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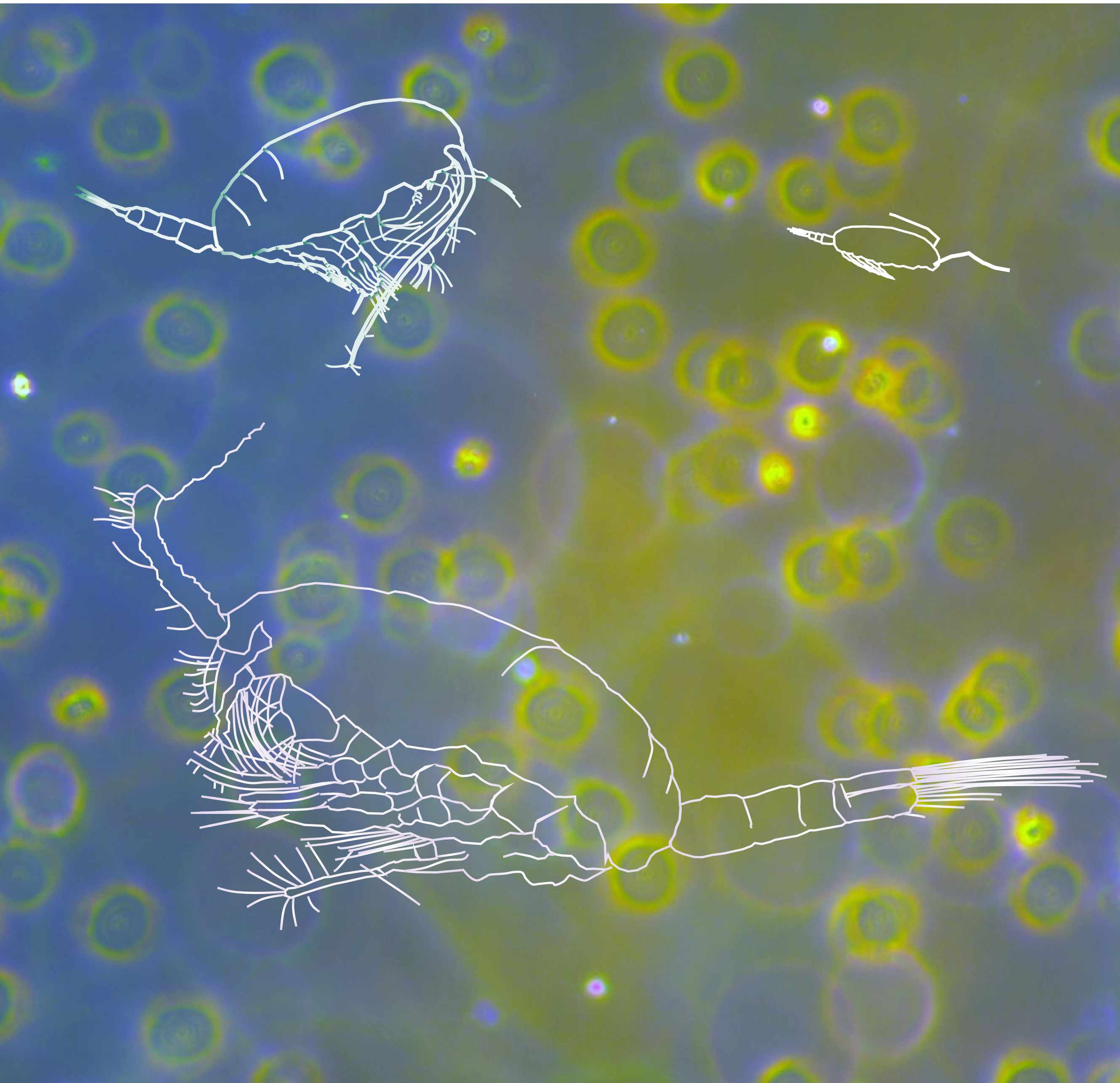
PhD Thesis



Institut de Ciències del Mar
EXCELENCIA SEVERO OCHOA
CSIC CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS
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Thermal compensation in marine copepods: long-term ecophysiological implications in *Paracartia grani*

Carlos de Juan Carbonell

Thesis presented to obtain the Doctoral Degree by the University of Barcelona (UB), Ph.D. Program in Marine Sciences

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A mis abuelas,
Amparo y Tinita

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En Barcelona a 7 de abril de 2024

SUMMARY

Ectothermic animals are highly sensitive to changes in the environmental temperature. As ocean temperatures rise, a major concern is the potential impact of warming on copepods, which are crucial contributors to marine food webs. However, our understanding of how copepods respond to chronic temperature changes and the underlying physiological mechanisms is limited due to a scarcity of long-term studies. This thesis presents a series of experiments investigating the direct effects of temperature on copepod phenotypic traits across multiple generations. We specifically focused on the calanoid copepod *Paracartia grani*, exposed at long-term to warmer conditions (+3°C and +6°C). We explored the implications of chronic thermal stress for production, energetics, life-history as well as the copepod's resilience to extreme events and low food availability.

The results showed that higher rearing temperatures accelerated copepod hatching and development rates, resulting in a reduction of the adult body size and elemental content (carbon, nitrogen and phosphorous). Initially, temperature increased the copepod main physiological rates (ingestion, respiration and reproduction), but these effects were compensated within a few generations under warmer conditions, with no relevant costs on multiple life-history traits, including the efficiency of food utilized for reproduction, energetics, nauplii hatching success or the sex ratio. Adult body stoichiometric ratios (C:N, C:P, and N:P) were either altered across generations. *P. grani* exhibited a broad thermal tolerance range (11-32ºC), and long-term warming (+6ºC) even expanded their tolerance to acute high temperatures (>30ºC). However, prolonged exposure to these temperatures reduced its survival after a week, diminishing the advantage gained from warm rearing. At these extreme temperatures, reproduction was reduced but sustained, thereby expanding the fitness of copepods reared under warming conditions. The thermal resilience of this species to extreme temperatures was highlighted when subjected to sub-lethal thermal stress (28ºC), which did not affect the metabolic balance of copepods regardless of the rearing temperatures (19, 22 and 25ºC). As a main trade-off, following chronic warming the food uptake under low food conditions of the prey *Rhodomonas salina* was highly reduced, potentially indicating a decline in feeding efficiency and subsequent reduction in reproductive activity.

Overall, the research carried out in this thesis suggests that *P. grani* has a large capability to adapt to temperature increases, maintaining key activities, like grazing and production. Nevertheless, an increase in thermal anomalies, low food availability, or the combination of both factors may limit their capacity to persist in their local environment. Given that the thermal effects on development were not compensated at long-term, size reductions in copepods might be a major consequence of ocean warming, with important ramifications for marine food webs and biogeochemical cycles.

RESUMEN

Los animales ectotermos son altamente sensibles a cambios de la temperatura ambiental. Ante el aumento de las temperaturas del océano, una de las principales cuestiones es su impacto en los copépodos, los cuales desempeñan un papel crucial a las redes tróficas pelágicas marinas. Sin embargo, debido a la escasez de estudios que aborden los efectos térmicos a largo plazo, nuestro conocimiento sobre su posible respuesta y de los procesos fisiológicos subyacentes es limitado. Esta tesis presenta una serie de estudios experimentales que investigan los efectos directos de la temperatura sobre los rasgos fenotípicos de los copépodos a lo largo de múltiples generaciones. Específicamente, se centra en el copépodo calanoide *Paracartia grani*, expuesto a largo plazo a temperaturas elevadas (+3 y +6ºC). Se exploran las implicaciones del estrés térmico para la producción, balance energético, historia vital, así como la resiliencia de los copépodos a eventos de calor extremo y a la baja disponibilidad de alimento.

Los resultados muestran que una mayor temperatura de cultivo acelera las tasas de eclosión y desarrollo de los copépodos, lo que resulta en una reducción del tamaño corporal de los adultos y del contenido elemental (carbono, nitrógeno y fósforo). Inicialmente, la temperatura aumentó las principales tasas fisiológicas de los copépodos (ingestión, respiración y reproducción) pero estos efectos se compensaron en unas pocas generaciones de exposición, sin que se produjeran costes relevantes en múltiples rasgos de su ciclo biológico, como la eficiencia de la utilización de alimento en la reproducción, el balance energético, la tasa de eclosión o la proporción de sexos. La relaciones estequiométricas de los adultos (C:N, C:P y N:P), tampoco fueron alteradas después de múltiples generaciones. *P. grani* mostró tolerancia a un amplio rango de temperaturas (11-32ºC) y después del calentamiento a largo plazo (+6ºC) incluso amplió su tolerancia a temperaturas extremas (>30ºC). Sin embargo, una exposición prolongada redujo su supervivencia después de una semana, disminuyendo la ventaja adquirida. A estas temperaturas extremas, la reproducción se redujo significativamente, pero manteniéndose y ampliando así la aptitud de los copépodos cultivados a mayores temperaturas. Asimismo, un estrés térmico subletal (28ºC) no afectó al equilibrio metabólico de los copépodos cultivados a diferentes temperaturas (19, 22 y 25ºC). Como principal desventaja, tras el calentamiento crónico la tasa de ingestión en bajas concentraciones de comida de la presa *Rhodomonas salina* se redujo significativamente, lo que podría indicar una reducción de la eficiencia de alimentación; y, consecuentemente, disminuyó su actividad reproductora.

En general, los resultados de esta tesis sugieren que *P. grani* posee la capacidad de adaptarse a un aumento gradual del calentamiento, manteniendo actividades clave, como la ingestión y la producción. Sin embargo, un aumento de las anomalías térmicas, una baja disponibilidad de alimento o la combinación de ambos factores pueden limitar su capacidad de persistir localmente. Dado que los efectos térmicos sobre el desarrollo no fueron compensados a largo plazo, la disminución de tamaño de los copépodos puede ser una de las mayores implicaciones del calentamiento del océano, con importantes ramificaciones en las redes tróficas y los ciclos biogeoquímicos marinos.

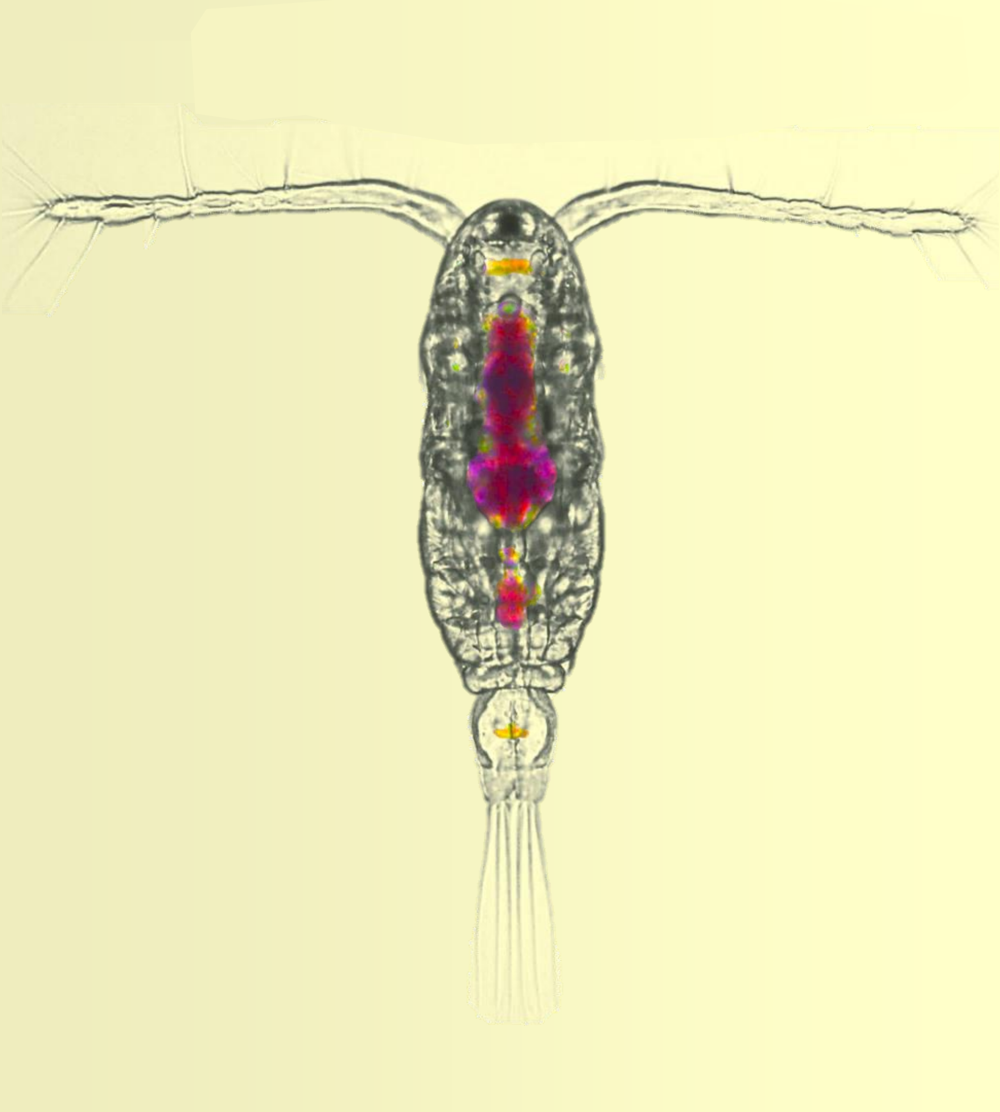
RESUM

L'activitat fisiològica dels animals ectoterms està estretament lligada a la temperatura ambiental. Per tant, davant l'escalfament de les temperatures de l'oceà, una de les principals preocupacions és el seu impacte en els copèpodes, els quals exerceixen un paper crucial a les xarxes tròfiques marines. Tot i això, a causa de l'escassetat d'estudis que aborden els efectes tèrmics a llarg termini, el nostre coneixement sobre la seva possible resposta i els processos fisiològics subjacents són encara limitats. Aquesta tesi presenta una sèrie d'estudis experimentals que investiguen els efectes directes de la temperatura sobre els trets fenotípics dels copèpodes al llarg de múltiples generacions. Específicament, ens centrem en el copèpode calanoide *Paracartia grani,* exposat a llarg termini a temperatures elevades (+3ºC i +6ºC). Explorem les implicacions de l'estrès tèrmic per a la producció, el balanç energètic, l'historia vital, així com la resiliència dels copèpodes a esdeveniments de calor extrems i a la baixa disponibilitat d'aliment.

Els resultats mostren que una major temperatura de cultiu accelera les taxes de reclutament i el desenvolupament dels copèpodes, cosa que resulta en una reducció de sa mida corporal dels adults i del seu contingut elemental (carboni, nitrogen, fòsfor). Inicialment, la temperatura va augmentar les principals taxes fisiològiques dels copèpodes (ingestió, respiració i reproducció), però aquests efectes es varen compensar en unes poques generacions d'exposició sense implicar costos rellevants en múltiple trets del seu cicle biològic, com l'eficiència de la utilització de l'aliment per la reproducció, l'èxit de reclutament de ses larves o la proporció de sexes. Tampoc no es van veure alterades les relacions estequiomètriques en els adults (C:N, C:P i N:P). *P. grani* va mostrar tolerància a un ampli rang de temperatures (11-32ºC) i el seu cultiu a temperatures més elevades (+6ºC) va ampliar la seva tolerància a temperatures extremes (>30ºC). Tot i això, una exposició perllongada va reduir la seva supervivència després d'una setmana, reduint l'avantatge adquirit. A aquestes temperatures extremes, la reproducció es va reduir però mantenint-se, ampliant així l'aptitud dels copèpodes cultivats a més temperatura. Així mateix, un estrès tèrmic subletal (28ºC) no va afectar l'equilibri metabòlic dels copèpodes en cap de les condicions de cultiu (19, 22 i 25ºC). Com a principal desavantatge, després de l'escalfament crònic la taxa d'ingestió en baixes concentracions de la presa *Rhodomonas salina* es va reduir significativament, lo que podria indicar una reducció de l'eficiència d'alimentació i, conseqüentment, una disminució de la seva activitat reproductora.

En general, els resultats d'aquesta tesi suggereixen que *P. grani* posseeix una gran capacitat d'adaptar-se a un augment gradual de la temperatura, mantenint activitats clau com la ingestió i la producció. Tot i això, un augment de les anomalies tèrmiques, una baixa disponibilitat d'aliment o la combinació de totes dues poden limitar la seva capacitat de persistir localment. Atès que els efectes sobre el desenvolupament no es compensen a llarg termini, la reducció de mida dels copèpodes pot suposar una de les implicacions més importants de l'escalfament de l'oceà, amb importants ramificacions a les xarxes tròfiques i als cicles biogeoquímics marins.

CONTENTS



1. GENERAL INTRODUCTION

Overview of the role of zooplankton in the marine pelagic ecosystem

The zooplankton constitutes a heterogeneous group of animals within the plankton (derived from the Greek word for “errant” or “drifter”) that comprises a broad array of organisms, including protozoans, crustaceans, molluscs, annelids, chaetognaths, echinoderms, appendicularians, cnidarians and ctenophores. Some zooplankton species are exclusively planktonic while others just spend part of their life-cycle as free forms. Their size ranges can vary by more than 2 orders of magnitude, hence, regarding size-classification, can be constituents of the nano- (2 - 20 μm), micro- (20 - 200 μm), meso- (200 μm - 20 mm), and macroplankton (> 20 mm; Sieburth et al., 1978). Despite their diversity, they all form a key group that relies on the primary production in the upper ocean layers, facilitating the transfer of nutrients and energy to higher trophic levels. Herbivorous microzooplankton (ciliates, dinoflagellates, foraminifera, copepod nauplii, etc.), are the main consumers of phytoplankton (Calbet & Landry, 2004); and mesozooplankton (copepods, cladocerans, etc.) feed on both phytoplankton and microzooplankton (Gifford, 1991; Calbet & Saiz, 2005). In turn, mesozooplankton are prey for bigger animals, such as fish larvae (Bachiller et al., 2016; Bouchard & Fortier, 2020).

Zooplankton plays a pivotal role not only in shaping secondary production and exerting a top-down control on phytoplankton, but also serve as important remineralizers of inorganic nutrients (Ikeda et al., 1982), supporting phytoplankton growth (e.g. Alcaraz et al., 1994). In oligotrophic areas or seasons, the available nutrients cannot support high phytoplankton concentrations, and therefore, primary productivity largely relies on the recycling of essential nutrients (“turnover”; Banse, 1995; Tamigneaux et al., 1997). The main source of these recycled nutrients is the dissolved organic matter released by phytoplankton itself, and the waste products (DOC from faecal pellets, “sloppy feeding” and excretion) of zooplankton, which are remineralized by heterotrophic bacteria (Azam et al., 1983).

While most of the organic matter egested by zooplankton ends up being respired or remineralized in the upper layers, particles that are not retained may sink – at increasing rates as they aggregate (Kiørboe, 2001). Across its vertical displacement, the sinking aggregates become a source of nutrients to bacteria and opportunistic species such as detritivorous zooplankton. Ultimately, a fraction of the initial aggregation will reach the ocean seabed, where it can be consumed by benthic organisms or buried in the sediments. This process, mediated by plankton organisms, is called the biological carbon pump, and it is considered an important sink of atmospheric carbon (Steinberg & Landry, 2017). The rates of carbon export might be fuelled by physical processes or actively by diel vertical migrators (zooplankton and other groups such as myctophid fishes; Saba et al., 2021), which feed in upper layers at night and spend the day-light hours below the euphotic zone (Longhurst et al., 1990; Jónasdóttir et al., 2015). The contribution of each player, the rates of export, and the timescales that carbon remains sequestered, however, can vary greatly regionally (Steinberg & Landry, 2017; DeVries, 2022; Nowicki et al., 2022).

Within the mesozooplankton, copepods are considered the most important group (Turner, 2004), as they often contribute to more than 50% of their abundance (Longhurst, 1985; Mauchline, 1998). These animals constitute a highly diverse group of crustaceans that, in fact, are extremely abundant and widespread in all aquatic environments with > 11500 species described (Humes, 1994), of which 2693 inhabit marine or brackish waters (Razouls et al., 2005-2024; **Fig. 1**). In the pelagic environment, there are three main orders of free-living copepods: Calanoid, Cyclopoida and Haparcticoida. Calanoid and cyclopoid copepods represent the most common groups found in pelagic environments, with important species representatives found worldwide or with particular ecological relevance. Given their abundance, planktonic copepods have been subject of study in oceanography since its beginnings (Damkaer & Mrozek-Dahl, 1980; Dolan, 2021, 2022). The accumulated knowledge on their morphologies, physiologies and life-history strategies have highlighted their capacity to succeed in a variety of environments and their crucial ecological role. Despite their diversity, several shared characteristics stands out to explain the capacity of pelagic copepods to thrive in the aquatic realm: their conserved torpedo-alike anatomies, a distinctive jump force to scape predators, and sensory capacities that allow to detect prey, predators and facilitate mate finding for sexual reproduction (Kiørboe, 2011).

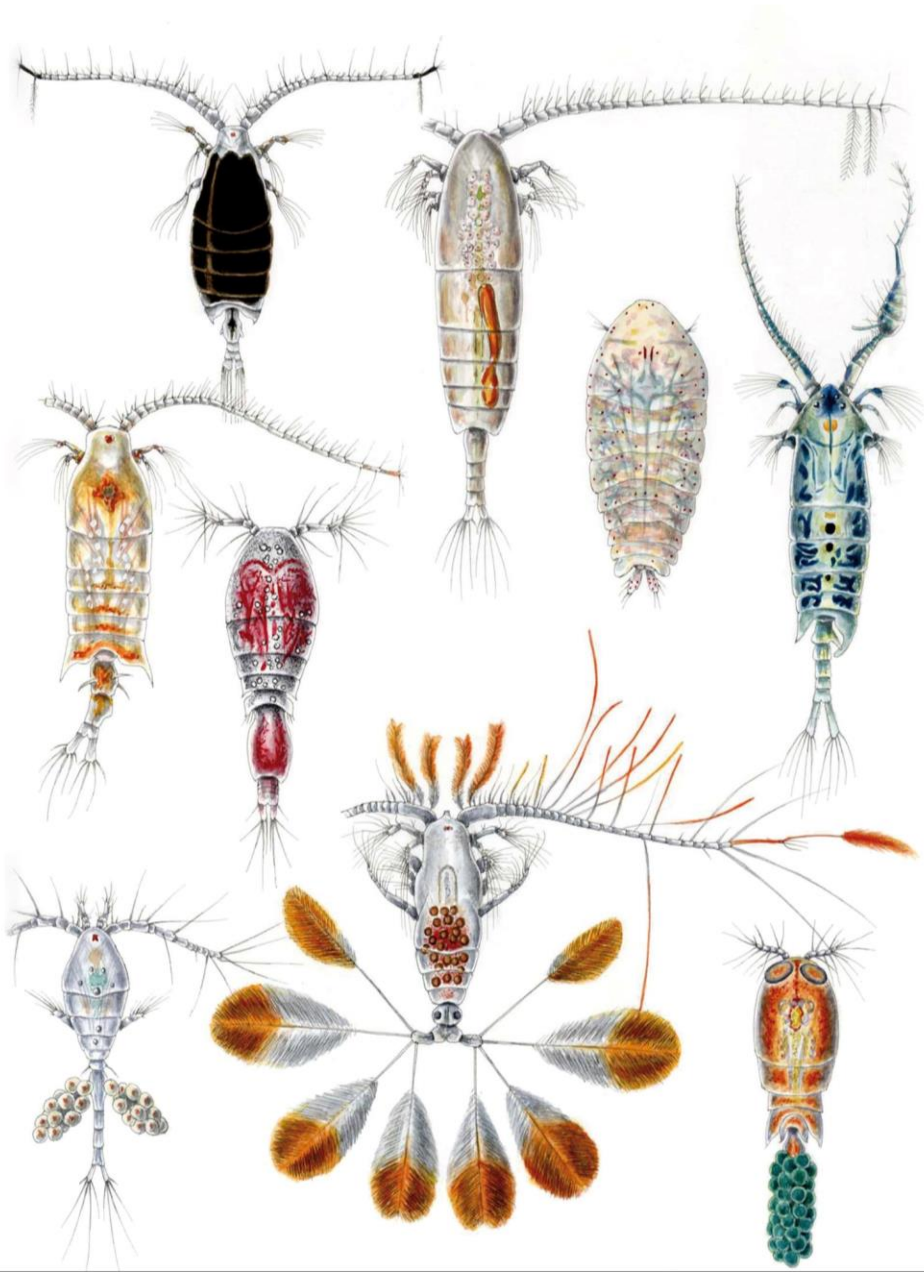


Fig. 1 | Diversity of morphological forms of planktonic copepods. Drawings from Prof. Miquel Alcaraz, some inspired by classical artwork from Ernst Haeckel (1834-1919), Wilhelm Giesbrecht (1853-1913) and Salvatore Lo Bianco (1860-1910).

The zooplankton in a changing environment

The ocean absorbs much of the excess of heat from the atmosphere, but not without repercussions. Over the past few decades, the ocean surface has been warming at a rate of 0.09 - 0.13ºC per decade, with some regions experiencing even a more accelerated warming, such as temperate areas in the Northern hemisphere (IPCC, 2019). This heat excess is not retained at the surface of the oceans, but it is even penetrating to depths up to 2000 meters (IPCC, 2019). Consequently, a primary challenge in contemporary oceanography is predicting the effects of rising temperatures on marine ecosystems, fisheries production and, particularly, on biogeochemical cycles. Direct observations gathered over recent decades, mostly derived from long-term temporal series, have revealed global patterns of impacts on marine (and terrestrial) life (Poloczanska et al., 2016). These impacts, also documented in marine copepods, include changes in phenology – species appearing earlier in spring and summer blooms and later in autumn – that can lead to trophic mismatches (Beaugrand et al., 2002; Edwards & Richardson, 2004); changes in biogeography – migration towards higher latitudes or deeper, colder layers potentially leading to the introduction of species to new habitats (Beaugrand et al., 2003; Feng et al., 2018); and the reduction in size of the organisms (Corona et al., 2024; Daufresne et al., 2009; Evans et al., 2020; Pitois and Fox, 2006).

Associated with the ocean heat uptake, there is an increasing confidence that extreme heat events – defined as anomalously high temperatures that persist 5 or more consecutive days – will increase in intensity, duration and frequency, especially when influenced by regional climate events (e.g. El Niño or the North Atlantic Oscillation; IPCC, 2019; Martínez et al., 2023; Oliver et al., 2021; Zhang et al., 2023). These so-called marine heatwaves are already impacting marine life (Smith et al., 2023), with notable examples of lethal effects on benthic organisms (those attached to the seafloor; Genevier et al., 2019; Leggat et al., 2019; Garrabou et al., 2022). Although the effects on zooplankton have received lesser attention, available evidences suggest that marine heatwaves can cause alterations in copepod population structure and trophic chain disruptions with widespread and lasting effects on marine ecosystems (Pitois & Fox, 2006; Evans et al., 2020; Batten et al., 2022; McKinstry et al., 2022). In addition, seasonal ocean stratification, intensified by the rise of the sea surface temperature and lower nutrient inputs from rivers, reduces the supply of nutrients to the photic zone (Agusti et al., 2017), thereby, negatively affecting primary productivity. In an already patchy environment, where small plankton (phytoplankton and microzooplankton) are heterogeneously distributed, the response of pelagic consumers, such as copepods, to thermal stress might be constrained by these increasing oligotrophic conditions (Lewandowska et al., 2014; Huey & Kingsolver, 2019).

It is important to note that some of the observed changes, regarding phenology and biogeography, may imply that some of the affected species conserve, to some degree, their thermal niche species (Corona et al., 2024). Nevertheless, chronic warming and increases in marine heatwaves are expected to directly determine the fate of organisms locally, especially in cases of low dispersal capacity (Sanford & Kelly, 2011). As most marine organisms are ectotherms (i.e., their body temperature closely matches that of the environment), changes in environmental temperature directly drives their activity. In copepods, from an ecological point of view the most important processes include grazing, respiration, egestion, excretion (Frangoulis et al., 2004) and the production of new offspring (**Fig. 2**). It is largely recognized that alterations in copepod fluxes might have important consequences for the transfer of energy and nutrients in the trophic chain, affecting the strength of the biological carbon pump (Steinberg & Landry, 2017; Brun et al., 2019). Under rising temperatures, virtually all these processes are expected to change, although the direction of these changes remains unclear, hindering our ability to predict their consequences for marine ecosystems (Ratnarajah et al., 2023).

The relationship between copepod activity and temperature have been extensively studied. Copepod physiological rates increase exponentially with a rise in ambient temperature, including ingestion (Kiørboe et al., 1982; Garrido et al., 2013), respiration (Ikeda et al., 2001; Cruz et al., 2013), egg production (Ban, 1994; Koski & Kuosa, 1999; Holste et al., 2009), egestion (Saiz et al., 2022), excretion (Almeda et al., 2011), growth and development (Uye, 1988; Almeda et al., 2010b; Forster & Hirst, 2012) and swimming activity (Moison et al., 2012; Svetlichny & Obertegger, 2022). Thus, from these studies, it could be inferred that environmental warming might cause an increase in the metabolic demands of copepods, thereby affecting the rates of feeding uptake and the outfluxes (egestion, respiration, excretion, growth/production). However, our knowledge of the thermal sensitivity of these processes is mainly based on short-term incubations, spanning from hours to few weeks, which could not fully capture the chronic nature of ocean warming (Hoffman & Sgrò, 2011; Dam, 2013). Indeed, the possible of the deployment of a long-term acclimation response (Seebacher et al., 2015), or evolutionary adaptation (Dam, 2013) that reduces the thermal sensitivity of these processes is not adequately addressed.

Our knowledge of copepod biology has advanced considerably with the cultivation of copepod species in the laboratory over the last decades, and this accumulated knowledge allows to perform long-term experiments with controlled conditions in the laboratory. These experimental evolution studies, despite their logistical complexity, allow to explore changes in phenotypes (and genotypes) across multiple generations of stress. Furthermore, copepods are a suitable model group in experimental evolution as most species have relatively short-generation times, and can be reared in large numbers for manipulation in laboratory setting to analyse adaptive changes in their phenotypes. In recent years, mounting evidence suggests that changes in copepod genotypes can be much more rapid than previously thought (Dam et al., 2021; Brennan et al., 2022; DeMayo et al., 2023). Therefore, in contrast to the classic short-term experiments, multigenerational approaches can unveil the potential for adaptive changes to mitigate thermal stress, and, consequently, reduce the thermal sensitivity of the physiological processes. Additionally, the effects of temperature on body size (Riccardi & Mariotto, 2000; Forster & Hirst, 2012) – a major factor governing their physiology – will overlap with the long-term thermal response, potentially influencing copepod activity (Runge, 1984; Koski & Kuosa, 1999; Saiz & Calbet, 2007; Olivares et al., 2019).

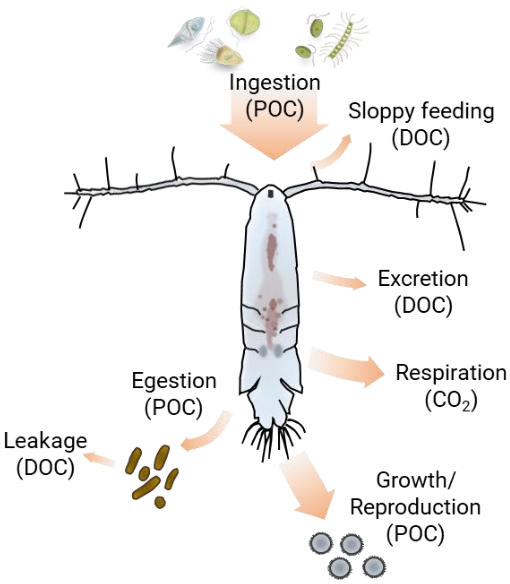


Fig. 2 | Main physiological processes of a copepod and the associated fluxes of organic carbon Based on Steinberg & Landry (2017). Drawings of microzooplankton and phytoplankton species kindly provided by A. Calbet.

The timescales of the thermal effects on physiology

Temperature affects function at all levels of biological organization, from molecules, enzymes, organs to whole-organismal and ecosystem processes. However, its effects are mostly described by the thermodynamics effects of a simple biochemical reaction (Schulte, 2015). Temperature increases the kinetic energy of molecules rising the number and the energy of the collisions between molecules, thus, increasing the speed of the reactions. This is described by the Boltzmann-Arrhenius equation (Arrhenius, 1915):

$$k = A\ e^{(-\frac{Ea}{RT})} \qquad \text{Eq. 1}$$

where k is the rate of the reaction, A is the pre-exponential factor (constant with same units as k), E_a is the mean activation energy coefficient of the reaction (related to the temperature sensitivity of the corresponding metabolic function), R is the universal gas constant (8.314 J mol^{-1} K^{-1}) and T is the absolute temperature (K). $e^{(-Ea/RT)}$ would be the fraction of molecules with enough energy to react.

Despite that at higher biological levels this approach would be a simplification of the truly multistep underlying processes (Bruggeman & Westerhoff, 2007), indeed, many physiological processes increase exponentially with temperature. Performance escalates with temperature up to a maximum (R_{max}), which is considered the thermal optimum (T_{opt}) of the process, from which function start declining. Further increases in temperature result in a rapid decay of function up to a certain temperature where function is lost – this upper thermal limit is often called critical thermal maximum (CT_{max}). Similarly, from a given starting temperature, cooling causes a deceleration of the process up to a lower limit of the process named critical thermal minimum (CT_{min}). The resulting curve – often represented as a bell-shape left-skewed – is named thermal performance curve (TPC, **Fig. 3**).

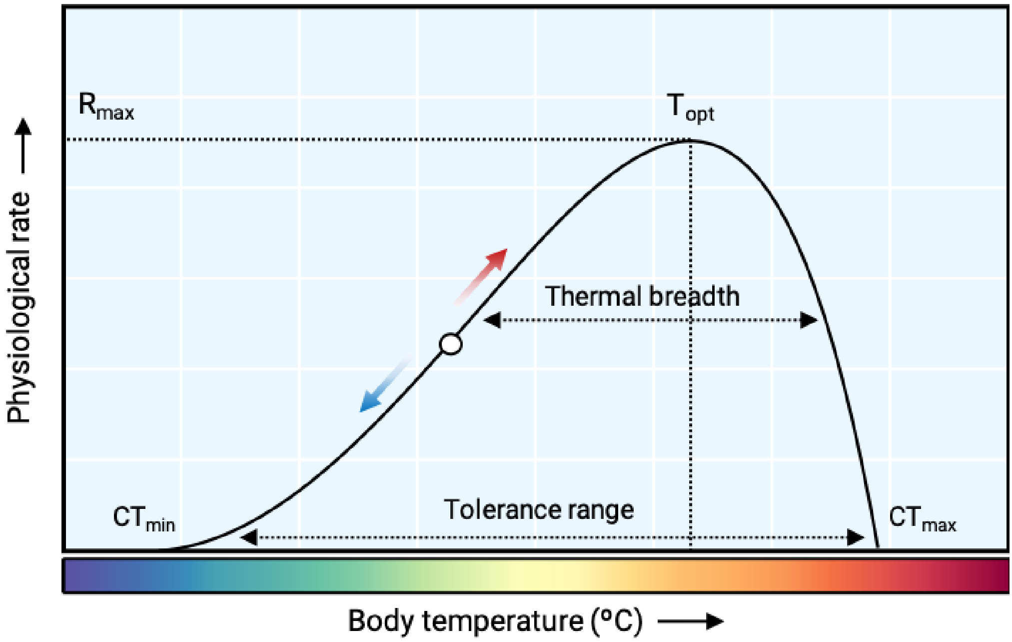


Fig. 3 | Model of a TPC highlighting the main parameters based on Pörtner (2002) and Sinclair et al. (2016). Curve was obtained from the data of *Paracartia grani* egg production rates (19°C) from **Results 3.2**.

TPCs are broadly used to characterize the relationship of organismal traits with temperature and, despite many recognized limitations (Sinclair et al., 2016), they are useful and informative of the thermal dependency of a given trait. Many parameters related with the TPC are used to predict the effects of global warming. For instance, Q_{10} coefficients determine the fold-change increase in rates of activity under 10ºC increase, seen as indicative of the thermal sensitivity of the process (it has relationship with the activation energy of the process). Q_{10} can be calculated as follows (Prosser, 1961):

$$Q_{10} = e^{\left(\frac{E_a}{R}\right)\left(\frac{10}{T^2}\right)} \qquad \text{Eq. 2}$$

Where T is the average temperature of the range of exposure, and E_a and R have been defined in Eq. 1.

These Q_{10} (or E_a, instead) values are widely used in predictive models to incorporate the effects of temperature on a given system. Q_{10} have been described for a variety of functions in many taxa. Based on observations of many biological processes, often extracted from short-term experiments, Q_{10} are typically assumed to be 2 - 3 or higher (Saiz et al., 2022).

Another metric derived from the TPC parameters is the thermal safety margin (TSM), which can be defined as the difference between either actual body temperature or T_{opt} to the CT_{max} (Sinclair et al., 2016). The TSM informs about the potential vulnerability of organisms (Deutsch et al., 2008; Gunderson et al., 2017; Pinsky et al., 2019) and is broadly used to make global comparisons of vulnerability between groups or latitudes. For example, global comparisons of the TSM suggest that tropical animals are particularly vulnerable to warming, as they already live near their T_{opt} (Deutsch et al., 2008; Huey et al., 2009); although this assertion is nuanced by other authors who highlight that temperate species are subjected to higher thermal variability, hence, experience higher extremes (Vasseur et al., 2014). CT_{max} is often used to inform about the limits of persistence of species as the thermal extremes might be more critical than the averages in determining their spatial distributions (Somero, 2010). Nonetheless, the equivalency between the CT_{max} and the tolerance limits depends on the choice of a trait related to the functional integrity of the organism. Further insights can be obtained from TPCs, such as comparisons of tolerance range, or thermal breadths, which could differentiate between generalist and specialist species (Buckley & Kingsolver, 2021).

The widespread application of TPCs forces to explain briefly the underlying mechanisms that shape them. It is often assumed that the exponential phase of the TPC is driven by Arrhenius effects (termed passive response or passive plasticity) and, at some stressful temperature, the physiological rate is constrained by reversible or irreversible denaturation of proteins. Yet, as mentioned earlier, TPCs are the result of multiple dynamic processes (Rohr et al., 2018) at molecular (e.g., changes in enzyme conformations that alters their activity, changes in enzyme-substrate interactions or membrane fluidity), at cellular (e.g., thermal dependency of the biochemical networks, reductions in the efficiency of ATP production due to increase proton leak), and up to organismal level (e.g. reduction in aerobic scope; reviewed by Schulte, 2015). However, it is thought that failures at organismal level may occur at narrower limits than the underlying mechanisms, thus, being a better predictor of the thermal limits of organisms (Sokolova et al., 2012; Alcaraz et al., 2014; Schulte, 2015; **Fig. 4**).

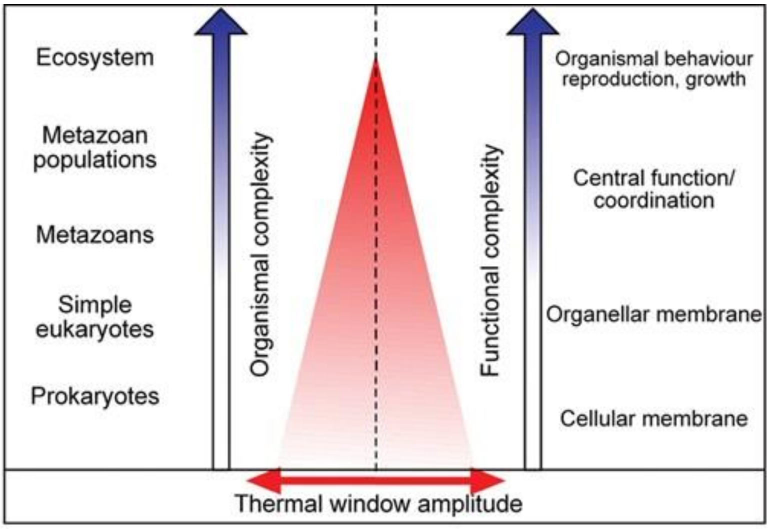


Fig. 4 | Thermal window amplitude in relation to organismal and functional complexity. Modified from Alcaraz et al. (2014) based on Pörtner (2002). Reproduced with permission.

Thermal stress causes disruptions at the molecular, cellular, and organismal levels. In response, the organisms possess a range of mechanisms across multiple levels to mitigate the impact of the stress (Angilletta et al., 2002; Schulte et al., 2011). For example, the increase in metabolic rates increases the formation of reactive oxygen species, which can potentially damage cellular macromolecules like lipids, proteins, or DNA (Sokolova et al., 2012). In response, the cellular machinery triggers the production of antioxidants that combat oxygen radicals and alleviate heat stress. Additionally, heat-shock proteins (HSPs), acting as molecular chaperones, assist in the proper folding of proteins and stabilize proteins and membranes. At more extreme temperatures, cellular machinery works on the repair of the potential damages, including the elimination of denatured proteins, DNA repair, removal of cellular or molecular debris, or induction of apoptosis (Sokolova et al., 2012). At organismal level, certain organisms suppress metabolic rates to reduce the damage caused by the metabolic waste (Schulte et al., 2011). This response, in contrast to the passive biophysical response, is considered active (Havird et al., 2020) and may occur relatively rapid, in hours or days (Hazel & Prosser, 1974; Scheffler et al., 2019). Importantly, since process measurements are conducted over a defined period, often 24 hours, what might initially appear as a passive response can also involve elements of an active response, which may be recognizable at a different time scale (Schulte et al., 2011).

The active response is energy-demanding (e.g., the process of production and action of HSPs are ATP-dependent; Schulte, 2015). This energetic expenditure implies an increase in the basal metabolic costs which might be followed by higher rates of food consumption. However, the difference in the thermal sensitivities between these processes and with other physiological rates (e.g., egestion or offspring production) can cause mismatches between rates, and ultimately affect fitness (Alcaraz et al., 2014). A bioenergetic framework offers an integrative perspective of the interdependency of the major physiological processes, and, thereby provides insights into energy allocation strategies of the organisms, that is, how the available energy is invested in each process (Sokolova et al., 2012). In copepods, food items need to be detected, captured, eaten, digested, assimilated and then utilized for growth or reproduction and metabolism

(Omori & Ikeda, 1984). A simplified carbon metabolic budget, assuming negligible carbon excretion, would be:

$$I = G + R + E \qquad \text{Eq. 3}$$

where I account for ingestion and G is either somatic growth or egg production. R is respiration rate, which can be estimated either in food depletion conditions and is assumed to be linked to the basal metabolism (called routine metabolism in the case of a free swimming copepod), or can be maximum respiration rates that include the process of nutrition (from food acquisition to biosynthesis; termed Specific Dynamic Action, SDA). The fraction that is not assimilated is egested (E).

At non-stressful temperatures, the basal metabolic costs are low and the net energy gain allows activity, growth, development, reproduction or storage in reserves. However, factors such as limited food availability, deficient assimilation of nutrients, or constraints on the delivery of oxygen to tissues may limit the energy available for these processes (Pörtner, 2002; Sokolova et al., 2012; Huey & Kingsolver, 2019). At moderate thermal stress levels, elevated metabolic costs can reduce the investments in reproduction or growth, impacting fitness. At more extreme temperatures, physiological homeostasis can be further disrupted allowing just short-term survival (Saiz et al., 2022). Importantly, low food resources during these thermal extremes would further constrain the energetic gain and narrow the thermal limits (Saiz et al., 2022; Rueda Moreno & Sasaki, 2023). The energy allocation strategies followed by the organisms, that is, the prioritization of maintenance over reproduction or vice versa, is representative of their life-history. Neritic copepods, such as the Acartia sp. females, often invest >30% of their carbon budget in reproduction and keep producing eggs nearly until natural death (Saiz et al., 2015). However, the oxidative stress associated with a higher activity levels can shorten their lifespan and decrease the hatching success of eggs produced at older ages (Saiz et al., 2015). Similarly, the effects of thermal stress may not be limited to the exposed individuals, but also can be manifested in other life-history traits, such as recruitment.

Active plasticity in organism can imply a change in the TPCs of the physiological processes following a period of acclimation (within the life of one individual). As a result, Q_{10} values will decline (**Fig. 5**). For example reductions in Q_{10} following some days of acclimation have been observed in copepods (Saiz et al., 2022). Depending on the species, acclimation can completely compensate the thermal effects on physiological rates (Scheffler et al., 2019). However, the degree of acclimation may not be the same for all physiological processes with potential impacts on bioenergetics.

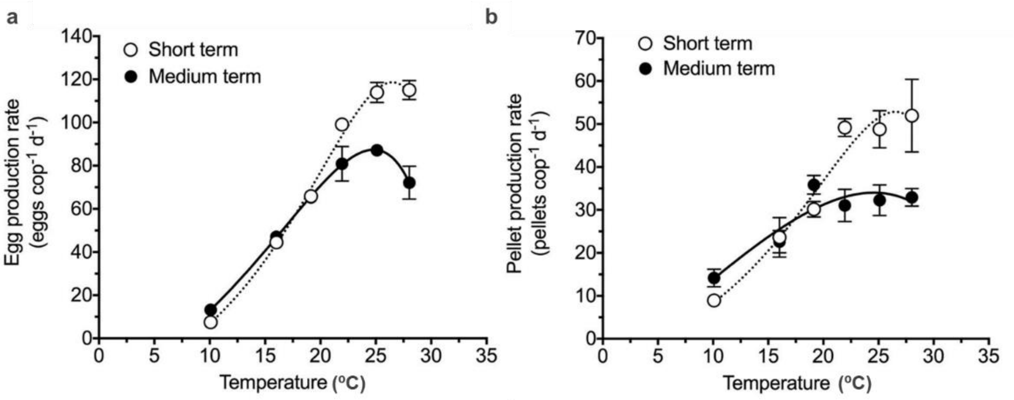


Fig. 5 | Acclimation response of adult female *P. grani* egg production (**a**) and egestion rates (**b**). Empty circles show response after 48h; black circles show response after 7 days. Modified from Saiz et al. (2022).

In many organisms, the tolerance limits can also be amplified following an acclimation period, demonstrating a degree of plasticity in this trait. In this regard, the acclimation response ratio (ARR), which measures the degree of increase in thermal tolerance per degree of increased acclimation temperature, is another widely-used metric for comparing the resilience of different groups and identify vulnerable ones (Morley et al., 2019). Yet, under prolonged warming conditions, organisms might fail to maintain functional integrity at most extreme temperatures, leading to mortality and a subsequent reduction in the tolerance limits. To what extend and for how long acclimation provides resistance to thermal extremes is, therefore, a crucial question to answer.

Prolonging the effects of warming, more reversible (plastic) changes in phenotypes can further shape the TPCs. For example, developmental plasticity - the response to temperature changes experienced during ontogeny - can determine the adult thermal sensitivity (Rebolledo et al., 2021). Plastic processes can persist beyond the life of the exposed individuals. Inter-generational (or trans-generational) effects (e.g., maternal provisioning, epigenetic processes) can modify the phenotypes of the offspring, for example, providing them more resistance, even if the thermal stress does not persist (Vehmaa et al., 2012; Thor & Dupont, 2015; Truong et al., 2022). These processes are gaining attention as mechanisms that buffer the effects of environmental variation (Donelson et al., 2018; Moore et al., 2019). Moreover, recent evidences show that adaptation, traditionally viewed as a slow process, can be more rapid than previously believed. For example, changes in allele frequency can take place in a some generations of exposure to moderate warming in copepods, manifesting in phenotypes with higher fitness (Dam et al., 2021; Brennan et al., 2022). Few amino acid replacements in essential enzymes can increase their stability and performance at warmer temperatures (Miller, 2003; Kingsolver, 2009). All these mechanisms can interact, leading to modifications in the TPCs (**Fig. 6**). It is noteworthy that some changes at genotype level can be irreversible, and, as a result, the plasticity in the trait under selection might be altered (enzymes can be selected to have higher stability at warmer temperatures and, in turn, lose flexibility; Hochachka & Somero, 2002). In this regard, it is not yet resolved if there is a trade-off between increases in the heat tolerance of an organism and the plasticity in this trait (van Heerwaarden & Kellermann, 2020). Reduced plasticity could limit the future capacity to cope with novel thermal stress, serving as a crucial factor determining resilience to environmental variation.

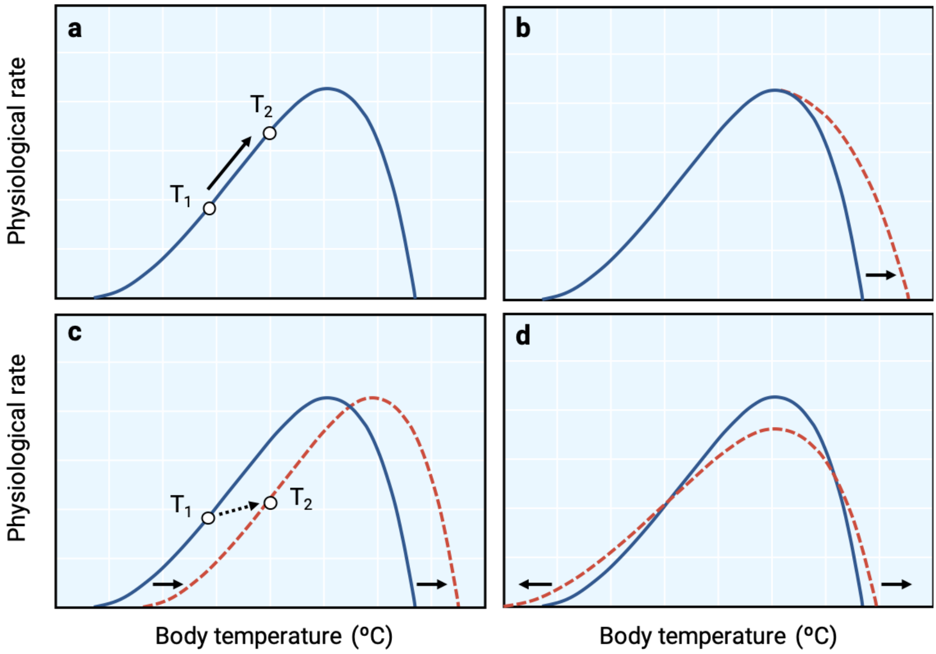


Fig. 6 | Examples of possible shifts in the thermal performance curves following long-term exposure. **a.** Conservation of the TPC. From an initial temperature (T_1), warming (T_2), cause an increase in physiological rates. **b**. Increase in performance just at high temperatures, resulting in an increase in the thermal breadth. **c**. Horizontal shift of the TPC due to an increase in performance at high temperature which is negatively correlated with performance at low temperatures. From T_1, warming (T_2) does not increase the physiological rate, in contrast to **a**. **d**. Increase in performance at high temperature and also at low temperature and decrease in maximum performance. Modified from Huey & Kingsolver (1993).

Effects of temperature on body size in ectotherms

A major implication of long-term exposures to thermal increase in ectotherms is the reduction in the body size of the organisms. Thus effect, arising from the different thermal sensitivities of growth and development during ontogeny (Forster & Hirst, 2012), is referred to as the temperature-size rule (TSR; Atkinson, 1994). The generality of this rule, extended to several taxa, is related to the increasing observations of negative trends in the size and the mass of species (Corona et al., 2024; Evans et al., 2020). The relevance of body size is broad because virtually all aspects of biology, structure, and function are size- (and, therefore, mass-) dependent (Gillooly et al., 2001; Speakman, 2005; Woodward et al., 2005). The control on the fluxes of energy and nutrients (metabolism), is one of the most important factors in ecology. A variable (*Y*) scales with mass (*M*) following a power function:

$$Y = a\ M^{b} \qquad \text{Eq. 4}$$

where *a* is the scaling coefficient and *b* is the allometric exponent. After Klieber's law, *b* is classically assumed to be ¾ (but see Dodds et al., 2001). In copepods, physiological rates scale with mass, including feeding (Saiz & Calbet, 2007; Olivares et al., 2019), egg production (Runge, 1984), and respiration (Ikeda, 1985) In turn, mass-specific rates scale by ¼. Therefore, after decades of studying the allometric effects and temperature, both factors are considered the most important drivers of the metabolism of organisms, and are the base of unifying theories aiming to explain general patterns in ecology (Gillooly et al., 2001; Brown et al., 2004). It has been argued that larger body size enhance survival (Kingsolver & Huey, 2008). Nonetheless, larger body size is also thought to reduce thermal tolerance (Peralta-Maraver & Rezende, 2021). In summary, beyond direct physiological effects, temperature's influence on organism size adds a complex layer to long-term thermal responses (**Fig. 7**).

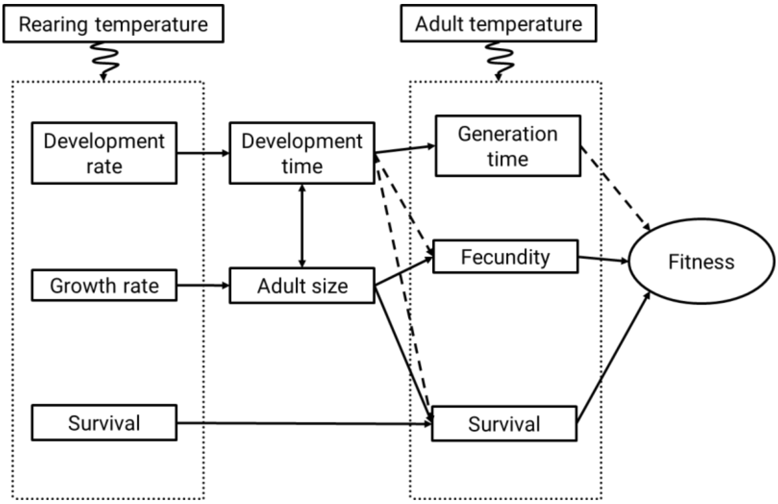


Fig. 7 | Interactions between rearing temperature and body size on fitness components. Modified from Kingsolver and Huey (2008). Solid lines indicate possible positive effects while dashed lines indicate possible negative ones.

Approach of this thesis

There are many models available for studying thermal adaptation of organisms (**Fig. 8**). One approach involves investigating the active response within an individual lifetime under controlled conditions, enabling exploration acclimation responses as a function of stress intensity and exposure time (Mayzaud, 1992; Saiz et al., 2022). Alternatively, experimental evolution studies consist in long-term experiments in manipulated conditions, either in the laboratory or in the field (Dam et al., 2021; DeMayo et al., 2023). Studies on recent invasions of organisms to new habitats can unveil genetic or phenotypic adaptations to these environments (Lee, 2016; Stern & Lee, 2020). Similarly, comparing the performance of "resurrected" organisms (e.g. eggs recovered from burial) with current populations can also indicate recent adaptation (Geerts et al., 2015). The comparison between different latitudinal populations of the same species shed light on local adaptation processes (Halsband-Lenk et al., 2002; Kelly et al., 2012). Finally, analyzing the thermal sensitivity of multiple species can inform about ecological successions (Sasaki et al., 2023).

This thesis presents long-term (multigenerational), experimentally-oriented studies focusing on the *direct* effects of temperature on phenotypic traits, using the neritic calanoid copepod *Paracartia grani* Sars, 1904 as a model species. Our aim is to gain new insights into the potential response of zooplankton, and the underlying physiological processes involved, to some of the key environmental changes predicted for the coming decades: warming and oligotrophy. The research addresses some relevant questions on this topic, including: Can copepods adapt to ecologically-relevant increases in temperature, and to what degree? Are there any fitness costs associated to these phenotypic changes? Can chronic warming increase the tolerance to extreme temperatures? Additionally, can low food availability constrain the thermal response? Overall, we explore the implications of long-term thermal stress for production, energetics, life-history as well as the copepod's resilience to extreme events and low food availability. Through the different studies conducted we provide valuable data on the biology of the species and address classical questions in ecology.

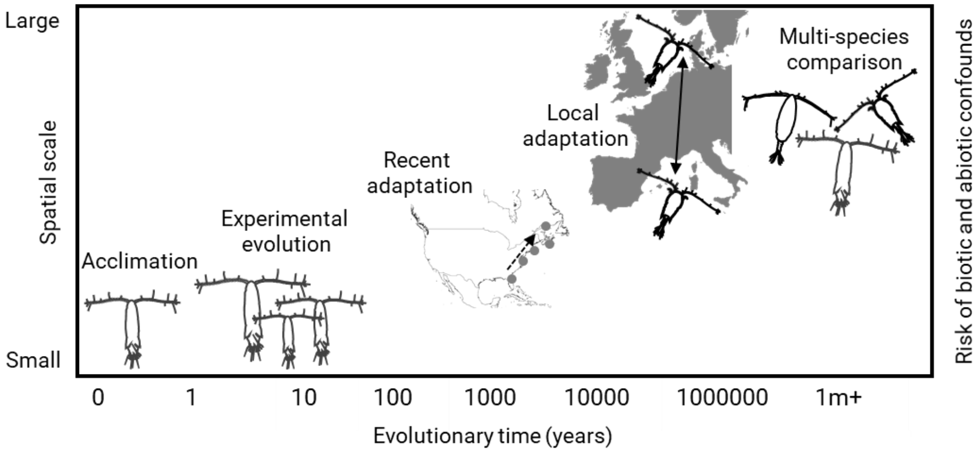


Fig. 8 | Examples of approaches to study thermal adaptation in copepods. Modified from Jutfelt (2020).

On the study species: the neritic copepod *Paracartia grani*

P. grani is a calanoid copepod belonging to the Acartidae family, a taxonomic group found in aquatic habitats worldwide (Belmonte, 2021). This species is commonly found in coastal and semi-enclosed waters of the North and South East Atlantic, as well as in the Mediterranean Sea (Rodríguez & Vives, 1984; S. Boyer et al., 2012; Razouls et al., 2005-2023). In the western Mediterranean, *P. grani* can be found year-round at a wide thermal range (8.3-23.8ºC), with peak abundances between spring and autumn (Rodriguez & Jiménez, 1990). In these regions, *P. grani* can coexist with other *Acartia* species (Alcaraz, 1977; Rodriguez & Jiménez, 1990). Due to their short-generation times, *P. grani* populations are expected to undergo multiple generations annually similar to other species (Christou and Verriopoulos, 1993).

The sexes of adult *P. grani* can be easily distinguished by morphology (Vilela, 1972). Females have a wing-life expansions on the last segment of the prosome and relatively short three-segmented abdomen (urosome). Males have a slender, five-segmented abdomen and possess modified 5th leg and a thickened right antenna used for mating. Females are larger than males (1050 µm and 930 µm, respectively, at 19ºC) and, generally exhibit higher metabolic rates (Olivares et al., 2019). The reproduction of the females is highly dependent on food intake (Calbet & Alcaraz, 1996). Food items can be detected by the mechano- and chemoreceptors present in their antennas and feeding appendages (Alcaraz et al., 1980; Tiselius et al., 2013). Depending on prey size and motility, the copepods can adopt ambush strategies or create feeding currents for feeding (Saiz & Kiorboe, 1995). Given the relatively small size and low motility of the cryptophyte *R. salina*, utilized as prey in our experiments, *P. grani* used the latter method, generating a current with their feeding appendages to bring food to their mouth (Saiz & Kiorboe, 1995). Similar to other zooplankton species, *P. grani* shows higher feeding activity at night (Bautista et al., 1988), but these rhythms are lost in the laboratory (Olivares et al., 2020).

The food is quickly digested, and given that the handling of the prey (time they spend eating the prey) is relatively short, the maximum capacity to eat is limited mainly by the gut volume and digestion time (time clearing the guts or gut passage time; Tiselius et al., 2013). At very low food concentrations, they can diminish their foraging activity to minimize the metabolic costs until higher concentrations are encountered, or they may switch to a more abundant prey (Holling Type III response; Kiørboe et al., 2018). Unassimilated food is egested in small faecal pellets, which consist of waste material enclosed by a peritrophic membrane that maintains their integrity (Mauchline, 1998). On an excess of food, *P. grani* can accumulate lipids visible as small oil droplets in the prosome (personal observation).

For copepods, mating in the water column relies on physical and chemical signals (Kiørboe & Bagøien, 2005) yet *P. grani* is closely related to species that uses hydrodynamic signals, such as *Acartia tonsa* (Bagøien & Kiørboe, 2005). Males detect the presence of the females and grasp them with the modified antennae and fertilizing them (Bagøien & Kiørboe, 2005). A single mating may fertilize the female for several days (Parrish & Wilson, 1978). *P. grani* females can lay >100 eggs per day (Saiz et al., 1997), while the male's spermatophores production may be similar to other copepod species, which can be around 1 per day (Burris & Dam, 2015), though for both the production of gametes is dependent on temperature and food availability (Calbet & Alcaraz, 1996; Burris & Dam, 2015). The eggs are released by females into the water column, and they are nearly spherical with a diameter of around 80 µm (at 19ºC).

Diapause eggs have been identified in *P. grani*, allowing this species to wait for favourable conditions (Boyer & Bonnet, 2018; Guerrero & Rodríguez, 1998). The embryonic development (and hatching) of copepod larvae (nauplii) is strongly influenced by temperature (Herrera et al., 2012; Sumares et al., 2013). At 19ºC, it may take up to two days for them to hatch (personal observation). The nauplii are morphologically different from advanced stages (copepodites), more rounded and shorter, and can be visually identified by the number and disposition of spines at the end of the prosome (Vilela, 1972). Nauplii will undergo five moults until reaching a certain size. The sixth moult determines the transition to a morphology similar to that of the adult, called copepodite (**Fig. 9**). Generally, males develop faster than females and are ready to mate soon after the adult moult (personal observation). For adult females it takes a couple of days to fully mature sexually (at 19ºC). Their development can be completed in 14 days (at 19ºC) reaching maturation. After this period, adults can survive for up to 90 days in laboratory, although the oxidative stress of a constantly high egg production (directly related to food availability) significantly reduces their lifespan (Saiz et al., 2015).

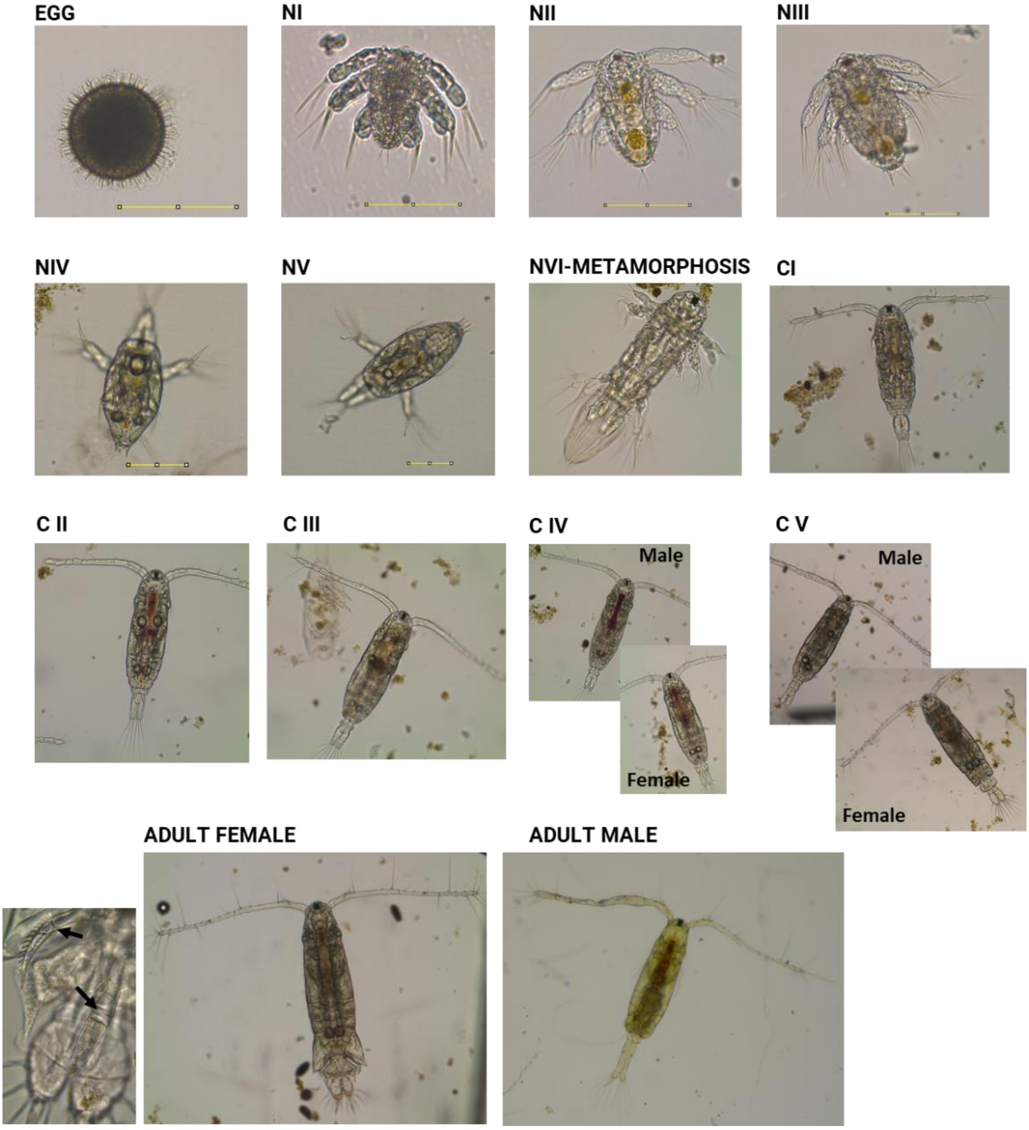


Fig. 9 | Developmental stages of *P. grani* (N: nauplii; C: copepodite). Arrows in adult female figure shows a modified 5th foot and spermatophore of the male attached to the genital segment.

Aims and objectives of this thesis

The capacity of organisms to adapt to changing thermal conditions has profound ecological and evolutionary implications. Temperature stands out as a major physical factor driving the activity of ectotherms. Consequently, the thermal effects on the life-history and physiology of marine zooplankton have been extensively studied in the last century. Nonetheless, studies evaluating the responses to thermal stress across multiple generations in zooplankton are very scarce. **This thesis aims to enhance our understanding of the response of marine copepods, the most abundant group in the zooplankton community, to long-term thermal stress.** We approach this subject experimentally, using the calanoid copepod *Paracartia grani* as a model species. *P. grani* is an ideal candidate for evolutionary studies because its relatively short generation times and biological and ecological similarities with other well-studied Acartia species. We focus on observing the changes in phenotypic traits of *P. grani* across multiple generations of exposure to warming, with the intent to evaluate its adaptive potential and contribute to predicting its ecological trends. It is worth noting that we solely investigated the direct effects of temperature and do not study the indirect effects, which, in nature, will certainly have profound influence on zooplankton performance through changes in phenology and abundance, cell size and composition of their prey.

The thesis is divided into four objectives which are addressed sequentially in Results.

Objective 1. To describe the changes in life-history traits of *P. grani* after multigenerational exposure to warming. Our initial hypothesis was that the overall thermal response (Q_{10} coefficient) of two main physiological processes (ingestion and egg production rates) would be diminished after multigenerational exposure to warming. We predicted that the mechanisms leading to this dampening would be 1) a decrease in thermal sensitivity of these processes after multigenerational exposure, and 2) a thermal-driven shrinkage of body size, which would result in a per capita decrease in metabolic rates. We also examined the possible costs of these adjustments in other life-history traits, such as sex ratio, hatching success, and gross-growth efficiency. This objective is mainly addressed in **Results 3.1** but it is also discussed in **Results 3.2**, **Results 3.3** and **Results 3.4** and further developed in the **Discussion**.

Objective 2. To assess the changes in thermal tolerance in *P. grani* following a prolonged exposure to temperature changes. Multigenerational thermal compensation can alter the tolerance limits and provide an advantage in the case of extreme heat events, such as marine heatwaves. Thus, we determined the thermal performance curves for survival and reproduction of this copepod species following multigenerational rearing under warming conditions (25°C). We hypothesized that multigenerational thermal exposure to warmer temperatures (25ºC) would increase the thermal tolerance of the copepod and also both the T_{opt} and CT_{max} in its reproductive response. This objective is addressed in **Results 3.2.**

Objective 3. To compare different thermal responses of the main physiological processes in *P. grani* and the implications for its metabolic balance. Physiological processes can have different thermal responses, leading to an internal disequilibrium that can limit their efficiency of food utilization. We hypothesized that copepods long-term reared under warmer conditions (>15 generations) would show a higher capacity to maintain their metabolic balance under acute thermal stress. This objective is addressed in **Results 3.3**. The discussion of this chapter involves the interpretation of the carbon budget of *P. grani* under these different warming conditions.

Objective 4. To assess the differences in feeding functional response parameters (I_{max} and K_{m}) and numerical responses between copepods exposed to short- and long-term warming. Based on the results of the previous experiments, we hypothesized that grazing and reproduction under different food concentrations would differ at two different timescales of exposure (4 days and 23 generations). Our working hypotheses were: 1) maximum ingestion rate (I_{max}) will be reduced following long-term rearing under warming compared to a short-term response; 2) half-saturation constant (K_{m}) will also be reduced because of the lower demands of their whole organism basal metabolism; and 3) numerical responses will match the functional responses at all thermal conditions; hence, no effects of thermal stress on gross-growth efficiency would be expected. We also evaluated the effects of changing ingestion and gross-growth efficiency on the stoichiometry of the copepod’s body content. This objective is addressed in **Results 3.4**.

2. CULTURING CONDITIONS

This section provides an overview of the methods used to establish and maintain the laboratory cultures employed throughout this research. Specific methodologies for each experiment are presented in detail in each respective section.

Paracartia grani specimens were obtained from a laboratory culture maintained at the Institut de Ciències del Mar (ICM, CSIC) facilities at 19°C. From this population, eggs were collected to initiate three subpopulations maintained at different rearing temperatures: 19°C (control), 22°C, and 25°C. These subpopulations were used throughout most of the research and were employed at different times to address specific objectives. We chose these temperatures based on increases predicted by IPCC scenarios (+3°C; IPCC, 2019) and extended the range to +6°C to better understand the effects of thermal stress.

The cultures were maintained in a 19ºC (± 1ºC) walk-in chamber in three 150-L baths, each containing two 20 L transparent polycarbonate tanks. The baths were filled with filtered seawater and equipped with TECO® chiller/heater units (TK 500/2000) to maintain constant temperature conditions (19, 22 and 25ºC). The temperature at each bath was continuously monitored with HOBOs (Onset HOBO® Dataloggers) submerged in the water (**Fig. 10A**) and routinely checked with manual thermometers. Inside the copepod tanks, temperature fluctuated at a maximum of ± 0.1ºC (**Fig. 10B**). Led lighting was positioned horizontally above the water baths at a calculated distance to provide a light intensity of 15 - 20 µE m^{-2} s^{-1}. The photoperiod (10:14 light/dark cycle) was synchronized with the walk-in chamber. All tanks were provided with constant low aeration. Food was periodically delivered either by hand or automatically, using dosing pumps (GroTech® TEC 4 NG) with silicon tubing fitted to the tank lids. Automated feeding proved crucial during periods of severe mobility restrictions in Barcelona (March–July 2020) due to the COVID-19 pandemic, ensuring the maintenance of the cultures.

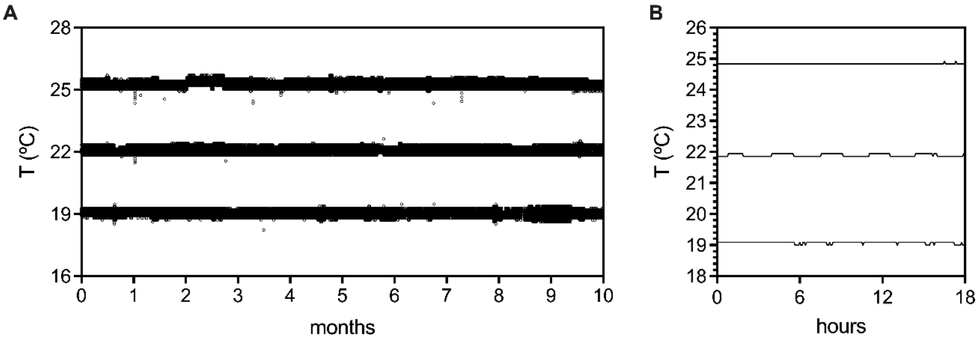


Fig. 10 | **A)** Water temperature inside each water bath (19ºC, 22ºC and 25ºC) during 10 months. **B)** Water temperature fluctuations inside the copepod tanks during 18 hours.

The 150 L water baths were also used to submerge the experimental bottles for incubation and physiological rate assessment during the experiments. When it was necessary to host several temperature treatments (**Results 3.2** and **3.3**) or a large number of experimental bottles (**Results 3.4**), additional water baths (50 L) were set up under the same conditions as described before.

Our experimental populations of *P. grani* originated from a laboratory culture established in 2007-2008 using *P. grani* collected from coastal waters off Barcelona (NW Mediterranean). To initiate the F0 culture, we collected freshly laid eggs (less than 24 hours old) from the stock. Eggs were obtained by siphoning the bottom of the rearing tank and sieving the collected water through 200 µm and 40 µm sieves to remove adult copepods and retain the eggs, respectively. Approximately 20.000 eggs were transferred to a 20-L tank submerged in the 19°C water bath. The initial number of eggs was established based on previous experience to ensure enough animals for sampling, experimentation and future recruitment. This amount was largely maintained (15.000-20.000) during the whole rearing period. Copepod cohorts were grown into adults under food satiation conditions with a mono-diet of the cryptophyte flagellate *Rhodomonas salina*. All food volume added to the rearing tanks, incubations and experiments was calculated based on previous knowledge (Helenius & Saiz, 2017; Olivares et al., 2019). Based on this information, the prey concentrations added in the cohort rearing ranged from 2 ppm for early-stage nauplii to 10 ppm for adults. Prey concentrations were checked three times a week using a particle counter coulter (Beckman Coulter Multisizer III). We routinely monitored, and according to life-stage and copepod density in the tanks, we calculated the volume of *R. salina* to be added. Upon copepods reaching sexual maturity, new produced eggs were collected using the previously described method. Batches of 20000 eggs were transferred to duplicated 20 L tanks filled with filtered seawater pre-acclimated to the corresponding experimental temperature (19ºC, 22ºC and 25ºC) to start the respective F1 at different temperature. We repeated these same procedures throughout the research to generate successive generations. As indicated before, these cultures were also used for experimentation at different generations of rearing (**Fig. 11**).

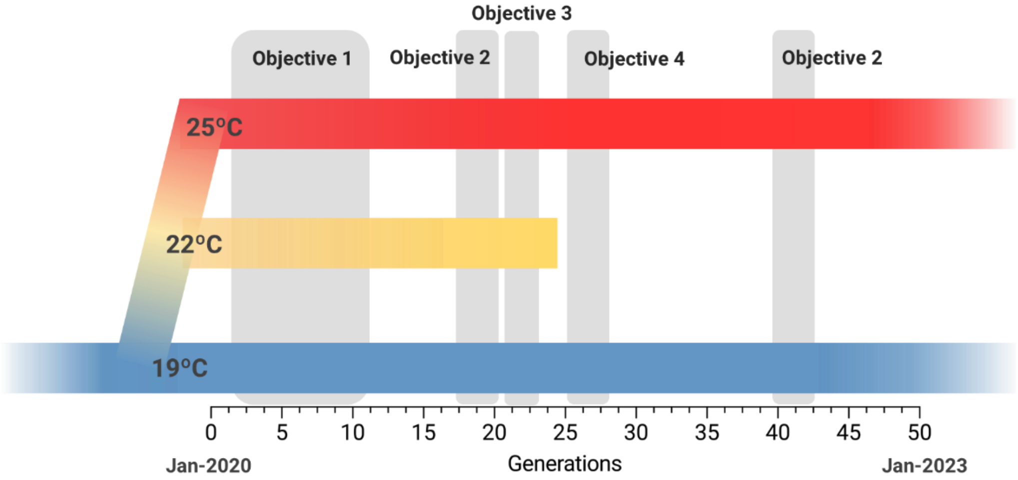


Figure 11 | Experimental design implemented in this thesis. It was observed that copepods developed faster at warmer temperatures, resulting in a progressively widening temporal mismatch between generations at each rearing temperature. The number of generations achieved by the warmer temperatures was used as a reference for the experiments and were compared with the nearer proximate generation of the control (19ºC) copepods available, which were also monitored but assumed to be steady. The 22ºC culture was stopped in March 2022 following the experimentations planned to address **Objective 1** and **3**.

3. RESULTS

3.1 Multigenerational thermal effects on copepod life-history traits

Based on:

de Juan, C., Griffell, K., Calbet, A. and Saiz, E. (2023) Multigenerational physiological compensation and body size reduction dampen the effects of warming on copepods. Limnol Oceanogr, 68: 1037-1047. DOI: 10.1002/lno.12327

ABSTRACT

Under the current ocean warming scenario, multigenerational studies are essential to address possible adaptive changes in phenotypic traits of copepod populations. In this study, we exposed the calanoid copepod *Paracartia grani*, reared in the laboratory at 19°C, to warmer conditions (22°C and 25°C) to investigate the changes in key phenotypic traits in the first, tenth and eleventh generations. Development rates and adult body size were inversely related to temperature in all generations. We also found a decline in copepod egg size at higher temperatures. Temperature had positive effects on the ingestion and egg production rate of females only in the first generation, but the thermal response of the copepods diminished significantly in the consecutive generations. The decrease in thermal effects on feeding and egg production rates after multigenerational exposure cannot be explained only by the shrinkage in body size at warmer temperatures, but also involves the action of physiological compensation. These adaptive processes did not appear to have a significant cost on other traits, such as egg hatching success, gross growth efficiency and sex ratio. Our findings have implications for the prediction of ocean warming effects on copepod activity rates and highlight the importance of physiological adaptation processes after multigenerational exposure.

INTRODUCTION

The response of marine zooplankton to the forecasted rise in ocean temperature is of crucial interest for predicting the functioning of marine pelagic systems (Hays et al., 2005; Richardson, 2008). Within zooplankton, copepods are the most abundant group (Longhurst, 1985). They have key roles in marine food webs as secondary consumers (Banse, 1995; Calbet, 2001) and remineralizers (Alcaraz et al., 1994, 2010), influencing the energy and biochemical fluxes in marine ecosystems (Steinberg & Landry, 2017).

As in most ectothermic animals, the physiological traits of zooplankton species are coupled with the environmental temperature in their habitats (Huey & Stevenson, 1979; Angilletta et al., 2002). The effects of temperature on physiological activity have been extensively studied, particularly for copepods (Kiørboe et al., 1982; Uye, 1988; Holste & Peck, 2006; Cruz et al., 2013). Within their thermal tolerance limits, vital rates of zooplankton respond to acute exposure to temperature according to the Arrhenius law (passive response), with Q_{10} coefficients (fold variation in rate for a 10°C increase) classically assumed to range between 2 and 4 (Saiz et al., 2022). Longer exposure allows the development of physiological compensatory processes (active plasticity) that diminish the phenotypic response of the organism to temperature, resulting in a decrease in Q_{10} values (Schulte et al., 2011; Scheffler et al., 2019; Havird et al., 2020). However, the acclimation capacity of an organism is limited and may have associated costs that challenge its persistence capacity (Angilletta et al., 2003; Alcaraz et al., 2014; Souissi et al., 2021). At longer timescales, comprising multiple generations, other compensatory mechanisms at phenotypic and genotypic levels can come into play and modify the thermal response of the species (Sanford & Kelly, 2011; Dam, 2013). Recent advances addressing the multigenerational effects of temperature on copepod traits (i.e. fecundity, recruitment, body size and heat tolerance) suggest that adaptive shifts can be indeed rather rapid, within a few generations (Souissi et al., 2016; Kelly et al., 2017; Dam et al., 2021).

Temperature also affects body size owing to the different shapes of the thermal responses of developmental and growth rates (Forster & Hirst, 2012). The shrinkage of body size driven by warming is generalized in nature (Sheridan & Bickford, 2011) and has also been observed in copepods (Rice et al., 2015; Evans et al., 2020). These shifts in size associated with temperature can affect trophic interactions and have important ecological consequences (Sheridan & Bickford, 2011; Brosset et al., 2016). Body size also influences metabolism (Gillooly et al., 2001). As body mass decreases, the metabolic rate declines and the mass-specific metabolic rate increases (Gillooly et al., 2001). In copepods, physiological rates such as feeding, egg production and respiration scale with body mass (Runge, 1984; Ban, 1994; Saiz & Calbet, 2011). Therefore, the direct effects of temperature on physiological rates are expected to overlap with those driven by the reduction in body size also induced by warming (Riemer et al., 2018).

In this study, we have addressed the multigenerational response of the marine calanoid copepod *Paracartia* (formerly *Acartia*) *grani* to temperature. *P. grani* is a well-studied small coastal species (ca. 1 mm; Calbet et al., 1999; Olivares et al., 2019) with broadcasting reproductive behaviour whose distribution extends along a thermal gradient from cold northeast Atlantic waters to the warmer Mediterranean Sea (Boyer et al., 2012). Because *P. grani* has a short lifespan, its populations undergo a few generations annually (Ianora, 1998), which make it suitable for multigenerational experiments. In our study, we exposed a parental population of *P. grani*, reared at 19°C in the laboratory, to warmer conditions (22°C and 25°C) for 11 generations and examined the direct effects of temperature on feeding, fecundity, development, population sex ratio and somatic traits (adult and egg sizes and adult carbon content). The range of increased temperatures used was based on the IPCC's extreme prospects for long-term ocean warming (IPCC, 2022) and also encompass shorter-period phenomena such as the severe, months-lasting marine heatwaves that have been increasingly present in the Mediterranean in recent years (Garrabou et al., 2022). We expected that the overall thermal response (Q_{10} coefficient) of two main physiological processes (ingestion and egg production rates) would be diminished after multigenerational exposures to warming. We hypothesized that the mechanisms of this dampening effect would be 1) a decrease in thermal sensitivity to these processes after multigenerational exposure, and 2) a thermal-driven shrinkage of body size, which would result in a per capita decrease in metabolic rates. We also examined the possible costs of these adjustments in other life-history traits, such as sex ratio, hatching success and gross growth efficiency.

MATERIAL AND METHODS

Experimental organisms, rearing conditions and thermal exposure

Specimens of *Paracartia grani* were originally collected in coastal waters near Barcelona (NW Mediterranean) and have been kept in culture at the Institut de Ciències del Mar (ICM-CSIC) for >14 years in 20 - 40 L tanks (Saiz et al., 2015). The copepod culture is kept in a temperature-controlled room at 19 ± 1°C with a 10:14 light/dark cycle and routinely fed ad libitum three times a week with the cryptophyte *Rhodomonas salina* (strain K-0294, Scandinavian Culture Collection of Algae and Protozoa) grown in f/2 medium.

To start the F0 culture, we collected eggs spawned during the past 24 h from the stock culture and transferred 20,000 of them into a 20-L tank submerged in a water bath at 19ºC, where eggs were allowed to hatch and copepods to grow under excess *R. salina*. The number of eggs was established on the basis of previous experience (Saiz et al., 2015; Olivares et al., 2019) to ensure enough animals for sampling, experimentation and future recruitment. The procedure for collecting the eggs consisted in siphoning the bottom of the tank and sieving the collected water through sieves of 200 μm (to remove any adults present) and 40 μm (to collect the eggs).

Once F0 reached adulthood, we collected eggs from the bottom of the tank and transferred them in batches of 20,000 eggs to duplicated 20-L tanks filled with filtered seawater previously acclimated to the corresponding experimental temperatures (19°C, 22°C and 25°C). These tanks were introduced into three 150-L water baths provided with TECO water-temperature conditions set at 19°C, 22°C and 25ºC and diode lighting (15 - 20 μE m^{-2} s^{-1}; 10:14 light/dark cycle), where they were kept throughout the multigenerational rearing study. The copepods were routinely fed with *R. salina*, supplied in different amounts to maintain satiation during development (i.e., concentrations ranging from 2 ppm for early-stage nauplii to 10 ppm for adults; Helenius and Saiz 2017; Olivares et al. 2019). We checked the prey concentration in the tanks three times per week using a Beckman Coulter Multisizer III particle counter and adjusted it to the desired concentrations according to parallel estimations of copepod abundance and stage composition. After the first thermal-exposed generation (F1) at each experimental temperature was raised and adults were present in the cohort, we repeated the aforementioned procedure to generate successive generations (F2, F3,…) for each thermal treatment until the 11th generation was reached (5 - 6 months). Previous evidence suggests that 3 - 6 generations might be enough for copepods to adapt to a new temperature in terms of reproduction and foraging capacities (Souissi et al., 2016; Dam et al., 2021). Therefore, we assumed that full thermal adjustment was reached after ten generations (F10) and repeated the experiments one generation later (F11) to better assess the differences from F1.

Adult development time and adult sex ratio

Adult development times at 19°C, 22°C and 25°C were assessed for the F1 and F10 generations. Throughout the development of the cohorts, we took water samples every 24 h from each rearing tank after thorough mixing; sample volumes ranged from 250 mL for nauplii to 500 mL for copepodites and 1000 mL for adults. The samples were sieved through a 40-μm sieve, and the copepods were preserved in 4% formaldehyde. Later, the specimens were counted and photographed under an Olympus SZX12 stereomicroscope and identified from copepodite IV to the adult stage. We calculated the adult male and female median development times by fitting a Hill sigmoidal function to the observed frequencies and then assessing the time required for 50% of the individuals to moult to that stage (Landry, 1983). The adult sex ratio was calculated as the quotient between the mean abundance of adult males and adult females estimated from the last three samplings, when adult stages dominated the population.

Feeding and fecundity experiments

Feeding and fecundity experiments were carried out on the F1, F10 and F11 generations. The experiments consisted in 24 h incubations to determine feeding and egg production rates and posterior incubation of the spawned eggs to assess hatching success. Prey concentrations and numbers of copepods in the incubations were chosen using previous feeding rate data to ensure feeding saturation conditions (i.e., maximum ingestion and egg production rates) and to produce a reduction of 20 - 30% of the prey concentration during the incubation (Olivares et al., 2019). To avoid ageing effects (Saiz et al., 2015), we started every experiment 2 - 5 days after the copepods had moulted into the adult stage; at that time, females were already fertilized and producing eggs. For each temperature treatment, we prepared four control and eight experimental 610-mL Pyrex bottles (four for females and four for males) filled with 7 ppm (approx. 1120 μg C L^{-1}) of *R. salina*. We sorted the copepods under the stereomicroscope, and with the aid of a wide-bore Pasteur pipette we introduced either 18 - 28 females or 45 - 80 males into each experimental bottle, depending on the treatment (Olivares et al., 2019). All bottles were amended with 5 mL of f/2 medium per litre. Then, the bottles were placed in the corresponding temperature-controlled baths and regularly rotated by hand end-over-end (2 - 3 times per day) to homogenize their contents. After 24 h, the bottle contents were sequentially screened through 100- and 20-μm sieves to retain the copepods and the eggs, respectively. Initial and final algae concentrations in the incubations were measured using the Coulter Multisizer. The copepods were checked for survival under the microscope and then preserved in 4% formaldehyde. Subsequently, we photographed random individuals ($n = 28$ for each duplicated tank, treatment, generation and sex; total $n = 1008$) under the stereomicroscope and measured adult prosome length using FIJI ImageJ software. The egg samples were divided into four aliquots. One of them was immediately preserved in 2% Lugol’s solution to determine egg production rates. To assess hatching success, we further incubated the remaining aliquots in 50-mL vials (filled with filtered seawater and 1 ppm of *R. salina*) at their respective temperatures and consecutively preserved them in 2% Lugol’s solution after 24, 48 and 72 h. Eggs, egg shells and nauplii were counted using an inverted microscope (Nikon Diaphot 200). Egg diameter was determined from pictures ($n = 30$ per treatment and generation; total $n = 270$) by fitting an ellipsoid using the ImageJ software and calculating the average of the fitted major and minor axes (Saiz et al., 2020).

Copepod ingestion rates were calculated using Frost’s equations (Frost, 1972), which consider the disappearance and growth of prey in experimental and control bottles, respectively, assuming exponential models. Egg production rates were computed as the total number of eggs and hatched nauplii divided by the number of females in the incubation and the duration of the incubation. Hatching success was calculated as the percentage of nauplii in relation to the total number of eggs laid. For 22°C and 25°C, we used the data for the 48-h incubation, whereas for 19°C the samples after 72 h were used to ensure that the hatching plateau was reached (**Supplementary Fig**. **S1**).

Carbon-specific rates

Carbon ingestion rates were obtained by computing ingestion rates in prey biovolume units from the Coulter counter and then using *R. salina* carbon contents estimated from cell volume using the factor 0.17 pg C μm^{-3} (Saiz et al., 2020). Similarly, egg production rates were transformed into carbon units from egg volume (estimated from the measured diameter) by applying the factor 0.129 pg C μm^{-3} (Saiz, Griffell and Calbet, 2020; C. de Juan, unpubl.). To normalize physiological rates in terms of copepod body carbon, parallel to the feeding and fecundity incubations, we took additional copepod samples to assess the adult carbon content for each treatment and generation. The copepods were left for approximately 30 minutes in 0.1-μm filtered seawater to empty their guts, and then they were narcotized with MS-222 (Saiz et al., 2015) and transferred into groups of 25 - 40 females and 60 males into precombusted 25-mm Whatman GF/C filters (450°C, 5 h). The filters were stored at -80°C until analysis. Prior to analysis, the filters were thawed and then dried for 48 h at 60°C and finally processed with a Thermo Finnigan Flash EA1112 CHNS analyser. Gross growth efficiency was obtained by dividing the carbon egg production rate by the respective carbon ingestion rate.

Statistical analysis

Simple linear regression analysis was performed to assess the statistical significance of the relationship between copepod physiological traits and temperature. When the relationship was anticipated to follow the Arrhenius law and be exponential (median development time, feeding and egg production; Almeda et al. 2010a; Saiz et al. 2022), the dependent variable was ln-transformed, and Q_{10} coefficients were estimated from the slopes as exp(10 × slope) (Ikeda, 1985). When the slopes were not statistically different from 0, meaning that no thermal effect was observed, we assigned to the Q_{10} a value of 1. Statistical analyses were conducted in Prism v.7. Unless otherwise stated, mean values ± SE are provided.

RESULTS

Median development times decreased exponentially with temperature (linear regression on ln-transformed data, $p < 0.01$ in all cases; **Table 1**); the 6°C difference between the lowest and the highest temperatures resulted in adult development times 4 - 5 d shorter. Covariance analysis showed that the rate of change with temperature did not differ between males and females in any of the generations (test for slope: $p > 0.6$ for both F1 and F10; **Table 1**); in F1, male median development times were slightly, but significantly, shorter (covariance analysis, test for intercept: $p = 0.33$), whereas this difference disappeared in F10 ($p > 0.7$) (**Table 1**). Overall, median development times for F10 were higher than those for F1 (on average 14 - 15% difference; covariance analysis, test for intercepts: $p < 0.001$ for both male and female comparisons; **Table 1**). Sex ratios were close to 1 (**Table 1**; t-tests, $p > 0.09$ in all cases) and did not show a significant relationship with temperature in either F1 or F10 (**Table 1**; linear regression, $p > 0.07$ in both cases).

Table 1 | Median development time (d) and sex ratio (male/female) of *P. grani* reared at 19ºC, 22ºC and 25ºC in F1 and F10 generations. Average ± SE are shown.

Generation	T(ºC)	MDT (d) Female	MDT (d) Male	Sex ratio
F1	19ºC	14.1 ± 0.08	13.9 ± 0.17	1.1 ± 0.07
	22ºC	11.2 ± 0.02	10.9 ± 0.07	1.1 ± 0.09
	25ºC	8.9 ± 0.01	8.8 ± 0.09	1.1 ± 0.05
F10	19ºC	15.5 ± 0.55	15.4 ± 0.85	0.9 ± 0.06
	22ºC	13.5 ± 0.20	13.2 ± 0.15	1. 1 ± 0.08
	25ºC	10.0	10.0	1.1 ± 0.06

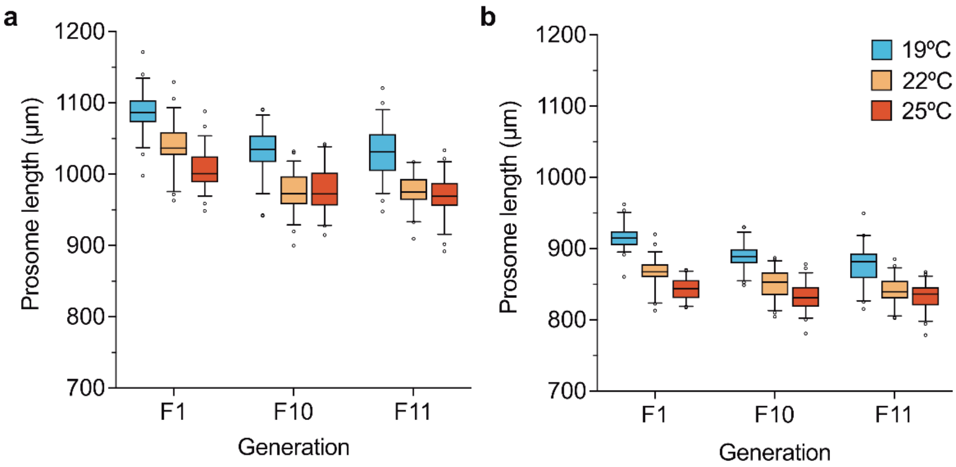


Figure 12 | Prosome length of adult female (**a**) and male (**b**) *P. grani* as a function of temperature and generation. Box plots show median values and 25 - 75 percentiles, while the whiskers show the 5 - 95 percentiles.

Figure 12 shows the prosome length of the female and male *P. grani* as a function of the experimental temperature and rearing generation. The copepods' prosome length was inversely related to temperature, both within gender and across generations (linear regression, $p < 0.001$ in all cases). On average, the 6°C temperature increase resulted in a body size decrease in F1 of 7.8% and 7.1% for males and females, respectively. The effect of temperature on size reduction decreased with time to 6.3% and 5.0% for males and to 5.1% and 5.9% for females in F10 and F11, respectively (**Fig. 12a,b**; covariance analysis, test for slopes: $p = 0.012$ and $p < 0.001$ for females and males, respectively). This pattern was linked to a generalized decrease in the adult prosome length over time, which was particularly evident when the male and female body sizes for the 19°C treatment were compared through generations (one-way ANOVA, $p < 0.001$ in both cases).

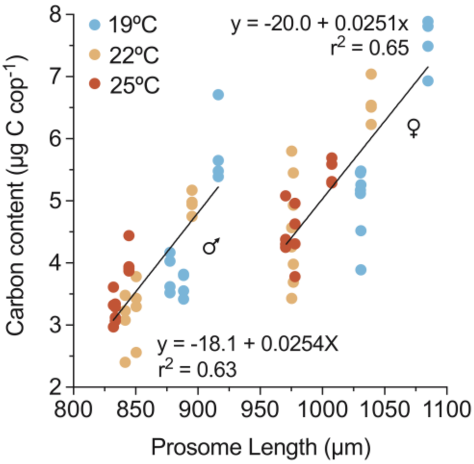


Fig. 13 | Relationship between prosome length and carbon content of adult female and male *P. grani*. Regression equations are shown. Data from all generations are combined.

The mean carbon content of *P. grani* ranged from 7.5 ± 0.2 *µ*g C at 19°C (F1) to 4.4 ± 0.3 *µ*g C at 25°C (F10) for females and from 5.8 ± 0.3 *µ*g C at 19°C (F1) to 3.1 ± 0.2 *µ*g C at 22°C (F11) for males (**Fig. 13**). The differences in copepod carbon content were positively related to prosome length (linear regression analysis; *p* < 0.001 for both males and females).

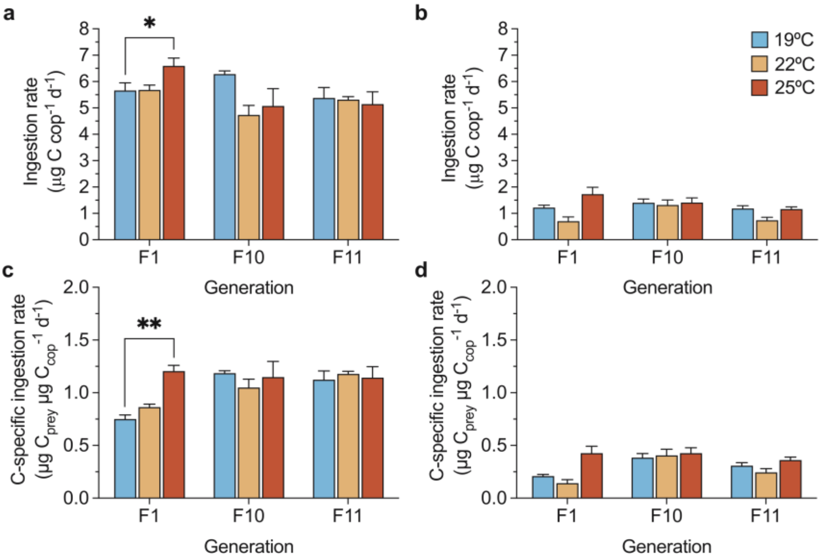


Fig. 14 | Per capita and carbon-specific ingestion rates of adult female (**a**,**c**) and male (**b**,**d**) *P. grani*. Error bars show standard error. Asterisks highlight statistically significant relationships with temperature (linear regression of ln-transformed data; *p < 0.05, **p < 0.001).

For copepod ingestion rates, expressed as μg C cop^{-1} d^{-1}, only females in F1 showed a significant trend with temperature (linear regression on ln-transformed data, $p = 0.034$), resulting in a Q_{10} coefficient of 1.28 (**Fig. 14a**). After multigenerational thermal exposure (F10, F11), however, neither female nor male feeding rates were related to temperature (linear regression on ln-transformed data, $p > 0.12$ in all cases) (**Fig. 14a,b**).

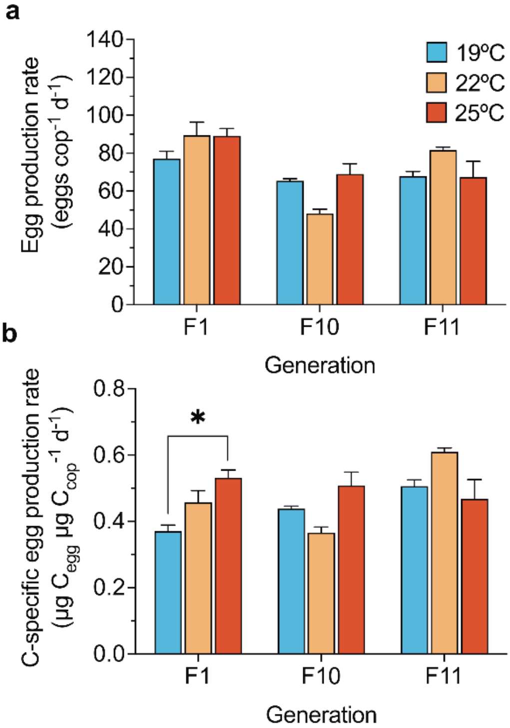


Fig. 15 | Per capita (**a**) and carbon-specific egg production rates (**b**) of *P. grani*. Error bars show SE. Asterisk highlights statistically significant relationships with temperature (linear regression of ln-transformed data; *p < 0.001).

Like the per capita rates, carbon-specific ingestion rates for females in F1 showed a statistically significant relationship with temperature ($Q_{10} = 2.15$; linear regression of ln-transformed data, $p < 0.001$; **Fig. 14c**). After multigenerational rearing (F10 and F11), however, carbon-specific female feeding rates showed no significant trend with temperature (linear regression of ln-transformed data, $p > 0.66$; **Fig. 14c**). For males, we found no statistically significant relationship with temperature in any generation (linear regression of ln-transformed data, $p > 0.17$ for F1, $p > 0.39$ for F10 and F11; **Fig. 14d**).

Egg production rates on a per capita basis showed no clear relationship with increasing temperature in any of the three generations (linear regression on ln-transformed data, $p > 0.12$ in all cases; **Fig. 15a**); mean values were 85 ± 3.1, 61 ± 3.3 and 72 ± 3.3 eggs cop^{-1} d^{-1} for F1, F10 and F11, respectively. However, carbon-specific egg production rates in F1 showed a significant trend with increasing temperature ($Q_{10} = 1.79$; linear regression on ln-transformed data, $p < 0.001$; **Fig. 15b**). We found no statistically significant trend with temperature in the successive generations ($p > 0.24$; **Fig. 15b**).

Table 2 | Hatching success of the eggs of *P. grani* reared at 19ºC, 22ºC and 25ºC in F1, F10 and F11. At 19°C, the hatching incubations lasted 72 h, whereas at 22°C and 25°C, they lasted 48 h (see text and **Supplementary Fig. S1**). Average ± SE are shown.

T(ºC)	F1	F10	F11
19ºC	91.1 ± 1.4	91.2 ± 2.0	89.9 ± 0.7
22ºC	85.7 ± 1.6	84.3 ± 3.3	94.5 ± 0.8
25ºC	83.8 ± 2.4	88.0 ± 2.6	88.6 ± 2.4

Egg diameter of *P. grani* declined overall with temperature, although differences were only statistically significant in F10 and F11 (linear regression analysis, $p = 0.037$ and $p = 0.034$ for F10 and F11, respectively; **Fig. 16a**). In addition to likely direct effects of temperature on egg diameter, we observed that egg diameter was positively related to female prosome length (linear regression analysis, $p = 0.028$; **Fig. 16b**), suggesting an adult size effect. In F1, hatching success was influenced by temperature and decreased by 8% for the 6ºC warming interval; this relationship with temperature was not observed in the successive generations (**Table 2**).

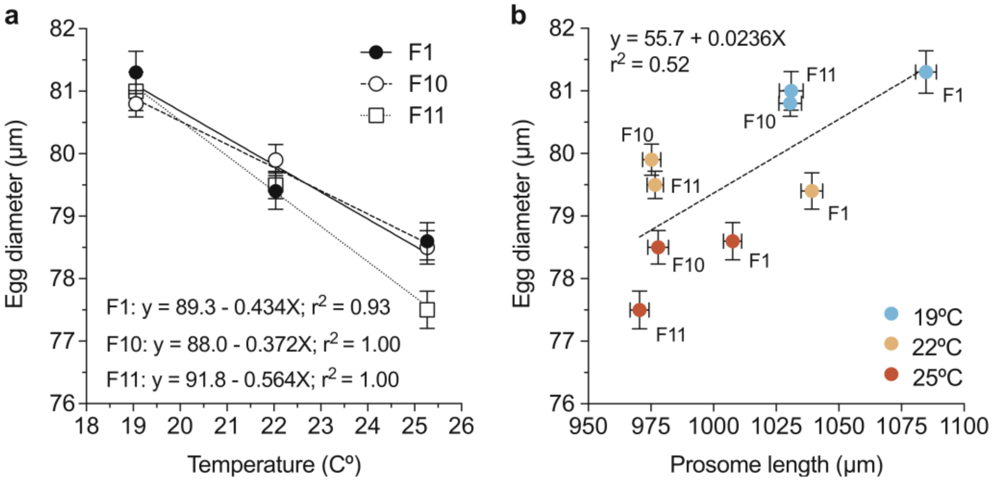


Fig. 16 | (**a**) Egg diameter of *P. grani* as a function of temperature and generation. (b) Relationship between egg diameter and adult female prosome length. Regressions equations are shown. Error bars show SE.

The carbon gross growth efficiency of female *P. grani* showed no statistically significant relationships with temperature in any of the generations (linear regression analysis, $p > 0.27$ in all cases; **Fig. 17**), with a grand mean value of 44 ± 1% across temperatures and generations.

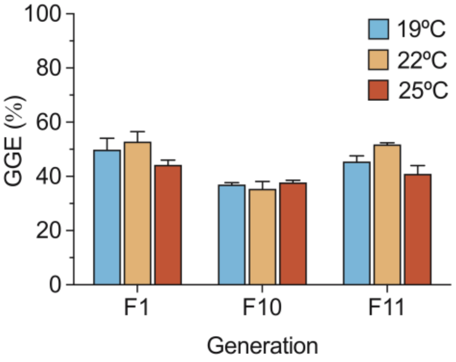


Fig. 17 | Gross growth efficiency of adult female *P. grani* as a function of temperature and generation. Error bars show SE.

DISCUSSION

Thermal effects on somatic traits and development times

The inverse relationship between body size and temperature in copepods has been well known for decades (McLaren, 1965; Klein Breteler & Gonzalez, 1988), and it has received renewed interest lately owing to ocean warming (Rice et al., 2015; Evans et al., 2020; Campbell et al., 2021). The decrease in copepod body size with warming is expected to have a great effect on the performance of the organisms because of its close relationship with metabolism (Gillooly et al., 2001). In our experiments, as expected, we found a strong relationship between copepod body size and temperature. The observed reduction in prosome length with temperature was, on average, 1.2% $^{\circ}C^{-1}$ and was associated with a decrease in body carbon content of 3.7% $^{\circ}C^{-1}$; these are similar to the values previously reported for calanoid copepods (Horne et al., 2016). The relationship between temperature and copepod body size is driven by the different thermal sensitivities between development and somatic growth rates (Forster & Hirst, 2012). In our case, mean development times showed a similar negative relationship with temperature, with no differences in the slope between F1 and F10. The development times of *P. grani* in our experiments were comparable to those reported for the same or other closely related species at similar temperatures (Landry 1975; Saiz and Alcaraz 1991, Calbet and Alcaraz 1997).

It was rather unexpected to observe that copepod body size decreased overall after multigenerational rearing at all temperatures. However, in some previous studies, similar observations for body size changes over long timescales in laboratory reared copepods have been reported (Klein Breteler & Gonzalez, 1988; Klein Breteler et al., 1990). Changes in food availability and crowding effects are among the main factors attributed to the shifts in body size (Franco et al., 2017). In our multigenerational rearing, however, the food satiating conditions (Olivares et al., 2019) and low copepod density in the rearing tanks (from 1000 eggs L^{-1} initially to 186 - 316 adults L^{-1}) at the time of carrying out incubation experiments and recruiting eggs for the next generation (Jepsen et al. 2007; Chintada et al. 2021; Wilson et al. 2022) make these factors unlikely to have influenced copepod body size. Another plausible explanation could be that the overall experimental setup, with a very regular rearing routine (more stable temperatures and satiating food conditions), cumulatively affected the copepod size in all treatments. Nevertheless, given that this influence was homogeneous in all treatments, the effects of temperature were still evident, as demonstrated by the similarity of the slopes of the relationship of body size with temperature between generations.

The effects of temperature also affected egg size. The reduction in egg diameter (0.6% $^{\circ}C^{-1}$), however, was smaller than the 1.2% $^{\circ}C^{-1}$ observed reduction in adult body size. Hansen et al. (2010), for a similar temperature range (17 - 24ºC), also reported variations in copepod body and egg sizes of the order of 1.4% $^{\circ}C^{-1}$ and 0.4% $^{\circ}C^{-1}$, respectively. It is worth noting, however, that temperature can also directly affect copepod egg size (Saiz et al. 2022), so the observed egg size is likely the result of the combined direct effects of temperature and adult size dependence.

Thermal effects on physiological rates

The positive effects of warmer temperatures on the physiological rates of females of *P. grani* only appeared in the first generation, and this influence dampened at longer thermal exposures. In the case of males, there was also a positive trend of feeding rates with temperature in F1 (albeit not significant), which in successive generations also dampened. The drop in thermal response found in males and females in our experiments was greater than that reported for the same species by Saiz et al. (2022), who found a decrease in the Q_{10} coefficients from 2.6 - 2.7 for an acute response (24 h) to 1.6 - 1.7 after a 7-d acclimation. It seems, therefore, that long-term exposure (multigenerational rearing) has deeper effects on the thermal compensation of physiological rates, levelling out the response to temperature and evidencing a notable phenotypic plasticity in the copepod species, which may be related to the coastal environments that constitute its habitat and the broad latitudinal distribution (Saiz et al. 2022).

Under a warming ocean scenario, chronic exposure to higher temperatures through multiple generations may allow other mechanisms, at phenotypic and genotypic levels, to modulate the thermal response (Angilletta et al., 2003; Dam, 2013). Thus, within-generation and transgenerational (maternal effects) plasticity are increasingly examined for their role in evolutionary potential in the face of climate change (Donelson et al., 2018). For instance, the temperature experienced at earlier development stages can “carry over” to the next one (Donelson et al., 2018; Moore et al., 2019), and females may produce offspring better fitted for their environmental conditions (Vehmaa et al., 2012). It has been shown that phenotypic traits of adaptive value can be inherited in a few generations (Lee et al. 2013; Souissi et al. 2016). More recently, Dam et al. (2021) suggested that modifications at the genotype level that change the thermal response can occur quite rapidly, in just three generations. In this regard, the F1 copepods in our experiments experienced a temperature rise during embryogenesis and development. This fact may explain some of the discrepancies found between F1 (raised from the stock culture F0) and later generations in our experiments.

Combined effects of thermal compensation and body size reduction

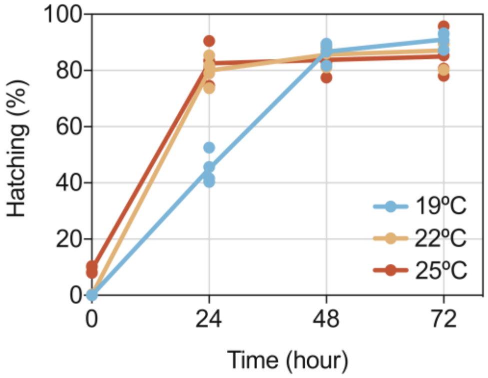
Body size influences copepod ingestion and egg production rates (Runge, 1984; Ban, 1994; Saiz & Calbet, 2011). Therefore, we would expect an interaction between the direct effects of temperature on metabolism and those mediated by the thermal-induced body size change (Riemer et al., 2018). Based on our results, we explored the relative contribution of body size change and physiological compensation to the dampening of physiological rates that we found in our experiments after multiple generational thermal exposure. To do this, we took the per capita female feeding rates at 19ºC for each generation and applied a Q_{10} value from literature for *P. grani* (Q_{10} = 1.6; Saiz et al. 2022) to estimate the expected temperature-mediated increase in ingestion. Subsequently, we corrected the obtained feeding rates for allometric effects applying a body weight scaling factor of 0.743 (Saiz & Calbet, 2007), in order to take into account the decrease in body size (carbon content) at warmer temperatures. In F1, the body size reduction could largely explain (70% at 22ºC and 129% at 25ºC) the copepod’s thermal response under warming conditions **(Supplementary Fig. S2).** In F10 and F11, however, the observed decline in body size at warmer temperatures could explain, respectively, only a 19 - 23% and 10 - 21% drop in the copepod feeding rates, the rest being attributed to the thermal compensation processes in the organism’s physiology.

We must note that, whether plastic or adaptive, the underlying processes adjusting the thermal response to new conditions can be beneficial for some traits but may have a cost for the organism in other traits (Angilletta et al., 2003). A trade-off commonly considered in life-history theory is the expenditure of resources by the basal metabolism and reproductive effort. Here, we have reported an enhancement of feeding and fecundity rates caused by warming in the first generation reared. However, we found no long-term negative effect of temperature on gross growth efficiency or sex ratios, which were never significantly different from one, as expected for copepod species with no seminal receptacles that require frequent remating (Kiørboe, 2006). Hatching success showed a significative negative trend with an 8% reduction in F1, but a recovery across generations. This result implied that thermal compensation after long-term exposure achieved homeostatic balance and that no detrimental effects appeared.

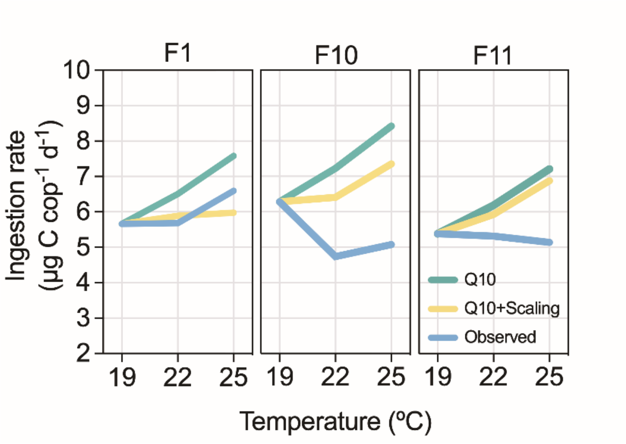
Implications

The thermal plasticity in body size played a significant role in buffering the thermal effects on the per capita ingestion and egg production rates in the first generation of exposure to warming. After multigenerational exposure, however, other processes (either plastic or adaptive) came into play and further reduced the thermal effects on physiological rates, with no apparent cost for the life-history traits studied. It seems evident from our study that long-lasting exposure to warming such as that expected under future global scenarios will not necessarily imply higher rates of metabolic activity by copepods. Therefore, the use of Q_{10} coefficients of 2 - 3 for climate change models could overestimate the actual impacts of thermal change in copepod-mediated biogeochemical fluxes (Saiz et al., 2022). However, the variability between generations calls for caution in extrapolating our results. Also, our study was carried out with copepods from a long-term laboratory culture, and field animals may have higher genetic variability that allows other adaptive responses. Previous reports have shown that laboratory-reared copepods can lose some adaptive traits (Tiselius et al., 1995; Calbet et al., 1999), even in a few generations (Olivares et al., 2020). We must also keep in mind that in nature the effects of global warming on marine copepod populations will also encompass more complex responses, involving biogeographical and phenological changes and interactions with other components of the pelagic ecosystem, such as predators and prey, which in turn may also be affected by temperature (Beaugrand et al., 2002; Richardson, 2008; Garzke et al., 2015).

SUPPLEMENTARY INFORMATION



Supplementary Fig. S1 | Hatching success of *P. grani* eggs as a function of time and temperature. Only F1 data are shown.



Supplementary Fig. S2 | Observed and predicted ingestion rates of adult female *P. grani* as a function of temperature and generation. Predicted values were calculated using either the temperature effect (Q_{10} = 1.6) alone or a combined effect of temperature and body size shrinkage.

3.2 Shifts in the thermal limits for copepod survival and reproduction after chronic warming

Based on:

de Juan, C., Calbet, A., and Saiz , E. (2023) Shifts in survival and reproduction after chronic warming enhance the potential of a marine copepod to persist under extreme heat events, *J. Plankton Res.*, Vol. 45, Issue 5, 751–762, DOI: 10.1093/plankt/fbad037

ABSTRACT

The study of a species' thermal tolerance and vital rates responses provides useful metrics to characterize its vulnerability to ocean warming. Under prolonged thermal stress, plastic and adaptive processes can adjust the physiology of organisms. Yet it is uncertain whether the species can expand their upper thermal limits to cope with rapid and extreme changes in environmental temperature. In this study, we reared the marine copepod *Paracartia grani* at control (19°C) and warmer conditions (25°C) for >18 generations and assessed their survival and fecundity under short-term exposure to a range of temperatures (11-34°C). After multigenerational warming, the upper tolerance to acute exposure (24 hours) increased by 1-1.3°C, although this enhancement decreased to 0.3-0.8°C after longer thermal stress (7 days). Warm-reared copepods were smaller and produced significantly fewer offspring at the optimum temperature. No shift in the thermal breadth of the reproductive response was observed. Yet the fecundity rates of the warm-reared copepods in the upper thermal range were up to 21-fold higher than the control. Our results show that chronic warming improved tolerance to stress temperatures and fecundity of *P. grani*, therefore enhancing its chances to persist under extreme heat events.

INTRODUCTION

Marine organisms are currently facing the combined effects of a gradual increase in the mean ocean heat content and an escalation in the frequency, duration, and intensity of extreme thermal events (Smith et al., 2023). Consequences of such phenomena are already being observed globally, with studies reporting severe reductions in species abundance (Garrabou et al., 2022; Edgar et al., 2023), phenological (Richardson, 2008), and distributional shifts (Evans et al., 2020) and re-organization of species assemblages (Benedetti et al., 2021) that result in profound and lasting alterations in marine ecosystems (Suryan et al., 2021; Batten et al., 2022). In this context, studying the thermal tolerance and temperature effects on the vital rates of species can help assess their responses to changing ocean temperatures and predict ecological trends (Pörtner & Farrell, 2008; Huey et al., 2012;).

Among marine organisms, copepods represent one of the most abundant and diverse groups, inhabiting almost all marine environments (Ibarbalz et al., 2019). Given their pivotal role in marine food webs and global biogeochemical cycles, they are particularly compelling subjects for investigation in the face of rising ocean temperatures (Steinberg & Landry, 2017). Global patterns in the heat tolerance of this group are primarily influenced by specific habitats and annual maximum temperatures (Sasaki & Dam, 2021). However, changes in habitat temperatures over time can lead to alterations in organismal heat tolerance. Copepods may exhibit a degree of plasticity in their thermal tolerance, allowing them to acclimate to rapid changes in ambient temperature (Jiang et al., 2008), similar to other groups of ectotherms (Gunderson et al., 2017; Morley et al., 2019). However, prolonged duration of stress strongly affects survival (Jiang et al., 2008; Rezende et al., 2014), although this factor is often overlooked when assessing species' heat tolerance. Long-term warming may result in an increase in heat tolerance through the action of intra- and intergenerational responses (Geerts et al., 2015). Nonetheless, this enhancement could be constrained by physiological limits (Kelly et al., 2012; Morgan et al., 2020). Furthermore, following an enhancement in heat tolerance the acclimation capacity to future extreme temperatures could be reduced (van Heerwaarden & Kellermann, 2020; Sasaki & Dam, 2021). In this regard, there is a scarcity of studies that experimentally address changes in heat tolerance through multiple generational exposure in marine zooplankton.

The range of temperatures within which vital processes, such as reproduction, can take place is often narrower than the range required for survival (Halsband-Lenk et al., 2002; Feng & Papes, 2017). Consequently, at sublethal temperatures, adverse effects on fecundity can impose stricter limits on local persistence, resulting in a decline in population size. Therefore, considering both survival and reproductive responses can offer valuable insights into an organism's capacity to withstand changing ocean temperatures.

Life-history characteristics, such as reproductive rates, typically show unimodal responses (Saiz et al., 2022), where rates increase exponentially as the temperature rises until reaching an optimum temperature (T_{opt}). At this T_{opt}, rates are at maximum (R_{max}), and then they decline rapidly until the loss of the function occurs (CT_{max}) (Angilletta et al., 2002). Under prolonged thermal stress, adjustments in the physiology of organisms can lower the R_{max} by reducing the thermal sensitivity of metabolic processes or displace the T_{opt} and CT_{max} along the thermal gradient (Schulte et al., 2011; Seebacher et al., 2015). Predictably, these adaptive responses in vital rates and thermal tolerance are expected to emerge from changes at the phenotypic and genotypic level and play a fundamental role in the thermal response of organisms to habitat conditions, especially for those that, as copepods, show fast development and short generation times (Vehmaa et al., 2012; Dam, 2013; Dam et al., 2021). However, it is uncertain if both limits can be expanded to an extent that allow these organisms to persist under changing ocean temperatures.

In this study, we compared the thermal tolerance and reproduction response to short-term thermal stress (15-34°C) of a parental population of the marine copepod *Paracartia grani*, which had been cultured in the laboratory under stable conditions (19°C) for over a decade, with a descendent line of *P. grani* reared at a warmer temperature (25°C) for more than 18 generations. *P. grani* is a calanoid copepod member of the Acartidae family, a taxonomic group found in aquatic habitats worldwide (Belmonte, 2021). This species is commonly found in coastal and semi-enclosed waters of the North and South East Atlantic, as well as in the Mediterranean Sea (Rodríguez & Vives, 1984; Boyer et al., 2012; Razouls et al., 2005-2023). In the western Mediterranean, this species can be found year-round over a wide thermal range (8.3-23.8°C), with peak occurrences between spring and autumn (Rodriguez & Jiménez, 1990).

In laboratory conditions, this species has been shown to tolerate an even broader range of temperatures, from 5.7 to 32°C (Saiz et al., 2022), and exhibit a high capacity to acclimate to thermal stress (Saiz et al., 2022; de Juan et al., 2023a). The main questions addressed in our study were whether survival and reproduction are enhanced by multigenerational rearing under warming conditions (25°C), and to what extent these shifts can provide an advantage for this species in case of extreme heat events. We hypothesized that multigenerational thermal exposure to warmer temperatures would enhance the thermal tolerance of this species and also improve both the T_{opt} and CT_{max} in its reproductive response. Additionally, building upon previous research (de Juan et al., 2023a), we investigated whether the magnitude of the effects of a rapid change in temperature on physiological processes would be diminished, resulting in lower Q_{10} values.

MATERIAL AND METHODS

Species rearing conditions

Paracartia grani (Sars, 1904) specimens were originally collected in coastal waters north of Barcelona (NW Mediterranean) in 2007-2008 and maintained at 19°C (± 1°C) with 10:14 light/cycle at the Institut de Ciències del Mar (ICM, CSIC) for >14 years. Copepods were reared in 20-40 L tanks and routinely fed *ad libitum* three times a week with the cryptophyte *Rhodomonas salina* (strain K-0294, Scandinavian Culture Collection of Algae and Protozoa), grown in f/2 medium. From this culture, two separated lines were established to be used for the experiments, one at 19°C ("Control") and another one at 25°C ("Warm"). The temperature was maintained stable (± 0.1°C) using two 150 L water baths with TECO chiller and heater units, and the light regime was set to 15-20 µE m^{-2} s^{-1} and 10:14h light/dark cycle. We monitored the development of the cohorts and, according to life stage and copepod density, we adjusted the volume of *R. salina* added to keep copepods in satiation or close to satiation conditions (from 2 ppm for early-stage nauplii to 10 ppm for adults; Olivares et al., 2019). Once the cultures were dominated by mature adults, freshly spawned eggs were collected by siphoning the bottom of the tank and transferred in batches of 10,000-20,000 into the new 20 L tanks with temperature-acclimated filtered (0.1 μm) seawater. The subsequent generations were then reared separately to ensure the conditioning of the lines to temperature. Experiments were carried out after being reared for >10 months (>18 generations).

Experimental setup

The survival and performance of adult female *Paracartia grani* reared under control (19°C) and warm conditions (25°C) were examined by exposing individuals to 11, 15, 19, 22, 25, 27, 29, 30, 31, 31.5, 32, 33 and 34°C (± 0.1°C) during 7 days. To achieve this, we conducted the following procedure: once the majority of individuals in the cohorts had reached the adult stage (both males and females), we ensured their nourishment by feeding them a satiating diet of *R. salina* for a period of 2-5 days to guarantee fertilization. Subsequently, we separated adults from the culture with a 250-μm sieve, and we transferred groups of 20 females into 1 L Pyrex bottles using a wide-mouth Pasteur pipette. These bottles were filled with a saturating suspension of *R. salina* (9 ppm, 1,144 μg C L^{-1}), which had been previously acclimated to the respective temperatures. To ensure that nutrient availability was not limited at higher temperatures, we supplemented the *R. salina* suspensions with f/2 medium, aiming to achieve a nutrient load equivalent to approximately f/10 medium in the final suspension. The concentration of prey and copepod density in the bottles were calculated based on previous knowledge to ensure food-saturating conditions during incubation (Olivares et al., 2019). The food concentration in the bottles was measured initially and during the experiment using a Beckman Coulter Multisizer III particle counter (aperture tube 100 μm). The experimental bottles were immersed in the water baths and regularly homogenized (three times a day) by gently turning the bottles upside down several times. We ran at least triplicate bottles for each test temperature and rearing condition. In total, 1,800 (900 control + 900 warm) individuals were used in the assays to assess the tolerance and reproductive thermal responses. After 24 hours, we filtered the content of the bottles through 100 μm and 20 μm sieves; adults, retained by the 100 μm mesh, were inspected and counted under the stereo-microscope and their survival was checked. Copepods were considered dead if they did not swim or react to physical stimulation. Alive individuals were returned to the same suspensions using a Pasteur pipette. The fraction between 100 and 20 μm, containing eggs, nauplii, and faecal pellets, was discarded. We renewed the food suspensions in the bottles every 48 hours by gently siphoning the old suspension using a pipette tip fitted with 100-μm mesh (reverse filtration) and then refilled the incubation bottles with the new temperature-conditioned suspension of *R. salina*. After 6 days, the bottle content was filtered through 100-μm and 20-μm sieves to separate, respectively, adults and eggs from the suspension and to check the survivorship as previously described. We refilled the bottles with fresh suspensions of *R. salina* and returned the adult females to the bottles to assess egg production rates. The following day, we repeated the procedure described before and ended the experiment. We checked the final survival of the copepods in the bottles and preserved the remaining alive individuals in 4% formaldehyde. The fraction containing the eggs and pellets (20-μm mesh) was fixed in a 2% Lugol solution. Posteriorly, we counted the eggs, egg shells, and hatched nauplii using an inverted microscope (Nikon Diaphot 200) and calculated egg production rates as the total number of eggs laid divided by the number of females in the incubation and the incubation time. To determine the effect of ambient temperature on egg diameter, for each test temperature and rearing condition we took pictures of 30 preserved eggs (all from a single replicate) under an inverted microscope. Additionally, we took pictures of the adults at each rearing condition initially and the preserved adults after the stress period (only in the upper thermal range; n = 30 for each test temperature and rearing condition) to check whether there was a relationship between the size of the female and the survival at the lethal temperatures. All pictures were processed using ImageJ software (v1.53v). The size of the adult was measured as the length of the prosome, which is the linear distance from the upper end of the cephalosome to the last somite of the metasome. The diameter of the egg was determined by adjusting an ellipse to the copepod egg and averaging the x and y axes.

Models and calculations

To assess the survival (S) of *P. grani* along the thermal gradient, we adjusted a sigmoidal curve following Tarapacki et al. (2021):

$$S = \frac{c}{1 + \exp\left(-a \times (T - b)\right)} \qquad \text{Eq. 5}$$

where a is the slope of the descending phase, T is the test temperature (°C), and b is the temperature at which 50% mortality is reached in relation to c, which is the maximum survival. The temperature causing 50% (LT_{50}) and 90% (LT_{90}) mortality was then extracted from the fitted curves.

Thermal performance curves of the egg production rates were modelled using the R package *rTPC* (Padfield et al., 2021). Based on the global score of the AIC among 9 fitted models (Padfield et al., 2021), we chose the empirical model by Rosso et al. (1993) to be fitted to the egg production data:

$$R = \frac{R_{max} \times ((T - T_{max})(T - T_{min}))^2}{(T_{opt} - T_{min})\left((T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)\right)} \qquad \text{Eq. 6}$$

where R is the rate of copepod egg production (eggs ind^{-1} d^{-1}), T is the test temperature (°C), T_{min} (°C) is the temperature at which the egg production rate is zero, T_{max} (°C) is the temperature at which the egg production rate is zero, and T_{opt} (°C) is the optimum temperature at which the maximum egg production rate, R_{max} (eggs ind^{-1} d^{-1}), occurs.

Parameter estimates and 95% confidence intervals were obtained using case resampling bootstrapping (R package *car*, v3.1-1; Fox & Weisberg, 2019). CT_{max} values represent the estimated rate closest to 0 at the upper limit and it is equivalent to the model T_{max}. Additionally, we also assessed the *thermal breadth*, defined as the range of temperatures where performance is 80% of the maximum rate, and the *thermal safety margin,* calculated as the difference between T_{opt} and the rearing temperature. We calculated the Q_{10} coefficients for egg production, i.e. the fold variation in a given rate for a 10°C increase, from the slopes of the linear regression between the natural logarithm of the estimated egg production rates and temperature, as $exp^{(10 \times slope)}$.

As a proxy for fitness, we multiplied the adult survival rate and the offspring produced (egg production rates), both taken from the fitted curves after 7 days of exposure, to determine the potential recruitment of both control and treatment at all test temperatures.

Data analysis

Estimates of the curve parameters were compared by examining the 95% confidence intervals and considered to differ significantly if they did not overlap. The differences in female prosome length under both rearing conditions were analyzed using a two-tailed Student's test. Additionally, two-way ANOVA and Dunnett post hoc comparisons were conducted to assess whether the body size of females that survived at higher temperatures (27°C and 32°C) differed from their respective controls.

As egg diameter and temperature followed an exponential relationship, a comparison between the intercepts and slopes of the control and treatment was carried out using ANCOVA analysis on ln-transformed egg diameter. All plots and statistical analyses were performed in R software (v4.1.1; R Core Team, 2021).

RESULTS

Heat tolerance

After 24 hours of exposure, survival was high, close to 100%, for the control (19°C) and warm-reared (25°C) treatments across the thermal range comprised between 11°C and 31.5°C (**Fig. 18A**). From 32°C, survival of the control population started to decrease, whereas in the warm-reared copepods, this drop appeared after 33°C. In the descending phase of the curve, survival was consistently higher for warm-reared copepods. Consequently, LT_{50} was 32.5°C (95% CI 32.47-32.59) for the control and 33.5°C (95% CI 33.37-33.56) for the warm-reared treatment. The LT_{90} after 24 hours was 33.0°C (95% CI: 32.98-33.10) and 34.3°C (95% CI: 34.20-34.39) for the control and warm-reared treatments, respectively.

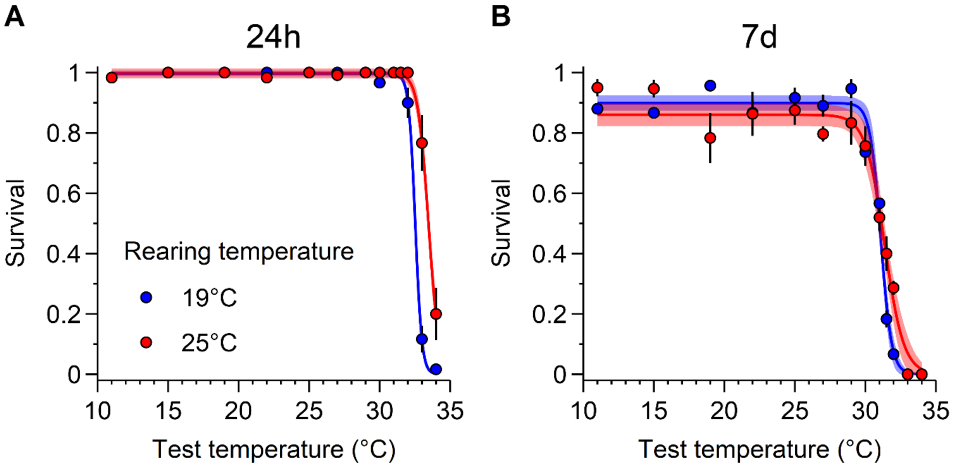


Fig. 18 | Survival curves for adult females of *P. grani* reared under control (19°C, blue) and warm (25°C, red) conditions after 24 hours (**A**) and 7 days (**B**) of exposure to a range of test temperatures. Each point represents the average of 3-6 replicate bottles for each test temperature, and the error bars show the standard errors. The lines and shades show the fitted sigmoidal curves and the corresponding 95% confidence intervals, respectively.

After 7 days, survival for both populations was still high, between 11 and 30°C with a maximum survival of 85% (**Fig. 18B**). However, the upper thermal tolerance was significantly reduced in the control and warm-reared treatments, and the differences between them smoothed, with LT_{50} values of 31.1°C (95% CI: 30.95-31.21) and 31.4°C (95% CI: 31.13-31.59), respectively. At the most extreme temperatures (31.5 and 32°C), however, the survival of warm-reared copepods was still 2.2 and 5.4 times higher, respectively, than that of the control treatment. LT_{90} also decreased in both treatments but remained significantly higher in warm-reared copepods (control: 31.9°C [95% CI: 31.68-32.11]; warm-reared: 32.7°C [95% CI: 32.28-33.15]).

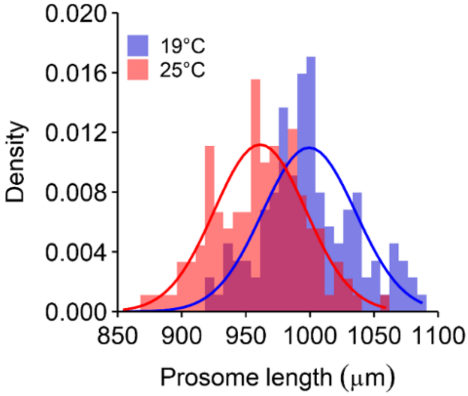


Fig. 19 | Density plot for prosome length of adult females of *P. grani* reared in control (19°C; blue) and warm (25°C, red) conditions. Lines show Gaussian fit.

Body size

Individuals reared at 19°C were generally larger than those reared at 25°C ($p < 0.001$), averaging 999.3 (± 3.3SE) and 961.1 (± 3.2SE) µm, respectively (**Fig. 19**). The homogeneity test did not show differences in body size variance between control and warm-reared treatments ($F_{121, 124} = 1.04$, $p = 0.841$), with similar coefficients of variation (control: 3.6%; warm-reared: 3.5%). The body length of adults who survived extreme temperatures (27-32°C) did not differ from that of their respective rearing temperature, neither for 19°C ($F_{1,216} = 0.528$, $p = 0.468$) nor for 25°C ($F_{1, 213} = 0.133$, $p = 0.716$).

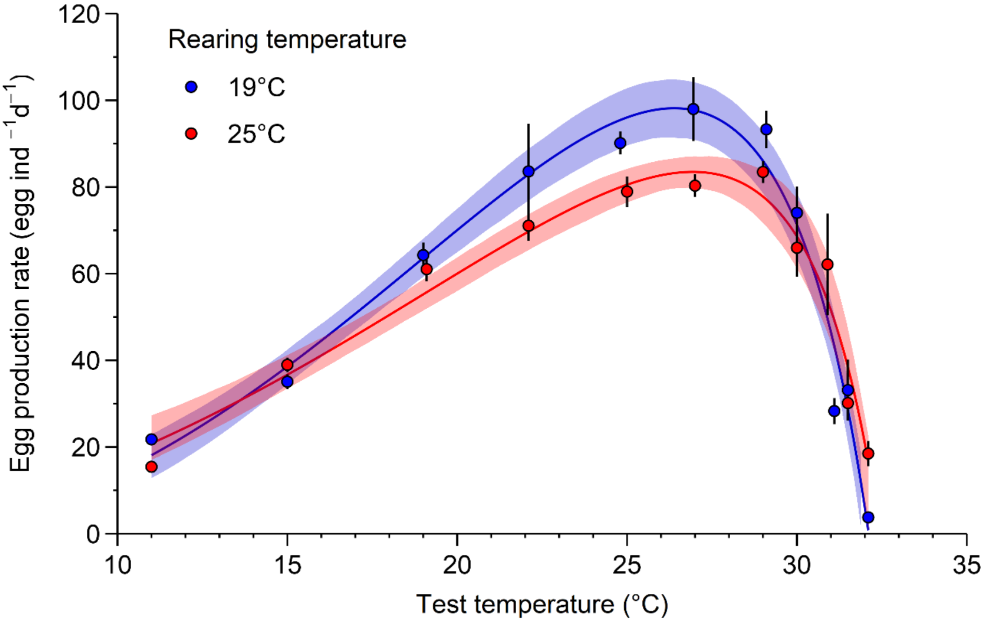


Fig. 20 | Thermal performance curves of the egg production rate of female *P. grani* reared under control (19°C, blue) and warm (25°C, red) conditions after 7 days of exposure. Each point shows averages (n = 3-6 for each test temperature), and error bars show standard errors. Lines show the model fit (see Methods) and shades show the 95% confidence intervals estimated using case resampling.

Table 3 | Parameter values of the fitted thermal performance curve of the *P. grani* egg production rate reared at control (19°C) and warm (25°C) conditions. Otherwise indicated, all units are °C.

	19°C			25°C		
		95% CI			95% CI	
Parameter	Estimate	Lower limit	Upper limit	Estimate	Lower limit	Upper limit
R_{max} (eggs ind^{-1} d^{-1})	98.2	91.1	106.5	83.5	78.8	86.6
T_{opt}	26.4	25.9	27.2	26.9	25.9	28.0
CT_{max}	32.1	31.9	32.4	32.4	32.3	33.0
Thermal safety margin	7.4	6.9	8.2	1.9	0.9	3.0
Thermal breadth	8.3	7.8	8.7	8.7	8.2	9.4

Egg production rate

Surviving females produced offspring at all temperatures tested (**Fig. 20**). The maximum observed egg production rate for the copepods reared at 19°C was 98 ± 7.4 eggs ind^{-1} d^{-1} when exposed to 27°C, while for the 25°C reared copepods the maximum recorded value was 83.4 ± 2.6 eggs ind^{-1} d^{-1} at 29°C. **Table 3** shows the estimated parameters of the fitted thermal performance curve. The $R_{\max}$ of the warm-reared copepods was significantly lower than the control. The T_{opt} was not significantly modified by multigenerational rearing, but the amplitude of the confidence intervals was larger under warm conditions (larger optimum plateau). $CT_{\max}$ was not significantly different from the control either, although the values found in the descending phase of the response curve of warm-reared copepods were up to 21 times higher than the control. Consequently, there was no significant difference in thermal breadth between the two rearing conditions. However, the thermal safety margin decreased after multigenerational warming, primarily due to the preservation of T_{opt}.

The Q_{10} coefficients for the curves fitted to the thermal response of the warm-reared copepods were consistently lower than those of the control treatment (**Table 4**). When considering the direction of the response, the higher Q_{10} coefficients were observed at the cooling phase at both temperatures.

Table 4 | Q_{10} coefficients for the ascending phase of the thermal windows of female *P. grani* reared at 19°C and 25°C. "Rearing T" refers to the rate registered at the test temperature equal to the respective rearing temperature. "Min. rate" refers to the rate at 11°C and "Max. rate" refers to the rate at the T_{opt} for each curve.

Curve phase	19°C	25°C
Global Q_{10} (Min. rate – Max. rate)	2.9	2.4
Cooling Q_{10} (Min. rate – Rearing T)	4.7	2.6
Warming Q_{10} (Rearing T – Max. rate)	1.8	1.2

The diameter of the egg showed a negative trend with the test temperature (**Fig. 21**) and no differences were found due to the rearing temperature (linear regression of the ln-transformed data; $F_{1,18} = 3.38$; $p = 0.08$).

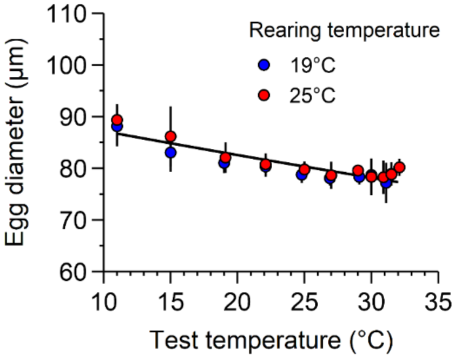


Fig. 21 | Egg diameter against exposure temperature under control (19°C; blue) and warm conditions (25°C; red). Individual points show the mean and standard deviation. The line show the fitted negative exponential.

Fitness proxy

The joint effects of temperature on survival and fecundity were analysed by comparing the potential recruitment index for the control and warm-reared copepods (**Fig. 22**). At most of the non-stress test temperatures (19-29°C), at which mortality at the control and warm-reared treatments was negligible, the higher fecundity of individuals reared at 19°C determined a higher recruitment potential. In the upper temperature range (30-32°C), at which substantial mortality occurs, the individuals reared at 25°C achieved higher survival and also produced higher fecundity shifting the trend.

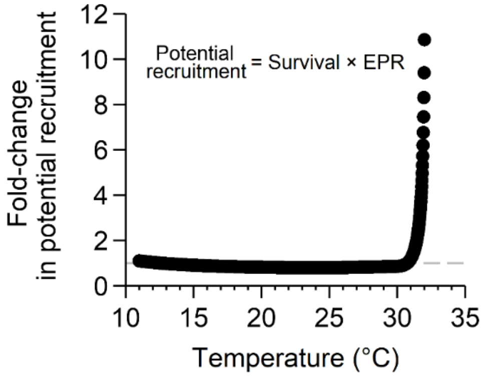


Fig. 22 | Fold-change in potential recruitment (estimated as the product of the survival and offspring production of females, based on the fitted curves in **Figs. 18B and 20**) of *P. grani* reared under warm conditions (25°C) relative to the control (19°C) after 7 days of exposure to a range of temperatures. The dashed grey line shows a fold-change of 1.0.

DISCUSSION

Thermal tolerance

We have shown that the temperate copepod *Paracartia grani* reared in warmer conditions (+6°C) for >18 generations increased the LT_{50} and LT_{90} in acute exposure (24 hours) by 1 and 1.3°C, respectively. However, this enhancement decreased to 0.3 and 0.8°C after longer exposure (7 days). From these results arise that both the duration of the exposure and the choice of metric used to measure thermal tolerance can significantly affect the outcomes obtained (**Supplementary Fig. S3**). Although the time of exposure to stress conditions is a key factor in determining the thermal tolerance of an organism (Terblanche et al., 2007; Rezende et al., 2014), it is often overlooked, which can result in significant uncertainty when conducting global comparisons of the vulnerability of ectotherms to extreme heat events (Gunderson & Stillman, 2015; Morley et al., 2019; Weaving et al., 2022). As such events, potentially exceeding the thermal tolerance limits, can persist for extended periods (Garrabou et al., 2022) and are expected to be more frequent and severe in the coming future (Oliver et al., 2021), it becomes more relevant to assess the organism's response to sustained thermal stress.

The available information on ectotherms (e.g. Gunderson & Stillman, 2015; Morley et al., 2019; Weaving et al., 2022) indicates a general limitation in the plasticity of heat tolerance, with the increase in tolerance per 1°C in short-term acclimation temperatures being much lower than 1°C. However, in contrast to acute exposures, the thermal response after multiple generations may involve mechanisms at phenotypic and genotypic level (Sasaki & Dam, 2019; Brennan et al., 2022; Dam et al., 2021) that could potentially lead to adaptive changes in heat tolerance. Nevertheless, the increase in heat tolerance of *P. grani* we observed in our study (from 0.05 up to 0.22°C per degree increase in the rearing temperatures), in which the copepod populations were conditioned to warmer sub-lethal temperatures over 18 generations, was, in fact, similar to or lower than those reported in the aforementioned acute-response studies. Similarly, Sasaki & Dam (2021) also found, after rearing 40 and 80 generations the related copepod *Acartia tonsa* at +4°C conditions, comparable increases in acute heat tolerance (ranging 0.1-0.5°C $°C^{-1}$). A stronger selective pressure than that used in our experiments during rearing (25°C) could favour the selection of the most heat-tolerant genotypes and thereby amplify the tolerance response. However, studies on experimental evolution report the presence of hard physiological limits to the increase in heat tolerance (Morgan et al., 2020). For instance, in the intertidal copepod *Tigriopus californicus*, a strong selection for tolerant phenotypes over 5 and 10 generations resulted in only a 0.5°C increase in heat tolerance, showing significant latitudinal differences (Kelly et al., 2012). Interestingly, in our experiments, the reduced survival capacity of the warm-reared copepods at extreme temperatures after a longer exposure could suggest an increase in their plasticity rather than a mean increase in their basal heat tolerance (**Supplementary Fig. S3;** van Heerwaarden & Kellermann, 2020).

Reproductive response

At sub-lethal temperatures, the direct effects of temperature on other life-history traits (i.e., growth and reproduction) can set narrower limits for the persistence of the species. It has long been recognized that energy investments in survival can be at the cost of reproductive efforts and vice versa (Stearns, 1989; Truong et al., 2022). In our study, *P. grani* reared under warm conditions could expand both survival and reproduction at extreme temperatures, providing further evidence of a strong coupling between both traits in this species under thermal stress (Saiz et al., 2015). Contrary to our hypothesis, we did not observe significant shifts in the T_{opt} of the reproductive response of *P. grani* following multigenerational warming, resulting in a decrease in the thermal safety margin; the thermal breadth of the reproductive response, however, was not altered by the rearing temperature. Moreover, the warm-reared copepods exhibited higher fecundity rates (up to 21-fold) at the thermal extremes despite their smaller size, revealing a shift in the limits of the reproductive response. The observed decrease in R_{max} of the warm-reared copepods can be attributed not only to their smaller size (Ban, 1994; Halsband-Lenk et al., 2002) but also to the action of physiological compensation processes (Saiz et al., 2022; de Juan et al., 2023a).

The decrease in the Q_{10} coefficients of the warm-reared copepods provides additional evidence of a reduction in the thermal sensitivity of the female reproductive rate (de Juan et al., 2023a). Depending on the phase of the curve considered in relation to the rearing temperature, the Q_{10} coefficients varied, suggesting a change in the shape of the curve (i.e., hysteresis) and emphasizing the importance of considering the interaction between previously experimented temperature and the direction of the thermal change (Sinclair et al., 2016). However, at lethal temperatures (30-33°C), the warm-reared copepods showed higher survival and produced more offspring than the control treatment **(Supplementary Fig. S4)**. Consequently, the improvement of survival and reproduction increased the potential recruitment of warm-reared copepods after 7 days at thermal extremes. Therefore, small shifts at the physiological level can scale up to the population level and have ecological consequences (Pörtner & Farrell, 2008).

Temperature and body size

Temperature also has well-recognized effects on body size (Forster & Hirst, 2012) and its occurrence complicates the evaluation of the thermal responses of ectotherms (Riemer et al., 2018). As a major trait that governs all energy and nutrient fluxes, body size effects overlap with thermal effects on physiology (de Juan et al., 2023a). Examples of thermally driven changes in size and distribution have been observed in marine ectotherms, including marine zooplankton (Evans et al., 2020). Shifts in biodiversity and abundance of this key group may affect its direct links in the trophic chain (Brosset et al., 2016) and, consequently, may ultimately alter the functioning of marine food webs and the carbon sequestration capacity for the oceans. Here, the reduction in adult body size at warmer temperatures had scaling effects on egg production, resulting in lower potential recruitment. Recent studies also suggest that animals with smaller body sizes could benefit from an increase in heat tolerance and, at the same time, exhibit lower endurance capacity to longer exposures (Peralta-Maraver & Rezende, 2021). However, we did not observe any significant differences between the body sizes of the females that survived the extreme temperatures that could support the occurrence of such phenomena. In addition, given the relatively small difference in adult size between the warm-reared and control populations (0.6% $°C^{-1}$), any benefits on the tolerance after prolonged exposition to lethal temperatures would possibly be undetectable. Likely, larger size differences, like those driven by interspecific or ontogenetic comparisons, would be required to discern the effect on tolerance. Female size did not affect egg size, which was found to be highly dependent on immediate ambient temperature, as suggested in previous studies (McLaren et al., 1969). This reduction of egg size driven by temperature may have important implications for copepod populations as it has been shown that it can influence egg viability, naupliar survival under starvation (McLaren et al., 1969), and ontogenetic development (Guisande & Harris, 1995).

Scaling our results to natural communities

Many copepod species experience important changes in water temperature throughout their seasonal presence (e.g., Horne et al., 2016). Therefore, we could expect an advantage for copepods reared in warmer waters over thermal extremes across population development since these heat spikes primarily occur during the warm seasons. An implication of this is that the plasticity of wild animals subjected to larger environmental fluctuations might be underestimated when using laboratory specimens (Morgan et al., 2022). *P. grani* is tolerant to a wide range of temperatures (5.7-32°C; Saiz et al., 2022). Before the experiments, this species had been reared for >14 years under steady thermal conditions (19 ± 1°C), yet it still exhibited notable physiological plasticity and acclimation capacity (Saiz et al., 2022; de Juan et al., 2023a). It was rather unexpected to find that this strong phenotypic plasticity, which in nature would help to cope with the intrinsic environmental variability of their habitat (coastal, estuaries and semi-enclosed waters), could be maintained after culturing in the laboratory for so long under stable conditions. Contrarily, other copepod life-history traits, such as feeding and egg production diel rhythms, can be less fixed and quickly affected after laboratory culturing (Tiselius et al., 1995; Olivares et al., 2020).

In nature, copepods employ various strategies to cope with environmental changes in temperature, either warming or cooling. For example, specific behaviours, such as vertical migration, can also help to reduce or avoid thermal stress. However, for copepods inhabiting coastal and semi-enclosed waters, such as *P. grani*, positive thermal anomalies can occur tens of meters down the water column, making exposure to thermal stress unavoidable. Many copepod species produce diapause eggs to overcome the unfavourable period. *P. grani* is typically considered a thermophile species and in nature may rely on the production of resting eggs to survive the winter season (Guerrero & Rodríguez, 1998). In this regard, we did not observe morphologically distinct eggs that could be categorized as diapause eggs throughout all the egg counts out under the inverted microscope.

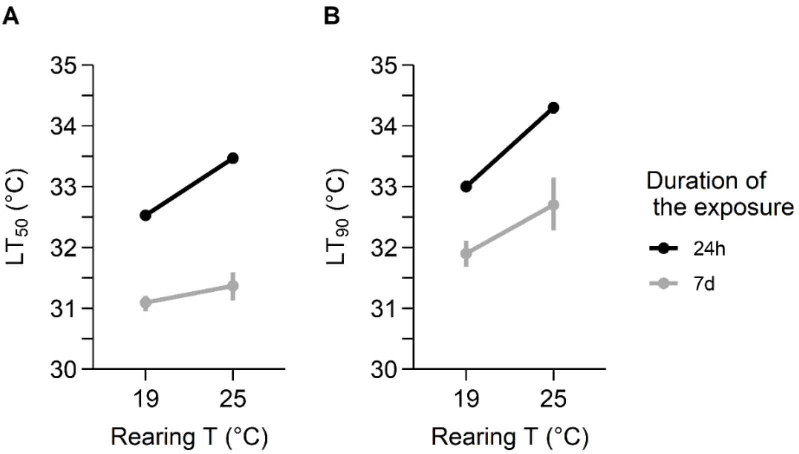
Our results show that the warm-reared copepod population may have the advantage to tolerate heat spikes at short time scales; nevertheless, under prolonged thermal stress situations like those under heatwave events (Smith et al., 2023), the differential advantage of the warm population still exists but diminish in magnitude. Our study also highlights the importance to include other key variables, besides survival, to assess the vulnerability of a species to thermal stress. Thus, the warm-reared copepods exhibited a much higher recruitment potential due to the combined effects on survival and fecundity. Further life-history traits should also be taken into account for a better comprehension of the thermal response of a species in nature.

Although we did not explore the viability of subsequent offspring at the test temperatures, some studies suggest that early stages may exhibit more plasticity than previously thought (Tangwancharoen & Burton, 2014; Klockmann et al., 2017; Holmes-Hackerd et al., 2023), and that temperature at development can improve the tolerance of adult stages (van Heerwaarden et al., 2016). Over long timescales, trans- or intergenerational mechanisms can emerge and provide further resilience to the organisms, increasing their chances to overcome extreme events. However, the evidences in this regard are equivocal. For instance, Geerts et al. (2015) reported that *Daphnia* recovered from a burial in an egg bank for over 40 years had lower thermal tolerance than some collected recently, subjected to current warming trends. Similarly, other studies show examples of recent local adaptation in heat tolerance in ectotherms (Brans et al., 2017; Diamond et al., 2017). Nevertheless, there is mounting evidence of the presence of physiological limits to heat tolerance that could also reduce the future acclimation capacity of species to extreme events (Hoffmann et al., 2013; Morgan et al., 2020; van Heerwaarden & Kellermann, 2020; Sasaki & Dam, 2021). The long-term changes in heat tolerance and vital rates in response will have a significant influence on the local persistence of a species, as well as the reorganization of marine assemblages.

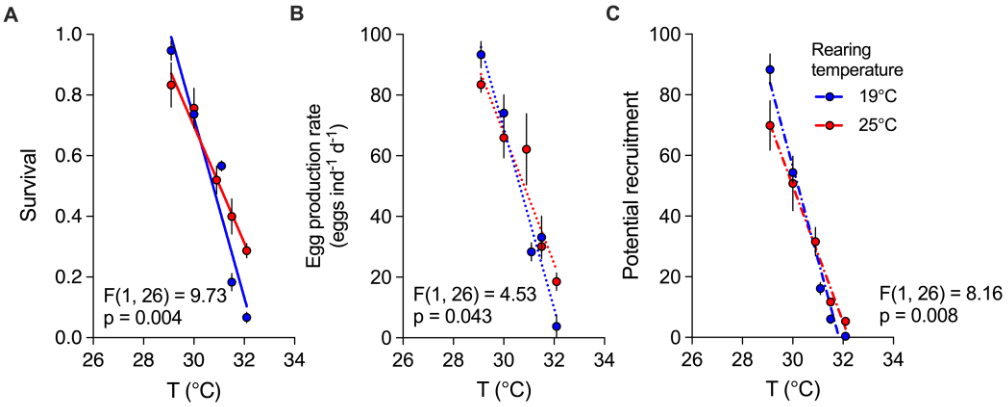
CONCLUSIONS

Long-term warming has been found to enhance tolerance to acute extreme temperatures in marine ectotherms. However, it is crucial to consider the duration of the stress and the choice of the metric used, as they can significantly affect our assessment of a species' thermal tolerance. When the calanoid copepod *Paracartia grani* was reared for multiple generations at elevated temperatures, it exhibited increased survival and reproduction rates at the upper thermal range. This response enhances the species' potential to persist during extreme heat events. While establishing and maintaining marine cultures and conducting long-term laboratory experiments with marine invertebrates can be challenging, the available data provides valuable insights that can contribute to our understanding of field conditions and help forecast ecological trends.

SUPPLEMENTARY INFORMATION



Supplementary Fig. S3 | (**A**) LT_{50} and (**B**) LT_{90} after 24 hours (black) and 7 days (grey) exposure to elevated temperatures of the *P. grani* females reared during >18 generations at control (19°C) and warm (25°C) conditions.



Suppl. Fig. S4 | (**A**) Survival, (**B**) egg production rate, and (**C**) potential recruitment after 7 days of exposure to extreme temperatures of *P. grani* reared at control (19°C) and warm conditions (25°C) during >18 generations. Potential recruitment was obtained by multiplying the survival rate after 7 days per the egg production rate at each rearing temperature. Each point shows the average of three replicates and standard error. Lines show linear regression of the descending phase of the thermal performance curve. p-value indicates significant differences between slopes.

3.3 Metabolic balance under chronic and acute warming scenarios

de Juan, C., Traboni, C, Calbet, A. & Saiz, E. Submitted

ABSTRACT

We investigated the impact of thermal conditions on physiological (ingestion, reproduction, respiration and egestion) and somatic (body size and carbon content) traits of the copepod *Paracartia grani*. The copepods, reared at 19ºC, were exposed for 23 generations to 22ºC and 25ºC, and posteriorly exposed for 7-d to stress temperature (28ºC). The copepod acclimation capacity was assessed by comparing metabolic balance at 28ºC against their respective controls. There was an inverse relationship between rearing temperature and body size and carbon content for the reared copepod lines. Weight-specific rates, except respiration, increased with rearing temperature, whereas per capita rate differences were levelled, partly due to differences in copepod size. Heat stress impact, as weight-specific rate fold-change, appeared inversely related to rearing temperature. Carbon gains were overall sufficient and slightly in excess to account for carbon losses. Gross-growth efficiency across warming scenarios was conserved, emphasizing the resilience of this species to environmental change.

INTRODUCTION

In ectotherms, an increase in ambient temperature accelerates metabolism. As the temperature exceeds optimal levels, the maintenance costs associated with the energetically demanding stress response might constrain other vital processes, such as growth or reproduction. Thus, the balance between individual energetic demands and food intake and assimilation can determine an organism's potential success in the face of environmental change (Sokolova et al., 2012; Huey & Kingsolver, 2019; Sokolova, 2021). In many species, the effects of temperature on physiology can be compensated, to some extent, following acclimation (Seebacher et al., 2015), thereby reducing the energetic expenditure of the stress response (Schulte, 2015). Moreover, adjustments of adaptive value might be inherited in a few generations in relatively fast-maturating organisms, maximizing their fitness in new environmental conditions (Dam et al., 2021; deMayo et al., 2023). Yet, concerns persist regarding whether these mechanisms can effectively facilitate adaptation to ongoing warming trends, where the gradual rise in heat ocean content is accompanied by an increasing number of extreme heat events (IPCC, 2019). It is particularly relevant to assess the impact of thermal anomalies on organisms that may struggle to avoid exposure to temperature fluctuations, as is the case with marine invertebrates inhabiting neritic areas (Smith et al., 2023).

Among marine invertebrates, planktonic copepods are a key group in the pelagic environment, both in terms of abundance and because of their crucial role in energy transfer and nutrient cycling due to their intermediate position in marine food webs (Steinberg & Landry, 2017). As ocean temperatures continue to rise, changes in copepod biomass and activities, such as grazing, respiration, egestion, and excretion are anticipated, although the direction of these changes remains uncertain (Ratnarajah et al., 2023). The assessment of impacts on carbon fluxes has to be considered in a context where temperature also favors the occurrence of smaller individuals due to uncoupling between growth and molting rates (Forster & Hirst, 2012), which generally show overall lower per capita activity rates (Riemer et al., 2018). Thus, in addition to extreme heat events leading to organism mortality, it is important to determine the alterations in copepod performance in the upper sub-lethal tolerance range, which may affect their thermal susceptibility, and to evaluate the buffering role of previous thermal history in this response.

This study aimed to assess the response of the main physiological rates of the marine calanoid copepod *Paracartia grani* (Sars, 1904) to evaluate potential changes in metabolic balance under thermal stress. Similar to other Acartidae species, *P. grani* is a small omnivorous copepod typically found in neritic environments, such as estuaries and semi-enclosed water masses (Boyer et al., 2012). Under controlled laboratory conditions, *P. grani* can tolerate acute changes in temperature up to 32-33ºC (Saiz et al., 2022) and multigenerational exposure to warming can strengthen their tolerance limits (de Juan et al., 2023b) and mitigate acute effects on main physiological rates (de Juan et al., 2023a), similarly to other copepod species (Rahlff et al., 2017; Sasaki & Dam, 2021).

We hypothesized that copepods reared under warmer conditions would show a higher capacity of maintaining their metabolic balance under thermal stress. To test our hypothesis, *P. grani* specimens were reared at different sub-lethal temperatures (19ºC, 22ºC and 25ºC) for 23 generations (~1 year) (**Fig. 23a**). Subsequently, individuals from each temperature line were exposed to a common stress temperature (28ºC) (**Fig 23a**). Measured variables included somatic traits (prosome length and carbon content) and the main physiological rates (ingestion, reproduction, respiration and egestion rates) (**Fig. 23b**). The discussion of the study involves the interpretation of the carbon budget of *P. grani* under these different warming conditions, elucidating the impact of thermal stress on its metabolic equilibrium.

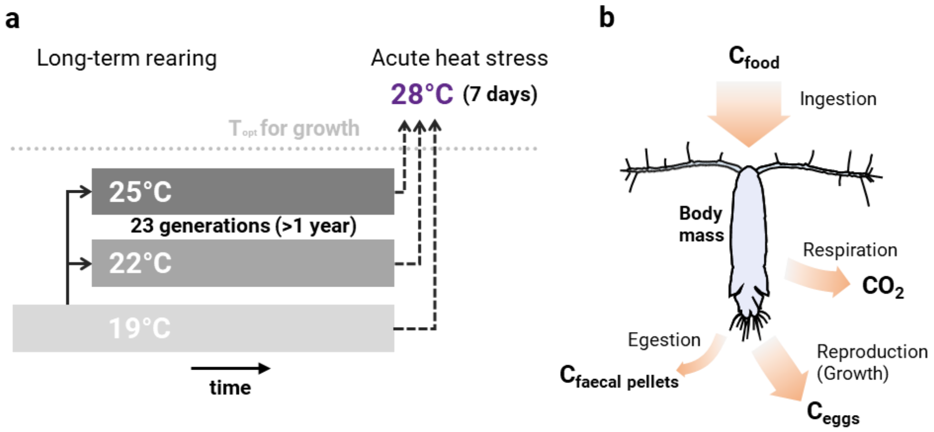


Figure 23 | Schematic representation of the experimental design. (**a**) From a laboratory culture maintained at 19ºC, two cultures were established at 22ºC and 25ºC to test the effects of long-term warming on copepod life history traits. Then, copepods reared at these conditions were exposed to a common sub-lethal heat stress (28ºC) to test their acclimation capacity. (**b**) Measured variables included somatic traits (prosome length and carbon content) and the main physiological rates (ingestion, reproduction, respiration and egestion), which were converted to carbon to evaluate the metabolic balance of adult *P. grani* females.

MATERIAL AND METHODS

Species and rearing conditions

Specimens of *Paracartia grani* were initially collected in waters off north of Barcelona (NW Mediterranean Sea) and have been maintained for more than 14 years at 19ºC (± 1ºC) with 10:14 light/cycle at the Institut de Ciències del Mar (CSIC). Copepods were cultivated in 20-L polycarbonate tanks and routinely fed *ad libitum* with the cryptophyte *Rhodomonas salina* (strain K-0294, Scandinavian Culture Collection of Algae and Protozoa). *R. salina* was grown exponentially in semi-batch cultures in a sterile f/2 medium (Guillard, 1975), in a light- and temperature-controlled room at 19ºC (± 1ºC), 10:14 light/cycle, 80 μmol photons m^{-2} s^{-1}.

To investigate the long-term response of *P. grani* to warming, three distinct lines were established at temperatures of 19ºC, 22ºC and 25ºC (± 0.1ºC). These conditions were created using three 150-L water baths equipped with TECO thermal conditioners and

LED lighting (15-20 µmol photons m^{-2} s^{-1}; 10:14 light/dark cycle). Eggs were collected from the original culture at 19ºC by siphoning the bottom of the rearing tank and transferred in batches of 10,000-20,000 into new 20-L tanks filled with temperature-acclimated 0.1-µm filtered seawater. Throughout the rearing routine, the development of the copepod cohorts was monitored, and the volume of *R. salina* provided to the cultures was adjusted based on the life-stage and copepod density to ensure nearly food-saturating conditions throughout ontogeny (Olivares et al., 2019). After obtaining mature adults in each culture, subsequent cohorts were generated following the same method. This procedure was repeated until 20-23 generations were reached, following 11-15 months of exposure to each rearing temperature.

Incubations prior to the experiments

Adult *P. grani* females designated for the experiments were harvested from the cultures within 2 to 5 days after molting to guarantee their sexual maturity and fertilization, as well as to minimize ageing effects (Rodríguez-Graña et al., 2010; Saiz et al., 2015). Approximately groups of ca. 300 individuals were placed into triplicated 4-L Nalgene bottles filled with saturating suspensions of *R. salina* (10 ppm, ~1160 µg C L^{-1}, with ~180 mL of f/2 medium per liter) previously acclimated to match the experimental temperature. The response of each copepod line (19ºC, 22ºC and 25ºC) to thermal stress (28ºC) was assessed in parallel to their respective control. The stress temperature (28ºC) was chosen based on previous studies that show it surpasses the thermal optimum of the reproduction (growth) response of *P. grani* while not causing significant mortality within 7 days of exposure (Saiz et al., 2022; de Juan et al., 2023b). Prey concentrations and copepod density in the incubation bottles were calculated based on previous data to ensure food-satiating conditions during the incubations (Olivares et al., 2019). Both the control and experimental bottles were submerged in the water baths and they were manually homogenized three times a day. After 24 hours, food concentrations were checked using a particle counter (Beckman Multisizer 3 Coulter Counter, 100 µm aperture tube) and adjusted to maintain satiation conditions. The food suspensions in the bottles were renewed every 48 hours through a careful reverse filtration of the old suspension using a 100-µm mesh. This procedure was repeated for 6 consecutive days. Then, the content of the Nalgene bottles was filtered through a 200-μm mesh to separate the adults from the suspension.

Vital rates measurements

Ingestion, reproduction and egestion rates of adult *P. grani* females were assessed through 24-hour incubations. After the 6-day conditioning, groups of 25 females at each experimental temperature were transferred using a wide-mouth Pasteur pipette to 610-mL Pyrex bottles filled with a saturating suspension of *R. salina* (7.5 ppm, ~1200 μg C L^{-1}) amended with inorganic nutrients (5-20 mL of f/2 medium per liter). Food concentration and copepod abundance were established to ensure a reduction of 20-30% of the prey concentration during the incubation (Olivares et al., 2019), enough to discern the copepod grazing while keeping the satiating conditions. For each rearing temperature, four replicates of control and grazing bottles were placed submerged in their respective water bath and homogenized by hand three times a day. Start bottles were also filled with the suspension to determine the prey concentration at the beginning of the incubation. After 24 hours, the bottles were sequentially filtered through 200-μm (to retain the copepods) and 20-μm (to retain eggs and faecal pellets) sieves. The final prey concentration in the bottles (<20 μm) was then measured using the particle counter. To estimate copepod survivorship in the bottles, individuals were concentrated in a Petri dish and counted under a stereomicroscope (Olympus SZX12) and subsequently preserved in 4% formaldehyde. Samples containing eggs and faecal pellets (20-200 μm) were preserved in 2% Lugol’s solution until they were processed.

Feeding rates, expressed as the total volume of *R. salina* ingested per individual and day, were calculated using Frost’s equations (Frost, 1972). Eggs, egg shells and hatched nauplii were counted using an inverted microscope (Nikon Diaphot 200), while faecal pellets were counted using a LEICA-MC170 HD microscope. The egg production rate was calculated as the total number of eggs laid daily divided by the number of alive females in the incubation. Egestion rates were calculated as the number of faecal pellets produced divided by the number of alive females throughout the incubation and expressed per day.

Respiration rates were determined in 24-h incubations. Adult females were sorted from the Nalgene bottles and let clear the guts in 0.1-μm filtered seawater acclimated to the corresponding experimental temperature. After 30-60 min, four groups of 15 individuals were transferred to 70-mL bottles filled with 0.1-μm filtered seawater; additional four bottles without copepods served as control. The bottles were covered with aluminum foil and then submerged in their respective water baths. After 24 hours, final oxygen concentration in the bottles was measured using an optode Oxy-4 Pre Sens®. After that, the experimental bottle content was gently filtered through a 20-μm sieve to collect the copepods, which were examined and counted under a stereomicroscope. Respiration rates were calculated from the difference in final oxygen concentration between the experimental and control bottles. An additional assay at only 19ºC and 25ºC, where oxygen changes were monitored continuously, was posteriorly carried out to assess the effects of incubation time under starvation conditions. These data will be presented as supplementary and examined in the **Discussion** section.

Somatic traits, carbon measurements and volume-carbon conversion factors

The preserved adults were photographed (n = 32) using a stereomicroscope (Olympus SZX12). Eggs and faecal pellets were photographed (35-48 randomly chosen from each replicate; for eggs just one replicate) using an inverted microscope (Nikon Diaphot 200 and Leica-MC170 HD, respectively). Pictures were then processed with ImageJ software (v1.53v). Adult size was measured as the length of the prosome, from the upper end of the cephalosome to the last somite of the metasome. The size of the eggs was determined by measuring their diameter. The size of faecal pellets was obtained by measuring the total surface of the pellets and approximating the measured surface to an ellipse. The x and y axis of this adjusted ellipse was used to approximate to an ellipsoid and determine the volume of the pellet.

In parallel to the incubations, we collected samples to determine the carbon content of copepods. For such purpose, we isolated adult females from the feeding incubation bottles and left them for approximately 30 min in 0.1-μm filtered seawater to clear their digestive tracts. Then, they were narcotized with MS-222 and gently transferred in groups of 40 individuals onto pre-combusted 25-mm Whatman GF/C filters (450ºC, 5 h).

About 4-5 replicate filters were obtained for each temperature treatment. The filters were dried for 48h at 60ºC and then stored in a desiccator until processing with a Thermo Finnigan Flash EA1112 CHNS analyzer. To convert feeding rates to carbon, the biovolume consumed was multiplied by a volume-carbon content estimate of 0.16 pg C μm^{-3} for *R. salina* (Saiz et al., 2020). To estimate the egg production rate in carbon terms, egg volume was calculated from the diameter assuming a spherical morphometry (Belmonte, 1998) and then converted to carbon using a common factor of 0.129 pg C μm^{-3} (Saiz et al., 2020; de Juan et al., 2023a). Oxygen consumption rates were transformed into carbon losses using a respiratory quotient of 0.97 (Omori & Ikeda, 1984).

In order to assess a carbon conversion factor for pellets, we collected fresh faecal pellets from the 19ºC and 25ºC cultures. To do that, we obtained adult copepods from the cultures using a 250-μm sieve. After briefly rinsing with filtered seawater, we transferred them to a new tank with a satiating suspension of *R. salina* at the corresponding temperature. The bottoms of the tanks were carefully siphoned after 1-3 hours, and the collected material filtered through 250- and 40-μm sieves to retain the adult copepods and pellets, respectively. To reduce the amount of other contaminant components (i.e., eggs, aggregates) in the pellet fraction, the samples were transferred to a 100 mL cylinder, left settle for ~5 minutes, and then the upper 30 mL, mainly pellets, collected. This process was repeated multiple times, and eventually any remaining eggs were manually removed. The cleaned pellet samples were then divided into aliquots and counted. Batches of 2,700-6,000 pellets were transferred to pre-combusted GF/C filters (n = 4 for each rearing temperature), dried at 60ºC for 48 hours, and stored in a desiccator for later analysis with a Thermo Finnigan CNHNS Analyzer. For each sample, 52-62 random pellets were photographed, and from their surface area, the volume was calculated using FIJI software by approximating them into an ellipsoid. The carbon-volume conversion factor was determined by dividing the average carbon per pellet (μg C) by the average pellet volume (μm^{3}). As no differences were found between rearing temperatures **(Suppl. Fig S5)**, a global carbon-volume factor of 0.13 pg C μm^{-3} was used.

Growth and assimilation efficiencies

Efficiencies were calculated following Omori and Ikeda (1984). Gross-growth efficiency (GGE) was calculated as the quotient between egg production and ingestion rates both expressed in carbon terms.

$$GGE = G/I \times 100 \qquad \text{Eq. 7}$$

where *G* is the egg production rates (µg C_{egg} ind^{-1} d^{-1}) and *I* is the ingestion rate (µg C_{prey} ind^{-1} d^{-1})

Assimilation efficiency is the percentage of ingested food that is digested, and it can be calculated from respiration and growth as AE_R:

$$AE_R = (G + R)/I \times 100 \qquad \text{Eq. 8}$$

Where *R* is the respiration rate (µg C ind^{-1} d^{-1}). Additionally, the assimilation efficiency was calculated from the egestion (AE_E) as:

$$AE_E = (1 - E/I) \times 100 \qquad \text{Eq. 9}$$

Where *E* is the egestion rate (µg C_{pellet} ind^{-1} d^{-1}). Net growth efficiency (*NGE*) represents the percentage of assimilated food converted into growth, expressed as:

$$NGE = G/(G + R) \times 100 \qquad \text{Eq. 10}$$

Statistics

One-way ANOVA tests on somatic traits and vital rates were carried out to analyze the simple main effects of rearing temperature and heat stress impacts. Dunnett's or Tukey's posthoc tests were conducted when necessary. Covariance analysis was used to assess differences in body carbon content as a function of body size and temperature. Statistical analyses were performed in R software (v4.1.1; R Core Team, 2021).

RESULTS

Body size and carbon content

The prosome length of adult females was negatively related to rearing temperature (one-way ANOVA, $F_{2,93} = 126.16$, $p < 0.001$) with an average ± SE of 1,075.8 ± 4.6 µm, 1,001.2 ± 6.1 µm, and 948.1 ± 5.5 µm for 19°C, 22°C and 25°C, respectively. According to the body size pattern, the body carbon content declined with increasing rearing temperature (one-way ANOVA, $p = 0.010$; **Fig. 24**). The exposure to the thermal stress significantly reduced the body carbon content of the individuals independent of their rearing temperatures ($p = 0.024$). The average reduction in body carbon content was 0.43 ± 0.013 µg C, representing a 10.9% decrease in body mass (**Fig. 24**). As expected, the prosome length of individuals exposed to the thermal stress (28°C) did not differ from that of the individuals at their respective rearing temperatures (one-way ANOVAs, $p > 0.09$ in all cases).

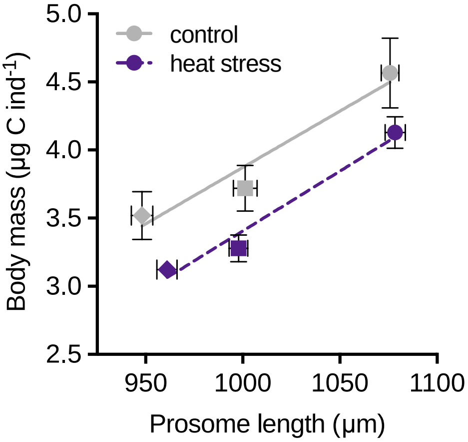


Fig. 24 | Carbon content of adult *P. grani* female after the 7-day exposure to control (19°C: ●; 22°C: ■; 25°C: ♦) and heat stress (28°C). Mean ± SE is shown.

Per capita vital rates

The effects of long-term warming and heat stress on the vital rates of *P. grani* are shown in **Fig. 25**. Feeding rates did not differ after multigenerational rearing at 19°C, 22°C and 25°C (one-way ANOVA, $F_{2,9} = 2.344$, $p = 0.152$), with an average of 5.4 ± 0.4 µg C ind^{-1} d^{-1} (**Fig. 25a**). Feeding rates under heat stress were 41% higher for the 19°C rearing population (one-way ANOVA, $F_{1,6} = 23.553$, p = 0.003). At the same time, no differences were found for the other rearing temperatures (one-way ANOVA, both $p > 0.47$).

In terms of carbon produced per individual, egg production rates were significantly affected by the rearing temperature (one-way ANOVA, $F_{2,9} = 6.400$, *p* = 0.019), with lower production for individuals from the 22°C population (Dunnett's test, *p* = 0.033) **(Fig. 25b)**. Heat stress only resulted in significantly higher carbon egg production rates for the 19°C culture (one-way ANOVA, $F_{1,6} = 10.204$, p = 0.019), whereas no significant effects were detected for 22°C and 25°C cultures (one-way ANOVA, both $p > 0.140$). Egg production in terms of egg numbers ranged between mean values of 69.7 ± 2.1 and 96.3 ± 4.9 eggs female^{-1} d^{-1}, and can be found as supplementary information **(Supplementary Fig. S6)**; mean egg size ranged between 78.2 ± 0.3 and 82.1 ± 0.5 µm and are also provided as supplementary (**Supplementary Table S1**).

Although we did not carry out specific experiments to assess the hatching success of the copepod populations, when counting eggs for egg production, we recorded differences in the number of eggs already hatched during the feeding incubation period (**Supplementary Fig S7a**); only at 19°C no hatching was observed. Interestingly, we found that among these early-hatched nauplii, some of them were in an advanced state of degradation, suggesting early mortality. Under heat stress, the affectation among offspring was twice as high for females reared at 19°C and 22°C (36.0 ± 4.0% and 36.1 ± 3.0%) than for those females reared at 25°C (14.6 ± 4.0%) (Tukey's tests after arcsin transformation, both p = 0.006; **Supplementary Fig S7b**).

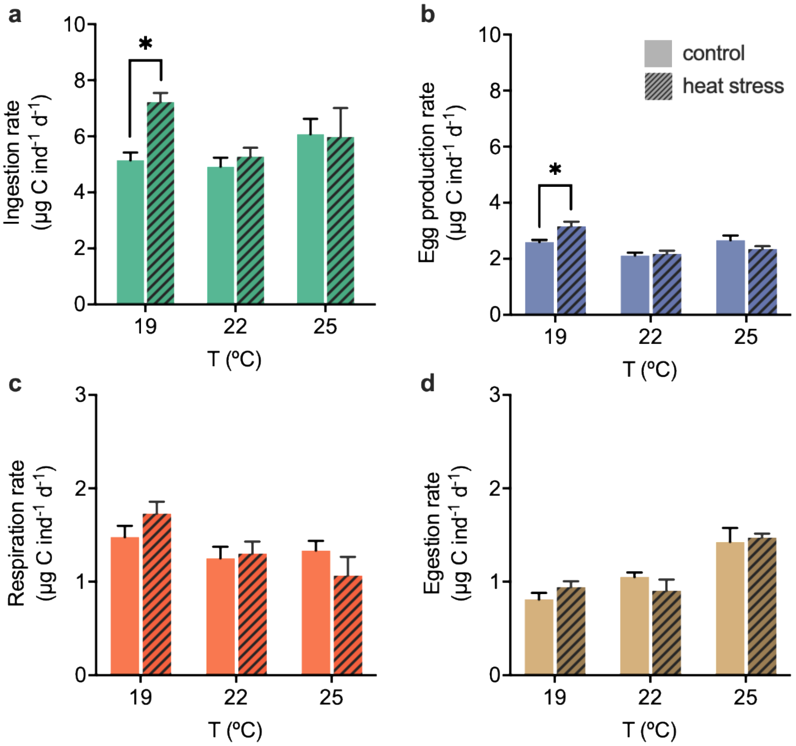


Fig. 25 | Comparison of **a**. feeding, **b**. reproduction, **c**. respiration and **d**. egestion rates of adult *P. grani* females under rearing conditions (19, 22 and 25°C) and exposed to heat stress (28°C). Mean ± SE is shown. Asterisks indicate statistically significant differences between vital rates under heat stress and the respective rearing temperature (one-way ANOVAs, *p < 0.02).

Mean respiration rates per individual did not differ after long-term rearing at warmer temperatures (one-way ANOVA, $F_{2,9}$ = 0.995, p = 0.407) (**Fig. 25c**). Moreover, we did not observe any significant effect of heat stress on copepod respiration rates (one-way ANOVA, p > 0.2 in all cases).

Rearing temperature had a significant effect on the carbon egestion rates with an increasing trend with warming (one-way ANOVA, $F_{2,9}$ = 9.85, p = 0.005; **Fig. 25d**). The response under heat stress, however, did not show any effects on carbon egestion rates when compared with their corresponding rearing temperature treatments (one-way ANOVA, p > 0.2 in all cases). No significant effects of rearing temperature or heat stress were observed on the volume of the faecal pellets (**Supplementary Table S1**, *p* > 0.37 in all cases).

Weight-specific vital rates

The effects of long-term warming and heat stress on weight-specific vital rates of *P. grani* are shown in **Fig. 26.** Rearing temperature significantly affected weight-specific ingestion rates (one-way ANOVA, $F_{2,9}$ = 7.98, *p* = 0.010), with higher rates found at 25ºC than 19ºC (Dunnett's test, *p* = 0.006) and no differences between 19ºC and 22ºC (Dunnett's test, *p* > 0.3) (**Fig. 26a**). Heat stress response of weight-specific ingestion rates were significantly higher for the 19ºC rearing treatment (one-way ANOVA, $F_{1,6}$ = 39.017, p < 0.001), while feeding enhancements were not significant for the 22ºC and 25ºC treatments (one-way ANOVA, p = 0.07 and p > 0.6, respectively).

Weight-specific egg production and egestion rates showed a similar pattern to that of feeding rates (**Fig. 26b,d**). Thus, both rates were influenced by rearing temperature, with an increasing trend (one-way ANOVA, egg production: $F_{2,9}$ = 11.406, *p* = 0.003; egestion: $F_{2,9}$ = 17.866, p < 0.001). Under heat stress, statistically significant enhancements of egg production were found for the 19ºC population (one-way ANOVA, $F_{1,6}$ = 21.874, p = 0.003), while differences were weakly significant for the 22º C treatment (one-way ANOVA, $F_{1,6}$ = 5.431, p = 0.059), and no differences were found for the 25ºC one (one-way ANOVA, $F_{1,6}$ = 0.004, p > 0.9). Regarding egestion rates, we found a weakly significant impact of heat stress for the 19ºC treatment (one-way ANOVA, $F_{1,6}$ = 5.388, p = 0.059), whereas no differences appeared for the other rearing temperatures (**Fig. 26b,d**; one-way ANOVAs, p > 0.18).

Finally, we found no effect of long-term rearing temperature on weight-specific respiration rates (one-way ANOVA, $F_{2,9}$ = 0.932, *p* > 0.4; **Fig. 26c**). Heat stress showed a small impact on the respiration rates of the 19ºC population, but differences were not statistically significant (one-way ANOVA, $F_{1,6}$ = 5.354, p = 0.060); the remaining treatments showed no effect (one-way ANOVAs, p > 0.29 in both cases).

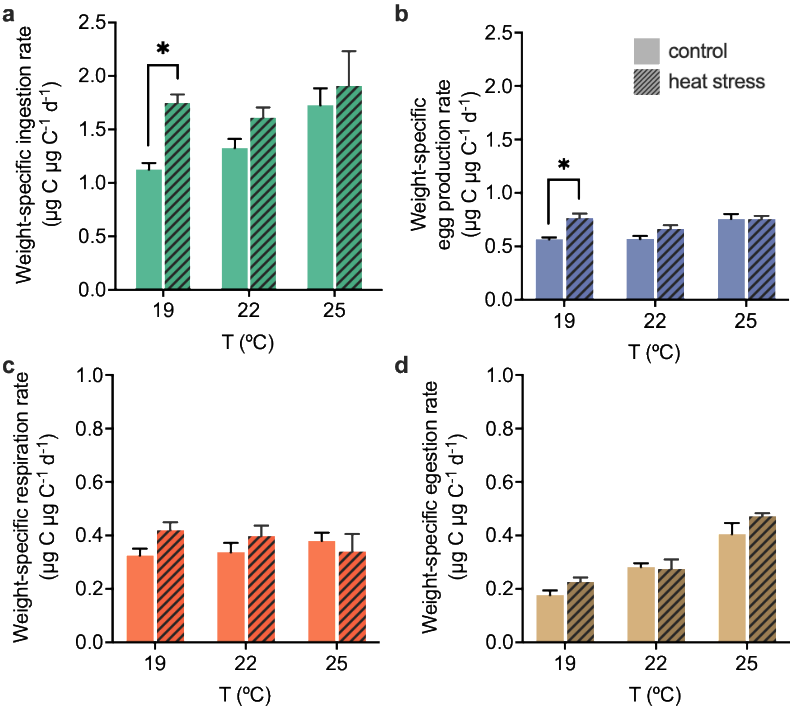


Fig. 26 | Comparison of weight-specific **a**. ingestion, **b**. reproduction, **c**. respiration, and **d**. egestion rates of adult *P. grani* females under rearing conditions (19ºC, 22ºC and 25ºC) and exposed to heat stress (28ºC). Mean ± SE is shown. Asterisks indicate statistically significant differences between vital rates under heat stress and the respective rearing temperature (one-way ANOVAs, *$p < 0.005$).

Growth and assimilation efficiencies

Table 5 shows the estimated growth and assimilation efficiencies. The grand mean GGE and NGE values were 44.6% and 64.8%, respectively, with no differences observed among rearing temperatures (one-way ANOVA, $p > 0.09$ in both cases) or heat stress conditions (one-way ANOVAs, $p > 0.10$ in all cases). Regarding assimilation efficiencies, grand mean values for AE_R and AE_E were, respectively, 69.0% and 80.1%. Similar to GGE and NGE, overall no differences in AE_R and AE_E were found among rearing temperatures (one-way ANOVA, $p > 0.065$ in both cases), or heat stress (one-way ANOVAs, $p > 0.10$), except for a weakly significant effect of heat stress in AE_R for the 19ºC population ($p = 0.060$).

Table 5 | Growth and assimilation efficiencies of adult *P. grani* females reared at 19ºC, 22ºC and 25ºC and exposed to heat stress (HS; 28ºC). Mean ± SE is shown.

Rearing temperature (ºC)	Treatment (ºC)	*GGE* (%) (*G*/*I*) × 100	AE_R (%) (*G*+*R*/*I*) × 100	AE_E (%) (1 - *E*/*I*) × 100	*NGE* (%) (*G*/*G*+*R*) × 100
19ºC	Control	51.0 ± 2.9	80.2 ± 4.3	84.2 ± 1.1	63.6 ± 0.7
	HS (28ºC)	44.0 ± 2.3	68.1 ± 2.9	86.9 ± 1.0	64.6 ± 1.2
22ºC	Control	43.3 ± 2.7	69.0 ± 3.9	78.4 ± 1.4	62.7 ± 1.1
	HS (28ºC)	42.1 ± 4.2	67.0 ± 5.7	82.7 ± 2.3	62.6 ± 1.2
25ºC	Control	44.3 ± 2.2	66.8 ± 4.1	75.9 ± 3.4	66.5 ± 1.4
	HS (28ºC)	43.0 ± 6.9	62.9 ± 11.0	72.2 ± 6.3	68.8 ± 0.9

Overall balance

Figure 27a shows a metabolic balance chart summarizing all the measured carbon pathways on a per capita basis in our experiments. We also provide a summary of the fold change effect of heat stress on the studied weight-specific vital rates, showing that the impact of heat stress appears inversely related to the rearing temperature and comparing the thermal response of vital processes (**Fig. 27b**).

The percentage of ingested carbon lost via respiration accounted for mean values of 26% among rearing temperatures and 22% for the heat stress treatments, whereas the percentage of carbon devoted to reproduction (i.e., GGE) varied between mean values of 46% among rearing temperatures and 43% for the heat stress treatments (**Fig. 27c**). Finally, egestion rates accounted for 20% and 18% of carbon intake for, respectively, the rearing temperature and heat stress treatments. The overall balance shows that the determined carbon losses represent, on average, 92.3% of the carbon intake (**Fig. 27c**). The influence of heat stress on metabolic balance resulted in a larger mismatch between carbon intake and losses (83.7%), particularly distinct for the 19ºC and 22ºC treatments.

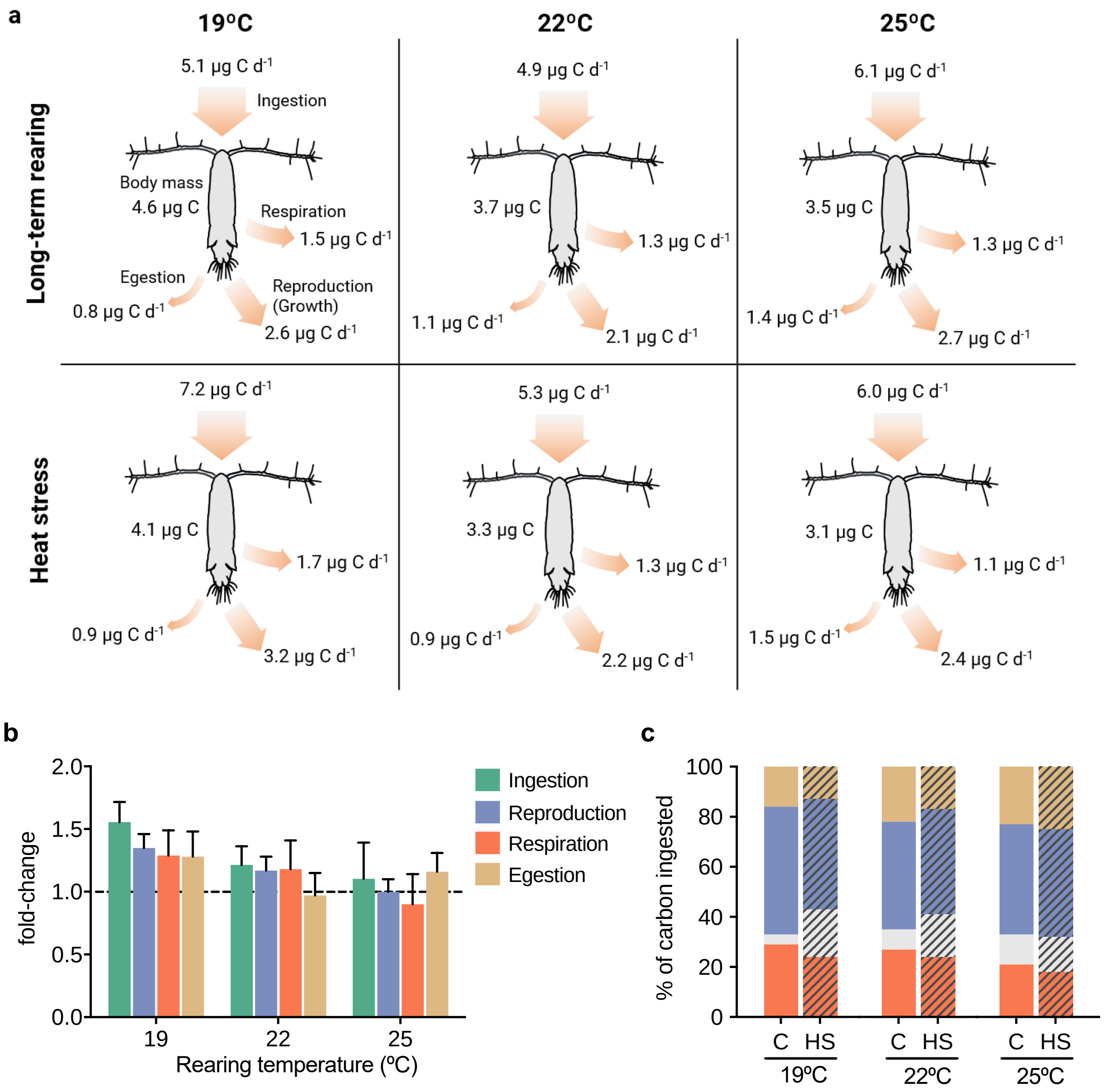


Fig. 27 | Summary of results. **a**. Measured carbon budget of adult *P. grani* female under rearing temperatures (19°C, 22°C and 25°C; upper row) and exposed to heat stress (28°C; lower row). Values indicate averages of per capita vital rates shown in **Fig. 25**. **b**. Fold-change in weight-specific vital rates under heat stress in relation to the respective rearing temperature. Average ± SE is shown. The dashed line indicates a fold-change of 1.0. **c.** Percentage of the ingested carbon devoted to respiration, reproduction and egestion under rearing conditions (C) and exposed to heat stress (HS; 28°C). Note that the fraction in grey represents the ingested carbon whose investment is not accounted for by the measured loss rates.

DISCUSSION

Long-term thermal response

In ectotherms, physiological processes typically exhibit exponential responses to a sudden rise in ambient temperature. Previous research by Saiz et al. (2022) found that short to medium-term thermal stress (19-25ºC, 24h - 7 days) within a single generation of *P. grani* significantly impacted ingestion, egg production, respiration and egestion rates. Our experiments, spanning multiple generations of exposure, revealed that the thermal responses of per capita ingestion and egg production rates in *P. grani* did not differ between rearing lines (19ºC, 22ºC and 25ºC), aligning with earlier findings (de Juan et al., 2023a). Moreover, in this study, no variations were observed in per capita respiration rates following chronic exposure; a finding corroborated in additional experiments (**Supplementary Fig. S8**). Conversely, egestion rates showed an increase with rearing temperature, contrasting with previous observations showing complete thermal compensation on this trait at medium-term thermal stress (Saiz et al., 2022).

A crucial factor explaining the discrepancies between short-term (Saiz et al., 2022) and multigenerational responses (this study) is the reduction in body size. Given the profound effects of body size on metabolism (Runge, 1984; Saiz & Calbet, 2011), its shrinkage at warmer temperatures is expected to overall reduce carbon inputs and outputs (Riemer et al., 2018). In addition to allometric constraints, the acute physiological response to new environmental conditions may be counteracted by reversible (Rahlff et al., 2017; Saiz et al., 2022) and adaptive adjustments (Vehmaa et al., 2012; Brennan et al., 2022). Notably, in organisms with relatively short life cycles, such as copepods, these adaptive responses can occur in just a few generations (Dam et al., 2021; de Juan et al., 2023a). Nevertheless, in our experiments none of the per capita physiological rates appear to be fully compensated, because no negative trends correlating to the reduction in body size were observed (**Fig. 25**). If thermal effects had been fully compensated, per capita vital rates would decrease with temperature following an allometric body size scaling (Saiz & Calbet, 2007). An incomplete thermal compensation also explains why, in general, weight-specific rates showed an increasing trend with rearing temperature larger than expected according to merely allometric scaling (-0.25; Saiz & Calbet, 2007).

Acclimation response to heat stress

Long-term exposure (rearing) of copepod to warming can imply changes in survival and thermal performance curves, making them more resilient to thermal stress and affecting fitness (de Juan et al., 2023b). In our experiments, copepods from the three rearing temperatures were exposed to a sub-lethal temperature (28ºC) known to exceed the optimum for growth (i.e., reproduction; de Juan et al., 2023b). Hence, our observations are the joint combination of the acclimation response to 28ºC and the thermal history of the population at each rearing temperature. We anticipated that copepods from the 22ºC and 25ºC treatments may exhibit increased resilience to heat stress. Corroborating our hypothesis, overall copepod vital rates of the populations reared at warmer temperatures showed reduced responses to common heat stress (**Fig. 27b**). A similar trend would be expected in a scenario of conservation of the thermal performance curve (i.e., 28ºC is thermally closer to 25ºC than 19ºC), but, as previously discussed, long-term warming included changes in copepod size and shifts in the shape of the thermal performance curve, which affected the relationship between current experienced temperature and the acute thermal stress (**Supplementary Fig. S9**). Egestion rates, strongly influenced by rearing temperature, remained unaffected by heat stress, whether expressed per individual or in weight-specific terms. These findings are consistent with those of Saiz et al. (2022), where acute thermal impacts on egestion rates were mitigated after 7 days in the same copepod species.

Metabolic balance

Different thermal responses of vital processes may result in a misbalance that alters homeostasis and constrains performance and/or survival (Alcaraz et al., 2014). When comparing the copepod populations with different thermal histories, we found that the sum of the observed carbon losses (respiration, reproduction and egestion) did not exceed the carbon gains (ingestion) in any case, suggesting that the copepods at each rearing temperature might have achieved a state of internal balance after the multigenerational exposure. This would be also supported by the GGE values obtained, 43-51%, similar to or even slightly higher than those usually found in the literature for this (e.g. Saiz et al., 2022; Traboni et al., 2021) and closely related species (Kiørboe et al., 1985). The surplus fraction of carbon (gains - losses) accounted for only 4, 8 and 11% of the ingested carbon for the 19ºC, 22ºC and 25ºC populations, respectively (**Fig. 27a,c**). Part, if not all, of this small inconsistency is explained because in our experiments we assessed routine respiration rates (i.e., under food depletion). Thus, our respiration measurements did not encompass the metabolic expenses associated with the entire process of nourishing (ingestion, digestion, absorption, transport, assimilation and, mainly, biosynthesis), namely the specific dynamic action (SDA). Ikeda (2021) in a metanalysis of respiration rates for marine metazooplankton, found that SDA represented 27% of total respiration costs. SDA can represent between 7% (closely related species, *A. tonsa*; (Kiørboe et al., 1985) and 9-10% (metanalysis metazooplankton; (Ikeda, 2021) of the total carbon ingested. Hence, these estimates notably align with the surplus of ingested carbon that remains unaccounted for in our experiments.

We expected that the thermal history of different copepod populations would show different sensitivity to heat stress exposure (28ºC, 7 days). Accordingly, we found that the highest carbon losses (via respiration, reproduction and egestion) were observed in copepods reared at 19ºC under heat stress. Nevertheless, these increased losses were accompanied by a corresponding rise in food intake. Consequently, the total carbon losses under heat stress did not surpass the gains in any case, similar to what was observed under rearing conditions. Under acute stress, we found a higher fraction (although still relatively small) of ingested carbon unexplained by the measured losses, particularly for the 19ºC and 22 ºC populations (from 4 to 19% for 19ºC; from 8 to 16% for 22ºC; and from 11 to 14% for 25ºC; **Fig. 27c**). The amount of unaccounted carbon may exceed the anticipated levels attributed to SDA according to aforementioned literature (Ikeda, 2021). However, considering that other carbon pathways, not accounted for in this study, such as dissolved carbon excretion (e.g. urea or amino acids) or sloppy feeding, are likely negligible for this species (Frangoulis et al., 2004; Møller, 2007; Steinberg & Landry, 2017), it can be inferred that SDA predominantly contributed to the unexplained carbon fraction under heat stress. In support of this, it has been seen that SDA may also increase under thermal stress (Luo & Xie, 2008; Mcgaw & Whiteley, 2012). Actually, the increase in the unaccounted fraction of carbon under thermal stress could also arise from the use of respiration rates based on one-point 24-h incubations, not taking into account potential non-linearity of the metabolic process (Thor, 2003; Almeda et al., 2011). This might also clarify why there were no differences in copepod respiration rates when exposed to heat stress compared to their rearing conditions — a surprising outcome given the assumed energetically costly nature of the heat stress response. In line with this, despite the measured carbon intakes were higher than losses (apparently resulting in net carbon gain), we observed a reduction of the 11% in the body mass of the copepods undergoing the 7-d thermal stress (28ºC) period. If this body mass reduction proportionally occurred over the duration of the heat stress, it would imply a daily loss rate of 1.7%. Yet, we cannot discard that the decline in body mass was the result of an acute effect more pronounced at the initial stages of exposure.

Assimilation and growth assimilation efficiencies

Our estimation of assimilation efficiency, whether based on routine respiration rates (AE_R) or egestion rates (AE_E), yielded notably different values. The grand mean of AE_R (69%) corresponded closely to the commonly assumed value for zooplankton (approximately 70%, see review by Steinberg and Landry, 2017). Supporting the aforementioned constraints in the assessment of respiration rates, NGE values were considerably higher (63-69%) than those in the literature (e.g., Ikeda et al. 2001: 21-54%). AE_E values were generally higher, exceeding >80%, however, are also sensitive to uncertainty, mainly driven by the use of the volume-carbon factor for faecal pellets (0.13 pg C μm^{-3}), which is comparable, on the lower side, to previous reports for copepods (Butler & Dam, 1994; B. Hansen et al., 1996). Hence, most likely, the true assimilation efficiency value would fall between our estimated values of AE_R and AE_E.

Regardless of the potential metabolic costs, heat stress did not impact the copepod reproductive effort; the resulting GGE values were similar to the ones found at the rearing temperature conditions. The absence of a trade-off in reproduction supports the idea that the strategy of maximizing reproduction might be inherent to this (de Juan et al., 2023b) and similar species (Kiørboe et al., 1985), although highly dependent on food availability and the type of diet (Traboni et al., 2021). However, the costs of heat stress may affect reproductive performance not only in terms of the number of eggs spawned but also in the offspring recruitment. In this context, our observations on nauplii viability suggest that early life stages were more sensitive to thermal stress when parental copepods originated from 19ºC and 22ºC than from 25ºC. This observation emphasizes the importance of parental thermal history in assessing the effects of heat stress on copepod population fitness (Vehmaa et al., 2012).

Concluding remarks

The consequences of prolonged exposure to warming encompassed shrinking body size and physiological compensation, resulting in an overall diminished impact of temperature on *Paracartia grani* physiological traits. Consequently, carbon gain (ingestion) and losses (respiration, reproduction and egestion) were closely linked among populations with different thermal histories (19ºC, 22ºC and 25ºC). However, when exposed to a common sub-lethal thermal environment (28ºC), the impact of heat stress on vital rates was lower on the warm-reared copepods. Conversely, for the copepod population reared at the lowest temperatures there was a higher mismatch (likely attributed to SDA costs) in the carbon balance under heat stress. Nonetheless, reproductive effort (GGE) remained unaffected under thermal stress exposure, highlighting the capacity of *P. grani* to endure acute sustained heat events on a medium-term temporal scale. Using ecologically-relevant shifts in temperature, this research contributes to our understanding of the physiological processes occurring at the onset of the decline in performance within the upper sub-lethal range and evaluates the impact of temperature on the carbon fluxes mediated by marine neritic copepods.

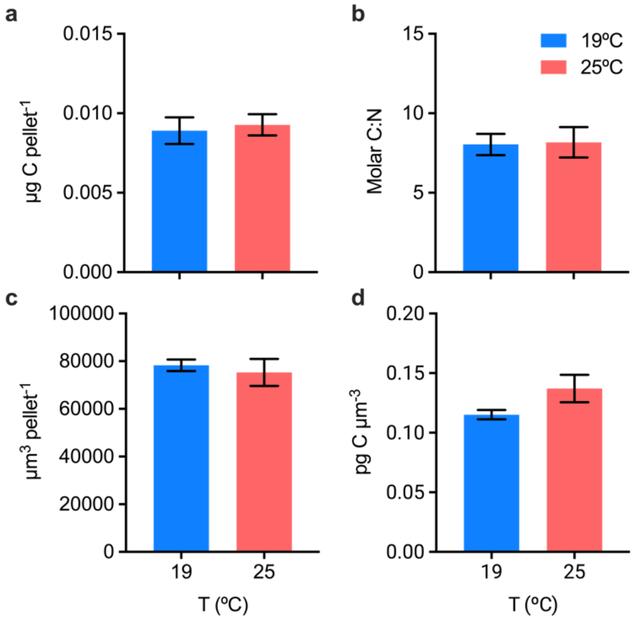
ACKNOWLEDGEMENTS

We thank M. Olivares for his assistance in the care of experimental cultures and respiration measurements.

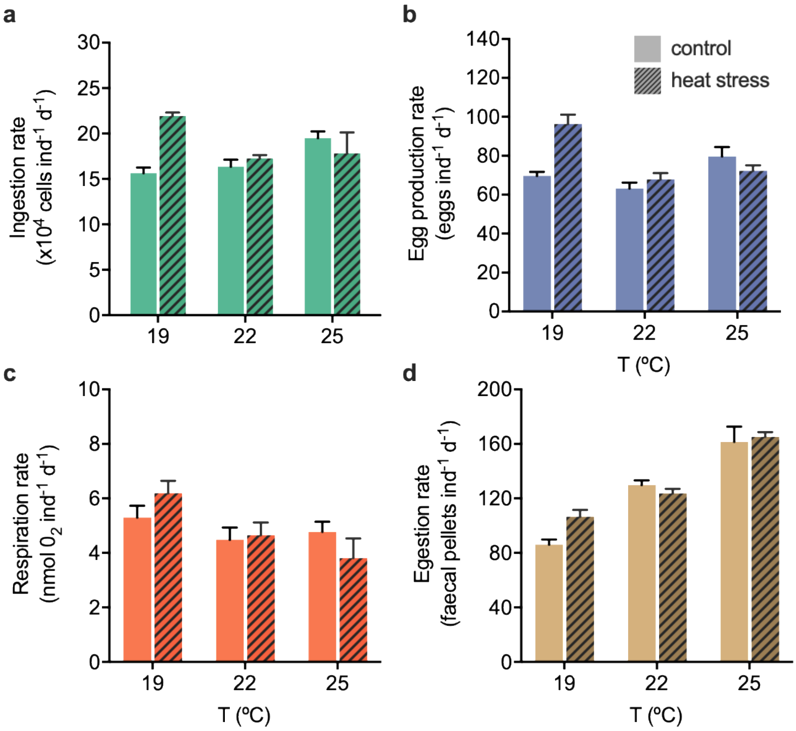
SUPPLEMENTARY INFORMATION

Supplementary Table S1 | Size (ESD) of prey *R. salina*, egg diameter and faecal pellet volume of adult female *P. grani* under exposure to their rearing conditions (19, 22 and 25ºC) and under thermal stress (28ºC).

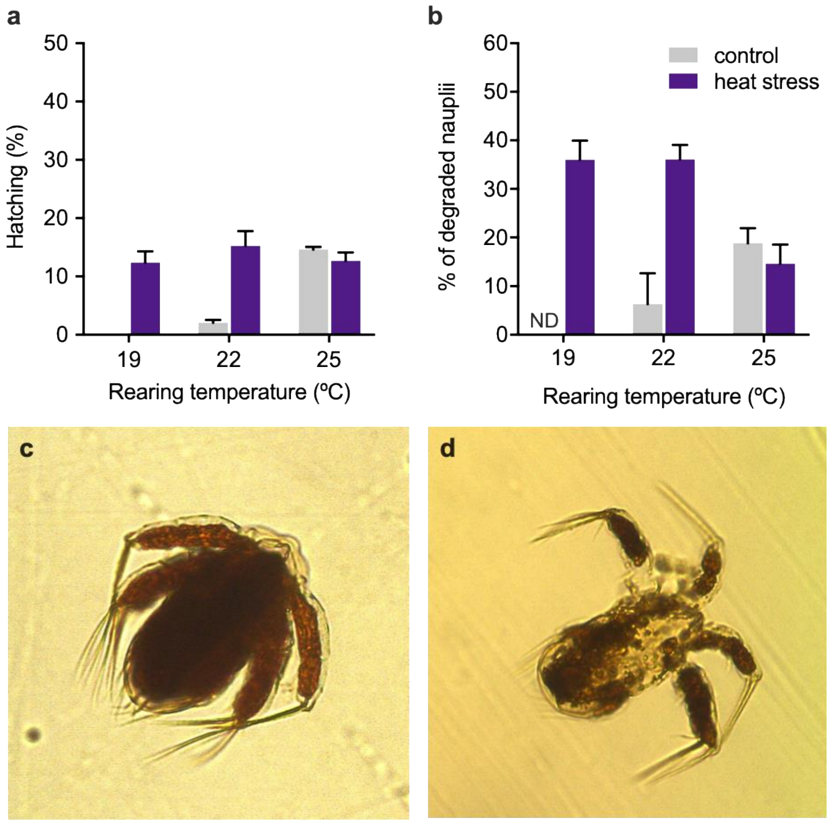
Growth temperature (ºC)	Exposure temperature (ºC)	*R. salina* cell ESD (μm)	Egg diameter (μm)	Faecal pellet volume (10^4 μm^3)
19ºC	19ºC	7.6 ± 0.015	82.1 ± 0.5	7.3 ± 0.8
	28ºC	7.3 ± 0.003	78.7 ± 0.4	6.8 ± 0.5
22ºC	22ºC	7.3 ± 0.002	79.1 ± 0.3	6.3 ± 0.3
	28ºC	7.3 ± 0.002	78.2 ± 0.3	5.6 ± 0.6
25ºC	25ºC	7.4 ± 0.006	79.1 ± 0.2	6.8 ± 0.3
	28ºC	7.4 ± 0.008	78.4 ± 0.3	6.9 ± 0.1



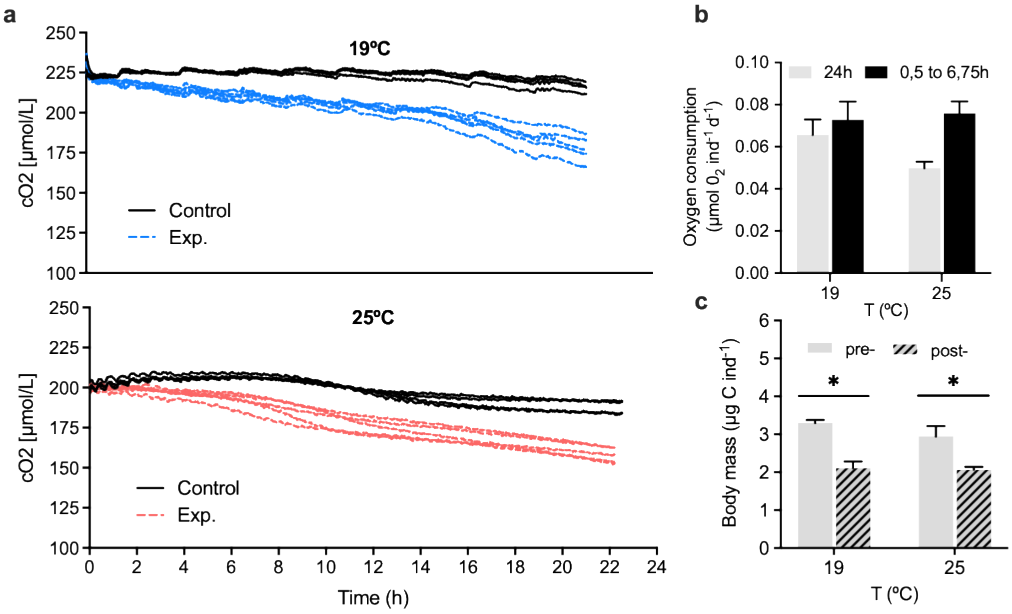
Supplementary Fig. S5 | **a**. Carbon, **b**. molar C:N, **c**. volume and **d.** carbon-volume factor of faecal pellets collected from cultures of *P. grani* reared at 19ºC and 25ºC and fed in satiation with *R. salina*.



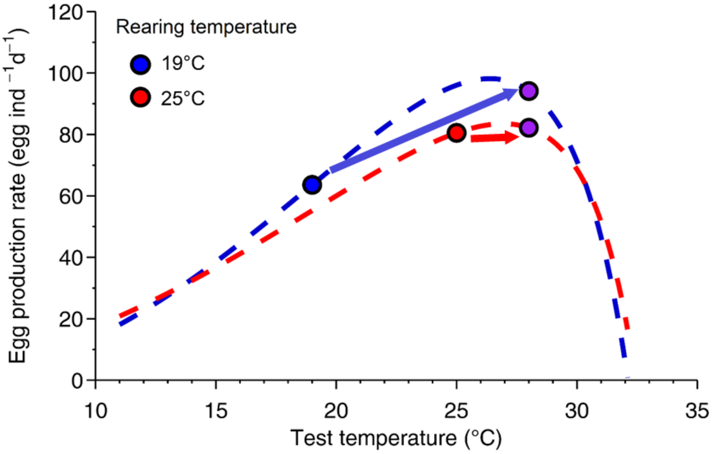
Supplementary Fig. S6 | Comparison of per capita **a**. ingestion, **b**. reproduction, **c**. respiration, and **d**. egestion rates, in terms of number of cells, eggs and faecal pellets, and nmols 0_2, of adult *P. grani* females under rearing conditions (19, 22 and 25ºC) and exposed to heat stress (28ºC). Mean ± SE is shown.



Supplementary Fig. S7 | **a.** Early hatching (%) of the deposited eggs during the 24h incubation at each temperature condition. **b.** Nauplii highly degraded (%) in relation to all nauplii hatched. Bars show average and error bars indicate standard error. N.D. is indicated for treatments for which there is not data available. Images show differences between **c.** normal nauplii and **d**. degraded nauplii.



Supplementary Fig. S8 | **a.** Oxygen concentration in control (0.1-µm filtered seawater) and experimental bottles (filtered seawater + 20 adult *P. grani* females) during 24 h incubation at rearing temperatures (19 and 25ºC) under starvation conditions. **b.** Comparison of respiration rates (µmol 0_2 ind^{-1} d^{-1}) calculated from one-point final measurement (24 h) and those derived from the slope of the first 6 hours (0.5 to 6.75 h) variation in oxygen concentration measured in continuum. Differences between both calculations at 25ºC evidence the non-linear response in individuals exposed to higher temperatures. Nonetheless, there are no differences in per capita respiration rates at 19 and 25ºC following >1 year of rearing at these conditions. **c.** Body mass of adult *P. grani* female before (pre-) and after (post-) 24 h continuous respiration measurements in starvation. Mean ± SE is shown. Asterisks show significance ($p < 0.001$).



Supplementary Fig. S9 | Thermal performance curve for egg production rate of *Paracartia grani* reared at 19ºC and 25ºC for >18 generations. Dashed lines show predicted egg production rates following 7 days of exposure at each test temperature (de Juan et al., 2023b). Blue and red symbols show predicted rates at the rearing temperatures. Purple symbols show predicted rates under heat stress (28ºC).

3.4 Effects of warming on the functional response of copepods: implications on body stoichiometry and growth efficiency

de Juan, C; Sotomayor-García, A., Calbet, A., Saiz, E. Manuscript in preparation

ABSTRACT

We assessed the thermal effects (+ 6ºC) on the functional response of the marine copepod *Paracartia grani* at short (4 days) and long-term (21 generations; 1 year) exposure. We show that basic parameters of functional response (maximum ingestion rate, I_{max}; half-saturation constant, K_m; and maximum clearance rates, F_{max}) differ significantly at the two different timescales. Short-term warming (4 days) resulted in a significantly increase in maximum ingestion rate. In contrast, after multiple generations of exposure, I_{max} did not differ from that of the control. Acclimated individuals required higher food concentrations to achieve saturation levels, suggesting elevated metabolic costs under thermal stress. Long-term warming also shifted satiating levels to higher concentrations, possibly due to a decrease in the foraging capacity (F_{max}) of the warm-reared copepods, potentially associated to their smaller body size. Consistent with body size, elemental composition (carbon, C; nitrogen, N; and phosphorus, P) was lower in warm-reared copepods at all food concentrations. At all thermal treatments, C, N and P body content dropped a 50% from satiation to nearly food depletion levels. The differences in C, N and P body content correlated with the decrease in egg production at decreasing food concentrations. Despite the changes in grazing, the stoichiometric relationships of adult females (C:N, N:P, C:P) remained unaltered by food concentration. Gross-growth efficiency (GGE) increased with food concentration up to an intermedium level, beyond which it gradually declined. Maximum GGE was lower in acclimated individuals, evidencing a higher mismatch between their functional and numerical responses. These results suggest that reduced food availability might constrain the food uptake of thermally-stressed copepods, affecting their thermal response.

INTRODUCTION

Functional responses quantitatively describe the predator prey-dynamics. Key parameters, including maximum clearance rates (F_{max}), half-saturating constant (K_m) and the maximum ingestion rate (I_{max}), characterize the feeding behaviour of the organisms and are widely used in plankton production models (Holling, 1959, 1965; Kiørboe et al., 2018), having a large influence on their stability (Gentleman & Neuheimer, 2008; Chenillat et al., 2021; Rohr et al., 2022). In turn, numerical responses describe the organismal growth as a function of prey density, relying on the rates of food intake and the efficiency of utilization for growth (Solomon, 1949; Holling, 1965). Given that copepods are often the most abundant group in zooplankton, hence, constituting a major link between planktonic primary producers and upper consumers, determining their functional and numerical responses are crucial to understand plankton system dynamics.

As in most ectotherms, physiological processes in copepods, such as feeding and reproduction rates, accelerate with temperature at initial stages of exposure (Ikeda et al., 2001; Almeda et al., 2010b). Under increased environmental temperature, the energetic requirements of organisms are expected to elevate and therefore the use of resources by consumers. Laboratory experiments assessing the thermal sensitivity of copepod physiological rates, usually report 2-3 fold-change increases in rates under 10ºC increase (Q_{10}; Prosser, 1961; Saiz et al., 2022). However, these relationships are fundamentally based in short-term exposures (hours – days), not encompassing possible adaptive changes in the physiology of organisms. Hence, more prolonged exposures, involving multiple generations, allow to complex processes to take place (Schulte et al., 2011; Dam, 2013); potentially compensating the thermal effects on metabolism and thus, also resources consumption (de Juan et al., 2023a). Furthermore, temperature inversely influences body size, which may constrain physiological processes (Riemer et al., 2018) and change the organism's relationship with its environment.

The previous mentioned changes in copepod activity rates might be true only when resources are not limiting. However, in marine environments, the resources are distributed heterogeneously and organisms face abrupt changes in food availability that most often do not reach saturating levels. Additionally, current warming trends are expected to affect primary productivity, by diminishing the nutrients available (Agusti et al., 2017), and altering the quantity and quality of prey items (Hixson & Arts, 2016). Organisms experiencing fluctuations in food availability often possess life-history strategies that allow them to feed efficiently during resources depletion or buffering against the effects of starvation periods (Calbet & Alcaraz, 1996, 1997). Yet, under thermal stress, insufficient food uptake and assimilation may fail to cover the elevated metabolic costs, constraining their response (Huey & Kingsolver, 2019). Furthermore, the specific energy allocation strategies under scarcity of resources might have costs on fitness components, such as growth and reproduction, as well as changing organismal elemental composition, with the subsequent scaling effects on the trophic chain.

Here, we compared the functional and numerical responses of the calanoid copepod *Paracartia grani* feeding on the autotrophic flagellate *Rhodomonas salina* at two different timescales (4 days and 23 generations) of exposure to warming (+ 6ºC). *P. grani* is an omnivorous suspensions-feeder, able to create feeding currents but also shift to ambush strategies. Functional responses have been described for their whole ontogeny (Olivares et al., 2019) and with different types of prey (Helenius & Saiz, 2017). However, the effects of warming on their feeding response has not yet been described. Our working hypothesis were: 1) I_{max} will be reduced following long-term rearing under warming compared to a short-term response; 2) K_{m} will also be reduced because of the lower demands of their whole organism basal metabolism; and 3) numerical responses will match the functional responses at all thermal conditions; hence, no effects of thermal stress on gross-growth efficiency will be expected. We also evaluate the effects of changing ingestion and gross-growth efficiency on their stoichiometric body content.

MATERIAL AND METHODS

Copepod and prey cultures

Paracartia grani (Sars, 1904) specimens were originally collected in coastal waters north of Barcelona (NW Mediterranean) in 2007-2008 and maintained at 19°C (± 1°C) in walk-in chambers at the Institut de Ciències del Mar (ICM, CSIC) for >15 years. From this culture, we stablished two separated lines at 19 and 25°C in 20L polycarbonate tanks submerged in 150L water baths connected to TECO cooler/heater units (± 0.1°C) and provided with artificial light at 15-20 µE m^{-2} s^{-1} in a 10:14h light/dark cycle. We routinely fed copepods *ad libitum* three times a week with the cryptophyte *Rhodomonas salina* (strain K-0294, Scandinavian Culture Collection of Algae and Protozoa). In turn, *R. salina* (equivalent spherical diameter, ESD = 8 µm) was grown exponentially in semi-batch cultures kept in a sterile f/2 medium (Guillard, 1975), in a light- and temperature-controlled room at 19°C (± 1°C), 10:14 light/cycle, 80 µmol photons m^{-2} s^{-1}. We ensured permanent satiation or close-to-satiation feeding conditions by monitoring life-stage and copepod density and adjusting the concentration of *R. salina* in the cultures (from 2 ppm for early-stage nauplii to 10 ppm for adults; Olivares et al., 2019). We reared successive generations of the two copepod lines under these conditions for more than 20 generations (>1 year).

Functional and numerical response experiments

We determined feeding and egg production rates at 10 prey concentrations (1020 - 48000 cell mL^{-1}; 0.2 - 9.5 ppm; 20 - 976 µg C mL^{-1}; **Supplementary Fig. S10**) in copepods reared at 19°C (*control*) and 25°C (*warm-reared*), and in 19°C individuals incubated at 25°C after 4 days of pre-exposure (*warm-acclimated*). Prior to the experiments, we collected copepods from each culture using a 250 µm sieve and transferred them in groups of 50-180 females (plus the corresponding males), depending on prey concentration, to single 4L Nalgene bottles filled with thermally acclimated suspension at each concentration and amended with nutrients (70-160 mL of f/2 medium per litre) to compensate for zooplankton excretion and ensure nutrient availability for growth. Prey concentration were determined using a Particle Counter (Beckman Multisizer 3

Coulter Counter, 100 μm aperture tube). We submerged the experimental Nalgene bottles in water baths at the corresponding temperature. The next two consecutive days, we completely renewed the suspensions from the Nalgenes. To do that, we filtered their content through a 100 μm sieve to retain the adult copepods (>100 μm) and discarded the old suspension containing also eggs, nauplii and faecal pellets (<100 μm). We returned the retained copepods to the Nalgene bottles filled with the fresh suspension, at their corresponding prey concentration and temperature. Further experimental details can be found in (Saiz et al., 2014; Olivares et al., 2019). On the fourth day, we extracted the copepods from the Nalgene bottles using a 160 μm sieve. Subsequently, we gently transferred them individually, in batches of 8 to 28 females, depending on prey concentration, using a Pasteur pipette, to 610 mL Pyrex bottles filled with new suspensions at the same concentrations and temperature and amended with nutrients (5mL of f/2 medium per litre). The number of copepods in each bottle was calculated based on Olivares et al. (2019) to obtain a 30% of prey depletion during the incubation and it was similar for all temperature treatments. Two control bottles (without copepods) and two experimental bottles (with copepods) were run for each prey concentration. Initial prey concentration in the bottles was assessed using the Particle Counter. Control and experimental bottles were submerged in the water baths at the corresponding temperature for 24 hours. Following the end of the incubation, all bottles were sequentially filtered through a 20 μm and 100 μm sieve. The small fraction (<20 μm), containing the suspension, was used to determined final algae concentration (in terms of cell and biovolume) in the bottles. Copepods (>100 μm) were transferred to Petri dishes and checked for survivorship and counted under a stereomicroscope microscope. The intermediate fraction (20-100 μm), containing eggs, was fixed in 2% Lugol's solution. Posteriorly, we counted the eggs, egg shells and eggs present in faecal pellets under a stereomicroscope to determine the egg production rate per female. Randomly, we photographed the eggs (n = 20) of one of the two replicates at each prey concentration to determine its size and we measured the diameter of the eggs using ImageJ (v1.53v) software.

Body size and CNP

Parallel to the experiments, we separated copepods from the Nalgene bottles to determine their body size and carbon (C), nitrogen (N) and phosphorous (P) content. For body size, we fixed females from the highest prey concentration of each thermal treatment with formaldehyde at 4% final concentration. Posteriorly, we photographed them (n = 32 per temperature treatment) and determined their prosome length under the microscope using ImageJ software. As no changes in prosome length of matured adults are expected to be caused by changes in food availability, these measurements were considered representative of the size of the copepods at each thermal treatment. For CNP analysis, females were left for approximately 30 min in 0.1 µm-filtered seawater to clear their digestive tracts. Then, they were transferred to Petri dishes, immobilized with MS-222, and transferred in groups (18-40 for CN, 7-30 for P) onto pre-combusted 25-mm Whatman GF/C filters (450ºC, 5h). For CN, we ran two replicate filters for each prey concentration; for P, we ran two-three replicates. The CN filters were dried for 48h at 60ºC and then stored in a desiccator until analysis with a Thermo Finnigan Flash EA1112 CHNS analyzer. Filters for phosphorous were frozen at -80ºC; filters were posteriorly treated with acid persulfate digestion to convert particulate organic P into inorganic dissolved P, and then processed using a Seal Analytical AA3 (Bran + Luebbe) analyzer.

Calculations

We determined feeding (cells ind^{-1} d^{-1}) and clearance (mL ind^{-1} d^{-1}) rates (i.e. the volume swept clear by the copepods during the incubations) at each average prey concentration (cells mL^{-1}) according to Frost's equations (Frost, 1972). To obtain feeding rates expressed in carbon terms, the algae biovolume (mm^3 L^{-1}) consumed, estimated from the Particle Counter measurements, was converted to carbon by applying a volume-carbon estimate of 0.16 pg µm^{-3} for *R. salina* (Saiz et al., 2020). To express the egg production rate in carbon terms, we used a factor of 0.13 pg µm^{-3} (Saiz et al., 2022; de Juan et al., 2023a). Weight-specific rates were then calculated by dividing the feeding and egg production rates both expressed in carbon terms, by the carbon content of the copepods. Gross-growth efficiency (GGE) was calculated as the quotient of egg production and feeding rates also both expressed in carbon terms. From C, N and P content we calculated the molar CN, CP and NP ratio. The standard error of C:P and N:P were calculated by error propagation.

Models

We assessed functional responses by fitting Holling type III models (Holling, 1965; Real, 1977):

$$F = I_{max} \times C / (C^q + K_m{}^q) \qquad \text{Eq. 11}$$

where F is clearance rate, I_{max} is maximum ingestion rate, C is prey concentration, q is an exponent (>1) and K_m is the half-saturation constant; and

$$I = I_{max} \times C^q / (C^q + K_m{}^q) \qquad \text{Eq. 12}$$

where I is ingestion rate. From these fits, we calculated clearance rates (F_{max}) following Helenius & Saiz (2017):

$$I = (F_{max} \times 2K_m \times C^q) / (C^q + Km^q) \qquad \text{Eq. 13}$$

Similarly, we determined numerical response by fitting a Holling Type III , changing I and I_{max} for G (egg production rates) and G_{max} (maximum egg production rate), respectively. To assess the relationship between C, N and P body content and prey concentration, we adjusted Michaelis-Menten models at each temperature treatment.

Statistics

Holling type III (for feeding and egg production rates) and Michaelis-Menten curves (for CNP) were fit with non-linear regression in GraphPad Prism (v. 9.0e) software, and differences in the fit parameters among temperature treatments were assessed by F tests. Post-hoc Tukey test was conducted to compare the parameters of each thermal condition. Given that GGE showed a non-linear response to food concentration with maximum values found at intermedium concentrations, we compared the effects of each thermal treatment using separate ANCOVAs for increasing and decreasing GGE values. Effects of food concentration and differences between thermal treatments on C,N and P body content and egg size were also assessed with ANCOVAs.

RESULTS

Functional response

Holling Type III ($q > 1$) models adjusted well to the observed feeding patterns at increasing *R. salina* concentration at 19ºC, 25ºC-acclimated and 25ºC-reared ($r^2 = 0.93$, $r^2 = 0.95$, and $r^2 = 0.94$, respectively; **Fig. 28a,b,c**). The parameters of the fitted functional responses are shown in **Table 6**. Clearance rates increased with food concentration, reached a peak at low-intermedium level and decreased at further increases in food concentration. The F_{max} value of the long-term exposed copepods showed a remarkable drop (7.7 mL^{-1} ind^{-1} d^{-1}) in comparison with F_{max} values for the control (26.8 mL^{-1} ind^{-1} d^{-1}) and warm-acclimated individuals (24.1 mL^{-1} ind^{-1} d^{-1}). K_m was lower for individuals reared at 19ºC than for those warm-acclimated and warm-reared ($p < 0.0002$ in both cases), while there were no differences between the latter ($p = 0.934$). The highest I_{max} was found in warm-acclimated individuals, and was significantly different that at control ($p < 0.0003$) and long-term treatments ($p = 0.01$). In turn, there were no significant differences in I_{max} between control and long-term acclimation ($p = 0.089$). The percentage of body carbon ingested was higher in warmed individuals than in the control ($p < 0.03$ in both cases), with no differences between them ($p = 0.068$) (**Table 6**; **Supplementary Fig. S11**).

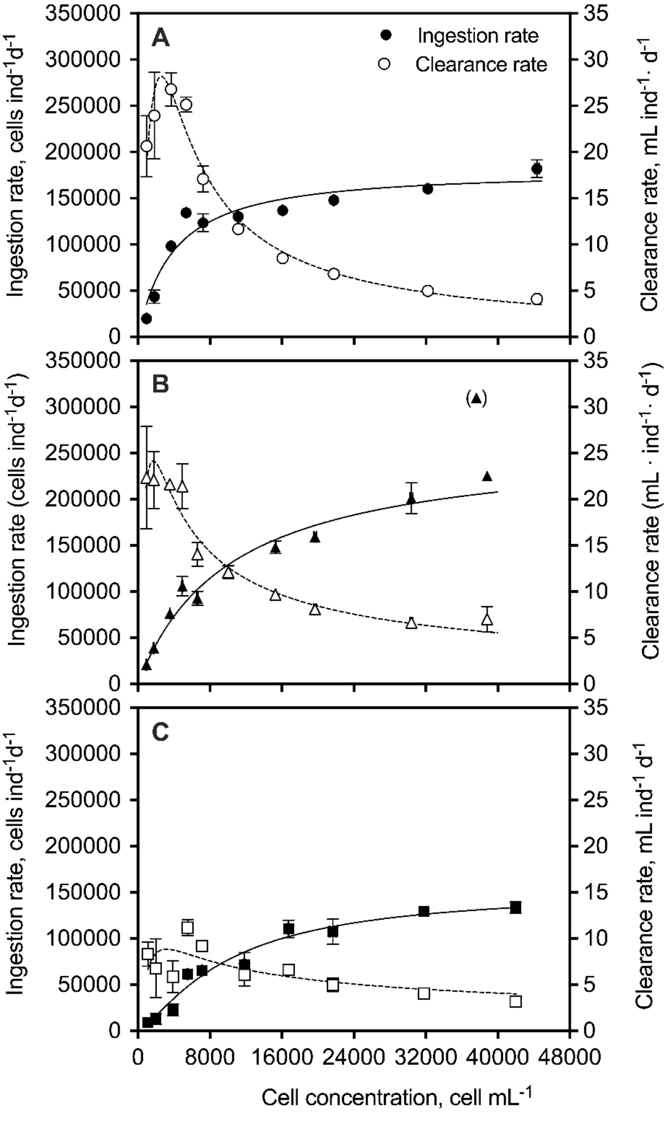


Fig. 28 | Feeding rates as a function of prey concentration of adult female *P. grani* exposed to **A**) control (19°C) **B**) warm-acclimated (25°C for 4 days) and **C**) warm-reared (25°C for 21 generations). Left axis indicates feeding rates and right-axis show clearance rates. Symbols indicate means; error bars indicate standard error. Lines represent fitted Holling Type III curves. The point between brackets is a value excluded from the analysis.

Table 6 | Summary of main parameters of the functional response of adult *P. grani* females feeding on *R. salina* at each temperature treatment.

Treament	I_{max} (cells ind^{-1} d^{-1})	I_{max} (µg C ind^{-1} d^{-1})	I_{max} (% body C$_{ing}$ d^{-1})	F_{max} (ml ind^{-1} d^{-1})	K_m (cells mL^{-1})
Control (19ºC)	162833 ± 7696	6.12 ± 0.59	170.4 ± 6.2	26.8 ± 2.7	3041 ± 377
Acclimated (4d)	261315 ± 43371	8.63 ± 1.55	250.6 ± 39.0	24.1 ± 2.9	10275 ± 4021
Reared (F21)	15304 ± 20467	7.38 ± 2.4	228.2 ± 36.8	7.7 ± 1.1	9983 ± 2673

Egg production rates and gross-growth efficiency

Holling Type III ($q > 1$) were also fitted to the observed egg production rates as a function of prey availability in the different treatments (**Fig. 29**). G_{max} was not significantly different among treatments ($p > 0.774$), with values of 57.2 ± 2.8 eggs ind^{-1} d^{-1}, 58.5 ± 5.0 eggs ind^{-1} d^{-1} and 53.8 ± 10.4 eggs ind^{-1} d^{-1} for control, acclimated and warm-reared copepods, respectively. Egg diameter was not affected by food concentrations (linear regression, $p > 0.06$ in all cases) (**Supplementary Fig. S12**). Eggs produced at 19ºC were larger (80.0 ± 0.2 µm) than those produced by the 25ºC reared copepods (78.3 ± 0.3 µm) and both were larger than those of the warm-acclimated individuals (76.9 ± 0.2 µm) (Tukey's post-hoc comparison, $p < 0.002$ in all cases).

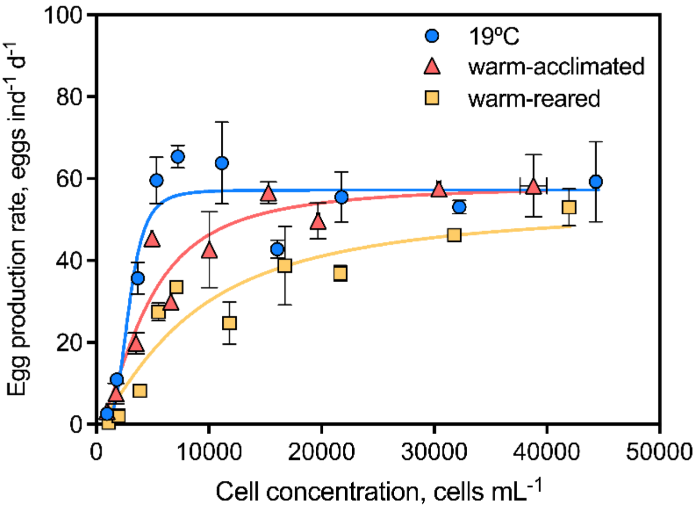


Fig. 29 | Egg production rates as a function of prey concentration of adult female *P. grani* exposed to control (19ºC), warm-acclimated (25ºC during 4 days), and warm-adapted (25ºC during 21 generations, 1 year). Symbols are means, and error bars are standard error. Lines represent fitted Holling Type III (q > 1) curves.

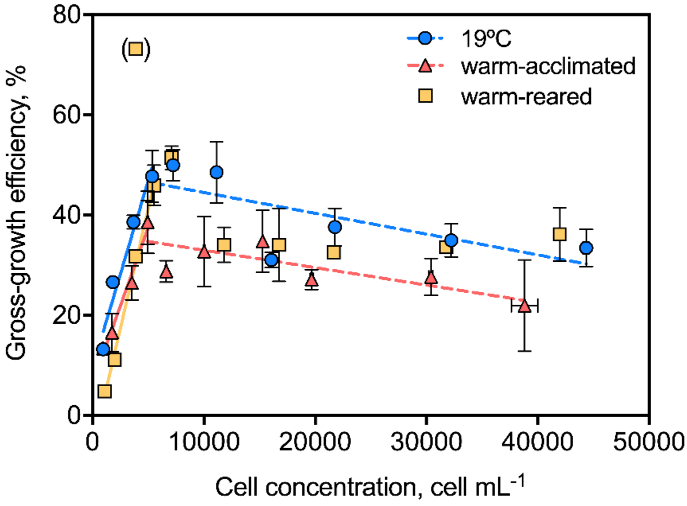


Fig. 30 | Gross-growth efficiency as a function of prey concentration of adult female *P. grani* exposed to control (19ºC), warm-acclimated (25ºC during 4 days), and warm-adapted (25ºC during 21 generations, 1 year). Symbols are means, and error bars are standard error. Lines show linear regressions at increasing GGE from low to intermedium concentrations (continuous lines) and decreasing GGE at higher concentrations (dashed lines). The point between brackets is excluded from analysis.

GGE increased from values <13% at the lowest concentrations (<1000 cells mL^{-1}) to a maximum of ca. 50% at intermedium concentrations (~5000 cells mL^{-1}) (**Fig. 30**). Within this range, the slopes did not differ between thermal treatments (ANCOVA, $p > 0.656$ in all cases) and the intercepts were equal in warm-acclimated and warm-reared copepods (ANCOVA, $p = 0.301$) and both lower than 19°C (ANCOVA, $p < 0.05$ in both cases; **Table 7**). At higher prey concentrations, GGEs declined smoothly in control and warm-acclimated individuals (linear regressions; $p < 0.02$ in both cases); in the warm-reared treatment the decline in GGE was not statistically significant (linear regression, $p = 0.07$). Within this range (>5000 cells mL^{-1}), GGE did not differ between control and warm-reared individuals (ANCOVA, $p = 0.656$) and the lowest GGEs were observed in the warm-acclimated copepods (ANCOVA; $p < 0.002$ in both cases).

Table 7 | Equations of linear regressions of GGE and food concentration at increasing and decreasing with food concentration.

Range	Treatment	Cell conc. (cells mL^{-1})	Equation	R^2	p-value
Low	19°C	953 - 5348	$y = 0.00746x + 9.56$	0.92	0.0002**
	25°C-acclimated	946 - 4954	$y = 0.00625x + 6.27$	0.88	0.0006**
	25°C-reared	1103 - 5518	$y = 0.00954x - 6.46$	0.92	0.0006**
High	19°C	5348 – 44330	$y = -0.000415x – 48.71$	0.42	0.0121*
	25°C-acclimated	4954 - 38801	$y = -0.000348x – 26.51$	0.39	0.0172*
	25°C-reared	5518 - 41956	$y = -0.000319x – 44.53$	0.25	0.0706

Body size and stoichiometric content

Body size of individuals reared at 19°C (986.4 ± 4.7 µm) was larger than that of individuals reared at 25°C (944.0 ± 4.7 µm; t.test, $p < 0.0001$). C, N and P body contents showed an assymptotic trend with food concentration, which fitted well to Michaelis-Menten models. C and N body content curves did not differ between the control individuals and those acclimated to 25°C for 4 days ($p > 0.20$ in both cases) (**Fig. 31AC**). Maximum C body content was 3.25 ± 0.13 µg at 19°C and 3.04 ± 0.12 µg C for warm-acclimated individuals, with no differences between them ($F_{1,42} = 1.337$; $p = 0.25$) (**Fig.**

31A). Through the prey concentration range, C body content was lower for individuals reared at 25ºC than control and acclimated individuals ($F_{1,39} = 14.47$, $p < 0.001$). Maximum C body content was 2.30 ± 0.16 µg C for the 25ºC adapted copepods; significantly lower than the control and acclimated individuals ($p < 0.005$ in both cases). Similarly, there were no statistically significant differences ($F_{1,42} = 1.641$; $p = 0.21$) between the maximum N body content in the control and acclimated individuals (0.74 ± 0.04 µg N and 0.67 ± 0.03 µg N, respectively); contrarily, the maximum N body content was lower (0.49 ± 0.04 µg N) for the warm-reared individuals ($p < 0.005$ in both cases) (**Fig. 31C**). P copepod content was 0.21 ± 0.003 µg P in the control conditions (19ºC) (**Fig. 31E**). Acclimated and warm-reared individuals had similar lower P body contents (0.15 ± 0.004 µg P and 0.14 ± 0.004 µg P, respectively; $F_{1,39} = 3.682$; $p = 0.06$), and both were significantly lower the P body content of the copepods in the control treatment ($p < 0.0001$ in both cases).

The molar C:N ratio of female *P. grani* ranged 4.6 - 6.3 at all treatments, with a grand average of 5.3 ± 0.05 (**Fig. 31B**). There were no differences between the average C:N of control, acclimated and adapted individuals (one-way ANOVA, $F_{2,27} = 1.618$, $p = 0.217$). The C:N of control and warm-acclimated copepods did not show any significant trend with temperature ($p > 0.10$ in both cases), whereas an increasing trend with food concentration was observed for the C:N of the warm-reared copepods (linear regression, $p = 0.004$). The molar N:P ratio of *P. grani* ranged 4.6 - 11.6 across treatments (**Fig. 31D**), and proved to be higher in the acclimated individuals (9.6 ± 0.45) compared to the control and adapted copepods (7.2 ± 0.33 and 7.2 ± 0.41, respectively; Tukey's post-hoc comparison, $p < 0.0008$ in both cases). We did not find, however, any significant relationship between the molar C:P ratios and food concentration (linear regressions, $p > 0.184$ in all cases). The copepod molar C:P ratio ranged 28.0 - 61.2 across food concentrations for the three thermal treatments (**Fig. 31F**). On average, the molar C:P ratio was higher in the warm-acclimated copepods (52.1 ± 1.66) than in the control (38.0 ± 1.17) and warm-adapted (37.1 ± 2.00) ones (Tukey's multiple comparisons, $p < 0.0001$, in both cases), which did not differ between them ($p = 0.91$). We found no significant relationship between the copepod molar C:P ratios and food concentrations in any of the treatments (linear regressions, $p > 0.06$ in all cases).

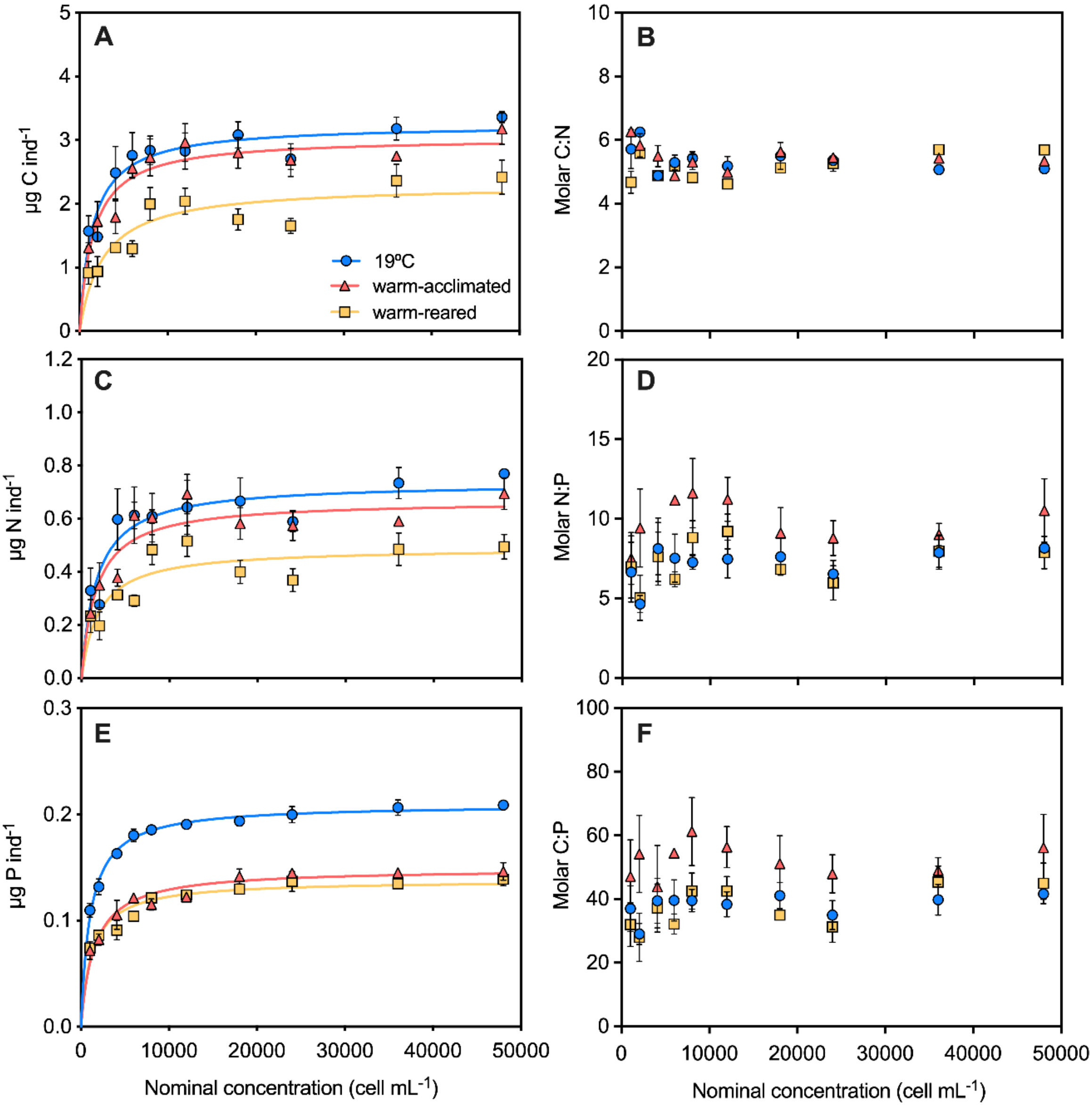


Fig. 31 | Carbon, nitrogen and phosphorous (**A, C, E**) body content, and molar C:N, N:P and C:P (**B, D, F**) body ratios of adult *P. grani* female exposed to control (19ºC), warm-acclimated (25ºC during 4 days) and warm-adapted (25ºC during >20 generations, 1 year) conditions after 4 days of exposure at each prey concentration. Symbols indicate means and error bars indicate standard error. Lines represent fitted Michaelis-Menten models.

DISCUSSION

Functional responses: maximum ingestion rates (I_{max})

Functional responses of ambush feeder copepods are thought to follow a Holling Type II curve ($q = 1$), while Type III ($q > 1$) responses are most attributed to suspension-feeders (Kiørboe et al., 2018). *P. grani*, as other Acartia-like copepods (Kiørboe et al., 1996) can switch between both feeding behaviours as a function of prey type. When offered algae like *Rhodomonas salina*, *P. grani* has been shown to follow type III across its life cycle (Olivares et al., 2019). As expected, in our experiments we reported type III responses for adult *P. grani* female at different timescales of warming (4-d acclimation and 23 generations of exposure).

At high concentrations, feeding rates reached a maximum (I_{max}) at which further increases in food concentrations did not translate into higher grazing. In many organisms, maximum feeding rates are constrained by the several process encompassing from prey detection to ingestion (i.e. handling time). However, in copepods like *P. grani* feeding on comparatively small sized prey, handling time does not seem to be an issue (Tiselius et al., 2013), and intake limitation seems to be driven by gut clearance and digestion capacity (Tiselius, 1998; Henriksen et al., 2007). Consequently, as temperature rises, gut clearance rates and I_{max} increase (Kiørboe et al., 1982). In our study, the increase in I_{max} under 4 days of warming was equivalent to a fold-change in a 10°C increase (Q_{10}) of 2.2, agreeing with the expected acute effect of temperature on physiological rates. Through thermal acclimation, the rates of feeding can be further dampened; however, total compensation (return to process rate prior exposure) was not observed for this species within the first week of exposure ($Q_{10} = 1.7$ after 7 days; Saiz et al., 2022). Yet, at more extended timescales, allowing for multigenerational processes to act, thermal effects on biological rates can be further dampened through plastic and genetic processes (Schulte et al., 2011). Thus, in our experiment, the warm-reared copepod line presented I_{max} values that did not differ from the control ones (i.e., $Q_{10} = 1$). This results are in accordance with previous studies (de Juan et al., 2023a; de Juan, in review) showing thermal compensation of feeding rates at long-term. In addition to eventual compensatory adaptive processes, the feeding rates under multigenerational exposure may have been also affected by the reduction in organismal body size, which constitute a major factor determining physiological processes (Ikeda et al., 2001).

Functional responses: maximum clearance rates (F_{max})

The size (and mass) of organisms also influences their clearance rates (i.e., the capacity of individuals to find and capture prey, Olivares et al., 2019). Accordingly, maximum clearance rates ($F_{\max}$) were markedly lower in individuals reared at 25ºC. However, whether all of the difference in $F_{\max}$ can be attributed to the differences in prosome length (- 40 µm) is uncertain. The resulting allometric exponent (*b*) of the clearance response is far higher (2.2 based on carbon content) than the normally observed (0.75-1.1; Olivares et al., 2019), suggesting that other processes than mere shrinking body size are dampening $F_{\max}$ after multigenerational exposure to warming. Another important factor affecting clearance rates is viscosity but, as it decrease with warming, it should act in the opposite direction, facilitating clearance (Tyrell & Fisher, 2019). Yet, at acute thermal stress, $F_{\max}$ did not differ from that of the control, perhaps indicating the maximum clearance capacity related to its size (Durbin & Durbin, 1992). At increasing concentrations, $F_{\max}$ of the warm-acclimated copepods was consistently higher than that of the controls ones, leading to the aforementioned higher grazing rates at satiating levels.

Functional responses: half-saturation constant (K_m)

Type III responses are characterized by low active search of prey at minimum food concentrations until food levels reach a density that better satisfies the implied energetic expenditure. Subsequently, feeding rates escalate until half-satiating concentrations (K_m) at which the feeding response starts deaccelerating. A raise in basal metabolic costs caused by acute heat stress might affect the threshold critical food concentration to trigger grazing (Frost, 1975), or might increase the food concentration needed to achieve satiation, therefore, shifting K_m to higher prey concentrations. Hence, we expected that warming may affect metabolic costs causing changes in the K_m. Conversely, compensation of thermal effects on metabolism after long-term exposure would reduce again K_m values. In addition, satiating thresholds are also expected to be lower in smaller organisms (Almeda et al., 2010a; Olivares et al., 2019). However, in contrast to our hypotheses, our determined K_m values increased both in the warm-acclimated (acute response) and warm-adapted (long-term exposure) individuals, evidencing at both timescales that copepods had to find higher food concentration levels to start satiating than those at 19ºC. While the anticipated explanation agrees with the observed changes in K_m of acclimated copepods, perhaps it does not apply for "adapted" individual. Copepods exposed to warming during multiple generations are not expected to have higher basal metabolic demands. It has been previously seen that routine respiration rates in adult *P. grani* female do not differ after multiple generations of thermal exposure (de Juan et al., in review).

The size relationship between predator and prey are considered one of the main factors affecting the feeding behaviour of copepods (Berggreen et al., 1988). *R. salina* is a small prey for *P. grani* (Helenius & Saiz, 2017) and therefore, the F_{max} and I_{max} of *P. grani* is lower in comparison to more optimum-sized prey (Olivares et al., 2019; Traboni et al., 2020). As copepod body size and its phytoplankton prey volume shrinks with rearing temperature (Ferreira et al., 2022) some differences in feeding rates could be explained by the predator-prey size ratio. However, the similar magnitude of the changes in *P. grani* body size (in prosome length: - 0.7% $ºC^{-1}$; in carbon: -4.9% $ºC^{-1}$) and *R. salina* volume (in ESD: -0.3 % $ºC^{-1}$, in carbon -1% $ºC^{-1}$, **Supplementary Fig. S13**) did not imply significant changes in the predator-prey size ratio (0.0077 - 0.0079 in size; 0.011 - 0.015 in carbon) that could explain the decrease in F_{max} of warm-adapted individuals.

In turn, the high K_m in the warm-reared copepods could be related to their relatively lower F_{max}, and this could suggest that smaller-sized copepods are affected by mechanical constraints that limit their capacity to forage and capture prey. However, further research is needed to confirm this hypothesis. In this regard, it could be interesting to explore if clearance capacity is influenced by allometric differences in copepod structures (e.g. feeding appendages).

Numerical response, gross-growth efficiency and elemental composition

Prolonged low food conditions significantly impact organisms' fitness. Copepods are less tolerant to acute temperatures when they are food depleted (Saiz et al., 2022; Rueda Moreno & Sasaki, 2023). However, before lethal effects manifest, the decreasing food concentrations have other consequences on copepod fitness: reduction of the maximum metabolic rates (partly due to a decrease in metabolic costs associated to feeding), growth suppression, and lower reproductive effort (Calbet & Alcaraz, 1996; Dagg, 1977). In our experiments, GGE was almost nil at the lowest concentrations and increased to maximum levels at intermedium concentrations. Within this range, warmed copepods seemed less efficient in producing eggs from the carbon ingested. However, this might reflect the differences in the % of body carbon ingested, lower for the warmed copepods at the same food concentrations than the control (**Supplementary Fig. S14**). In fact, for all thermal treatments GGE was equal (11-13%) at a 50% of body carbon ingested and reached maximum values at 100-130% of body carbon ingested at which the warm-reared exhibited the highest GGE (**Supplementary Fig. S15**). Hence, their lower GGE at minimum food concentrations seem to arise from the limitations in feeding described above that constrains the weight-relative food requirements.

At maximum GGE values (>100% of body carbon ingested), the warm-acclimated copepods had significantly reduced GGE compared to the control and warm-reared copepods, indicating a mismatch between their ingestion and the egg production response. In this regard, it is worth noticing that copepods were preconditioned at each food concentration for 4 days, a period of time considered sufficient for the egg production rates to stabilize (Tester & Turner, 1990). Additionally, previous studies have shown that *P. grani* have phenotypic plasticity on egg production rates, including active compensation for thermal effects on this trait. However, none of these studies suggest that thermal compensation can be achieved in 4 days (Saiz et al., 2022; de Juan et al., 2023b; de Juan, in review), nor do they find alterations in the GGE under the same thermal conditions and satiation. In this case, the relatively lower P body content in comparison to copepods of the same size under control conditions might be affecting their reproductive capacity, as P limitation constrains egg production (Isari et al., 2013).

A general decline in GGE was observed in all thermal treatments (although it was not significant at 25ºC), from maximum values observed an intermedium, non-satiating food concentration. This study is not the first to report reductions in GGE at increasing food concentrations in copepods (Paffenhöfer, 1976). Given that ingestion seems to reach maximum levels at higher concentrations than egg production (K_m was lower in egg production), the mismatch between the rates at satiating condition might be determined by a limit in the capacity of egg production. GGEs seem to be maximum at 100-160% of body carbon ingested and descended at above this value. In turn, CNP reached a maximum at non-satiating conditions (~10000 cells mL^{-1}). Thus, within four days under satiating conditions, excess of food that did not translate into eggs, neither accumulated in the body, suggesting a decline in assimilation efficiency and higher egestion. Aligning with this, previous studies report that assimilation efficiency can decrease with some types of prey (Landry et al., 1984; Besiktepe & Dam, 2002).

It is known that *P. grani* accumulates lipids, although not to the extent of other copepod groups, which are mobilized to cover energetic requirements when necessary. However, the drop in C, N and P body content at decreasing food concentrations was correlated with the estimated reduction of elemental content associated to the fewer eggs produced (**Supplementary Fig. S16**). Despite important possible caveats (copepods can exhibit different spawning cycles during a day, e.g. higher reproductive activity at night; Calbet & Alcaraz, 1996; and oocytes can be at different developmental stages; Eckelbarger & Blades-Eckelbarger, 2005), the number of mature oocytes in the gonads can match the deposited eggs within a given period (Niehoff & Hirche, 1996). Additionally, molar ratios were not altered at any thermal treatment within the 4 days of acclimation to food concentration, at least in the range used. Food availability did affect the recruitment of early eggs under warmer conditions; from <5% at low food levels to 15-20% at satiating levels (**Supplementary Fig. 17**).

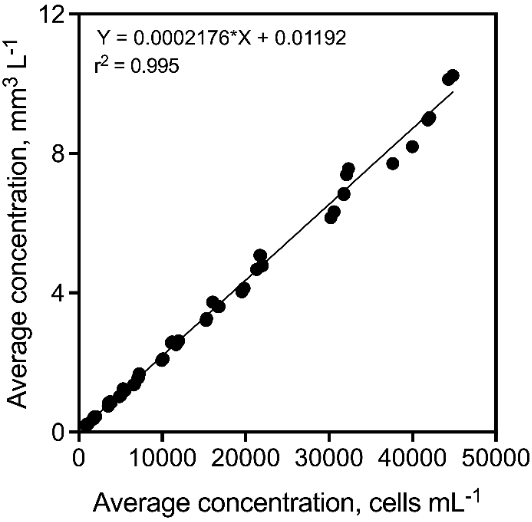
CONCLUSIONS

Holling Types III responses were described for a calanoid copepod exposed to varying thermal conditions (19 and 25ºC) at two timescales (4 days and 21 generations). Maximum grazing rates (I_{max}) on phytoplankton prey increased significantly at short-term exposure to warming but decreased to previous levels following multigenerational exposure, indicating the action of compensatory processes. Maximum clearance rates (F_{max}) did not increase at short-term and were markedly lower in individuals reared for multiple generations at 25ºC. As the latter were significantly smaller than in the other treatments, we attributed the reduction in F_{max} to allometric constraints that reduced their foraging capacity. Warming increased the half-saturation constant (K_m) at both timescales; being attributed, in the case of acclimated individuals, to the higher basal metabolic costs; and in warm-reared to the reduction in F_{max}. These results suggest that reduced food availability might have important affectations on resources uptake in copepods under warming, even after thermal adaptation. Likewise, an increase in the food required to achieve satiation might reduce their reproductive activity. Our study has important implications for the parametrization (I_{max}; F_{max}; K_m) of predictive models on a warmer, more oligotrophic ocean.

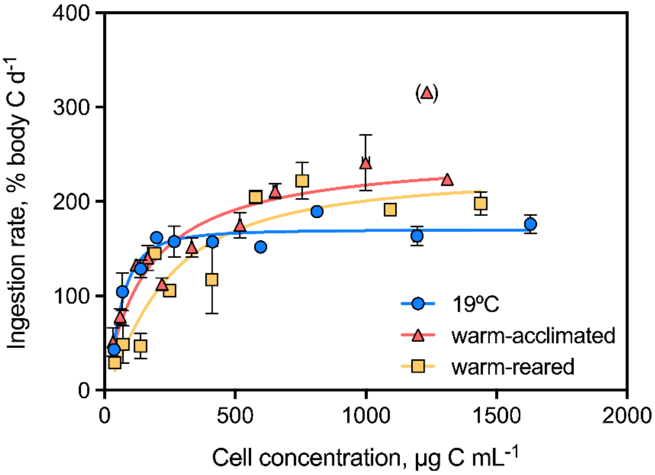
ACKNOWLEDGEMENTS

We thank Claudia Traboni for processing the phosphorous samples prior analysis.

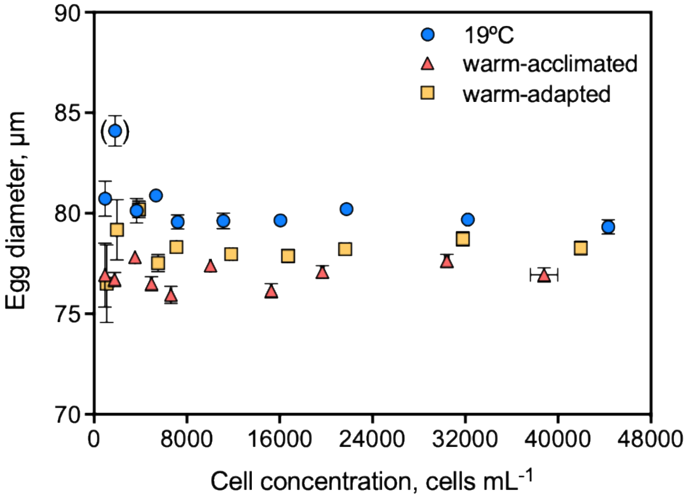
SUPPLEMENTARY INFORMATION



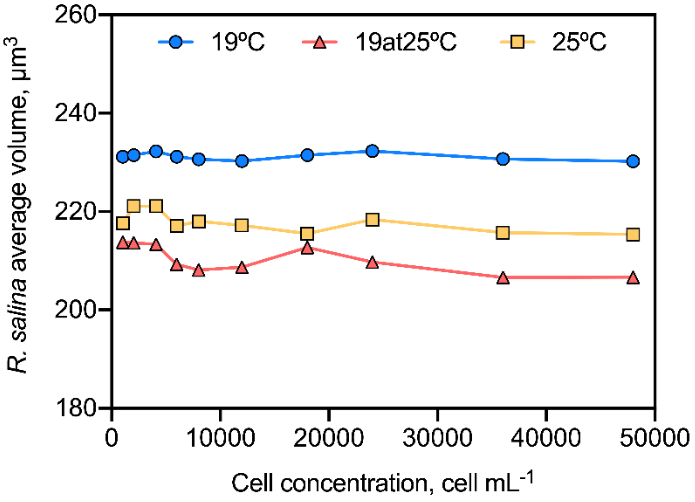
Supplementary Fig. S10 | Relationship between average concentration expressed in cells (cells mL^{-1}) and in biovolume (mm^3 L^{-1}). All temperature treatments are plotted together. Equation and r^2 of the linear regression is shown.



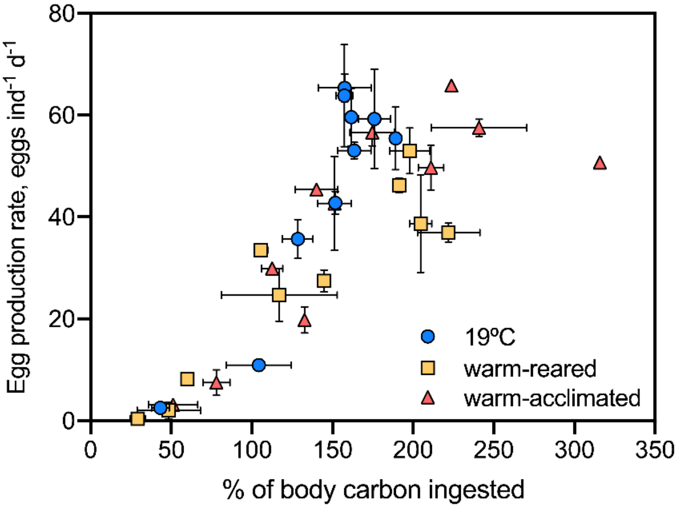
Supplementary Fig. S11 | Percentage of body carbon ingested by adult *P. grani* female as a function of prey concentration when exposed to control (19°C), warm-acclimated (25°C for 4 days) and warm-reared (25°C for 21 generations). Symbols are means and error bars standard errors.



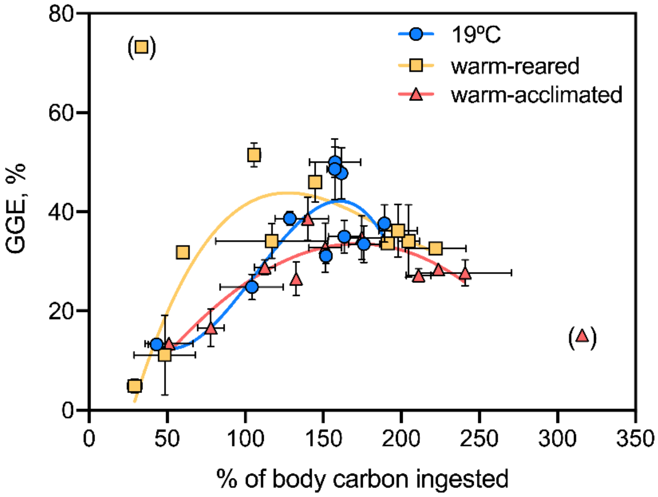
Supplementary Fig. S12 | Diameter of eggs produced by adult *P. grani* females at control (19ºC), warm-acclimated (25ºC during 5 days) and warm-adapted (25ºC during >20 generations, 1 year) conditions as a function of prey concentration. The point between brackets was ignored in the analysis.



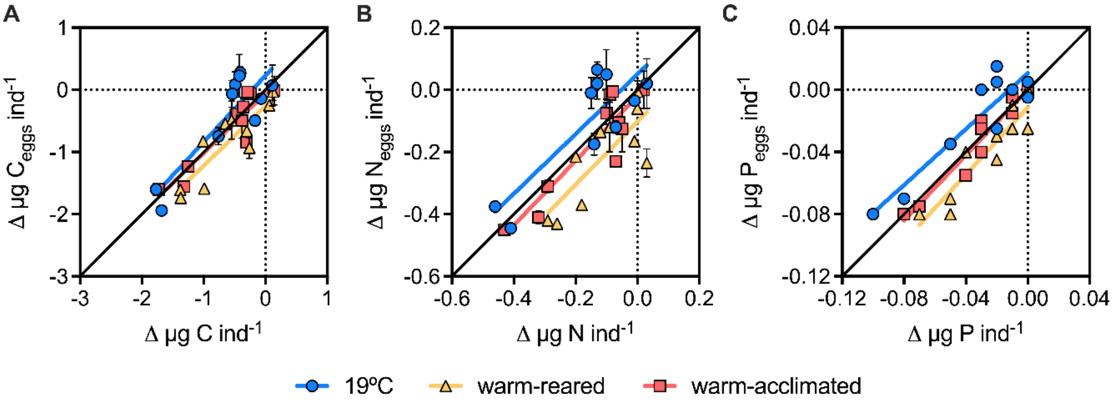
Supplementary Fig. S13 | Average volume of *R. salina* cells during the incubation at each thermal treatment (19ºC, control; 19at25ºC, warm-acclimated; and 25ºC, warm-reared).



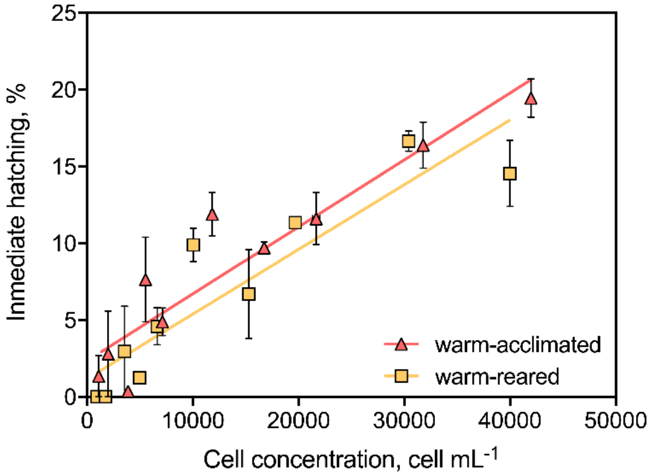
Supplementary Fig. S14 | Egg production rate of adult *P. grani* female as a function of the percentage of body carbon ingested. Symbols are means and error bars standard errors.



Supplementary Fig. S15 | Gross-growth efficiency (GGE) as a percentage of body carbon ingested in adult *P. grani* female exposed to control (19ºC), warm-acclimated (25ºC during 4 days), and warm-adapted (25ºC during 21 generations, 1 year). Symbols are means, and error bars are standard error. Lines show cubic polynomial fits. Points between brackets are excluded.



Supplementary Fig. S16 | Relationship between the losses in **A**) carbon **B**) nitrogen and **C**) phosphorous body content and the differences in the total estimated carbon, nitrogen and phosphorous content of the clutch produced. Maximum values of the fitted models for elemental content and egg production were used as reference points to assess the differences at decreasing food concentrations. The elemental content of the eggs of *P. grani* (32.5 ng C, 8.1 ng N and 1.6 ng P per egg) was extracted from previous works (Saiz et al. 2020; de Juan et al. 2023a) and unpublished data.



Supplementary Fig. S17 | Hatching success (%) within the 24-h incubation for the assessment of ingestion and egg production rates. Symbols are means and error bars standard errors. Lines are simple linear regressions ($p < 0.0001$ in both cases). Intercepts does not significantly differ (ANCOVA, $p = 0.14$).

SUMMARY OF RESULTS

Results 3.1 Multigenerational thermal effects on copepod life-history traits

This chapter explored the effects of multigenerational rearing under warmer temperatures (19ºC, +3 and +6ºC) on the ingestion and egg production rates of *P. grani* males and females, its relationship with body size and the potential associated costs on other life-history traits. Development times in both sexes decreased significantly with warmer temperatures, from 14 days at 19ºC to 9 days at 25ºC in the first generation. At this generation (F1), the body size of adults decreased by -1.2% ºC^{-1} and the carbon content declined by -3.7% ºC^{-1}. The effects of temperature on development, body size and carbon content persisted after ten and eleven generations of rearing. Temperature had positive effects on the ingestion and egg production rates of females only in the first generation, but the thermal response diminished significantly in the subsequent generations. The ingestion rates of the males did not show any significant trend with rearing temperature at any generation. We estimated that the decline in body size could largely explain the reduction in the feeding rates of the females in the first generation of rearing (70% and 129% for, respectively, 22ºC and 25ºC). In the tenth and eleventh generations, however, the drop in body size explained a small fraction of the reduction in feeding rates (10-23%), the rest being attributed to thermal compensation processes. Sex ratio, gross-growth efficiency and egg hatching success remained unaltered across generations and treatments.

Results 3.2 Shifts in the thermal limits for copepod survival and reproduction after chronic warming

This chapter analysed how multigenerational rearing at warmer temperatures (19ºC and +6ºC) affects the tolerance and fecundity of *P. grani* females under short-term exposure to a range of temperatures (11-34ºC). Survival after 24 hours of exposure was close to 100% at the range between 11 and 31.5ºC for both rearing temperatures. The median lethal temperature (LT_{50}) was 32.5ºC for 19ºC and 33.5 for 25ºC. The temperature causing a 90% mortality was 33ºC for 19ºC and 34.3ºC for 25ºC. Overall, multigenerational rearing increased the upper tolerance to acute exposure (24 hours) by 1 – 1.3°C. However, at prolonged stress (7 days) copepod survival decreased at the upper extreme for both the control and warm-reared treatments, and the differences between rearing treatments narrowed to 0.3 - 0.8°C. At most extreme temperatures (31.5 and 32°C) the survival of warm-reared copepods was still 2.2 and 5.4 times higher, respectively, than the control treatments. Surviving females of both rearing temperatures produced eggs at all temperatures tested. Maximum egg production rates were higher for 19°C (98.2 eggs ind^{-1}; 95%CI [91.1, 106.5]) than 25°C (83.5 eggs ind^{-1} d^{-1}; 95%CI [78.8, 86.6]), in part attributed to the smaller body size of the latter (999.3 and 961.1 µm, respectively). The thermal optimum and the critical thermal maximum of the thermal performance curve of reproduction did not differ between rearing temperatures; nevertheless, egg production rates were up to 21 times higher in the warm-reared copepods. Q_{10} coefficients (fold-change increases in rates under 10°C increase) were in general lower in the warm-reared copepods. Overall, differences in survival and fecundity at extreme temperatures suggest a higher potential recruitment of the copepods reared during multiple generations at warmer temperatures.

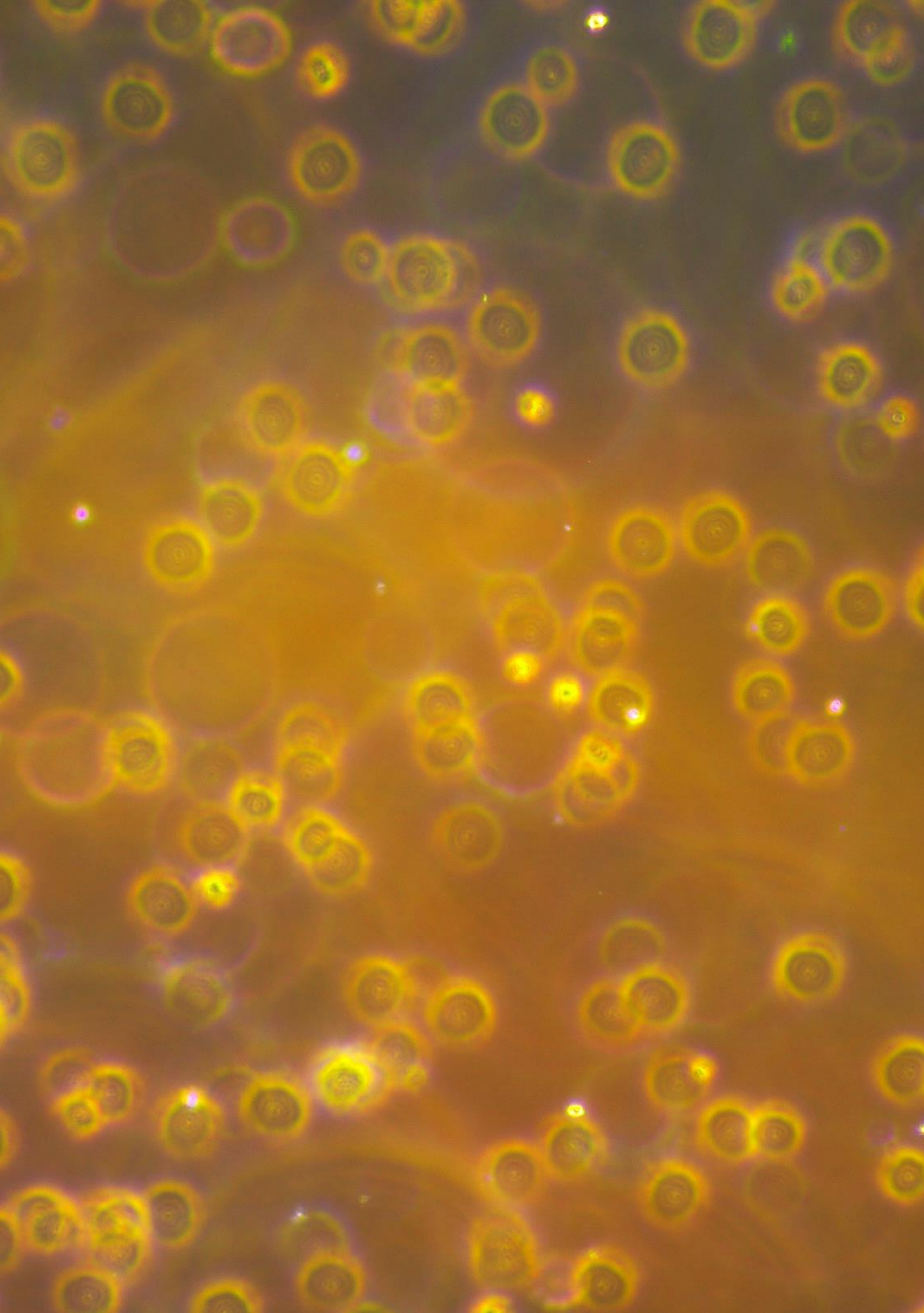
Results 3.3 Metabolic balance under chronic and acute warming scenarios

This chapter investigated the impact of sub-lethal thermal stress (28°C) on the main physiological rates (ingestion, reproduction, respiration and egestion) and somatic traits (body size and carbon content) of *P. grani* cultivated during multiple generations at warmer temperatures (+3°C and +6°C). Body size and carbon content of female adults were inversely related to the rearing temperature. Thermal stress significantly reduced the body carbon content (-11%) of the individuals, regardless of the rearing temperatures. Weight-specific rates, except respiration, increased with rearing temperature. However, per capita rates were similar across rearing temperatures, except for egestion. This difference can be explained, in part, due to the variations in copepod size. Heat stress impact, measured as weight-specific rate fold-change, appeared inversely related to the rearing temperature. The carbon gains (ingestion) were overall sufficient and slightly in excess to account for the measured carbon losses (reproduction, routine metabolism and egestion) at all temperature treatments.

Assimilation and net-growth efficiencies remained unaltered across thermal treatments, averaging 69-80% and 64.8%, respectively. Gross-growth efficiency (conversion of ingested food into growth or egg production) was also conserved across warming scenarios, with an average of 44.6%. Observations on the mortality of the early-hatched nauplii during heat stress may suggest a higher tolerance of those produced by warm-reared copepods (25ºC).

Results 3.4 Effects of warming on the functional response of copepods: implications on body stoichiometry and growth efficiency

This chapter explored the effects of warming (+ 6ºC) on the functional and numerical responses and its relationship with body stoichiometry in adult female *P. grani* at short (4 days) and long-term (21 generations, i.e. 1 year) exposures. In all treatments, the functional response followed a Holling Type III curve. Maximum clearance rates (F_{max}) were not affected by short-term exposure to warming and decreased significantly in long-term acclimated copepods. Short-term warming (4 days) resulted in a significantly increase in maximum ingestion rate (I_{max}) in comparison to the control. Conversely, after multiple generations of exposure, maximum ingestion rates (I_{max}) did not differ from that of the control. Both short-term and long-term acclimated individuals required higher food concentrations to achieve saturation levels (half-saturating constant, K_m). Consistent with the observed reduction in body size, the elemental composition (carbon, C; nitrogen, N; and phosphorus, P) was lower in warm-reared copepods at all food concentrations. In all treatments, C, N and P body contents dropped by 50% from satiation conditions to nearly food depletion, which correlates with the decrease in egg production at decreasing food concentrations. Despite the changes in grazing, the stoichiometric molar ratios of adult females (C:N, N:P, C:P) remained unaltered by food concentration. Finally, gross-growth efficiency (GGE) increased with food concentration up to certain point, beyond which it gradually declined in all treatments. The maximum GGE was lower in the individuals exposed to short-term warming but did not differ between the control and the long-term acclimated copepods.



4. GENERAL DISCUSSION

Through the experimentation carried out in this thesis, we have evaluated several key copepod traits at different temporal scales, encompassing intragenerational and multigenerational effects, and distinct thermal conditions. The general experimental design of the thesis, using the same reared species under controlled conditions, facilitates an integrative view of the results. Thus, in this General Discussion, I will put together the data from the different sections and search for more general patterns through cross-comparisons, in an attempt to refine or reinforce the conclusions that arise individually from each chapter. Finally, I will also discuss the results obtained in this thesis within a more ecological context and wider scope, in relation to climate change-driven warming effects on zooplankton and the expected consequences for the pelagic ecosystem.

Body size across generations: intergenerational variability and thermal effects

Perhaps the most noticeable aspect of the long-term thermal response of copepods, spanning multiple generations, was the decrease in size of the individuals with rearing temperature, a phenomenon commonly known as the temperature-size rule (TSR; D. Atkinson, 1994; Forster & Hirst, 2012). In recent decades, the TSR is regaining interest due to the observation of body size reductions in a wide spectrum of animal groups associated with global warming in the last decades, particularly evident in aquatic systems (Daufresne et al., 2009). The TSR emerges from the different thermal sensitivities of growth and development (i.e., moulting) during ontogeny (Forster & Hirst, 2012; Horne et al., 2019). Warming accelerates development more than growth, and this uncoupling results in reaching a certain ontogenetic stage with a smaller size and mass. Accordingly, warming also provokes a faster emergence of mature adults, shortening population generation times. In **Results 3.1**, we reported a trend towards shorter ontogenetic development times (including embryonic development) and declining body size in adult female and male copepods exposed to warmer conditions. This response, already manifested in the first generation of exposure, persisted beyond 40 generations (**Results 3.1, 3.2, 3.3 and 3.4**). **Fig. 32** shows average trends of the adult female prosome length with rearing temperature across generations and its relationship with body mass. Inter-generation variability was present at each rearing temperature and can be attributed to fluctuations associated with long-term caring of laboratory cultures (e.g., changes in copepod density that alter the actual predator: prey abundance ratio; (Klein Breteler & Gonzalez, 1988).

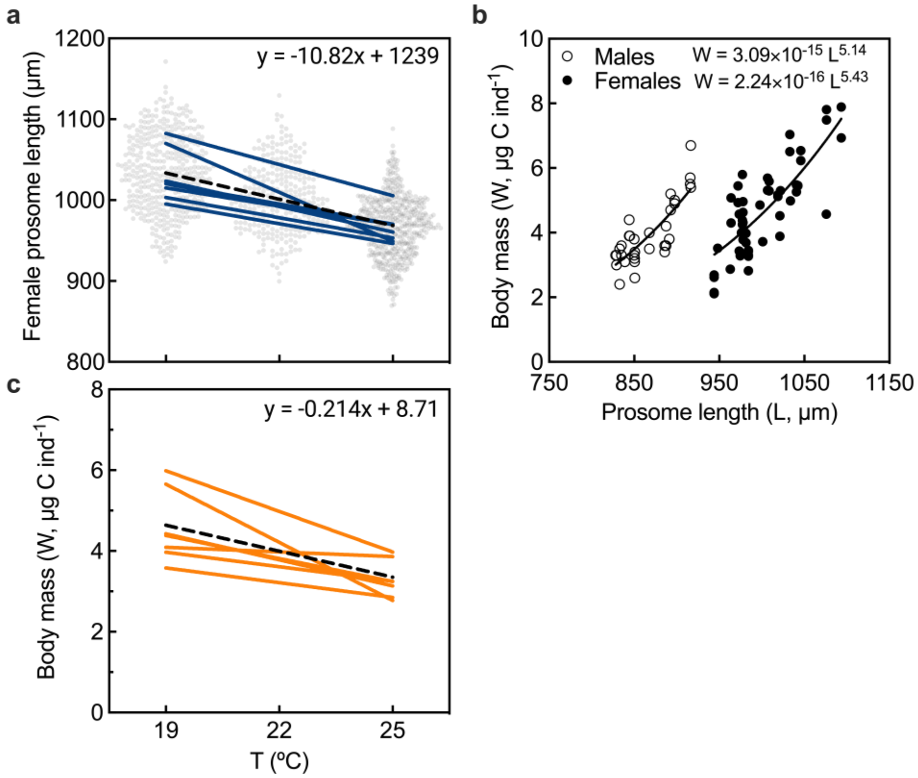


Fig. 32 | **a.** Relationship between prosome length and rearing temperature across generations. The dashed line shows the average trend across generations. **b.** Length-weight relationship in adult male (o) and female (●) *P. grani*. Data was compiled from this thesis (males n = 35, females n = 54) and also includes data from Saiz et al. (2022) and Traboni et al. (2021). Equations show carbon-length power relationships. **c.** Estimated carbon content of adult female *P. grani* against rearing temperature across generations using the carbon-length relationship shown in **b.**. The dashed line shows the average trend across generations.

The temperature-driven decline in the prosome length of *P. grani* was, on average, -10.8 µm $^{\circ}C^{-1}$, which implies a reduction of -1.2% $^{\circ}C^{-1}$. The magnitude of the thermal effects on the body size of *P. grani* females was similar to the seasonal changes in body size in

P. grani in its natural habitat (-6.8 µm $^{o}C^{-1}$; -0.6% $^{o}C^{-1}$) reported by Rodriguez & Jiménez (1990). Therefore, the expected gradual rise in ocean temperatures would result in an additional size shrinking to the usual seasonal fluctuations in copepod body size – larger individuals in winter, smaller in summer. In this regard, decadal reductions in body size have been reported in other Acartidae species, *Acartia tonsa* and *Acartia hudsonica,* in the northwest Atlantic by Rice et al. (2015). Similarly, Corona et al. (2021) found a long-term reduction in copepod mass in *Acartia clausi* and *Centropages typicus* in the Western English Channel. Importantly, the strength of the TSR might differ between taxonomic ranks or be influenced by other factors. In a meta-analysis, Horne et al., (2016) found that calanoid copepods (such as *P. grani*) show a stronger TSR response than cyclopoids or harpacticoids. Notably, temperature was a better descriptor of size changes than food concentration (Horne et al., 2016).

Changes in body size will also imply changes in body mass. Due the volume-surface scaling relationship, however, we could expect a higher magnitude of body mass reduction, although subjected to higher variability given that same-sized individuals can vary significantly in mass (Rey-Rassat et al., 2004). For instance, Corona et al., (2021) reported seasonal shifts in mass varying from -10.1 to -2.9% $^{o}C^{-1}$ in 7 copepod species, differences being attributed mainly to population density. In our experiments, body mass declined by a -4.6% $^{o}C^{-1}$ (**Fig. 32c**) and our results in **Results 3.4** indicated that nitrogen and phosphorus copepod body content declined at similar rates than carbon (-5.3%$^{o}C^{-1}$) with increasing temperature. Consequently, warming may affect not only size, but also the elemental composition and nutritional quality of zooplankton assemblages that constitutes the prey for planktivorous consumers (Helenius et al., 2023). Changes in body size and mass are expected in a warming ocean across the different components of plankton and their consumers, potentially disrupting the size structure and transfer of energy across trophic levels (Heneghan et al., 2023).

As mentioned previously, the long-term rearing of the copepod cultures in the laboratory under controlled conditions did not prevent phenotypic variability. Thus, the size and body mass of the copepods in the control populations (19ºC) suffered variations over the 4.5 years of study. As we repeatedly measured egg production rates of the control

(19ºC) copepods for every experiment, we can describe the relationship between the body size of the copepods and their egg production rate (**Fig. 33**). Within the range of variation in the prosome length of females reared at 19ºC (934.3 - 1085.8 µm C), the number of eggs produced per female increased from 54 to 73.5 eggs ind^{-1} d^{-1}. From here, using the equations in **Fig. 32a** and **Fig. 33**, we can derive that the decrease in body size driven by temperature could lead to a decline of 2% in egg production rates per degree increase. For example, average prosome length at 19ºC was 1033.4 µm at 19ºC and 968.5 µm at 25ºC, and the egg production rate associated to each size was 66.7 and 58.2, respectively. This gross estimation indicates that just the reduction in body size (-7%) under +6ºC increase could explained the observed 12% decline in egg production rates. Therefore, we can conclude that the allometric constraints on physiological rates, driven indirectly by warming-driven shrinkage, are noteworthy and necessarily will play a role in the long-term responses of organisms (Ikeda et al., 2001; Saiz & Calbet, 2007; Riemer et al., 2018).

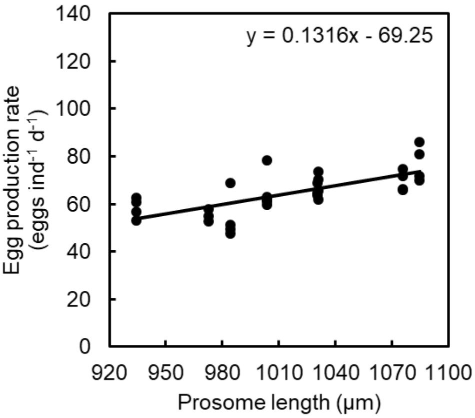


Fig. 33 | Egg production rate as a function of body length in adult female *P. grani* exposed to 19ºC.

Thermal compensation in copepods: the case of egg production rates

In ectotherms, warming initially causes an exponential increase in the activity of rate processes. This change in phenotypic traits is considered to be driven by "purely" physical effects mediated by temperature (Arrhenius effect). As the stress persists, the machinery of the organism may make adjustments to counteract the deleterious effects of this passive response and diminishes the rates of activity (this acclimation response is then termed *active plasticity*; Havird et al., 2020) and may involve changes in gene expression, membrane composition or enzyme concentration (Schulte et al., 2011). Over the long-term, a more diverse array of processes, at phenotypic and genotypic level, might come into play further mitigating the thermal effects on physiological rates. Depending on the strength of the response , the thermal effects can be partially, completely, or over-compensated (Havird et al., 2020). Thermal compensation processes at different timescales are readily apparent in the observed changes in *P. grani* egg production rates, where the initial acute thermal response is dampened at long-term (**Fig. 34**).

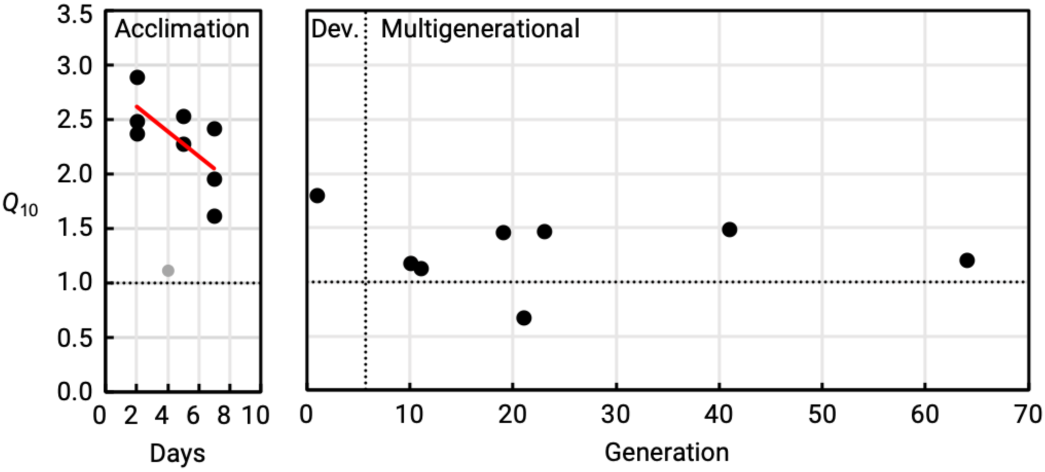


Fig. 34 | Q_{10} coefficients for egg production rate of adult female *P. grani* at different timescales of exposure to different thermal conditions (19ºC, 22ºC and 25ºC). The figure combines the results obtained in this thesis with additional data for short-term responses extracted from Saiz et al. (2022) and unpublished data. The line in red shows the acclimation responses of female adults exposed to warming during 48 h, 5 d and 7 days. The grey point corresponds to the experiment in **Results 3.4**, which was excluded from this regression. Acclimation (adult exposure) and multigenerational (inter-generations) are separated by a vertical dashed black line. Note that F1 was exposed to increased temperature only during its development (Dev.).

Thus, we observed a continued reduction in the magnitude of the thermal effects through time. Within intra-generational effects, the Q_{10} coefficients declined through time, from the initial acute response (on average $Q_{10} = 2.6$) to exposures of 5 days ($Q_{10} = 2.4$) and 7 days ($Q_{10} = 2$). The thermal response of the F1 copepods, only exposed to warming during ontogenetic development, was of similar magnitude to that of the acclimated individuals ($Q_{10} = 1.8$). In contrast, in subsequent generations (>F10) the egg production rates seemed to be further compensated, with Q_{10} coefficients close to 1 (on average $Q_{10} = 1.2$). The increase in Q_{10} coefficients at some generations (>F19) perhaps reflects the variability among egg production determinations, and might also indicate some persistence of the thermal effects. In **Results 3.3** we noted that thermal compensation was not fully achieved even after multiple generations of exposure.

As mentioned earlier, the decrease in body size also constrains egg production rates in addition to the reduction in the thermal sensitivity of the rate process, further contributing to the decrease in Q_{10} coefficients. The thermal compensation patterns observed in physiological rates contrasts with the effects on body size, which as mentioned earlier, was not recovered following multiple generations. It appears, therefore, that the uncoupling in the thermal response of ontogenetic development (moulting) and physiological rates (growth, feeding, egg production) must be related to different fundamental processes in the organism.

Q_{10} coefficients are often assumed to range between 2 and 3 for most physiological processes and many models often use it (or alternatively, the activation energy E_a of the Arrhenius equation) as a fixed parameter (Stock et al., 2014; Bisson et al., 2020; Krumhardt et al., 2022). For example, Karakuş et al., (2022) assumed a $Q_{10} > 2$ for mesozooplankton respiration, or Archibald et al., (2022) applied a $Q_{10} = 1.88$ to zooplankton grazing. Variations in the thermal sensitivity in zooplankton activity, however, will have profound implications for the outputs obtained (Rohr et al., 2023). Actually, the commonly used values of Q_{10} coefficients are often extracted from studies assessing only short-term thermal responses. Considering the results from this thesis, which highlights processes of acclimatization or local adaptation (including reduction in body size and compensatory mechanisms), combined with the evidences showing that species may conserve, to some degree, their thermal niches (e.g., due to changes in phenology or distribution that maintain the same environmental temperature; Corona et al., 2024), it is reasonable to assume that response of some copepod species will not follow a simple passive or acclimation response. Therefore, the use of a fixed Q_{10} or E_a in predictive models should be reconsidered taking into account the effects of adaptive processes (**Fig. 35**) to improve the prediction of the trends in carbon fluxes mediated by zooplankton. Similarly, other groups across trophic levels might show adaptive capacity to temperature. Protozoans also exhibit adaptive capacity in their growth and grazing dynamics (Strock & Menden-Deuer, 2021; Calbet & Saiz, 2022; Ferreira et al., 2022; Liu et al., 2022). It is also worth noticing that one has to be careful with the indiscriminate use of Q_{10} coefficients because often the 10°C variation is larger than the range of temperatures tested for its calculation, or it could encompass the decline phase in thermal performance curves. For instance, in fish studies experimental Q_{10} values often do not align with those predicted by Arrhenius equations (van Denderen et al., 2020).

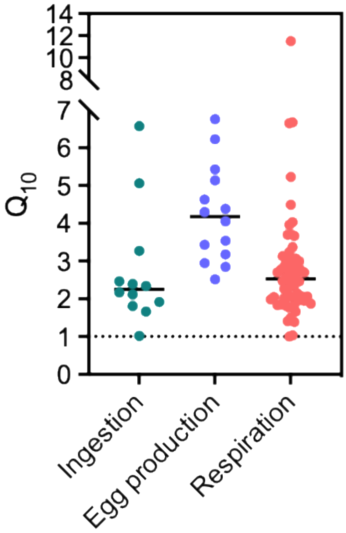


Fig. 35 | Q_{10} coefficients for ingestion, egg production, and respiration for 46 copepod species compiled from literature by Saiz et al. (2022). The dotted line indicates a Q_{10} equivalent to 1.

Bioenergetics and thermal effects on other life-history traits

Egg production rates are especially interesting in copepods because, contrarily to other crustaceans, such as most decapods, copepods do not moult after they achieve the adult stage; hence, egg production rate can be associated with their growth, although with some limitations (Berggreen et al., 1988; Hirst & McKinnon, 2001). On the other hand, the egg production rate is highly correlated with the ingestion rate in this and similar species (*A. tonsa*; Kiørboe et al., 1985). Thus, similarly to the pattern observed with egg production rates, we did not find differences in ingestion between copepods reared at each temperature (**Results 3.1, 3.3 and 3.4**). This coupling between ingestion and egg production rates across generations at the three rearing temperatures resulted in conserved GGE (**Fig. 36**). The average trends show that in *P. grani* almost half of the carbon ingested was invested in the offspring production. Equally to GGE, in **Results 3.3** we did not find significant differences in AE and NGE between rearing temperatures. Overall, the bioenergetics at the three rearing temperatures were remarkably consistent. Moreover, as previously mentioned, the decrease in elemental content observed in the smaller-size warm-reared copepods was similar for carbon, nitrogen and phosphorous, indicating that the stoichiometry of copepods remained unchanged.

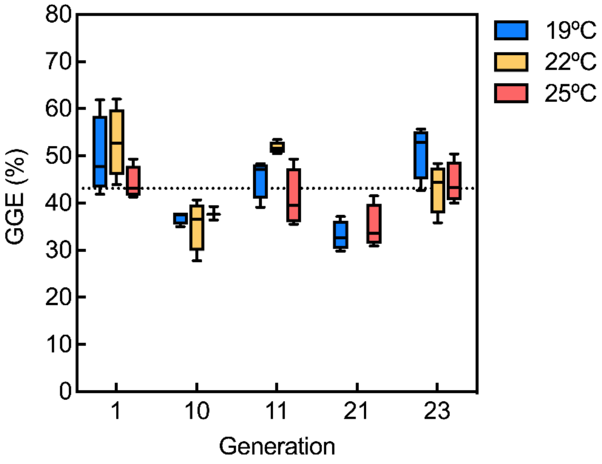


Fig. 36 | Gross-growth efficiency in *P. grani* across generations exposed to different thermal conditions (19ºC, 22ºC and 25ºC). The dotted line indicates the global average.

Then, have copepods *adapted* to the new environmental temperatures? The term adaptation implies not only changes in a trait or the underlying genes but needs to be looked through its evolutive history to evaluate its adaptive capacity (S.J. Gould cited in Hochachka & Somero, 2002). Acknowledging the complexity and the profound implications on assuming adaptation, across this thesis I have been cautious with the use of the term *adapted*. Instead, I have used the terms *adaptive capacity* or *adaptive value* to describe changes in traits that, being modified with time of exposure, enhanced or maximize evolutionary fitness, that is, the capacity of organisms to generate offspring by surviving and sustaining reproduction. Overall, the obtained results throughout this thesis suggest that the changes in phenotypic traits would maximize the fitness of the warm-reared copepods in comparison with the original line at 19ºC. Thus, we found that reproduction was similar at each rearing condition, despite the decrease in size of the copepods with temperature. Moreover, the higher development rates (i.e., lower developmental times) would hypothetically increase the chances of arrival to the adult stage without being predated. In turn, lower development times also reduces the age at maturity (i.e. generation time) facilitating an increase in population growth rate (Kingsolver & Huey, 2008; see **Fig. 7** of the **General Introduction**). Obviously, this is an oversimplification and there are other key life-history traits affecting fitness that were not assessed in this thesis and might also be influenced by temperature. For instance, negative effects on longevity (Gophen, 1976; Beyrend-Dur et al., 2011) might reduce the net reproductive rate (the total offspring produced during the lifetime of one individual). Another key population feature, the proportion of males and females has been related to temperature (Katona, 1970), but in our experiments the sex ratio was not altered by warmer rearing temperatures (**Results 3.1**). The full recruitment success, from eggs to adults, at each thermal condition was not measured in our experiments, yet, we did not find relevant effects on the egg hatching success (**Results 3.1**). Moreover, the fact that the copepods could be cultivated for more than 4 years might indicate a high success in the viability of the cohorts reared at warmer temperatures (**Results 3.1**). Altogether, it appears that *P. grani* can thrive at warmer sub-lethal temperatures (+6ºC) without apparent changes on its life-history traits.

Copepod response to thermal extremes: tolerance limits and metabolic balance

Up until now, we have observed that organisms reared at warmer temperatures counteract the effects of moderate thermal stress on physiological rates. Nonetheless, in parallel to the gradual ocean warming, the severity of marine heatwaves is predicted to increase in coming decades (Smith et al., 2023). These thermal extremes might impose a higher stress to organisms which can prioritize protective mechanisms to maintain homeostasis over other traits (e.g. reproduction), yet, such protection might not be sustained for long periods and ultimately can cause mortality (Rezende et al., 2014; Saiz et al., 2022). Organisms also exhibit some plasticity in their tolerance limits manifested following a period of acclimation. However, this enhancement of the organism's upper thermal limits, whether after short- or long-term (multigenerational) exposure, is considered to be limited (Kelly et al., 2012; Morley et al., 2019). In **Results 3.2** we found that after 18 generations, copepods reared at warmer temperatures (25ºC) did indeed display higher temperature tolerance. However, the magnitude of this regulation was limited and depended greatly on the time of exposure. Copepods reared at 25ºC showed greater resistance than those reared at 19ºC within the first two days of exposure to lethal temperatures; afterwards, however, mortality increased and the gained tolerance margin diminished. It remains uncertain if a stronger selection pressure, such as the exposure to lethal temperatures during one or more generations could have favoured the selection of the most resistant genotypes and result in an further increase in thermal tolerance (Dam, 2013). In this regard, we lack data on the enhancement of thermal tolerance following a short-term acclimation experiment in *P. grani*. However, the resulting margin of increase in thermal limits (0.3 - 0.8ºC) after a 1 year of exposure to +6ºC and supports the idea of a firm upper limit to thermal tolerance in many organisms (van Heerwaarden et al., 2016). For example, in a similar multigenerational study, Sasaki & Dam (2021) found increases in tolerance of 0.1-0.5 in *Acartia tonsa* following 40 and 80 generations of moderate warming. Importantly, in this species development plasticity could show higher strength than long-term acclimation (Ashlock et al. 2024). In the brackish copepod species *Tigriopus californicus* a continuous selection of the most resistant genotypes, increased the tolerance only by 0.5ºC (Kelly et al., 2012). Hence, adaptive increases in upper tolerance seem slow in evolutionary terms and might be a major constraint for the response of marine organisms to extreme heat events. Moreover, there is growing body of literature examining the possible negative relationship between increases in tolerance and the reduction of the plasticity in this trait (van Heerwaarden et al., 2016; van Heerwaarden & Kellermann, 2020; Sasaki & Dam, 2021). Such trade-offs could reduce the capability of the organisms to face future environmental changes; in this view, the reduction of phenotypic plasticity acts also as an evolutionary constraint. In *P. grani* the thermal limits for copepod survival were coupled with that of the fecundity, that is, the copepod lines from both temperatures, 19ºC and 25ºC, exhibited sustained reproductive activity at lethal temperatures under satiating conditions. For example, at temperatures causing 50% mortality, the 19ºC copepods produced an average of 60 eggs per day, similar to the control conditions. As the tolerance to extreme temperatures increased in the 25ºC-copepods in comparison of those reared at 19ºC, the limits for offspring production also shifted to higher temperatures. In this regard, the statement of Kiørboe et al. (1985) on the energy allocation strategy in *Acartia tonsa* that this species "seems to be very efficient in transforming ingested material into eggs" could also be applied to *P. grani*. However, this also calls for caution on the experimental design when using egg production rate to assess possible thermal effects on these copepods, as it might not be the most sensitive proxy to environmental change, at least when satiating food supply is guaranteed.

Trade-offs between rates at whole-organismal level are often used to search for a mechanistical basis in thermal tolerance (Pörtner, 2002; Sokolova et al., 2012; Alcaraz et al., 2014; Pörtner et al., 2017). For example, the concept of oxygen- and capacity-limited thermal tolerance theory assumes that thermal limits are defined by a reduction in aerobic scope driven by higher basal metabolism and constraints in oxygen supply at increasing temperatures (Pörtner et al., 2017). Alternatively, other authors propose that differences in thermal sensitivities of key physiological processes for energetic gains and expenses, such as ingestion and respiration, might constrain the survival of organisms (Alcaraz et al., 2014). Building upon these theories, with the bioenergetics approach used in **Results 3.3** we hypothesized that the misbalance between the thermal responses of vital rates would precede the lethal effects of temperature. However, our results did not show relevant differences in the efficiencies of food utilization. Chronic warming diminished the impacts of sub-lethal thermal stress (28ºC) on the main physiological rates in *P. grani*, but no effects were observed on efficiencies of carbon utilization across different rearing temperatures. Despite potential variations in the magnitude of SDA, the carbon budget of the copepods at heat stress was remarkably consistent among rearing temperature. Moreover, a decrease in body mass of the same magnitude was observed in all rearing temperatures. Therefore, the increases in thermal tolerance (**Results 3.2**) could not be attributed to differences in thermal sensitivities. Given the proximity of the used thermal stress temperature (28ºC) to the T_{opt} for egg production rates (which did not shift significantly following chronic warming), a more stressful thermal treatment (>28ºC) would have likely revealed larger differences in the acclimation response at the extremes of the performance curves (**Results 3.2**). The main positive effects of chronic warming prior to heat stress events might affect the survival of the offspring produced. Our findings (**Result 3.3**) suggested that a proportion of the early-hatched nauplii under stress at 28ºC struggled to survive, with those originating from the 25ºC population surviving more than the ones from the colder treatment (19ºC). In this regard, while the assumption that early life stages are more vulnerable to heat stress is not widely supported (Tangwancharoen & Burton, 2014), the benefits of maternal provisioning (including epigenetics) are increasingly being acknowledged as a major factor providing resilience to organisms in response to environmental change (Vehmaa et al., 2012; Thor & Dupont, 2015; Donelson et al., 2018). However, the observations in our experiments were not conclusive, and additional studies addressing this issue specifically would be needed to confirm the presence of maternal effects on heat tolerance in this species.

Oligotrophy: role of food availability

The long-term compensation in physiological rates and tolerance increase were observed under satiating conditions (necessary to avoid confounding factors). However, as introduced in **Results 3.4,** food resources in the ocean are distributed heterogeneously and might not reach satiating levels. In a warmer ocean, a higher stratification and lower inputs from rivers are predicted to increase oligotrophy, lowering primary productivity (Agusti et al., 2017). Decreased food resources might constrain the thermal response or reveal hidden costs of compensation. With this in mind, we assessed the feeding and numerical responses at two timescales of exposure to warming. Unexpectedly, we found an increase in the half-saturation constant (K_m) in "adapted" copepods for which we previously had not found evidence of higher basal metabolic costs (**Results 3.3**). In turn, the increase of K_m may be related to the lower foraging capacity of these copepods, as evidenced by the remarkable decrease in clearance rate (F_{max}). In this regard, additional studies are needed to reveal if allometric constraints are the ultimate cause of the decrease. Importantly, at the same food level concentrations, warm-reared copepods eat a lesser proportion of carbon in relation to their body mass, affecting their gross-growth efficiency. Thus, from these results, we conclude that warm-reared copepods, despite the apparent benefits of being smaller in an oligotrophic environment, might be more affected by low food availability.

Ecological implications

The thermal tolerance range found for adult *P. grani* females in **Results 3.2** matches with its natural distribution. *P. grani* inhabits neritic waters across a broad latitudinal range, from colder North East Atlantic, the Mediterranean Sea and some tropical locations (Boyer et al., 2012). As far as I know, this copepod species was first observed in the Mediterranean in Barcelona harbour by Alcaraz (1977) in the 70s and seems to have expanded towards eastern waters (e.g. Ligurian Sea, Sei et al., 1999; Thau Lagoon in the coast of France, Boyer et al., 2012; or the Gulf of Napols, Mazzocchi et al., 2012). It appears to be more abundant in estuaries or harbours, where it can be found all year-round with peaks in summer (Rodriguez & Jiménez, 1990). Nowadays, sea surface temperatures in some of the habitats of *P. grani* can be well above the T_{opt} measured in our experiments (~27ºC) and close to its lethal temperatures (~31ºC). For example, in western Mediterranean areas such as in the Alboran Sea in Málaga, in the mouth of Ebro estuary, or waters off Barcelona can reach 28ºC in August, while inside the harbours in Málaga or Castellón sea temperatures can exceed 30ºC (Puertos del Estado, n.d.). Therefore, it seems that the range of temperatures we used in our experiments is not far from the natural thermal range *P. grani* can be likely exposed under the current warming scenario.

From our results, some considerations can be made. First, the relatively low plasticity in thermal tolerance observed in *P. grani* following multiple generations under warming (LT_{50} = 0.3°C after 7 days) seem insufficient to provide enough temporal resilience to cope with the observed and predicted trends of extreme heat events (Martínez et al., 2023). Therefore, we would expect a low potential of the species to increase their tolerance range. However, other aspects are also relevant for the persistence and propagation of populations. For example, in temperate regions, which are characterized by strong seasonality (see Saiz et al., 2014 for the Mediterranean Sea), a higher ocean stratification in summer and lower input of nutrients from rivers and streams that lead to poor nutrient influx and reduced primary productivity in the upper layers, resulting in lower food availability (Agusti et al., 2017) that could limit their reproduction and survival. Conversely, copepod species like *P. grani* can rely on resistance eggs produced in the previous year, helping populations to persist when more favourable conditions are present (Guerrero & Rodríguez, 1998; Boyer et al., 2013; Boyer & Bonnet, 2013). As a result of the interaction between changes in environmental conditions and copepod life strategy traits, peaks of species occurrence in natural populations might shift. At the community level, involving multiple species, differences in the strength of the phenological response to temperature could lead to higher overlapping of peaks of abundances of concurrent species (Alcaraz, 1983; Rodriguez & Jiménez, 1990), enhancing competition in which differences in physiological performance might determine the dominant species. However, extrapolations of the thermal biology of the species to ecosystem level should be taken with caution. For example, the winter species *A. hundsonica* did not yield ground to *A. tonsa* in a global warming context, in contrast to what authors predicted (Sullivan et al., 2007). A number of biological and physical factors, particular for every environment and season, might open windows of opportunity for the species.

Future directions

Despite that experimental evolution studies are scarce in zooplankton, these can help evaluating the adaptive potential of these key marine organisms (Dam, 2013), and due to the feasible manipulation in laboratory and short generation times, copepods have been proved to be good models for this long-term studies. In recent years, some notable examples have revealed the relatively speediness of adaptive changes in fitness in response to environmental change (Dam et al., 2021; DeMayo et al., 2023). However, these experiments require significant maintenance and logistic efforts, and the emergence the of more long-term studies with this model group would be facilitated by a higher degree of automation. These laboratory experiments can provide an integrative view of the effects of warming on fitness components such as survival, reproduction, lifespan, or the sensitivity of other earlier life-stages. The conduction, in parallel, of genomic, transcriptomic and biomarkers analysis might help bridge the gap between genotype and phenotype, revealing the underlying mechanisms of thermal compensation or adaptation, and their reversibility. In this regard, maternal effects (e.g., epigenetics) are gaining attention as key mechanisms conferring resilience to organisms in face of environmental change. A number of other approaches can provide information about the adaptation potential of species (see **General Introduction**). Importantly, our knowledge of the thermal biology of copepods might be biased towards temperate and neritic species, and efforts should be put in the study of the thermal biology of oceanic species in poles and tropical regions (Sasaki & Dam, 2021). Also, in their natural habitats, organisms are most likely to face several stressors at a time. An increase in complexity of the experiments could reveal synergistic effects of temperature with other factors (e.g., nutrient deficiency, low oxygen, acidification, low salinity or predation). With the increase in ocean temperatures, time-series have re-emerged as key sources of information of the long-term effects of natural populations (Fernández De Puelles & Molinero, 2008; García-Comas et al., 2011). Regular monitoring can inform about population changes including changes in abundance, richness, or in the size of the organisms across trophic levels. Regarding the latter, studies addressing the changes in body size, the allometry and the implication for the food web and the transfer of energy is crucial. Also, in addition to the spatially limited stationary time-series, autonomous vehicles or other infraestructures as in-situ sensors are seeing as promising to provide a finer spatial and temporal resolution (Ohman et al., 2019). They could be useful to address physical drivers of the zooplankton biogeographic boundaries as well to assess direct impacts of marine heatwaves on planktonic communities. All these studies would not only enlighten the actual and predicted ecological trends but also expand our knowledge on the processes that have modulated and keeps shaping the adaptation of zooplankton species to their habitats.

5. CONCLUSIONS

1. Higher rearing temperature accelerated copepod hatching and development rates, resulting in a reduction of adult body size of 1.2% per ºC increase. Additionally, adult elemental content (carbon, nitrogen and phosphorous) decreased by 5.2% $ºC^{-1}$.

2. At short-term, a rise in temperature increased the copepod main physiological rates (ingestion, respiration and reproduction), but these Arrhenius thermal effects on were partially compensated following multiple generations of exposure.

3. The physiological thermal compensation and the effects of reduced body size on the ingestion, reproduction and respiration rates resulted in Q_{10} values equivalent to 1.

4. After chronic warming, the grazing impact (I_{max}) on phytoplankton prey was similar or lower than the non-acclimated copepods.

5. The thermal adaptation of copepods to warming did not result in a fitness cost in recruitment, nor affected the population sex ratio. Moreover, the efficiency of food utilized for reproduction was either altered.

6. In terms of weight-specific rates, the main physiological rates increased with rearing temperature accordingly to the lower body mass of the specimens.

7. Stoichiometric ratios (C:N, C:P, and N:P) of the copepod body content remained unaltered by temperature following thermal compensation.

8. The exposure to warming during multiple generations in copepods increased their thermal tolerance limits by 1-1.3ºC. However, prolonged exposure resulted in diminished advantages, reducing tolerance to 0.3-0.8ºC after a week.

9. At extreme temperatures, reproduction was reduced but sustained, maximizing copepods' fitness under warming conditions.

10. Copepods reared under warming conditions increased their capacity to acclimate to a sub-lethal acute heat stress. However, the balance between the main physiological rates (ingestion, respiration, reproduction and egestion) was unaffected by this acute stress despite the different rearing conditions. All copepods equally lost body mass during the heat stress..

11. Copepods reared under warming exhibited reduced maximum clearance rate (F_{max}), potentially limiting its food uptake at low concentration and subsequently reducing their reproductive activity.

12. *P. grani* has a large capability to adapt to temperature increases, maintaining key activities, like grazing and production. Nevertheless, it may be vulnerable to an increase in thermal anomalies, low food availability, or the combination of both factors

13. Experimental evolution studies provide crucial information of the adaptive capacity of copepods, contributing to the reduction of uncertainty in predictive models.

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