

RESEARCH ARTICLE OPEN ACCESS

These Boots Are Made for Walking: Sex-Specific Physiological and Metabolomic Strategies Reflect Male-Skewed Vulnerability to Ocean Warming in a Keystone Amphipod

Joana Filipa Fernandes^{1,2,3,4}  | Lauric Feugere²  | Mário S. Diniz^{3,4}  | Piero Calosi²  | Diana Madeira¹ 

¹ECOMARE – Laboratory for Innovation and Sustainability of Marine Biological Resources, CESAM – Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, Gafanha da Nazaré, Portugal | ²Marine Ecological and Evolutionary Physiology Laboratory, Département de Biologie, Chimie et Géographie, Université du Québec à Rimouski, Rimouski, Quebec, Canada | ³UCIBIO – Applied Molecular Biosciences Unit, Department of Chemistry, School of Science and Technology, NOVA University Lisbon, Caparica, Portugal | ⁴Associate Laboratory i4HB Institute for Health and Bioeconomy, School of Science and Technology, NOVA University Lisbon, Caparica, Portugal

Correspondence: Joana Filipa Fernandes (joanafcf@ua.pt; joanafilipa.dacunhafernandes@uqar.ca) | Diana Madeira (d.madeira@ua.pt)

Received: 31 August 2025 | **Revised:** 15 May 2026 | **Accepted:** 15 May 2026

Keywords: acclimation capacity | climate change | energy metabolism | global change | invertebrate | metabolomics | plasticity | temperature | thermal tolerance

ABSTRACT

Sex-specific variation can critically shape species' physiological responses to environmental change, with potentially strong implications during reproduction. Yet this source of variation is often overlooked. To help address this paucity, we investigated fitness and whole-organism traits and metabolomic profiles to elevated temperature in males and females of *Gammarus locusta*, a keystone intertidal amphipod inhabiting thermally dynamic coastal environments. Individuals were gradually acclimated to four ecologically relevant temperatures (16°C, 21°C, 26°C and 31°C) and maintained at these conditions for 21 d under controlled laboratory settings. Survival declined at 26°C and 31°C in both sexes, and females exhibited consistently higher upper thermal limits, broader thermal safety margins, but lower thermal acclimation capacity compared to males. Whilst reproductive output was lower in elevated temperatures (26°C and 31°C), juveniles' body length increased, indicating a “quality-over-quantity” reproduction strategy. At the metabolomic level, females exhibited greater plasticity, with enhanced pathways linked to energy metabolism, amino acid biosynthesis, and cellular stress defence, suggesting adaptive activation rather than heightened vulnerability, consistent with their higher thermal tolerance and survival. Elevated temperatures also impaired amphipods' energetic status, with both glucose and ATP:ADP ratio decreasing, particularly at 31°C. Overall, we show that sex-specific metabolomic strategies define different sex thermal tolerance levels in *G. locusta*. As ocean warming intensifies, males' greater vulnerability could skew population sex ratios toward females. This imbalance may limit mating opportunities and alter population operational sex ratio and therefore dynamics, if the number of males is insufficient to fertilise all females. Our study highlights the importance of considering sex-specific physiological responses by integrating them with life-history and fitness measures to build a mechanistic understanding of how thermal stress may shape reproductive dynamics and, ultimately, affect population viability. By linking cellular metabolism with organismal fitness, we provide the cross-scale insight needed to more accurately forecast marine invertebrates' responses to global change.

This is an open access article under the terms of the [Creative Commons Attribution-NonCommercial-NoDerivs](https://creativecommons.org/licenses/by-nc-nd/4.0/) License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2026 The Author(s). *Global Change Biology* published by John Wiley & Sons Ltd.

1 | Introduction

Biological variation between sexes is a deeply rooted feature of animal life, influencing not only individual traits but also population structures, dynamics and ecological functions (Edmands 2021; Gissi et al. 2023). Males and females often differ in their morphology, endocrine function, and physiological abilities (Berns 2013; Valencak et al. 2017). Particularly, there is growing evidence that females and males show distinct responses to environmental challenges across taxa (Gissi et al. 2023; Pérez-Cerezales et al. 2018). For instance, females typically allocate more energy toward somatic maintenance and reproductive investment, while males often exhibit a greater investment in growth, development, and mating behaviours (Hinojosa and Thiel 2003; Merrill and Collins 2015; Raymond et al. 2004). Despite these fundamental differences, to date, only a few ecological and physiological studies considered sex differences when characterising species' tolerance and adaptability to environmental stressors (Ellis et al. 2017; Iwasa and Yamaguchi 2023), thus potentially obscuring a key source of biological variation. Understanding the extent and direction of sex-specific responses, as well as the mechanisms underpinning such differences, is therefore essential for predicting population-level tolerance and resilience, identifying the mechanisms underlying vulnerability, and anticipating demographic or reproductive shifts under future warming scenarios.

Ocean warming represents one of the most pervasive and multifaceted stressors threatening marine ecosystems (Bopp et al. 2013; Venegas et al. 2023). It is characterised not only by a chronic increase in baseline seawater temperatures, but also by the increasing frequency, intensity, and duration of acute thermal anomalies, such as marine heatwaves (MHWs; Hobday et al. 2016; IPCC 2022). MHWs also reduce oxygen solubility (Shepherd et al. 2017), alter the water chemistry (Orr 2011), disrupt seasonal thermal cues (Bennett et al. 2019), and shift metabolic baselines in marine organisms. Therefore, the effects of MHWs may ripple across all levels of biological organisation, from molecular pathways to whole-organism physiological traits, species interactions and population dynamics (Sokolova et al. 2011; Venegas et al. 2023). Moreover, a chronic increase in temperature can drive long-term shifts in habitat suitability and alter phenology, whereas episodic MHWs can rapidly push organisms beyond their physiological limits, triggering mortality events, reproductive failure, and community collapse (Smith et al. 2023; Venegas et al. 2023). This particularly applies to marine ectotherms, which often present narrower thermal safety margins than terrestrial ectotherms (Villeneuve and White 2024). Because thermal stress affects energetic balance, development, and reproductive success, sex-specific physiological strategies may modulate how well males and females can cope with warming. Therefore, disentangling sex differences becomes fundamental to forecast population trajectories under climate change. In this context, thermal plasticity, defined as the capacity of an individual to adjust its thermal limits through acclimation (Ghalambor et al. 2007), emerges as a crucial adaptive mechanism (Bennett et al. 2021). By shifting their thermal limits upward, organisms can expand their thermal tolerance window to alleviate the risk of acute thermal injury during sudden or sustained heat exposure (Magozzi and Calosi 2015; Whiteley and Mackenzie 2016). Growing scientific evidence suggests that

thermal plasticity may differ between sexes. Despite this, a large proportion of studies (ranging from 75% to 90% – Edmands 2021; Pottier et al. 2021) still do not report or consider sex, making it difficult to assess the role of sex-specific differences in species' responses to global changes. Therefore, identifying when, why, and how sexes differ in their thermal responses is critical for understanding population tolerance and for generating mechanistic insights that can inform broader, cross-species predictions.

The few studies that analysed sex reported differences in energy allocation strategies, reproductive roles, and life-history pacing, evidenced that males and females exhibit distinct physiological responses to elevated temperatures (Tarka et al. 2018). While consistent sex-specific heat tolerance patterns remain elusive, several studies report that females exhibit greater thermal plasticity, and may possess inherently higher heat tolerance across taxa (Edmands 2021; Pottier et al. 2021). This enhanced plasticity in females may stem from physiological adaptations enabling them to sustain core functions under elevated temperatures. This is particularly true not only during the energetically-demanding reproductive phase, where oocyte formation substantially shapes female metabolic allocation and influences other physiological processes (Edmands 2021; Hayward and Gillooly 2011; Lipaeva et al. 2025), but also during post-extrusion, as an important fraction of their energy metabolism is diverted to eggs' ventilation and/or parental cares (Marochi et al. 2021). Conversely, males often show a more limited thermal performance capacity (Sasaki et al. 2019; Vermandele et al. 2024), suggesting that they are less plastic and more vulnerable to ocean warming. However, the cellular physiological adaptations explaining these differences are still poorly understood. Most notably, the disproportional vulnerability to increased temperature reported between sexes may disrupt sex ratios, and reduce reproductive output, brood size and egg viability (Donelson et al. 2010; Edmands 2021). Ultimately, this can have dramatic demographic effects (Brunel and Boucher 2007). Furthermore, the presence of sex-specific responses to warming may cause chronic warming to exacerbate existing sex-dependent differences in developmental timing (Sasaki et al. 2019), moult cycles (Nelson 1990), or reproductive maturation (Spinks et al. 2021), which could lead to population-level mismatches.

Whole-organism thermal tolerance and plasticity are underlied by complex molecular mechanisms. For instance, elevated temperatures can impair enzymes, destabilise cellular membranes, and trigger the accumulation of reactive oxygen species (ROS), all of which compromise homeostasis (Benedetti et al. 2021; Lesser 2006; Sokolova et al. 2011). Organisms may counteract these effects by adjusting biochemical pathways, for example by producing antioxidant defences (Vinagre et al. 2014), reshaping energy metabolism (Beaudreau et al. 2024; Guscetti et al. 2023; Lemieux and Blier 2022; Li et al. 2017), modulating osmolytes production (Madeira et al. 2021), and inducing chaperones (e.g., heat-shock proteins) (Madeira et al. 2014; Shatilina et al. 2011). These molecular fingerprints reflect marine ectotherms' ability for cellular rewiring and can be harnessed to describe their thermal tolerance. Although several studies have examined isolated temperatures or specific physiological traits (e.g., Neuparth et al. 2002; Rastrick and Whiteley 2013), a mechanistic, integrated multi-level sex-specific perspective on molecular, physiological, and reproductive thermal tolerance is surprisingly missing.

To address such a gap, we investigated the sex-specific fitness response, thermal tolerance and metabolomic plasticity across a broad thermal window in females and males of a keystone intertidal amphipod, *Gammarus locusta*, used as a model species. Estuarine and intertidal amphipods inhabit some of the most thermally variable environments (Bozinovic et al. 2011), undergoing daily cycles of desiccation, high diurnal heating, and temperature fluctuations (Eckersley and Scrosati 2012; Vaz et al. 2005). In this context, amphipods must cope with a wide range of temperatures, including both marine extreme events and chronic ocean warming trends. We hypothesise that sex-specific differences will emerge across multiple biological levels of *G. locusta* in response to thermal stress. Based on existing literature (Gissi et al. 2024; Vermandele et al. 2024), we expect males to be more vulnerable than females to elevated temperatures because their faster growth rates and larger body size may impose higher energetic demands and a lesser scope for energy reallocating toward heat-stress defences. Conversely, we expect females to maintain performance under high temperatures through enhanced plasticity and/or energy production mechanisms.

2 | Material and Methods

2.1 | Study Area and Field Environmental Data Collection

We selected as a study area the coastal lagoon of the Ria de Aveiro in Portugal (Mira channel, Costa Nova, 40°36' 59.3" N 8°44' 25.3" W) (Figure S1). Here, to characterise the thermal environment of the habitat of *G. locusta*, intertidal temperature data was collected every 30 min using data loggers (with a resolution of 0.1°C; v.2.4 27 mm, Envlogger ElectricBlue, Porto, Portugal). Temperature data was acquired during summer months, i.e., between May 30th and October 17th, 2022. Data loggers ($n=3$) were deployed, ensuring that they were always submerged at low tide, in three tide pools at the collection site. After that, descriptive statistics (means and standard deviation—SD, minimum and maximum values) were computed and data were plotted using the R package *macleish* vR 4.4.0. These data were used to inform and to calculate thermal safety margins (TSM).

2.2 | Specimens' Collection, Transport and Husbandry

Gammarus locusta individuals were collected in the Mira channel in July 2022 ($n_{\text{total}}=858$ adults including ovigerous females and males; females 0.021 ± 0.003 g and males 0.038 ± 0.012 g of weight measured on a subsample of 50 indiv.). At the time of collection, environmental conditions were: seawater temperature = $20.4^\circ\text{C} \pm 0.5^\circ\text{C}$, salinity = 30.7 ± 0.3 , dissolved oxygen = 8.96 ± 0.11 mg L⁻¹, and pH = 7.98 ± 0.28 . Specimens were transported (over a 7 min drive) to the *ECOMARE Laboratory for Innovation and Sustainability of Marine Biological Resources*, Gafanha da Nazaré, Portugal (40°38'17.3" N 8°43'43.8" W) inside 20 L buckets filled with local, constantly aerated, seawater. After arriving at the facilities, individuals were haphazardly

placed inside forty 20 L plastic tanks ($n=40$ amphipods *per* tank). Tanks contained artificial seawater (made with reverse osmosis water and artificial sea salt; Pro-Reef, Tropic Marin, Lisboa, Portugal), and a 40-mm layer of sand (grain size ± 0.3 mm) collected from a nearby beach (Praia de Biarritz, Costa Nova, Ria de Aveiro; 40°62'87.47" N, 8°74'58.88" W) to mimic habitat conditions. In addition, constant aeration was provided, and the photoperiod was set to 14 L:10 D to mimic summer season light exposure. Seawater temperature and salinity were measured daily with a multi-parameter probe (MU 6100H, VWR, Leuven, Belgium). Mean $\pm$ SD conditions were: temperature $20^\circ\text{C} \pm 1^\circ\text{C}$, salinity 30 ± 3 . Other seawater parameters were measured twice weekly: pH at 8.1 ± 0.1 , total ammonia < 0.1 mg L⁻¹, nitrites < 0.05 mg L⁻¹, nitrates < 0.1 mg L⁻¹, and dissolved oxygen $> 8.5 \pm 0.3$ mg L⁻¹. To ensure suitable water quality, we conducted partial water changes (50% of the total volume) every 2 days. Amphipods were kept under these conditions for 15 days prior to the beginning of the experiment to allow them to adjust to laboratory conditions and recover from any possible stress due to collection and transportation. Mortality during this acclimation period was low, with only six individuals ($< 1\%$ of total $n=858$) dying across all tanks. Only females carrying unfertilised, dark-coloured eggs were used for *posteriori* experiment; egg colour was monitored daily during the husbandry period to ensure synchronised reproductive status. Individuals were fed *ad libitum* with *Ulva* spp. collected at the sampling site.

2.3 | Experimental Design, System and Protocol

To investigate sex-specific thermal responses, as well as fitness, in the amphipod *G. locusta*, adult females and males ($n=400$ of each sex) were exposed for 21 d to one of four temperatures: 16°C, 21°C, 26°C and 31°C. These temperatures were selected to reflect a thermal gradient including optimal (16°C), supra-optimal (21°C and 26°C), and near-critical (31°C), based on natural temperature recordings (Figure 1) and future climatic predictions for the end of the century (IPCC 2022). These temperatures span the full thermal range experienced in the species' intertidal habitat—from typical summer conditions ($\sim 16^\circ\text{C}$ – 21°C) to the upper extremes recorded during low tide in warm days (up to $\sim 28^\circ\text{C}$ – 29°C), with 31°C representing projected near-future summer maxima under warming scenarios (Figure 1).

Temperature treatments were achieved independently in individual closed plastic aquariums, with a total of 20 replicate tanks (five replicates *per* temperature treatment; box size = $300 \times 200 \times 170$ mm, 6.5 L), set up on two shelves in a climatized 20°C room. Briefly, five replicate tanks *per* treatment were set up and randomly distributed on a bench, with approx. 20 male and 20 female individuals *per* tank. Each tank was individually regulated by a submersible heater (Thermocontrol 3612 50 W, Eheim, Deizisau, Germany) or chiller (HC-300A, Hailea, Guangdong, China), depending on the experimental temperature group. Tank water parameters were monitored daily using a multiparameter probe (MU 6100H, VWR, Leuven, Belgium) to measure temperature, pH, salinity, and oxygen. Additionally, ammonia, nitrates, and nitrites were measured with colorimetric test kits twice a week. Artificial seawater (prepared as described above)

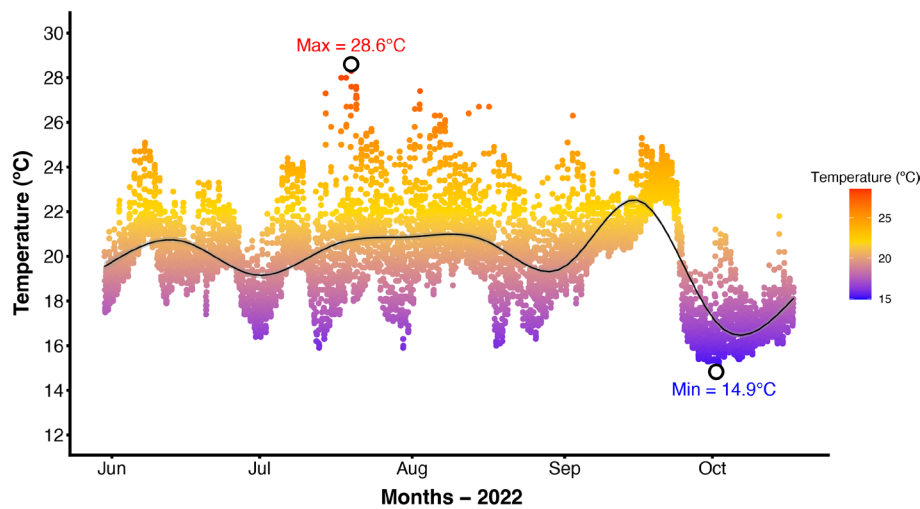


FIGURE 1 | Daily seawater temperature in Ria de Aveiro, collected every 30 min, from June to October 2022. Each point represents an individual temperature measurement. The black line shows a smoothed trend (LOESS). The maximum recorded temperature (28.6°C) and minimum (14.9°C) are highlighted with circled markers and labelled.

was supplied to each tank at a constant salinity of 30. The experimental bench was equipped with fluorescent lamps (V165 LED, ViparSpectra, CA, USA), and the photoperiod was set to a 14L:10 D (as described above). Each tank included approximately 40 mm of sand (as described above) and one oyster shell (to create and mimic tide pool refuges), and amphipods were fed *ad libitum* with *Ulva* spp. to simulate natural habitat conditions as closely as possible during the 21 d exposure period. To prevent thermal shock, temperature in each tank was gradually adjusted from a starting point of 20°C to the target experimental temperature by 1°C h⁻¹ increments or decreases (according to Fernandes et al. 2023; Missionário et al. 2022). Oxygen levels in each tank were maintained through continuous aeration provided by an air stone connected via silicone tubing to an air compressor (LP-100, Resun, Guangdong, China). After 21 d of exposure (D21), one female and one male *per* tank ($n = \text{five tanks per temperature treatment}$) were collected, quickly weighed, and immediately flash-frozen in liquid nitrogen, then stored in a -80°C freezer for metabolomic analyses (see details below). Additionally, three individuals *per* tank ($n = 15 \text{ per temperature treatment}$) were haphazardly collected for Critical Thermal Maximum (CTmax) assays. No feeding occurred 24 h prior to the sampling for metabolomics and physiological assays to avoid the effect of specific dynamic action (SDA) and reduce variability due to recent food intake.

2.4 | Survival

Mortality was observed at the end of the experiment and calculated as the proportion of surviving individuals relative to the initial number placed in each tank. Sex was not included as a factor in survival models because, at the end of the exposure period, the sex of some deceased individuals (especially at higher temperatures) could not be reliably identified due to rapid deterioration. An individual was considered dead when it showed no movement, no response to gentle stimulation, no pleopod or appendage activity, and typically remained motionless at the

bottom of the tank, consistent with standard criteria for mortality assessment in amphipods well established for this species (Costa and Costa 1999).

2.5 | Upper Thermal Tolerance Limits, Heat Tolerance Plasticity and Thermal Safety Margins

Thermal tolerance was determined using a dynamic temperature assay to define specimens' Critical Thermal Maximum (CTmax), which is commonly used in ectothermic animals (Madeira, Narciso, Cabral, and Vinagre 2012; Vinagre et al. 2016). CTmax was determined using a temperature-controlled water bath, where individuals were exposed to a constant increase of seawater temperature at a rate of 2°C h⁻¹ (Fernandes et al. 2023) until they reached the pre-defined endpoint of loss of locomotor performance. This was assessed by gently tapping or tilting the beaker to elicit a swimming response, allowing us to measure individuals' CTmax, defined as the temperature at which the individual failed to respond and passively sank to the bottom. Three individuals from the same tank were carefully transferred from the tanks to 250 mL glass beakers filled with artificial seawater (prepared as described above) and a standardised 20-mm thin layer of sand. These beakers (five *per* temperature, 20 in total, each with the three individuals from the same tank) were then placed in the temperature-controlled water bath (Optima T100, Grant Instruments, Cambridge, UK). The CTmax trial for each group began at its respective acclimation temperature, ensuring continuity between pre-exposure and testing conditions. This procedure follows the latest recommendations to avoid differential thermal shock and injury that would otherwise occur if CTmax trials all started at a common temperature (Raby et al. 2025).

A total of 15 individuals *per* exposure temperature ($n = 7\text{--}8$ females and 7–8 males *per* temperature treatment) were tested. To accurately track water temperature during the assay without disturbing the amphipods, we used a multiparameter probe (MU 6100H, VWR).

The acclimation capacity, a measure of absolute heat tolerance plasticity (Gunderson and Stillman 2015; Magozzi and Calosi 2015), was calculated following Equation (1):

$$\text{Acclimation capacity} = \text{CTmax}_T^{\text{ind}} - \text{CTmax}_{16}^{\text{H}} \quad (1)$$

where individual CTmax temperature $\text{CTmax}_T^{\text{ind}}$ represents the CTmax of each individual in a given temperature group subtracted by $\text{CTmax}_{16}^{\text{H}}$, the mean CTmax at the optimum temperature (16°C). Mean acclimation capacity indicates the absolute magnitude of the plasticity of the species' thermal tolerance limits, regardless of the breadth of the temperature window tested.

Acclimation response ratio (ARR) is a standardised measure of heat tolerance plasticity, indicating how much thermal tolerance limits change *per* degree of temperature change. ARR is calculated in Equation (2) as the change in heat tolerance (Acclimation capacity) for a given change in acclimation temperature (ΔTacc) (Claussen 1977):

$$\text{ARR} = \frac{\text{Acclimation capacity}}{\Delta\text{Tacc}} \quad (2)$$

where ΔTacc is the acclimation temperature difference.

To assess intraspecific variability of CTmax, the coefficient of variation (CV, expressed in%) was calculated for (i) species, (ii) sex and (iii) temperature treatments following Equation (3) (Fernandes et al. 2023):

$$\text{CV} = \left(\frac{\text{SD}}{\text{Mean}} \right) \times 100, \quad (3)$$

where SD is the standard deviation of CTmax and Mean is the average CTmax at a given temperature/sex.

The Thermal Safety Margin (TSM) was calculated in Equation (4) as the difference between the CTmax previously determined for each individual, within each sex and temperature, and the Maximum Habitat Temperature (MHT) determined through intertidal water temperature data measured at the sampling site (Sunday et al. 2014):

$$\text{TSM} = \text{individual CTmax temperature} - \text{MHT}. \quad (4)$$

2.6 | Quantification of Reproductive Output and Juveniles' Body Length Measurement

At the end of the experimental period, reproductive output was assessed by counting all offspring present in each tank. Juveniles were preserved in 96% ethanol for later measurements. A representative subsample of juveniles (30 *per* tank, five tanks *per* temperature, $n_{\text{total}} = 600$) was used to assess mean juvenile size *per* temperature using a stereomicroscope (DMS 300, Leica, Wetzlar, Germany) in conjunction with Leica LasX software, following the method described in Costa and Costa (1999). Briefly, metasomatic length (Lm) was measured, and is defined as the distance between the anterior end of the rostrum and the posterior end of the last metasomatic segment. The relation between total length (Lt) and Lm was previously determined for this species (Costa and Costa 1999), and was calculated following the linear regression Equation (5):

$$\text{Lt} = -0.153 + 1.218 \times \text{Lm} \quad (5)$$

2.7 | Characterisation of Metabolomic Profiles

The metabolomics profiles of female and male amphipods exposed to different temperatures were characterised using a targeted method developed by *Les Laboratoires Iso-BioKem Inc.* (modified from Beaudreau et al. 2024; Guscetti et al. 2023). Briefly, for metabolite extraction, samples were freeze-dried for 24 h, weighed on a microscale (XPR6UD5, Columbus, Mettler Toledo, OH, USA) before being homogenised in a bead-resistant tube with 2.8 mm glass beads (Fisherbrand Bulk Beads, cat. number 15-340-160, Fisher Scientific, Waltham, MA, USA) at 6000 rpm for 30 s (Precellys 24, Bertin corporation, Montigny-le Bretonneux, France). Once the extraction solution (v/v mixture of 2,2,2-Trifluoroethanol/LC-MS grade water) was added, samples were vortexed for 10 s and centrifuged at 30,130 $\times g$ for 5 min at 5°C to collect and transfer 250 mL of supernatant in an amber HPLC vial with insert (Wheaton, NJ, USA) to analyse the dried extract. Finally, 10 μL of the supernatant were injected in a high-performance liquid chromatography system (HPLC 1260 Infinity II, Agilent Technologies, Santa Clara, CA, USA) coupled to a mass spectrometer (6420 Triple Quad, Agilent Technologies, CA, USA) to detect targeted metabolites (metabolite list provided in Table S1). The absolute quantification of these metabolites (in ng mL⁻¹) was assessed with the MassHunter QQQ quantitative (Quant-my-Way) software (Agilent Technologies, CA, USA) using a calibration curve previously created using Valine-d8 and Pyruvate-d3 standards (Cambridge Isotope Laboratories, Tewksbury, MA, USA). Raw data matrices were normalised by dry weight and final concentration values were expressed as ng of metabolite mg⁻¹ dry weight.

2.8 | Data Analyses and Bioinformatics

All analyses were performed in the R software (R Core Team 2024) (v4.4.2) and plots produced with the *ggplot2* package and edited on Adobe Illustrator (29.2.1). Assumptions of normality and homogeneity of variances were validated using the Shapiro-Wilk test *shapiro.test* and Levene's test *leveneTest* from the *car* package, respectively.

2.8.1 | Survival

To test whether elevated temperatures negatively impact amphipods' survival, the proportion of surviving individuals at the end of the exposure time was analysed using a one-way ANOVA test with *Temperature* as a fixed factor using the *aov* function from the *stats* package. When significant effects were detected, post hoc pairwise comparisons between temperature groups were conducted using the Tukey's HSD test via the *TukeyHSD* function.

2.8.2 | CTmax

To determine if the CTmax data followed a normal distribution, a histogram, a QQ-plot, and Shapiro-Wilk tests were carried

out using the package *stats*. CTmax was analysed using a linear mixed-effects model (LMM; package *lme4*), with *Temperature* and *Sex* as fixed factors, wet weight as a covariate and *Tank* as a random effect. The models were ranked according to the Akaike's Information Criterion (AIC), and the model with the lowest AIC was reported. Quality of fit was determined considering the Null deviance and Residual deviance values. Random effects and covariate (wet weight) were found not to have a significant effect ($p=0.532$ and $p=0.101$ respectively) on CTmax and were removed from analyses.

2.8.3 | Reproductive Output and Juveniles Body Length

The effect of temperature on reproductive output was assessed using a generalised linear model (GLM, using the *glm* function from the *stat* R package) with a negative binomial distribution to account for overdispersion in count data. *Temperature* was included as a fixed factor and the final number of females in each tank as a covariate. Juveniles' body length was analysed using a linear mixed-effects model (*lmer* function from the *lme4* package), with *Temperature* as a fixed factor and *Tank* as a random effect. Post hoc comparisons were performed, where appropriate, using estimated marginal means via the *emmeans* function from the *emmeans* package.

2.8.4 | Targeted Metabolomics Data

As metabolomics datasets can contain a high number of missing values, the matrices were first screened and processed for missing data imputation. Across the 49 metabolites analysed (27 amino acids, 9 organic acids, 3 sugars and alcohols, 1 fatty acyl and 9 other metabolites; see for the full list and details Table S1) in 40 specimens, a total of 70 values were missing due to concentrations falling below the limit of quantification (LOQ), corresponding to approximately 3.6% of the dataset. Given the nature of these missing values—classified as 'Missing Not at Random' (MNAR)—we followed the protocol proposed by (Wei et al. 2018) using the online web-tool (<https://metabolomics.cc.hawaii.edu/software/MetImp/>; last accessed April 2025). Briefly, we applied the QRILC algorithm (Quantile Regression Imputation of Left-Censored Data) after filtering out features with more than 30% group-wise missing data (seed=1234). This procedure led to the exclusion of two metabolites out of the 49 originally targeted. Finally, the database was imported into Metaboanalyst 6.0 (<http://www.metaboanalyst.ca/>, accessed in April 2025) (Pang et al. 2024) to be cube-root transformed, to follow normality and homoscedasticity assumptions, and pareto-scaled, to adjust for differences in abundances between metabolites. These pre-processing steps are commonly used transformations for metabolomic data (Pang et al. 2021; van den Berg et al. 2006).

Metabolomics data were analysed in multiple steps using a suite of multivariate, univariate and functional analysis tools. First, transformed metabolite data were analysed by principal component analysis (PCA) using singular value decomposition with the *prcomp* base function. The *screeplot* R function helped evaluate the proportion of variation among temperature

and sex groups explained by each component and selected the first two PCA components. The first two PCA components explained most of the variation (> 50% of total) and were plotted using the *ggplot2* package as a function of *Temperature*- and/or *Sex*-groupings. A permutational multivariate analysis of variance (PERMANOVA) was applied to all 40 samples to test for the effects of *Temperature*, *Sex* and the interaction between *Temperature* × *Sex* on the whole metabolome (response of 47 targeted metabolites) using the *adonis2* function (number of permutations=9999, distance method=Euclidean) from the *vegan* package (Oksanen et al. 2020). The fixed independent variables were assumed to be categorical for this analysis (*Temperature* and *Sex*). The temperature treatment was considered as a categorical variable to facilitate multivariate analysis and for ease of interpretation. Individual dry weight was used as a proxy for body size and included as a covariate to account for the sexual dimorphism in size, females being smaller than males. To further investigate differences in metabolomic profiles across experimental temperatures and between sex, a supervised sparsified partial least squares discriminant analysis (sPLS-DA) was conducted using the *splsda* function from the *mixOmics* R package (Rohart et al. 2017). Separate sPLS-DA models were constructed for: (i) all samples grouped by treatment *Temperature*, (ii) all samples grouped by *Sex*, and (iii) within-sex subsets (females and males separately), with treatment temperature as the grouping factor. For each model, individual sample scores were visualised in the first two latent dimensions (components) using *plotIndiv*, with ellipses (95% confidence intervals) overlaid to indicate group separation. Component 1 (PLS1) scores were extracted for downstream visualisation and linear modelling. The discriminatory performance of the sPLS-DA models was evaluated using area under the receiver operating characteristic curve (AUROC) metrics. The *auROC* function from *mixOmics* was used to compute AUC values for all pairwise group comparisons within the first two sPLS-DA components. Associated *p*-values were also computed to assess statistical significance (Table S4). To identify differentially abundant metabolites (DAM) between *Temperature* and *Sex* groups, we applied Limma (Linear Models for Microarray Data), using the *limma* package (Ritchie et al. 2015). The modelling was performed with the *lmFit*, *eBayes*, and *topTable* functions. Contrasts between two groups were compared with *makeContrasts*. Metabolites with adjusted *p*-values (considering a conservative false discovery rate of FDR < 0.05 and a more exploratory FDR < 0.1) and absolute log₂ fold-change > 1.5 were considered differentially abundant. Results from the Limma analysis were visualised using volcano plots, generated with the *EnhancedVolcano* package (Blighe et al. 2024), which displays significance ($-\log_{10}$ *p*-value) versus magnitude (log₂ fold-change) of change across conditions.

Pathway analyses were performed using the *FELLA* package, a diffusion-based algorithm that considers a list of DAM and places them into knowledge-based biological networks that allow for pathway crosstalk (Picart-Armada et al. 2018). The algorithm was set to 10,000 iterations and *P*-scores and the number of nodes were limited to 0.05 and 1000, respectively. *Daphnia pulex*, a model crustacean available in the Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa and Goto 2000), was used to build the reference network. In addition to housing the

original input metabolites, the resulting network generated a list of significantly affected metabolites, enzymes, reactions, modules, and pathways. The visual representation of this network was trimmed according to the elements of interest, namely the input metabolites and pathways, and then improved aesthetically using Cytoscape 3.10.3 version (Java 17.0.5) (Shannon et al. 2003).

Finally, ATP:ADP ratios calculated a posteriori as an indicator of cellular energetic status (Feidantsis et al. 2020) were analysed using linear models with *Temperature* (treated as a continuous predictor) and *Sex* as fixed factors. Both the full model, including the *Temperature* $\times$ *Sex* interaction, and a simplified additive model were fitted to evaluate the effects of *Temperature* and *Sex* on ATP:ADP ratios.

3 | Results

3.1 | Survival

Survival at the end of the 21-d exposure period decreased significantly with increasing temperature ($F_{3,16} = 17.27$, $p < 0.0001$). Post hoc comparisons revealed that mean survival at 16°C (66.9%) and 21°C (62.3%) was comparable, both being significantly higher than survival at 26°C (49.1 A%) and 31°C (38.9%), with the lowest survival observed at 31°C (Figure 2).

3.2 | Upper Thermal Tolerance Limits, Heat Tolerance Plasticity and Thermal Safety Margins

The minimum CTmax value observed was 32.6°C for males exposed to 16°C, while the highest one was 35.1°C for females exposed to 31°C. CTmax increased non-linearly with increasing

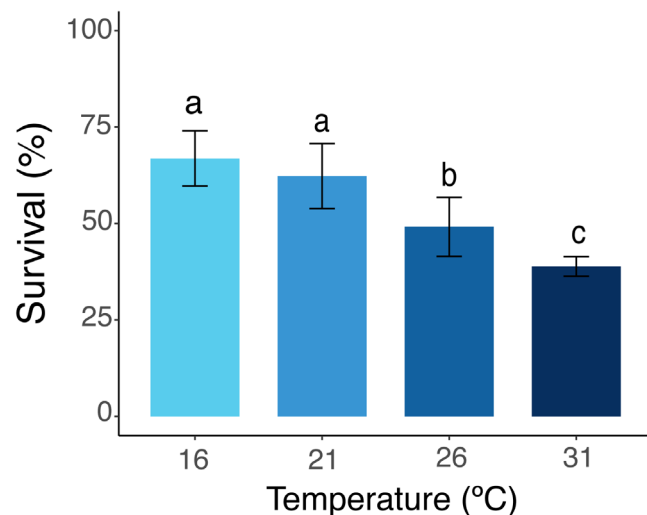


FIGURE 2 | Effect of elevated temperature on mean $\pm$ SD survival in the amphipod *Gammarus locusta* after 21 days of exposure to 16°C, 21°C, 26°C and 31°C ($n = 5$ tanks per temperature, 20 tanks total). The bar graphs represent the mean and the vertical lines their associated SD. Different letters denote significant differences ($p\text{-adj} < 0.05$) between means.

exposure temperature, but differently for females and for males, as indicated by the presence of a significant interaction between the terms *Sex* and *Temperature* ($\chi^2 = 15.367$, $df = 3$, $p < 0.0015$, Table 1). In more detail, the increase in mean CTmax between 16°C and 21°C was greater in males than in females: 2.73% versus 1.49%, respectively. Further, females displayed higher mean CTmax values when compared to males at 16°C and 31°C (2.12% and 1.16% higher, at 16°C and 31°C, respectively, Figure 3A). Moreover, variation within each group was low, as shown by coefficients of variation (CV) ranging from 0.64% to 1.37% (Table S3). ARR values decreased with increasing temperature, in females and males, ranging from [0.11–0.18] at 21°C, to [0.10–0.14] at 26°C and [0.08–0.10] at 31°C, respectively, with males generally showing slightly higher values than females (Table S3).

The species overall CTmax CV was 1.81%. Between sexes, CV ranged from 0.68% to 0.84% (at 16°C and 31°C) in females, and from 0.64% to 1.37% in males (at 16°C and 31°C, respectively). Finally, the highest TSM was 5.87°C for males and 6.20°C for females (Table S3).

TABLE 1 | Summary of the results of the ANOVA type III test used to investigate the effect of *temperature* on amphipods' survival, reproductive output and juveniles' body length, and for *temperature*, *sex* and their interaction on amphipods' critical thermal limit (CTmax) following a 21-day exposure to a temperature gradient. Factors for which we have a significant effect ($p < 0.05$) are highlighted in bold. For each variable, models were selected and reported based on their lowest Akaike Information Criterion. Df: Degrees of freedom. Sum sq.: Sums of squares.

Survival				
Factor	Sum sq	df	F value	Pr(> F)
<i>Temperature</i>	2433	3	17.27	<0.0001
Residuals	751	16		
Critical Thermal Maximum (CTmax)				
Factor		df	χ^2	Pr(> χ^2)
(Intercept)		1	155,500	<0.0001
<i>Sex</i>		1	32.891	<0.0001
<i>Temperature</i>		3	98.845	<0.0001
<i>Sex</i> × <i>Temperature</i>		3	15.367	0.0015
Residuals				
Reproductive output				
Factor		df	χ^2	Pr(> χ^2)
<i>Temperature</i>		3	136.34	<0.0001
Juveniles body length				
Factor		df	χ^2	Pr(> χ^2)
(Intercept)		1	367.46	<0.0001
<i>Temperature</i>		3	106.14	<0.0001

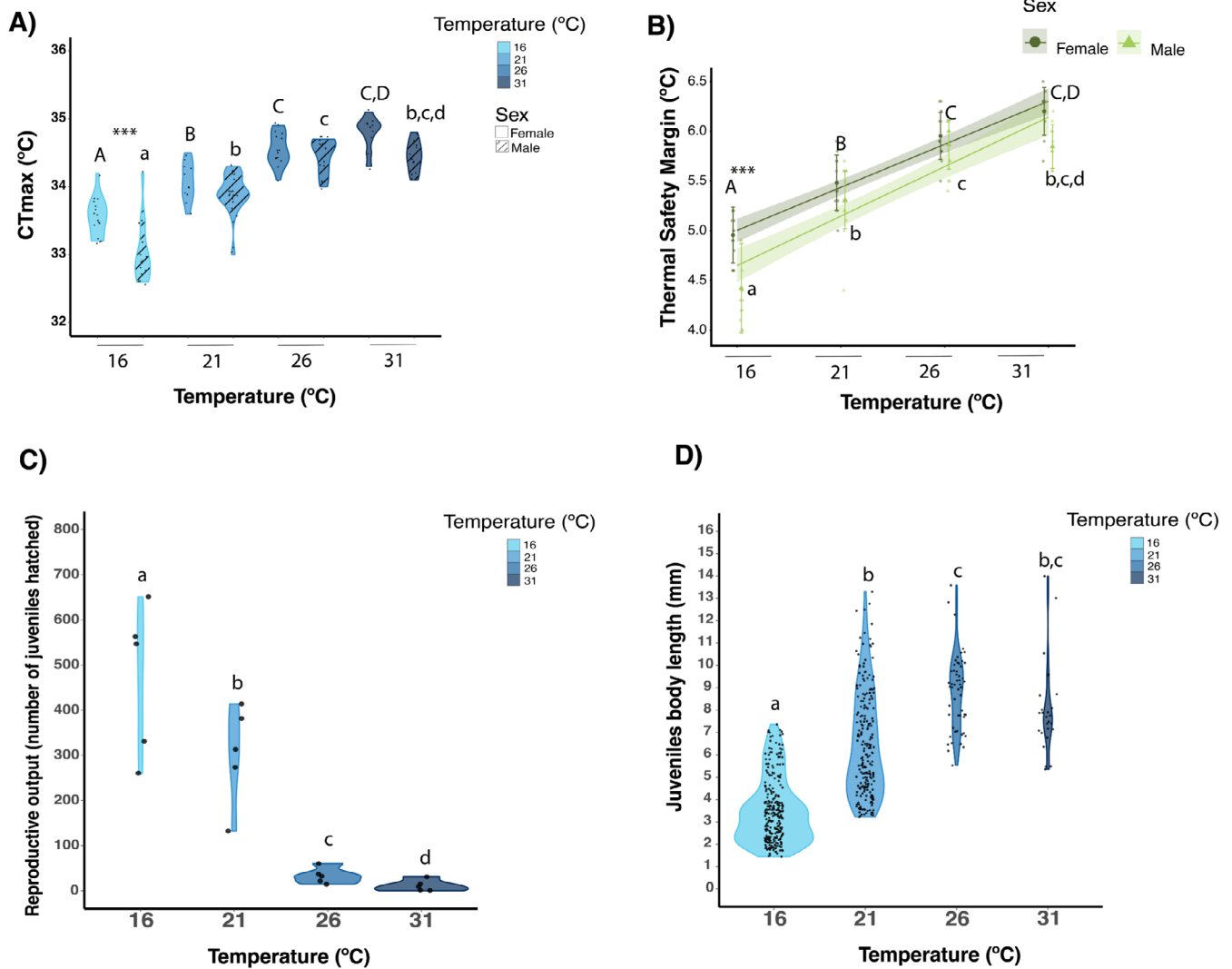


FIGURE 3 | The effect of elevated temperatures (16°C, 21°C, 26°C, and 31°C) for both males and females of *G. locusta* after exposure of 21 days in different traits. (A) Critical Thermal maximum (CTmax) values; (B) Mean $\pm$ SE (standard error) of Thermal Safety Margin (TSM); (C) Reproductive output; and (D) Juveniles' body length. In sex-specific graphs (A and B), uppercase letters indicate significant differences between females across temperatures; lowercase letters indicate significant differences between males across temperatures; asterisks indicate differences between females and males within each temperature. Data in A, C and D are violin plots showing metrics' distribution and jittered points show individual data.

3.3 | Reproductive Output and Juveniles Body Length

Reproductive output significantly decreased non-linearly with increasing exposure temperature, from 470.4 hatched juveniles at 16°C to 11.2 at 31°C (Figure 3C). Juvenile body length significantly increased with increasing exposure temperature, visibly in a non-linear fashion, reaching a *plateau* at 26°C. At all elevated temperatures, juveniles were significantly longer when compared to the optimum (16°C). The non-linearity of the thermal response was supported by the fact that juvenile mean size increased linearly from 16°C to 26°C (from 3.50 to 8.98 mm, respectively) but plateaued at the highest temperature treatment (31°C, 8.05 mm, being comparable to that at 26°C; Figure 3D).

3.4 | Metabolomic Profiles in Response to Sex and Temperature

The amphipods' whole targeted metabolome (i.e., 47 metabolites) primarily differed based on *Sex* and *Temperature*, but not their interaction (Table 2), which was visually confirmed through the sPLS-DA (Figure 4A,B). Despite the interaction between *Temperature* and *Sex* was not significant in the PERMANOVA of the whole targeted metabolome (Table 2), as males and females exhibited different thermal tolerance levels across the tested temperature gradient, and metabolomic responses were shown to strongly relate to changes at the whole-organism level (e.g., Calosi et al. 2017), we dissected the thermal response *per sex*. To do so, we used specific contrasts to identify possible differences in metabolite concentrations underlying males and

TABLE 2 | Summary of results from the permutation analysis of variance (PERMANOVA) with the effects of the fixed variables *Temperature* and *Sex* and their interaction on *G. locusta* metabolome (47 metabolites) ($N=40$). Degrees of freedom (df), R^2 , F -statistic and associated p -values for each term are reported, with significant results highlighted in bold.

Factor	df	Sum sq	R^2	F value	p ($> F$)
Sex	1	654	0.166	8.11	0.001
Temperature	3	452	0.115	1.87	0.030
Sex $\times$ Temperature	3	241.7	0.061	0.99	0.415
Residual	32	2579.4	0.657		
Total	39	3927.1	1		

females' responses to different temperatures. Accordingly, when *Sex* had a significant effect (main or interactive), traits were re-analysed separately for each sex to avoid masking temperature effects (Vermandele et al. 2025). These within-sex analyses revealed that females and males' whole metabolome clustered based on temperature (Figure 4C,D).

We also revealed distinctive metabolomic signatures across *Sex* and *Temperature* groups using the *limma* analyses (Figure 5, 6). Briefly, 21 differentially abundant metabolites (DAMs) were identified, all present at lower concentrations in males compared to females (Figure 6A). Furthermore, six and five DAMs had lower abundances at 26°C and 31°C, respectively, compared to 16°C (Figure 5A). Comparing sexes within each temperature, we identified four DAMs at the lowest temperature (16°C) (Figure 6B).

Males and females showed more pronounced differences at 21°C (seven DAMs) and particularly at 26°C (18 DAMs), with males showing lower metabolite abundances compared to females (Figure 6B). Contrastingly, no significant DAMs were identified at 31°C between sexes (Figure 6B). The complete list of DAMs is provided in Table S5, and summary boxplots of metabolite fold changes and significance are available in Figures S3–S5.

Network-based pathway analyses identified a total of nine significantly enriched pathways, differentially utilised by males and females, from all temperatures tested (Figure 7A). Sex-specific analyses comparing females and males at each temperature separately showed one enriched pathway at 16°C (Figure 7B), four at 21°C (Figure 7C), and eight at 26°C (Figure 7D). This represents a 700% increase in the number of enriched pathways from 16°C to 26°C. Noteworthy, *Alanine, aspartate and glutamate metabolism* was the one common pathway utilised across all comparisons. Unique pathways characterising sex differences at 21°C were *One carbon pool by folate* and at 26°C were *β -Alanine metabolism*, *ABC transporters*, *Arginine and proline metabolism*, *Glutathione metabolism* and *Non-homologous end-joining*. Comparisons between temperatures, irrespective of *Sex*, revealed five enriched pathways at 26°C and four at 31°C, with three pathways overlapping between these two conditions (*mTOR signaling*, *ABC transporters* and *Lysine degradation*) (Figure 5B,C). Metabolites did not significantly vary at 21°C

when compared to 16°C, and therefore no enrichment pathway analysis was performed for this comparison (Figure 5).

3.5 | ATP:ADP Ratio

The interaction between *Temperature* and *Sex* was not significant ($p=0.13$), indicating that males and females showed similar thermal responses. Given the lack of interaction, we therefore fitted an additive model, which showed that ATP:ADP ratios declined significantly with increasing temperature ($p=0.011$), while *Sex* had a marginal effect, with males exhibiting slightly higher ATP:ADP ratios than females ($p=0.063$; Figure 8).

4 | Discussion

Our study uncovers pronounced sex-specific variation in thermal sensitivity and physiological responses in the amphipod *G. locusta*, a keystone species in coastal marine ecosystems. Females display a higher upper thermal tolerance, but lower plasticity for heat tolerance, when compared to males. This was coupled with subtle metabolite-level sex-specific patterns, with differences in metabolite abundance between the sexes increasing along the temperature gradient, before converging close to the species' near-critical upper thermal limits. This indicates that near-critical conditions may override or suppress sex-specific metabolomic variation. Particularly, females consistently showed higher abundance of metabolites associated with cellular energy balance and redox regulation (such as *NAD*, *FAD*, *ATP*, *ADP*, *Glutamate*, *Aspartate*, *Betaine*, *Cystine* and *Serine*) when compared to males. This highlights females' greater ability to maintain physiological functions and cellular metabolism under moderate warming conditions, but less so when extreme temperatures occur. Exposure to intermediate and high temperatures imposes marked negative effects on *G. locusta*, reducing their survival rates, reproductive output and cellular energy status. By combining the characterisation of fitness, juveniles' growth, physiological performances and molecular mechanisms, our study provides a unique perspective on the thermal abilities of females and males of a keystone marine ectotherm exposed to a thermal gradient. Overall, we evidence a complex interaction between sex and exposure to ocean warming, underscoring the need to consider sex as a fundamental source of phenotypic variation.

In fact, for key traits (i.e., CTmax and Thermal Safety Margins) we show sex-specific thermal responses. However, this pattern is not ubiquitous, with traits such as ATP:ADP ratio not differing or showing just a marginal difference between females and males. Altogether our results highlight that sex effects are ecologically extremely relevant, despite being trait specific. We also show that integrating molecular approaches, such as metabolomics, to the characterisation of life history and whole organisms' responses provides valuable mechanistic insight into species' sensitivity to global change drivers. We call for the wide implementation of sex as a biological variable (Clayton 2018; Farrukh and Becker 2025; Shansky and Murphy 2021), as well as the characterisation of the mechanisms underpinning such differences, as critical steps toward improving future biodiversity projections under global changes.

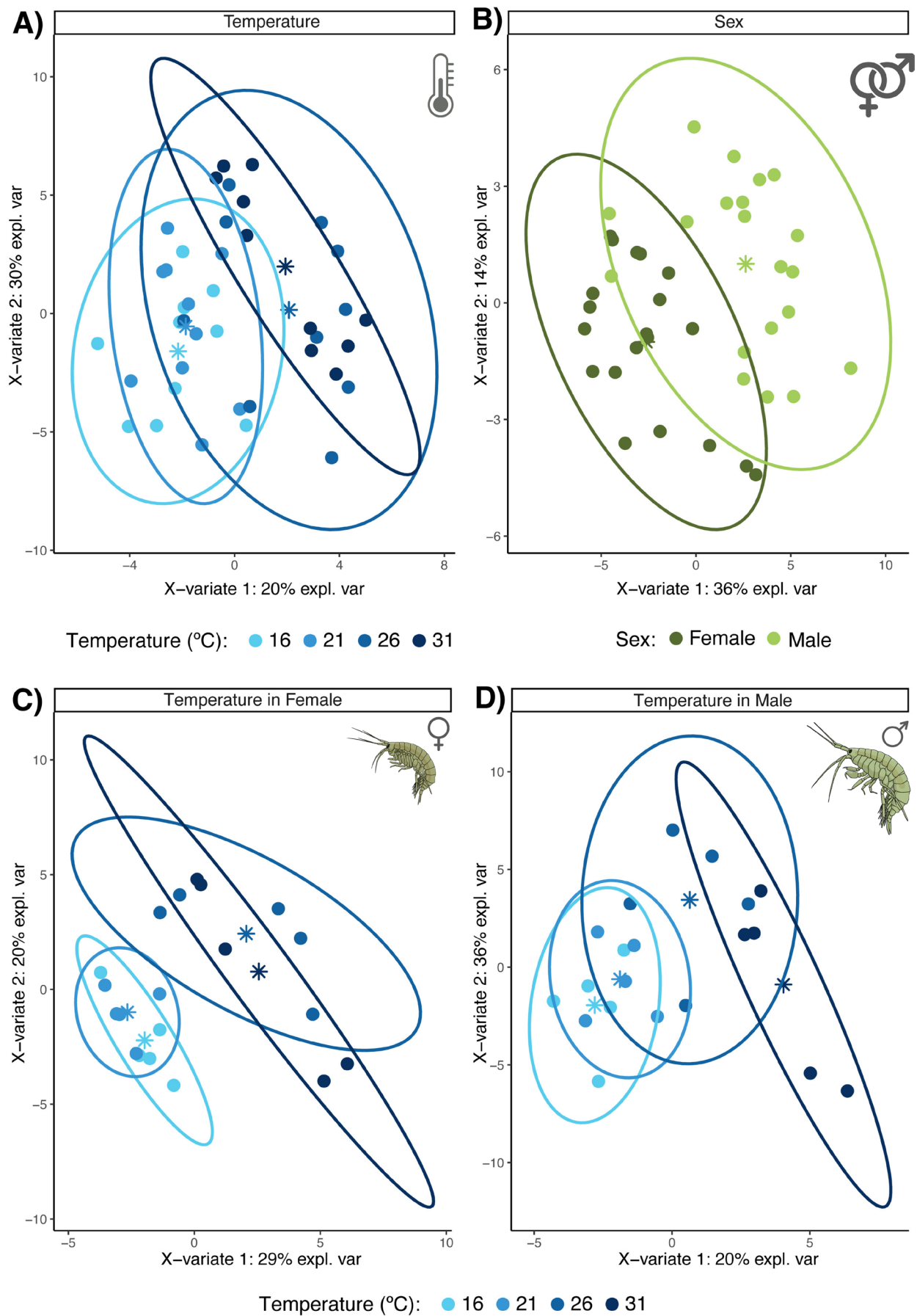


FIGURE 4 | Legend on next page.

FIGURE 4 | Supervised sparse Partial Least Squares Discriminant Analyses (sPLS-DA) of *G. locusta* metabolomic profiles as a function of: (A) temperature treatments across all individuals from both sexes combined; (B) sexes across all individuals from all temperature treatments; (C) Temperature treatments separation within females; (D) Temperature treatments separation within males. Each plot shows the ellipses representing 95% confidence regions around group centroids (asterisks). The percentage of variance explained by each component is indicated on the axes. High AUC values and associated p -values for all pairwise comparisons confirmed strong classification performance across most models (see Table S5).

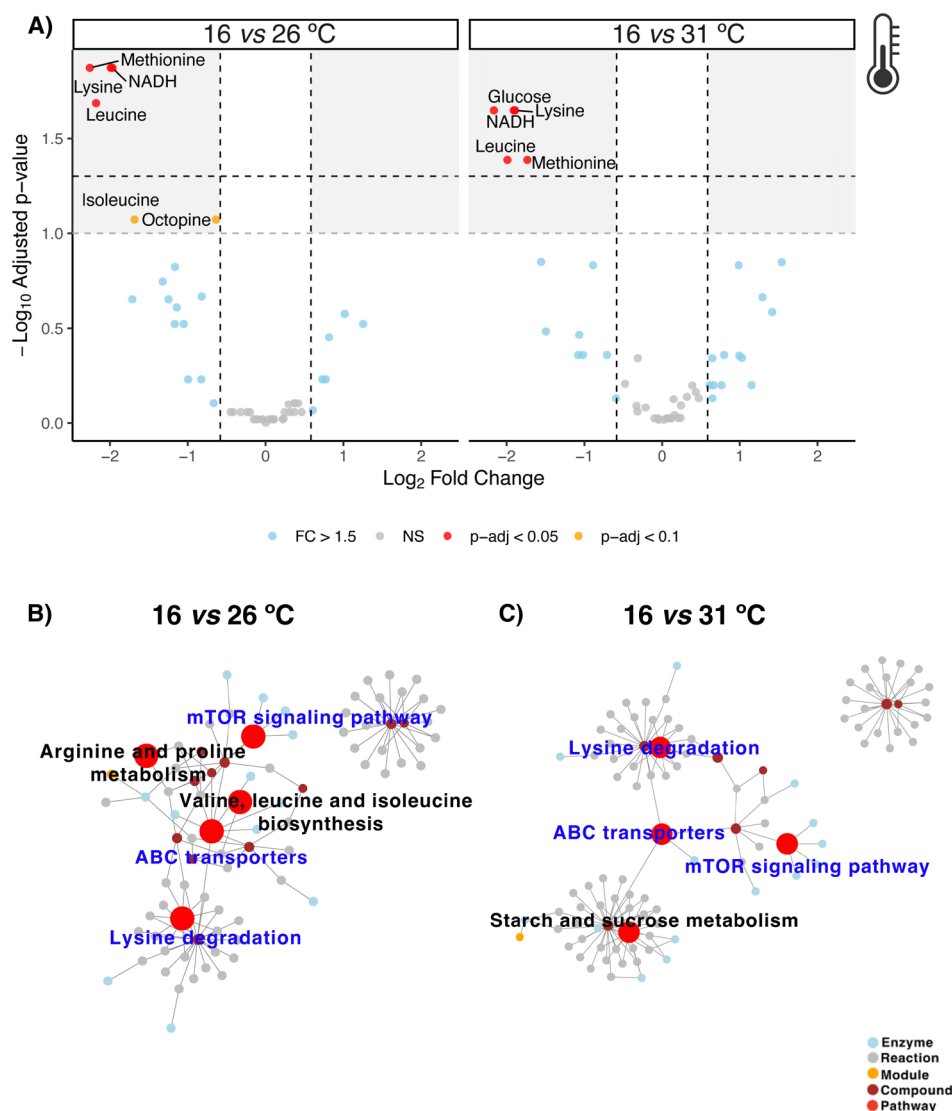


FIGURE 5 | Differential abundant metabolites (DAMs) and pathway-level responses across temperatures in relation to the optimum temperature (16°C): (A) Volcano plots showing DAMs between 16°C and 26°C and 16°C and 31°C, based on *limma* analyses. Each point represents a metabolite; significantly altered metabolites (adjusted $p < 0.05$ and < 0.1) are highlighted. No significant differences were detected at 21°C vs. 16°C. NS: Non-significant. FC: Fold change. Horizontal dotted lines show the significance threshold ($-\log_{10}$ adjusted p -value), and vertical dotted lines show the fold-change cutoffs ($\log_2$ FC); (B, C) Network-based pathway enrichment analyses for the same comparisons: 16°C vs. 26°C (B) and 16°C vs. 31°C (C). Networks show significantly enriched pathways (large light red nodes) and their associated compounds or metabolites (dark red nodes), enzymes, modules and reactions. Shared pathways are highlighted in blue and unique pathways in black.

4.1 | Sexual Dimorphism: From Metabolites to Physiology

Here, we show that spent females and males exhibit distinct metabolomic profiles, with nine pathways differentially utilised between sexes. Notably, males under-utilise amino acid biosynthesis and metabolism, which are central for energy

production, as well as folate transport and metabolism, which are key for anabolic reactions (DNA, RNA and amino acid synthesis) (Zarou et al. 2021). Additionally, males show the de-activation of two key pathways when compared to females. First, males rely less on ATP-binding Cassette (ABC) transporters, which are essential for molecule and ion transport across membranes (Dean et al. 2001; Jeong et al. 2017) and possibly

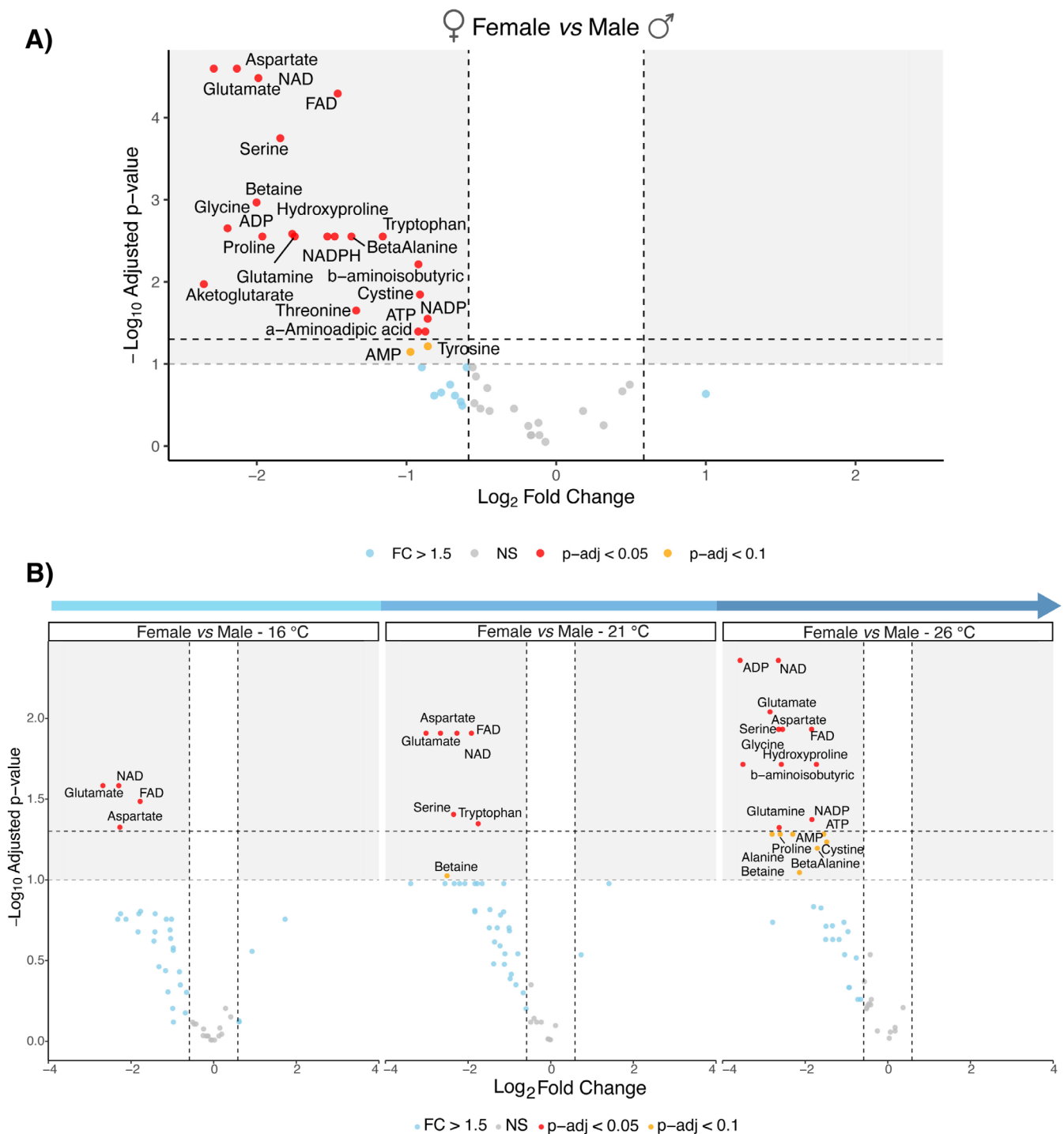


FIGURE 6 | Differential abundant metabolites (DAMs) between sexes (A) Volcano plot showing differential metabolite abundance in males when compared to females (all temperatures combined) and (B) within each temperature (16°C, 21°C and 26°C). No plot is shown for 31°C as no significant DAMs were identified between sexes at this temperature. Each point represents an individual metabolite; those with significant differences between sexes (adjusted $p < 0.05$ in red and < 0.1 in orange) are highlighted. NS: Non-significant. FC: Fold change. Horizontal dotted lines show the significance threshold ($-\log_{10}$ adjusted p -value), and vertical dotted lines show the fold-change cutoffs ($\log_2$ FC).

heat stress tolerance (Duan et al. 2025). ABC transporters also play a key role in regulating cellular homeostasis under stress by mediating the active export of metabolites, osmolytes, and xenobiotics, and their lower expression has been associated with reduced stress tolerance in invertebrates under thermal challenge (Duan et al. 2025). Second, males rely less on glutathione metabolism, a pathway important for managing oxidative stress

and contributing to cellular protection during thermal exposure (Cabral et al. 2025). These results suggest that males rely on a more energy-conserving cellular metabolic strategy to sustain basal functions when compared to spent females, with males also displaying lower baseline levels of adenylates (including AMP, ADP and ATP) when compared to females. However, weak differences in females and males' ATP:ADP ratio are

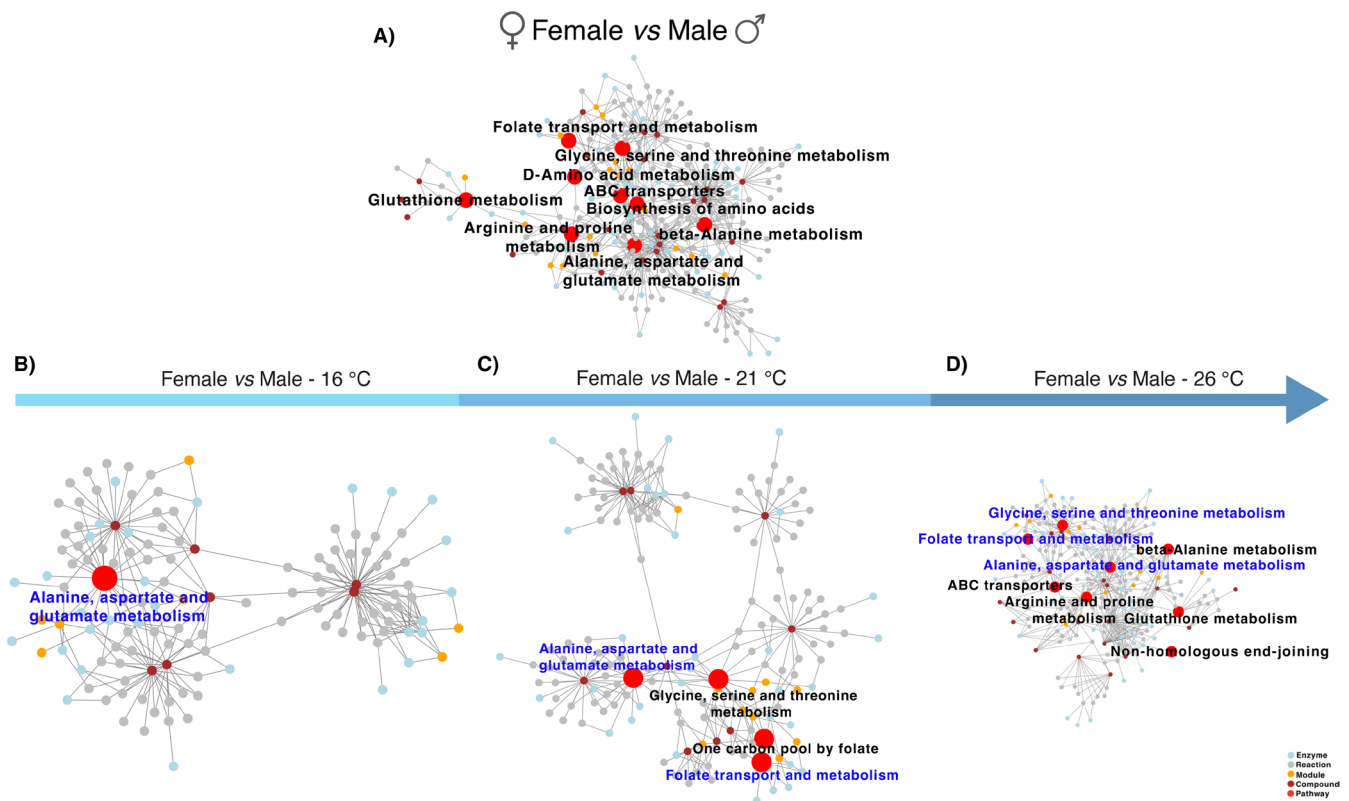


FIGURE 7 | Network-based enriched pathways for *G. locusta*: (A) between females and males all temperatures combined; (B) at 16°C, (C) at 21°C, (D) at 26°C. No graph is shown for 31°C as no significant DAMs or pathways were identified between sexes at this temperature. Nodes represent compounds (metabolites, dark red points), reactions, modules, enzymes, and pathways (large light red points) from the KEGG database with node colour indicating functional categories. Shared significant pathways are highlighted in blue and unique pathways in black.

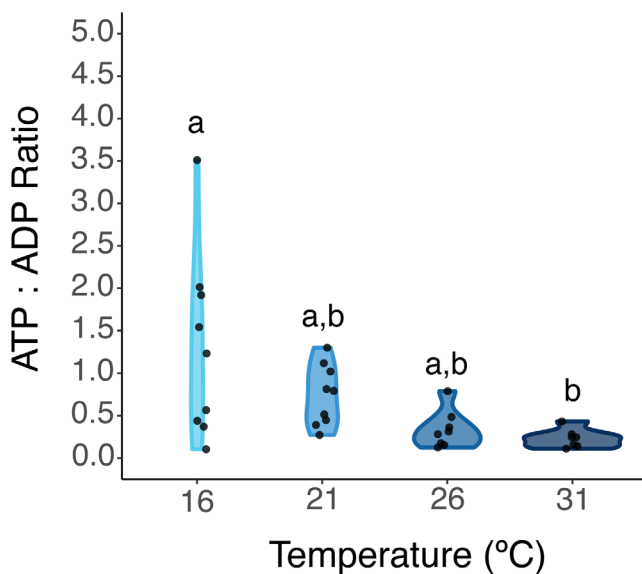


FIGURE 8 | Relationship between temperature and ATP:ADP ratio. Violin plots represent the distribution of ATP:ADP ratio under different temperatures (16°C, 21°C, 26°C and 31°C) in *G. locusta*. Points indicate individual observations. Different letters denote statistical differences ($p\text{-adj} < 0.05$).

found, with males displaying a slightly higher ratio than females. This suggests that despite overall adenylate levels being lower, males have abundant energy and remain in a state of low

activation of ATP-generating pathways. This energetic profile points to a strategy of maintenance, rather than energetically costly biosynthetic processes. A lower ATP:ADP ratio in females may suggest constitutive higher energy turnover and higher activation of ATP producing pathways. This divergence in metabolomic profiles is likely underpinned by the distinct biological roles of each sex within the species life-history strategy and populations dynamics, with females needing to allocate resources for processes like egg production, accumulation of egg's reserves to fuel embryogenesis, and brooding, processes that are energetically demanding (Hayward and Gillooly 2011). Given that *G. locusta* has a short generation time and reproduces continuously across seasons (Costa and Costa 1999), females likely maintain a sustained reproductive investment year-round. This could amplify sex-specific differences in energetic demands and help explain the consistent metabolic differences we report. By contrast, males often invest more in mate searching and guarding or clasping females, which may require intense but less constant energetic costs. These different behavioural demands between sexes align well with the metabolomic patterns we observe, suggesting that males rely more on immediate energy-mobilising pathways, whereas females channel resources into biosynthetic and antioxidant processes associated with egg production and brooding (Alonzo and Warner 2000; Rotimi et al. 2024). As the females used in our experiment were ovigerous at the time of collection from the field but had released their eggs prior to experimental sampling, the observed differences likely reflect post-reproductive physiological states. This recent investment in reproduction may help explain the enhanced biosynthetic

and antioxidant activity seen in females, in contrast to males' reliance on more transient energy pathways. Also, in our study, we observed clear sexual dimorphism in CT_{max}, with females showing higher thermal tolerance than males. As expected, body mass also differed between sexes, with males being larger, a factor often linked to reduced heat tolerance in ectotherms (Brans et al. 2017; Leiva et al. 2019). However, our analyses showed that body mass did not significantly explain variation in CT_{max}, indicating that the observed sex differences in thermal tolerance are not attributable to size but instead reflect genuine physiological dimorphism.

4.2 | From Divergence to Convergence: How Temperature Modulates Sex-Based Physiological Strategies

Besides the general metabolome differences between sexes, when investigating molecular sexual dimorphism at each exposure temperature, the key finding of our study is that there are metabolome-level and functional-level differences in the thermal response of males and females. At the optimal temperature (16°C), only alanine, aspartate and glutamate metabolism is lower in males when compared to females. This indicates that, compared to males, females may maintain higher baseline levels of amino acid pools for energy production or biosynthesis to fuel their higher reproductive energetic demand (Raymond et al. 2004). However, as temperature increases, the differences between males and females become more marked, being maximum at 26°C. At 21°C, three pathways are lower in males than females, including glycine, serine and threonine metabolism, as well as one-carbon pool by folate and folate transport and metabolism. This suggests a progressive decline in the ability of males to perform anabolic reactions (biosynthesis and turnover of proteins, DNA, RNA) as temperature increases. Folate metabolism is essential for anabolic processes, cell division and embryogenesis (Wangensteen et al. 2016), and differs between males and females at 21°C, in line with a prior study on crustaceans (Jiang et al. 2023). At 26°C, males have lower β -alanine metabolism, a key process in cell volume regulation and stress responses (Kasschau et al. 1984), and lower ABC transporters, indicating reduced translocation of molecules and ions across membranes, potentially impairing cellular homeostasis (Thomas and Tamp   2020). Concomitantly, males show a reduction in metabolites associated with arginine and proline metabolism, which support cellular energy production (Saibi et al. 2015), as well as in pathways linked to redox regulation (Jefferies et al. 2003) and non-homologous end-joining, a vital DNA repair mechanism (Barnes 2001). At 31°C, these sex-specific metabolomic differences largely disappear, with males and females displaying overlapping metabolomic profiles. This convergence likely reflects a breakdown in sex-dependent physiological strategies under extreme thermal stress. At a high near-critical temperature, both sexes may face comparable energetic and cellular constraints, leading to a shared metabolic suppression or collapse in regulation. This could result from the failure of aerobic metabolism and mitochondrial function, leading to a loss of ATP production capacity and forcing both sexes to rely on similar anaerobic or emergency metabolic pathways. Moreover, essential regulatory mechanisms (such as redox balance, ion transport, and protein repair) may become saturated or impaired, narrowing

the scope for sex-specific metabolic adjustments. Such convergence has been proposed as a hallmark of approaching upper thermal limits, where organismal systems lose the capacity for differentiated responses (Bawa et al. 2021). This loss of metabolomic sexual dimorphism at high temperature represents a key finding, underscoring that sex-specific resilience is most evident under moderate stress but may vanish when survival thresholds are exceeded (Weaving et al. 2022).

Thermal tolerance limits show sex-specific differences, with females consistently exhibiting higher upper thermal limits but lower ARR than males across treatments. This supports growing evidence of sex-specific tolerance to heat in marine arthropods (Gissi et al. 2023; Lam et al. 2022; Mills and Fish 1980; Mission  rio et al. 2022; Vermandele et al. 2024). Altogether, this suggests that females can have higher baseline tolerance but lower plasticity for this trait. Interindividual variation in tolerance to heat is greater among males at the control temperature. However, this variation diminishes at higher temperatures, and approaches that of females, suggesting that thermal stress narrows phenotypic differences as both males and females reach shared physiological limits. A similar trend of decreased phenotypic variability at extreme temperatures has also been found for other intertidal organisms (Madeira et al. 2021). This said, the selective death of the most thermally sensitive phenotypes cannot be entirely excluded. The progressive increase and eventual *plateau* of CT_{max} with acclimation temperature is consistent with the 'Concrete Ceiling' hypothesis (Sandblom et al. 2016; Stillman 2002; Schulte et al. 2011), which proposes that acclimation capacity diminishes at higher temperatures, even if plasticity is evident at moderate ranges. Together, these patterns suggest that both plasticity and selective filtering shape thermal responses under chronic warming.

In the present study, thermal safety margins (TSM) are also broader in females, suggesting that they may cope better with marine heatwaves (Vermandele et al. 2024), whereas males may face greater mortality risk. Such risk could be mitigated (at least in part) by the males' greater heat tolerance plasticity. However, the fact that their tolerance reaches a *plateau* at 26°C and does not further improve at 31°C in both sexes may indicate a limited window for thermal damage risk mitigation if warming exceeds these limits. The maximum habitat temperature (MHT) used in our TSM estimates was recorded in 2022. However, ocean temperatures are very likely to have increased since then (Monteiro et al. 2025), potentially narrowing *G. locusta*'s thermal safety margins under real-world conditions. Still, estimating interannual variability in thermal limits and MHT could be an important avenue of research to refine TSM predictions. Beyond thermal limits and fitness, temperature also affects other physiological processes, with elevated temperatures disrupting immune regulation in gammarids (Labaude et al. 2017), hampering chemosensory mate detection across generations (Pereira et al. 2024) and increasing metabolic rates (Aarset and Aunaas 1990). Thermal tolerance is also species-specific (Bedulina et al. 2017; Magozzi and Calosi 2015; Vermandele et al. 2024), which as we show here depends on sexes' differential capacity to modulate cellular stress responses and metabolism when faced with heat stress (Foley et al. 2019; Madeira, Narciso, Cabral, Diniz, and Vinagre 2012).

4.3 | Linking Sex-Specific Thermal and Metabolomic Plasticity With Fitness Outcomes

The sex-specific divergence in metabolomic strategies we report here highlights that sex and associated reproductive features significantly influence thermal stress tolerance, with females generally demonstrating a greater metabolic plasticity, higher energetic capacity and greater ability to keep homeostasis. In *G. locusta*, elevated temperatures reduced both survival and reproductive output, corroborating the strong negative effects of warming on amphipod fitness (Tsoi et al. 2005; Xue et al. 2018). Although we could not estimate sex-specific survival, other studies also report that differential survival between sexes may alter populations sex ratio, thereby affecting reproductive success and ultimately populations' dynamics, especially as warming trends continue to intensify (Edmands 2021). Concomitant to a decline in survival, fitness also decreases at elevated temperatures, suggesting that ocean warming may severely compromise *G. locusta*'s reproductive capacity and thus its persistence locally, as shown in other species (Bai et al. 2023; Gissi et al. 2023). Crucially, while male-biased vulnerability to heat has been reported before (Pottier et al. 2021; Vermandele et al. 2024), our study is the first to identify the molecular and physiological mechanisms underpinning this pattern, specifically in *G. locusta*, and link them to fitness. By combining organismal, physiological and metabolomic analyses within a single experimental framework, we move beyond descriptive comparisons and provide mechanistic insight into the processes driving sex-specific thermal tolerance. This includes the identification of key metabolic pathways (e.g., glutathione and ABC transporters) that are differentially regulated between sexes and directly linked to their performance under heat stress. Interestingly, more numerous but smaller juveniles are produced at lower temperatures, whereas at higher temperatures, fewer but larger juveniles are recorded. This shift may reflect a temperature-induced adaptive trade-off in brooding strategy, wherein females prioritise investment in egg quality over quantity to increase survival under stressful conditions, a pattern observed in other invertebrates (Gibbin et al. 2017; Maranhão and Marques 2003; Massamba-N'Siala et al. 2014). Such thermal impacts on fitness may carry population- and ecosystem-level consequences, particularly for coastal species, such as *G. locusta*, playing key trophic roles in such transitional ecosystems. However, the sex-specific responses we report here may not be ubiquitous being potentially dependent on taxa, life-stages, populations and generations (Ascione et al. 2024; Cachet et al. 2015; Guscetti et al. 2023; Marcellin-Gros et al. 2020; Verberk et al. 2013). However, Gissi et al. (2023) report that sex-specific differences were detected in 89.7% of the studies accounting for sex. Nevertheless, our integrative design, linking thermal tolerance, reproduction and metabolism, provides a transferable approach to uncover sex-dependent vulnerabilities in other ectotherms. We provide an empirical demonstration of how sex shapes thermal physiology and metabolism to influence fitness outcomes, underscoring the importance of incorporating sex as a fundamental axis of biological variation to improve predictions of thermal sensitivity under climate change. Further, we show the importance of acquiring a critical understanding on the molecular underpinnings of females and males' responses to ocean warming. While integrative approaches such

as metabolomics, lipidomics, and even *de novo* transcriptomics are increasingly accessible and applicable beyond traditional model species, they still face challenges (particularly regarding annotation depth and pathway resolution). Nonetheless, these tools offer valuable mechanistic insight when applied thoughtfully, even in non-model organisms. We evidence that different metabolomic strategies emerge between sexes, driving differences in their thermal tolerance and overall fitness under heat stress. For estuarine species, which inhabit extremely dynamic thermal environments (Costa and Costa 1999), and act as key nodes in coastal food webs (Vinagre and Gastón 2024), such integrative approaches are highly relevant. We highlight that when ATP production is compromised under heat stress, organisms are left with insufficient resources to maintain vital cellular processes, eventually leading to their death. Moreover, we report a strong male-skewed vulnerability to ocean warming, underpinned by their lower ability to maintain homeostasis, energy production or activate stress mitigation pathways when compared to females. We call for a greater effort in defining sex-specific patterns in thermal biology experiments, to be able to draw where possible and applicable generalities (or lack of) which can help drive our understanding of the ecology and evolution of females and males of the same species. In addition, integrating sex as a key biological variable will strengthen predictions on marine ectotherms' tolerance and ability to adapt to an increasingly variable and harsh environment, particularly when the molecular underpinnings of sexual variation are elucidated.

Author Contributions

Joana Filipa Fernandes: conceptualization, methodology, formal analysis, investigation, data curation, writing – original draft, writing – review and editing, visualization. **Lauric Feugere:** writing – review and editing, visualization. **Mário S. Diniz:** conceptualization, methodology, writing – review and editing, supervision. **Piero Calosi:** conceptualization, methodology, resources, writing – review and editing, visualization, supervision, project administration, funding acquisition. **Diana Madeira:** conceptualization, methodology, resources, writing – review and editing, visualization, supervision, project administration, funding acquisition.

Acknowledgements

P.C. is a member of the inter-institutional strategic research networks Québec-Océan and Ressources Aquatiques Québec (RAQ).

Funding

This work was supported by FCT – Fundação para a Ciência e Tecnologia, I.P., via strategic projects to (a) UCIBIO (UID/04378/2025+UID/PRR/04378/2025, DOI: 10.54499/UID/04378/2025 and UID/PRR/04378/2025, DOI: 10.54499/UID/PRR/04378/2025), (b) i4HB (LA/P/0140/2020, DOI: 10.54499/LA/P/0140/2020), and (c) CESAM under the project/grant (UID/50017/2025, DOI: 10.54499/UID/50017/2025 and UID/50017/2025+LA/P/0094/2020, DOI: 10.54499/LA/P/0094/2020). We also thank FCT, I.P. for the research contract awarded to D.M. via the Scientific Employment Stimulus (2022.00153.CEECIND, DOI: 10.54499/2022.00153.CEECIND/CP1720/CT0016, and the PhD scholarship awarded to J.F.F. (2021.04675.BD, DOI: 10.54499/2021.04675.BD). This work was supported by project ExtremeOceans (PTDC/BIA-BMA/1494/2020, DOI: 10.54499/PTDC/BIA-BMA/1494/2020) funded by FCT, I.P. through national funds. This work was also supported by a Canada Foundation for Innovation

(CFI) grant and a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant (RGPIN-2020-05627), both granted to P.C. L.F. also acknowledges funding from MITACS Accelerate grants (IT35557; IT47018) and Fonds de recherche du Québec – Nature et technologies (FRQNT) (PBEEE#350373).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The raw data supporting this study are available in Zenodo at: <https://doi.org/10.5281/zenodo.20347473>.

References

- Aarset, A. V., and T. Aunaas. 1990. "Metabolic Responses of the Sympagic Amphipods *Gammarus wilkitzkii* and *Onisimus glacialis* to Acute Temperature Variations." *Marine Biology* 107, no. 3: 433–438. <https://doi.org/10.1007/BF01313425>.
- Alonzo, S. H., and R. R. Warner. 2000. "Allocation to Mate Guarding or Increased Sperm Production in a Mediterranean Wrasse." *American Naturalist* 156, no. 3: 266–275. <https://doi.org/10.1086/303391>.
- Ascione, D., Y. Carotenuto, C. Lauritano, and V. Roncalli. 2024. "Folate-Mediated One-Carbon Metabolism in the Crustacean Copepod *Calanus finmarchicus*: Identification of Transcripts and Relative Expression Across Development." *Journal of Marine Science and Engineering* 12, no. 5: 5. <https://doi.org/10.3390/jmse12050786>.
- Bai, Z., N. Wang, M.-S. Kim, et al. 2023. "Physiological and Molecular Responses of a Marine Copepod Under Multigenerational Exposure to Coastal Warming and Mercury at an Environmentally Realistic Concentration." *Environmental Science & Technology Letters* 10, no. 3: 240–246. <https://doi.org/10.1021/acs.estlett.3c00010>.
- Barnes, D. E. 2001. "Non-Homologous End Joining as a Mechanism of DNA Repair." *Current Biology* 11, no. 12: R455–R457. [https://doi.org/10.1016/S0960-9822\(01\)00279-2](https://doi.org/10.1016/S0960-9822(01)00279-2).
- Bawa, S. A., P. C. Gregg, A. P. D. Soccoro, C. Miller, and N. R. Andrew. 2021. "Estimating the Differences in Critical Thermal Maximum and Metabolic Rate of *Helicoverpa punctigera* (Wallengren) (Lepidoptera: Noctuidae) Across Life Stages." *PeerJ* 9: e12479. <https://doi.org/10.7717/peerj.12479>.
- Beaudreau, N., T. M. Page, D. Drolet, C. W. McKindsey, K. L. Howland, and P. Calosi. 2024. "Using a Metabolomics Approach to Investigate the Sensitivity of a Potential Arctic Invader and Its Arctic Sister-Species to Marine Heatwaves and Traditional Harvesting Disturbances." *Science of the Total Environment* 917: 170167. <https://doi.org/10.1016/j.scitotenv.2024.170167>.
- Bedulina, D., M. F. Meyer, A. Gurkov, et al. 2017. "Intersexual Differences of Heat Shock Response Between Two Amphipods (*Eulimnogammarus verrucosus* and *Eulimnogammarus cyaneus*) in Lake Baikal." *PeerJ* 5: e2864. <https://doi.org/10.7717/peerj.2864>.
- Benedetti, M., M. Giuliani, M. Mezzelani, et al. 2021. "Emerging Environmental Stressors and Oxidative Pathways in Marine Organisms: Current Knowledge on Regulation Mechanisms and Functional Effects." *Biocell* 46, no. 1: 37–49. <https://doi.org/10.32604/biocell.2022.017507>.
- Bennett, J. M., J. Sunday, P. Calosi, et al. 2021. "The Evolution of Critical Thermal Limits of Life on Earth." *Nature Communications* 12, no. 1: 1198. <https://doi.org/10.1038/s41467-021-21263-8>.
- Bennett, S., C. M. Duarte, N. Marbà, and T. Wernberg. 2019. "Integrating Within-Species Variation in Thermal Physiology Into Climate Change Ecology." *Philosophical Transactions of the Royal Society, B: Biological Sciences* 374, no. 1778: 20180550. <https://doi.org/10.1098/rstb.2018.0550>.
- Berns, C. M. 2013. "The Evolution of Sexual Dimorphism: Understanding Mechanisms of Sexual Shape Differences." In *Sexual Dimorphism*, edited by H. Moryama. IntechOpen. <https://doi.org/10.5772/55154>.
- Blighe, K., S. Rana, and M. Lewis. 2024. "Enhanced Volcano: Publication-Ready Volcano Plots With Enhanced Colouring and Labeling." R package version 1.22.0, <https://github.com/kevinblighe/EnhancedVolcano>.
- Bopp, L., L. Resplandy, J. C. Orr, et al. 2013. "Multiple Stressors of Ocean Ecosystems in the 21st Century: Projections With CMIP5 Models." *Biogeosciences* 10, no. 10: 6225–6245. <https://doi.org/10.5194/bg-10-6225-2013>.
- Bozinovic, F., P. Calosi, and J. I. Spicer. 2011. "Physiological Correlates of Geographic Range in Animals." *Annual Review of Ecology, Evolution, and Systematics* 42: 155–179. <https://doi.org/10.1146/annurev-ecolsys-102710-145055>.
- Brans, K. I., M. Jansen, J. Vanoverbeke, N. Tüzün, R. Stoks, and L. De Meester. 2017. "The Heat Is on: Genetic Adaptation to Urbanization Mediated by Thermal Tolerance and Body Size." *Global Change Biology* 23, no. 12: 5218–5227. <https://doi.org/10.1111/gcb.13784>.
- Brunel, T., and J. Boucher. 2007. "Long-Term Trends in Fish Recruitment in the North-East Atlantic Related to Climate Change." *Fisheries Oceanography* 16, no. 4: 336–349. <https://doi.org/10.1111/j.1365-2419.2007.00435.x>.
- Cabral, H. N., D. Madeira, M. Diniz, et al. 2025. "Climate Change Impacts on Coastal Biological Communities." In *Volume 2: Marine Ecology*, edited by J.M. Molina, G.E. Blasina. CRC Press.
- Cachet, N., G. Genta-Jouve, J. Ivanisevic, et al. 2015. "Metabolomic Profiling Reveals Deep Chemical Divergence Between Two Morphotypes of the Zoanthid *Parazoanthus axinellae*." *Scientific Reports* 5, no. 1: 8282. <https://doi.org/10.1038/srep08282>.
- Calosi, P., S. Melatunan, L. M. Turner, et al. 2017. "Regional Adaptation Defines Sensitivity to Future Ocean Acidification." *Nature Communications* 8: 13994. <https://doi.org/10.1038/ncomms13994>.
- Claussen, D. 1977. "Thermal Acclimation in Ambystomatid Salamanders." *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology* 58, no. 4: 333–340. [https://doi.org/10.1016/0300-9629\(77\)90150-5](https://doi.org/10.1016/0300-9629(77)90150-5).
- Clayton, J. A. 2018. "Applying the New SABV (Sex as a Biological Variable) Policy to Research and Clinical Care." *Physiology & Behavior, the Proceedings of the American University Symposium on Sex Differences: From Neuroscience to the Clinic and Beyond* 187: 2–5. <https://doi.org/10.1016/j.physbeh.2017.08.012>.
- Costa, F. O., and M. H. Costa. 1999. "Life History of the Amphipod *Gammarus locusta* in the Sado Estuary (Portugal)." *Acta Oecologica* 20, no. 4: 305–314. [https://doi.org/10.1016/S1146-609X\(99\)00136-8](https://doi.org/10.1016/S1146-609X(99)00136-8).
- Dean, M., A. Rzhetsky, and R. Allikmets. 2001. "The Human ATP-Binding Cassette (ABC) Transporter Superfamily." *Genome Research* 11, no. 7: 1156–1166. <https://doi.org/10.1101/gr.184901>.
- Donelson, J. M., P. L. Munday, M. I. McCormick, N. W. Pankhurst, and P. M. Pankhurst. 2010. "Effects of Elevated Water Temperature and Food Availability on the Reproductive Performance of a Coral Reef Fish." *Marine Ecology Progress Series* 401: 233–243. <https://doi.org/10.3354/meps08366>.
- Duan, S., X. Meng, H. Zhang, et al. 2025. "The Effect of Heat Stress on Wheat Flag Leaves Revealed by Metabolome and Transcriptome Analyses During the Reproductive Stage." *International Journal of Molecular Sciences* 26, no. 4: 4. <https://doi.org/10.3390/ijms26041468>.
- Eckersley, L., and R. Scrosati. 2012. *Temperature, Desiccation, and Species Performance Trends Along an Intertidal Elevation Gradient*. OSF. <https://doi.org/10.31230/osf.io/y9ph3>.
- Edmands, S. 2021. "Sex Ratios in a Warming World: Thermal Effects on Sex-Biased Survival, Sex Determination, and Sex Reversal."

- Journal of Heredity* 112, no. 2: 155–164. <https://doi.org/10.1093/jhered/esab006>.
- Ellis, R. P., W. Davison, A. M. Queirós, et al. 2017. “Does Sex Really Matter? Explaining Intraspecific Variation in Ocean Acidification Responses.” *Biology Letters* 13, no. 2: 20160761. <https://doi.org/10.1098/rsbl.2016.0761>.
- Farrukh, F., and R. C. Becker. 2025. “Sex as a Biological Variable: A Contemporary Perspective.” *American Heart Journal* 287: 10–15. <https://doi.org/10.1016/j.ahj.2025.03.012>.
- Feidantsis, K., I. Georgoulis, A. Zachariou, et al. 2020. “Energetic, Antioxidant, Inflammatory and Cell Death Responses in the Red Muscle of Thermally Stressed *Sparus aurata*.” *Journal of Comparative Physiology B* 190, no. 4: 403–418. <https://doi.org/10.1007/s00360-020-01278-1>.
- Fernandes, J. F., R. Calado, D. Jerónimo, and D. Madeira. 2023. “Thermal Tolerance Limits and Physiological Traits as Indicators of *Hediste diversicolor*'s Acclimation Capacity to Global and Local Change Drivers.” *Journal of Thermal Biology* 114: 103577. <https://doi.org/10.1016/j.jtherbio.2023.103577>.
- Foley, H. B., P. Y. Sun, R. Ramirez, et al. 2019. “Sex-Specific Stress Tolerance, Proteolysis, and Lifespan in the Invertebrate *Tigriopus californicus*.” *Experimental Gerontology* 119: 146–156. <https://doi.org/10.1016/j.exger.2019.02.006>.
- Ghalambor, C. K., J. K. McKAY, S. P. Carroll, and D. N. Reznick. 2007. “Adaptive Versus Non-Adaptive Phenotypic Plasticity and the Potential for Contemporary Adaptation in New Environments.” *Functional Ecology* 21, no. 3: 394–407. <https://doi.org/10.1111/j.1365-2435.2007.01283.x>.
- Gibbin, E. M., L. J. Chakravarti, M. D. Jarrold, et al. 2017. “Can Multi-Generational Exposure to Ocean Warming and Acidification Lead to the Adaptation of Life-History and Physiology in a Marine Metazoan.” *Journal of Experimental Biology* 220: 551–563. <https://doi.org/10.1242/jeb.149989>.
- Gissi, E., M. C. Goodman, R. Elahi, et al. 2024. “Sex-Specific Variation in Species Interactions Matters in Ecological Communities.” *Trends in Ecology & Evolution* 39, no. 11: 1004–1013. <https://doi.org/10.1016/j.tree.2024.07.006>.
- Gissi, E., L. Schiebinger, R. Santoleri, and F. Micheli. 2023. “Sex Analysis in Marine Biological Systems: Insights and Opportunities.” *Frontiers in Ecology and the Environment* 21, no. 7: 324–332. <https://doi.org/10.1002/fee.2652>.
- Gunderson, A. R., and J. H. Stillman. 2015. “Plasticity in Thermal Tolerance Has Limited Potential to Buffer Ectotherms From Global Warming.” *Proceedings of the Royal Society B: Biological Sciences* 282, no. 1808: 20150401. <https://doi.org/10.1098/rspb.2015.0401>.
- Guscelli, E., D. Chabot, F. Vermandele, D. Madeira, and P. Calosi. 2023. “All Roads Lead to Rome: Inter-Origin Variation in Metabolomics Reprogramming of the Northern Shrimp Exposed to Global Changes Leads to a Comparable Physiological Status.” *Frontiers in Marine Science* 10:1170451. <https://doi.org/10.3389/fmars.2023.1170451>.
- Hayward, A., and J. F. Gillooly. 2011. “The Cost of Sex: Quantifying Energetic Investment in Gamete Production by Males and Females.” *PLoS One* 6, no. 1: e16557. <https://doi.org/10.1371/journal.pone.0016557>.
- Hinojosa, I., and M. Thiel. 2003. “Somatic and Gametic Resources in Male Rock Shrimp, *Rhynchocinetes typus*: Effect of Mating Potential and Ontogenetic Male Stage.” *Animal Behaviour* 66, no. 3: 449–458. <https://doi.org/10.1006/anbe.2003.2220>.
- Hobday, A. J., L. V. Alexander, S. E. Perkins, et al. 2016. “A Hierarchical Approach to Defining Marine Heatwaves.” *Progress in Oceanography* 141: 227–238. <https://doi.org/10.1016/j.pocean.2015.12.014>.
- IPCC. 2022. “Climate Change 2022: Impacts, Adaptation, and Vulnerability.” In *Contribution of Working Group II to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change*, edited by H.-O. Pörtner, D. C. Roberts, M. Tignor, et al., 3056. Cambridge University Press. <https://doi.org/10.1017/9781009325844>.
- Iwasa, Y., and S. Yamaguchi. 2023. “Theoretical Studies of Diverse Sexual Patterns in Marine Animals.” *Proceedings of the Royal Society B: Biological Sciences* 290: 20222229. <https://doi.org/10.1098/rspb.2022.2229>.
- Jefferies, H., J. Coster, A. Khalil, J. Bot, R. D. McCauley, and J. C. Hall. 2003. “Glutathione.” *ANZ Journal of Surgery* 73, no. 7: 517–522. <https://doi.org/10.1046/j.1445-1433.2003.02682.x>.
- Jeong, C.-B., H.-S. Kim, H.-M. Kang, and J.-S. Lee. 2017. “ATP-Binding Cassette (ABC) Proteins in Aquatic Invertebrates: Evolutionary Significance and Application in Marine Ecotoxicology.” *Aquatic Toxicology* 185: 29–39. <https://doi.org/10.1016/j.aquatox.2017.01.013>.
- Jiang, G., Y. Xue, and X. Huang. 2023. “Effects of Dietary Folic Acid Supplementation on Sex Differences in Oriental River Prawn, *Macrobrachium nipponense*.” *Animals: An Open Access Journal From MDPI* 13, no. 23: 3677. <https://doi.org/10.3390/ani13233677>.
- Kanehisa, M., and S. Goto. 2000. “KEGG: Kyoto Encyclopedia of Genes and Genomes.” *Nucleic Acids Research* 28: 27–30. <https://doi.org/10.1093/nar/28.1.27>.
- Kasschau, M. R., C. M. Skisak, J. P. Cook, and W. R. Mills. 1984. “ β -Alanine Metabolism and High Salinity Stress in the Sea Anemone, *Bunodosoma cavernata*.” *Journal of Comparative Physiology B* 154, no. 2: 181–186. <https://doi.org/10.1007/BF00684143>.
- Labaude, S., Y. Moret, F. Cézilly, C. Reuland, and T. Rigaud. 2017. “Variation in the Immune State of *Gammarus pulex* (Crustacea, Amphipoda) According to Temperature: Are Extreme Temperatures a Stress?” *Developmental & Comparative Immunology* 76: 25–33. <https://doi.org/10.1016/j.dci.2017.05.013>.
- Lam, E. K., M. Abegaz, A. R. Gunderson, B. Tsukimura, and J. H. Stillman. 2022. “Interactions Between Temperature Variability and Reproductive Physiology Across Traits in an Intertidal Crab.” *Frontiers in Physiology* 13: 796125. <https://doi.org/10.3389/fphys.2022.796125>.
- Leiva, F. P., P. Calosi, and W. C. E. P. Verberk. 2019. “Scaling of Thermal Tolerance With Body Mass and Genome Size in Ectotherms: A Comparison Between Water- and Air-Breathers.” *Philosophical Transactions of the Royal Society, B: Biological Sciences* 374, no. 1778: 20190035. <https://doi.org/10.1098/rstb.2019.0035>.
- Lemieux, H., and P. U. Blier. 2022. “Exploring Thermal Sensitivities and Adaptations of Oxidative Phosphorylation Pathways.” *Metabolites* 12: 360. <https://doi.org/10.3390/metabo12040360>.
- Lesser, M. P. 2006. “Oxidative Stress in Marine Environments: Biochemistry and Physiological Ecology.” *Annual Review of Physiology* 68: 253–278. <https://doi.org/10.1146/annurev.physiol.68.040104.110001>.
- Li, A., L. Li, K. Song, W. Wang, and G. Zhang. 2017. “Temperature, Energy Metabolism, and Adaptive Divergence in Two Oyster Subspecies.” *Ecology and Evolution* 7, no. 16: 6151–6162. <https://doi.org/10.1002/ece3.3085>.
- Lipaeva, P., P. Drozdova, K. Vereshchagina, et al. 2025. “How to Reproduce in the Siberian Winter: Proteome Dynamics Reveals the Timing of Reproduction-Related Processes in an Amphipod Species Endemic to Lake Baikal.” *Ecology and Evolution* 15, no. 7: e71675. <https://doi.org/10.1002/ece3.71675>.
- Madeira, D., J. F. Fernandes, D. Jerónimo, et al. 2021. “Calcium Homeostasis and Stable Fatty Acid Composition Underpin Heatwave Tolerance of the Keystone Polychaete *Hediste diversicolor*.” *Environmental Research* 195: 110885. <https://doi.org/10.1016/j.envres.2021.110885>.
- Madeira, D., L. Narciso, H. N. Cabral, M. S. Diniz, and C. Vinagre. 2012. “Thermal Tolerance of the Crab *Pachygrapsus marmoratus*:

- Intraspecific Differences at a Physiological (CTMax) and Molecular Level (Hsp70)." *Cell Stress and Chaperones* 17, no. 6: 707–716. <https://doi.org/10.1007/s12192-012-0345-3>.
- Madeira, D., L. Narciso, H. N. Cabral, M. S. Diniz, and C. Vinagre. 2014. "Role of Thermal Niche in the Cellular Response to Thermal Stress: Lipid Peroxidation and HSP70 Expression in Coastal Crabs." *Ecological Indicators* 36: 601–606. <https://doi.org/10.1016/j.ecolind.2013.09.023>.
- Madeira, D., L. Narciso, H. N. Cabral, and C. Vinagre. 2012. "Thermal Tolerance and Potential Impacts of Climate Change on Coastal and Estuarine Organisms." *Journal of Sea Research* 70: 32–41. <https://doi.org/10.1016/j.seares.2012.03.002>.
- Magozzi, S., and P. Calosi. 2015. "Integrating Metabolic Performance, Thermal Tolerance, and Plasticity Enables for More Accurate Predictions on Species Vulnerability to Acute and Chronic Effects of Global Warming." *Global Change Biology* 21, no. 1: 181–194. <https://doi.org/10.1111/gcb.12695>.
- Maranhão, P., and J. C. Marques. 2003. "The Influence of Temperature and Salinity on the Duration of Embryonic Development, Fecundity and Growth of the Amphipod *Echinogammarus marinus* Leach (Gammaridae)." *Acta Oecologica* 24, no. 1: 5–13. [https://doi.org/10.1016/S1146-609X\(02\)00003-6](https://doi.org/10.1016/S1146-609X(02)00003-6).
- Marcellin-Gros, R., G. Piganeau, and D. Stien. 2020. "Metabolomic Insights Into Marine Phytoplankton Diversity." *Marine Drugs* 18, no. 2: 2. <https://doi.org/10.3390/md18020078>.
- Marochi, M. Z., G. C. Castellano, C. A. Freire, and S. Masunari. 2021. "Carrying Eggs in a Semi-Terrestrial Environment: Physiological Responses to Water Deprivation of Mothers and Embryos of the Tree-Climbing Crab *Aratus pisonii*." *Journal of Experimental Marine Biology and Ecology* 540: 151547. <https://doi.org/10.1016/j.jembe.2021.151547>.
- Massamba-N'Siala, G., D. Prevedelli, and R. Simonini. 2014. "Trans-Generational Plasticity in Physiological Thermal Tolerance Is Modulated by Maternal Pre-Reproductive Environment in the Polychaete *Ophryotrocha labronica*." *Journal of Experimental Biology* 217, no. 11: 2004–2012. <https://doi.org/10.1242/jeb.094474>.
- Merrill, L., and P. M. Collins. 2015. "Environment-Specific and Sex-Specific Allocation Strategies Among Gonadal, Somatic, and Immune Indices in a Marine Fish." *Canadian Journal of Zoology* 93, no. 3: 207–212. <https://doi.org/10.1139/cjz-2014-0281>.
- Mills, A., and J. D. Fish. 1980. "Effects of Salinity and Temperature on *Corophium volutator* and *C. arenarium* (Crustacea: Amphipoda), With Particular Reference to Distribution." *Marine Biology* 58, no. 2: 153–161. <https://doi.org/10.1007/BF00396127>.
- Missionário, M., J. F. Fernandes, M. Travesso, E. Freitas, R. Calado, and D. Madeira. 2022. "Sex-Specific Thermal Tolerance Limits in the Ditch Shrimp *Palaemon varians*: Eco-Evolutionary Implications Under a Warming Ocean." *Journal of Thermal Biology* 103: 103151. <https://doi.org/10.1016/j.jtherbio.2021.103151>.
- Monteiro, M., N. Vaz, S. M. Cotrim, R. Freitas, U. M. Azeiteiro, and J. M. Dias. 2025. "Trends and Variability of Marine Heatwaves in Portuguese Coastal Waters." *Science of the Total Environment* 973: 179161. <https://doi.org/10.1016/j.scitotenv.2025.179161>.
- Nelson, K. 1990. "Scheduling of Reproduction in Relation to Molting and Growth in Malacostracan Crustaceans." In *Crustacean Egg Production*, edited by A. Kuris. CRC Press.
- Neuparth, T., F. O. Costa, and M. H. Costa. 2002. "Effects of Temperature and Salinity on Life History of the Marine Amphipod *Gammarus locusta*. Implications for Ecotoxicological Testing." *Ecotoxicology* 11: 61–73. <https://doi.org/10.1023/A:1013797130740>.
- Oksanen, J., F. G. Blanchet, M. Friendly, et al. 2020. "Vegan: Community Ecology Package. R package version 2.5-6." <https://CRAN.R-project.org/package=vegan>.
- Orr, J. C. 2011. "Recent and Future Changes in Ocean Carbonate Chemistry." In *Ocean Acidification*, edited by J.-P. Gattuso and L. Hansson. Oxford University Press. <https://doi.org/10.1093/oso/9780199591091.003.0008>.
- Pang, Z., J. Chong, G. Zhou, et al. 2021. "MetaboAnalyst 5.0: Narrowing the Gap Between Raw Spectra and Functional Insights." *Nucleic Acids Research* 49, no. W1: W388–W396. <https://doi.org/10.1093/nar/gkab382>.
- Pang, Z., Y. Lu, G. Zhou, et al. 2024. "MetaboAnalyst 6.0: Towards a Unified Platform for Metabolomics Data Processing, Analysis and Interpretation." *Nucleic Acids Research* 52, no. W1: W398–W406. <https://doi.org/10.1093/nar/gkac253>.
- Pereira, B. P., S. Neff, F. O. Borges, et al. 2024. "Transgenerational Exposure to Deoxygenation and Warming Disrupts Mate Detection in *Gammarus locusta*." *Behavioral Ecology* 35, no. 1: arad102. <https://doi.org/10.1093/beheco/arad102>.
- Pérez-Cerezales, S., P. Ramos-Ibeas, D. Rizo, P. Lonergan, P. Bermejo-Alvarez, and A. Gutiérrez-Adán. 2018. "Early Sex-Dependent Differences in Response to Environmental Stress." *Reproduction* 155, no. 1: R39–R51. <https://doi.org/10.1530/REP-17-0466>.
- Picart-Armada, S., F. Fernández-Albert, M. Vinaixa, O. Yanes, and A. Perera-Lluna. 2018. "FELLA: An R Package to Enrich Metabolomics Data." *BMC Bioinformatics* 19: 538. <https://doi.org/10.1186/s12859-018-2487-5>.
- Pottier, P., S. Burke, S. M. Drobnik, M. Lagisz, and S. Nakagawa. 2021. "Sexual (In)equality? A Meta-Analysis of Sex Differences in Thermal Acclimation Capacity Across Ectotherms." *Functional Ecology* 35, no. 12: 2663–2678. <https://doi.org/10.1111/1365-2435.13899>.
- R Core Team. 2024. *R: A Language and Environment for Statistical Computing*. <https://www.R-project.org/>.
- Raby, G. D., R. Morgan, A. H. Andreassen, et al. 2025. "Measuring Critical Thermal Maximum in Aquatic Ectotherms: A Practical Guide." *Methods in Ecology and Evolution* 16: 2208–2228. <https://doi.org/10.1111/2041-210X.70103>.
- Raistrick, S. P. S., and N. M. Whiteley. 2013. "Influence of Natural Thermal Gradients on Whole Animal Rates of Protein Synthesis in Marine Gammarid Amphipods." *PLoS One* 8: e60050. <https://doi.org/10.1371/journal.pone.0060050>.
- Raymond, J.-F., J. H. Himmelman, and H. E. Guderley. 2004. "Sex Differences in Biochemical Composition, Energy Content and Allocation to Reproductive Effort in the Brooding Sea Star *Leptasterias polaris*." *Marine Ecology Progress Series* 283: 179–190. <https://doi.org/10.3354/meps283179>.
- Ritchie, M. E., B. Phipson, D. Wu, et al. 2015. "Limma Powers Differential Expression Analyses for RNA-Sequencing and Microarray Studies." *Nucleic Acids Research* 43: e47. <https://doi.org/10.1093/nar/gkv007>.
- Rohart, F., B. Gautier, A. Singh, and K.-A. L. Cao. 2017. "mixOmics: An R Package for 'Omics Feature Selection and Multiple Data Integration." *PLoS Computational Biology* 13, no. 11: e1005752. <https://doi.org/10.1371/journal.pcbi.1005752>.
- Rotimi, D. E., M. Iyobhebe, E. T. Oluwayemi, et al. 2024. "Energy Metabolism and Spermatogenesis." *Heliyon* 10, no. 19: e38591. <https://doi.org/10.1016/j.heliyon.2024.e38591>.
- Saibi, W., K. Feki, I. Yacoubi, and F. Brini. 2015. "Bridging Between Proline Structure, Functions, Metabolism, and Involvement in Organism Physiology." *Applied Biochemistry and Biotechnology* 176, no. 8: 2107–2119. <https://doi.org/10.1007/s12010-015-1713-0>.
- Sandblom, E., T. D. Clark, A. Gräns, et al. 2016. "Physiological Constraints to Climate Warming in Fish Follow Principles of Plastic Floors and Concrete Ceilings." *Nature Communications* 7: 11447. <https://doi.org/10.1038/ncomms11447>.

- Sasaki, M., S. Hedberg, K. Richardson, and H. G. Dam. 2019. "Complex Interactions Between Local Adaptation, Phenotypic Plasticity and Sex Affect Vulnerability to Warming in a Widespread Marine Copepod." *Royal Society Open Science* 6, no. 3: 182115. <https://doi.org/10.1098/rsos.182115>.
- Schulte, P. M., T. M. Healy, and N. A. Fanguie. 2011. "Thermal Performance Curves, Phenotypic Plasticity, and the Time Scales of Temperature Exposure." *Integrative and Comparative Biology* 51: 691–702. <https://doi.org/10.1093/icb/ict097>.
- Shannon, P., A. Markiel, O. Ozier, et al. 2003. "Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks." *Genome Research* 13: 2498–2504. <https://doi.org/10.1101/gr.1239303>.
- Shansky, R. M., and A. Z. Murphy. 2021. "Considering Sex as a Biological Variable Will Require a Global Shift in Science Culture." *Nature Neuroscience* 24, no. 4: 457–464. <https://doi.org/10.1038/s41593-021-00806-8>.
- Shatilina, Z. M., H. Wolfgang Riss, M. V. Protopopova, et al. 2011. "The Role of the Heat Shock Proteins (HSP70 and sHSP) in the Thermotolerance of Freshwater Amphipods From Contrasting Habitats." *Journal of Thermal Biology* 36, no. 2: 142–149. <https://doi.org/10.1016/j.jtherbio.2010.12.008>.
- Shepherd, J. G., P. G. Brewer, A. Oschlies, and A. J. Watson. 2017. "Ocean Ventilation and Deoxygenation in a Warming World: Introduction and Overview." *Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences* 375, no. 2102: 20170240. <https://doi.org/10.1098/rsta.2017.0240>.
- Smith, K. E., M. T. Burrows, A. J. Hobday, et al. 2023. "Biological Impacts of Marine Heatwaves." *Annual Review of Marine Science* 15: 119–145. <https://doi.org/10.1146/annurev-marine-032122-121437>.
- Sokolova, I. M., A. A. Sukhotin, and G. Lannig. 2011. "Stress Effects on Metabolism and Energy Budgets in Mollusks." In *Oxidative Stress in Aquatic Ecosystems*, edited by D. Abele, J.P. Vázquez-Medina, T. Zenteno-Savín, 261–280. John Wiley & Sons, Ltd. <https://doi.org/10.1002/9781444345988.ch19>.
- Spinks, R. K., L. C. Bonzi, T. Ravasi, P. L. Munday, and J. M. Donelson. 2021. "Sex- and Time-Specific Parental Effects of Warming on Reproduction and Offspring Quality in a Coral Reef Fish." *Evolutionary Applications* 14, no. 4: 1145–1158. <https://doi.org/10.1111/eva.13187>.
- Stillman, J. H. 2002. "Causes and Consequences of Thermal Tolerance Limits in Rocky Intertidal Porcelain Crabs, Genus *Petrolisthes*." *Integrative and Comparative Biology* 42: 790–796. <https://doi.org/10.1093/icb/42.4.790>.
- Sunday, J. M., A. E. Bates, M. R. Kearney, et al. 2014. "Thermal-Safety Margins and the Necessity of Thermoregulatory Behavior Across Latitude and Elevation." *Proceedings of the National Academy of Sciences* 111, no. 15: 5610–5615. <https://doi.org/10.1073/pnas.1316145111>.
- Tarka, M., A. Guenther, P. T. Niemelä, S. Nakagawa, and D. W. A. Noble. 2018. "Sex Differences in Life History, Behavior, and Physiology Along a Slow-Fast Continuum: A Meta-Analysis." *Behavioral Ecology and Sociobiology* 72, no. 8: 132. <https://doi.org/10.1007/s00265-018-2534-2>.
- Thomas, C., and R. Tampé. 2020. "Structural and Mechanistic Principles of ABC Transporters." *Annual Review of Biochemistry* 89: 605–636. <https://doi.org/10.1146/annurev-biochem-011520-105201>.
- Tsoi, K. H., K. M. Chiu, and K. H. Chu. 2005. "Effects of Temperature and Salinity on Survival and Growth of the Amphipod *Hyale crassicornis* (Gammaridea, Hyalidae)." *Journal of Natural History* 39, no. 4: 325–336. <https://doi.org/10.1080/0022293042000200077>.
- Valencak, T. G., A. Osterrieder, and T. J. Schulz. 2017. "Sex Matters: The Effects of Biological Sex on Adipose Tissue Biology and Energy Metabolism." *Redox Biology* 12: 806–813. <https://doi.org/10.1016/j.redox.2017.04.012>.
- van den Berg, R. A., H. C. Hoefsloot, J. A. Westerhuis, A. K. Smilde, and M. J. van der Werf. 2006. "Centering, Scaling, and Transformations: Improving the Biological Information Content of Metabolomics Data." *BMC Genomics* 7, no. 1: 142. <https://doi.org/10.1186/1471-2164-7-142>.
- Vaz, N., J. M. Dias, P. Leitão, and I. Martins. 2005. "Horizontal Patterns of Water Temperature and Salinity in an Estuarine Tidal Channel: Ria de Aveiro." *Ocean Dynamics* 55, no. 5: 416–429. <https://doi.org/10.1007/s10236-005-0015-4>.
- Venegas, R. M., J. Acevedo, and E. A. Trembl. 2023. "Three Decades of Ocean Warming Impacts on Marine Ecosystems: A Review and Perspective." *Deep Sea Research Part II: Topical Studies in Oceanography* 212: 105318. <https://doi.org/10.1016/j.dsr2.2023.105318>.
- Verberk, W. C. E. P., U. Sommer, R. L. Davidson, and M. R. Viant. 2013. "Anaerobic Metabolism at Thermal Extremes: A Metabolomic Test of the Oxygen Limitation Hypothesis in an Aquatic Insect." *Integrative and Comparative Biology* 53, no. 4: 609–619. <https://doi.org/10.1093/icb/ict015>.
- Vermandele, F., E. Roy, M. Sasaki, et al. 2025. "You Are on Your Own, Kid: Parental Effects Impair a Ubiquitous Copepod's Ability to Recover From Extreme Events." *Marine Environmental Research* 210: 107286. <https://doi.org/10.1016/j.marenvres.2025.107286>.
- Vermandele, F., M. Sasaki, G. Winkler, H. G. Dam, D. Madeira, and P. Calosi. 2024. "When the Going Gets Tough, the Females Get Going: Sex-Specific Physiological Responses to Simultaneous Exposure to Hypoxia and Marine Heatwave Events in a Ubiquitous Copepod." *Global Change Biology* 30, no. 10: e17553. <https://doi.org/10.1111/gcb.17553>.
- Villeneuve, A. R., and E. R. White. 2024. "Predicting Organismal Response to Marine Heatwaves Using Dynamic Thermal Tolerance Landscape Models." *Journal of Animal Ecology* 94, no. 8: 1515–1527. <https://doi.org/10.1111/1365-2656.14120>.
- Vinagre, C., and C. L. Gastón. 2024. "Short Food Chains, Highly Diverse and Complex Food Web Networks in Coastal Lagoons." *Food Webs* 38: e00341. <https://doi.org/10.1016/j.fooweb.2024.e00341>.
- Vinagre, C., I. Leal, V. Mendonça, et al. 2016. "Vulnerability to Climate Warming and Acclimation Capacity of Tropical and Temperate Coastal Organisms." *Ecological Indicators* 62: 317–327. <https://doi.org/10.1016/j.ecolind.2015.11.010>.
- Vinagre, C., D. Madeira, V. Mendonça, M. Dias, J. Roma, and M. S. Diniz. 2014. "Effect of Temperature in Multiple Biomarkers of Oxidative Stress in Coastal Shrimp." *Journal of Thermal Biology* 41: 38–42. <https://doi.org/10.1016/j.jtherbio.2014.02.005>.
- Wangensteen, O. S., X. Turon, and C. Palacín. 2016. "Reproductive Strategies in Marine Invertebrates and the Structuring of Marine Animal Forests." In *Marine Animal Forests: The Ecology of Benthic Biodiversity Hotspots*, edited by S. Rossi, L. Bramanti, A. Gori, C. Orejas, 1–24. Springer. https://doi.org/10.1007/978-3-319-17001-5_52-1.
- Weaving, H., J. S. Terblanche, P. Pottier, and S. English. 2022. "Meta-Analysis Reveals Weak but Pervasive Plasticity in Insect Thermal Limits." *Nature Communications* 13, no. 1: 5292. <https://doi.org/10.1038/s41467-022-32953-2>.
- Wei, R., J. Wang, M. Su, et al. 2018. "Missing Value Imputation Approach for Mass Spectrometry-Based Metabolomics Data." *Scientific Reports* 8, no. 1: 663. <https://doi.org/10.1038/s41598-017-19120-0>.
- Whiteley, N. M., and C. L. Mackenzie. 2016. "Physiological Responses of Marine Invertebrates to Thermal Stress." In *Stressors in the Marine Environment: Physiological and Ecological Responses; Societal Implications*, edited by M. Solan and N. Whiteley. Oxford University Press. <https://doi.org/10.1093/acprof:oso/9780198718826.003.0004>.
- Xue, S., Y. Mao, J. Li, L. Zhu, J. Fang, and F. Zhao. 2018. "Life History Responses to Variations in Temperature by the Marine Amphipod *Eogammarus posseticus* (Gammaridae) and Their Implications for Productivity in Aquaculture." *Hydrobiologia* 814, no. 1: 133–145. <https://doi.org/10.1007/s10750-018-3524-0>.

Zarou, M. M., A. Vazquez, and G. Vignir Helgason. 2021. "Folate Metabolism: A Re-Emerging Therapeutic Target in Haematological Cancers." *Leukemia* 35, no. 6: 1539–1551. <https://doi.org/10.1038/s41375-021-01189-2>.

Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** (A) Map of Ria de Aveiro with the sampling site (highlighted with a black dot and written in blue) in the Mira channel (Costa Nova, Ria de Aveiro, Portugal—40°36' 59.3" N 8°44' 25.3" W) and (B) Water temperature recorded at the sampling site between May 2022 and October 2022: temperatures were recorded every 30 min with EnvLoggers. **Figure S2:** (A) Acclimation capacity calculated as the difference between individual CTmax values at each acclimation temperature (21°C, 26°C and 31°C) and the mean CTmax at 16°C, shown for females and males. Different letters indicate significant differences among temperatures within each sex (uppercase for females and lowercase for males); asterisks indicate significant differences between sexes and (B) Coefficient of variation (CV %) of CTmax across acclimation temperatures for females and males. **Figure S3:** Principal Component Analyses (PCA) plots displaying the PC1 and PC2 of the targeted metabolome (47 metabolites) of *Gammarus locusta* comparing (A) tested temperatures (16°C, 21°C, 26°C, and 31°C) and the (B) females and males. Points represent individuals. Ellipses show 95% confidence intervals. Variance contribution is given on the axes. **Figure S4:** Log₂-transformed mean abundances of metabolites that are differentially accumulated (according to Limma tests) in *G. locusta* adults between females and males after 21 days of exposure in all temperatures. Dots in the boxplots represent the individual data. **Figure S5:** Log₂-transformed mean abundances of metabolites that are differentially accumulated (according to Limma tests) in *G. locusta* adults between (A) 16°C and 26°C, and (B) 16°C and 31°C after 21 days of exposure. Dots in the boxplots represent the individual data. **Figure S6:** Log₂-transformed mean abundances of metabolites that are differentially accumulated (according to Limma tests) in *G. locusta* adults between females and males at: (A) 16°C, (B) 21°C, and (C) 26°C after 21 days of exposure. Dots in the boxplots represent the individual data. **Table S1:** List of all 49 metabolites analysed in this study, including their compound identifiers, chemical classification, and associated biological pathways. For each metabolite, we provide the Metabolite ID, chemical taxonomy based on classification in the Human Metabolome Database (HMDB), corresponding KEGG and HMDB accession numbers, and the primary biological pathways in which the metabolite is involved. **Table S2:** Summary of precursor ions, product ions, retention time, and QQQ parameters for each compound in positive mode (left) and negative mode (right). These tables were provided by the analytical facility Laboratoires Iso-BioKem (Rimouski, QC, Canada). For some compounds, multiple ion transitions (e.g., quantifier and qualifier ions) are listed to enhance detection confidence and accuracy; however, each metabolite was only counted once in subsequent data analyses. **Table S3:** List of stock metabolites manufacturers and corresponding KEGG identifiers for positive analysis (left) and negative analysis (right). **Table S4:** Summary of CTmax (Critical Thermal Maximum) values (minimum, maximum and mean ± SD), CV (Coefficient of Variation), TSM (Thermal Safety Margin), where TSM is the difference between CTMax mean from each treatment and MHT (Maximum Habitat Temperature), Acclimation capacity (CTMax treatment—CTMax optimum temperature—16°C), ARR (Acclimation Response Ratio) for temperatures tested in females and males of the amphipod *Gammarus locusta*. **Table S5:** Summary of the area under the curve (AUC) and associated *p*-values resulting from sparse partial least square discriminant analyses (sPLS-DA) AUROC curves for the sPLS-DA represented in the main manuscript (Figure 4). Component 1 and 2 refer to the first and second latent variables extracted by the sPLS-DA model, which are linear combinations of metabolites that maximise group separation. The AUROC values indicate the discriminatory ability of each component individually for the classification of treatment or sex groups. Statistical significance was assessed using permutation tests, with *p*-values <0.05 considered significant. **Table S6:** List of differentially abundant metabolites (DAMs) (*padj.* <0.05 and 0.1)

between sex and among all temperatures in *G. locusta* resulting from a Limma analyses. **Table S7:** List of differentially abundant metabolites (DAMs) (*padj.* <0.05 and 0.1) between sex within temperature in *G. locusta* resulting from a Limma analyses.