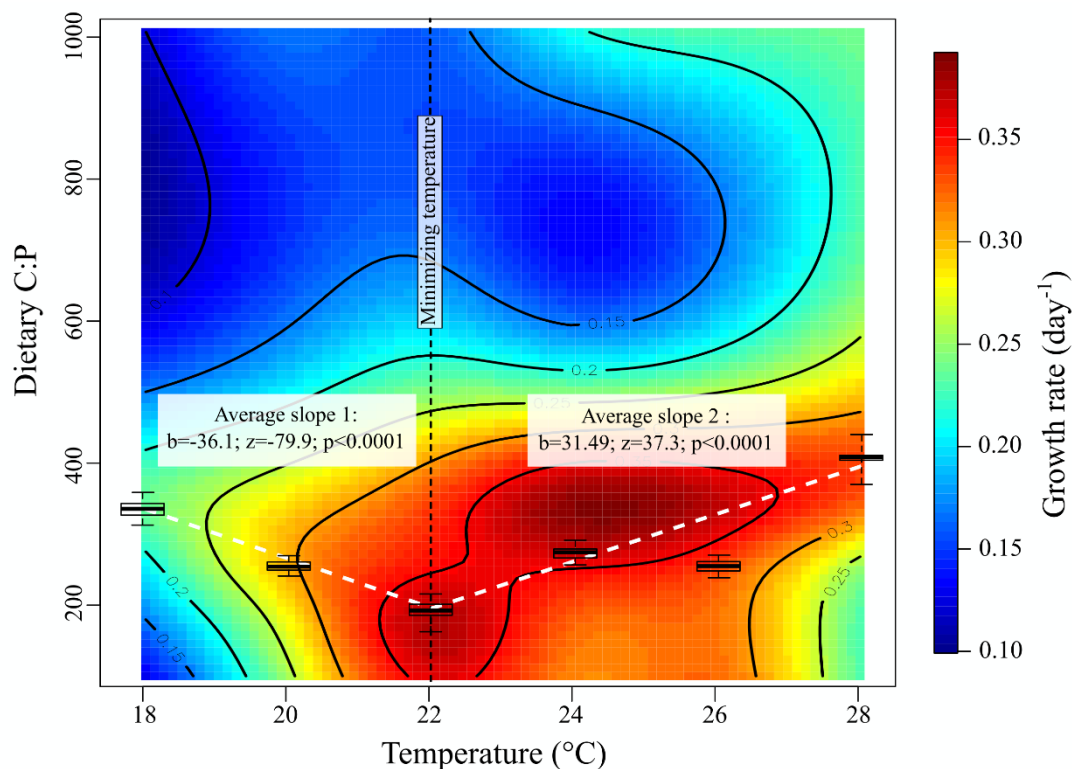


**Figure 3:** Frequency of occurrence of  $TER_{C:P}$  thermal reaction norms across 1000 random model parameter combinations. Parameter values are sampled within the ranges given in table 1 assuming uniform distributions. A U-shape is considered “strong” when both lines have a slope above 10%. Decrease and Increase indicate a monotonically increasing or regressing reaction norms, respectively. Error on slopes estimates is set at 5%. See methods for details. See **Figure S4** for detailed parameter distributions of each pattern.

More precisely, softly convex U-shapes occur in 26.2% of observations and strongly convex U-shapes in 51.4%. The remainder of simulation outcomes are monotonic increases (13.4%) or decreases (9%) of  $TER_{C:P}$  with temperature. These monotonic thermal reactions of the  $TER_{C:P}$  only emerge when  $I_C$  thermal response is the less concave or when  $R_C$  thermal reaction is flattened. In such situations, it is the thermal reaction norm of  $Q_{C:P}$  that dictates the direction of the  $TER_{C:P}$  thermal response (**Figure S4**).

### 2.4.2 Experiments:

The surface response of daphnids growth across the factorial combination of dietary treatment and temperature is given in **Figure 4**.



**Figure 4:** Generalized Additive Model (GAM;  $n=72$ , see also **Fig.S3**) of *D.magna* growth rate exposed to a factorial combination of dietary molar C:P ratio and temperature. The boxplots at each temperature are the bootstrapped  $TER_{C:P}$  mean values and confidence intervals ( $n=1000$ ). The shape of  $TER_{C:P}$  thermal reaction norm (white line) has been assessed with the two-line test (Simonsohn, 2018). Decreasing and increasing sections are both significant ( $p<0.0001$ ).

The GAM used to generate the surface fit well with our data explaining 97.9% of total variance (**Figure S5-S6**). The GAM also indicates a significant effect of the temperature and dietary C:P interaction on *D.magna* growth ( $n=72$ ;  $F=15.9$ ;  $p<0.001$ ). The response of growth to temperature is typically unimodal for diet C:P ratios ranging from 95 to 331, with an optimum ranging from 0.36 at 22°C to 0.39 at 24°C, respectively (**Figure 4**, **Figure S4**). For C:P ratio above 500, the response of growth appears to be almost flat. Growth rate responded



unimodally to diet at all temperatures (**Figure 4, Figure S4**) but the  $TER_{C:P}$  (i.e. the peak of the unimodal response) changed across temperatures forming a U-shape with a minimum value at 22°C. The  $TER_{C:P}$  decreased from 338.4 (CI<sub>95%</sub>[326.9, 349.9]) at 18°C down to 193.4 (CI<sub>95%</sub>[176.6, 210.2]) at 22°C. The latter can be considered as our *Daphnia* clone's TMT since it represents the lowest  $TER_{C:P}$  observed in our thermal range. Further temperature increases above this TMT lead to an increase of the  $TER_{C:P}$  up to 414 (CI<sub>95%</sub> [370.8, 457.2]) at 28°C (**Figure 4**). The two-line test of Simonsohn (2018) confirmed that  $TER_{C:P}$  significantly decreases between 18 and 22°C ( $p < 0.0001$ ), reaches a breakpoint at 22°C after which  $TER_{C:P}$  significantly increases up to 28°C ( $p < 0.0001$ ) (**Figure 4**) thus generating a significant U-shape.

## **2.5 Discussion:**

Both our model and growth experiments showed that the  $TER_{C:P}$  of *D.magna* responds non-monotonically to temperature generating a “U-shaped” thermal reaction norm. The  $TER_{C:P}$  decreases between 18°C and 22°C indicating a rising demand in P with rising temperature. At 22°C, the  $TER_{C:P}$  reaches its minimal value, the Threshold Minimizing Temperature (TMT) where the dietary C requirements relative to those of P are minimized. Above the TMT, and at least up to 28°C, the  $TER_{C:P}$  increases again indicating a rising demand in dietary C. Consequently, for the same *D.magna* clone, both temperature-driven decrease and increase of the  $TER$  are possible, depending on the temperature range within which observations are performed. Provided that this  $TER_{C:P}$  U-shaped reaction norm can be generalised across consumers, our results suggest that previous apparent contradictory predictions of ectotherm consumers requirements in a warming context (Boersma et al., 2016; Persson et al., 2011; Wojewodzic et al., 2011) could be reconciled within a single framework.

Our model differs from those based on MTE that often assume that consumption is a monotonous function of temperature that has the same or higher  $Q_{10}$  than metabolism

(Anderson et al., 2017a). Such assumptions can only be valid within the lower part of consumer's thermal tolerance range. All else kept equal in our model, such assumptions will yield a monotonously decreasing  $TER_{C:P}$  thermal reaction norm. On the other hand, focusing only on the upper part of the consumers thermal tolerance range where metabolism increases faster while consumption decreases will yield a monotonously increasing  $TER_{C:P}$  thermal reaction norms.

However, understanding ectotherm consumers' responses to climate change requires considering their full thermal tolerance range (Kingsolver and Buckley, 2017). Within this range, C ingestion ( $I_c$ ) and respiration rates ( $R_c$ ) respond differently to temperature, both in terms of shape and scaling. It is these differential responses of  $I_c$  and  $R_c$  that generate the U-shaped  $TER$  reaction norm in our model. In agreement with empirical data on *Daphnia* (Kibby, 1971; McMahon, 1969; Yurista, 1999) and numerous other ectotherms (Dell et al., 2011; Lemoine and Burkepile, 2012), we assume that within the full thermal tolerance range, the reaction norms of  $I_c$  and  $R_c$  are unimodal and exponential, respectively. At the lower temperature range of the modelled  $I_c$  reaction norm, ingestion rate rises faster than metabolic rate does (Lemoine and Burkepile, 2012; Yurista, 1999). Under these circumstances and given a constant C assimilation efficiency ( $AE_C$ ), higher temperatures increase C gross growth efficiency ( $GGE_C$ ; **eq.2**). The biological interpretation is that at higher body temperatures, the consumer "perceives" the food as being richer in C relative to P. This manifests as a decrease in the  $TER_{C:P}$  with temperature until it reaches the TMT. In the model, the TMT indicates the tipping point where  $R_c$  begins to rise faster than  $I_c$ . From this point, the  $GGE_C$  starts decreasing with temperature and the consumer "perceives" the food as being increasingly "poorer" in C relative to P as temperature rises. Consequently, above the TMT, the  $TER_{C:P}$  rises with

temperature. Hence, when considering a sufficiently wide thermal tolerance range, the  $TER_{C:P}$  exhibits a U-shaped response to temperature.

The ingestion and respiration rates might not be the only model components affected by temperature. Although much less consensual, there is evidence that the assimilation efficiencies for C and P ( $AE_C$  and  $AE_P$ ) as well as the consumer's body stoichiometry ( $Q_{C:P}$ ) may also respond to temperature ((Bobka et al., 1981; Prater et al., 2018; Tsai et al., 2008)). Depending on whether they increase or decrease with temperature, strong thermal responses could either “flatten” the U-shape, or one of its sides thereby yielding a quasi-monotonous  $TER_{C:P}$  thermal reaction norms. For example, unlike our *D.magna* clone Prater et al (2018) found that the body stoichiometry of *D.magna* in two lakes tends to decrease with increasing temperature. In the model, such a decrease tends to flatten the rising part of the  $TER_{C:P}$  reaction norm. Yet, the range of  $Q_{C:P}$  in Prater et al. (2018)(from 100 at 15°C to 65 at 25°C) is not enough to cause a monotonous decrease of  $TER_{C:P}$  with temperature in the model (**Figure 2.E**).

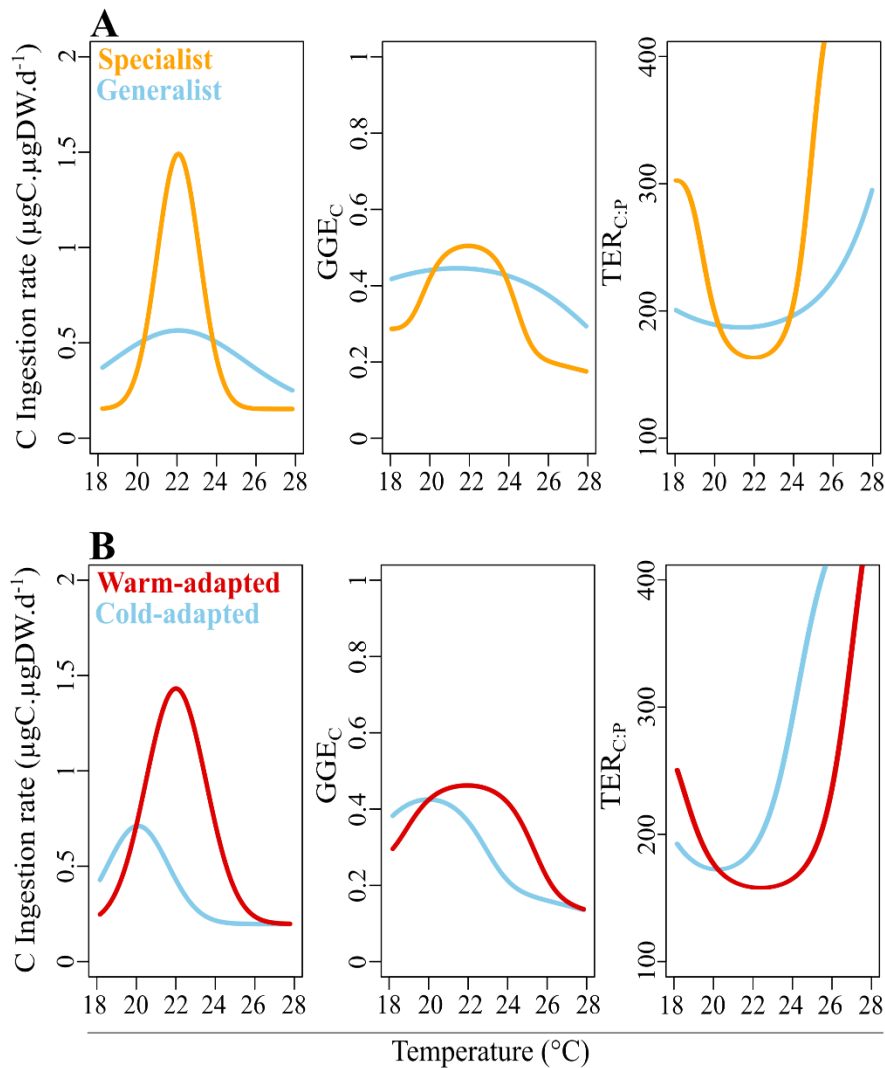
More generally, based on the model parameter ranges from literature, the simulation shows that 77.6% of random parameter combinations result in a U-shaped  $TER_{C:P}$  thermal reaction norm with 51.4% producing strongly convex U-shapes. This result suggests that U-shaped  $TER$  thermal reaction norms might be quite common among ectotherm consumers. Obviously, this is a rough estimate and it needs be considered with caution. First, given the scarcity of data in literature, we assumed a uniform distribution for all parameter values. Improving the estimates of parameters distribution may potentially lead to different predictions but this is beyond the scope of the present study. Second, parameter combinations in our simulation are random, and some of them might be biologically aberrant. For example, we could expect that in nature, higher ingestion rates are systematically associated with higher

respiration rates in agreement with the increased intake hypothesis ((Burton et al., 2011) and lower  $Q_{C:P}$  in agreement with growth rate hypothesis (Elser et al., 2003). Keeping only the biologically relevant combinations could alter the predicted frequency of occurrence of a U-shaped  $TER_{C:P}$ .

A fundamental challenge in experimental ecology is to capture nonlinearities of ecological responses to environmental drivers. Our results underscore the importance of carefully selecting the temperature range and increments in any experimental assessment of temperature-dietary stoichiometry interaction on ectotherm consumers. In the case of strongly convex  $TER_{C:P}$  responses, large temperature increments ( $>4^{\circ}\text{C}$ ) might miss the U-shape and thus yield misleading conclusions. From an ecological perspective, strongly convex  $TER_{C:P}$  responses imply that small changes in temperature can drastically change consumer nutritional requirements. A diet of a given stoichiometry can shift towards one or the other side of  $TER_{C:P}$  within a range of few degrees Celsius. In agreement with the “knife-edge” theory (Elser et al., 2016), our experimental data shows that both sides of the  $TER_{C:P}$  imply physiological costs which manifest as decrease in growth rate. Hence, consumers with strongly convex  $TER_{C:P}$  responses to temperature might be more susceptible to thermally-induced changes in their dietary preferences (Boersma et al., 2016a). Following the same reasoning, such consumers might also be more prone to behavioral thermoregulation in order to optimize nutritional outcomes (Clissold et al., 2013; Coggan et al., 2011). Furthermore, the U-shape of the  $TER_{C:P}$  thermal reaction norm implies that, for a given resource C:P, climate change-induced nutritional limitations could differ at the borders of the species geographical range. At the lower latitudinal border where the species is at the “warmer” part of its thermal breadth, an increase in environmental temperature could drive it towards a limitation by C. On the other hand, the

same temperature increase could lead to P limitation at the higher latitudinal border where the species is at the “colder” part of its thermal breadth.

In the model, the U-shape of the  $TER_{C:P}$  is strongly influenced by the shape of the  $I_c$  thermal reaction norm. A wide  $I_c$  thermal reaction norm breadth (*thermal generalist*) will lead to a “flat”  $TER_{C:P}$  U-shape. From this point on, one can explore possible links between thermal and stoichiometric traits. For example, thermal biology tells us that in some cases the wide thermal breadth of a trait (here,  $I_c$ ) could trade-off with a low maximum performance (i.e. *specialist-generalist trade-off* (Angilletta, 2006)). All else kept equal in model, a low maximum  $I_c$  implies a low maximum consumer growth rate. On the other hand, reaching a high  $I_c$  peak performance and consequently a higher maximum growth rate, could come at the cost of a narrow thermal performance breadth (*thermal specialist*) and a strongly convex  $TER_{C:P}$  reaching lower values (**Figure 5**). Hence, the higher growth rate potential of a thermal specialist (reached at the TMT) has also a lower  $TER_{C:P}$  than that of the generalist. Biologically, this means that the specialist may require relatively more P to reach its full growth potential as compared to the thermal generalist. It is interesting to note how this prediction connecting high growth rate potential to higher P demand resembles that of the growth rate hypothesis (GRH- (Elser et al., 2003)). The GRH is based on the observation that taxa with high growth rate potential have lower body C:P ratios. Here, we show that this positive relationship between growth rate potential and P requirements persists even when assuming a constant body C:P ratio. Another common pattern found in thermal biology is the “hotter is better” continuum (Angilletta et al., 2010)) in which warm-acclimated organisms reach higher maximal performances (here  $I_c$ ) than cold-acclimated ones. Applying this into our  $TER_{C:P}$  model also yields a prediction that reminds the GRH: warm-acclimated organisms achieve higher peak  $I_c$  and growth rates at lower  $TER_{C:P}$  than cold adapted ones (**Figure 5**).



**Figure 5:** Comparison of hypothetical  $GGE_C$  and  $TER_{C:P}$  thermal reaction norms of: (A) thermal specialist (orange lines) versus thermal generalist (blue lines) consumers and (B) cold-acclimated (blue line) versus warm-acclimated (red line) consumers. Changes are induced by modifying parameters  $b$   $\Delta I_{C_{MAX}}$  and  $T_{OPT}$  (see **table 2**) while keeping all other parameters constant at the values for *D.magna*. See text for discussion.

Another influential model component is  $AE_C$ . A low  $AE_C$  shifts the  $TER_{C:P}$  towards higher C:P ratios and increases the convexity of the U-shaped response to temperature. Biologically,  $AE_C$  reflects the digestibility of ingested C. Detritivores or terrestrial herbivores feeding on diets rich in refractory carbon should have lower  $AE_C$  than aquatic herbivores

consuming microalgae. Hence, when considering only  $AE_C$ , it is tempting to speculate that detritivores and terrestrial herbivores might be more susceptible to thermally-induced dietary preference shifts or dietary-induced behavioral thermoregulation than aquatic herbivores because of their more convex  $TER_{C:P}$  U-shape. However, further studies are necessary in order to determine how the other model components vary among feeding guilds of terrestrial and aquatic ectotherms and whether they can compensate predicted effects of  $AE_C$  on the  $TER_{C:P}$  U-shape.

In conclusion, we demonstrate that in *D.magna* the  $TER_{C:P}$  responds to temperature in a U-shaped fashion. Our model simulations suggest that such a U-shaped reaction norm might be common among ectotherm consumers thereby offering a potential common explanation to previous contradictory results indicating either increases or decreases in consumer P demand at higher temperatures. However, the shape of thermal responses can be highly variable among traits and species (Dell et al., 2011) and the generality of the U-shaped TER remains to be tested for other species having different thermal response shapes and positions than the one provided in table 2. Caution is also required when transposing our finding to other nutrients. The present formulation of  $TER_{C:P}$  assumes negligible P metabolic losses (Frost et al., 2006). This does not hold for N for which nitrogenous metabolic waste excretion represents an important fraction of the nutrient budget. Temperature accelerates metabolism and N excretion with unknown consequences on the  $TER_{C:N}$  thermal reaction norm (Anderson et al., 2017a). Finally, body size will also differentially affect the different model components and thus potentially the shape of the  $TER_{C:P}$  response. Including the allometric size scaling in the model is clearly a promising perspective. Despite these potential caveats, our study unveils novel potential links between variation in thermal and stoichiometric traits and contributes to the ongoing unification of thermal ecology and ecological stoichiometry (Cross et al., 2015; Hillebrand et al., 2009; Rosenblatt and Schmitz, 2016).

## **Transition :**

Les résultats présentés dans le chapitre précédent ont mis en évidence le lien étroit entre température et besoins nutritionnels élémentaires (ici C et P) des consommateurs ectothermes. La réponse en U démontrée ici semble capable de réunir les observations contrastées de la littérature prévoyant d'une part une augmentation des besoins en P et d'autre part une augmentation des besoins en C en cas de réchauffement. Si la tendance générale de cette réponse s'est montrée relativement robuste, la formulation du TER sur laquelle repose le modèle établi dans ce chapitre soulève néanmoins une question essentielle. En effet, si la formulation du TER proposée par Frost et al (2004) prend en compte le métabolisme du consommateur via la perte de C induite par la respiration de l'organisme, cette respiration est considérée constante quelle que soit la nourriture disponible pour le consommateur. Or, au cours de ces 20 dernières années plusieurs études ont suggéré une potentielle variabilité de ce taux respiratoire en fonction du ratio C:P de la nourriture. Plus précisément, elles suggèrent qu'une élévation du taux respiratoire permettrait d'éliminer l'excès de C alimentaire via une augmentation de relargage de CO<sub>2</sub> (Anderson et al., 2017). Si cette hypothèse semble être appuyée par la littérature, notamment chez *Daphnia* (Darchambeau et al., 2003; Jensen and Hessen, 2007), la présence d'importants biais techniques liés à la mesure du taux respiratoire chez des organismes de taille très réduite persiste, laissant place à une certaine incertitude.

Dans ce contexte, le chapitre suivant de ce manuscrit vise à développer une méthode permettant de mesurer et de suivre de façon précise le métabolisme d'organismes de petite taille. Cette méthode sera ici appliquée pour définir les effets d'une nourriture excédentaire en C sur le métabolisme de *Daphnia* afin de déterminer si ce dernier peut s'élever pour faire face à une telle contrainte stœchiométrique.



**Chapitre 3 : Une méthode par microcalorimétrie  
pour explorer les conséquences d'une contrainte  
stœchiométrique sur le taux métabolique standard  
d'invertébrés de petite taille**

# **A microcalorimetric approach for investigating stoichiometric constraints on the standard metabolic rate of a small invertebrate**

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## **3.1 Abstract:**

Understanding the determinants of metabolism is a core ecological topic since it permits to link individual energetic requirements to the ecology of communities and ecosystems. Yet, besides temperature, metabolic responses to environmental factors remain poorly understood. For example, it is commonly assumed that dietary stoichiometric constraints increase metabolism of small invertebrates despite scarce experimental support. Here, we used microcalorimetric measurements to determine the resting metabolic rate (RMR) of *Daphnia magna* fed stoichiometrically balanced (C/P: 166) or imbalanced (C/P: 1439). *Daphnids* fed imbalanced maintained their stoichiometric homeostasis within narrow boundaries. However, they consistently increased their RMR while decreasing their growth rate. Our measurements demonstrate that homeostatic regulation implies higher metabolic costs thereby reducing available energy for growth. We demonstrate that microcalorimetry is a powerful and precise tool for measuring small-sized organisms' metabolic rate, thus opening promising perspectives for understanding how environmental factors, such as nutritional constraints, affect organismal metabolism.

### **3.2 Introduction:**

Metabolism fuels all physiological processes in organisms and appears as the most fundamental mechanism reflecting the energetic cost of living (Hulbert and Else, 2000). Individual metabolic rate sets the “pace of life” of a population through its generation time and maximal growth rate (Brown et al., 2004; Savage et al., 2004). Therefore, understanding how environmental factors constrain individual metabolic rate, and how such individual-level constraints influence population dynamics within interacting communities, is central for understanding ecosystems functioning (Pawar et al., 2015). Resting metabolic rate (RMR) represents the minimum energy that ectotherms must expend on tissue maintenance but also on homeostatic mechanisms needed to sustain life (Auer et al., 2015; Fry, 1947). The maintenance of homeostasis is one of the core concepts of ecological stoichiometry (ES) and implies that the imbalance of elements, such as carbon, nitrogen or phosphorus between consumers requirements and nutritional resources, force consumers to activate specific regulatory processes. Ecological stoichiometry posits that such processes are energetically costly and may thereby affect RMR (Hessen et al., 2013).

A large number of studies investigated the effects of nutrient limitation on invertebrate performance (Cross et al., 2005; Elser et al., 2000; Frost et al., 2002; Hessen et al., 2013; Sterner, 1992), yet only few elucidated the physiological processes involved in maintaining homeostasis and even less considered effects on metabolic rate. However, Sterner and Hessen (1994) already hypothesized that consumers could cope with a dietary C excess by increasing their respiration rate. This hypothesis is well accepted despite the fact that only a handful of studies attempted to unravel it experimentally (Darchambeau et al., 2003a; Jensen and Hessen, 2007; Jeyasingh, 2007; Zanutto et al., 1997). The main difficulties for testing this hypothesis rely on the low sensitivity and the numerous biases related to respirometric methods (Walsberg,

2005). These issues are further exacerbated when considering relatively small animals such as insects or zooplankton species, often used as models in nutritional ecology but generating very low O<sub>2</sub> and CO<sub>2</sub> fluxes. Recent technical and methodological improvements (Rosewarne et al., 2016) might help to reduce these biases to some extent, yet a careful interpretation of the results is needed (Clark et al., 2013). More recently, Lukas and Wacker (2014) and Elser *et al.* (2016) confirmed that mass-specific respiration rates increased when organisms consumed imbalanced diets. However, both of these studies were performed on feeding animals and did not attempt to discriminate the specific dynamic action (SDA, i.e. the increase in respiration associated with feeding) from resting metabolic rate. SDA can be highly variable and represent up to 30% of total metabolic rate (McCue, 2006) and thereby needs to be excluded in order to determine RMR. Finally, despite the increasing precision of respirometric equipments, the conversion of respirometric measurements into metabolic energy equivalents still relies on a set of strong assumptions (Walsberg, 2005). First, this conversion relies on Thornton's rule which states that the enthalpy of organic compound combustion by oxygen is approximately constant when expressed as heat per mole of oxygen consumed (Wadsö and Hansen, 2015). The Thornton's constant however, referred to as the oxycaloric equivalent, is actually variable: different values have been published, ranging from -430 to -480 kJ mol<sup>-1</sup> with a commonly admitted average value of -455 kJ mol<sup>-1</sup> (Maskow et al., 2010; Wadsö and Hansen, 2015). Further assumptions also include: (1) the nature of the primary substrate that is catabolized (i.e. lipids, proteins or carbohydrates), (2) its degree of oxidation and (3) the absence of O<sub>2</sub>-consuming or CO<sub>2</sub>-producing synthetic processes. All of these assumptions can be violated depending on the nutritional status of the assayed individuals (Walsberg, 2005; Walsberg and Wolf, 1995) thereby hindering up to now a clear assessment of the relationship between dietary stoichiometric constraints and RMR.

From a methodological perspective, calorimetry constitutes the “*golden standard*” for quantifying metabolic rates (Kaiyala and Ramsay, 2011). It allows the direct and non-invasive assessment of the metabolic energy produced by an organism measured in terms of heat dissipated by all chemical reactions and energy transformations taking place in the cells. Considering this, calorimetry should enable a precise determination of organismal metabolic rate. Quite surprisingly, despite its early success (Lamprecht and Schmolz, 1999) and high potential, calorimetry remains largely underused in more recent ecological research (but see Braissant et al., 2010; Bricheux et al., 2013; Lescure et al., 2013; Normant-Saremba et al., 2015). This might be explained by the complexity and limited throughput of older instruments (Normant et al., 2007) but also by the increased availability of affordable and user-friendly respirometers (Kaiyala and Ramsay, 2011). Fortunately, modern microcalorimeters, which have become extremely sensitive and easier to use, now enable direct measurement of the heat produced by organisms as small as tiny insects or zooplankton species.

Here, we experimentally illustrate the high potential of microcalorimetry in ecological studies by determining the metabolic response of a homeostatic consumer undergoing a dietary stoichiometric constraint. We hypothesize that the necessary maintenance of homeostasis of consumers induces a higher RMR in organisms fed a stoichiometrically imbalanced diet. To test this hypothesis, we compare, in a standardized growth experiment, the individual heat flow produced versus mass of a clonal line of *Daphnia magna* submitted or not to a stoichiometric constraint during its ontogenetic growth.

### **3.3 Material and Methods:**

#### 3.3.1 Origin and maintenance of daphnids:

The planktonic cladoceran *Daphnia magna* used in this study is a classical model organism in ecological stoichiometry approaches (Hessen et al., 2013) and biology in general (Altshuler et al., 2011) due to its short development cycle and parthenogenetic reproduction allowing to work with clonal population. *Daphnia* is a non-selective filter feeder preponderant in freshwater communities and considered as a keystone species in planktonic food webs (Dodson and Hanazato, 1995). The *Daphnia magna* clone used here was initially collected from a pond of the Allier River floodplain (Puy-de-Dôme, France). To minimize any environmental contamination or parasites, individuals were maintained in laboratory in 2 L glass container of bacteria-free Volvic© water on a 14:10 h light:dark cycle at 20°C and with a maximum of 15 individuals per litre. They were fed daily *ad libitum* (3mg C.L<sup>-1</sup> well above the incipient limiting level that is reported to be approximately 0.5 mg C.L<sup>-1</sup>; Lampert 1978) with a suspension of the green algae *Chlamydomonas reinhardtii*.

#### 3.3.2 Algae cultures and preparation:

*Chlamydomonas reinhardtii* was used as food during all the experiment. Algae were grown on modified WC medium with vitamins (Von Elert and Wolffrom, 2001) with 7.1 mg.L<sup>-1</sup> or 0.35 mg.L<sup>-1</sup> phosphates (PO<sub>4</sub><sup>3-</sup>) providing respectively a control algae (P+) or a P-deficient algae (P-). Cultures were maintained at 20°C under permanent light at a dilution rate of 0.10 per day using aerated 5 L vessels. To exclude any risk of contamination of the cultures, medium used was systematically sterilised and inflows and outflows of air were filtered with 0.2µm filters. Food suspensions were daily prepared from monospecific stock solution of algae by centrifugation and resuspension of the cultured cells in bacteria-free Volvic© water. The carbon concentrations of the algal food suspensions were estimated from photometric light

extinction (800 nm) and pre-established carbon concentration - light extinction regressions. Algal culture subsamples were taken at the beginning, the middle and the end of the experiment, filtered and dried to measure their carbon and phosphorus content.

### 3.3.3 Experimental setup:

Before the experiment, one individual was isolated from the stock culture of *Daphnia magna* and maintained in the same conditions as described earlier. When this clone produced offspring, the neonates (first generation) were kept and the mother removed. This step was repeated twice to keep females from the third generation. After they released their first and second clutch, females from the third generation were kept and the neonates discarded. In order to minimize inter-individual variability, neonates from the third clutch and less than 8h-old were used for the experiments

During the first 24h, the daphnids were fed on a suspension of P-sufficient *Chlamydomonas reinhardtii* (3 mgC.L<sup>-1</sup>) and then randomly distributed into 4 replicate flasks for each treatment (P+ and P-). In each of these 500mL flasks, organisms were raised in Volvic© water renewed each day and had a daily *ad libitum* food supply (3 mgC.L<sup>-1</sup>) to maintain optimal feeding conditions during the 9 days of the experiment. Each day of measurement, one individual was randomly selected from three out of the four replicate flasks to determine its metabolic rate by microcalorimetry resulting in triplicate measurements per dietary treatment and per day of measurement (except for day 9 studied in quadruplicate). After measuring their heat flow, the organisms were sacrificed and individually placed into pre-weighed aluminium containers, dried 48 h at 60°C and weighed (Sartorius; ME36S; Goettingen-Germany) to determine their dry weight. Simultaneously, in the same replicate flasks, a pool of five organisms was removed for somatic growth rate, C and P content measurements.

### 3.3.4 Carbon and Phosphorus analysis:

After drying 48 h at 60°C, filtered algae were crushed and placed with 10mL ultra-pure water in 20mL vials. Daphnids were dried, reduced to powder and placed with 10 mL ultra-pure water in 20 mL vials. The following steps are similar for both daphnid and algal samples: Carbon content was measured using a CHN elemental analyzer (NA 2100 Protein, ThermoQuest CE Instruments, Milano, Italy). Phosphorus content was estimated using the potassium persulfate method as describe by Gales et al., (1966). Briefly, potassium persulfate and heating transform organic phosphorus in  $\text{PO}_4^{3-}$  and then a solution of sulfuric acid, ammonium molybdate, ascorbic acid and antimony-potassium tartrate transform  $\text{PO}_4^{3-}$  in a blue solution of phosphomolibdic acid. The intensity of the colour is proportional to the quantity of  $\text{PO}_4^{3-}$  transformed allowing a spectrophotometric measurement of initial P content of the sample. Carbon and phosphorus content are then related to dry weigh to establish C and P proportion in organisms and their molar C/P ratio.

### 3.3.5 Growth rate measurement:

After 48 h drying, organisms were weighed with a precision of 0.001 mg using a micro-balance (Sartorius ME36S; Goettingen-Germany). They were then placed at 60°C one more day and re-weighed to ensure their total desiccation and a maximum precision of the measurement. At the beginning of the experiment, triplicates of 20 randomly selected neonates were weighed in the same conditions to determine the initial dry weigh. Growth rate was calculated as follow:

$$\text{Growth rate} = \frac{\ln(W_t) - \ln(W_0)}{t} \quad \text{eq.9}$$

where  $W_t$  is the weight (in  $\mu\text{g}$ ) at age  $t$  (in days) and  $W_0$  the weight (in  $\mu\text{g}$ ) of neonates.

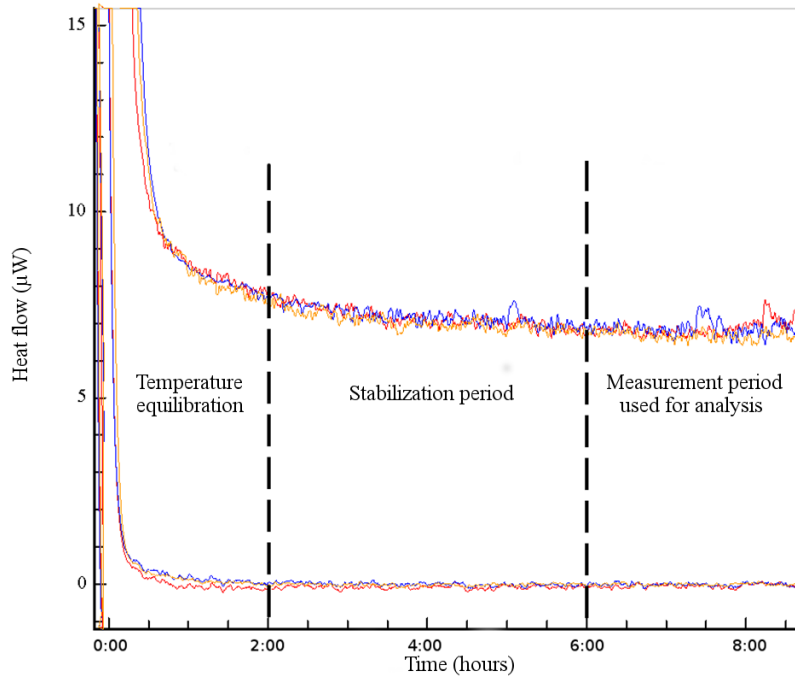


### 3.3.6 Heat flow measurement by microcalorimetry:

Isothermal microcalorimeters of the heat-conduction type are particularly interesting because they allow direct record of the heat flow as a function of time. Here, a TAM III (Waters, TA Instruments ; Newcastle, United States) equipped with two multicalorimeters, each holding six independent minicalorimeters, was used to record continuously and simultaneously the heat flow of 12 samples, with a precision of  $\pm 0.2 \mu\text{W}$ . The temperature of the bath was set at  $20^\circ\text{C}$  and its long-term stability was  $\pm 1 \times 10^{-4} \text{ }^\circ\text{C}$  over 24 h. The minicalorimeters were electrically calibrated. Specific disposable 4 mL ampoules, capped with butyl rubber stoppers and sealed with aluminium crimps, were used in each minicalorimeter. Daphnids were rinsed three times with Volvic® water to remove all food particles and then were kept one hour in Volvic® water to exclude excretory products. After that, they were placed individually into the microcalorimetric ampoules containing 2 mL of Volvic® water. In preliminary experiments, we verified that oxygen diffusion from the 2 mL of air present in each ampoule was sufficient to ensure aerobic conditions during the whole measurement duration. Three ampoules were prepared for each experimental condition. Some ampoules were also prepared with only water in order to carry out blank experiments. The closed ampoules were introduced into the 12 minicalorimeters and the data collection was simultaneously launched using the TAM Assistant Software (Waters, TA Instruments; Newcastle-United States). A heat flow value was collected every second during 8 hours for each of the samples studied.

Blank experiments showed that after 2 hours of temperature equilibration the microcalorimetric signal reaches a plateau at zero, confirming that the water used was bacteria-free (**Figure 6**). In the presence of daphnids, the experiments showed that the signal still decreases slowly after 2 hours until it reaches a plateau after a maximum of about 6 hours for 9 days old daphnids. The heat flow value corresponding to the steady-state was thus

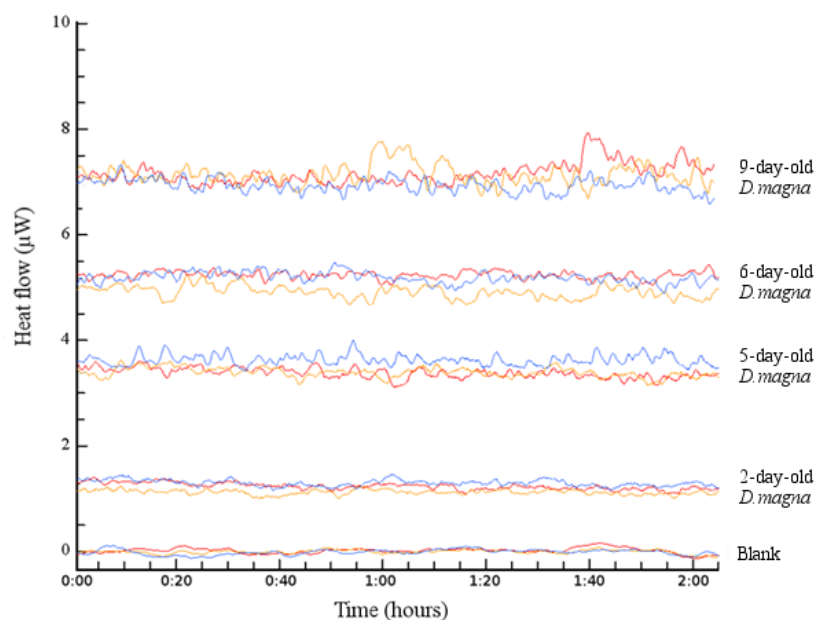
determined, in all cases, by fitting linearly the signal between the 6<sup>th</sup> and 8<sup>th</sup> hours to ensure signal stability whatever the size of daphnids (**Figure 6**).



**Figure 6:** Examples of individual Heat Flow ( $\mu\text{W}$ ) versus Time (hours) curves: blank experiments (lower lines) ; experiments with 9-day-old control fasting daphnids (upper lines). Each color represents an individual ( $n=3$ )

This 6h delay before signal stabilization should be interpreted as a gradual reduction of energy allocated to digestion (a.k.a. specific dynamic action; SDA) during fasting period until digestion processes stops. This observation provides a useful temporal range for the arrest of digestive activity following food removal in *Daphnia*. With this method we were able to determine precisely the heat flow produced by individuals of different sizes, even those weighing only a few  $\mu\text{g}$  (**Figure 7**). Moreover, we showed that the heat produced by individuals bred in the same conditions but of different sizes were clearly distinguishable and reproducible (**Figure 7**). Furthermore, the heat flow - time curves of **Figure 7** clearly show that the signal

noise increases slightly with the daphnids size, which is consistent with the more vigorous motion and greater friction with water for larger individuals (Braissant et al., 2015)



**Figure 7:** Individual Heat flow ( $\mu\text{W}$ ) versus Time (hours) curves observed in the absence of daphnids (blank) and in the presence of 2-, 5-, 6- or 9-day-old control fasting daphnids. Each color represents an individual ( $n=3$ )

### 3.3.7 Data analysis:

Prior to analysis, all data were  $\log_{10}$  transformed in order to meet ANOVA assumptions of normality and homoscedasticity. Shapiro-Wilk tests confirmed the normality of our transformed data and Fisher-snedecor tests confirmed the homogeneity of their variances. Algae and daphnid C and P content as well as C/P ratio of the two dietary treatments were compared for each experimental duration using a two way ANOVA. Differences in daphnid individual metabolic rate (i.e. heat production) between stoichiometrically balanced or imbalanced diets were compared by ANCOVA using individual mass as covariate. The effect size of the treatments on metabolic rate was estimated from their partial  $\omega^2$  (Kotrlik *et al.* 2011; Olejnik & Algina 2000). All statistical analysis were performed using R v.3.4.3 (R Core Team 2018) with an  $\alpha$  error set at 0.05.

### 3.4 Results:

#### 3.4.1 Organisms' mineral content:

The green algae *Chlamydomonas reinhardtii* of the P+ treatment had a ten-fold lower C/P than in the P- treatment ( $F_{(1;4)}=1032.3$ ;  $p<0.001$ ) but no temporal variability was observed during the experiment ( $F_{(1;4)}=1.88$ ;  $p>0.1$ ). The carbon relative content was similar in both treatments ( $F_{(1;4)}=10.73$ ;  $p>0.05$ ) and the variation of C/P ratio was only explained by a ten-fold reduction of P in P- algae ( $F_{(1;4)}=1296.9$ ;  $<0.001$ ) (**Table 3**).

		<i>Chlamydomonas reinhardtii</i>	<i>Daphnia magna</i>			
			Day 2	Day 5	Day 6	Day 9
P+	%C	47 ( $\pm 0,6$ )	41,1 ( $\pm /$ )	41,9 ( $\pm 2,1$ )	42,2 ( $\pm 2,45$ )	44 ( $\pm 2,33$ )
	%P	0,731 ( $\pm 0,026$ )	1,42 ( $\pm 0,028$ )	1,27 ( $\pm 0,065$ )	1,19 ( $\pm 0,205$ )	1,23 ( $\pm 0,067$ )
	Molar C/P	166,3 ( $\pm 3,9$ )	74,6 ( $\pm /$ )	85,63 ( $\pm 3,3$ )	93 ( $\pm 12,7$ )	92,4 ( $\pm 6,2$ )
P-	%C	49,1 ( $\pm 2,6$ )	36,8 ( $\pm /$ )	38,8 ( $\pm 2,47$ )	39,7 ( $\pm 2,83$ )	46,3 ( $\pm 1,1$ )
	%P	0,089 ( $\pm 0,007$ )	1,45 ( $\pm 0,02$ )	1,53 ( $\pm 0,034$ )	1,2 ( $\pm 0,39$ )	1,23 ( $\pm 0,083$ )
	Molar C/P	1439,6 ( $\pm 174,6$ )	65,8 ( $\pm /$ )	65,6 ( $\pm 4,6$ )	69,6 ( $\pm 4,1$ )	97,5 ( $\pm 5,97$ )

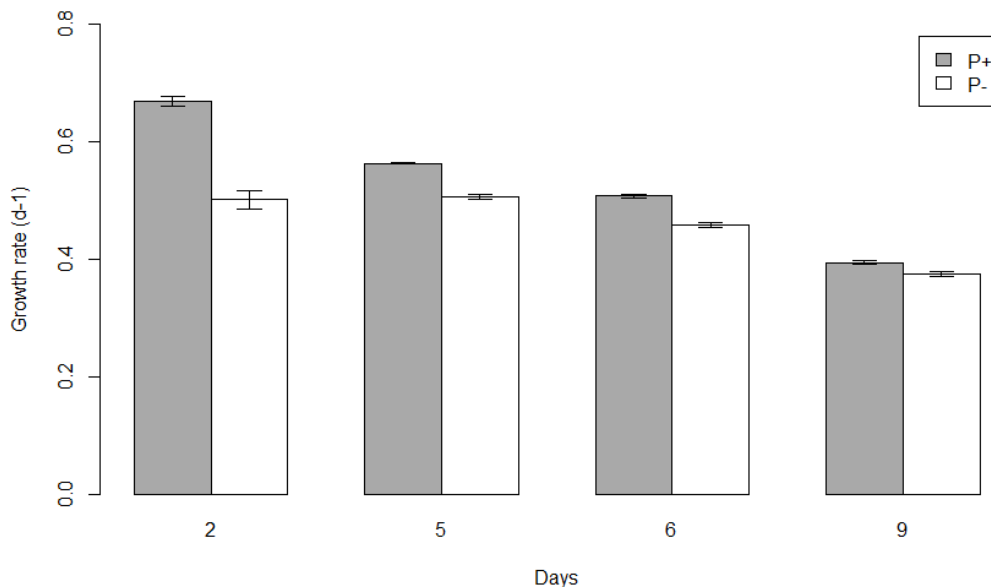
**Table 3:** Mean ( $\pm$  s.e.,  $n=6$ )\_Carbon, Phosphorus content and C/P molar ratio of algae (*Chlamydomonas reinhardtii*) and experimental organisms (*D.magna*) in the control (P+) or P depleted (P-) treatments. C and P are given in % of total dry mass.

Treatment (P+ or P-) had no significant effect on the C in daphnids which accounted for ca. 40% of their dry weight ( $F_{(1;20)}=1.63$ ;  $p>0.1$ ). Daphnids fed P- had a significantly higher proportion of phosphorus during the experiment ( $F_{(1;20)}=9.37$ ;  $p<0.01$ ). Molar C/P ratios of daphnids were lower in the P- than in the P+ treatment ( $F_{(1;20)}=17.9$ ;  $p<0.001$ ). Diet and age interactively affected daphnid C/P ratio ( $F_{(3;20)}=7.2$ ;  $p<0.01$ ) with a stronger influence of diet C/P on younger individuals (**Table 3**). We estimated the homeostatic capacity of daphnids by calculating their stoichiometric flexibility coefficient (1/H - Elser & Sterner, 2002). The 1/H

value represent the variability of consumers versus food stoichiometry, the more 1/H approach 0 the more homeostatic is the consumer. It appears that, in our experiment, 1/H ranged from -0.13 (day 6) to 0.025 (day 9) and the average 1/H value across all experiment ( $-0.072 \pm 0.07$ ) did not significantly differ from 0 ( $t_{(3)} = -1.983$ ;  $p > 0.1$ ) indicating a strict homeostasis of our *Daphnia* clone (See **Figure S7** in supporting information).

### 3.4.2 *Daphnia* growth:

Organisms from P- treatments exhibited a significantly lower growth rate than those from P+ (**Figure 8**) ( $F_{(1;24)} = 23.68$ ;  $p < 0.001$ ). *Daphnids'* age also induced a significant variation in growth rate ( $F_{(3;24)} = 46.82$ ;  $p < 0.001$ ) where younger organisms exhibited a higher growth rate than the older ones (**Figure 8**). Dietary stoichiometry and organisms age also had an interactive effect on growth ( $F_{(3;24)} = 3.94$ ;  $p < 0.05$ ) with younger individuals being more responsive to the dietary C/P ratio.



**Figure 8:** Mean ( $\pm$  s.e.,  $n=3$  except day 9 where  $n=4$ ) *D. magna* growth rate ( $\text{day}^{-1}$ ) versus age (days) for the control (P+) and the P-depleted (P-) treatments.

### 3.4.3 Heat flow produced by daphnids:

The regression lines linking heat-flow as a function of mass appears highly significant for both treatments (P+:  $R^2$  0.96; P-:  $R^2$  0.97). The regression model of heat versus mass and treatment was also highly significant ( $F_{(3,22)}=220.7$ ; p-value <0.001; Multiple  $R^2 = 0.9678$ ) (**Figure 9**). Based on an ANCOVA analysis of log transformed data of **Figure 9.A**, we found that individual mass had induced a significant variation of the heat produced by organisms ( $F_{(1;23)}=616.02$ ; p<0.001). The dietary treatment also induced a significant variation of individual metabolic rate after controlling for individual body mass ( $F_{(1;23)}=36.03$ ; p<0.001), organisms from the P- treatment showing a higher heat flow than those from the P+ treatment. Results of the ANCOVA analysis are presented in **Table 4**. As revealed by the partial  $\omega^2$  of 0.577 estimating the effect size of the treatments, the dietary stoichiometric constraint should explain more than half of the variations observed in metabolic rate when mass effect is excluded. No interactive effect of treatment and mass has however been found ( $F_{(1;23)}=1.31$ ; p>0.05) (**Table 4**).

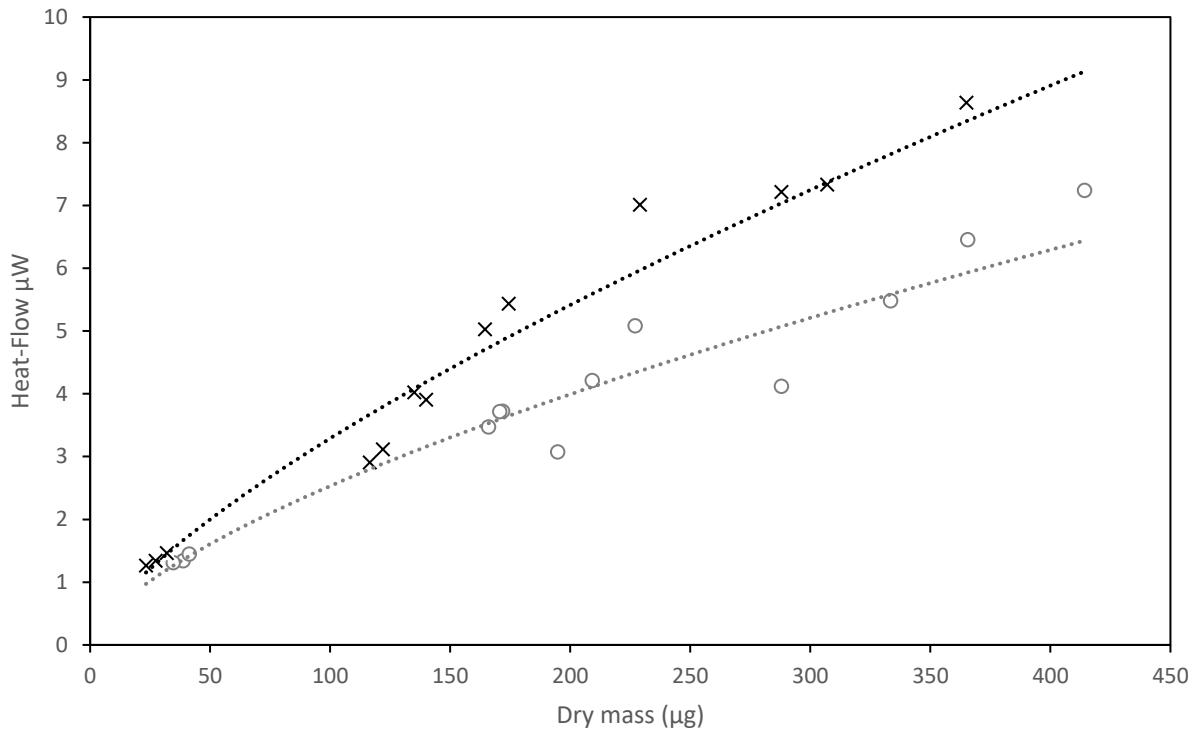
Response: Heat-Flow					
	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Mass	1	1,63235	1,63235	624,3191	< 2,2e-16
Treatment	1	0,09547	0,09547	36,5132	4,41E-06
Mass:Treatment	1	0,00342	0,00342	1,3097	0,2647
Residuals	22	0,05752	0,00261		

**Table 4:** Summary of the ANCOVA analysis performed to determine effects of mass and treatments on individual heat flow.

### 3.5 Discussion:

We conducted a growth experiment on a clone of *D. magna* to evaluate how a dietary stoichiometric constraint could affect its resting metabolic rate (RMR). Isothermal microcalorimetry enabled high precision and repeatability of RMR measurements (**Figure 6-**

7). We were therefore able to demonstrate clearly the age and diet-related changes in the RMR of *D. magna* (**Figure 9**).



**Figure 9:** Individual heat flow ( $\mu\text{W}$ ) versus body dry mass ( $\mu\text{g}$ ) for treatment P+ (grey circles; regression :  $y=0.12x^{0.66}$ ;  $R^2=0.96$ ) or P- (black cross; regression :  $y=0.12x^{0.72}$ ;  $R^2=0.97$ ).

As RMR is the most fundamental component of organismal energy budget (Sibly et al., 2013), its variation should affect the energy that can be allocated to other functions such as growth or reproduction (Burton et al., 2011; Sibly et al., 2013). At species level, most studies on the variation of RMR have focused on inter-genotype differences and their consequences on individual fitness. Some studies suggested that a higher RMR confers a fitness advantage by maintaining larger metabolic machinery facilitating higher resources intake (Careau et al., 2013; Metcalfe et al., 1995; Yamamoto et al., 1998). Conversely, others found that the

energetic cost of RMR is a burden to the organismal energy budget and that a low RMR leaves a larger amount of energy available for growth or reproduction (Alvarez & Niecieza 2005; Blackmer *et al.* 2005; Reid *et al.* 2011). However, as environmental factors are likely to affect individuals metabolic rate (Glazier, 2010), interpreting individual variation in RMR requires an understanding of the relative importance of environmental factors in comparison to genetic variations of RMR (Glazier, 2010; Rosenfeld *et al.*, 2015). Thus, our focus here is not on genotype differences in RMR but on the link between RMR and an environmental factor (diet quality). We therefore used a single *Daphnia* clone to minimise inter-individual genetic differences.

In our experiment, body C/P ratio of *D. magna* showed significant variations depending on both organisms age (size) and dietary stoichiometry. This interactive effect is not surprising since younger *Daphnia* have typically higher phosphorus contents than adults (DeMott, 2003; Wagner *et al.*, 2017). This observation has been explained by the higher growth rate of juveniles compared to adults leading to higher P-requirements (Growth Rate Hypothesis, (Elser *et al.*, 2003). In our experiment, the lower C/P ratio of organisms fed low-P resources could thus be explained by their smaller sizes thereby confirming that P requirements can change with organismal ontogeny (Halvorson and Small, 2016). Despite age-related changes, our daphnia clone showed a nearly strict homeostasis throughout the 9d experiment with a stoichiometric flexibility coefficient (1/H) not significantly different from 0. This value indicates that our clone is particularly homeostatic compared to other values reported for *Daphnia magna* (0.2-0.25) (DeMott and Pape, 2005; Hood and Sterner, 2014). This result clearly showed that, regardless of their size, daphnids facing an imbalanced diet had to regulate their body stoichiometry by releasing excess C. Under certain conditions, such stoichiometric regulation can occur through pre-absorptive pathways, through adjustments in ingestion or



assimilation rate (DeMott et al., 1998; Plath and Boersma, 2001). However, a previous study (Darchambeau et al., 2003) showed that when fed *ad-libitum* as in our experiment, the ingestion or assimilation rates of *Daphnia* are less affected by dietary stoichiometry. Growth of algal resources in P-limited conditions might also have changed their digestibility for consumers and, in turn, the assimilation rate, as proposed by Van Donk *et al.* (1997). Yet, Boersma and Wiltshire (2006) showed that only 2 to 4% of ingested P-limited algae pass intact through the gut indicating that digestion resistance effects are marginal. Hence, we can consider that in our study, homeostatic regulation was probably mainly explained by post-absorptive processes. Such processes involve an active release of C, either through a higher excretion of DOC and/or a greater respiratory release of CO<sub>2</sub> (Jensen and Hessen, 2007; Lukas and Wacker, 2014)) leading to the hypothesis that a dietary stoichiometric constraint increases RMR (Hessen et al., 2013).

Resting metabolic rate represents the very minimum energy required for organism's maintenance corresponding to the metabolic activity of a resting, non-feeding organism at a given temperature (Auer et al., 2015; Fry, 1947). However, measuring the exact value of RMR is usually difficult due to some unavoidable organismal routine activity (Willmer *et al.* 2005). As an example, pelagic zooplankton species, such as *Daphnia*, must remain continuously active to prevent sedimentation. This swimming activity is constant and essential for survival. We therefore argue that it should be considered as part of the maintenance processes. On the other hand, specific dynamic action (SDA, see McCue 2006) might complicate metabolic data interpretation. Previous studies reported that depending on food quality, daphnids are able to show some plasticity in their feeding behaviour (Plath and Boersma, 2001; Sterner, 1993) or variations in assimilation capacity (Van Donk *et al.* 1997 but see Boersma & Wiltshire 2006) which might affect SDA. However such consideration are irrelevant here since SDA is

excluded from our measurements. Microcalorimetry enables a temporally unconstrained, real-time monitoring of the organismal heat flow. This makes it possible to determine precisely when SDA becomes negligible to ensure that only RMR is measured. Hence, by eliminating all the sources of bias discussed above, our experiment demonstrate that daphnids facing a dietary stoichiometric constraint face higher maintenance costs (RMR) potentially due to homeostatic body stoichiometry regulation. These higher costs for structural and reproductive maintenance should thus reduce the remaining energy allocable to net growth and reproductive output (Kooijman, 2009) and explain the reduced somatic growth rate on P- diet in our experiment.

### **3.6 Conclusion and perspectives:**

We showed that heat-conduction isothermal microcalorimetry enables a precise, reproducible, continuous and real-time monitoring of the metabolic rate of small invertebrates. By using a multichannel instrument, several samples can be processed simultaneously. For each sample, a heat flow value can be collected every second during several hours, leading to a high resolution of the heat flow – time curve. The possibility of precise, unconstrained real-time monitoring of metabolism and opens novel perspectives for understanding metabolic rate variability and physiological processes in small ectotherms. Below, we provide a non-exhaustive and subjective list of exciting perspectives:

(A) *The cost of SDA*: The implications of dietary stoichiometry on consumer ingestion and/or assimilation rate remain debated (DeMott *et al.* 1998; Plath & Boersma 2001; Darchambeau *et al.* 2003). Quantifying the food processing costs (SDA -(Secor, 2009)) of stoichiometrically imbalanced diets should unravel the behavioural response of consumers to a reduced food quality. (B) *The cost of homeostasis*: The degree of stoichiometric flexibility is highly variable both between and within *Daphnia* species (DeMott & Pape 2005; Hood &

Sterner 2010; this study). Being able to link the stoichiometric flexibility to the metabolic costs induced by unbalanced diets should improve our understanding of the consequences of stoichiometric regulation on individual fitness and the potential physiological trade-offs involved. (C) *The links between RMR and growth*: The growth and reproduction rates are the basis of population dynamics. The relationship between RMR and growth remains, however, largely debated hindering extrapolations from metabolism to population dynamics (Burton et al., 2011; Moyano et al., 2018). It is likely that the nature of the correlation between the two factors depends on whether it is considered within or between genotypes. Microcalorimetry offers the necessary precision needed to clarify this central question. (D) *Temperature-stoichiometry interactions on metabolism*: In ectotherms, temperature drives all physiological processes through its direct effect on metabolic activity (Auer et al., 2018). Temperature also strongly interacts with nutrition because it modulates the nutritional needs (in terms of energy and specific nutrients) of ectotherm consumers (Kendrick and Benstead, 2013; Woods et al., 2003). However, the direction towards which nutritional requirements change with temperature remains unclear and actively debated (Boersma et al., 2016a; Winder et al., 2016a). (E) *Aerobic versus anaerobic metabolism*: Combining calorimetric with respirometric measurements could enable the delineation of aerobic and anaerobic metabolism. Recently, such “calorespirometric” approaches (Brueckner et al., 2017; Wadsö and Hansen, 2015) showed that in goldfish the relative contribution of anaerobic processes to the metabolic budget increases with temperature (Regan et al., 2017). However, as explained above sensitivity issues in respirometric equipments hinder for instance the applicability of the method to small aquatic invertebrates such as *Daphnia*.

Microcalorimetry enables testing the existing hypotheses on how consumer metabolism is affected by temperature and dietary stoichiometry. Such data would greatly contribute to the

current effort to merge ecological stoichiometry with metabolic frameworks into a unified ecological theory (Allen and Gillooly, 2009; Barnes et al., 2018, 2014; Hillebrand et al., 2009; Ott et al., 2014; Wang and Brose, 2018).

## **Transition :**

Dans le chapitre 3 nous avons mis en place et démontré l'efficacité d'une méthode par microcalorimétrie permettant de mesurer de façon précise et individuelle le taux métabolique au repos chez *Daphnia*. L'utilisation de cette méthode nous a permis de confirmer expérimentalement l'hypothèse selon laquelle une élévation du taux respiratoire (i.e. proxy du taux métabolique) permettait à l'organisme de rejeter l'excès de C alimentaire afin de maintenir l'homéostasie. Ce premier résultat semble démontrer l'existence d'un lien entre qualité nutritionnelle de la ressource et taux métabolique au repos du consommateur ainsi que de potentielles conséquences pour la croissance de ce dernier. Cependant, les conclusions apportées dans ce chapitre sont ancrées à une approche stœchiométrique de la qualité nutritionnelle et ne reflètent donc que partiellement les conséquences de cette qualité sur le métabolisme des consommateurs.

Afin d'établir les liens qualité-métabolisme de façon plus générale, le chapitre suivant a élargi le gradient de qualités nutritionnelles pour intégrer une large gamme de composés couvrant à la fois une vision stœchiométrique (C, P) mais aussi biochimique (stéroïdes, AGPI) des apports alimentaires. Au-delà de chercher à établir les liens entre qualité et métabolisme ainsi que leur conséquence pour la croissance individuelle, ce chapitre s'est également attelé à comprendre les processus physiologiques sous-jacents qui y sont associés.

**Chapitre 4 : Conséquences des contraintes  
nutritionnelles sur le métabolisme des  
consommateurs et la production de biomasse**

# **Linking nutritional constraints to consumer metabolism and biomass production.**

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## **En préparation**

### **4.1 Abstract:**

Two biological currencies have been the focus of much theoretical work in ecology: energy, which is required to fuel the metabolic processes of survival, growth and reproduction in organisms; and material (i.e. dietary intake of specific compounds or elements) which is required to construct biomass. These currencies have been mostly studied independently but the integration of food quality and energetics is increasingly requested. Here, using a clonal line of *Daphnia magna*, we explored the effects of food quality on individual metabolic rate (energy flux dissipated by metabolism) and the resulting consequences on biomass production (material flux). We showed that both biochemical and stoichiometric dietary constraints reduce consumers' growth rate while elevating their resting metabolic rate (RMR). These results show the effect of nutritional constraints on consumers' biomass production cost which can increase by up to 400% between optimal or poor food quality. Along ontogeny the decline of food quality also elevates the scaling exponent linking RMR to body mass from 0.46 (optimal food quality) to 1 (poor food quality). Such differences in scaling may reflect the variations of the ratio between structural or reproductive tissues along ontogenetic development thus capturing

the qualitative difference in biomass production. Given its level of integration covering both individual growth and reproductive capacities, but also due to its repeatability regardless of the specific element or compounds limiting consumer performances, here we showed that the direct measurement of individuals RMR may constitute an attractive indicator for population growth and persistence in natural habitats.

## **4.2 Introduction:**

Resting metabolic rate (RMR) is a central component of individuals energetics since it reflects the incompressible cost of self-maintenance subsequently constraining the energy allocation to other functions such as growth or reproduction. This key role made of RMR a central component of metabolic ecology since the Kleiber's power law (1932) and up to the more recent metabolic theory of ecology (MTE – (Brown et al., 2004)). MTE uses the allometric scaling of RMR with body size and temperature to assess the rates at which organisms expend (metabolism) and store energy as biomass (growth and reproduction). Thus, MTE constitutes a quantitative approach that aims to predict the fluxes and stores of energy between and within trophic levels (Brown et al., 2004). Yet, focusing on energy as sole currency fails to explain the high spatial and temporal variability in energy transfer efficiencies observed between trophic levels, particularly at the plant-animal interface (Brett and Goldman, 1997).

To explain this variability, one needs to consider that energy transfer is coupled to and driven by material transfer. Material can obviously be converted into energy to fuel metabolism but is also required to build structure and insure vital functions. The non-substitutability of some essential compounds in organism's physiology coupled with their inability to synthesize them *de-novo* require the availability- at appropriate proportions- of such compounds in food resources. The disparity between the consumer's nutritional demand for and the resource's



supply in essential compounds -or alternatively, *food quality*- drives the energy transfer between trophic levels via its effect on consumer growth and reproductive rates. At the end of 20<sup>th</sup> century, identifying quantitative markers of food quality became an important task in ecological research (Brett and Muller-Navarra, 1997; Elser et al., 2000). For example, a highly unsaturated fatty acid (HUFA) has been suggested to predict secondary production in lakes (Müller-Navarra et al., 2000). At the same time, Elser et al (2000) showed that the C:N:P stoichiometry of food can also constrain herbivore growth efficiency. Following these early works, the biochemical view of trophic interactions unveiled a great variety of dietary compounds such as n-3 and n-6 polyunsaturated fatty acids (PUFA) (Wacker and von Elert, 2001) and phytosterols (Martin-Creuzburg et al., 2005; Von Elert et al., 2003) as important determinants of food quality limiting aquatic consumer growth either alone or in co-limitation (Lukas et al., 2011; Marzetz et al., 2017; Sperfeld et al., 2012). In parallel, the development of nutritional geometry highlighted the importance of food macronutrient proportions (i.e. Carbohydrate:Protein:Lipids) as major food quality determinants for terrestrial consumers (Raubenheimer et al., 2009; Raubenheimer and Simpson, 1999). While revealing the complexity of food quality, nutritional ecology turned away from the energetic perspective of early trophic ecology work (Lindeman, 1942) increasingly focusing on material transfer only. Several authors identify this segregation as an obstacle to our understanding of ecosystem structure and functioning and call for the integration of these two currencies into a common framework (Allen and Gillooly, 2009; Barnes et al., 2018; Hillebrand et al., 2009; Kodric-Brown et al., 2012; Ott et al., 2014). We argue that the first step towards this goal is to understand how limitation by material transfer (food quality) affect the way consumers dissipate (RMR) and store energy (growth / reproduction).

In that aim, we used *Daphnia magna* to test whether and how individual RMR can be affected by food quality. Besides allowing the exclusion of genetic differences of RMR, the use of *Daphnia magna* is advantageous since the nutrition of this key aquatic organism has been extensively studied over the last decades. Thanks to this rich literature we know that daphnids commonly face both stoichiometric (C:P) and biochemical (PUFA, sterols) dietary limitations in the field. Here, we measured individual resting metabolic rate (RMR) and growth from birth to sexual maturity along a gradient of realistic food qualities incorporating various degrees of biochemical and stoichiometric limitation as well as their combination. We used a direct microcalorimetric method to precisely measure how energy is dissipated by RMR (Ruiz et al., 2018). We hypothesized that independently of its proximal cause (*i.e.* PUFA, Sterol or stoichiometry) sub-optimal food quality increases individual RMR proportionally to the measured decrease in individual growth. We argue that quantifying the energetic cost imposed by food quality on individual RMR may constitute a common currency enabling the integration of food quality and energetics.

### **4.3 Material and Method:**

#### **4.3.1 Preparation of food suspension:**

To explore how dietary constraints affect *D.magna* growth we established an ecologically relevant dietary gradient from the lowest to the highest food quality for daphnids. In order to obtain a biochemical nutritional constraint, we used 3 different phytoplankton species: *Cryptomonas sp.* a cryptophyceae rich in highly unsaturated fatty acid (HUFA - especially 20-5w3) constituting an excellent food quality for daphnids, *Chlamydomonas reinhardtii* considered as a good food quality due to its polyunsaturated fatty acids content (PUFA - especially 18-3w3) and *Synechococcus sp.* a cyanobacteria of poor dietary quality due to its lack of PUFA and sterols (Martin-Creuzburg et al., 2009; Müller-Navarra et al., 2000;

Von Elert et al., 2003). As *Synechococcus sp.* alone do not allows satisfactory *D.magna* growth we supplemented it with different proportions of liposomes containing cholesterols (see below liposomes preparation) to create a gradient of cholesterol availability with 2, 4, 8 and 12 $\mu$ g cholesterol per mgC in the final food suspension. In parallel, to obtain a stoichiometric constraint we grown *Cryptomonas sp.* and *Chlamydomonas sp.* on a P-limited medium providing P-limited diets. In addition to these first monospecific dietary treatments, we used a mixture of *Chlamydomonas sp.* (20%) and *Synechococcus sp.* (80%) as well as a sample of natural lake seston to explore how diets with more complex composition may affect the response noted on monospecific diets. All these dietary treatments are summarized in **Table 5**.

#### 4.3.2 Daphnia maintenance:

We used a clonal line of *Daphnia magna* in our experiment to exclude any genotypic variability. The stock cultures of daphnids were kept in Volvic water© at 20°C on a 14:10h day:night cycle and fed with *Chlamydomonas reinhardtii* (at 3mgC.L<sup>-1</sup>) for more than 3 generations before the beginning of the experiment.

#### 4.3.3 Experimental setup:

To reduce inter-individual differences, daphnids used in the experiment came from a same clonal offspring and were born in a time interval of less than 8 hours. During the first 24 h, daphnids were fed on a suspension of *C. reinhardtii* (3 mg C L<sup>-1</sup>) to ensure a minimal growth. After this first day, daphnids were randomly distributed into three replicates 500 mL jar for each dietary treatment. Each of these jars were filled with 500mL Volvic© water and with ad-libitum food supply (3mgC.L<sup>-1</sup>). This breeding medium was renewed daily to maintain optimal conditions during all the experiment duration. Each day of measurement, one individual was randomly selected in each replicate of each dietary treatment to determine its RMR by microcalorimetry (see below). After measuring their RMR, individuals were then individually

placed into pre-weighed aluminum containers, dried 48 h at 60 °C and weighed (Sartorius; ME36S; Goettingen-Germany). Dry weight of each individual was then related to its RMR. In each treatment, the experiment stops when daphnids reached maturity to avoid misleading measurement of metabolic rate due to the development from eggs to juvenile in daphnids brood pouch. Mature organisms were weighted as described above and the growth rate at maturity was calculated as follow:

$$\text{Growth rate} = \frac{\ln(Wt) - \ln(W0)}{t} \quad \text{eq.10}$$

where  $Wt$  is the weight (in  $\mu\text{g}$ ) at age  $t$  (in days) and  $W0$  the weight (in  $\mu\text{g}$ ) of neonates.

#### 4.3.4 Resting metabolic rate measurement by microcalorimetry:

Resting metabolic rate (RMR) of daphnids was determined individually through the measurement of individual heat-production using a TAM III microcalorimeter of the heat-conduction type (Waters, TA Instruments; Newcastle, United States). Before RMR measurement, daphnids were rinsed three times with Volvic© water to remove all food particles and they were kept 1h in clean water to exclude excretory products. They were then placed into 4mL ampoules containing 2mL of Volvic© water and 2mL of air. Ampoules were then sealed and introduced in the microcalorimeter. The data collection was performed with the TAM assistant Software (Water, TA Instruments; Newcastle, United States) which collected a heat-flow value every second during 8h. This long-time measurement allows to monitor the activity of individuals and ensure that the value reported as RMR exclude residual digestive activity (see Ruiz et al 2018 for more details).

#### 4.3.5 Liposome preparation:

Liposome suspensions were prepared according to Denoux & al (2017). The 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG, Sigma-Aldrich) and 1-palmitoyl-2-oleoyl-

phosphatidylcholin (POPC; Sigma-Aldrich) were dissolved in ethanol at respective concentration of 3 and 7mg.mL<sup>-1</sup>. In parallel cholesterol was dissolved in ethanol at a concentration of 20mg.mL<sup>-1</sup>. A mix containing 1mL of POPG and POPC solution plus 400µL of cholesterol solution was dried using a rotary evaporator and re-suspended in 10 mL buffer (20 mmol.L<sup>-1</sup> NaPi , 150 mmol L<sup>-1</sup> NaCl). This suspension was then sonicated and centrifuged (150 000 g, 90 min, 4°C). The pellet was collected and resuspended in buffer to remove free cholesterol from the liposome suspension. Concentration of cholesterol was measured on the final liposomes' concentration used to supplement dietary treatment.

#### 4.3.6 Mineral and Biochemical analyses of food treatment:

Carbon and Nitrogen were directly measured with a CHN analyser (NA-2100-Protein, ThermoQuest-CE Instruments, Italy). Phosphorus content was estimated using a colorimetric method based on potassium persulfate reaction (Ruiz et al., 2018). Total lipids were extracted from analysed solution using chloroform/methanol method. Lipids extract used to fatty acid analysis were then submitted to an acid-catalysed transesterification producing fatty acid methyl esters (Denoux et al., 2017). For sterol analysis, lipids extract undergone an alkaline hydrolysis (saponification) to specifically extract sterols. The fatty acid methyl esters and sterols extracts were analysed by gas chromatography (6850-Network-GC, Agilent-technologies, USA).

#### 4.3.7 Statistical analysis:

The between-treatment differences of daphnids' growth rates at maturity were log transformed to meet ANOVA assumptions and then compared by ANOVA analysis followed by a post hoc Tukey test.

The RMR of daphnids was plotted against body mass following the power law commonly used by MTE as:

$$RMR = a * M^b \quad eq.11$$

Where  $a$  constitute the proportionality constant and  $b$  the scaling exponent linking RMR to body mass ( $M$ ). ANCOVA analyses were performed after a log transformation of data to define the effect of dietary treatment and body mass, as well as their interaction, on individual RMR. These analyses were performed on the entire dietary gradient or in different subsets of dietary treatments to cover different sections of the gradient (i.e. biochemical limitations, stoichiometric limitation, cholesterol supplementation). In addition, for each dietary treatment, the data was resampled a thousand times and the scaling exponent  $b$  was extracted for each iteration. This non-parametric bootstrapping (Efron and Tibshirani, 1986) allowed to generate 95% confidence intervals around  $b$  for each dietary treatment. In absence of overlap between these 95%CI we considered the differences reported for  $b$  significant.

We also explored the links between dietary-induced variation of individual RMR and individual growth. For doing so we plotted the mass-specific RMR of individuals, including all dietary treatments, versus growth rate. The use of mass-specific RMR allows the harmonization of values for individuals of different body sizes. To erase variability induced by individuals developmental stage, we performed the comparison for each day independently. We fitted linear regressions displaying individual growth rate versus daphnids RMR and age. The regressions were individually tested using a Pearson correlation test.

Finally, using our data displaying RMR and growth of consumers on a daily basis, we model the biomass production cost (BPC) as:

$$BPC = \frac{\int_{t_0}^t RMR}{W_t - W_{t_0}} \quad eq.12$$

where  $BPC$  is the biomass production cost,  $RMR$  is individuals resting metabolic rate (in  $\mu W$ ) its integration between  $t_0$  and  $t$  reflecting the gross energy dissipated in the time interval (in J).  $W_t$  is the weight of individuals at time  $t$  and  $W_{t_0}$  the weight of neonats (at  $t_0$ ). BPC is thus

reported in  $\text{J}\cdot\mu\text{g}^{-1}$  and reflect the gross energy dissipated through RMR between  $t_0$  and  $t$  per unit of biomass increment on the same time range.

## **4.4 Results:**

### 4.4.1 Dietary resources composition:

The phytoplankton species used as diets for *Daphnia magna* differ in their biochemical composition, especially in term of sterols, polyunsaturated fatty acids (PUFA) and highly unsaturated fatty acids (HUFA) (**Table 5**). The four lowest quality diets were composed of *Synechococcus sp.*, a cyanobacterium deprived of sterols, PUFA and HUFA, as a base diet supplemented with cholesterol at four concentrations ranging from 2 to  $12\mu\text{g}\cdot\text{mgC}^{-1}$ . Of slightly better quality, was a 80:20 (carbon:carbon) mixture of *Synechococcus* with *Chlamydomonas reinhardtii* a Chlorophyceae which naturally contains sterols and PUFA (18:2n-6 and 18:3n-3) but not HUFA (hereafter, *mixed diet*). Next, was a diet of intermediate biochemical quality composed of pure *Chlamydomonas*. The highest biochemical quality *Cryptomonas sp.*, a Cryptophyceae that is rich in sterols, PUFA and HUFA (20:5n-3 and 22:6n-3) (**Table 5**). Within intermediate to high quality biochemical gradient (i.e. *Chlamydomonas-Cryptomonas*), we also manipulated the stoichiometric (C:P ratio) quality. Hence, the C:P ratio of *Chlamydomonas sp.* and *Cryptomonas sp.* ranged from 166 and 112 (high quality), respectively, when cultured in classical medium and to 1439 and 453 (low quality), respectively, in P depleted conditions. In order to have a comparison of the growth and metabolic responses to artificially controlled diets with natural conditions we also used a natural seston treatment. Diets compositions are given in **Table 5**.

Dietary Treatment	HUFA		PUFA		Sterol	C:P ratio
	20:5n-3	22:6n-3	18:2n-6	18:3n-3		
<i>Cryptomonas sp. P+</i>	8.8	2.7	7.9	8.4	10-12 <sup>1</sup>	111.9
<i>Chlamydomonas sp. P+</i>	Und.	Und.	14.5	10.9	4-6 <sup>1</sup>	166.3
<i>Synechococcus</i> + 12µg cholesterol / mgC	Und.	Und.	Und.	Und.	12	195
<i>Synechococcus</i> + 8µg cholesterol / mgC	Und.	Und.	Und.	Und.	8	195
<i>Synechococcus</i> + 4µg cholesterol / mgC	Und.	Und.	Und.	Und.	4	195
<i>Synechococcus</i> + 2µg cholesterol / mgC	Und.	Und.	Und.	Und.	2	195
<i>Cryptomonas sp. P-</i>	7.6	2.1	8.3	8	10-12 <sup>1</sup>	453.5
<i>Chlamydomonas sp. P-</i>	Und.	Und.	27.4	11.2	4-6 <sup>1</sup>	1439.6
Mixed diet	Und.	Und.	2.9	2.2	3-4	171.8
Natural seston	Und.	Und.	4.7	3.2	N.A.	76.4

**Table 5:** Dietary treatments used in the experiment and their main characteristics in term of biochemical and stoichiometric composition. Fatty acid and sterol content are given in µg.mgC<sup>-1</sup>. Und. reflect undetected compounds. N.A. are not measured parameters. <sup>1</sup>Martin-Creuzburg & Merkel (2016)

#### 4.4.2 Effects of dietary treatments on growth:

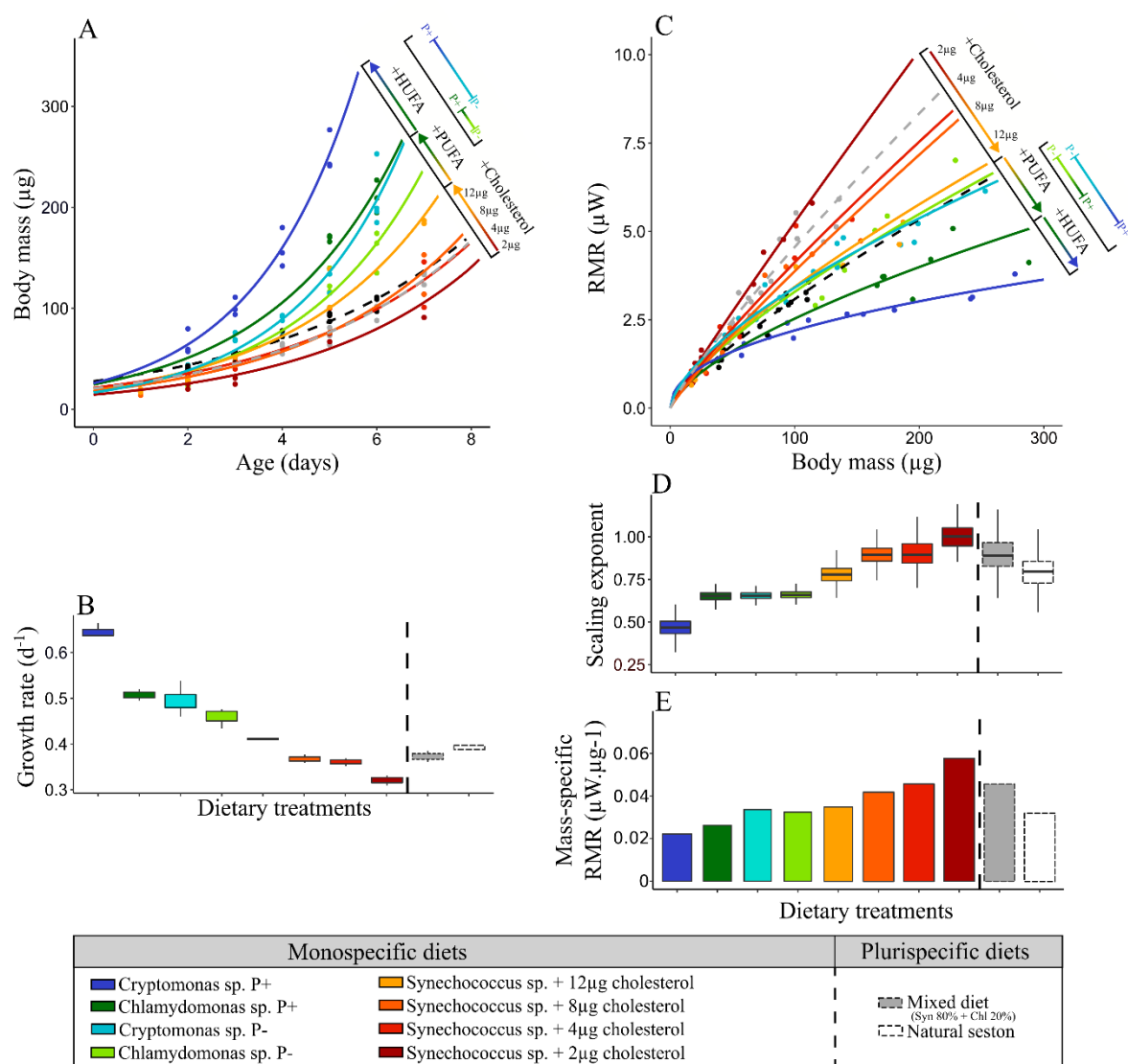
The progressive increase in availability of essential biochemical compounds across diets induced significant increases of individuals growth rate (ANOVA,  $F_{(9,110)}=9.94$ ;  $p<0.001$ , **Figure 10.B**). The lowest reported growth rate was  $0.32d^{-1}$  for individuals fed with *Synechococcus sp.* supplemented with  $2\mu g.mgC^{-1}$  cholesterol. The gradual supplementation of *Synechococcus* with cholesterol concentrations up to  $12\mu g.mgC^{-1}$  alone, significantly increased individual's growth rate up to  $0.41d^{-1}$  (Tukey post-hoc test,  $p<0.001$ ). Individual's growth rate further increased to  $0.5d^{-1}$  when PUFAs were available (*Chlamydomonas reinhardtii*, Tukey post-hoc test,  $p<0.001$ ) and  $0.64d^{-1}$  when both PUFAs and HUFAs were available (*Cryptomonas sp.* Tukey post-hoc test,  $p<0.001$ ). From a stoichiometric viewpoint, the deficiency of nutrient (here phosphorus) significantly reduced individuals growth rate in *Cryptomonas sp.* treatments (containing sterols, PUFA and HUFA; growth decline from  $0.64d^{-1}$  (P+) to  $0.49d^{-1}$  (P-), Tukey post-hoc test,  $p<0.001$ ) but not in *Chlamydomonas reinhardtii* treatments (containing sterols and PUFA; growth decline from  $0.5d^{-1}$  (P+) to  $0.45d^{-1}$  (P-), Tukey post-hoc test,  $p=0.06$ ). These results also underlined the co-limitation between HUFA



and P since the lack of both these compounds induced a greater reduction of growth rate (from  $0.64\text{d}^{-1}$  in *Cryptomonas sp.* P+ to  $0.45\text{d}^{-1}$  in *Chlamydomonas reinhardtii* P-, Tukey post-hoc test,  $p<0.001$ ) than the limitation by HUFA (growth rate from  $0.64\text{d}^{-1}$  to  $0.5\text{d}^{-1}$ , Tukey post-hoc test,  $p<0.001$ ) or P (growth rate from  $0.64\text{d}^{-1}$  to  $0.49\text{d}^{-1}$ , Tukey post-hoc test,  $p<0.001$ ) alone. Individuals fed with more complex diets exhibited growth rates of  $0.38\text{d}^{-1}$  on the mixed diet and  $0.39\text{d}^{-1}$  on the natural seston which correspond to the lower range of growth rates reported (*i.e.* treatment *Synechococcus sp.* + $8\mu\text{g Chol/ mg C}$  **Figure 10.A-B**).

#### 4.4.3 Effects of dietary treatments on resting metabolic rate:

The progressive increase in availability of essential biochemical compounds induced significant decrease of individuals resting metabolic rate (ANCOVA,  $F_{(5,53)}=30.691$ ;  $p<0.001$ , **Figure 10.C-D**). Conversely, to growth, the worst dietary treatment (*Synechococcus sp.* with  $2\mu\text{g.mgC}^{-1}$  cholesterol) resulted in the highest RMR with a mass-specific RMR of  $0.054\mu\text{W}.\mu\text{g}^{-1}$  for individuals of  $100\mu\text{g}$  (**Figure 10.E**). The successive supplementation of *Synechococcus sp.* with cholesterol (from  $2\mu\text{g.mgC}$  to  $12\mu\text{g.mgC}$ ) gradually alleviate the sterol deficiency with the consequence of decreasing the mass-specific RMR down to  $0.033\mu\text{W}.\mu\text{g}^{-1}$ . The mass-specific RMR reached values of  $0.026\mu\text{W}.\mu\text{g}^{-1}$  when PUFA were available and  $0.022\mu\text{W}.\mu\text{g}^{-1}$  when both PUFA and HUFA were available. Decreasing dietary quality resulting from a deficiency of phosphorus also significantly increased individuals RMR (ANCOVA,  $F_{(3,49)}=59.9$ ;  $p<0.001$ ). Mass-specific RMR at  $100\mu\text{g}$  shifted from  $0.026\mu\text{W}.\mu\text{g}^{-1}$  (P+) to  $0.032\mu\text{W}.\mu\text{g}^{-1}$  (P-) in treatment with PUFA and from  $0.022\mu\text{W}.\mu\text{g}^{-1}$  (P+) to  $0.034\mu\text{W}.\mu\text{g}^{-1}$  (P-) in treatment with PUFA and HUFA. As reported for growth rates, complex diets exhibited intermediate value with  $0.045\mu\text{W}.\mu\text{g}^{-1}$  for the mixed diet and  $0.031\mu\text{W}.\mu\text{g}^{-1}$  for the natural seston.



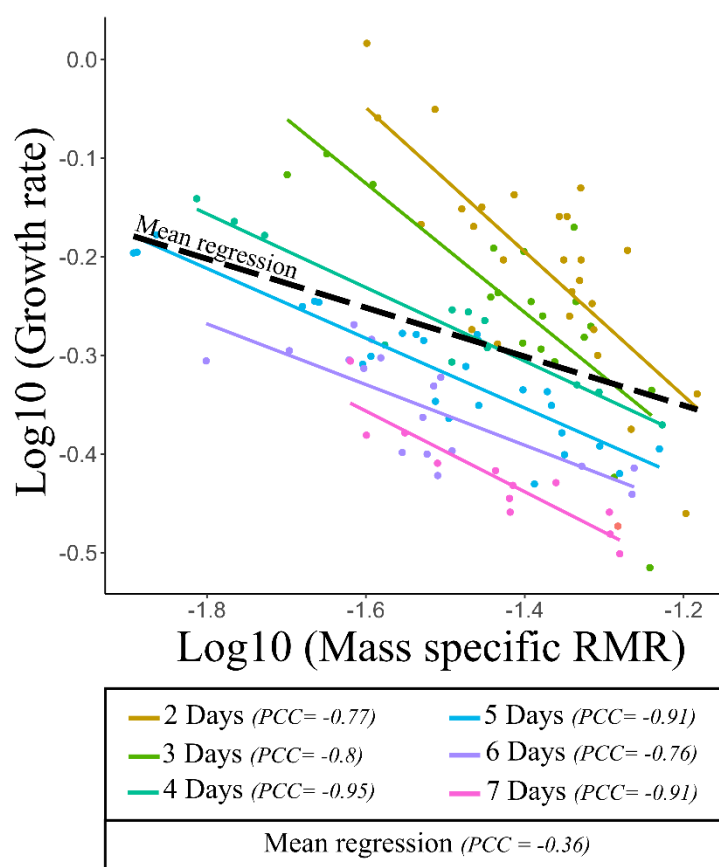
**Figure 10:** Individual growth and resting metabolic rate measured along dietary gradient. Left side panels show the evolution of individuals body mass versus age depending on dietary treatments (A) and the corresponding growth rate at maturity (B). Right side panels show the resting metabolic rate (RMR) of individual depending on their body mass and diet (C), the coefficients defining the RMR scaling (D) and the mass-specific RMR of an individual of 100µg (E). In each panel, colors represent dietary treatments. Error bar on boxplot present 95% confidence interval.

In addition to the direct effect of dietary quality on individual RMR, we also reported a significant variation of the mass-specific RMR scaling during ontogeny in response to dietary quality (ANCOVA,  $F_{(9,110)}=6.9$ ;  $p<0.001$  **Figure 10.C**). Here, the worst diet composed of *Synechococcus sp.* with 2µg.mgC<sup>-1</sup> cholesterol exhibited a scaling exponent (coefficient *b*) of

1 (95% CI [0.85; 1.21]) which is the highest value reported. The successive supplementation of *Synechococcus sp.* with cholesterol slightly reduces the value of  $b$  which, however, remains not significantly different from 1 at  $4\mu\text{g}\cdot\text{mgC}^{-1}$  and  $8\mu\text{g}\cdot\text{mgC}^{-1}$  cholesterol supplementation and remain close to 1 at  $12\mu\text{g}\cdot\text{mgC}^{-1}$  cholesterol supplementation (95%CI [ 0.65; 0.95]). The treatments composed of *Chamydomonas rainhardtii* which contains PUFA exhibited  $b$  value of 0.65 (95%CI [0.57; 0.72]). The lowest value for  $b$  was 0.46 (95% CI [0.33; 0.59]) and was reported for daphnids fed on *Cryptomonas sp.* (**Figure 10.D**). The values of coefficient  $b$  starting at 0.46 for individuals fed with *Cryptomonas sp.* and approaching 1 for individuals fed on *Synechococcus sp.* suggest that the scaling of RMR versus body mass tends toward isometry when food quality decline.

#### 4.4.4 Individual RMR and growth rate:

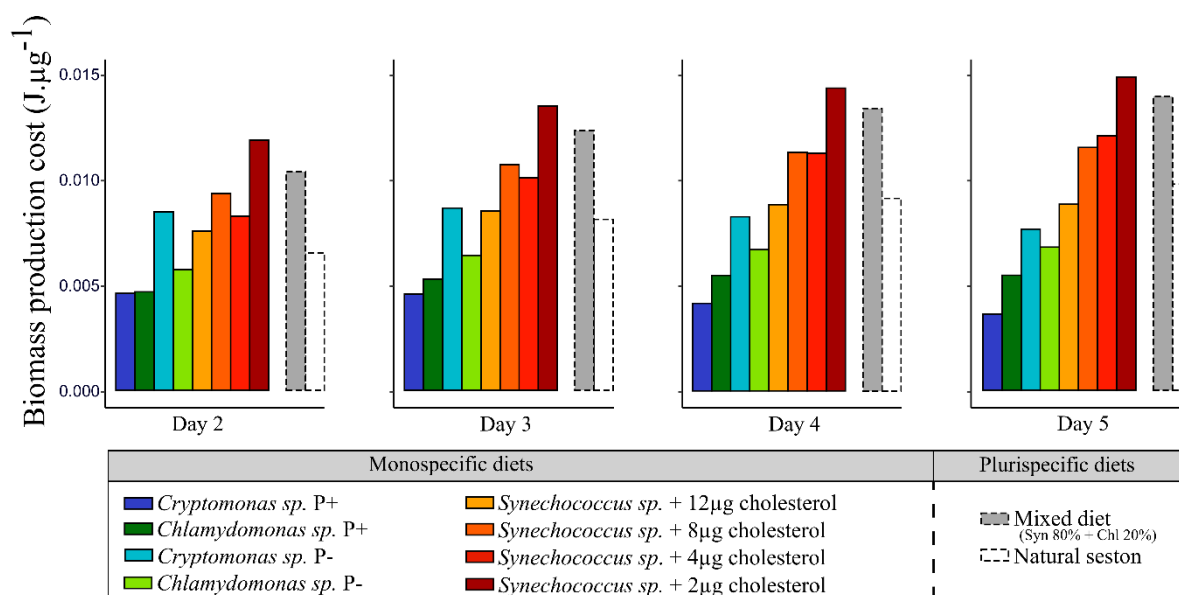
The correlations between RMR, induced by dietary quality, and growth were assessed daily all along ontogeny. Here, all dietary treatments were pooled to provide a RMR-growth correlation regardless of dietary intakes. In addition, RMR values were reported as mass specific RMR to allow comparison between individuals of different body sizes. Along ontogeny, the Pearson Correlation Coefficient (PCC) linking RMR to growth at each day remains systematically lower than -0.76 showing that RMR and growth were tightly negatively correlated (**Figure 11**). The strength of the correlation between RMR and growth reported here across all dietary treatments showed that this correlation was comparable regardless of the limiting element or compounds in food.



**Figure 11:** Mass-specific RMR versus individual growth rate correlation at different age during ontogeny. All dietary treatments are grouped to show the effect of diet on RMR-growth correlation for individuals of a comparable age. Data is log10 transformed for better readability. The Pearson correlation coefficient (PCC) is given for each day and reflect the correlation between mass-specific RMR and growth.

#### 4.4.5 Integration of energy dissipation versus biomass production:

Based on our daily measurement of growth and RMR we modelled the relation between energy dissipated through RMR versus biomass increment of individuals along ontogeny to estimate the Biomass Production Cost (BPC). BPC reflects the total of energy dissipated through RMR from daphnid birth to age at time  $t$  divided by individual biomass increment over the same period. Here we show that, after 5 days, the BPC can be up to 400% higher for individuals fed with the worst quality diet, *Synechococcus sp.* ( $0.015 \text{ J} \cdot \mu\text{g}^{-1}$ ) in comparison to the one fed with the best quality diet, *Cryptomonas sp.* ( $0.0037 \text{ J} \cdot \mu\text{g}^{-1}$ ) (**Figure 12**). The availability of one single compound, here cholesterol going from  $2 \mu\text{g}/\text{mgC}$  to  $12 \mu\text{g}/\text{mgC}$ , can alone increase the energy dissipated by consumers by almost 170%.



**Figure 12:** Model of biomass production cost (BPC) during ontogeny versus dietary treatments. BPC reflect the gross energy dissipated through RMR from time  $t_0$  (birth) to time  $t$  in Joules (J) divided by individual biomass increment over the same period ( $\mu\text{g}$ ).

#### 4.5 Discussion:

Here, we show that regardless of the biochemical and/or elemental composition of the diet the instantaneous measurement of *Daphnia magna* resting metabolic rate (RMR) determines the strength of the nutritional constraint and accurately predicts individual growth. The cyanobacterium *Synechococcus sp.* which is of very low quality for *Daphnia* due to its deficiency in sterols, polyunsaturated fatty acids (PUFA) and highly unsaturated fatty acids (HUFA), induced the lowest growth rate yet the highest RMR reported here. The stepwise supply of sterols, PUFA and HUFA gradually improved food quality, increasing consumer growth rate and decreasing RMR. From a stoichiometric viewpoint, low food C:P values also enhanced consumer growth while reducing their RMR compared to P-limited (high C:P) diets.

The negative relationship between RMR and growth persisted throughout ontogeny thereby enabling the estimation of the energetic cost imposed by nutritional constraints on biomass production. Here, we define biomass production cost (BPC) as the energy dissipated by RMR per unit of produced biomass during a given time interval. A high BPC reflects high dissipation of energy and low production of biomass while a low BPC reflects the opposite. Our results show that decreasing food quality can drastically elevate individual BPC. After 5 days, BPC can be up to 400% higher for individuals fed with *Synechococcus sp.* (BPC: 0.015 J.μg<sup>-1</sup>) in comparison to those fed with *Cryptomonas sp.* (BPC: 0.0037 J.μg<sup>-1</sup>). A rise of BPC reflects the consequences of decreasing food quality on the energy budget of the organism. Under a resting state, half of the energy used by organisms is dedicated to maintain ionic equilibrium across cellular membranes by fueling membrane transport proteins (NaKATPase, Ca<sup>2+</sup>ATPase,...) (Rolfe and Brown, 1997). The efficiency of such transporters is highly dependent on membrane composition, especially in terms of sterols, PUFA or HUFA (Bell et al., 1986; Pepe et al., 1999). Given that dietary lipid supply (HUFA, PUFA, Sterols) can strongly alter membrane composition (Clamp et al., 1997; Geiser, 1990), low quality diets (in terms of lipids) may thus increase ion leaks and reduce transporter efficiency. Under such circumstances, a higher proportion of the energy budget may be allocated to maintenance and thus dissipated as RMR. This hypothesis is in line with observation on mammals where higher nutritional HUFA intakes have shown to improve mitochondrial membrane functioning and energetic efficiency (Stanley et al., 2012). Regarding stoichiometric constraints, high C:P diets induce an excess in terms of C intake. To maintain tissue homeostasis, excess C must be actively released by the organism through respiration and excretion (Darchambeau et al., 2003; Jensen and Hessen, 2007). Such an active regulation of C body content also comes at the cost of higher energetic requirements for maintenance (Ruiz et al., 2018).

Our results also highlight important differences in the RMR-mass scaling coefficient  $b$  ranging from 1 (*Synechococcus* diets) to 0.45 (*Cryptomonas* P+ diet). These differences likely reflect shifts in the evolution of body mass composition along ontogeny. At the onset of sexual maturity, *Daphnia* incorporates reproductive tissues (ovaries/eggs) in addition to the structural tissue that constitute its body mass. Compared to structural tissues, the production and maintenance of reproductive tissues require considerably less energy *per* unit mass (Kooijman, 2009). Hence, the increasing contribution of reproductive tissues to body mass during ontogeny gradually decreases the whole body mass-specific energy requirements ultimately reducing the scaling of RMR to body mass (coefficient  $b$ ). Our results here support this hypothesis. In the dietary treatments composed of *Synechococcus* *sp.* solely supplemented with cholesterol, reproduction is not possible or very reduced in *Daphnia* (Martin-Creuzburg et al., 2005). In these treatments, *Daphnia* exhibit a nearly isometric RMR-mass scaling ( $b=1$ ) which is what is expected when the observed ponderal growth is only due to an increase of structural tissue (Glazier 2005). To be able to reproduce, *Daphnia* requires HUFA or at least their PUFA precursors (Martin-Creuzburg and von Elert, 2009; Schlotz et al., 2012). In our experiment, *Chlamydomonas*-based diets contain PUFA thereby enabling reproduction, yet to a moderate scale compared the HUFA-rich *Cryptomonas* *sp.*, which usually maximizes fecundity in *Daphnia* (Bec et al., 2006; Denoux et al., 2017; Müller-Navarra et al., 2004). Interestingly, the decrease of observed  $b$  values follows this gradient of fecundity, with the lowest  $b$  found for the *Cryptomonas* P+ diet. This trend suggests that by capturing the qualitative differences in biomass production (structural: reproductive tissues ratio),  $b$  could ultimately constitute a relatively easily measurable proxy for population growth potential.

In conclusion, we provide a first quantification of the energetic cost (in joules) of various nutritional constraints (HUFA, PUFA, Sterols, C:P). We show that in *Daphnia*, the

mass-specific RMR at a given size predicts food quality constraints on individual growth rate. We also show that, when RMR is measured over ontogeny, its scaling with body mass ( $b$ ) may constitute a proxy of food quality constraints on the reproductive potential and thereby population growth rate. Obviously, prior to applying our results in the field, further studies should scale-up our experiments to multiclonal populations while including other environmental factors such as temperature, competition, predation or contaminants potentially affecting RMR. Furthermore, it has to be kept in mind that by increasing in RMR, food quality constraints also increase the organismal oxygen demand. Aquatic animals often face hypoxia, a situation that is expected to worsen due to temperature rises (Coutant, 1990; Jenny et al., 2014; Verberk and Bilton, 2013). Our results suggest that the anticipated decreases in phytoplankton food quality (De Senerpont Domis et al., 2014; Elliott, 2010; Galloway and Winder, 2015; Kwiatkowski et al., 2018) could exacerbate the effects of hypoxic stress in aquatic systems. This is urging ecologists to explore how the combination of temperature, oxygen and nutritional constraints will shape future aquatic communities.



## **Transition :**

Le chapitre 4 a permis de mettre en évidence les liens étroits entre qualité nutritionnelle, taux métabolique au repos et croissance des individus. En effet, nous avons démontré qu'une diminution de la qualité nutritionnelle s'accompagne systématiquement d'une élévation du taux métabolique au repos (RMR) des individus induisant une diminution de leur croissance. La mesure instantanée du RMR individuel pourrait donc constituer une devise commune permettant de quantifier le coût énergétique de la contrainte nutritionnelle. Une telle quantification des contraintes nutritionnelles pourrait permettre l'intégration de la qualité nutritionnelle dans les approches métaboliques visant à prédire la production secondaire et la structure des écosystèmes.

Au-delà de cette conclusion principale, le chapitre 4 souligne également le rôle central joué par l'activité membranaire, notamment le maintien de l'équilibre ionique, dans cette interaction qualité-métabolisme. Ainsi les différences de RMR observées pourraient être liées à une modification de l'efficacité énergétique de l'osmorégulation par les apports alimentaires. Au vu de la nécessité pour les organismes de maintenir une osmolarité stable, une telle modification du coût énergétique de l'osmorégulation pourrait avoir des conséquences drastiques sur les performances des organismes. Dans un contexte de changement global où les phénomènes de salinisation des eaux douces se font de plus en plus courant (Kaushal et al., 2018), la tolérance et la survie des organismes dulcicoles face à ce stress salin dépend directement de leur capacité d'osmorégulation. Ainsi, il semble naturel de s'interroger sur la façon dont l'interaction qualité-métabolisme peut affecter les capacités d'osmorégulation des organismes et leur tolérance face à un stress salin.

Le chapitre 5 de ce manuscrit s'est donc attaché à déterminer comment la réponse à un stress salin pouvait affecter, ou être affectée, par la relation qualité-métabolisme précédemment établie. Dans ce chapitre nous avons donc cherché à comprendre dans un premier temps comment le contexte nutritionnel pouvait affecter la tolérance à la salinité des organismes puis, dans un second temps, les implications au niveau métabolique d'une telle interaction.

**Chapitre 5 : Tolérance au stress osmotique et qualité  
nutritionnelle : des interactions au niveau  
métabolique**

# **Nutritional context modulates the salinity tolerance of freshwater invertebrates.**

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**En préparation**

## **5.1 Abstract:**

Salinization of freshwater systems is increasingly recognized as a serious threat for freshwater animals worldwide. While the effect of saline stress alone on freshwater species are well documented, their interaction with other ecological factors are rarely assessed complicating the anticipation of saline stress consequences in natural habitats. Here we explored the interaction between salinity tolerance and dietary quality on the key aquatic specie *Daphnia magna*. Our results showed that higher dietary quality can increase the salinity tolerance range of daphnids by almost 45%. It appears that, through its effect on the energetic efficiency of osmoregulation, poor dietary quality increases the energetic cost of body maintenance when suffering from saline stress. This higher energetic requirements for body maintenance accordingly reduce the energy allocable to growth and reproduction ultimately reducing consumers performances. Under the effects of global change, freshwater systems regularly suffer simultaneously from salinization and decreasing producer's dietary quality. In such context, the interaction between dietary quality and salinity tolerance should be assessed more systematically to avoid miss-estimation of consumers performances.

## **5.2 Introduction:**

The salinization of freshwater ecosystems has been studied tardily while it threatens ecological diversity, community structure and ecosystem services worldwide (Kaushal et al., 2018). The mechanisms of salinization are diverse, including land clearing, agriculture, resource mining and the use of de-icing salts (Schuler et al., 2019) and every type of freshwater ecosystem has been affected by salinization, from small wetland to large lakes. In most of these freshwater systems, organisms do not have physiological capacities to face high salinity, especially filter feeder zooplankton species (Castillo et al., 2018) which, however, constitute an essential component of freshwater food webs, responsible for transferring energy from primary producers to higher trophic levels. Consequently, it is necessary to identify the ecological impacts of freshwater salinization on organism functioning and life history traits.

Recent studies show that salinization can substantially reduce the abundance of zooplankton grazers such as *Daphnia* (Searle et al., 2016). Indeed, high external salinity lead to Na<sup>+</sup> and Cl<sup>-</sup> accumulation in cells, disrupting the uptake of other essential ions or water. Mechanisms to exclude excess ions from cell should be metabolically expensive and have been suggested to come at the cost of a reduced growth or reproduction and may ultimately lead to the mortality of freshwater species (Herbert et al., 2015). However, studies concerning salinization have especially focused on the single effect of salinity on *Daphnia* growth or reproduction ignoring other ecological factors that may interact with saline stress. An exception is the paper of Hintz et al (2017) which showed that the effects of salinity and predation stress interactively alters zooplankton growth and reproduction highlighting the existence of such interactions. Surprisingly, no studies have been devoted to the links between salinity and food quality at zooplankton level while reasonable hypotheses about their interactions can be drawn. Nutritional studies, especially biochemical approaches, consider the availability of sterols and

polyunsaturated fatty acids (PUFA) in food as major determinants of consumers growth due to their essential role in organisms functioning (Martin-Creuzburg et al., 2009). Indeed, sterols and PUFA are major constituents of cellular membranes, regulating their fluidity and permeability (Clamp et al., 1997). Yet, given that salinity tolerance is tightly linked to the ability of organism to maintain osmotic gradient across these membranes (Gonzalez, 2012), any alteration of their composition may have consequences on consumers response to salinization. Increasing membrane permeability may facilitate the direct transfers of ions in cell increasing the potential accumulation of toxic  $\text{Na}^+$  and  $\text{Cl}^-$  but also facilitating the uptake of other essential ions. However, increasing the proportion of sterols and PUFA in membranes may also improve their functioning, potentially facilitating the deposition of excess ions through active transfers. Consequently, it remains unclear if an increase of sterols and PUFA intakes in diet, and their consequences on membranes composition, can be advantageous or disadvantageous for individuals facing saline stress. Moreover, freshwater salinization and phytoplankton communities are tightly linked on different levels. On the one hand, the underlying mechanisms leading to freshwater salinization (land clearing, urbanization, agricultural practices) also lead to a nutrient enrichment of freshwater system favoring the proliferation of cyanobacteria (Wang et al., 2010), a dietary resource of poor quality for zooplankton due to its lack of sterols and PUFAs (Von Elert et al., 2003; Von Elert and Wolffrom, 2001). On the other hand, salinization of freshwater has been shown as a direct driver affecting the diversity of phytoplankton communities. Increasing salinity tends to impede the development of diatoms which are replaced by species more tolerant to saline stress, especially cyanobacteria (Ballot et al., 2009). Consequently, in natural habitats, organisms exposed to saline stress generally also undergone shifts in dietary quality. In this context, it appears critically important to understand the interaction between these two factors to avoid erroneous prediction of consumers responses to freshwater systems anthropization.

Here we hypothesized that dietary quality may affect the salinity tolerance of organisms, in terms of survival, growth and reproduction, especially due to an increasing cost of osmoregulation for individuals fed with inadequate diets. To test this hypothesis, the aim of our study is twofold. In a first part, we explored the tolerance of *Daphnia magna* to a large salinity gradient under different food conditions to understand the interactive effects of these two factors on individual growth, reproduction and survival. In a second part, we selected three specific salinity (optimal salinity, hypersaline and hyposaline conditions) to explore the metabolic consequences of such saline stress on *Daphnia* physiology. We especially focus on individuals' capacities to maintain a stable NaCl body content as well as the metabolic cost involved by this mechanism.

### **5.3 Materiel and Method:**

#### 5.3.1 Preparation of food suspensions:

We used three phytoplankton species as dietary sources in our experiments. *Synechococcus sp.* a cyanobacteria of poor quality for daphnia growth due to its lack of sterols and PUFA, *Chlamydomonas reinhardtii* a chlorophyceae classically used to grow daphnids and *Cryptomonas sp.* constituting a rich diet due to its content of highly unsaturated fatty acids (HUFA). Each phytoplankton specie was cultured in WC medium (Von Elert and Wolffrom, 2001) in 5L aerated vessels and under constant light. When sufficient biomass was produced, cultures were concentrated and freeze-dried. During the experiment, food suspensions were prepared daily by suspending freeze-dried stocks in Volvic© water. Despite a slight reduction in individual performance, freeze-dried algae were shown to be adequate food for *D.magna* for several generations (Naylor et al., 1993). Moreover, the use of freeze-dried resources was unavoidable to ensure that our salinity treatments did not alter phytoplankton composition during the experiment.

### 5.3.2 *Daphnia* maintenance:

We used *Daphnia magna* as model organisms since their parthenogenetic reproduction allows the exclusion of genetic variability. The stock cultures of daphnids were kept in Volvic water© at 20°C on a 14:10h day:night cycle and fed with *Chlamydomonas reinhardtii* (3mgC.L<sup>-1</sup>) for more than 3 generations.

### 5.3.3 Experimental procedure:

#### 5.3.3.1: Salinity tolerance:

To explore the salinity tolerance of daphnids depending on dietary treatments we exposed a clonal line of *Daphnia magna* to a combination of two dietary and salinity treatment. Daphnids were bred on a combination of two food sources constituting a rich diet (*Cryptomonas sp.*) or a poor diet (mixed diet: 70% *Synechococcus sp.* + 30% *Chlamydomonas sp.*) and 12 different concentrations of NaCl (0, 0.07, 0.17, 0.35, 1, 2, 2.5, 3, 3.5, 4, 4.5 and 5g.L<sup>-1</sup>). At the beginning of the experiment, 10h old neonates were randomly distributed into 3 replicate glass jar (250mL) per treatments. The breeding medium was constituted of Volvic© water (adjusted to each salinity by NaCl adjunction or dilution with deionized water) and ad-libitum food supply (6mg dry-weight.L<sup>-1</sup>). This medium was renewed daily to maintain optimal food quantity and stable salinity during all the juvenile growth phase of daphnids. During daphnids growth, survival rate in each replicate was measured daily. When daphnids reached maturity, the experiment stops, and individuals were pooled by jar. Each of these pools were used to determine reproductive rate (numbers of egg by female), then dried at 60°C during 48h and weighted (Sartorius-ME36S; Germany; precision:0.001mg) to determine their growth rate at maturity. The average growth rate in each jar was calculated as follows:

$$\text{Growth rate} = \frac{\ln(W_t) - \ln(W_0)}{t} \quad \text{eq.13}$$

where  $W_t$  is the average weight (µg) at age of maturity  $t$  (days) and  $W_0$  the average weight (µg) of neonates.

### *5.3.3.2: Metabolic implications:*

To define the metabolic response of daphnids to saline stress we selected three specific salinity (0.017g, 1g and 3.5g NaCl.L<sup>-1</sup>), based on the result of part 1, providing a control salinity treatment (1gNaCl.L<sup>-1</sup>), an hyposaline stress (0.017g NaCl.L<sup>-1</sup>) and an hypersaline, but sublethal, stress (3.5g NaCl.L<sup>-1</sup>). Daphnids were grown on two dietary treatment on each of these salinities, a rich diet (*Cryptomonas sp.*) and an intermediate diet (*Chlamydomonas sp.* 70% + *Synechococcus sp.* 30%). We choose an intermediate diet rather than a poor diet in this section because technical issues require sufficient growth to assess metabolic rate and NaCl content of individuals. Growth experiment was similar as described in part 1 at the difference that we used 4 replicates. When daphnids reaches maturity, they were individually submitted to a measurement of their metabolic rate (see Ruiz et al. 2018) and weighted. A subset of organisms was pooled by jar, freeze-dried, and submitted to the measurement of their NaCl content.

### 5.3.4 *Daphnia NaCl measurements:*

Freeze-dried daphnids were crushed and suspended in pure water. The resulting solution was used to determine the sodium concentration by atomic absorption spectrophotometry (Perkin Elmer Analyst 100) and chloride concentration by ionic chromatography (Dionex 4500i equipped with a Ion Pac AS4A column). The total NaCl content of daphnids was then related to the initial freeze-dried body mass.

### 5.3.5 *Data analyses:*

To assess the survival of daphnids to salinity we calculated the NaCl concentration where half the population died after 96h. Unlike most toxicant, daphnids response to salinity is unimodal. To assess both the hyposaline and hypersaline stress we thus statistically analyzed each section of the curve individually. In each case, we used the R package “MASS” to fit a



logistic regression to our data and extract the NaCl concentrations (and associated CI<sub>95%</sub>) inducing 50% mortality. We concluded to significance differences in salinity tolerance in response to dietary treatment in the absence of overlapping of CI<sub>95%</sub> between treatments.

Growth and fecundity responses to salinity were analyzed with a gradient approach. We first LOG transformed the salinity data (x-axis) which erased the asymmetry of the growth response thus facilitating readability and model fitting. We then fit a modified gaussian to our data as:

$$\mathbf{Variable} = \mathbf{a} * e^{-1 * \frac{(\mathbf{Salinity} - \mathbf{b})^2}{\mathbf{c}^2}} \quad \mathbf{eq.14}$$

Where the *variable* is growth rate or fecundity, *Salinity* the concentration of NaCl in g.L<sup>-1</sup>, *a* the proportionality constant defining the higher level of the curve, *b* is the position on x axis where y is maximized and *c* the parameter defining the slope of the curve around *b*. Biologically, *a* define the maximal growth rate reached at optimal salinity thus characterizing the effect of dietary treatment, *b* constitute the optimal salinity and *c* the decrease speed of the curve when salinity derive from its optimum. Assessing the dietary-salinity interactive effect can be done by comparing these decrease speed (i.e. *c* parameter). A similar *c* between treatments suggests no interactive effects while a significant variation of *c* confirms that a same variation of salinity induces differential response of growth, confirming an interactive effect between diet and salinity. The various parameters (*a*, *b* and *c*) were estimated by nonlinear least squared regression. Comparison between dietary treatments were performed by comparing CI<sub>95%</sub> of each parameter.

For the second experimental part, the metabolic rate of individual where mass-corrected, with previously determined metabolism-mass regression for each dietary treatment, in order to exclude any effect of mass. We first log transformed our data to meet the ANOVA

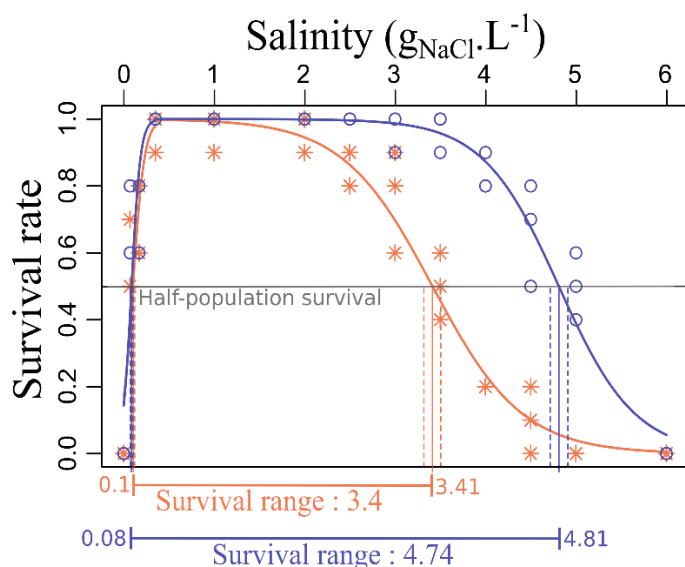
assumption of normality and homogeneity. NaCl content was analyzed through a two-way ANOVA analysis followed by TUKEY HSD test to find significant differences induced by salinity and dietary treatment. Metabolic rate was analyzed in two steps, we first applied a two-way ANOVA to our data to test the effect of dietary treatment and salinity interaction on individual RMR. We then test the response to salinity for each dietary treatment independently with ANOVA analysis. All analyses were performed using the software R v.3.6.3 (R-Core Team 2020) with the alpha error set at 0.05.

## **5.4 Results:**

### 5.4.1 Salinity Tolerance:

#### 5.4.1.1 Survival:

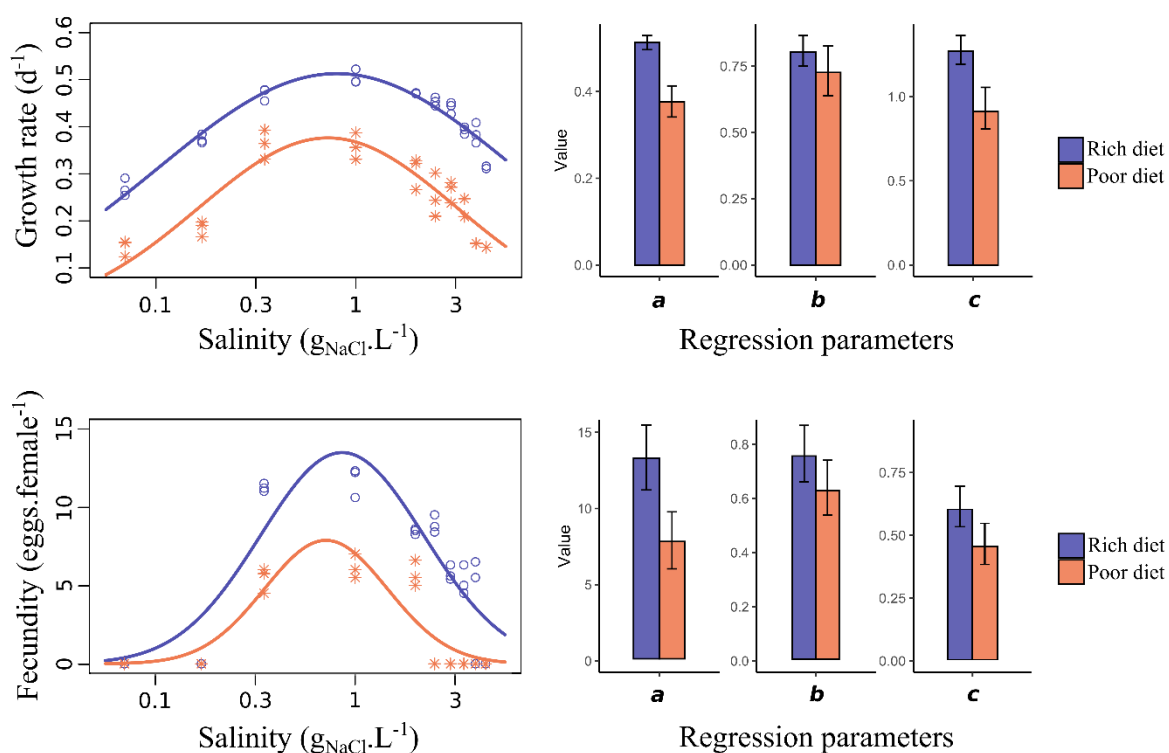
After 96h exposition, the minimal NaCl concentration allowing 50% survival remains similar in both treatment, 0.086g.L<sup>-1</sup> (+/- 0.013) and 0.102g.L<sup>-1</sup> (+/- 0.015) for the rich and the poor diet respectively. Conversely, the higher salinity level allowing 50% survival appears significantly different between dietary treatments. Daphnids fed with the rich diet exhibit a LD50 at 4.91g.L<sup>-1</sup> (+/- 0.09) which is significantly higher than daphnids fed with the poor diet exhibiting a LD50 at 3.41g.L<sup>-1</sup> (+/- 0.09) (**Figure 13**). Thus, the range of salinity allowing 50% survival is 42% higher for well-fed daphnids after 96h exposition (**Figure 13**).



**Figure 13:** *Daphnia magna* survival after 96h exposition to salinity gradient. Blue circles are replicates fed with rich diet and orange crosses are replicates fed with poor diet. Each replicate constitutes the survival rate of 10 individuals. Lines are logistic regressions used to calculate NaCl lower and upper concentration allowing 50% survival and defining the survival range.

#### 5.4.1.2 Growth:

The first thing to note is that both for rich and poor diet quality, the modified gaussian model adjusted to our data fit efficiently and confirms the unimodal response of individual growth. The significant variation of parameter  $a$  between treatments confirms the effect of dietary quality on individuals growth which reach a maximum of 0.51 (CI<sub>95%</sub> [0.49; 0.53]) for the rich diet and 0.38 (CI<sub>95%</sub> [0.34; 0.41]) for the poor diet. This maximum growth rate in each treatment is reached at a same optimal salinity as revealed by the parameters  $b$  which is not significantly different between dietary treatment. The rich diet presenting an optimum at 0.8g.L<sup>-1</sup> NaCl (CI<sub>95%</sub>[0.75; 0.87]) and the poor diet at 0.72g.L<sup>-1</sup> NaCl (CI<sub>95%</sub>[0.64; 0.83]) (Figure 14).



**Figure 14:** Growth and Fecundity of daphnids exposed to a salinity gradient. Blue circles are replicates fed with rich diet; orange crosses are replicates fed with poor diet. Each point constitutes the mean observed value for a replicate of 6 individual. X-axis is log-scaled to facilitate readability. Parameters given in right panels are estimated by plotting eq.14 to our data. Error bars are  $CI_{95\%}$  for each parameter.

Concerning dietary-salinity interaction it appears that the parameter *c* presents significant differences between the two treatments being 1.27 ( $CI_{95\%}$  [1.192; 1.363]) for the rich diet and 0.91 ( $CI_{95\%}$  [0.8; 1.05]) for the poor diet (**Figure 14**). Attention is required since, due to the equation of the fitted model, the higher is *c* the flatten in the response. Thereby here, the growth rate of individuals fed with the rich diet appears noticeably less affected by salinity than the growth rate of individuals fed with the poor diet.

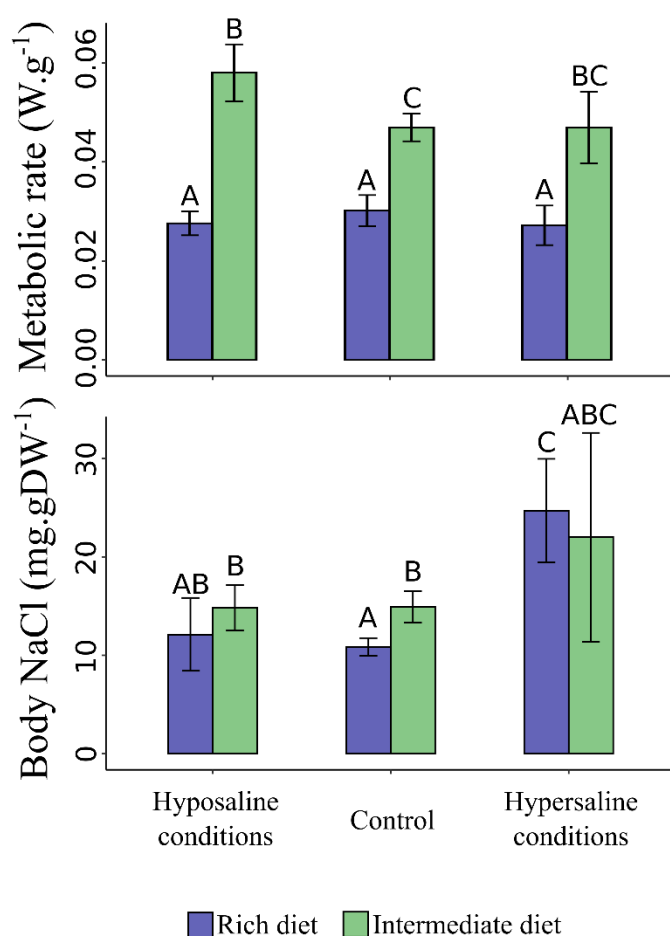
#### 5.4.1.3 Fecundity:

Fecundity responds to saline stress in a same fashion that growth. Dietary quality directly constraints the maximum fecundity (i.e. **a** parameter) reached during the experiment with an average 13.5 eggs per female (CI<sub>95%</sub> [11.3; 15.7]) in the rich diet and 7.9 (CI<sub>95%</sub> [6; 9.9]) in the poor diet (**Figure 14**). As noted for growth rate, the optimal salinity (**b** parameter) is not significantly different between diets, nor with the optimum noted for growth. Optimal salinities are 0.86 (CI<sub>95%</sub> [0.75; 0.99]) and 0.72 (CI<sub>95%</sub> [0.61; 0.84]) for the rich and poor diet respectively. Conversely to growth, the parameters **c** for fecundity presents slight, but not significant, differences between treatments being respectively 0.578 (CI<sub>95%</sub> [0.51; 0.67]) and 0.435 (CI<sub>95%</sub> [0.37; 0.52]) for the rich and the poor diets (**Figure 14**). This result suggests the absence of interaction between diet and salinity on fecundity but should be taken carefully regarding the high variance of fecundity measurements.

#### 5.4.2 Metabolic implications:

##### 5.4.2.1 Metabolic rate:

It appears that the dietary-salinity interaction significantly affect individual metabolic rate (ANOVA,  $F_{(1;24)}=3.94$ ;  $p<0.05$ ). In the rich diet salinity does not induce significant variations of metabolic rate (ANOVA,  $F_{(2;11)}=1.54$ ;  $p>0.05$ ). However, for the intermediate quality, metabolic rate is significantly affected by salinity (ANOVA,  $F_{(2;11)}=4.27$ ;  $p<0.05$ ). In detail, metabolic rate significantly increases from control to hyposaline conditions (Tukey test,  $p<0.05$ ). In hypersaline conditions, despite a slight increase of metabolic rate, the important increase of inter-individual variability erase the significance of this variation (Tukey test,  $p<0.5$ ) (**Figure 15**).



**Figure 15:** *Physiological responses of Daphnids in term of metabolic rate and NaCl body content after exposition to control, hyposaline or hypersaline conditions during all their juvenile phase. Error bars presents standard errors and letters constitutes significantly different groups.*

#### 5.4.2.2 NaCl body content:

ANOVA analysis showed that water salinity significantly constraints the NaCl body content of daphnids ( $F_{(2,14)}=9.25$ ;  $p<0.005$ ). Yet, only hypersaline conditions induced differences in individuals NaCl body content. Here again, a higher interindividual variability is observed at high salinity. In that case, for individuals fed with intermediate diet, this variability partly erased the significance of the differences noted in NaCl body content which, however, increased by up to 50%. Individual from control treatment and hyposaline conditions kept a similar NaCl content. Despite a slight difference in the NaCl body content of individual in response to diet in the control treatment, dietary quality has generally not significant effect on NaCl content of individuals (ANOVA;  $F_{(1,15)}=0.56$ ;  $p>0.45$ ) (**Figure 15**).

## **5.5 Discussion:**

The effect of food quality on salinity tolerance of zooplankton consumers has rarely been assessed. However, it is increasingly clear that anthropization of freshwater systems affected both these factors in parallel. First, a common observed response of freshwater to human activity is nutrient enrichment, which lead to eutrophication, and ultimately tends to favor the proliferations of cyanobacteria over diatoms or green algae (Fastner et al., 2016; Riedinger-Whitmore et al., 2005). Cyanobacteria constitute a poor dietary resource for consumers strongly constraining their performances and life traits (Von Elert et al., 2003). Second, recent studies highlight the *freshwater salinization syndrome* which become a major ecological problem, not limited to estuaries or coastal aquatic system, as it was initially expected, but also importantly affecting inland waters (Kaushal et al., 2018). The rapid salinization, induced by human activity, of inland water bodies can importantly constraint the performances of organisms which are not adapted to face such stress (Castillo et al., 2018). In additions, it has also been showed that salinization of freshwater systems alters phytoplankton communities especially shifting species from diatoms and chlorophytes to cyanobacteria (Ballot et al., 2009). Given this context, and the increasing recognition of the freshwater salinization syndrome, it appeared necessary to understand how nutritional context may affect organisms' salinity tolerance.

Here we showed that nutritional context significantly constraints the salinity tolerance of *Daphnia magna*. The first consequence of nutrition-salinity interaction is the survival rate of daphnids exposed to saline gradient. The survival range, defined as salinity range allowing at least 50% survival, appears up to 45% higher for individuals fed with a rich diet. This difference, almost entirely explained by the shift of the upper tolerance limits of individuals, suggests that the effects of nutrition are particularly important in high salinity conditions.

Besides consequences on individual survival, the combined effects of nutrition and salinity also constraint organisms' growth and fecundity inside of the survival range defined above. First, regardless of water salinity, the effect of dietary quality on individual's growth and fecundity is significant. Individuals fed with the rich diet exhibiting, as expected, higher growth and fecundity rate than individuals fed with poor diet. Similarly, the effect of salinity alone significantly affects consumers performances (i.e. growth and fecundity) which presents an optimum below and above which the performance decrease. Yet, integrating nutritional and salinity contexts revealed that, while the salinity allowing optimal performance remains unchanged by nutritional context, the declining speed of performance around this optimum depends on dietary quality. It appears that the growth and fecundity of individuals fed with poor diet reduce slightly faster than for well-fed individuals when salinity derives from the optimum, highlighting an interactive effect of salinity and nutrition on consumers performances. Yet, in our experimental conditions, this interactive effect on growth and fecundity remains reduced in comparison to the direct additive effects of nutrition and salinity taken independently. Overall, it appears that the interactive effects of nutrition and salinity have important consequences for consumers populations dynamics, mainly due to their strong effects on individual survival.

A step further to understand the interaction between nutrition and water salinity is to understand how nutritional context can affect the underlying mechanisms involved in osmoregulation. A key element controlling organisms' osmoregulation is the active regulation of ionic equilibrium across cellular membrane (Shaw, 2012). However, it is well recognized that nutritional constraints, especially in terms of biochemical compounds such as polyunsaturated fatty acids, have important consequences on membranes functioning (Clamp et al., 1997; Stanley et al., 2012) potentially affecting the ability of organisms to maintain ionic



gradient through these membranes. Here we showed that, regardless of dietary treatment, organisms can keep a constant NaCl body content when water salinity drop from control treatment to hyposaline conditions. Conversely, however, increasing salinity from control to hypersaline conditions led the NaCl body content of organisms to rise, underlying the inability of these freshwater specie to cope with an excess water salinity. While the effect of dietary quality had no significant effect on the NaCl body content of organisms, their metabolic rate is differentially affected. Overall, metabolic rate tends to increase in hyposaline condition for individuals fed with the intermediate dietary quality. In that case, the increased metabolic rate is paired with the strict maintenance of NaCl body content of organism. The link between these two processes suggests that the maintenance of strict osmoregulation in hyposaline conditions had an important energetic cost for individuals which reflects on their metabolic rate. However, interestingly, individuals fed with a rich dietary quality maintain a strictly similar NaCl body content without increasing their RMR. This can potentially be explained by a better membrane functioning in well-fed individuals facilitating active transfers of ions, ultimately increasing the energetic efficiency of osmoregulation (Stanley et al., 2012). This result showed that the energetic cost required to maintain a similar degree of osmoregulation is highly dependent of dietary quality, an increasing proportion of polyunsaturated fatty acids facilitating the process.

In hypersaline conditions, no significant variations of metabolic rate were noted but the intra-group variance slightly increased. Yet, interestingly, this increase of metabolic rate variance is tightly paired with an increased variance in NaCl body content. This may suggest a similar correlation between metabolic rate and NaCl body content than the one observed in hyposaline conditions. However, this increased variance may also be used as an indicator of system instability. Indeed, as suggested by (Carpenter and Brock, 2006), the increase of variance in ecological system can be seen as an early warning signal before breakdown. The variance of numerous ecological processes has been shown to gradually increases with system

instability up to the breaking point (Beck et al., 2018; Carpenter et al., 2011). The application of this theory to our data suggests that, in hypersaline condition, individuals are unable to regulate their NaCl body content leading to an important stress resulting in the increased variance reported. Interestingly, it appears that the intra-group variance is markedly higher for individuals fed with intermediate diet in comparison to the rich diet. Following the early warning signal theory, this shows that, at similar salinity, individuals fed with a poorer diet had a greater instability. This greater instability suggesting that they are closer the breaking point than individuals fed with a richer diet. Thus, while freshwater organisms cannot cope with an excess water salinity, a better dietary intake seems to increase their tolerance to higher NaCl body content. This conclusion strikingly matches with the dietary-induced differences of individual survival noted above in hypersaline conditions and confirm the important dependency of salinity tolerance to dietary status.

In conclusion, we showed that dietary quality and salinity tolerance of freshwater organisms are tightly linked at different physiological levels. First, it appeared that the energetic cost to maintain osmoregulation increases with declining dietary quality. Such interaction results in a faster decline of individual growth when facing non-optimal salinity and poor dietary intakes. Second, we showed that the effect of diet on salinity tolerance is accentuated in conditions where individuals are not able to maintain efficient osmoregulation. In this context, it appeared that a rich diet allows a tolerance to higher body NaCl content, improving the survival rate of organisms, especially in hypersaline conditions. Overall, our study highlights the strong interaction between dietary quality and salinity tolerance, showing that the deleterious consequences of increasing water salinity are accentuated by reducing dietary quality. In natural habitat where freshwater salinization is generally paired with decreasing nutritional quality (Lind et al., 2018; MacLeod et al., 2011), such interactions will strongly constraints consumers populations dynamics and should not be neglected.

## **Chapitre 6 : Discussion générale et Perspectives**

La pression anthropique grandissante exercée sur les milieux naturels requiert plus que jamais une gestion adaptée de ceux-ci afin d'assurer leur pérennité à long terme. Prédire la production secondaire pourrait constituer un proxy important permettant d'anticiper les altérations de la structure et du fonctionnement écosystémique (Dolbeth et al., 2012) et apparaît donc comme un enjeu crucial. Cependant, prédire cette production secondaire peut s'avérer problématique, en particulier à l'interface producteur-consommateur où la qualité nutritionnelle des autotrophes est susceptible de moduler de façon importante les transferts d'énergie vers les niveaux trophiques supérieurs (Brett and Goldman, 1997). Pour surmonter cette difficulté, l'intégration de l'écologie nutritionnelle et de l'écologie métabolique pourrait constituer une avancée importante permettant, à termes, de mieux anticiper les réponses des écosystèmes au changement global (Allen and Gillooly, 2009; Barnes et al., 2018; Hillebrand et al., 2009; Wang and Brose, 2018). A ce titre, ce travail de thèse s'est attaché à explorer les liens existants entre qualité nutritionnelle et taux métabolique des consommateurs. Il a également cherché à comprendre comment des facteurs environnementaux associés au changement global pouvaient affecter ou être affectés par ces liens et les conséquences découlant sur la croissance des organismes.

### **6.1 Synthèse des résultats :**

Avant de chercher à établir des liens entre qualité nutritionnelle et taux métabolique, il semblait essentiel de comprendre comment les besoins nutritionnels des consommateurs (et donc la qualité relative de leurs ressources) pouvaient être affectés par des facteurs environnementaux. A ce titre, le chapitre 2 de ce manuscrit a pu démontrer, via une approche combinant modélisation et expérimentation, la forte dépendance thermique des besoins nutritionnels de *Daphnia*. Sur une large gamme de températures, il est apparu que l'évolution du  $TER_{C:P}$  (ratio nutritionnel C:P optimal) présente une forme en U dont le point de

basculément dépend de l'acclimatation thermique de l'organisme. Ce résultat, au-delà d'harmoniser les observations contradictoires de la littérature (Anderson et al., 2017; Boersma et al., 2016a; Persson et al., 2011; Winder et al., 2016), démontre surtout l'importance que joue le contexte thermique de l'organisme sur ses besoins élémentaires (C, P). En effet, une élévation des températures comparable sur la partie froide ou sur la partie chaude de la niche thermique de l'individu tendrait à augmenter ses besoins en P ou en C, respectivement. Cette conclusion démontre donc l'importance d'intégrer la stœchiométrie écologique et l'écologie thermique afin de pouvoir anticiper l'évolution des besoins nutritionnels des consommateurs dans un contexte de changement global.

Le chapitre 2 semble également démontrer que les besoins nutritionnels stœchiométriques de l'organisme sont largement régulés par ses besoins énergétiques (C). Cette observation suggère donc l'existence d'une interaction directe entre le taux métabolique de l'individu (proxy de son statut énergétique) et la qualité nutritionnelle de ses ressources. La mise au point d'une méthode de mesure individualisée du taux métabolique de *Daphnia* par microcalorimétrie (Chapitre 3) a permis d'explorer les interactions existantes entre taux métabolique des consommateurs et qualité nutritionnelle. En se focalisant dans un premier temps sur une approche stœchiométrique (chapitre 3) puis en intégrant une large gamme de composés biochimiques essentiels (Chapitre 4), nous avons pu démontrer une forte corrélation entre qualité nutritionnelle et taux métabolique, et ce, quelle que soit l'origine de la contrainte nutritionnelle. Il est apparu qu'une diminution de la qualité nutritionnelle induisait systématiquement une élévation du taux métabolique au repos (RMR) de l'organisme. L'élévation du RMR s'est avérée fortement corrélée avec une diminution de la croissance des individus. Cette corrélation négative suggère que l'accroissement du RMR constitue un surcoût énergétique se répercutant sur la croissance des organismes. En d'autres termes, l'élévation du

RMR reflèterait le coût énergétique de la contrainte nutritionnelle et permettrait donc sa quantification en termes d'énergie. Ainsi, le RMR de l'organisme pourrait constituer une devise transversale entre l'écologie nutritionnelle et l'écologie métabolique permettant d'entrevoir leur intégration. De plus, nous avons également démontré ici le rôle central que joue le taux de fécondité de l'organisme sur la relation liant RMR et masse durant l'ontogénie. Ainsi, en intégrant à la fois croissance et reproduction, la mesure du RMR de l'organisme pourrait constituer un proxy direct permettant de prédire la croissance des populations de consommateurs. Cependant, les conclusions avancées ici proviennent d'observations réalisées en microcosmes où les conditions sont strictement contrôlées. En milieu naturel, les facteurs environnementaux pouvant affecter la qualité nutritionnelle d'une part, ou le RMR des consommateurs d'autre part, sont nombreux (température, prédation, polluants, ...). A titre d'exemple, la température, démontrée ici comme modulateur des besoins nutritionnels des organismes ectothermes (chapitre 2), régule également leur taux métabolique et pourrait donc altérer la relation RMR-croissance établie ici. Ainsi, si l'utilisation du RMR comme proxy de la croissance populationnelle semble une perspective prometteuse, elle doit encore être vérifiée à plus large échelle et ses interactions avec divers facteurs environnementaux se doivent d'être explorées.

A ce titre, nous avons ici cherché à comprendre comment le lien RMR-qualité nutritionnelle pouvait être affecté par une salinisation du milieu, qui constitue un problème émergent pour nombre de masses d'eaux continentales (Kaushal et al., 2018). Nous avons démontré (chapitre 5) une interaction forte entre la qualité nutritionnelle et la tolérance des consommateurs à un stress osmotique (hypo- ou hyper-salinité). Le coût énergétique requis afin d'assurer l'osmorégulation semble être à l'origine du lien qualité-salinité observé ici. La diminution du RMR chez les organismes nourris avec une qualité nutritionnelle supérieure

dénote d'une meilleure efficacité énergétique des protéines de transferts membranaires. L'efficacité accrue de ces protéines facilite l'osmorégulation pour l'organisme, lui conférant ainsi une tolérance plus importante à la salinité. On constate ici une relation RMR-croissance comparable à celle observée lors de variations de qualité nutritionnelle. L'élévation du RMR s'associe à une réduction de croissance mais aussi à une mortalité plus précoce. De façon plus générale l'élévation du RMR semble donc, ici encore, corrélée à une diminution de la croissance de la population, voire à son déclin.

## **6.2 Discussion générale :**

De manière transversale, ce travail de thèse a démontré l'importance des interactions existantes entre différents facteurs affectant la production secondaire au sein des écosystèmes aquatiques (température, qualité nutritionnelle, salinité). La prise en compte de ces interactions est aujourd'hui nécessaire afin de prédire au mieux la réponse des organismes face aux changements globaux. Pourtant, la fragmentation des théories écologiques en un nombre important de sous-disciplines (écologie nutritionnelle, écologie métabolique, écologie thermique, écotoxicologie, ...) constitue un frein important à la prise en compte de telles interactions (Levin, 1992). Ces difficultés sont à l'origine d'une volonté grandissante d'harmoniser les cadres disparates de l'écologie afin de permettre une vision plus globale (Allen and Gillooly, 2009; Barnes et al., 2018; Hillebrand et al., 2009; Ott et al., 2014). Nous avons ici démontré le rôle central que joue le taux métabolique individuel dans la réponse des organismes face aux contraintes environnementales. L'accroissement systématique du RMR des organismes soumis à divers stress environnementaux semble refléter le coût énergétique induit par ces stress. Ainsi, la détermination du RMR, en constituant une vision globale des besoins énergétiques de l'organisme, pourrait constituer une mesure intégrative permettant de quantifier le coût énergétique induit par les conditions environnementales dans leur globalité.

De plus, les conséquences de ce coût énergétique se reflétant directement sur la croissance, la reproduction ou la survie des organismes, la mesure directe du RMR, ici réalisée par microcalorimétrie, pourrait constituer un proxy intégratif permettant de prédire la dynamique des populations de consommateurs, et donc d'anticiper la production secondaire.

Si cette conclusion semble prometteuse, elle se doit néanmoins d'être observée avec précaution pour plusieurs raisons. D'une part, les résultats présentés ici se basent sur la réponse de *Daphnia magna* dans des conditions strictement contrôlées. En milieu naturel, la présence d'un nombre important de facteurs confondant pourrait brouiller les relations avancées dans cette thèse. D'autre part, ces résultats reflètent les réponses d'une lignée clonale de *Daphnia magna* excluant donc toute variabilité intra et interspécifique. Cette simplification était essentielle afin de comprendre l'effet direct d'un stress sur le RMR d'organismes présentant un taux métabolique maximum comparable. Nous avons ainsi été en mesure de caractériser, de façon simplifiée, le budget énergétique de l'organisme pour comprendre les conséquences des variations de RMR sur la croissance des consommateurs. Néanmoins, en milieu naturel les assemblages de populations sont bien plus complexes et de futures études seront nécessaires afin de comprendre dans quelles mesures variabilité génétique du RMR et variabilité induite par les conditions environnementales peuvent interagir. Plus généralement, les conclusions établies ici ne constituent qu'une première étape vers l'utilisation du RMR comme proxy à la dynamique des populations et se doivent encore d'être testées à plus large échelle, notamment en milieu naturel. Néanmoins, si cette variabilité du RMR en réponse aux conditions environnementales persiste à large échelle, la mesure du RMR pourrait constituer une devise énergétique commune, transversale à de nombreux pans de l'écologie, permettant d'harmoniser ses cadres disparates sous une théorie unifiée.

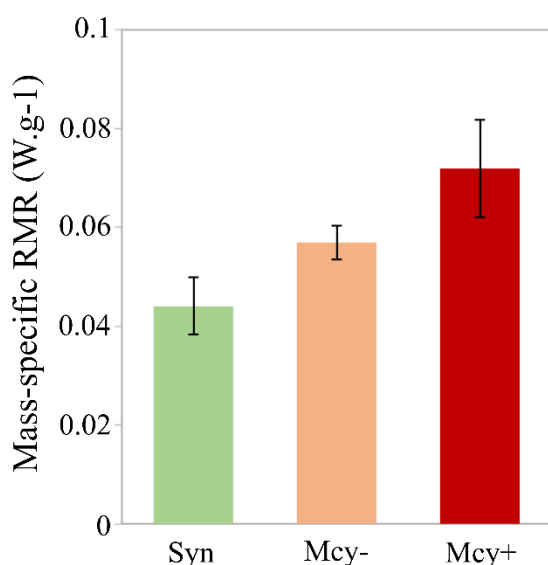


### **6.3 Perspectives :**

A la suite de ce travail de thèse un certain nombre de résultats soulève d'intéressantes perspectives permettant d'améliorer notre compréhension de la réponse métabolique des organismes face à divers stress environnementaux et ainsi d'anticiper la dynamique de leurs populations dans un contexte de changement global.

#### 6.3.1 L'utilisation de la microcalorimétrie comme outil pour étudier la réponse métabolique des ectothermes de taille réduite :

D'un point de vue méthodologique, nous avons, au cours de cette thèse, développé une méthode directe via microcalorimétrie permettant de définir précisément et individuellement le taux métabolique au repos (RMR) de *Daphnia*. Si cette méthode a ici été utilisée afin de déterminer le coût énergétique de contraintes nutritionnelles ou d'un stress osmotique, sa grande précision, en comparaison à des méthodes plus classiques de respirométrie (Walsberg, 2005), et sa capacité à suivre le métabolisme individuel en temps réel pourraient être mises à profit afin de déterminer la réponse métabolique d'invertébrés de petite taille (aquatiques ou terrestres) à une grande variété de facteurs environnementaux. A titre d'exemple, la récurrence de proliférations cyanobactériennes dans les milieux aquatiques fortement anthropisés, au-delà de constituer une ressource nutritionnelle de qualité médiocre, peuvent également induire une toxicité directe pour les consommateurs du fait de la synthèse de cyanotoxines (Ferrão-Filho and Kozlowsky-Suzuki, 2011). Une telle toxicité pourrait se répercuter sur le RMR des organismes et impacter, potentiellement drastiquement, le budget énergétique des consommateurs. Des résultats obtenus en parallèle à cette thèse (**Figure 16**) semblent démontrer un lien direct entre la présence de cyanotoxines et l'élévation du RMR des organismes.



**Figure 16 :** RMR masse-spécifique de *Daphnia* exposée à des cyanobactéries aux niveaux de toxicité différents.

*Synechococcus* sp. (Syn) cyanobactérie non toxique, *Microcystis* sp. cyanobactérie productrice (Mcy+) ou non (Mcy-) de cyanotoxines. Les barres d'erreur représentent l'écart type.

Bien que ces résultats ne soient que préliminaires ils laissent présager, une nouvelle fois, que la prise en compte du RMR des organismes pourrait permettre de quantifier le coût énergétique induit par cette toxicité.

### 6.3.2 Les effets de la température sur la relation Qualité nutritionnelle - RMR :

Au cours de cette thèse nous avons démontré, d'une part la forte dépendance thermique des besoins nutritionnels des consommateurs (chapitre 2) et, d'autre part, le lien étroit entre qualité nutritionnelle et RMR des consommateurs (chapitre 3 & 4). Cependant, en milieu aquatique la grande majorité des consommateurs sont des organismes ectothermes dont le taux métabolique est directement contrôlé par la température. Ainsi, les liens qualité nutritionnelle-RMR, observés ici à une température donnée, pourraient être modulés de façon importante dans un contexte thermique différent. Il semblerait donc intéressant d'explorer la dépendance thermique de ces liens et leurs conséquences sur la croissance des consommateurs. De tels résultats pourraient permettre de prédire plus précisément la production secondaire dans un contexte de réchauffement climatique et ainsi de mieux anticiper les conséquences de ce dernier sur la structure et le fonctionnement écosystémique.

### 6.3.3 L'élévation du RMR en réponse à un stress et l'augmentation du risque hypoxique :

La prise en compte du risque hypoxique pour les consommateurs est sans doute l'une des perspectives les plus pertinentes à poursuivre après ce travail de thèse. En effet, nous avons ici démontré que l'augmentation du taux métabolique individuel apparaissait comme une réponse récurrente de l'organisme face aux stress environnementaux (déséquilibre homéostatique, qualité nutritionnelle, stress osmotique). Cette observation soulève une question capitale quant à la capacité des organismes à soutenir une telle élévation de leur RMR. Chez les organismes aérobies, le taux métabolique est directement corrélé au taux respiratoire des individus et donc à leurs besoins en O<sub>2</sub>. Or, si le taux d'oxygène de l'atmosphère est extrêmement stable, il n'en va pas de même en milieux aquatiques (Verberk et al., 2011). Au sein de ces derniers, la disponibilité en oxygène dissout peut fluctuer de façon importante. D'une part, la saturation en oxygène de l'eau, bien qu'elle tende généralement vers les 100%, n'attend pas nécessairement cette valeur, notamment dans les milieux lenthiques présentant un brassage de surface très faible limitant les échanges gazeux avec l'air. D'autre part, une saturation d'oxygène de 100% ne reflète pas nécessairement une concentration similaire dans le milieu. En effet la dissolution de l'O<sub>2</sub> dans l'eau diminue avec la température, réduisant donc sa disponibilité lorsque l'eau se réchauffe (Verberk et al., 2011). Le réchauffement climatique actuel tend donc à réduire la disponibilité en O<sub>2</sub> dans les milieux aquatiques augmentant d'autant le risque d'hypoxie pour les organismes (Blumberg et al., 1990; Verberk et al., 2011). Ainsi, dans un contexte de changement global où les organismes se voient régulièrement soumis à divers stress environnementaux (Woodward et al., 2010), ceux-ci peuvent être forcés à élever leur taux métabolique, et donc leurs besoins en O<sub>2</sub>. Dans le même temps, la disponibilité en O<sub>2</sub> dans le milieu est susceptible de diminuer (Burns et al., 2005), augmentant potentiellement le risque d'hypoxie chez ces consommateurs. Il serait intéressant d'explorer plus en détail le risque hypoxique chez des organismes soumis à diverses contraintes

environnementales afin de mieux comprendre si la disponibilité en O<sub>2</sub> dans le milieu peut limiter leur tolérance à ces stress.

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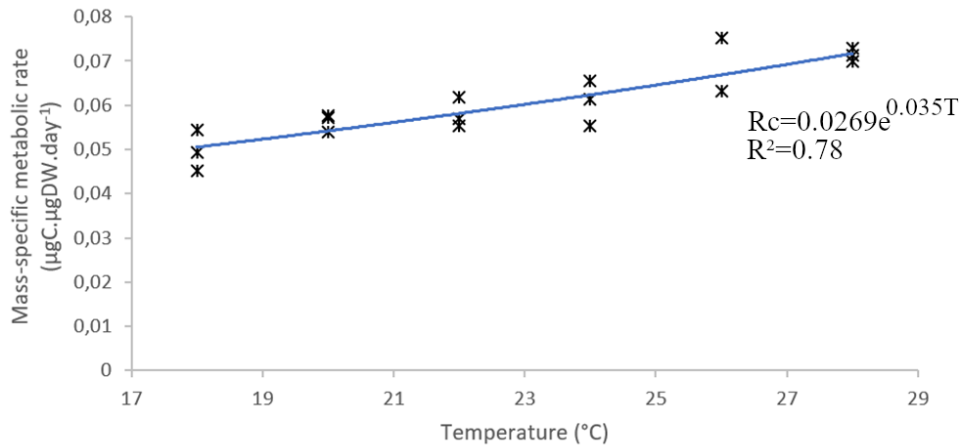
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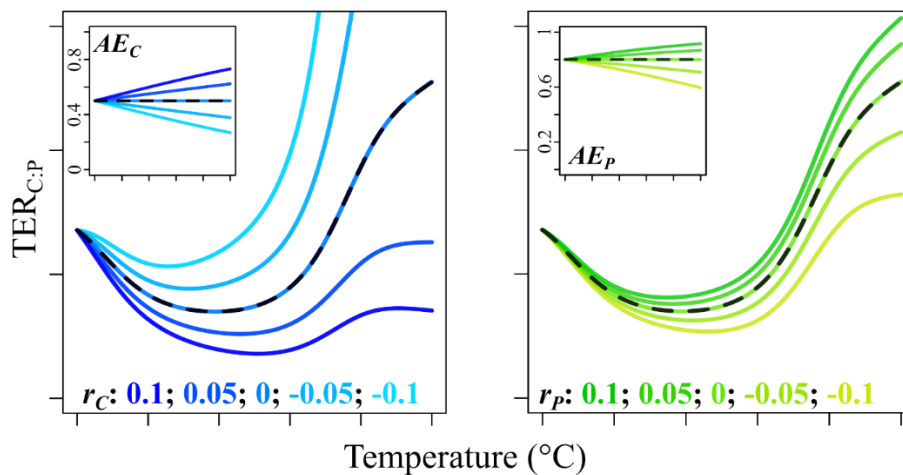
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## Annexes

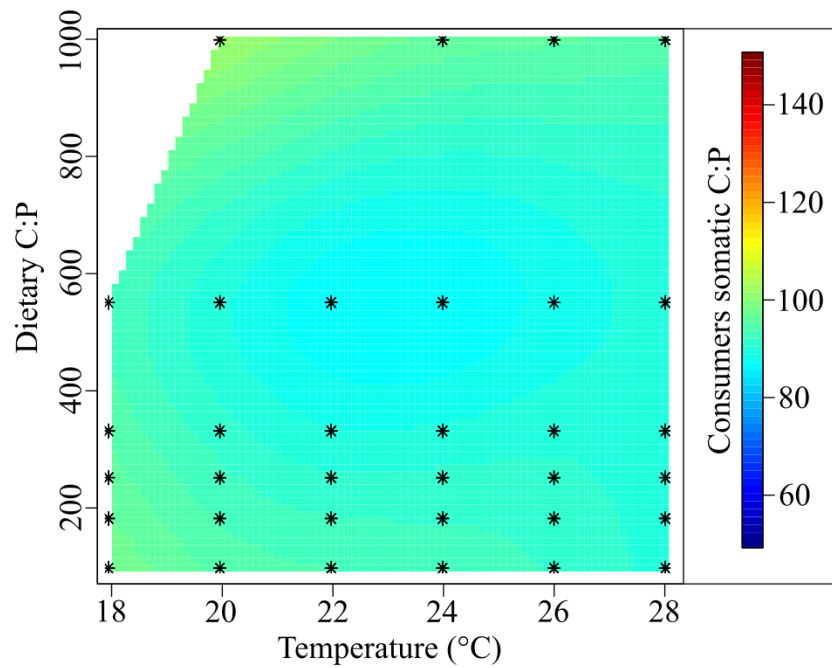


**Figure S1:** Daphnid mass-specific metabolic rate (proxy for respiratory rate) in  $\mu\text{gC} \cdot \mu\text{g dry weight} \cdot \text{day}^{-1}$ . The regression line linking  $R_c$  and temperature is fitted with Eq. 4 and defines the value of the proportionality constant ( $a = 0.0269$ ) and the scaling exponent ( $d = 0.035$ ). The equation fit correctly with data ( $R^2:0.78$ )

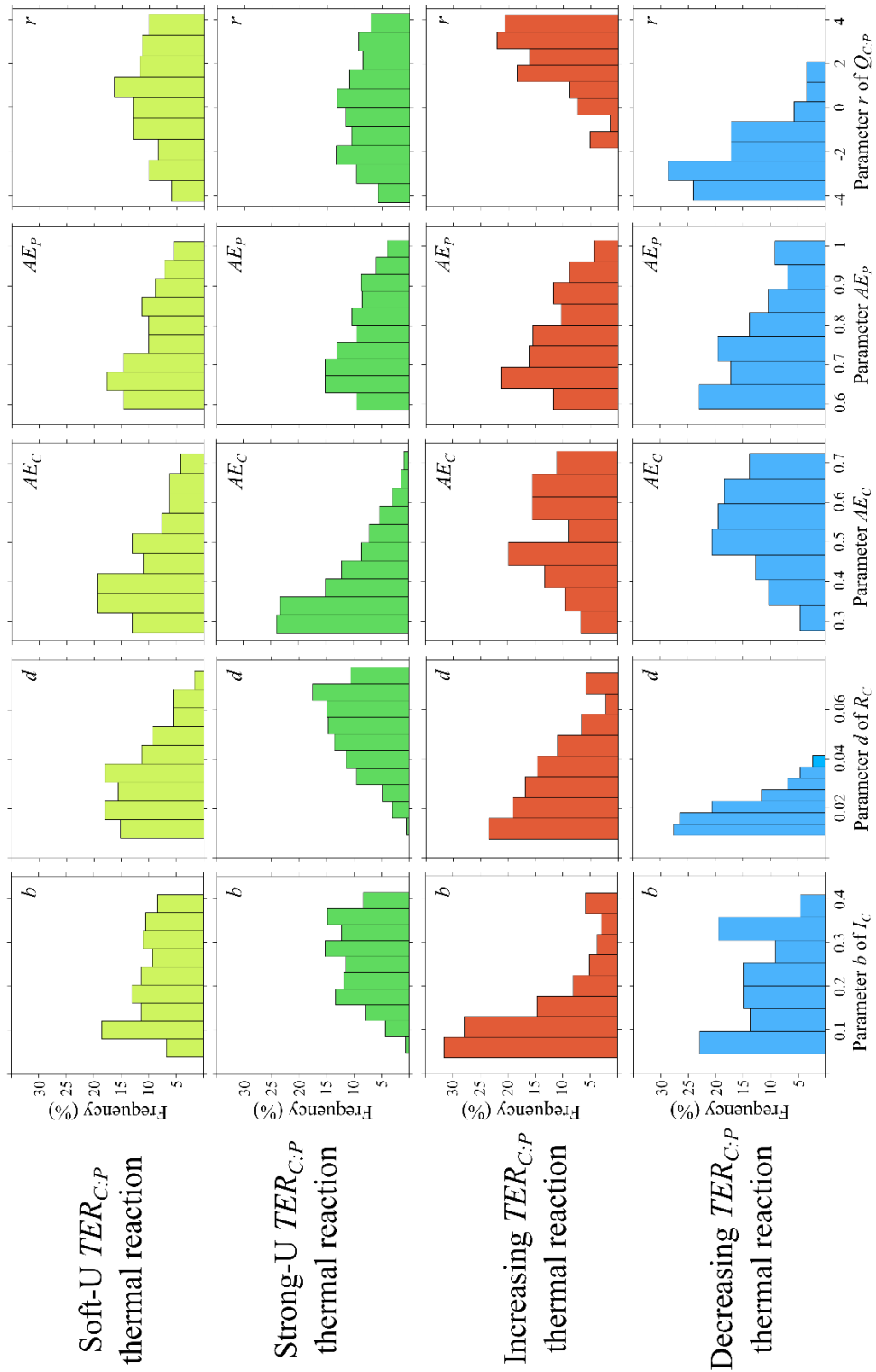


**Figure S2:** Logistic model of  $AE_C$  and  $AE_P$  thermal reaction norms and their consequences on  $TER_{C:P}$  predictions. Logistic responses modelled as:  $AE(T) = \frac{1}{1+(A_0 e^{-r(T-T_{MIN})})}$  eq.15

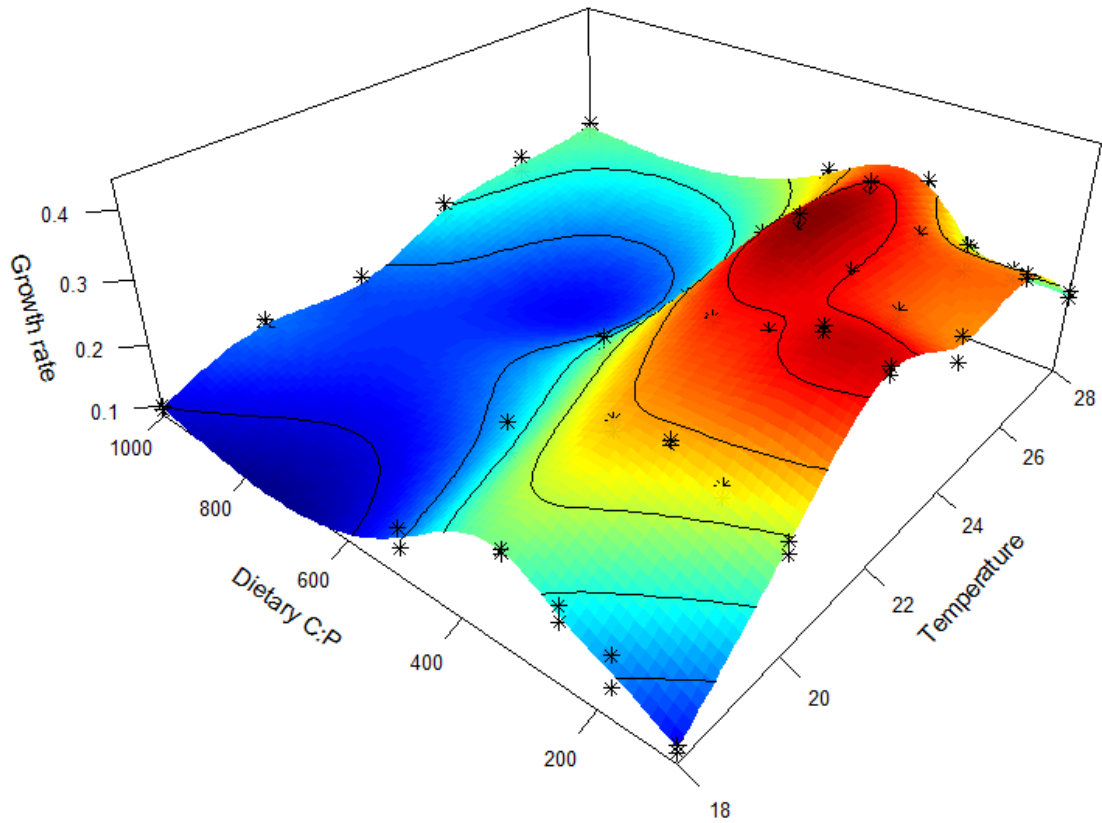
where  $A_0$  define the intercept and  $r$  the slope of the reaction and  $T_{MIN}$  is the minimal temperature of the thermal range observed. Logistic model allows the confinement of Assimilation efficiency within the 0 to 1 range with increasing or decreasing thermal reaction as observed for amphibians or reptiles (Tsai et al. 2008; Bobka et al. 1981).



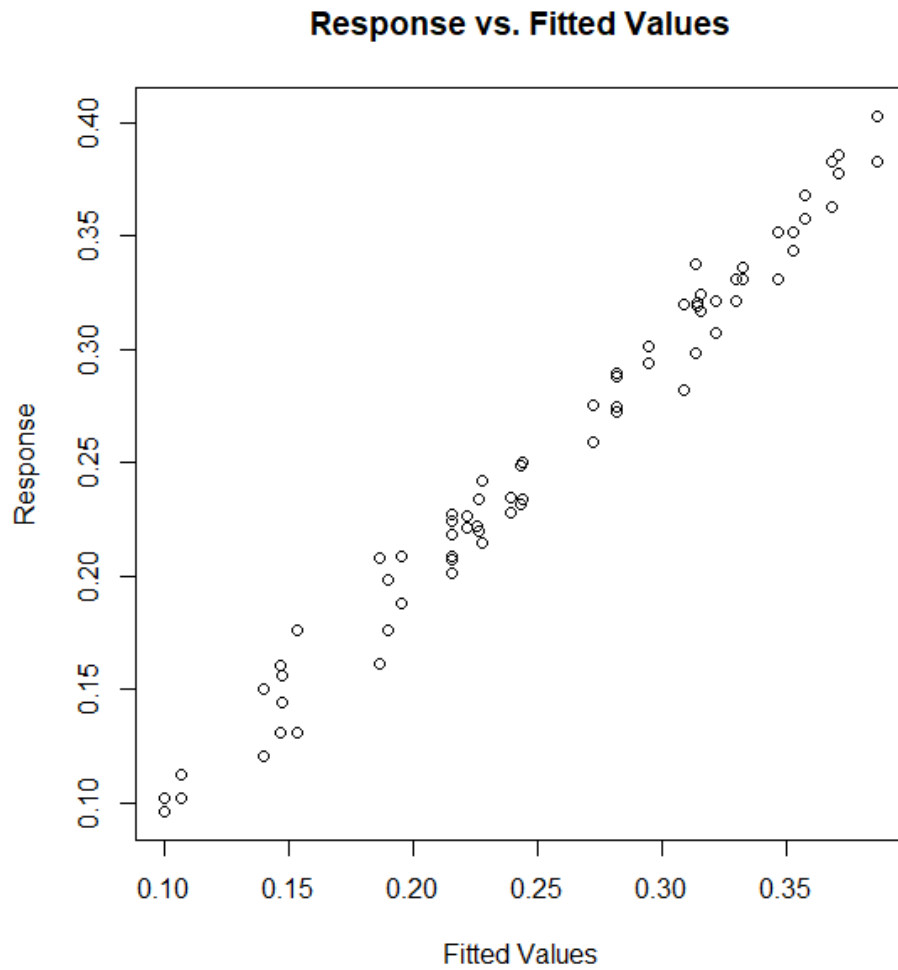
**Figure S3:** *Daphnids* somatic C:P versus dietary C:P and temperature. ANOVA analysis shows no significant changes in the somatic C:P ratio of *daphnids* neither in response to dietary C:P ( $F_{(5,124)}=0.763$ ,  $p=0.384$ ) or to temperature ( $F_{(5,124)}=0.139$ ,  $p=0.710$ ) nor due to the interactive effects of these factors ( $F_{(5,124)}=1.553$ ,  $p=0.215$ ). This result support the constant somatic C:P with temperature assumed in the model. Asterisks indicate the measurements point (measurement performed in triplicates)



**Figure S4:** Distribution of parameters values for each observed pattern of  $TER_{C:P}$  thermal reaction norm (in % of total distribution for each panel). The distribution of parameters creating a soft U-shape  $TER_{C:P}$  thermal reaction appears uniform suggesting that the pattern is not strongly associated with one specific parameter value. Rapid thermal scaling of  $R_C$  (high value for  $d$  parameters) and lower  $AE_C$  tend to result in more convex  $TER_{C:P}$  thermal reaction norm. Increasing  $TER_{C:P}$  thermal reaction arises when  $b$  is reduced (flatter  $I_C$ ) and high values for  $r$  (Increasing  $Q_{C:P}$  thermal reaction norm). Decreasing  $TER_{C:P}$  thermal reactions are associated to lower levels of  $R_C$  (low  $d$  parameter) and low values for  $r$  (decreasing  $Q_{C:P}$  thermal reaction norm).



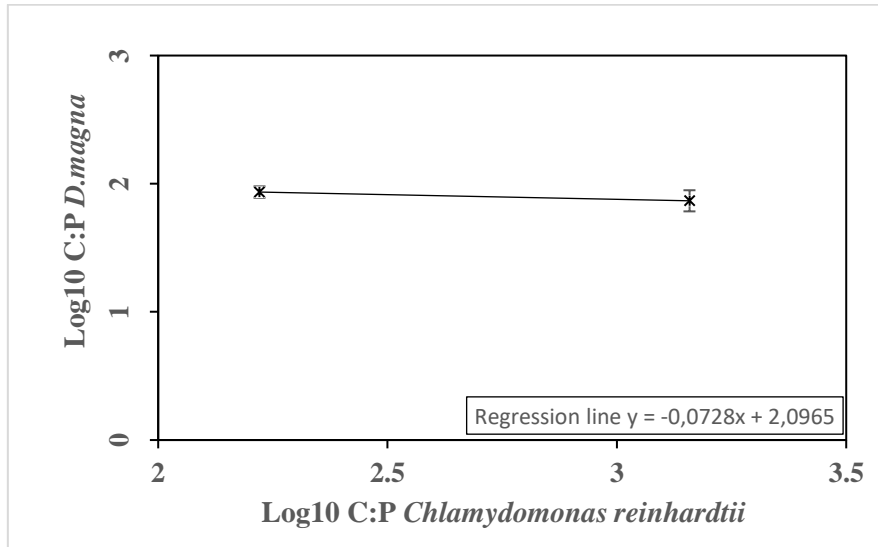
**Figure S5:** Surface response of *Daphnia magna* growth rate versus temperature and dietary C:P. Contour plot generated by generalized additive models and package mgcv on R software. Black crosses are experimental data values.



Basis dimension ( $k$ ) checking results. Low  $p$ -value ( $k$ -index $<1$ ) may indicate that  $k$  is too low, especially if edf is close to  $k'$ .

	$k'$	edf	$k$ -index	$p$ -value
s(Temp)	5.00	4.68	1.36	1.00
s(CP)	5.00	4.77	1.22	0.95
ti(Temp,CP)	25.00	18.31	1.30	1.00

**Figure S6:** Experimental data response versus Fitted value from generalized additive models ( $R^2$ : 0.96) and Gam.check table. The model reaches full convergence confirming its realism. Random distribution of residuals for each smooth term, as revealed by high  $p$ -values, showed that the number of basic function underlying the general model ( $k$ ) is appropriate.



**Figure S7:** Log10 Carbon / Phosphorus ratio of algae (*Chlamydomonas reinhardtii*) versus mean Log10 C/P of daphnids (*Daphnia magna*) across all the experiment duration (9 days). Error bars represent the standard errors of mean between days. Slope of the curve represent the 1/H value (-0.0728 not significantly different from 0).

Stock Algae C/P	Dietary Treatments C/P					
	95	172	250	331	553	1014
95	100%	66%	33%	0%	0%	0%
331	0%	33%	66%	100%	66%	0%
1014	0%	0%	0%	0%	33%	100%

**Table S1:** C:P ratio of dietary treatment and proportions of freeze-dried algal stock mixed for their preparation. Algae stock were grown during 2 months on modified WC medium with 14.2mg.L<sup>-1</sup>, 7.1mg.L<sup>-1</sup> or 0.35mg.L<sup>-1</sup> phosphates (PO<sub>4</sub><sup>3-</sup>) providing the 3 cultures with C:P 95, 331 and 1014 respectively. When enough algal biomass was available for the experiment, cultures were stopped, concentrated and freeze-dried to constitute 3 algal stocks. The non-selective feeding of *D.magna* allows to work with mixed diet without inducing bias.

Fatty Acid	Chlamydomonas P+	Chlamydomonas P-
14: 0	0,21	0,22
16: 0	18,1	22,6
18: 0	2,59	2,89
18: 1n-9	14,43	13,91
18: 2n-6	19,31	18,85
18: 3n-6	4,84	5,19
18: 3n-3	7,88	8,44

**Table S2:** Fatty acids composition of *Chlamydomonas reinhardtii* with (P+) or without (P-) phosphorus. Only main fatty acids are shown, results are given in percentage of total fatty acids.

Number of iterations	n=50		n=250		n=1000		n=2000	
	TER	s.d.	TER	s.d.	TER	s.d.	TER	s.d.
18	340,5	11,8	339,1	11,5	338,4	11,5	338,6	11,2
20	255,3	9,4	256,3	9,8	255,8	9,5	256,3	9,8
22	195,3	18,76	196,1	14,4	193,4	16,8	193,9	15,8
24	275,6	7,97	276,7	7,69	275,9	8,37	275,9	8
26	255,7	9,3	256,4	9,2	255,3	8,9	255,7	9,3
28	420,9	52,4	412,4	40,9	414	43,2	411,4	39,6

**Table S3:** Robustness of TER predictions for the numbers of iteration in bootstrap. TER and standard deviation appear highly robust regardless of the number of iterations of the bootstrap.



*“We did it, we did it, Yeah”*

T.E. Dora