Interplay of diet, heat stress, and the microbiome shapes health and escape behavior in amphibian larvae

Paula C. Eterovick[1,5], Julian Glos[2], Franziska Burkart[1], Jörg Overmann[1,3], Katharina Ruthsatz[4,5]

[1]*Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH, Inhoffenstraße 7B, 38124 Braunschweig, Germany*

[2]*Institute of Cell and System Biology, Universität Hamburg, Martin-Luther-King-Platz 3, 20146, Hamburg, Germany*

[3] *Present address: SNSB – Staatliche Naturwissenschaftliche Sammlungen Bayerns, Menzinger Str. 71, 80638 München, Germany, and Chair of Molecular Biodiversity Research, Ludwig-Maximilians-Universität München*

[4]*Ecology, Evolution, and Development Group, Department of Wetland Ecology, Doñana Biological Station, CSIC, C. Américo Vespucio 26, 41092 Seville, Spain*

[5]*Zoological Institute, Technische Universität Braunschweig, Mendelssohnstraße 4, 38106 Braunschweig, Germany*

Running title: Diet and heat shape tadpole microbiomes

Corresponding author: Paula C. Eterovick, paula.cabral.eterovick@dsmz.de

---

Data availability statement

Raw data and R scripts are deposited in FigShare (https://doi.org/10.6084/m9.figshare.29447390). Raw sequences are deposited in the NCBI (BioProject PRJNA1304763).

Acknowledgements

We are thankful to Miguel Vences, Sven Gippner, and Janina Rudolph for field assistance, Maileen Weidner, Maline Türk, Fabian Bartels, and Ben Oetken for help with animal husbandry, and Christoph Reisdorff for support in the isotope analyses conducted in his laboratory. We also thank Frank Suhling for identification of the dragonfly naiads, Selma Vieira and Johannes Sikorski for helpful advice during bioinformatic analyses, and Robin Schmidt for providing the picture of the mating pair of *Rana temporaria* used in Fig. 1. We especially thank Richard Wassersug for enlightening conversations about amphibian larvae behavior and its analysis. We acknowledge financial support from the Open Access Publication Fund of the Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH.

Funding

DNA analyses were supported by the Deutsche Forschungsgemeinschaft (DFG; GZ: CA 3427/2-1, project number: 546565602 granted to PCE). Experimental work at the Technical University of Braunschweig was funded by the DFG (Project number: 459850971; granted to KR). KR was supported by Marie-Curie Actions (Grant Number: 101151070-AMPHISTRESS).

Conflict of interest statement

Not applicable.

Ethics statement

Permits for the experiments were obtained from the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Germany (Gz. 33.19-42502-04-20/3590 and 33.19-42502-04-22-00274). Fieldwork was carried out with permits from the Stadt Braunschweig (Stadt Braunschweig - Fachbereich Umwelt und Naturschutz, Willy-Brandt-Platz 13, 38102 Braunschweig; Gz. 68.11-11.8-3.3).

Author contributions

P.C.E. and K.R. were responsible for funding acquisition, conceptualization, methodology, investigation, data curation, project administration, writing —review and editing; P.C.E. was also responsible for formal analysis, validation, visualization, writing—original draft. J. G., F. B. and J. O. contributed to investigation. All authors participated in writing —review and editing.

Abstract

What animals eat modulates their microbiome and is fundamental to their health. Microbiomes can improve hosts' ability to cope with environmental stressors, including increased temperatures and altered food quantity and quality associated with climate change. Using a multifactorial experimental design, we tested whether three diets with increasing amounts of protein, fat, and components of animal origin (designated low-, intermediate-, and high-quality), two rearing temperatures (18 °C or 24.5 °C), and exposure or not to a heatwave (28 °C for 48 h) shaped the gut bacterial community of amphibian larvae (*Rana temporaria*). We then examined how the treatments, associated shifts in gut bacterial communities, and predicted metabolic pathways related to larvae nutrient assimilation (isotopic signatures), health (body condition and developmental rate), and escape behavior. Larvae maintained their body condition and developed faster at 24.5 °C, with higher diet quality (i.e., reduced herbivory) further accelerating development at this temperature. The intermediate-quality diet reduced the ability of larvae to react to an aversive stimulus at 24.5 °C, but this effect did not occur in larvae exposed to the heatwave. The heatwave may have triggered an increase in the abundance of *Klebsiella*, together with a predicted increase in the myo-inositol degradation pathway, which influences cell membrane fluidity and signaling and may increase attention levels. Similar outcomes in host performance under most experimental conditions highlight the potential plasticity of the bacterial community and the presence of alternative enterotypes with functionally redundant metabolic capacities compatible with host health.

Key-words: Food quality, thermal stress, bacteria, escape behavior, developmental plasticity, behavioral plasticity, gut-brain-axis, *Rana temporaria*

Introduction

What animals eat shapes their available energy, growth, and development, ultimately affecting their likelihood of survival (e.g., Kupferberg, 1997; Wang et al., 2015; Llobat and Marín-García, 2022). Beyond its nutritional value, food intake also influences the microbiome - the diverse community of microorganisms (Archaea, Bacteria, Fungi, Protists, Viruses), their metabolites, and interactions (Berg et al., 2020) - that inhabit animal guts (Tuddernham and Sears, 2015) and contribute critically to nutrient assimilation and host health (McFall-Ngai et al., 2013). Animals and their mutualistic or commensal microbial partners have likely been co-evolving since the origin of the animal kingdom (McFall-Ngai et al., 2013). This long-standing association expanded the metabolic potential of animals, enabling the use of otherwise inaccessible food resources and tightly linking host and microbial genomes (McFall-Ngai et al., 2013). The gut microbiome supports digestion and the assimilation of end products by host cells (Perry et al., 2020) and may further influence the host's ability to cope with environmental stress by regulating specific metabolic pathways (Fontaine and Kohl, 2023). Because microbiomes respond more rapidly to changing conditions than host genomes, they act as key mediators of animal resilience to environmental stress (Voolstra and Ziegler, 2020). On the other hand, microbial communities may also respond to environmental conditions in a way that compromises host health (Douglas and Werren, 2016), what is usually associated to stressful conditions for their hosts (e.g., Fontaine and Kohl, 2023; Guo et al., 2024). Thus, it is important to keep in mind that microbial communities have their own dynamics with varying consequences for host health, and certain environmental conditions can lead to unbalance of host-microbiome interactions or dysbiosis (Zaneveld et al., 2017).

Human activities and resulting climate change have created a world in which wildlife faces multiple stressors that compromise individual health, drive population declines, and can ultimately trigger species extinctions (Ruddiman, 2013; McCallum, 2015). Climate change encompasses not only increasing mean temperatures but also transient temperature extremes, altered precipitation patterns, droughts, and shifts in food webs, food quality, and food availability (IPCC, 2023; Hardison and Eliason, 2024). Animals are exposed to both prolonged elevated temperatures and short-term heatwaves, with responses varying according to the intensity and duration of thermal stress (Carreira et al., 2016; Staniek et al., 2025; Xiao and Wang, 2025). The increasing occurrence of heatwaves in Europe, Asia, and Australia (IPCC, 2023) highlights their likely importance for the fate of species under climate change.

Emerging evidence suggests that gut microbes play a role in mediating heat tolerance in ectotherms. Conversely, impoverished microbiomes may reduce ectotherm tolerance to thermal extremes (Fontaine et al., 2022; Fontaine and Kohl, 2023). Shifts in microbiome composition can modify host thermal resilience by influencing metabolic pathways, oxidative stress resistance, and energy balance. For example, pathways linked to amino acid metabolism - often enriched in hosts with diverse microbiomes - may allow hosts to use bacteria-derived amino acids as additional energy sources during thermal stress (Fontaine and Kohl, 2023).

Even species with comparatively high warming tolerance may experience costs at higher temperatures (Duarte et al., 2012). Temperature changes can alter predator–prey dynamics (Seifert et al., 2014), affect key physiological processes, and influence nutrient assimilation

(Croll and Watts, 2004). This can lead to shifts in foraging behavior and food preferences (Carreira et al., 2016) and ultimately influence food webs (Seifert et al., 2014). For example, omnivorous amphibian larvae increase their consumption of plant material relative to animal food at higher temperatures, improving growth and performance (Carreira et al., 2016). In crayfish, increased temperatures reduce protein absorption but increase soluble carbohydrate absorption (Croll and Watts, 2004), helping explain reduced consumption of animal-based foods at high temperatures. Thus, diet preferences respond to temperature (Behrens and Lafferty, 2007; Devries and Appel, 2014; Carreira et al., 2016), while the microbiome responds to diet (Tuddernham and Sears, 2015) and can itself influence food intake and behavior (Miri et al., 2023). Food quality and availability, as well as the abundance of key microbial groups, are influenced by the same environmental stressors that affect host survival and recruitment, making these interactions important determinants of species success or failure (e.g., Manning and Sullivan, 2021; Yan et al., 2024; Videvall et al., 2023). Yet, the combined effects of diet and temperature on the microbiome remain poorly understood (Hardison and Eliason, 2024).

Behavioral changes influenced by the microbiome extend beyond shifts in foraging behavior (Wong et al., 2015; Miri et al., 2023). Gut microbes produce and regulate numerous neuroactive substances - hormones, neuropeptides, neurotransmitters, and many metabolites that affect host metabolic pathways (Lynch & Hsiao, 2019). These microbial compounds influence neuronal signaling and neural development (Bercik et al., 2012) and include enzymes that synthesize key neuroactive molecules involved in behavioral regulation (Dinan et al., 2015; Chen et al., 2013). This modulation is coordinated through the gut-brain axis - a bidirectional network operating through neural (especially via the vagus nerve), endocrine, and immune pathways (Miri et al., 2023; Silva et al., 2020). Short-chain fatty acids (SCFAs) exemplify influential microbial metabolites that maintain gut integrity, modulate immune and endocrine function, and cross the blood-brain barrier to affect neurotransmission, neurotrophic factors, and microglial activity (Silva et al., 2020).

Much research on microbiome-driven behavior has focused on humans or mice as model organisms (Sampson and Mazmanian, 2015), yet understanding the microbiome’s role in wildlife evolution and survival is urgently needed (Hird, 2017). In house sparrows, microbiome diversity correlates with exploratory behavior, which in turn promotes greater microbiome diversity (Florkowski and Yorzinski, 2023). The microbiome also influences mate choice and social behavior, with implications for individual fitness and evolutionary success (Sharon et al., 2010; Archie and Theis, 2011). Studies on microbiome-ectotherm interactions are especially important given the sensitivity of ectotherms to climate change and the potential role of their microbiome in mitigating associated stressors (Fontaine and Kohl, 2023).

Among ectotherms, amphibians are particularly vulnerable to climate change and other stressors (Collins and Storfer, 2003; Hayes et al., 2010; Luedtke et al., 2023), making them the most threatened vertebrate group globally (Wake & Vredenburg, 2008; Borzée et al., 2025). They are therefore valuable model organisms for studying interactions among climate change, diet, microbiome, and behavior. Amphibian diet shapes larval growth and development (Kupferberg, 1997; Carreira et al., 2016; Ruthsatz et al., 2019), while the microbiome affects larval thermal stress tolerance (Fontaine and Kohl, 2023). Altered foraging behavior may reduce thermal stress impacts (Carreira et al., 2016), yet amphibian larvae often exhibit lower thermal tolerance than their predators, potentially increasing their vulnerability to predation (Bastiani, 2023). For instance, larvae of the treefrog *Pithecopus rusticus* showed reduced thermal acclimation capacity and thermal tolerance compared to a co-occurring dragonfly predator, losing locomotor capacity at temperatures at which predators remained active (Bastiani, 2023). Because predation is a major source of mortality during larval development (McDiarmid and Altig, 1999; Wells, 2019), the ability to avoid predators is essential for survival. Predator avoidance behavior depends on both immobility in response to predator cues (Relyea, 2001; Preston and Forstner, 2015; Eterovick et al., 2020) and rapid escape responses once detected (Hébert et al., 2019). Diet can influence this behavior: nutrient-rich diets enhance growth and escape performance (Kloh et al., 2024), whereas ingestion of toxic cyanobacteria impairs locomotor performance (Moura et al., 2023). Low-quality diets may therefore compromise escape responses, increasing predation risk.

Here, we investigated the interconnected and potentially synergistic effects of diet, temperature, and the gut microbiome on the health and behavior of larvae of the European Common Frog (*Rana temporaria*), an ectothermic model organism. Using a multifactorial experimental design, we tested whether three diets differing in amounts of protein, fat, and animal-derived components (low-, intermediate-, and high-quality), two rearing temperatures (18 °C and 24.5 °C), and exposure to a heatwave (28 °C for 48 h) shaped the gut bacterial communities of *R. temporaria* larvae. We then linked these experimental conditions - and the resulting bacterial communities - to food assimilation (isotopic signatures), health biomarkers (body condition and developmental rate), and behavior, focusing on escape responses to an aversive stimulus as a proxy for predator avoidance.

We tested three hypotheses: (1) diet quality, sustained elevated rearing temperature, and/or transient heatwaves affect gut bacterial diversity and composition; (2) diet, temperature treatments, and/or altered gut bacterial communities influence larvae’s carbon and nitrogen isotopic signatures and affect health biomarkers; and (3) diet, temperature treatments, and/or altered gut bacterial communities lead to differences in behavioral responses to a simulated predator attack. Finally, we predicted metabolic pathways enriched in bacteria that increased in abundance under each treatment to identify potential links between microbial activity and amphibian larval performance.

Materials and methods

Experimental design

Five egg clutches of the European Common Frog (*Rana temporaria*) were collected on 25 March 2023 in the Kleiwiesen (52.328°N, 10.582°E; Braunschweig, Lower Saxony, Germany) and transported to the Zoological Institute of the Technische Universität Braunschweig. When hatched larvae reached developmental stage 25 (*sensu* Gosner, 1960) they were distributed among three food treatments and two controlled-temperature rearing environments (4 larvae per clutch × 5 clutches × 3 food treatments × 2 rearing temperatures = 120 larvae; Fig. 1).

The food treatments were prepared using soluble powdered foods that differed in protein and fat content, as well as in the diversity of nutrient sources. The diet with the lowest protein and fat levels and the lowest diversity of components (hereafter “low-quality”) consisted of an organic grass powder (NaturaleBio®; *Hordeum vulgare*) containing 3% lipid, 11% carbohydrate, and 32% protein. The diet with the highest protein and fat content and the greatest diversity of components (hereafter “high-quality”) was Sera Micron Nature® fish food, which contains 7.2% lipid, 10.3% carbohydrate, and 56.6% protein. The intermediate diet (“intermediate-quality”) was a thoroughly blended 1:1 mixture of the powders used for the low- and high-quality diets.

The energy content of each diet was determined by bomb calorimetry (6200 Isoperobol Calorimeter, Parr Instruments, Moline, Illinois) at the laboratory for chemical analyses at the University of Hamburg. Mean (± SD) caloric values were 17.13 ± 0.04 kJ/g ($n = 3$) for the low-quality diet, 18.72 ± 0.03 kJ/g ($n = 3$) for the intermediate-quality diet, and 20.35 ± 0.06 kJ/g ($n = 4$) for the high-quality diet.

The lower temperature (18 °C) represented typical ambient conditions during *R. temporaria* larval development. The higher temperature (24.5 °C) was chosen to fall within the range of 22-26 °C, in which *R. temporaria* larvae exhibit elevated stress levels but can still maintain body condition, likely supported by adjustments in their gut bacterial communities (Eterovick et al., 2024).

When larvae reached developmental stages 34–37 (pre-metamorphic stages; digit development in the hind limbs; *sensu* Gosner 1960), approximately half of the surviving individuals from each treatment were exposed to a heatwave protocol to test the effects of temperature extremes on escape behavior, as well as potential interactions with diet quality and rearing temperature (Fig. 1). Larvae were kept at 28 °C for 48 h, after which temperature was decreased at the same rate back to the original rearing temperature. Larvae remained in their individual buckets throughout the procedure. Additional details on animal husbandry and experimental setup are available in the supplementary material.

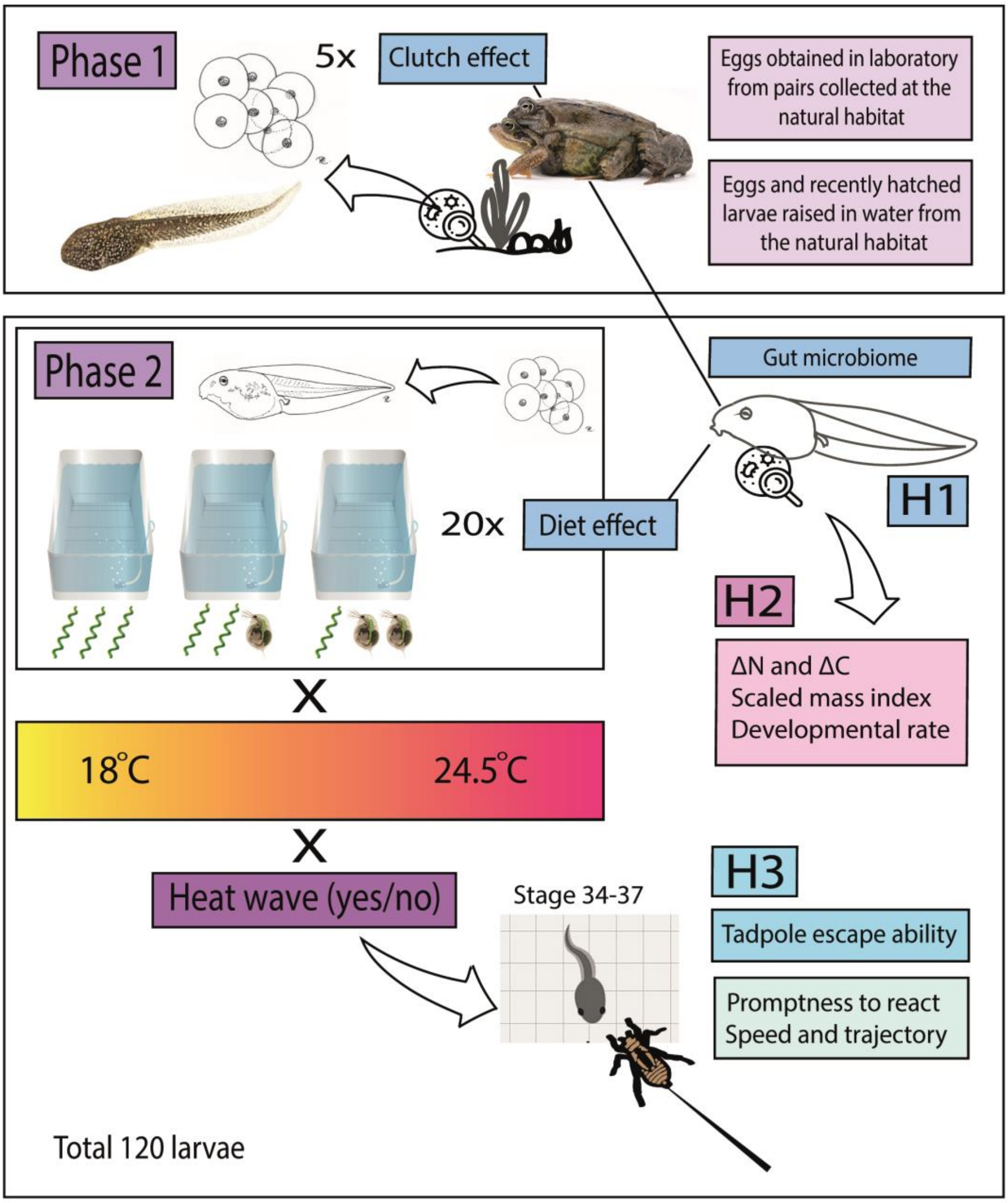

Fig. 1. Graphical summary of the experimental design representing acquisition of offspring (larvae) from five different egg clutches from *Rana temporaria* and the experiment itself. The experiment structure is shown based on three main hypotheses to be tested: whether diet and temperatures experienced during development affect assemblage of gut bacteria (H1), nutrient assimilation and biomarkers (body condition and developmental rate; H2), as well as escape ability of *R. temporaria* larvae (H3).

Behavioral trials

One day after larvae exposed to the heatwave had returned to their original rearing temperature, behavioral trials were conducted with both heatwave and control (non-exposed) larvae. A white plastic tray (35 × 35 cm) was filled with 1.3 L of rested tap water at the rearing temperature of the tested larva (18 °C or 24.5 °C), reaching a water depth of 1 cm. A laminated paper marked with 5 × 5 cm squares was placed at the bottom, and an adjustable metal frame delineated the arena (Fig. 2). The tray was surrounded by white cardboard to shield larvae from the experimenter, and a high-definition webcam (Logitech C920s HD Pro, Logitech, Lausanne, Switzerland) was mounted on a tripod directly above the arena.

Each larva was gently captured from its bucket using a spoon, placed in the central square of the arena, and covered with a glass funnel (Fig. 2A). Handling was minimized and conducted as gently as possible. After a three-minute acclimation period (following Eterovick et al., 2018), the funnel was removed, and a dragonfly naiad (*Libellula*, Libellulidae, Anisoptera) model was presented as a potentially aversive stimulus. The stimulus consisted of a transparent plastic pipette containing 4 mL of water assumed to hold chemical predator cues. This water was obtained from a 500 mL container where ten dragonfly naiads (*Libellula depressa*; returned to their habitat after use) from the same frog habitat had been held for 4 h. Each stock of water was used for two hours after removal of the naiads, with water temperatures matched to the larva's rearing temperature. A life-size predator model, made from non-toxic modeling clay and ink, was attached to the pipette tip.

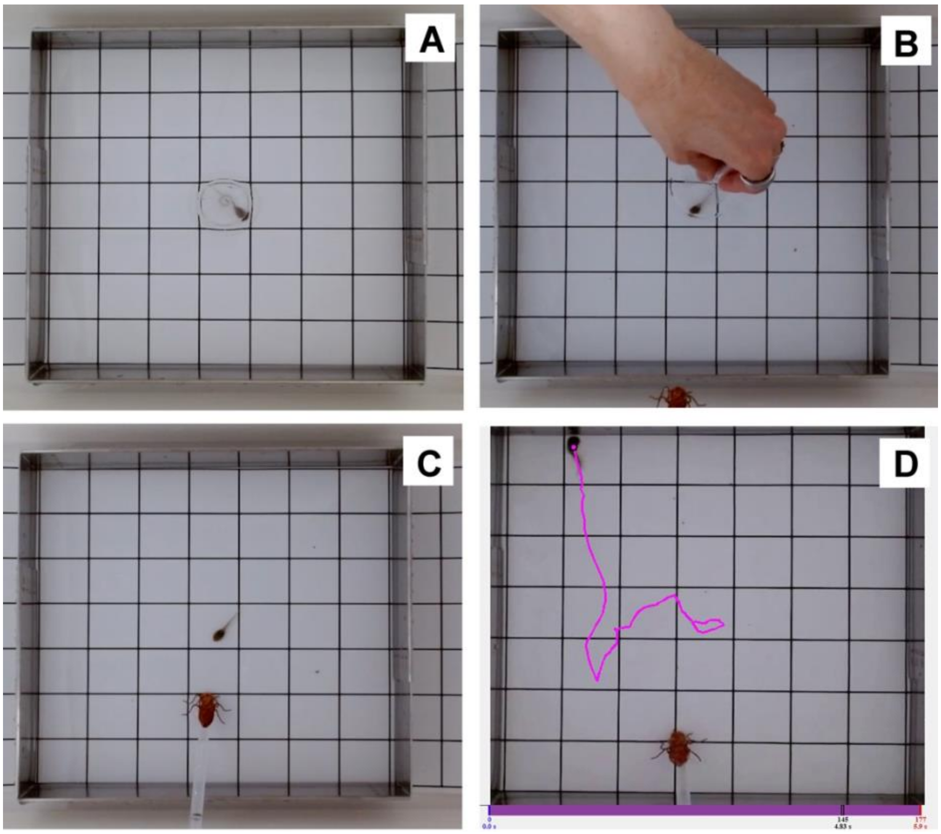

Fig. 2. Experimental setup for behavioral tests. Plastic trays filled up to 1 cm with rested tap water at larvae rearing temperature were lined with a grid of 5 x 5 cm squares. A space of 35 x 35 cm was delimited with a metal frame and the larva to be tested was placed at the central square, where it was retained for 3 minutes under a glass funnel (A). After careful removal of the funnel without disturbing the larva (B), a predator model was approached (C) and the reaction of the larva was filmed to evaluate the escape response (see text for details). Fleeing trajectories of the larva were tracked with the software AnimalTA (Chiara and Kim, 2023; D).

Immediately after funnel removal, the pipette was inserted at ~45° relative to the larva's frontal direction, touching the water two grid squares (10 cm) away. Water containing predator cues was slowly released, and the predator model was gradually moved toward the larva until it elicited an escape response or gently touched it. Because amphibian larvae perceive varied cues from predators (Melo et al., 2021), this combined stimulus was designed to engage visual (model), mechanical (approach and water flow), and chemical (predator exposed water) cues, as the most relevant cue for *R. temporaria* larvae is unknown. Trials ended once the larva attempted to flee or if the model touched the larva without eliciting any escape movement. Video recordings were captured using OBS Studio (Open Broadcaster Software, Version 29.1; https://obsproject.com/). Larvae were tested in random order, blind to their heatwave exposure and rearing conditions.

Videos were analyzed in random order and without treatment information using AnimalTA software (Chiara and Kim, 2023). Occasionally, larvae moved during funnel removal and had to be repositioned in the arena's center. We recorded the "number of attempts" (times a larva was repositioned) as an additional variable, reflecting early movements that could contribute to energy expenditure and stress. To ensure uniformity, we quantified the elapsed time between funnel removal and the trial start (when the predator model contacted the water) and found it to be strongly correlated with the number of repositioning attempts (Spearman's Rs = 0.9, $p < 0.001$; Fig. S1), indicating no significant variation in attempt durations.

Escape behavior was quantified using the following variables: (1) whether the larva reacted (fleeing) or not, with no reaction defined as remaining stationary even when touched by the model; (2) reaction time, measured from the moment the predator model touched the water until the larva's flee response; (3) whether the larva reacted before or after contact with the predator model; (4) average speed; and (5) trajectory linearity ("meander" function, Chiara and Kim, 2023) during fleeing. Variables 2–5 were analyzed only for larvae exhibiting escape responses. Speed and trajectory linearity were measured until the larva stopped or touched a wall, as such a barrier would otherwise bias the metrics.

This behavioral test protocol was used to examine the effects of diet, rearing temperature, and heatwave exposure on larval kinematics. We expected larvae fed higher-quality diets, reared at 18 °C, and not exposed to the heatwave to be more alert and reactive, fleeing earlier and at higher speed. Escape trajectories were expected to be more curved, reflecting the typical anti-predator strategy of anuran larvae, which rely on rapid turns with small radii rather than straight-line swimming (Wassersug, 1989). Simply stated, tadpoles typically escape from predatory attacks by turning away from the approaching predator rather than trying to outrun it (Wassersug, 1989).

Sample collection

Within 12 hours after the behavioral trials, each tadpole was euthanized using 2 g × L^{-1} tricaine methanesulfonate (MS-222; Ethyl 3-aminobenzoate methanesulfonate; Sigma-Aldrich). The developmental stage of each larva was confirmed under a stereomicroscope according to Gosner (1960). Snout-vent length (SVL) was measured to the nearest 0.5 mm using a digital caliper. Larvae were then gently dry-blotted and weighed to the nearest 0.001 g using an electronic balance (Sartorius A200 S, Germany).

A sterile scalpel was used to excise the tail for subsequent isotopic analysis. Using a sterile scalpel and tweezers, the ventral skin was cut to remove the entire gut for bacterial DNA extraction. The tail, gut, and remaining body were placed in three separate tubes, all stored at -80 °C until further analysis.

Isotope analyses

Stable isotope analyses were conducted to assess differences in absorption and incorporation of food components by larvae subjected to different diets, based on isotopic signatures. Analyses were performed at the Biozentrum Klein Flottbek, University of Hamburg, Germany, following the methods of Glos et al. (2020), as detailed in the supplementary material.

Body condition and developmental rate assessment

Body condition was estimated using the scaled mass index (SMI), calculated from the slope of the regression of log-transformed snout-vent length (SVL) and log-transformed body mass (standardized major axis, SMA) as: SMI = [individual Mass × (mean SVL of population/individual SVL) ^{SMA}] (Peig and Green, 2009; 2010).

This index has been previously applied to *R. temporaria* larvae (Dittrich et al., 2018; Ruthsatz et al., 2020; Eterovick et al., 2024). In the present study, SMA was 2.742.

Developmental rate was calculated as the number of Gosner (1960) stages advanced by each larva divided by the number of days from hatching to the end of the experiment.

Bacterial 16S rRNA gene library preparation

DNA was extracted from whole guts of larvae using the QIAamp Fast DNA Stool Mini Kit (QIAGEN) following the manufacturer's instructions. Extractions were performed over five days, with one negative control included per day to monitor for contamination. A ZymoBIOMICS™ microbial community standard (Zymo Research Europe GmbH) was used as a positive extraction control on the first and last days of the extraction process.

The V4 region of the 16S rRNA gene was amplified using the forward primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′; Caporaso et al., 2011). Each sample was tagged with a unique combination of forward and reverse primers from a stock of 24 forward and 24 reverse primer tags. Two PCR plates were prepared, each including one negative control. A positive control consisting of ZymoBIOMICS™ microbial community DNA standard was also included. The Zymo microbial community and DNA standards, which contain known species compositions and abundances, were used to verify the precision of extraction and PCR protocols, respectively.

PCR products were pooled and purified. Aliquots were electrophoresed on a 2% agarose gel, and the desired 251 bp fragment was extracted using the Monarch DNA Gel Extraction Kit (New England BioLabs, GmbH, Germany) following the manufacturer's protocol. Purified DNA was quantified with a Qubit™ fluorometer (Invitrogen) and sequenced using the MiSeq500 Illumina platform (paired-end 2 × 250 bp, v2 chemistry) at the Leibniz-Institut DSMZ - German Collection of Microorganisms and Cell Cultures GmbH.

Bioinformatic analyses

Sequence denoising, filtering, and beta diversity calculations were performed in QIIME2 (Bolyen et al., 2019), as detailed in the supplementary material.

Beta diversity was assessed using unweighted UniFrac distances (sensitive to low-abundance microbes; Lozupone and Knight, 2005) and compared among treatments using PERMANOVA with pairwise post hoc tests. Metagenomic predictions of metabolic pathways of the gut microbiota were generated using PICRUSt2 (Douglas et al., 2020), based on the MetaCyc database (Caspi et al., 2016).

Statistical analyses

Isotopic signatures were compared among diet treatments and between rearing temperatures using the R package nicheROVER (Swanson et al., 2015; R Core Team, 2024). This approach estimates the probability that the isotopic niches of individuals from one group overlap with those of another, based on quantitative variables such as $\delta^{15}N$ and $\delta^{13}C$. We ran 1,000 simulations to calculate niche breadth and overlap. Isotopic signatures of the three diets were based on six replicate samples per food type (see Suplementary Material).

The effects of food treatment, rearing temperature, and heatwave exposure - including all two- and three-way interactions - on larval mass, body condition, and developmental rate were analyzed with GLMMs in the afex package (Singmann et al., 2024), with clutch identity included as a random factor.

Before analyzing behavioral traits, we screened for outliers in the time elapsed between the moment the predator model touched the water and when it touched the larva (where applicable). Two outliers with unusually long times (Fig. S2) were removed. For the remaining data, this interval averaged 7.44 ± 1.78 s. Mixed models were then built to test the influence of food treatment, rearing temperature, and heatwave exposure (fixed variables), including their interactions, on: (1) whether the larva reacted (binary), (2) reaction time, (3) whether the reaction occurred before or after being touched (binary), (4) average speed, and (5) trajectory linearity (see "Behavioral trials"). Trial day and clutch identity were included as random effects nested within food treatment. When full models failed to converge due to model complexity, we simplified random-effect structures or analyzed likely interactions separately (Singmann et al., 2024). For binary outcomes, singular-fit warnings were expected, but results were considered robust when outcomes were consistent across full and simplified models (Singmann & Kellen, 2019; Singmann et al., 2024). Post hoc tests were performed with emmeans (Lenth, 2017).

For each behavioral variable, we first tested whether larval mass, body condition, or number of positioning attempts influenced results (Pearson or Spearman correlations for quantitative variables; Wilcoxon tests for binary outcomes). When relevant, these variables were incorporated into the models (e.g., number of attempts as a random factor). We expected larvae in better condition to respond more rapidly and before being touched, and to escape with higher speed and less linear trajectories. Positioning attempts were considered problematic if they were associated with reduced responsiveness, delayed reactions, increased likelihood of being touched, slower speeds, or more linear escapes.

Microbiome bacteria alpha-diversity was calculated using Hill numbers with order of diversity q1 (based on Shannon diversity index) (Alberdi and Gilbert, 2019) using the R package iNext (version 4.5.1; R Core Team, 2025; Chao et al., 2014). Hill numbers represent a number of taxonomic entities that would give the same diversity value if they all had the same abundance (Hill, 1973; Alberdi and Gilbert, 2019). A GLMM was posteriorly built in afex, using food treatment, rearing temperature, and heatwave exposure (and all interactions) as fixed effects and clutch identity as a random effect to explain bacteria alpha diversity.

To assess microbiome composition, we constructed a phyloseq object (McMurdie & Holmes, 2013) normalized via Total Sum Scaling (TSS) and tested for microbial markers that differed across the 12 treatment combinations (3 diets × 2 rearing temperatures × heatwave vs. no heatwave). Variance homogeneity among groups was evaluated with betadisper (vegan; Oksanen et al., 2013), and ASV abundances were ordinated using PCoA. Microbiome biomarkers were identified through LEfSe (Segata et al., 2011) using the R package microbiomeMarker (Cao et al., 2022), with an LDA score threshold of 4. LEfSe identifies taxa most likely to explain group-level differences while accounting for statistical significance.

Predicted microbial metabolic pathways were compared among all 12 treatment combinations using ggpicrust2 (Yang et al., 2023), applying the ALDEx2 method for multi-group comparisons.

Results

Isotope analyses

The three diets produced markedly different isotopic signatures in *Rana temporaria* larvae, whereas isotopic niches of the two temperature treatments largely overlapped within each diet (Figs. 3, S3). For the low- and intermediate-quality diets, higher temperature reduced isotopic niche breadth. In contrast, for the high-quality diet, niche breadth was narrower at 18 °C and increased at 24.5 °C (Fig. 3).

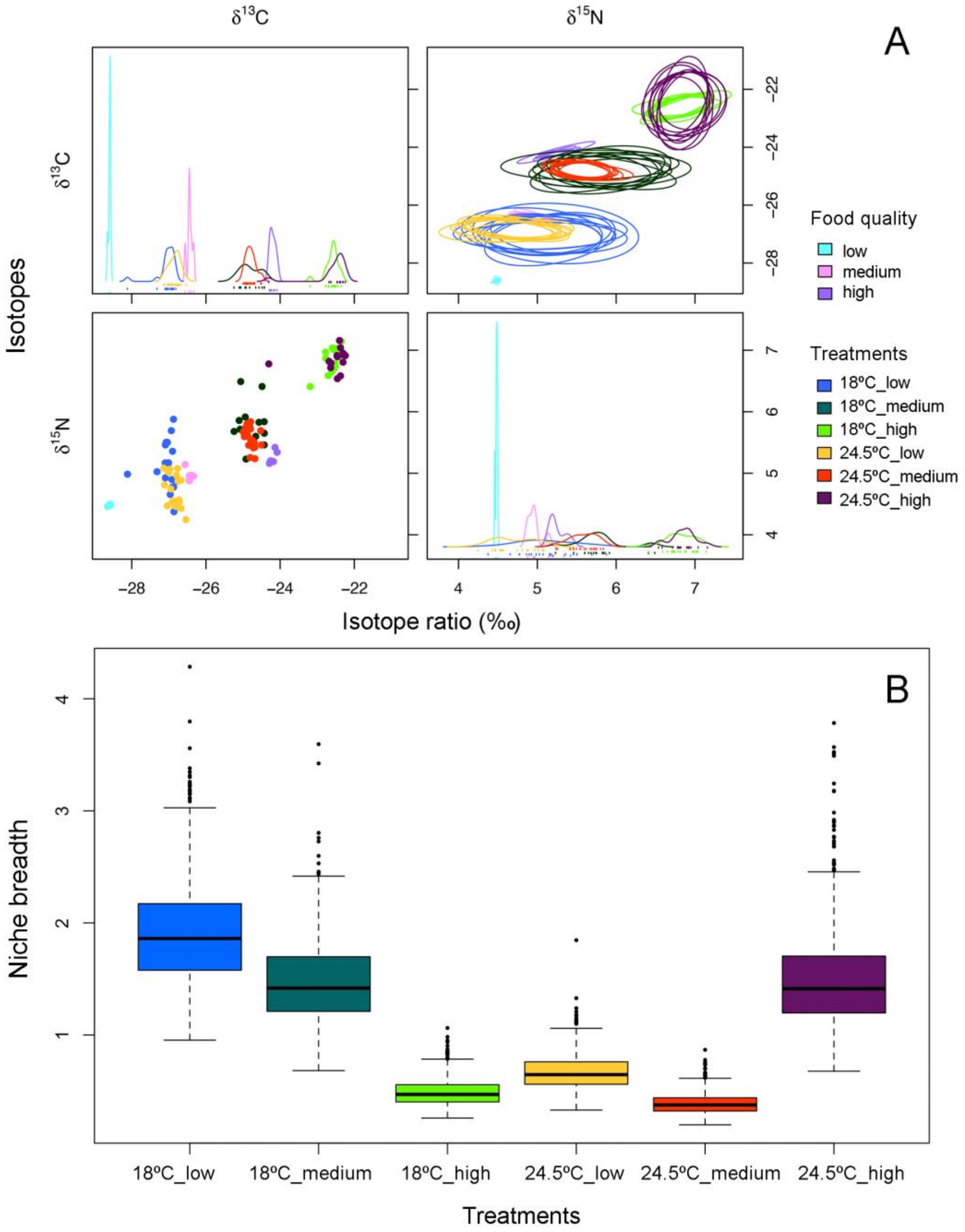

Fig. 3. Isotopic signatures (A) and niche breadth (B) of *Rana temporaria* larvae reared with three food treatments and two temperatures in a crossed experimental design. Food treatments correspond to diets with increasing levels of protein, fat, and components of animal origin (their isotopic signatures are also included in A). Rearing temperatures were 18 ºC and 24.5 ºC. The isotopic signatures are represented as lines in one-dimensional density plots (top left and bottom right graphs, A), two-dimensional scatterplots (bottom left graph; A) and ten random elliptical simulated projections of trophic niches corresponding to each diet and each treatment (top right graph; A).

Survivorship, development, and body condition

Of the 120 larvae used in the experiment, 12 died: six in the 18 °C treatment (five with intermediate- and one with high-quality food) and six in the 24.5 °C treatment (five with high- and one with intermediate-quality food). Five of these deaths occurred during or after the heatwave phase (three heatwave larvae and two controls). One larva developed hydrops and was excluded.

Larval body condition (SMI) did not differ among food treatments, rearing temperatures, or heatwave exposure; the same was true for body mass alone (Table 1, Figs. S4, S5). In contrast, developmental rate was higher at 24.5 °C than at 18 °C (Table 1). At 24.5 °C, developmental rate also increased with intermediate-quality food (Kenward–Roger post-hoc: estimate = –0.029, SE = 0.010, df = 91.5, t = –2.874, p = 0.025) and with high-quality food (estimate = –0.054, SE = 0.011, df = 92.0, t = –4.804, p < 0.001) compared with low-quality food. These effects were absent at 18 °C (Table 1; Figs. 4A, S6).

Behavioral trials

Escape-behavior trials were conducted with 102 *R. temporaria* larvae. Of the 108 surviving larvae, one showed hydrops and four displayed abnormal behavior (lethargy or irregular swimming) and were therefore excluded. In addition, one video file was accidentally lost. Of the 102 larvae tested, 81 responded to the aversive stimulus (61 before being touched by the predator model and 20 upon contact), whereas 21 did not react even when gently touched.

Larvae likeliness to react

Larval response (reacted vs. did not react) was unrelated to mass (W = 711.5, p = 0.252; Fig. S7) or body condition (W = 936, p = 0.482; Fig. S8). The number of attempts needed to position a larva before the trial differed between responders and non-responders (W = 601, p = 0.022; Fig. S9); however, larvae requiring more positioning attempts were also more likely to react, indicating that repositioning did not impair their ability to respond (Fig. S9). For this reason, number of attempts was included as an additional random effect in the models assessing reaction likelihood.

Reaction likelihood was not explained by any fixed factor alone but by interactions among them (Table 2). The full mixed-effects model with random structure did not converge, so we ran a model without random effect structure using the *glmer* function, which also did not converge. Simpler models including only individual predictors and single interactions yielded consistent results using the mixed function (Table 2).

Heatwave exposure increased larvae reaction likelihood at 24.5 °C (log odds scale post-hoc tests: z = -2.309, p = 0.021), whereas at 18 °C it reduced the likelihood of reacting (z = 2.454, p = 0.014). Heatwave exposure increased reaction likelihood at medium food quality (z = -2.017, p = 0.043), but not in the other food treatments (z = 0.475, p = 0.635 for low quality food and z = 0.037, p = 0.970 for high quality food; Fig. 4B, Table 2).

Table 1. Models built to explain variability in body condition (SMI), mass, developmental rate (dev_rate) and gut bacteria diversity of *Rana temporaria* larvae reared at two temperatures (either 18 °C or 24.5 °C) and receiving one of three food treatments considered as of low-, medium-, and high-quality (based on increasing content of protein, fat, and animal components), being exposed or not to a heatwave (HW) in a crossed experimental design. Developmental rate was calculated as the number of Gosner's (1960) developmental stages advanced during the experiment divided by the number of days from hatching to the end of the experiment. Significant effects are boldfaced and marked with an *. *mixed* refer to function employed to run the models.

Dependent variable / GLMM model	Fixed effects	df	F	p	n
Body condition (SMI)					
mixed (SMI ~ diet*temperature*HW + (1|Clutch))	diet	2; 91.72	2.196	0.117	107
	temperature	1; 91.66	0.067	0.797	
	HW	1; 94.99	0.236	0.628	
	diet:temperature	2; 93.03	1.001	0.372	
	diet:HW	2; 93.99	0.594	0.554	
	temperature:HW	1; 94.31	1.551	0.216	
	diet:temperature:HW	2; 90.66	0.190	0.827	
Mass (mg)					
mixed (mass ~ diet*temperature*HW + (1|Clutch))	diet	2; 91.72	2.291	0.107	107
	temperature	1; 91.66	0.124	0.725	
	HW	1; 94.99	0.106	0.745	
	diet:temperature	2; 93.03	0.322	0.272	
	diet:HW	2; 93.99	0.947	0.057	
	temperature:HW	1; 94.31	0.124	0.725	
	diet:temperature:HW	2; 90.66	1.705	0.188	
Developmental rate (dev_rate)					
mixed (dev_rate ~ diet*temperature*HW + (1|Clutch))	diet	2; 91.72	8.428	**<0.001***	107
	temperature	1; 91.65	412.706	**<0.001***	
	HW	1; 94.99	0.865	0.354	

	diet:temperature	2; 93.03	4.404	**0.015***
	diet:HW	2; 93.99	0.281	0.756
	temperature:HW	1; 94.31	3.364	0.070
	diet:temperature:HW	2; 90.66	0.036	0.965
Gut bacteria diversity (Hill numbers)				
mixed (Hill_q1 ~ diet*temperature*HW + (1|Clutch))	diet	2; 77.31	1.851	0.164
	temperature	1; 78.60	8.537	**0.005***
	HW	1; 79.95	0.393	0.533
	diet:temperature	2; 78.43	3.905	**0.024***
	diet:HW	2; 79.09	1.266	0.287
	temperature:HW	1; 77.97	0.077	0.783
	diet:temperature:HW	2; 69.18	3.347	0.103

Table 2. Models built to explain variability in five dependent variables describing *Rana temporaria* larvae escaping behavior when exposed to an aversive stimulus consisting of an approaching transparent plastic pipette with a predator model glued to the top releasing 4 ml of water previously exposed to predators. Analyzed escape responses were: (1) whether the larva reacted or not (no reaction meant not moving even when touched by the model), (2) larvae reaction time (time elapsed from the moment the predator model touched the water to the fleeing response of the larva), (3) whether the larva reacted before or after being touched by the predator model, (4) average speed and (5) trajectory linearity while fleeing. *Rana temporaria* larvae were reared at two temperatures (either 18 °C or 24.5 °C) and received one of three food treatments considered as of low, medium, and high quality (based on increasing levels of protein, fat, and components of animal origin), being exposed or not to a heatwave (HW) in a crossed experimental design. Significant effects are boldfaced and marked with an *. *mixed* and *lmer* refer to functions employed to run the models.

Dependent variable / GLMM model	Fixed effects	df	F	p	n
Reaction to the aversive stimulus (binary)					
lmer (reaction ~ diet*temperature*HW + (1|day_filmed) + (1|Clutch) + (1|attempts))		-	-	-	102
mixed (reaction ~ diet*temperature + (1|day_filmed) + (1|Clutch) + (1|attempts))	diet	7; 2	3.010	0.222	
	temperature	8; 1	0.788	0.374	
	diet:temperature	7; 2	10.520	**0.005***	
mixed (reaction ~ temperature*HW + (1|day_filmed) + (1|Clutch) + (1|attempts))	temperature	6; 1	0.309	0.580	
	HW	6; 1	0.212	0.654	
	temperature:HW	6; 1	16.366	**<0.001***	
mixed (reaction ~ diet*HW + (1|day_filmed) + (1|Clutch) + (1|attempts))	diet	7; 2	3.483	0.175	
	HW	8; 1	1.116	0.290	
	diet:HW	7; 2	13.397	**0.001***	
Reaction time					

mixed (reaction_time ~ diet*temperature*HW + (diet||day_filmed+clutch))	diet	2; 0.29	0.087	0.934	81
	temperature	1; 1.24	0.290	0.670	
	HW	1; 64.31	0.880	0.352	
	diet:temperature	2; 0.55	0.148	0.888	
	diet:HW	2; 51.67	1.638	0.204	
	temperature:HW	1; 62.73	0.870	0.355	
	diet:temperature:HW	2; 63.25	1.078	0.346	
Touch by the predator model before reaction (binary)			Chisq		
mixed (touch ~ diet*temperature*HW + (1|day_filmed) + (1|Clutch))		-	-	-	81
mixed (touch ~ diet* HW + (1|day_filmed) + (1|Clutch))	diet	6; 2	2.125	0.345	
	HW	7; 1	0.478	0.489	
	diet:HW	6; 2	4.919	0.085	
Speed while fleeing (log)			F		
mixed (logspeed ~ diet*temperature*HW + (diet||day_filmed+clutch))	diet	2; 0.86	0.364	0.768	81
	temperature	1; 0.85	0.079	0.831	
	HW	1; 61.65	0.087	0.769	
	diet:temperature	2; 0.84	0.268	0.813	
	diet:HW	2; 52.33	1.900	0.160	
	temperature:HW	1; 61.27	0.012	0.913	
	diet:temperature:HW	2; 61.42	1.697	0.192	
Trajectory non-linearity while fleeing or “meander” (log)					
mixed (logmeander ~ diet*temperature*HW + (diet||day_filmed+clutch))	diet	2; 0.61	0.137	0.893	81
	temperature	1; 0.93	0.908	0.525	
	HW	1; 63.74	0.502	0.481	
	diet:temperature	2; 1.04	0.106	0.908	
	diet:HW	2; 51.23	0.614	0.545	

	temperature:HW	1; 62.44	1.625	0.207
	diet:temperature:HW	2; 63.22	1.203	0.307

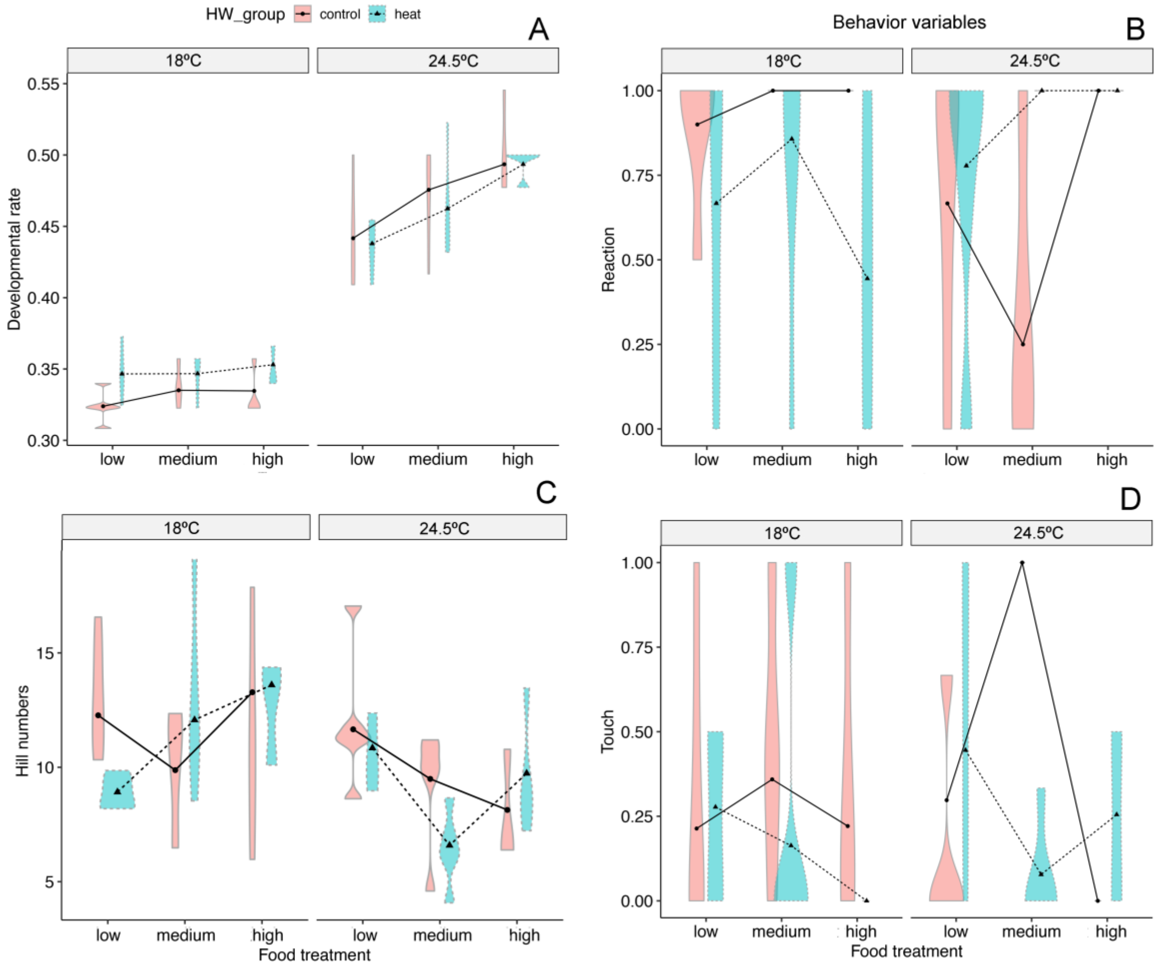

Fig. 4. Interactive effects among food quality, rearing temperature, and exposure to a heatwave in *Rana temporaria* larvae developmental rate (A), variables describing behavior (B, D) and gut bacteria diversity (C). Food quality refers to increasing levels of protein, fat, and components of animal origin. Rearing temperatures were 18 ºC and 24.5 ºC. The heatwave (HW) corresponded to increasing temperature at a ramping rate of 0.5 ºC per hour until 28 ºC, maintenance at 28 ºC for 48 h and subsequent temperature decrease of 0.5 ºC per hour until original rearing temperature. Variables describing behavior are larvae likeliness to react (fleeing) to an aversive stimulus (B) and to be touched by an approaching predator model before reacting (D). Graphs correspond to violin plots of estimated marginal means from the model including all fixed variables (see Table 1).

Larvae reaction time

Reaction time, measured for the 81 larvae that responded to the stimulus, was not influenced by mass (Adjusted $R^2 = 0.030$, $F_{79} = 3.487$, $p = 0.066$; Fig. S10), body condition (Adjusted $R^2 = -0.013$, $F_{79} = 0.005$, $p = 0.946$; Fig. S11), or the number of positioning attempts before the trial ($\rho = -0.125$, $p = 0.263$; Fig. S12). Reaction time was also unaffected by any experimental factor - food treatment, rearing temperature, heatwave exposure - or by their interactions (Table 2).

Larvae likeliness of being touched

Whether larvae reacted before or after being touched by the predator model was unrelated to mass (W = 577, p = 0.722; Fig. S13), SMI (W = 697, p = 0.343; Fig. S14), or the number of attempts needed to position them (W = 533, p = 0.366; Fig. S15). Reaction likeliness tended to increase in larvae fed medium quality food when exposed to the heatwave (Table 1, Figs. 2D).

Larvae escape speed and trajectory

Escape speed and movement non-linearity (“meander”; Chiara & Kim, 2023) were quantified for the 81 larvae that fled, with both variables log-transformed to meet normality assumptions. Neither metric was affected by mass, body condition, or the number of positioning attempts (speed: Adjusted $R^2 = -0.013$, $F_{79} = 0.004$, p = 0.949; Fig. S16; Adjusted $R^2 = -0.013$, $F_{79} = 0.009$, p = 0.923; Fig. S17; $\rho = -0.104$, p = 0.354; Fig. S18; meander: Adjusted $R^2 = 0.003$, $F_{79} = 1.271$, p = 0.263; Fig. S19; Adjusted $R^2 = -0.009$, $F_{79} = 0.247$, p = 0.620; Fig. S20; $\rho = 0.050$, p = 0.657; Fig. S21). Food treatment, rearing temperature, heatwave exposure, and their interactions likewise had no effect on larval escape speed or trajectory (Table 2).

Gut bacteria diversity and composition

Gut bacterial diversity was influenced by rearing temperature and its interaction with food treatment (Table 1). Both larvae fed medium-quality and high-quality food exhibited reduced gut bacterial diversity when reared at 24.5 °C compared to 18 °C, with no difference among temperatures for low-quality food (Fig. 4C, S22).

The two positive extraction controls (ZymoBIOMICS™ microbial community standard) and the positive PCR control (ZymoBIOMICS™ microbial community DNA standard) displayed identical species compositions but differed in the relative abundances of taxa compared with the manufacturer’s expected profile (Fig. S23). The two extraction controls yielded consistent results (Fig. S23), indicating that any deviations in relative abundances were systematic rather than random.

In total, 207 Amplicon Sequence Variants (ASVs) were recovered from the gut microbiomes of 92 *R. temporaria* larvae. The dominant phyla across treatments were *Pseudomonadota* and *Bacteroidota* (Fig. S24). Most treatment pairs differed significantly in gut bacterial community composition, with a few exceptions. No differences were detected between medium-quality food with heatwave exposure and high-quality food without exposure at 18 °C. At 24.5 °C, larvae fed low-quality food with heatwave exposure did not differ from those fed medium-quality food (with or without heatwave exposure) or high-quality food (with or without heatwave exposure) (Fig. 5A; Table S1).

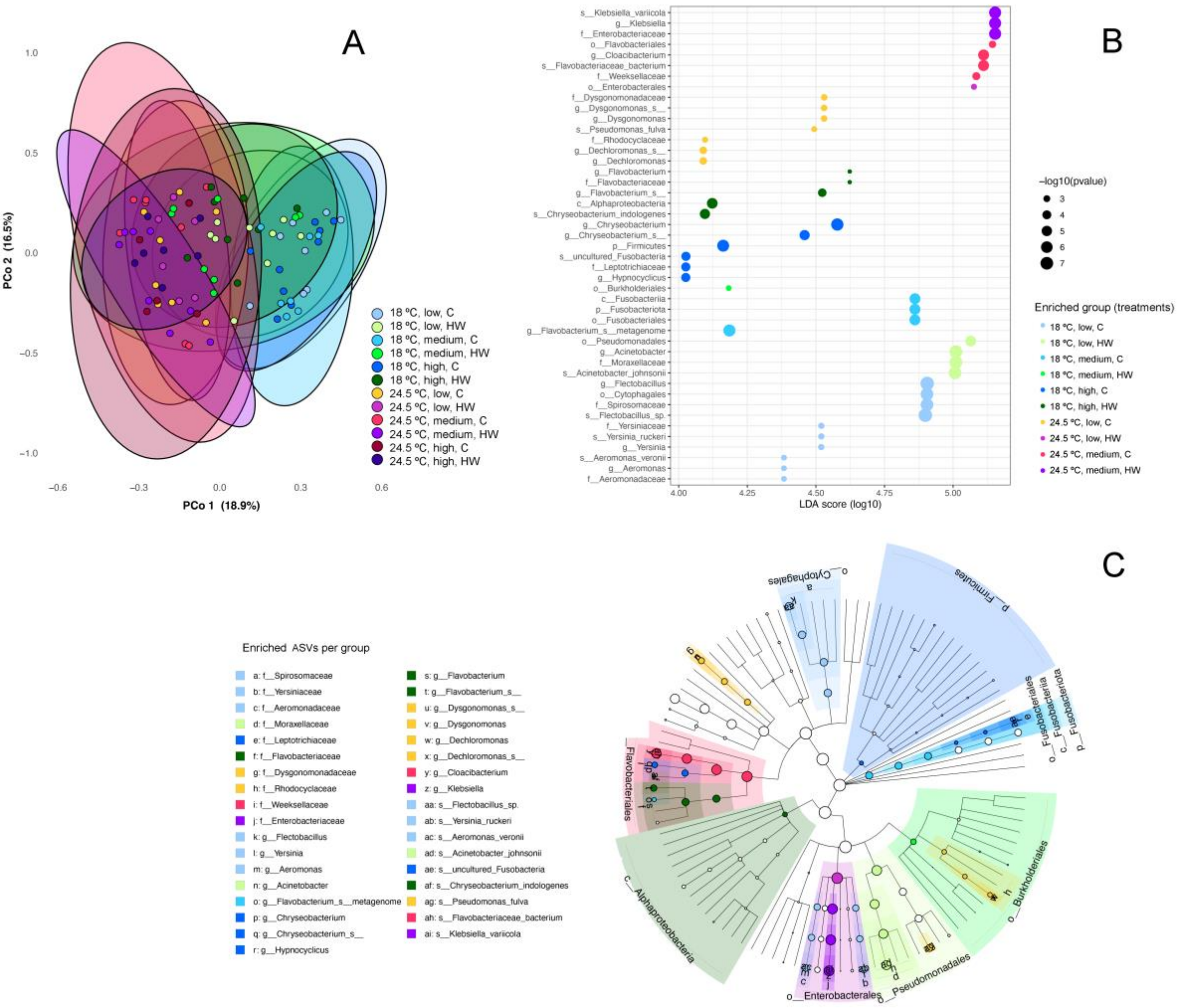

Fig. 5. Gut bacteria community composition (A) and enriched Amplicon Sequence Variants (ASVs; B) according to treatments imposed to larvae of *Rana temporaria*, corresponding to three diets with increasing levels of protein, fat, and components of animal origin (low-, medium-, and high-quality), two rearing temperatures (18 ºC and 24.5 ºC) and exposure or not to a heatwave (HW vs. C = control). Clustering of taxa with differences in abundance among treatments is also shown (C). Colors of ASVs correspond to colors of treatments in which they were the most abundant, cold colors (blue-green) correspond to 18 ºC and warm colors (yellow-purple) to 24.5 ºC rearing temperatures. Color intensity increases with food quality.

All treatment combinations except those involving high-quality food at 24.5 °C (regardless of heatwave exposure) had ASVs identified as biomarkers, totaling 45 ASVs (Fig. 5). At low food quality, the main biomarkers at 18 °C without heatwave exposure were *Flectobacillus* (*Spirosomaceae*, *Cytophagales*), *Yersinia ruckeri* (*Yersinaceae*), and *Aeromonas veronii* (*Aeromonadaceae*). When exposed to a heatwave, *Acinetobacter johnsonii* (*Moraxellaceae*) was predominant. At 24.5 °C, *Dysgonomonas* (*Dysgonomonadaceae*), *Pseudomonas fulva*, and *Dechloromonas* (*Rhodocyclaceae*) dominated without heatwave exposure, whereas *Enterobacterales* predominated under heatwave exposure.

At intermediate food quality, *Fusobacteriales* (*Fusobacteriia*, *Fusobacteriota*) and *Flavobacterium* were characteristic at 18 °C without heatwave exposure, while

Burkholderiales dominated with heatwave exposure. At 24.5 °C, *Cloacibacterium* (*Weeksellaceae*, *Flavobacteriales*) predominated without heatwave exposure, whereas *Klebsiella variicola* (*Enterobacteriaceae*) was selected as a biomarker under heatwave exposure.

At high food quality and 18 °C, *Chryseobacterium*, *Bacillota*, and *Hypnocyclicus* (*Leptotrichiaceae*) were biomarkers without heatwave exposure, and *Cryseobacterium indologenes*, *Flavobacterium* (*Flavobacteriaceae*), and *Alphaproteobacteria* predominated under heatwave exposure (Fig. 5).

In total, 357 unique metabolic pathways were predicted, of which 289 differed significantly among experimental treatments. The most significantly affected pathways included degradation of myo-inositol, D-glucarate, fructose, and various aromatic compounds (catechol, gallate, toluene, 3-phenylpropanoate, and 3-(3-hydroxyphenyl) propanoate), and conversion of amino acids into putrescine, as well as synthesis of L-tryptophan and aerobactin, (Fig. 6). The treatments with the highest expression of these pathways were those with larvae reared at 24.5 °C, exposed to a heatwave, or both (Fig. 6).

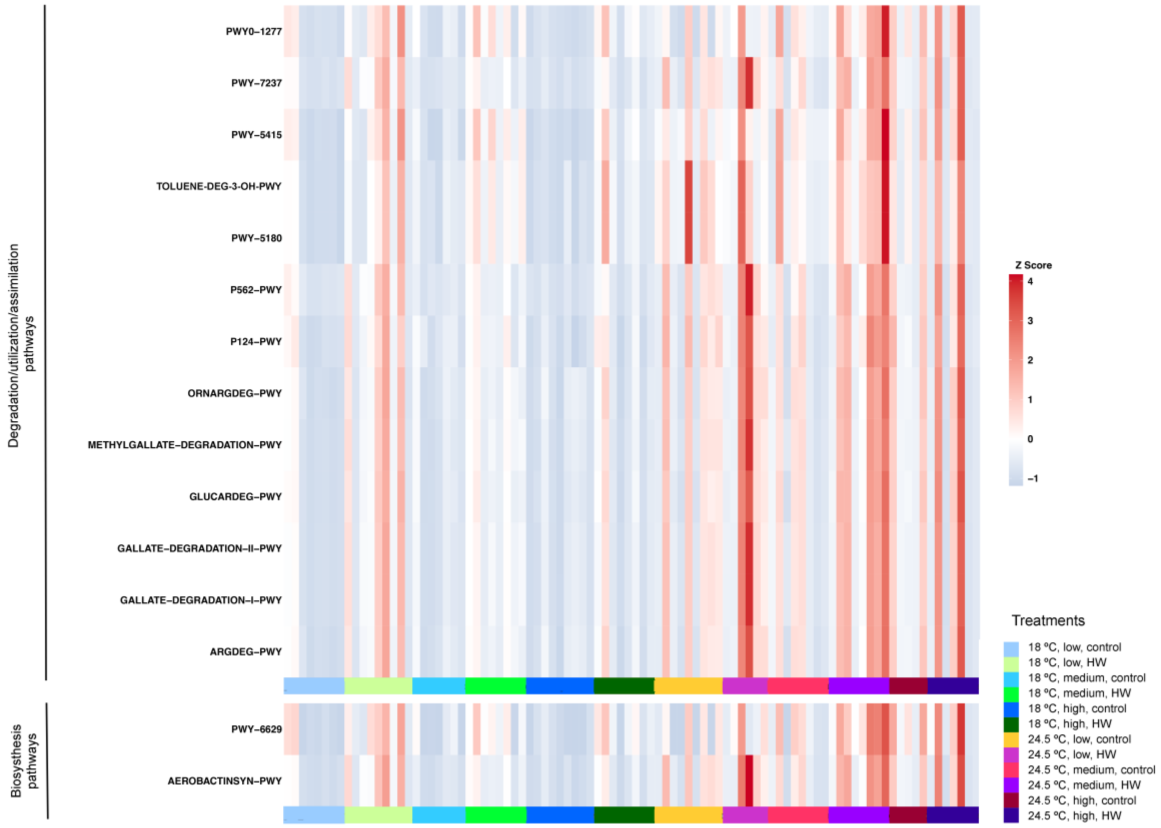

Fig. 6. The predicted 15 most significant pathways influenced by gut bacteria from larvae of *Rana temporaria* and their expression in treatments corresponding to a multifactorial experimental design of three diets with increasing levels of protein, fat, and components of animal origin (low-, medium-, and high-quality), two rearing temperatures (18 ºC and 24.5 ºC) and exposure or not to a heatwave (HW vs. C = control). Treatment colors are as in Fig. 5. Pathways follow the MetaCyc database (Caspi et al., 2016). In the order of appearance: PWY0-1277 = pathway 3-phenylpropanoate and 3-(3-hydroxyphenyl) propanoate degradation; PWY-7237 = pathway myo-, chiro- and scyllo-inositol

degradation; PWY-5415 = pathway catechol degradation I (meta-cleavage pathway); TOLUENE-DEG-3-OH-PWY = toluene degradation II (aerobic) (via 4-methylcatechol); PWY-5180 = pathway toluene degradation I (aerobic) (via o-cresol); P562-PWY = pathway myo-inositol degradation I; P124-PWY = pathway *Bifidobacterium shunt*; ORNARGDEG-PWY = superpathway of L-arginine and L-ornithine degradation; METHYLGALLATE-DEGRADATION-PWY = pathway methylgallate degradation; GLUCARDEG-PWY = pathway D-glucarate degradation I; GALLATE-DEGRADATION I and II-PWY = pathways gallate degradation I and II; ARGDEG-PWY = superpathway of L-arginine, putrescine, and 4-aminobutanoate degradation; PWY-6629 = superpathway of L-tryptophan biosynthesis; AEROBACTINSYN-PWY = pathway aerobactin biosynthesis.

Discussion

The gut microbiome plays key roles in many aspects of animal biology, from nutrient assimilation to immune defense and ultimately behavior (McFall-Ngai et al., 2013; Tuddernham and Sears, 2015). Animals respond to environmental conditions and their gut microorganisms are also expected to respond, potentially in ways that are adaptive and enhance the animals' ability to cope with both natural environmental fluctuations (Park and Do, 2024) and human-driven environmental challenges (Lynch and Hsiao, 2019; Fontaine and Kohl, 2023). Under changing conditions, microbial taxa favored by the new environment may increase in abundance and help maintain host metabolic functions, provided that the microbial community has sufficient functional redundancy (Louca et al., 2018).

In our study, larvae of *R. temporaria* exposed to different temperatures and diets exhibited shifts in gut bacterial diversity and composition, likely enabling them to maintain body condition and still develop faster under higher temperatures. At elevated temperatures, diet quality became a decisive factor for larval development and escape performance.

Larvae nutrient assimilation, growth, and development

Larvae of *Rana temporaria* exhibited clearly distinguishable stable isotope signatures depending on food treatment, reflecting expected differences in nutrient acquisition from the diets provided. However, body condition did not differ among food treatments, regardless of rearing temperature. Development, on the other hand, was faster at 24.5 ºC, and at this temperature, higher food quality further increased developmental rate. Because temperature determines the metabolic rate of ectotherms (Álvarez and Nicieza, 2002) and higher metabolism requires more energy (Arendt, 1997), the improved food quality likely enabled *R. temporaria* larvae to grow faster while maintaining good body condition.

The "macronutrient ratio hypothesis" predicts that ectotherms prefer increased carbohydrate/protein ratios at higher temperatures to meet the energetic demands of elevated metabolism, because excreting nitrogen from protein catabolism incurs a cost (Hardison and Eliason, 2024). Similarly, the "temperature metabolic stoichiometry hypothesis" proposes that ectotherms prefer diets with a higher carbon-to-nitrogen ratio under elevated temperatures (Hardison and Eliason, 2024). Nitrogen excretion rates, however, usually increase with temperature, reducing the cost of protein-rich diets (Hardison and Eliason, 2024). This increase in nitrogen excretion may have allowed *R. temporaria* larvae to maintain body condition and develop faster at higher temperatures when fed high-protein diets with relatively constant carbohydrate content. A proportional increase in protein consumption at higher temperatures has been observed in arthropods (Devries and Appel, 2014; Schmitz et al., 2016), and invertebrates can maintain stable carbon-to-nitrogen ratios if food intake increases with temperature (Anderson et al., 2017). In our study, this was likely the case because larvae were fed *ad libitum*.

Niche breadth, estimated from stable isotope analyses, was higher at 18 °C with low to intermediate food quality and at 24.5 °C with high food quality, but lower at 18 °C with high food quality and at 24.5 °C with intermediate food quality. Because diet was uniform within treatments, variation in niche breadth reflects individual differences in nutrient assimilation, potentially mediated by the microbiome.

The microbiome, shaped by diet and host genetics, can influence nutrient absorption and metabolism (Huda et al., 2022; Corbin et al., 2023). Thus, broader niche breadths may indicate greater plasticity of the holobiont in adjusting nutrient assimilation at the individual level. More diverse microbial communities often exhibit higher functional redundancy, maintaining metabolic functions despite taxonomic shifts (Louca et al., 2018). In humans, distinct host–microbial symbiotic states respond differently to diet (Arumugam et al., 2011), suggesting that such plasticity can help meet host nutritional demands. As host genetic variation was controlled across treatments (equal representation of five clutches), the larger niche breadths observed likely reflect greater microbiome-mediated adaptive capacity, potentially enhancing host performance.

If this hypothesis holds, higher efficiency in individual food assimilation could be achieved at 24.5 ºC when larvae consume high-quality food, as suggested by the observed faster development without detriment to body condition or escape performance (discussed below). However, in natural habitats, *ad libitum* access to the highest-quality food at elevated temperatures may not be realistic. In such circumstances, herbivorous diets - which resulted in broader niches than diets with intermediate animal components in our experiment - may represent the best available solution. Therefore, dietary preferences toward herbivory under heat stress could be subject to selection. In the wild, plant material has been associated with higher nutritional value for fish at warmer temperatures and is thought to influence latitudinal diversity gradients in herbivorous versus carnivorous fishes, with consumption of plant-based food increasing with temperature (Behrens and Lafferty, 2007; González-Bergonzoni et al., 2012). Choice experiments with ectotherms have similarly shown selection for more herbivorous diets at higher temperatures (Vejříková et al., 2016; Zhang et al., 2020). Yet, in some cases, herbivorous fish abundance did not increase with temperature in the southern hemisphere (Trip et al., 2014), and grasshoppers increased preference for protein under higher temperatures (Schmitz et al., 2016), indicating that increased plant consumption is not the only strategy for coping with heat. The availability of suitable microorganisms to aid digestion and assimilation of different nutrients, along with their own response to temperature, may be critical for host success at varying temperatures and food qualities (Vejříková et al., 2016).

Increasing temperatures can alter the diets of ectothermic animals by affecting both food availability and quality or by triggering dietary shifts (Hardison and Eliason, 2024). For instance, lipid content of algae decreases at higher temperatures (20-28 ºC vs. 12 ºC), which also reduces the growth of *Daphnia* fed on them (Tseng et al., 2021). Altered temperatures impose different nutrient demands, and species may adjust foraging behavior accordingly. Thus, understanding the nutrients ectotherms can actually access in natural habitats is crucial for interpreting laboratory results; otherwise, we risk overestimating their capacity to improve performance based on animals kept in unrealistic conditions (Hardison and Eliason, 2024).

Larvae escape behavior

Larval ability to react was influenced by experimental conditions, whereas reaction time, speed, and meander were not. At the higher rearing temperature (24.5 ºC), not all diets were sufficient to maintain an effective escape response in *R. temporaria* larvae. Diets with high protein content and greater representation of animal-derived components, as well as an herbivorous diet, resulted in efficient escape performance. Interestingly, the diet assumed to be of lowest quality produced intermediate results in terms of larval reactivity, whereas larvae receiving intermediate-quality food at 24.5 ºC and not exposed to a heatwave exhibited the poorest performance. These results align with observed patterns in larvae niche breadth, suggesting a relationship between nutrient assimilation plasticity (i.e., broader isotopic niches) and escape ability.

Using a combined visual, tactile, and chemical stimulus may have obscured cue-specific differences in reaction time, as responses can vary by sensory modality (Melo et al., 2021). We nevertheless combined cues to ensure stimulus detection by all larvae. Individuals that failed to respond, or responded only upon contact, were considered less responsive, as physical contact with a predator would likely result in capture in nature. *Rana temporaria* larvae in the Kleiwiesen develop in small ponds with dragonfly naiads but without predatory fish. High escape speed is adaptive against active predators such as fish, but less so against ambush predators like Odonata (Teplitsky et al., 2005). Under these conditions, rapid threat detection and initiation of escape likely have greater fitness consequences than speed or escape trajectory (Staudinger et al., 2011).

Gut bacteria, predicted metabolic pathways, and their potential influence on larvae performance

Variations in gut bacterial abundance and predicted metabolic pathways may have contributed to differences in *R. temporaria* larvae performance under the experimental conditions. Escape responses were markedly reduced in larvae reared at 24.5 ºC with an intermediate-quality diet and not exposed to a heatwave. In these larvae, *Cloacibacterium* showed increased abundance. Interestingly, *Cloacibacterium* was also abundant in the control group compared to elevated temperatures in rainbow trout (Zhou et al., 2022), although it remains unclear whether this taxon contributed directly to the reduced reactivity in larvae.

In contrast, larvae exposed to a heatwave under the same dietary and rearing temperature conditions showed improved escape performance and a higher abundance of *Klebsiella* (*Enterobacteriaceae*, *Enterobacterales*). This suggests that the heatwave may have triggered proliferation of *Klebsiella*, which in turn could have contributed to enhanced performance. However, this shift in microbial composition came with additional reduction in gut microbiome diversity, which was already low at medium quality food and 24.5 ºC, and may reduce host capacity to cope with additional stressors (Henry et al., 2021).

Klebsiella may influence host performance through multiple metabolic pathways. Pathways such as P562-PWY and PWY-7237, involved in myo-inositol and related inositol derivatives degradation (Berman and Magasanik, 1966a, 1966b; Anderson and Magasanik, 1971; Karp et al., 2019), were relatively increased in treatments with higher temperatures. Myo-inositol is essential in eukaryotes for membrane phospholipids and cell signaling, and its metabolism may help maintain membrane fluidity and protein activity - which are influenced by temperature (Hazel, 1995) - under thermal stress. Additionally, *Klebsiella* may influence behavior through neuromodulatory signals, as related species (*K. pneumoniae*) affect food intake and attention in humans via serotonin and dopamine signaling (Miri et al., 2023). Other upregulated pathways recorded for *Klebsiella*, such as GLUCARDEG-PWY (D-glucarate degradation) and AEROBACTINSYN-PWY (aerobactin biosynthesis; Karp et al., 2019), support bacterial growth by enabling carbon use and iron acquisition, which may indirectly benefit host performance.

Other taxa also contributed to larvae performance under specific conditions. *Yersinia* (*Yersiniaceae*, *Enterobacterales*) increased in abundance in larvae reared at low-quality food and 18 ºC under heatwave exposure, although performance did not differ from controls. *Chryseobacterium*, associated with lipid absorption (Semova et al., 2012), predominated in larvae fed high-quality food at 18 ºC. In larvae fed high-quality food at 24.5 ºC, no dominant biomarkers were detected, yet these individuals developed the fastest and exhibited effective escape responses, which could be linked to functional redundancy in the microbial community.

Predicted metabolic pathways suggest that microbial plasticity may provide alternative solutions for nutrient acquisition under different temperatures. For example, in larvae reared at 24.5 ºC with low-quality (herbivorous) diets, *Pseudomonas* and *Dysgonomonas* were abundant in non-heatwave conditions, supporting aerobic aromatic catabolism pathways (GALLATE-DEGRADATION-I-PWY, GALLATE-DEGRADATION-II-PWY, METYLGALLATE-DEGRADATION) that enable degradation of plant lignin and tannins (Karp et al., 2019). At the same time, increased abundance of *Enterobacterales* under heatwave exposure likely allowed efficient carbon utilization and maintenance of membrane function, supporting effective escape responses despite low-quality diets. In the fish *Plectropomus leopardus* dominant gut bacterial taxa were shown to change within 12 h and maintain estimated microbial functional capacity constant under different environmental conditions (Mekuchi et al., 2018).

Protein absorption efficiency may decline with increasing temperature in ectotherms (Croll and Watts, 2004). In fish, low-protein diets lead to gut microbiomes with altered composition and reduced diversity, which are less efficient at absorbing protein—likely due to the influence of specific bacterial strains on enterocyte protein uptake (Childers et al., 2025). For instance, strains of *Acetinobacter*, *Aeromonas*, and *Pseudomonas* can reduce protein absorption in the fish gut (Childers et al., 2025; Ye et al., 2019). Besides *Pseudomonas*, *Dysgonomonas* may also be disadvantageous to the host at elevated temperatures. Members of *Bacteroidales* (the order that includes *Dysgonomonas*) use putrescine to produce GABA (gamma-aminobutyric acid), a molecule that modulates stress responsiveness in humans (Miri et al., 2023). Thus, increased putrescine degradation may impair stress responses. In our study, the superpathway of L-arginine, putrescine, and 4-aminobutanoate degradation (ARGDEG-PWY) was upregulated in larvae reared at 24.5 ºC on low-quality food and exposed to a heatwave. However, these larvae also showed increased abundances of *Enterobacterales* (the order that includes *Klebsiella*), which may have facilitated the degradation of diverse carbon sources from the herbivorous diet and regulated membrane functions (as discussed above), ultimately allowing larvae to maintain an effective escape response.

Some pathways also suggest potential benefits for coping with environmental pollutants. PWY-5180 and TOLUENE-DEG-3-OH-PWY, corresponding to toluene degradation, were associated with *Pseudomonas* (Fishman et al., 2004; Karp et al., 2019) and may help larvae survive in polluted habitats. Additionally, PWY-6629, the L-tryptophan biosynthesis pathway, increased under higher temperatures. In other ectotherms, dietary L-tryptophan improves growth and thermic stress resistance (Akthar et al., 2013), suggesting possible similar benefits mediated by the microbiome, although this pathway has only been documented for *E. coli* due to limited ectotherm microbiome studies (Legrand et al., 2020; Eterovick et al., 2024).

Overall, exposure to elevated temperatures - either long-term or as short-term heatwaves - was associated with increases in the most significant metabolic pathways, though not uniformly across treatments. This variability aligns with individual differences in microbiome-host interactions and may underlie observed variation in larvae performance under different environmental conditions.

Concluding remarks

At a temperature equivalent to that naturally experienced by *R. temporaria* (18 ºC), food quality - defined by high protein, fat, and animal component content - did not appear to be a decisive factor for larval performance, including developmental rate and the ability to detect and escape from threats. Under these conditions, food provided the necessary nutrients for larvae performance and the gut bacterial community may have adjusted to variations in food quality and exposure to short-term heat stress, maintaining nutrient assimilation for host metabolic functions.

However, at elevated rearing temperatures, food quality became a key determinant of developmental rate and interacted with additional temperature fluctuations, such as heatwaves, to shape both the microbiome and behavioral outcomes. Larvae fed the diet richest in protein, fat, and animal components developed the fastest and were among the most likely to respond early to threats. Such traits would increase survival likelihood, allowing these larvae to leave warming and potentially drying habitats quickly and to escape predators efficiently. Interestingly, larvae fed an herbivorous diet - low in protein, fat, and component diversity - also exhibited effective escape responses. The comparable outcomes of these markedly different diets suggest that the different bacterial communities associated to them may provide functional redundancy, supporting host performance.

Larvae receiving intermediate-quality diets, with moderate inclusion of animal components, showed variable outcomes depending on heatwave exposure. This variability indicates that a more herbivorous diet may represent a safer strategy in unpredictable environments where high-quality animal food may not be consistently available. Temperature-modulated microbial growth may further favor the consumption of specific food types, as microbes play a key role in nutrient assimilation (Newsome et al., 2011; Vejříková et al., 2016). Supporting this, studies across diverse ectotherms - from insects to vertebrates - have often documented increased herbivory under elevated temperatures (Behrens and Lafferty, 2007; Carreira et al., 2016; Brankatschk et al., 2018; Zhang et al., 2020), although exceptions exist (Trip et al., 2014; Schmitz et al., 2016). To better understand these patterns, future research should investigate wild ectotherms' microbiomes, isotopic signatures, and health biomarkers, linking diet composition, microbiome-mediated nutrient assimilation, and host condition in natural habitats.

As human activities increase the intensity and frequency of environmental changes, accelerating species extinction rates (IPCC, 2023), understanding the role of the microbiome in animal resilience becomes increasingly important. Microbiomes are dynamic communities (Louca et al., 2018) that respond to environmental fluctuations (Mekuchi et al., 2018). Therefore, studies integrating multifactorial interactions among host, microbiome, and environment, and collecting data from animals under natural conditions, are essential to accurately interpret laboratory findings and predict ecological outcomes.

References

Akhtar, M. S., Pal, A. K., Sahu, N. P., Ciji, A., Meena, D. K., & Das, P. (2013). Physiological responses of dietary tryptophan fed *Labeo rohita* to temperature and salinity stress. Journal of Animal Physiology and Animal Nutrition, 97(6), 1075-1083. doi: 10.1111/jpn.12017

Alberdi, A., & Gilbert, M. T. P. (2019). A guide to the application of Hill numbers to DNA-based diversity analyses. Molecular Ecology Resources, 19(4), 804-817. doi: 10.1111/1755-0998.13014

Álvarez, D., & Nicieza, A. G. (2002). Effects of induced variation in anuran larval development on postmetamorphic energy reserves and locomotion. Oecologia, 131, 186-195. doi: 10.1007/s00442-002-0876-x

Anderson, T. R., Hessen, D. O., Boersma, M., Urabe, J., & Mayor, D. J. (2017). Will invertebrates require increasingly carbon-rich food in a warming world?. The American Naturalist, 190(6), 725-742. doi: 10.1086/694122

Anderson, W. A., & Magasanik, B. (1971). The pathway of myo-inositol degradation in *Aerobacter aerogenes*. Conversion of 2-deoxy-5-keto-D-gluconic acid to glycolytic intermediates. The Journal of Biological Chemistry, 246(18), 5662–5675. doi: 10.1016/S0021-9258(18)61857-5

Archie, E. A., & Theis, K. R. (2011). Animal behaviour meets microbial ecology. Animal Behaviour, 82(3), 425-436. doi: 10.1016/j.anbehav.2011.05.029

Arendt, J. D. (1997). Adaptive intrinsic growth rates: an integration across taxa. The Quarterly Review of Biology, 72(2), 149-177. doi:10.1086/419764

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D. R., ... & Bork, P. (2011). Enterotypes of the human gut microbiome. Nature, 473(7346), 174-180. doi: 10.1038/nature09944

Bastiani, V. I. M. (2023). Anuran amphibians in highland grasslands in southern Brazil: Effects of habitat, degradation, sensitivity and thermal tolerance. PhD Dissertation, Universidade Federal de Santa Maria, Brazil.

Behrens, M. D., & Lafferty, K. D. (2007). Temperature and diet effects on omnivorous fish performance: implications for the latitudinal diversity gradient in herbivorous fishes. Canadian Journal of Fisheries and Aquatic Sciences, 64(6), 867-873. doi: 10.1139/f07-063

Bercik, P., Collins, S. M., & Verdu, E. F. (2012). Microbes and the gut-brain axis. Neurogastroenterology & Motility, 24(5), 405-413. doi: 10.1111/j.1365-2982.2012.01906.x

Berg, G., Rybakova, D., Fischer, D., Cernava, T., Vergès, M. C. C., Charles, T., ... & Schloter, M. (2020). Microbiome definition re-visited: old concepts and new challenges. Microbiome, 8, 1-22. doi: 10.1186/s40168-020-00875-0

Berman, T., & Magasanik, B. (1966a). The pathway of myo-inositol degradation in *Aerobacter aerogenes*. Dehydrogenation and dehydration. The Journal of Biological Chemistry, 241(4), 800–806. doi: 10.1016/S0021-9258(18)96836-5

Berman, T., & Magasanik, B. (1966b). The pathway of myo-inositol degradation in Aerobacter aerogenes. Ring scission. The Journal of Biological Chemistry, 241(4), 807–813. doi: 10.1016/S0021-9258(18)96837-7

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., ... & Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nature Biotechnology, 37(8), 852-857. doi: 10.1038/s41587-019-0209-9

Borzée, A., Prasad, V. K., Neam, K., Tarrant, J., Kosch, T. A., Barata, I. M., … & Wren, S. (2025). Conservation priorities for global amphibian biodiversity. Nature Reviews Biodiversity. doi: 10.1038/s44358-025-00101-5

Brankatschk, M., Gutmann, T., Knittelfelder, O., Palladini, A., Prince, E., Grzybek, M., ... & Eaton, S. (2018). A temperature-dependent switch in feeding preference improves *Drosophila* development and survival in the cold. Developmental Cell, 46(6), 781-793. doi: 10.1016/j.devcel.2018.05.028

Cao, Y., Dong, Q., Wang, D., Zhang, P., Liu, Y., & Niu, C. (2022). microbiomeMarker: an R/Bioconductor package for microbiome marker identification and visualization. Bioinformatics, 38(16), 4027-4029. doi: 10.1093/bioinformatics/btac438

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., ... & Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences, 108(supplement_1), 4516-4522. doi: 10.1073/pnas.1000080107

Carreira, B. M., Segurado, P., Orizaola, G., Gonçalves, N., Pinto, V., Laurila, A., & Rebelo, R. (2016). Warm vegetarians? Heat waves and diet shifts in tadpoles. Ecology, 97(11), 2964-2974. doi: 10.1002/ecy.1541

Caspi, R., Billington, R., Ferrer, L., Foerster, H., Fulcher, C. A., Keseler, I. M., ... & Karp, P. D. (2016). The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. Nucleic acids research, 44(D1), D471-D480. doi: 10.1093/nar/gkv1164

Chen, X., D’Souza, R., & Hong, S. T. (2013). The role of gut microbiota in the gut-brain axis: current challenges and perspectives. Protein & Cell, 4, 403-414. doi: 10.1007/s13238-013-3017-x

Chiara, V., & Kim, S. Y. (2023). AnimalTA: A highly flexible and easy-to-use program for tracking and analysing animal movement in different environments. Methods in Ecology and Evolution 14: 1699–1707. doi: 10.1111/2041-210X.14115

Childers, L., Park, J., Wang, S., Liu, R., Barry, R., Watts, S. A., ... & Bagnat, M. (2025). Protein absorption in the zebrafish gut is regulated by interactions between lysosome rich enterocytes and the microbiome. eLife 13:RP100611. doi: 10.7554/eLife.100611

Collins, J. P., & Storfer, A. (2003). Global amphibian declines: sorting the hypotheses. Diversity and Distributions, 9(2), 89-98. doi: 10.1046/j.1472-4642.2003.00012.x

Corbin, K. D., Carnero, E. A., Dirks, B., Igudesman, D., Yi, F., Marcus, A., ... & Smith, S. R. (2023). Host-diet-gut microbiome interactions influence human energy balance: a randomized clinical trial. Nature Communications, 14(1), 3161. doi: 10.1038/s41467-023-38778-x

Croll, S. L., & Watts, S. A. (2004). The effect of temperature on feed consumption and nutrient absorption in *Procambarus clarkii* and *Procambarus zonangulus*. Journal of the World Aquaculture Society, 35(4), 478-488. doi: 10.1111/j.1749-7345.2004.tb00113.x

Devries, Z. C. & Appel, A. G. (2014). Effects of temperature on nutrient self-selection in the silverfish *Lepisma saccharina*. Physiological Entomology 39, 217–221. doi:

10.1111/phen.12064

Dinan, T. G., Stilling, R. M., Stanton, C., & Cryan, J. F. (2015). Collective unconscious: how gut microbes shape human behavior. Journal of Psychiatric Research, 63, 1-9. doi: 10.1016/j.jpsychires.2015.02.021

Dittrich, C., Rodríguez, A., Segev, O., Drakulić, S., Feldhaar, H., Vences, M., & Rödel, M. O. (2018). Temporal migration patterns and mating tactics influence size-assortative mating in *Rana temporaria*. Behavioral Ecology, 29(2), 418-428. doi:10.1093/beheco/arx188

Douglas, G. M., Maffei, V. J., Zaneveld, J. R., Yurgel, S. N., Brown, J. R., Taylor, C. M., ... & Langille, M. G. (2020). PICRUSt2 for prediction of metagenome functions. Nature Biotechnology, 38(6), 685-688. doi: 10.1038/s41587-020-0548-6

Douglas, A. E., & Werren, J. H. (2016). Holes in the hologenome: why host-microbe symbioses are not holobionts. MBio, 7(2), 10-1128. doi:10.1128/mBio.02099-15

Duarte, H., Tejedo, M., Katzenberger, M., Marangoni, F., Baldo, D., Beltrán, J. F., ... & Gonzalez-Voyer, A. (2012). Can amphibians take the heat? Vulnerability to climate warming in subtropical and temperate larval amphibian communities. Global Change Biology, 18(2), 412-421. doi: 10.1111/j.1365-2486.2011.02518.x

Eterovick, P. C., Mendes, I. S., Kloh, J. S., Pinheiro, L. T., Václav, A. B. H. P., Santos, T., & Gontijo, A. S. B. (2018). Tadpoles respond to background colour under threat. Scientific Reports, 8(1), 4085. doi: 10.1038/s41598-018-22315-8

Eterovick, P. C., Kloh, J. S., Figueredo, C. C., Viana, P. I. M., Goulart, M., Milan, D. T., ... & Vences, M. (2020). Background choice and immobility as context dependent tadpole responses to perceived predation risk. Scientific Reports, 10(1), 13577. doi: 10.1038/s41598-020-70274-w

Eterovick, P. C., Schmidt, R., Sabino-Pinto, J., Yang, C., Künzel, S., & Ruthsatz, K. (2024). The microbiome at the interface between environmental stress and animal health: an example from the most threatened vertebrate group. Proceedings of the Royal Society B, 291(2031), 20240917. doi:10.1038/s41598-018-22315-8

Fishman, A., Tao, Y., & Wood, T. K. (2004). Toluene 3-monooxygenase of *Ralstonia pickettii* PKO1 is a para-hydroxylating enzyme. Journal of Bacteriology, 186(10), 3117-3123. doi: 10.1128/jb.186.10.3117-3123.2004

Florkowski, M. R., & Yorzinski, J. L. (2023). Gut microbiome diversity and composition is associated with exploratory behavior in a wild-caught songbird. Animal Microbiome, 5(1), 8. doi: 10.1186/s42523-023-00227-x

Fontaine, S. S., & Kohl, K. D. (2023). The microbiome buffers tadpole hosts from heat stress: a hologenomic approach to understand host–microbe interactions under warming. Journal of Experimental Biology, 226(1), jeb245191. doi:10.1242/jeb.245191

Fontaine, S. S., Mineo, P. M., & Kohl, K. D. (2022). Experimental manipulation of microbiota reduces host thermal tolerance and fitness under heat stress in a vertebrate ectotherm. Nature Ecology & Evolution, 6(4), 405-417. doi: 10.1038/s41559-022-01686-2

Glos, J., Ruthsatz, K., Schröder, D., & Riemann, J. C. (2020). Food source determines stable isotope discrimination factors ΔN and ΔC in tadpoles. Amphibia-Reptilia, 41(4): 501-507. doi:10.1163/15685381-bja10020

González-Bergonzoni, I., Meerhoff, M., Davidson, T. A., Teixeira-de Mello, F., Baattrup-Pedersen, A., & Jeppesen, E. (2012). Meta-analysis shows a consistent and strong latitudinal pattern in fish omnivory across ecosystems. Ecosystems, 15, 492-503. doi:

10.1007/s10021-012-9524-4

Gosner, K.L., 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16: 183–190.

Guo, J., Li, Z., Liu, X., Jin, Y., Sun, Y., Yuan, Z., ... & Zhang, M. (2024). Response of the gut microbiota to changes in the nutritional status of red deer during winter. Scientific Reports, 14(1), 24961. doi: 10.1038/s41598-024-76142-1

Hardison, E. A., & Eliason, E. J. (2024). Diet effects on ectotherm thermal performance. Biological Reviews, 99(4), 1537-1555. doi: 10.1111/brv.13081

Hayes, T. B., Falso, P., Gallipeau, S., & Stice, M. (2010). The cause of global amphibian declines: a developmental endocrinologist's perspective. Journal of Experimental Biology, 213(6), 921-933. doi: 10.1242/jeb.040865

Hazel, J. R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation?. Annual Review of Physiology, 57(1), 19-42.

Hébert, M., Versace, E., & Vallortigara, G. (2019). Inexperienced preys know when to flee or to freeze in front of a threat. Proceedings of the National Academy of Sciences, 116(46), 22918-22920. doi: 10.1073/pnas.191550411

Henry, L. P., Bruijning, M., Forsberg, S. K., & Ayroles, J. F. (2021). The microbiome extends host evolutionary potential. Nature Communications, 12(1), 5141. doi: 10.1038/s41467-021-25315-x

Hill, M. O. (1973). Diversity and evenness: a unifying notation and its consequences. Ecology, 54(2), 427-432. doi: 10.2307/1934352

Hird, S. M. (2017). Evolutionary biology needs wild microbiomes. Front Microbiol 8: 725. doi: 10.3389/fmicb.2017.00725

Huda, M. N., Salvador, A. C., Barrington, W. T., Gacasan, C. A., D'Souza, E. M., Deus Ramirez, L., ... & Bennett, B. J. (2022). Gut microbiota and host genetics modulate the effect of diverse diet patterns on metabolic health. Frontiers in Nutrition, 9, 896348. doi: 10.3389/fnut.2022.896348

IPCC (2023). Summary for Policymakers. In: Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, H. Lee and J. Romero (eds.)]. IPCC, Geneva, Switzerland, pp. 1-34. doi: 10.59327/IPCC/AR6-9789291691647.001

Karp, P. D., Billington, R., Caspi, R., Fulcher, C. A., Latendresse, M., Kothari, A., Keseler, I. M., Krummenacker, M., Midford, P. E., Ong, Q., Ong, W. K., Paley, S. M., & Subhraveti, P. (2019). The BioCyc collection of microbial genomes and metabolic pathways. Briefings in Bioinformatics, 20(4), 1085–1093. doi: 10.1093/bib/bbx085

Kloh, J. S., Figueredo, C. C., Calaça, P., & Eterovick, P. C. (2024). Pollen as food: effects of consumption on tadpole growth, development, and mobility. Hydrobiologia, 851(8), 2071-2080. doi: 10.1007/s10750-023-05439-5

Kupferberg, S. J. (1997). The role of larval diet in anuran metamorphosis. American Zoologist 37, 146–159.

Legrand, T. P., Wynne, J. W., Weyrich, L. S., & Oxley, A. P. (2020). A microbial sea of possibilities: current knowledge and prospects for an improved understanding of the fish microbiome. Reviews in Aquaculture, 12(2), 1101-1134. doi: 10.1111/raq.12375

Lenth, R. (2017). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 0.9.1. https://CRAN.R-project.org/package=emmeans

Llobat, L., & Marín-García, P. J. (2022). Application of protein nutrition in natural ecosystem management for European rabbit (*Oryctolagus cuniculus*) conservation. Biodiversity and Conservation, 31(5), 1435-1444. doi: 10.1007/s10531-022-02426-5

Louca, S., Polz, M. F., Mazel, F., Albright, M. B., Huber, J. A., O'Connor, M. I., ... & Parfrey, L. W. (2018). Function and functional redundancy in microbial systems. Nature Ecology & Evolution, 2(6), 936-943. doi: 10.1038/s41559-018-0519-1

Lozupone, C., & Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. Applied and environmental microbiology, 71(12), 8228-8235. doi:10.1128/AEM.71.12.8228–8235.2005

Luedtke, J. A., Chanson, J., Neam, K., Hobin, L., Maciel, A. O., Catenazzi, A., ... & Stuart, S. N. (2023). Ongoing declines for the world's amphibians in the face of emerging threats. Nature, 622(7982), 308–314. https://doi.org/10.1038/s41586-023-06578-4

Lynch, J. B., & Hsiao, E. Y. (2019). Microbiomes as sources of emergent host phenotypes. Science, 365(6460), 1405-1409. Science 365: 1405–1409. doi: 10.1126/science.aay0240

Manning, D. W., & Sullivan, S. M. P. (2021). Conservation across aquatic-terrestrial boundaries: Linking continental-scale water quality to emergent aquatic insects and declining aerial insectivorous birds. Frontiers in Ecology and Evolution, 9, 633160. doi: 10.3389/fevo.2021.633160

McCallum, M. L. 2015. Vertebrate biodiversity losses point to a sixth mass extinction. Biodiversity and Conservation 24, 2497–2519. doi: 10.1007/s10531-015-0940-6

McDiarmid, R. W., & Altig, R. (Eds.). (1999). Tadpoles: the biology of anuran larvae. University of Chicago Press.

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., ... & Wernegreen, J. J. (2013). Animals in a bacterial world, a new imperative for the life sciences. Proceedings of the National Academy of Sciences, 110(9), 3229-3236. doi: 10.1073/pnas.1218525110

McMurdie, P. J., & Holmes, S. (2013). phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PloS one, 8(4), e61217. doi: 10.1371/journal.pone.0061217

Mekuchi, M., Asakura, T., Sakata, K., Yamaguchi, T., Teruya, K., & Kikuchi, J. (2018). Intestinal microbiota composition is altered according to nutritional biorhythms in the leopard coral grouper (*Plectropomus leopardus*). PloS one, 13(6), e0197256. doi: 10.1371/journal.pone.0197256

Melo, G. R., Solé, M., & Eterovick, P. C. (2021). Invisible or fearless: tadpole response to predator cues depends on color. Ethology Ecology & Evolution, 33(2), 99-107. doi: 10.1080/03949370.2020.1830859

Miri, S., Yeo, J., Abubaker, S., & Hammami, R. (2023). Neuromicrobiology, an emerging neurometabolic facet of the gut microbiome?. Frontiers in Microbiology, 14, 1098412. doi: 10.3389/fmicb.2023.1098412

Moura, S., Kloh, J. S., Figueredo, C. C., & Eterovick, P. C. (2023). An empty stomach is not a good adviser: avoiding toxic Cyanobacteria can compromise tadpole antipredator defenses. Amphibia-Reptilia, 44(4), 457-465. doi: 10.1163/15685381-bja10153

Newsome, S. D., Fogel, M. L., Kelly, L., & del Rio, C. M. (2011). Contributions of direct incorporation from diet and microbial amino acids to protein synthesis in Nile tilapia. Functional Ecology, 25(5), 1051-1062. doi: 10.1111/j.1365-2435.2011.01866.x

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'hara, R. B., ... & Wagner, H. (2013). Community ecology package. R package version, 2(0), 321-326.

Park, J. K., & Do, Y. (2024). Combined effect of seasons and life history in an anuran strengthens the response and relationship between their physiology and gut microbiota. Scientific Reports, 14(1), 10137. doi: 10.1038/s41598-024-60105-7

Peig, J., & Green, A. J. (2009). New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method. Oikos, 118(12), 1883-1891. doi:10.1111/j.1600-0706.2009.17643.x

Peig, J., & Green, A. J. (2010). The paradigm of body condition: a critical reappraisal of current methods based on mass and length. Functional Ecology, 24(6), 1323-1332. doi:10.1111/j.1365-2435.2010.01751.x

Perry, W. B., Lindsay, E., Payne, C. J., Brodie, C., & Kazlauskaite, R. (2020). The role of the gut microbiome in sustainable teleost aquaculture. Proceedings of the Royal Society B, 287(1926), 20200184. doi: 10.1098/rspb.2020.0184

Preston, D. B., & Forstner, M. R. (2015). Houston Toad (*Bufo (Anaxyrus) houstonensis*) tadpoles decrease their activity in response to chemical cues produced from the predation of conspecifics and congeneric (*Bufo (Incilius) nebulifer*) tadpoles. Journal of Herpetology, 49(2), 170-175. doi: 10.1670/13-059

R Core Team. 2024. R: A language and Environment for Statistical Computing. R Foundation for Statistical Computing. Version 4.4.2, Vienna, Austria. https://cran.r-project.org/

Relyea, R. A. (2001). Morphological and behavioral plasticity of larval anurans in response to different predators. Ecology, 82(2), 523-540. doi: 10.1890/0012-9658(2001)082[0523:MABPOL]2.0.CO;2

Ruddiman, W. F. (2013). The anthropocene. Annual Review of Earth and Planetary Sciences, 41(1), 45-68. doi: 10.1146/annurev-earth-050212-123944

Ruthsatz, K., Dausmann, K. H., Paesler, K., Babos, P., Sabatino, N. M., Peck, M. A., & Glos, J. (2020). Shifts in sensitivity of amphibian metamorphosis to endocrine disruption: the common frog (*Rana temporaria*) as a case study. Conservation Physiology, 8(1), coaa100. doi:10.1093/conphys/coaa100

Ruthsatz, K., Giertz, L. M., Schröder, D., & Glos, J. (2019). Chemical composition of food induces plasticity in digestive morphology in larvae of *Rana temporaria*. Biology Open, 8(12), bio048041. doi: 10.1242/bio.048041

Sampson, T. R., & Mazmanian, S. K. (2015). Control of brain development, function, and behavior by the microbiome. Cell Host & Microbe, 17(5), 565-576. doi: 10.1016/j.chom.2015.04.011

Schmitz, O. J., Rosenblatt, A. E. & Smylie, M. (2016). Temperature dependence of predation stress and the nutritional ecology of a generalist herbivore. Ecology 97, 3119–3130. doi: 10.1002/ecy.1524

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. Genome Biology, 12, 1-18. doi: 10.1186/gb-2011-12-6-r60

Seifert, L. I., de Castro, F., Marquart, A., Gaedke, U., Weithoff, G., & Vos, M. (2014). Heated relations: temperature-mediated shifts in consumption across trophic levels. PLoS One, 9(5), e95046. doi:10.1371/journal.pone.0095046

Semova, I., Carten, J. D., Stombaugh, J., Mackey, L. C., Knight, R., Farber, S. A., & Rawls, J. F. (2012). Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. Cell Host & Microbe, 12(3), 277-288. doi: 10.1016/j.chom.2012.08.003

Sharon, G., Segal, D., Ringo, J. M., Hefetz, A., Zilber-Rosenberg, I., & Rosenberg, E. (2010). Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. Proceedings of the National Academy of Sciences, 107(46), 20051-20056. doi: 10.1073/pnas.1009906107

Silva, Y. P., Bernardi, A., & Frozza, R. L. (2020). The role of short-chain fatty acids from gut microbiota in gut-brain communication. Frontiers in Endocrinology, 11, 508738. doi: 10.3389/fendo.2020.00025

Singmann H, Kellen D. (2019). An introduction to mixed models for experimental psychology. In New methods in cognitive psychology (eds DH Spieler, E Schumacher). Hove, UK: Psychology Press. doi:10.4324/9780429318405-2

Singmann H, Bolker B, Westfall J, Aust F, Ben-Shachar M (2024). _afex: Analysis of Factorial Experiments. doi: 10.32614/CRAN.package.afex <https://doi.org/10.32614/CRAN.package.afex>, R package version 1.4-1, https://CRAN.R-project.org/package=afex

Staniek, M. A., Pansch, C., Shama, L. N., Mehler, K., Steinmann, A., Middelburg, J. J., & Meysick, L. (2025). Heatwave intensity drives eco-physiological responses in infaunal bivalves: a mesocosm experiment. Limnology and Oceanography, 70, S417-S431. doi: 10.1002/lno.70012

Staudinger, M. D., Hanlon, R. T., & Juanes, F. (2011). Primary and secondary defences of squid to cruising and ambush fish predators: variable tactics and their survival value. Animal Behaviour, 81(3), 585-594. doi:10.1016/j.anbehav.2010.12.002

Swanson, H. K., Lysy, M., Power, M., Stasko, A. D., Johnson, J. D., & Reist, J. D. (2015). A new probabilistic method for quantifying n-dimensional ecological niches and niche overlap. Ecology, 96(2), 318-324. doi: 10.1890/14-0235.1

Teplitsky, C., Plénet, S., Léna, J. P., Mermet, N., Malet, E., & Joly, P. (2005). Escape behaviour and ultimate causes of specific induced defences in an anuran tadpole. Journal of Evolutionary Biology, 18(1), 180-190. doi: 10.1111/j.1420-9101.2004.00790.x

Trip, E. D. L., Clements, K. D., Raubenheimer, D., & Choat, J. H. (2014). Temperature-related variation in growth rate, size, maturation and life span in a marine herbivorous fish over a latitudinal gradient. Journal of Animal Ecology, 83(4), 866-875. doi: 10.1111/1365-2656.12183

Tseng, M., Di Filippo, C. M., Fung, M., Kim, J. O., Forster, I. P., & Zhou, Y. (2021). Cascading effects of algal warming in a freshwater community. Functional Ecology, 35(4), 920-929. doi: 10.1111/1365-2435.13752

Tuddenham, S., & Sears, C. L. (2015). The intestinal microbiome and health. Current Opinion in Infectious Diseases, 28(5), 464-470. doi: 10.1097/QCO.0000000000000196

Vejříková, I., Vejřík, L., Syväranta, J., Kiljunen, M., Čech, M., Blabolil, P., ... & Peterka, J. (2016). Distribution of herbivorous fish is frozen by low temperature. Scientific Reports, 6(1), 39600. doi: 10.1038/srep39600

Videvall, E., Burraco, P., & Orizaola, G. (2023). Impact of ionizing radiation on the environmental microbiomes of Chornobyl wetlands. Environmental Pollution, 330, 121774. doi: 10.1016/j.envpol.2023.121774

Voolstra, C. R., & Ziegler, M. (2020). Adapting with microbial help: microbiome flexibility facilitates rapid responses to environmental change. BioEssays, 42(7), 2000004. doi: 10.1002/bies.202000004

Wake, D. B., & Vredenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences, 105(supplement_1), 11466-11473. doi: 10.1073/pnas.0801921105

Wang, W., Zhou, R., He, L., Liu, S., Zhou, J., Qi, L., ... & Hu, D. (2015). The progress in nutrition research of musk deer: Implication for conservation. Applied Animal Behaviour Science, 172, 1-8. doi: 10.1016/j.applanim.2015.09.006

Wassersug, R. J. (1989). Locomotion in amphibian larvae (or" Why aren't tadpoles built like fishes?"). American Zoologist, 65-84.

Wells, K. D. (2019). The ecology and behavior of amphibians. University of Chicago press.

Wong, A. C. N., Holmes, A., Ponton, F., Lihoreau, M., Wilson, K., Raubenheimer, D., & Simpson, S. J. (2015). Behavioral microbiomics: a multi-dimensional approach to microbial influence on behavior. Frontiers in Microbiology, 6, 1359. doi: 10.3389/fmicb.2015.01359

Xiao, J., & Wang, W. X. (2025). Complex and lasting impacts of heatwaves on life-history traits and fitness in Daphnia magna. Journal of Experimental Biology, 228(18), jeb250837. doi:10.1242/jeb.250837

Yan, K., Guo, F., Kainz, M. J., Li, F., Gao, W., Bunn, S. E., & Zhang, Y. (2024). The importance of omega-3 polyunsaturated fatty acids as high-quality food in freshwater ecosystems with implications of global change. Biological Reviews, 99(1), 200-218. doi: 10.1111/brv.13017

Yang, C., Mai, J., Cao, X., Burberry, A., Cominelli, F., & Zhang, L. (2023). ggpicrust2: an R package for PICRUSt2 predicted functional profile analysis and visualization. Bioinformatics, 39(8), btad470. doi:10.1093/bioinformatics/btad470

Ye, L., Mueller, O., Bagwell, J., Bagnat, M., Liddle, R. A., & Rawls, J. F. (2019). High fat diet induces microbiota-dependent silencing of enteroendocrine cells. Elife, 8, e48479. doi: 10.7554/eLife.48479.

Zaneveld, J. R., McMinds, R., & Vega Thurber, R. (2017). Stress and stability: applying the Anna Karenina principle to animal microbiomes. Nature microbiology, 2(9), 1-8. doi: 10.1038/nmicrobiol.2017.121

Zhang, P., van Leeuwen, C. H. A., Bogers, D., Poelma, M., Xu, J. & Bakker, E. S. (2020). Ectothermic omnivores increase herbivory in response to rising temperature. Oikos 129, 1–12. doi: 10.1111/oik.07082

Zhou, C., Yang, S., Ka, W., Gao, P., Li, Y., Long, R., & Wang, J. (2022). Association of gut microbiota with metabolism in rainbow trout under acute heat stress. Frontiers in Microbiology, 13, 846336. doi: 10.3389/fmicb.2022.846336

Supplementary material

Animal husbandry and experimental setup

The transport of egg clutches took approximately 30 minutes. Upon arrival, clutches were carefully transferred to separate trays containing about 10 L of water from the original habitat and equipped with aerators. Larvae hatched on 2 April 2023. Both clutches and newly hatched larvae were maintained in a large room with windows along two walls, which were kept open to expose the animals as closely as possible to natural light and temperature conditions. Approximately one third of the water was replaced every two days with fresh water from the original habitat. This replacement water was collected every three days and stored at 4 °C in buckets. Before use, buckets were placed in the same room as the animals until the water reached the same temperature as that in the rearing containers (14 ± 0.2 °C).

Nine days after hatching, larvae reached developmental stage 25 (*sensu* Gosner, 1960), the point at which they deplete yolk reserves and begin feeding independently. At this stage, 120 larvae were placed individually into 1.2-L buckets containing 1 L of filtered, rested tap water and kept under a 14:10 h light:dark cycle. Larvae were randomly assigned to three food treatments (Fig. 1). Buckets for the 18 °C treatment were placed in a climate chamber (Kälte-Klimatechnik-Frauenstein GmbH, Germany). For the 24.5 °C treatment, buckets were placed inside a water bath housed within large plastic boxes (Surplus Systems Eurobox, 60 × 40 × 22 cm) in a different room, with temperature regulated by two adjustable heating elements (JBL PROTEMP S 25, 25 W, JBL GmbH & Co. KG, Germany). Water temperature in the buckets was gradually increased at a rate of 0.5 °C per hour until the target temperature was reached.

Diet quality was classified based on component diversity, protein and fat levels, and caloric content. The organic grass powder contains only one plant species and has lower caloric, protein, and fat content, whereas the fish food contains a wide range of ingredients (algae, zooplankton, plant and animal products) and is higher in calories, protein, and fat.

The powders used in all three diets have similar texture and solubility. They remain suspended in water for a short time before settling, ensuring that the feeding mechanisms of frog larvae - filtering and scraping surfaces - provide equal access to both powders when mixed at a 50:50 ratio. All diets were provided *ad libitum*. Buckets were cleaned at least every three days by completely replacing the water with rested tap water at the same temperature, during which each larva was briefly (<1 min) transferred to a sieve placed in a separate bucket of clean water.

Buckets assigned to the heatwave treatment were placed in a water bath inside plastic boxes (60 × 40 × 22 cm) containing two adjustable heating elements. The setup was housed in a warmer room (29 °C air temperature). Prior to the experiment, the heating system was calibrated to ensure accurate temperature ramping, and water temperatures were monitored hourly. Water temperature in the buckets was increased at a rate of 0.5 °C per hour until reaching 28 °C. Because ramping protocols were identical and final temperatures (i.e., original rearing temperatures) differed, larvae reared at 18 °C required more time to reach 28 °C and return (20 h total) than larvae reared at 24.5 °C (7 h total). Buckets assigned to the control treatment (no heatwave) were also moved and returned to their original positions during treatment allocation so that handling was standardized across experimental groups.

Methods for isotope analyses

Larval tails were dried in an oven at 60 °C for at least 24 hours. Subsequently, tail muscle tissue samples weighing 0.38-0.93 mg (mean = 0.76 mg) were taken in duplicate for each larva and placed in 4 × 6 mm tin cups (HEKAtech, Germany). The powdered foods corresponding to the three dietary treatments (NaturaleBio® grass powder, Sera Micron Nature® fish food, and a 50:50 mixture of both) were also analyzed, with six replicates per diet.

Samples were combusted in a mass spectrometer (EURO-EA 3000, Euro Vector, Italy) using BBOT (2,5-Bis-(5-tert-butyl-2-benzoxazolyl)-thiophen; 6.51% N; 72.52% C; HEKAtech, Germany), KNO_3, and caffeine as standards. Isotope ratios are reported in δ notation (‰) relative to atmospheric nitrogen (AIR) for $\delta^{15}N$ and Pee Dee Belemnite (PDB) for $\delta^{13}C$, following international reference standards (Fry, 2006).

Sequence quality filtering, sample depth, and taxonomic assignment

Paired-end demultiplexed FASTQ files were imported into QIIME2 and denoised using the q2-deblur algorithm, which applies quality filtering based on Bokulich et al. (2013), associates erroneous sequences with their true biological sequences, and removes chimeras. Forward and reverse reads were paired, quality filtered, and trimmed to a high-quality length (median Illumina Q30), resulting in 250 bp sequences. Of the initial 2,737,481 reads, 148,401 remained after filtering, with sequencing depths between 207 and 5,451 reads per sample. All negative controls (five extraction controls and two PCR controls) yielded zero reads after filtering.

A phylogenetic tree was constructed using the Greengenes 16S rRNA backbone tree (version gg-13-8; McDonald et al., 2012). Taxonomic classification was performed using a custom-trained classifier built with reference sequences, taxonomy, and animal proximal gut–specific sequence weights (SILVA release 138.1, 515F/806R) from Kaehler et al. (2019; https://github.com/BenKaehler/readytowear). Positive controls were evaluated separately via BLAST (NCBI; Sayers et al., 2025) because they do not represent animal gut samples.

Amplicon Sequence Variants (ASVs) represented by fewer than eight reads (~0.005% of total remaining sequences) were removed to minimize artifacts from amplification errors (Bokulich et al., 2013). The remaining reads were used to calculate Shannon entropy, which reached saturation at 556 reads. Thus, samples with fewer than 556 reads (15 samples, one to three per treatment) were excluded from further analyses. Because a very high number of samples was removed during quality filtering, we also estimated sample coverage and tested for curve stabilization using rarefaction in iNext (Chao et al., 2014). Sample coverage values were close to one and diversity curves stabilized for all maintained samples, indicating that our results were robust despite low sample depth.

References

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., … Caporaso, J. G. (2013). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nature Methods, 10(1), 57-59. doi: 10.1038/nmeth.2276

Chao, A., Gotelli, N. J., Hsieh, T. C., Sander, E. L., Ma, K. H., Colwell, R. K., & Ellison, A. M. (2014). Rarefaction and extrapolation with Hill numbers: a framework for sampling and estimation in species diversity studies. Ecological Monographs, 84(1), 45-67. doi: 10.1890/13-0133.1

Fry, B.G. (2006): Stable Isotope Ecology. Springer, New York.

Gosner, K.L., 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16: 183–190.

Kaehler, B. D., Bokulich, N. A., McDonald, D., Knight, R., Caporaso, J. G., & Huttley, G. A. (2019). Species-level microbial sequence classification is improved by source-environment information. Nature Communications 10: 4643. doi: 10.1038/s41467-019-12669-6

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., DeSantis, T. Z., Probst, A., ... & Hugenholtz, P. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. The ISME Journal, 6(3), 610-618. ISME J. 6, 610–618. doi: 10.1038/ismej.2011.139

Sayers, E. W., Beck, J., Bolton, E. E., Brister, J. R., Chan, J., Connor, R., ... & Pruitt, K. D. (2025). Database resources of the National Center for Biotechnology Information in 2025. Nucleic Acids Research, 53(D1), D20-D29. doi: 10.1093/nar/gkae979

Supplementary figures

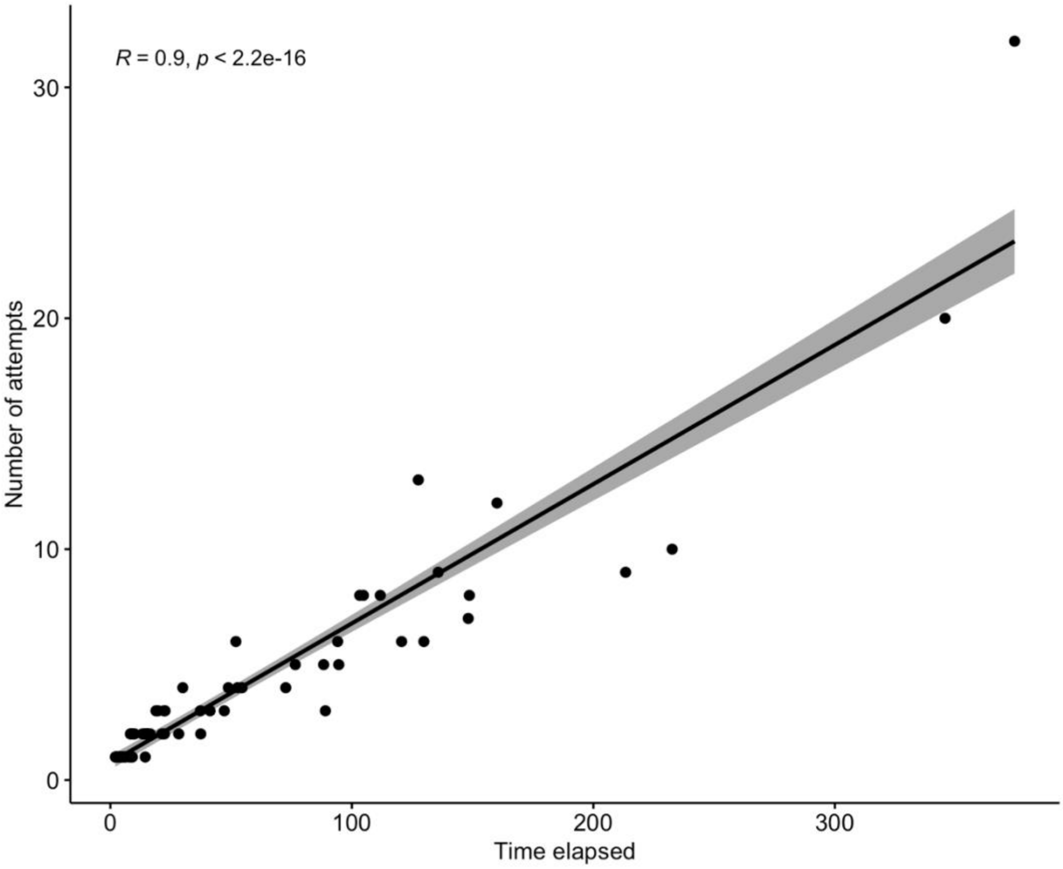

Fig. S1. Correlation between time elapsed from the end of the 3 minutes larvae remained under the funnel and the actual start of the behavioral trial (when the dragonfly naiad model touched the water) and number of attempts (number of times the larva had to be repositioned on the center of the tray). Refer to the section “Behavioral trials” for a detailed description of escape behavior trials of *Rana temporaria* larvae.

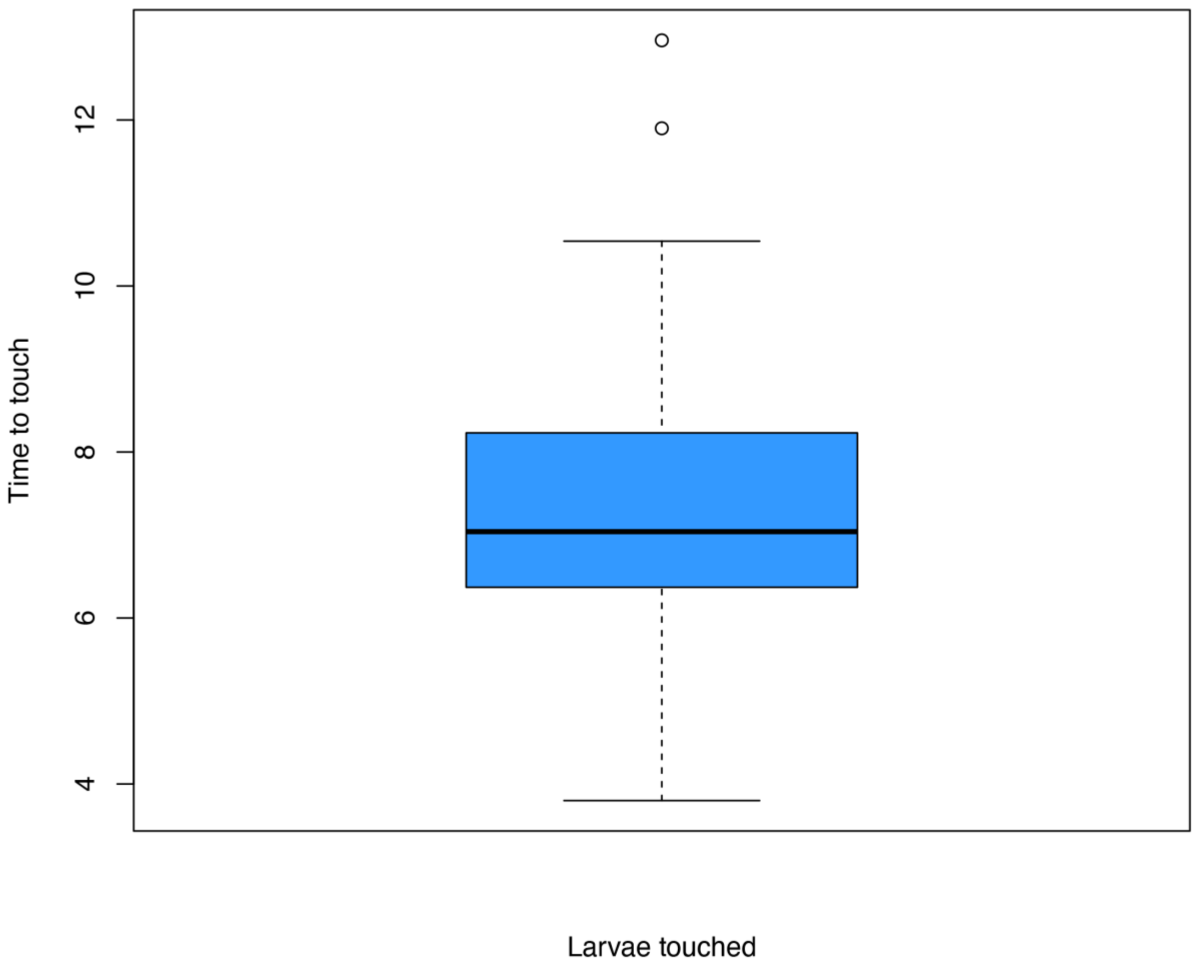

Fig. S2. *Rana temporaria* larvae were tested for escape behavior to an aversive stimulus represented by an approaching transparent plastic pipette filled with 4 ml of water containing chemical predator cues to be released and a predator model glued to the top of the pipette. The graph shows the time elapsed from the moment the predator model touched the water to the moment it touched the larvae (when it happened) in behavioral trials (n = 102 trials). The two outliers above were excluded from posterior analyses.

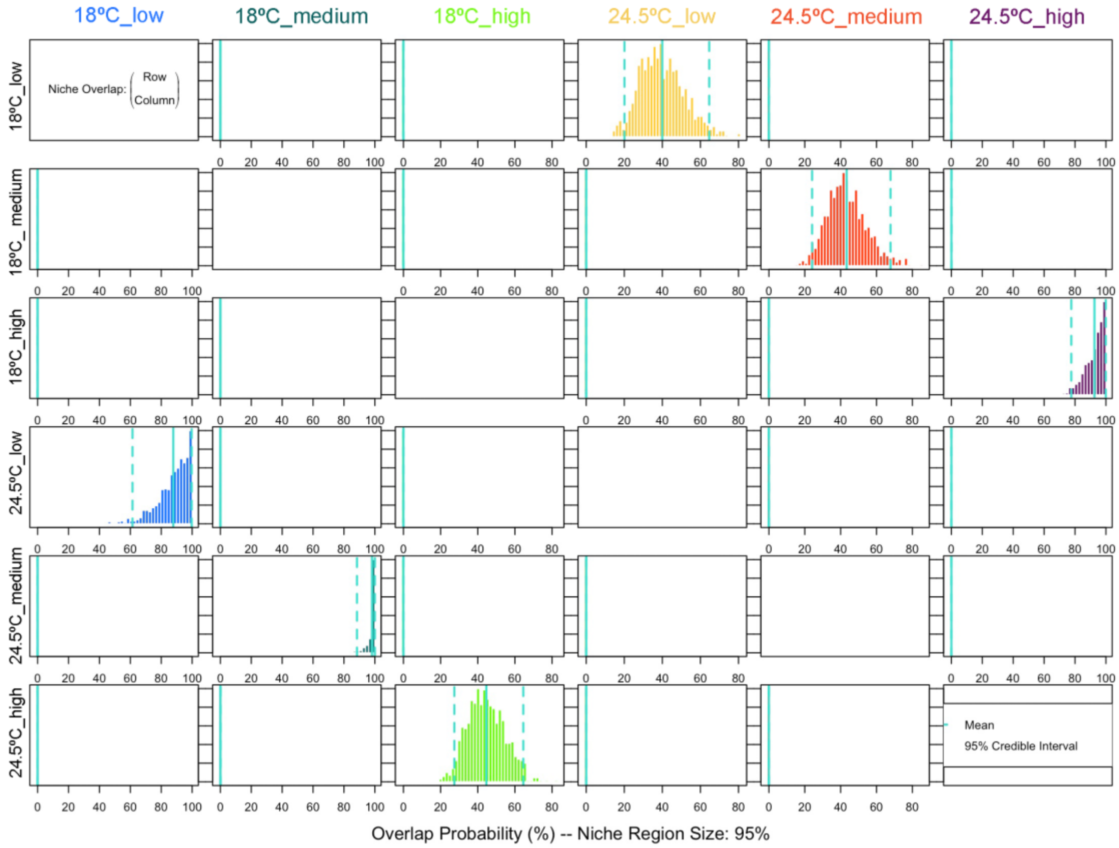

Fig. S3. Posterior distribution of the probabilistic niche overlap of *Rana temporaria* larvae reared with three different diets varying in nutritional quality and two temperatures in a crossed experimental design (colors correspond to treatments on the columns). Niche overlap metrics were generated by the package nicheROVER (Swanson et al., 2015). The probability distribution of species displayed in rows overlapping onto those displayed in columns is presented as well as posterior means (turquoise continuous lines) and 95% credible intervals (turquoise dashed lines).

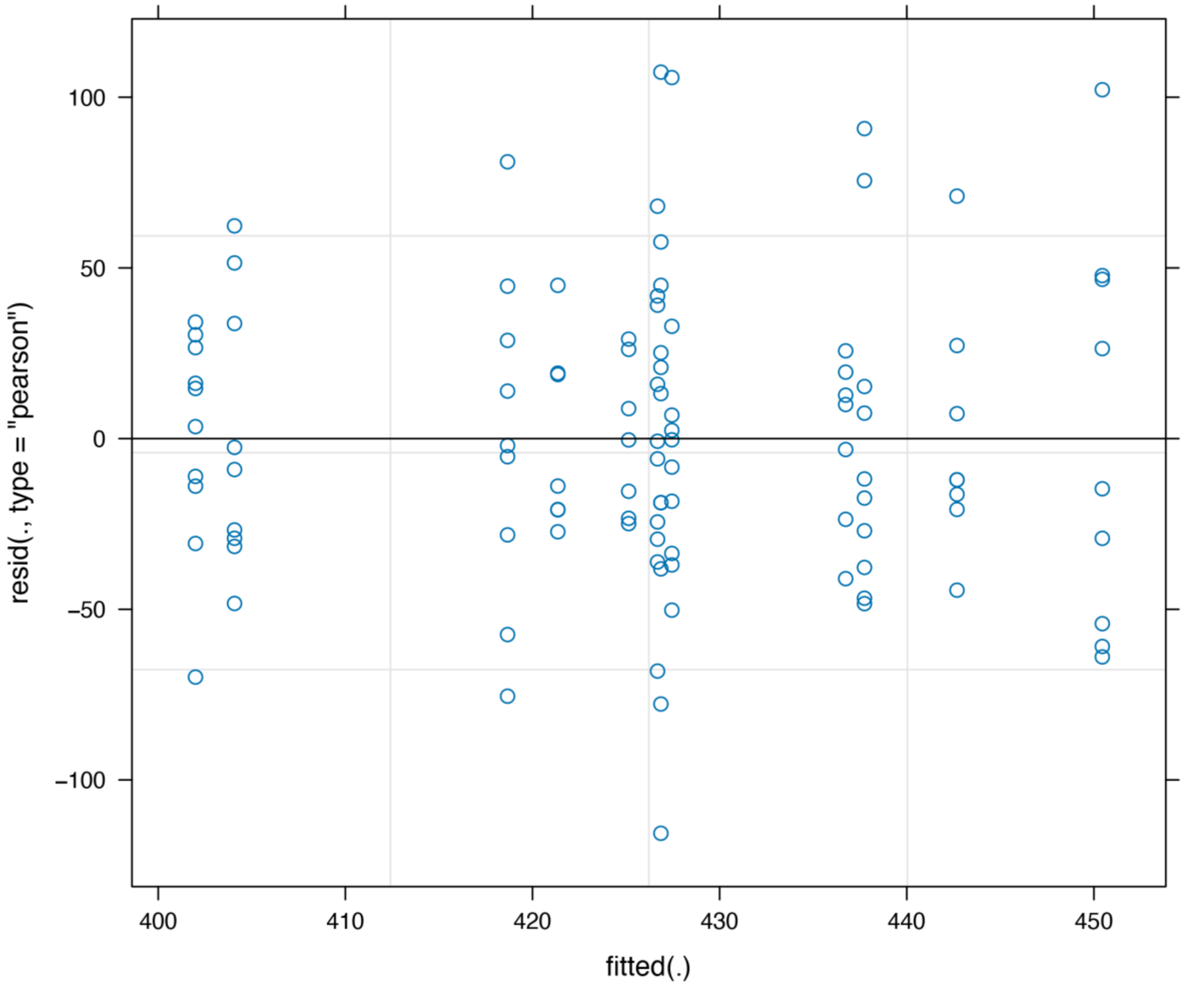

Fig. S4. Residual distribution of the model testing the effects of food treatment, rearing temperature, and exposure or not to a heatwave on body condition (SMI) of *Rana temporaria* larvae (see Table 1 for model description).

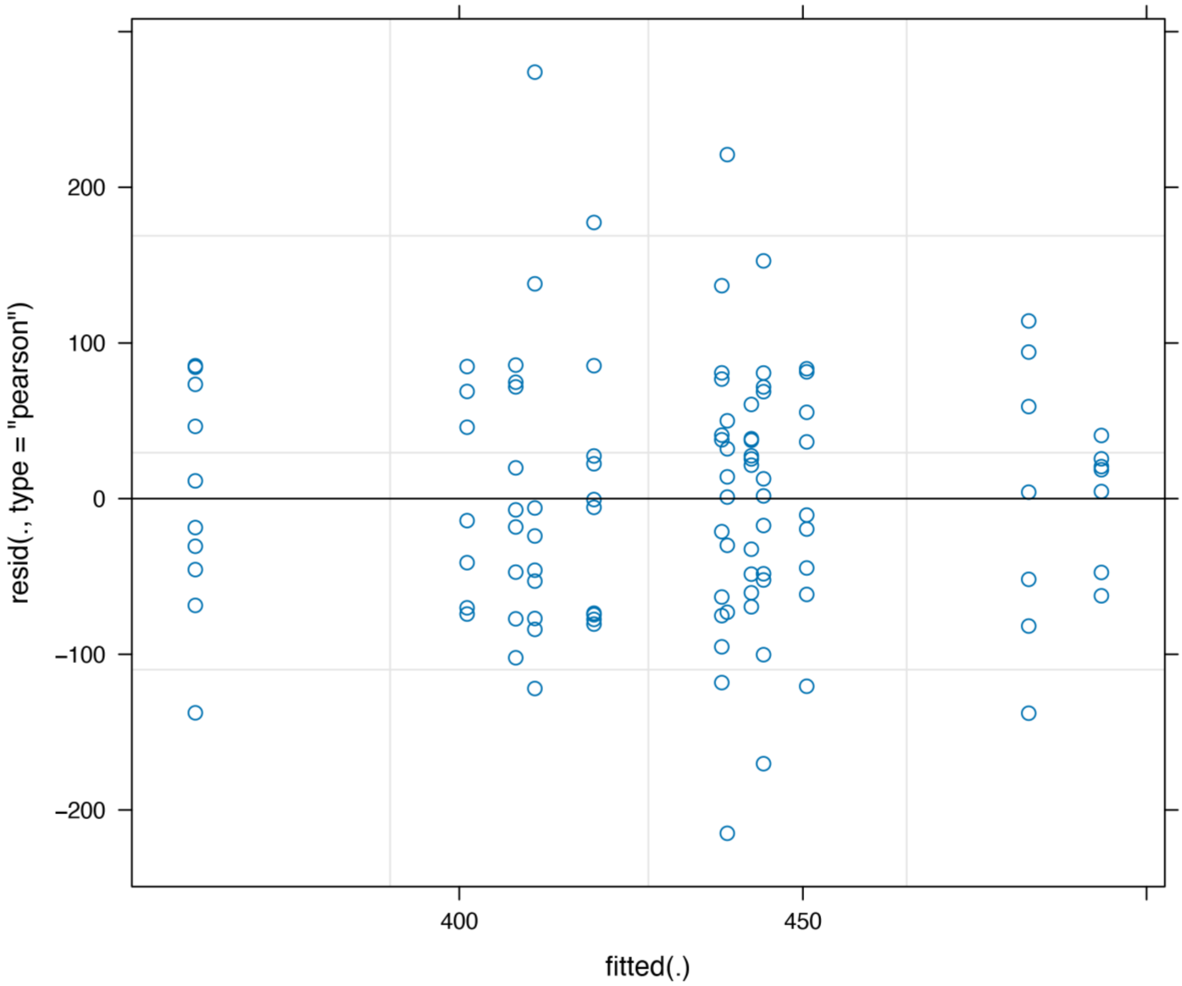

Fig. S5. Residual distribution of the model testing the effects of food treatment, rearing temperature, and exposure or not to a heatwave on mass of *Rana temporaria* larvae (see Table 1 for model description).

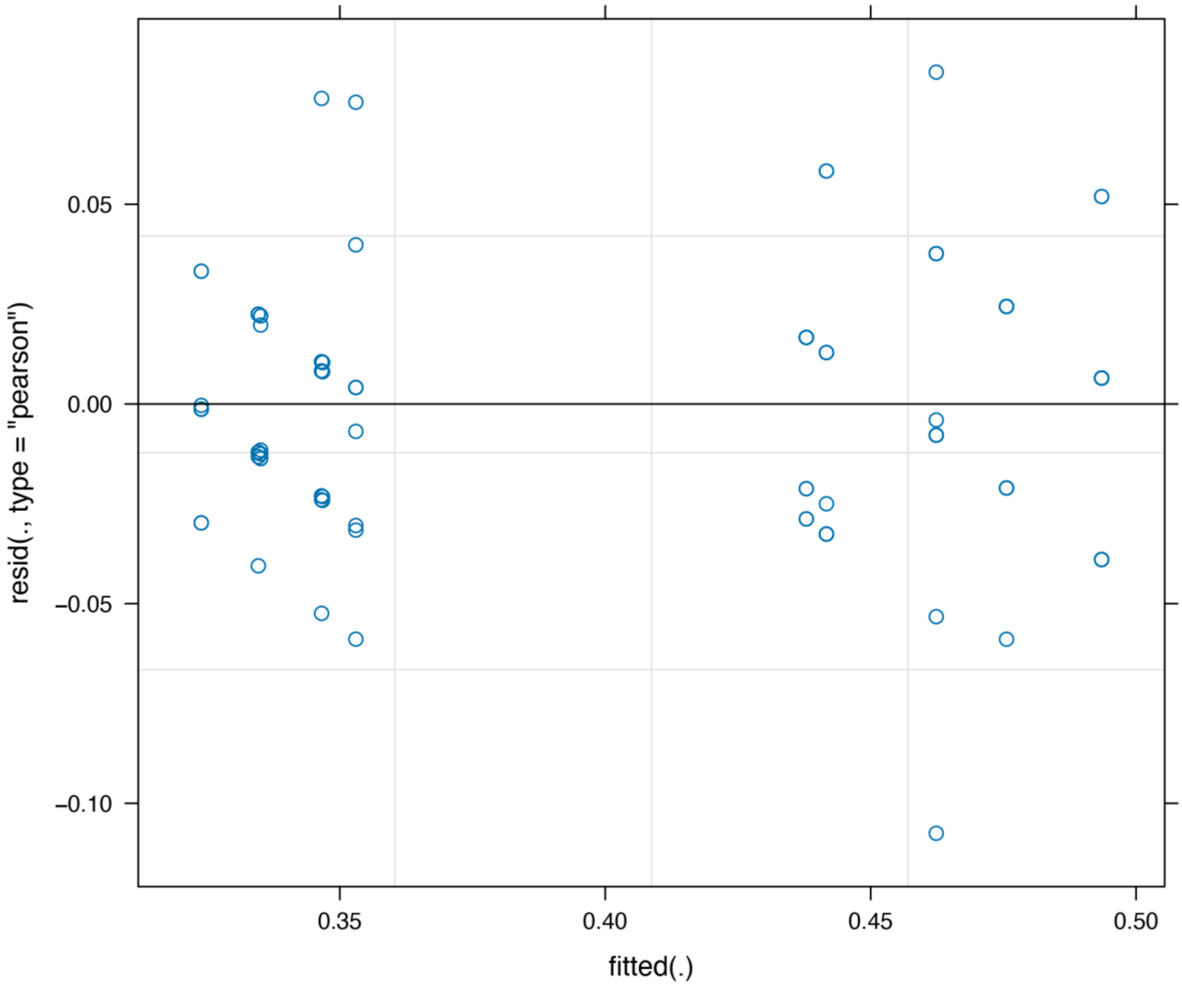

Fig. S6. Residual distribution of the model testing the effects of food treatment, rearing temperature, and exposure or not to a heatwave on developmental rate of *Rana temporaria* larvae (see Table 1 for model description).

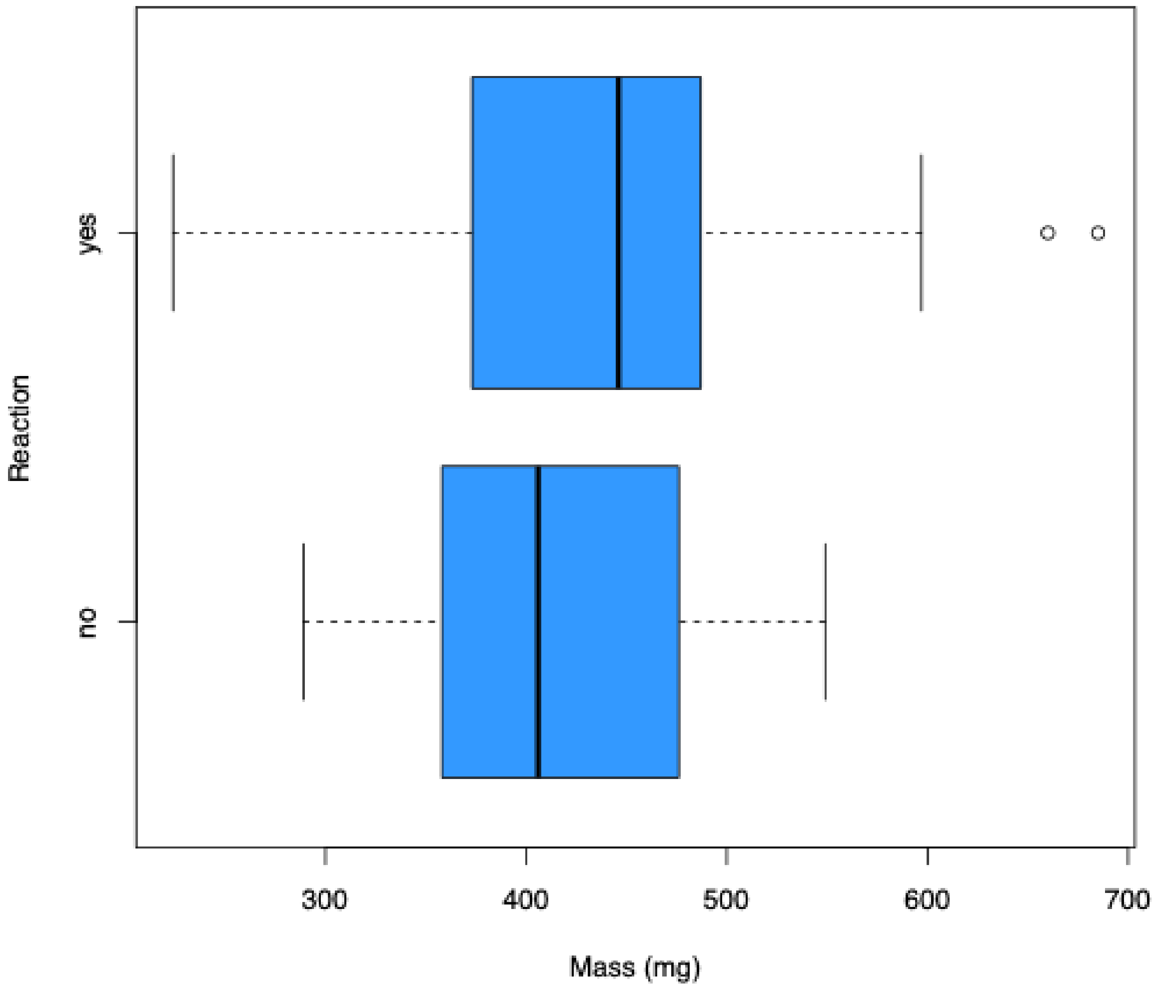

Fig. S7. Mass (mg) of *Rana temporaria* larvae that either reacted to the aversive stimulus presented in behavioral trials (1) or not (0). Wilcoxon-test: W = 711.5, p = 0.252.

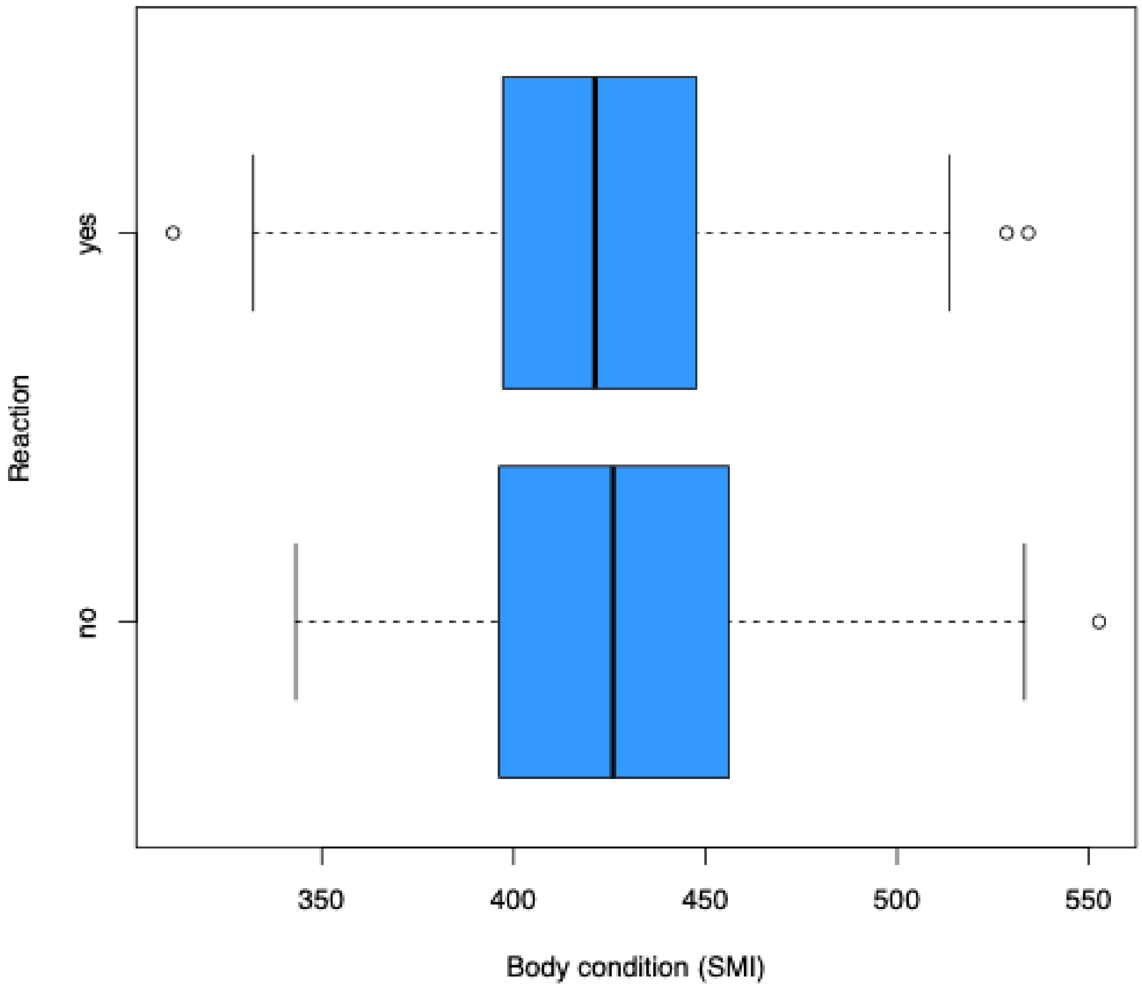

Fig. S8. Body condition (SMI) of *Rana temporaria* larvae that either reacted to the aversive stimulus presented in behavioral trials (1) or not (0). Wilcoxon-test: W = 936, p = 0.482.

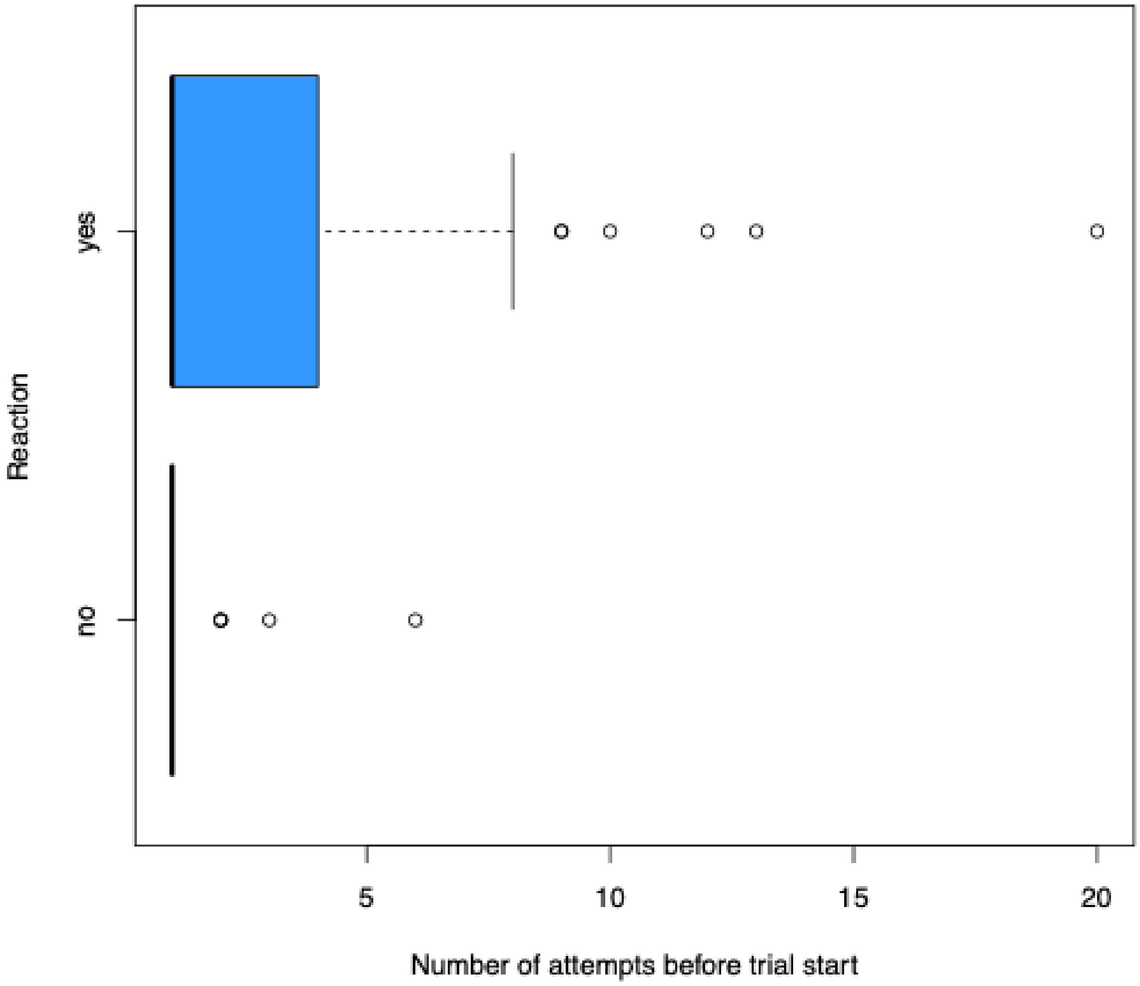

Fig. S9. Number of attempts to position *Rana temporaria* larvae before the start of the behavioral trials compared between larvae that either reacted to the aversive stimulus presented in behavioral trials (1) or not (0). Wilcoxon-test: W = 601, p = 0.022.

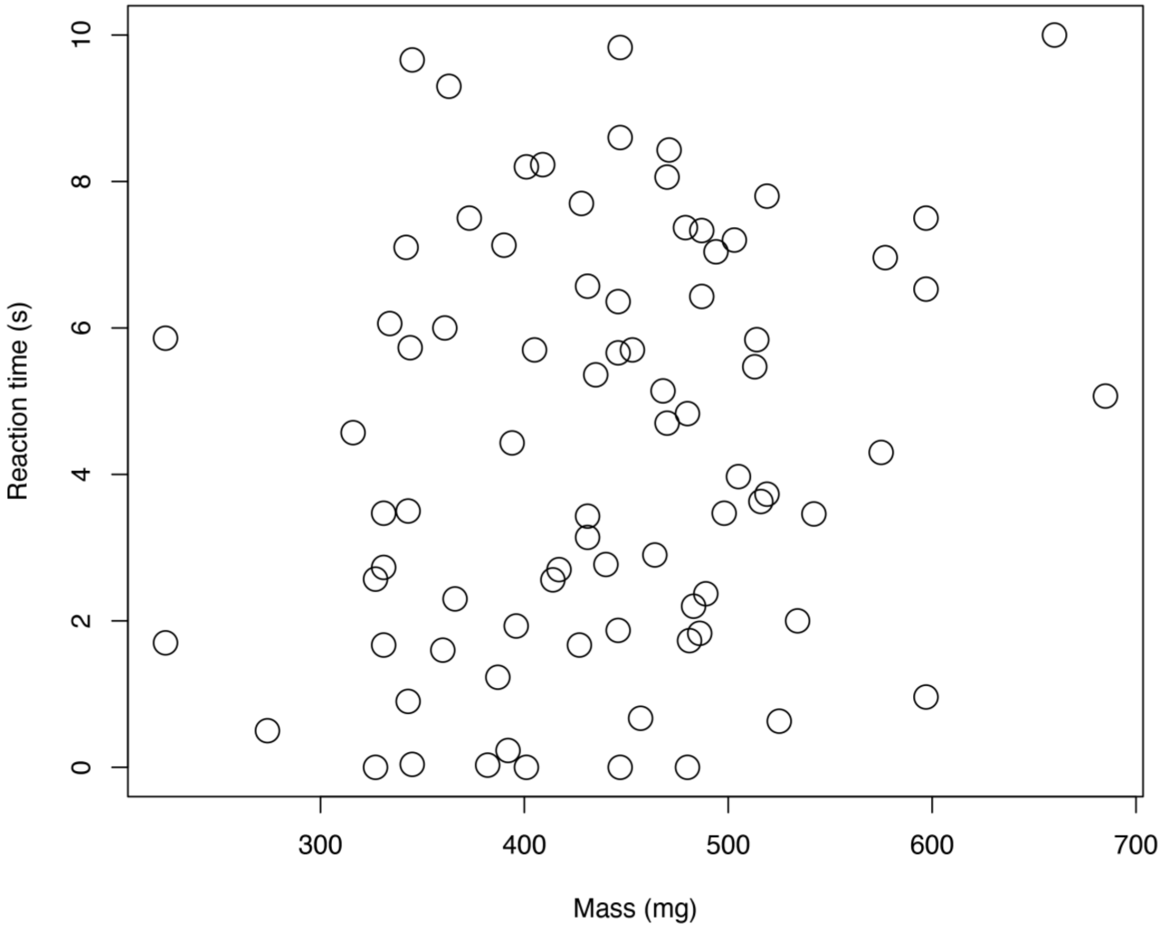

Fig. S10. Relationship between mass (mg) of *Rana temporaria* larvae and time to react to the aversive stimulus presented in behavioral trials. Adjusted R-squared = 0.030, F = 3.487, df = 79, p = 0.066.

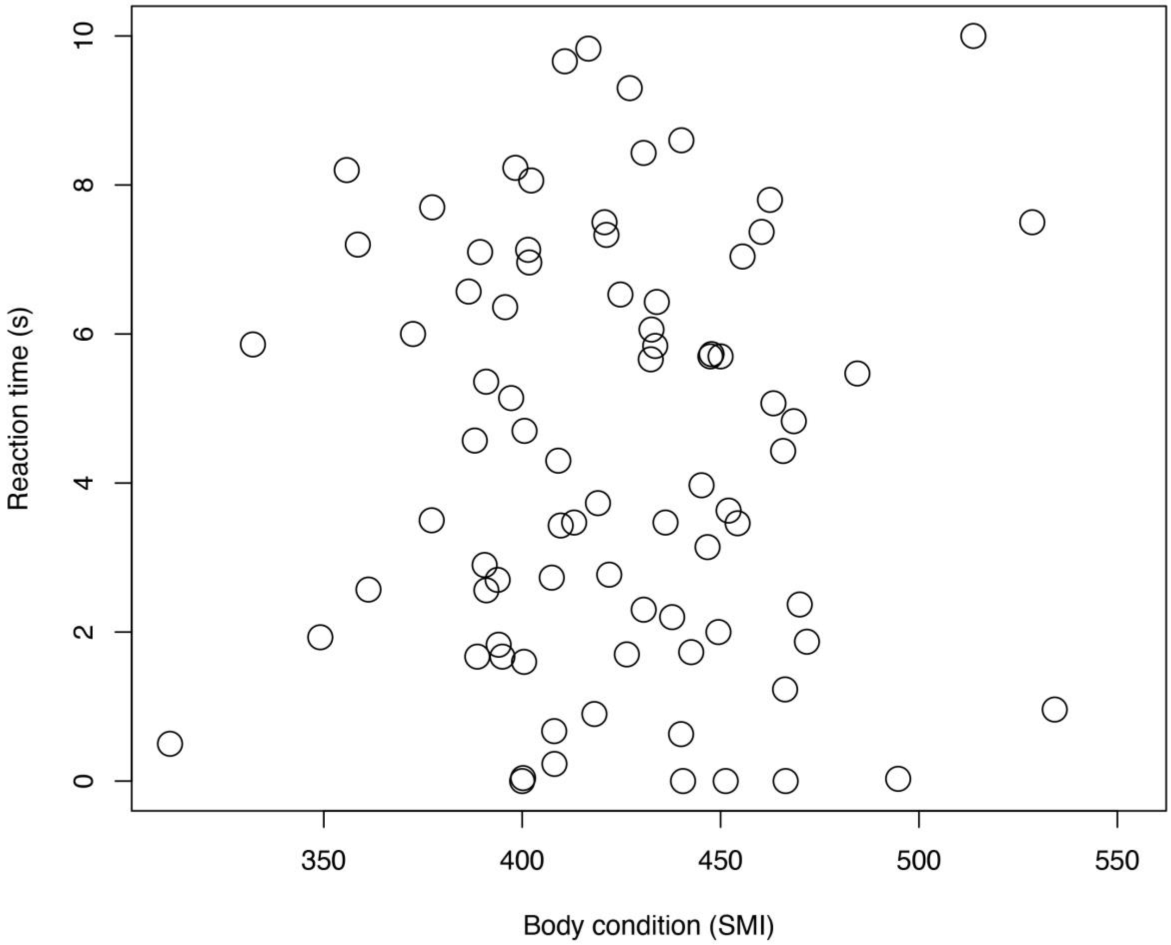

Fig. S11. Relationship between body condition (SMI) of *Rana temporaria* larvae and time to react to the aversive stimulus presented in behavioral trials. Adjusted R-squared = -0.013, F = 0.005, df = 79, p = 0.946.

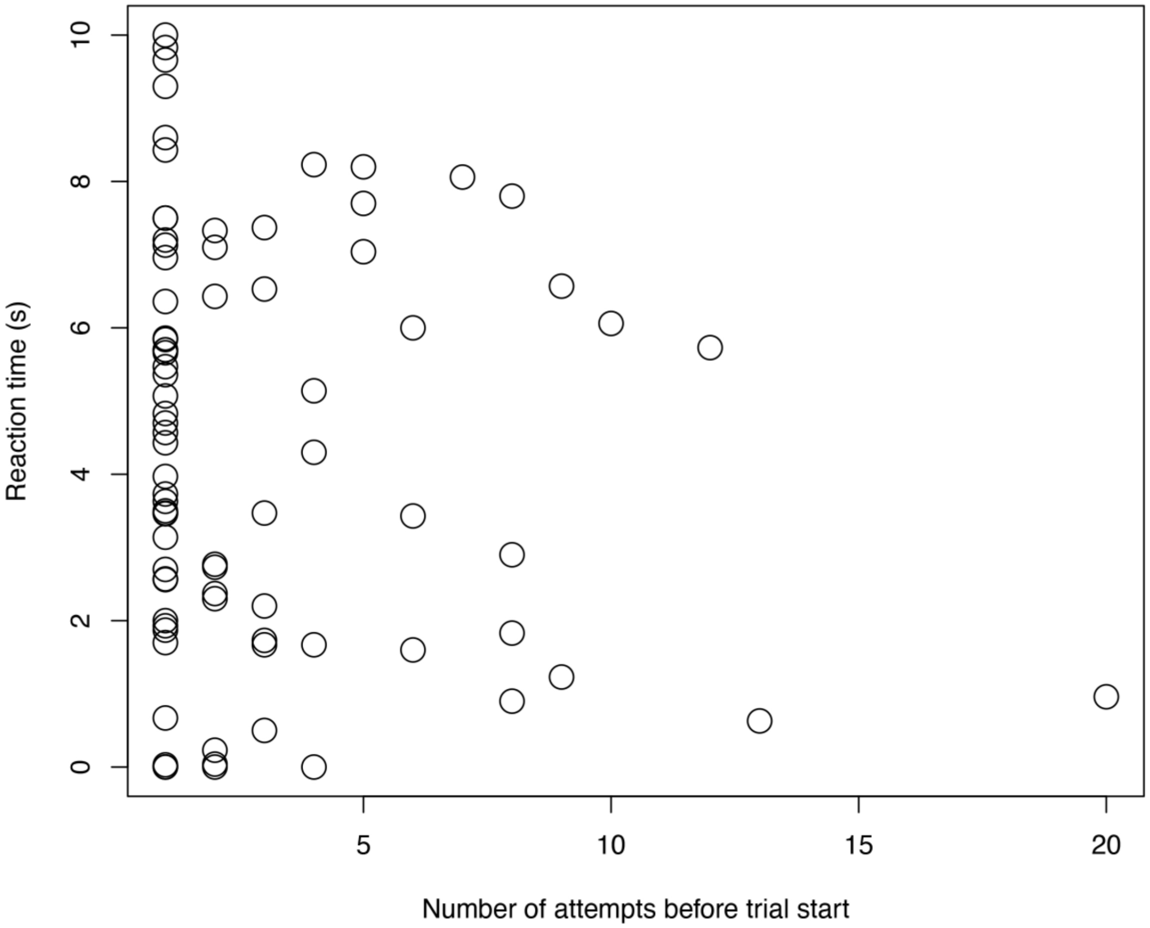

Fig. S12. Relationship between number of attempts to position *Rana temporaria* larvae before the start of the behavioral trials and time (s) the larvae took to react to the aversive stimulus presented. rho = -0.125, p = 0.263.

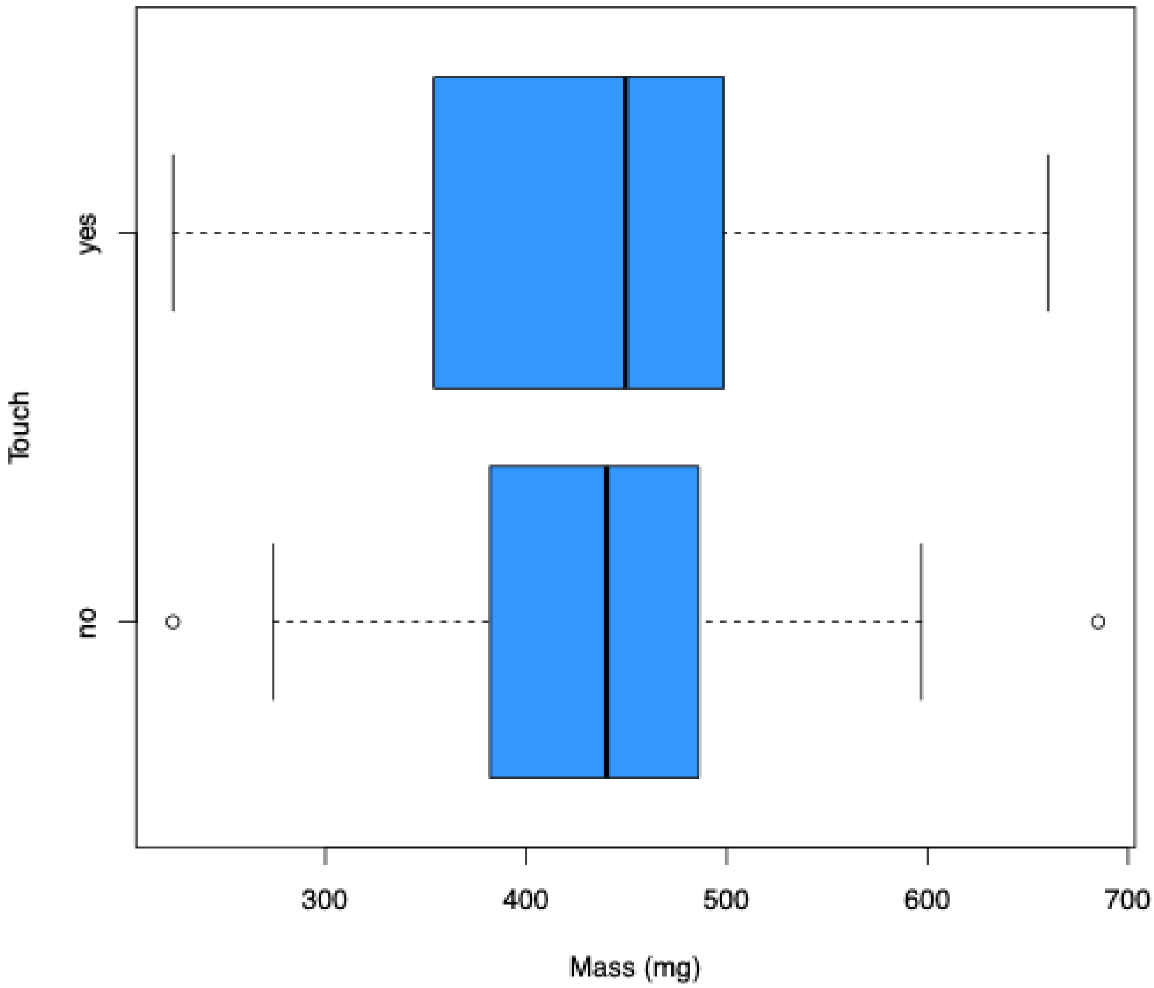

Fig. S13. Mass (mg) of reacting *Rana temporaria* larvae that either were touched by the predator model approached to them in behavioral trials (1) or not (0) before fleeing. Wilcoxon-test: W = 577, p = 0.722.

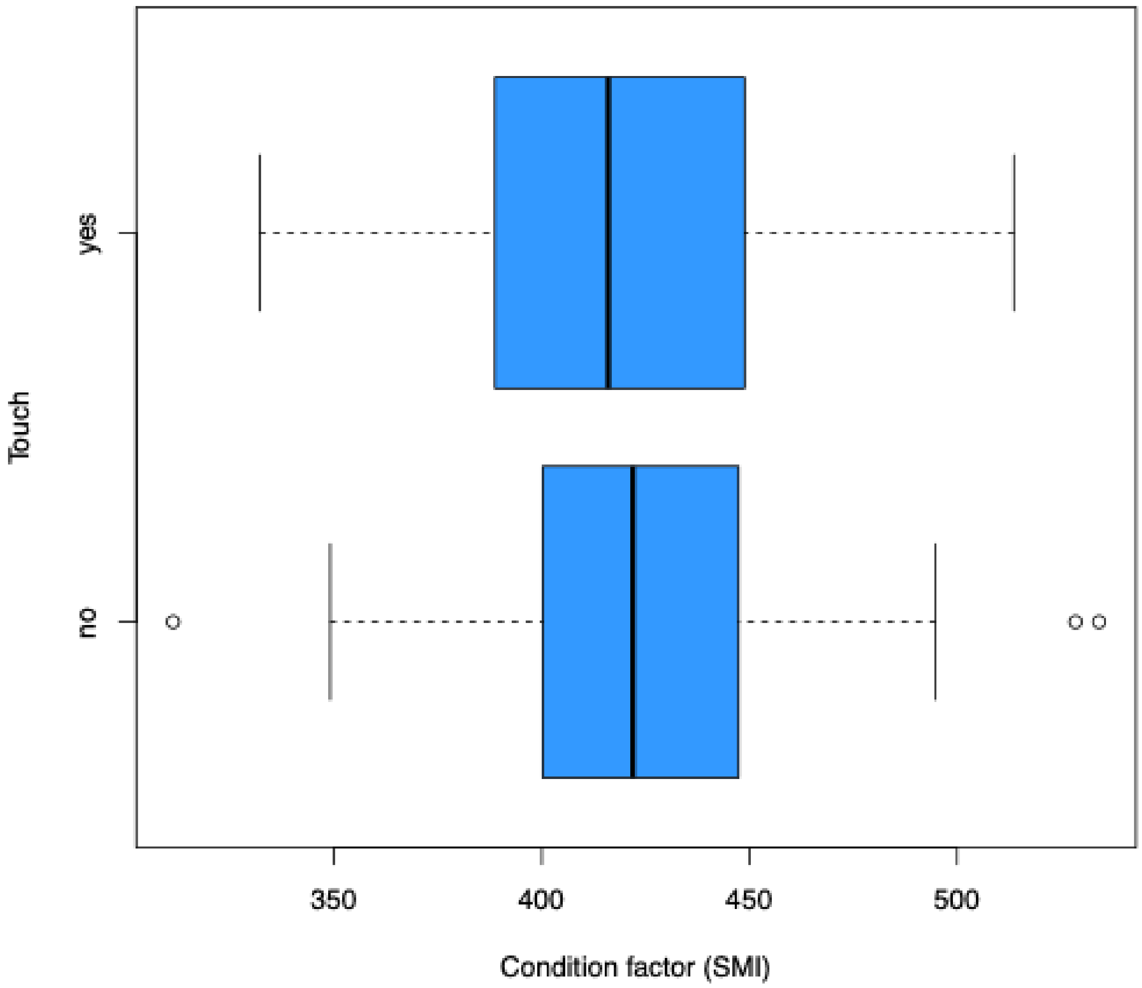

Fig. S14. Condition factor (SMI) of reacting *Rana temporaria* larvae that either were touched by the predator model approached to them in behavioral trials (1) or not (0) before fleeing. Wilcoxon-test: W = 697, p = 0.343.

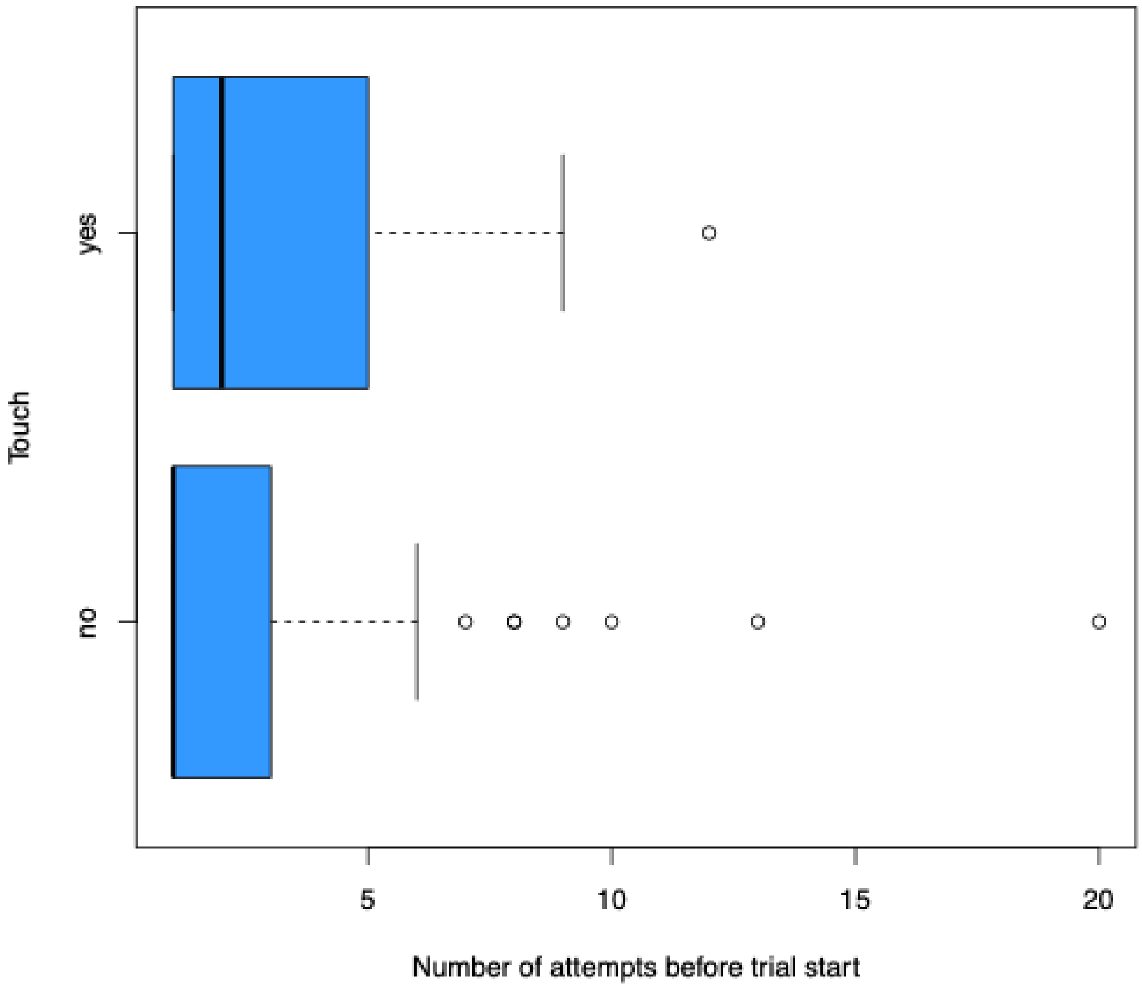

Fig. S15. Number of attempts to position *Rana temporaria* larvae before the start of the behavioral trials compared between larvae that either were touched by the predator model approached to them in the behavioral trials (1) or not (0) before fleeing. Wilcoxon-test: W = 533, p = 0.366.

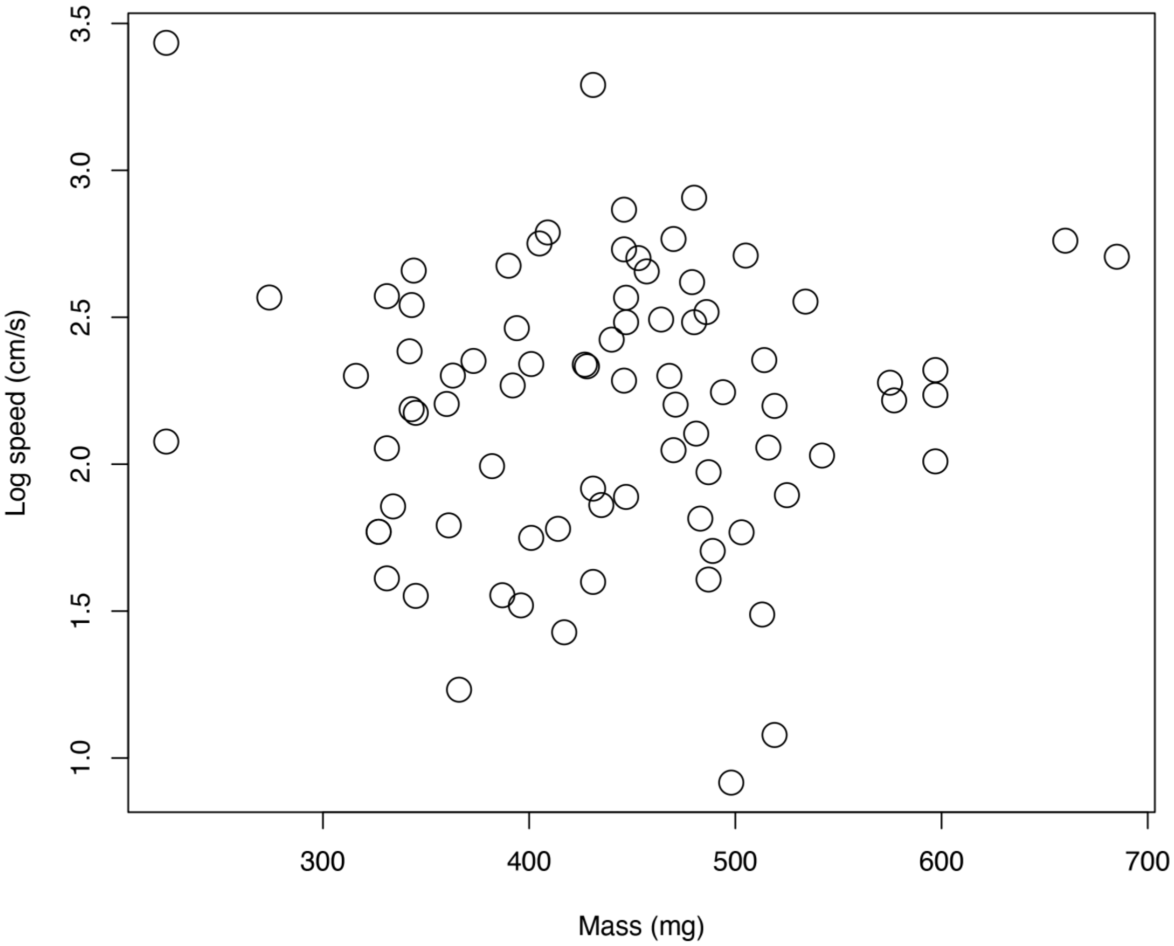

Fig. S16. Relationship between mass (mg) of *Rana temporaria* larvae and average speed (in cm/s, log transformed) while fleeing from the aversive stimulus presented in behavioral trials. Adjusted R-squared = -0.013, F = 0.004, df = 79, p = 0.949.

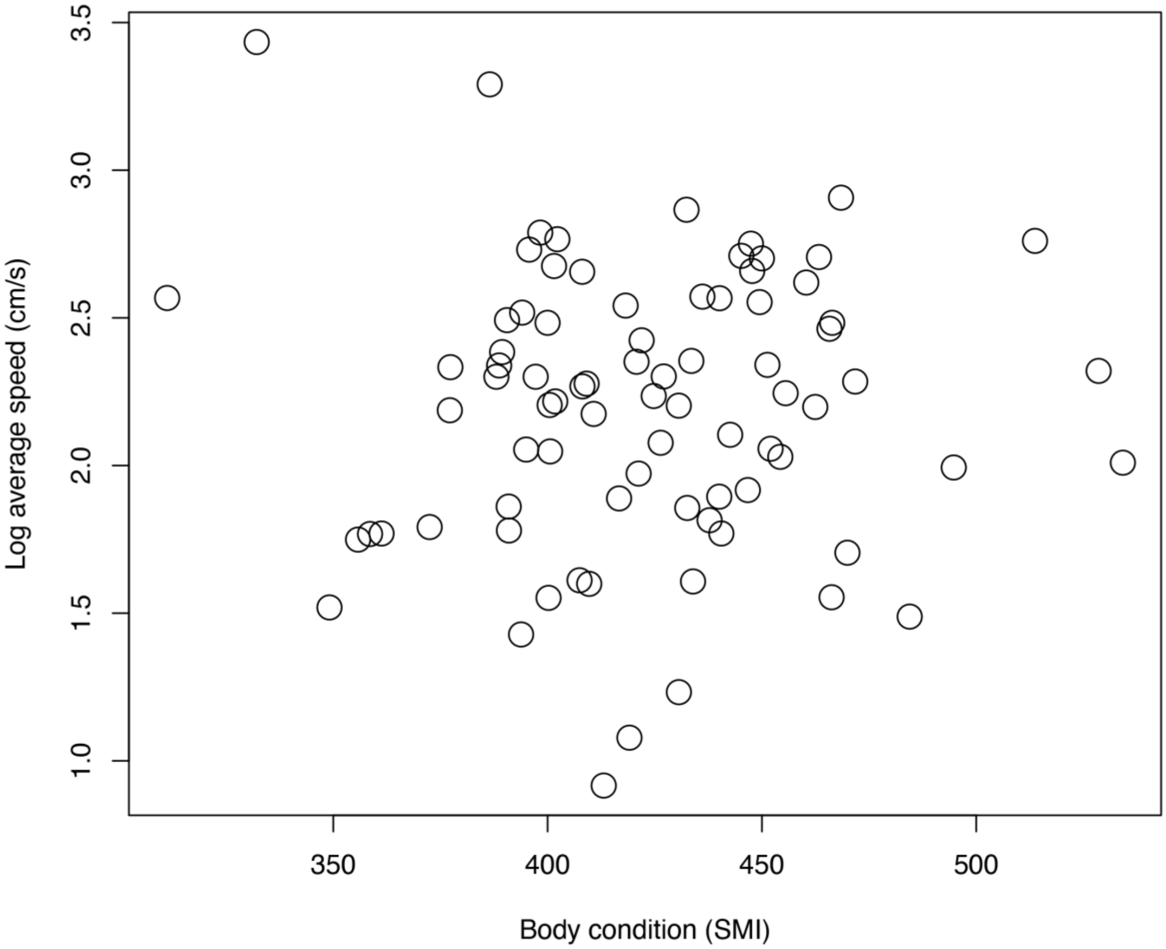

Fig. S17. Relationship between body condition (SMI) of *Rana temporaria* larvae and average speed (in cm/s, log transformed) while fleeing from the aversive stimulus presented in behavioral trials. Adjusted R-squared = -0.013, F = 0.009, df = 79, p = 0.923.

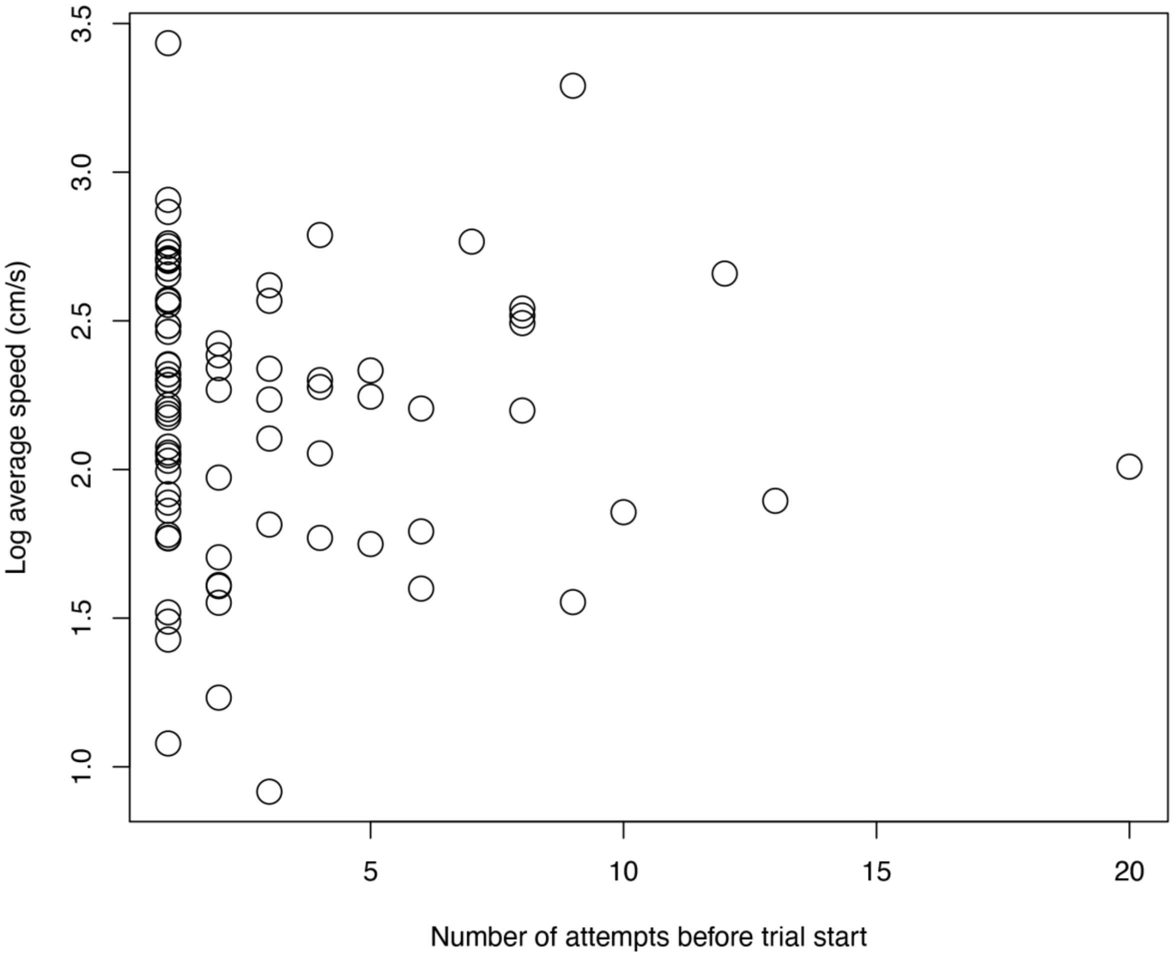

Fig. S18. Relationship between number of attempts to position *Rana temporaria* larvae before the start of the behavioral trials and average speed (in cm/s, log transformed) of the larvae while fleeing from the aversive stimulus presented. rho = -0.104, p = 0.354.

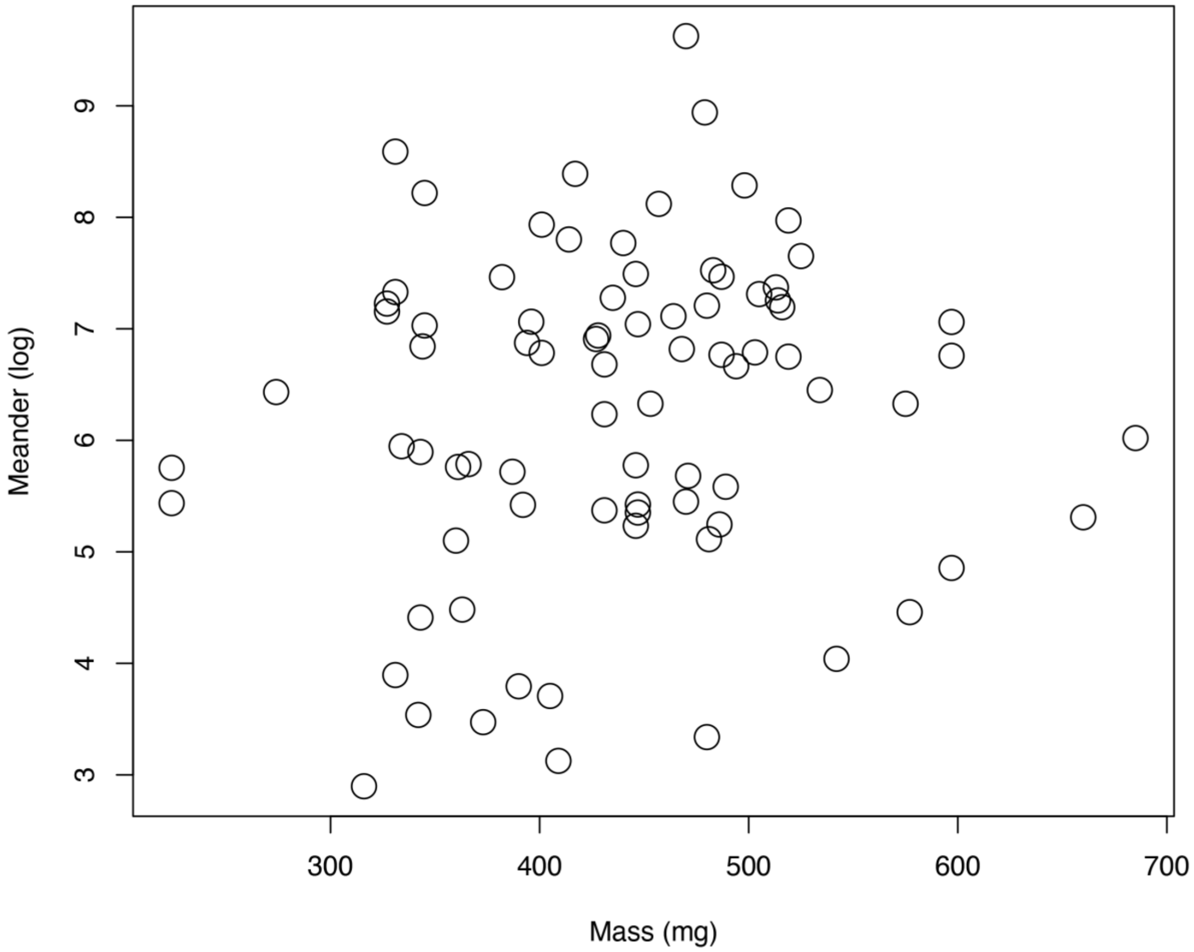

Fig. S19. Relationship between mass (mg) of *Rana temporaria* larvae and trajectory non-linearity (“meander”, log transformed) while fleeing from the aversive stimulus presented in behavioral trials. Adjusted R-squared = 0.003, F = 1.271, df = 79, p = 0.263.

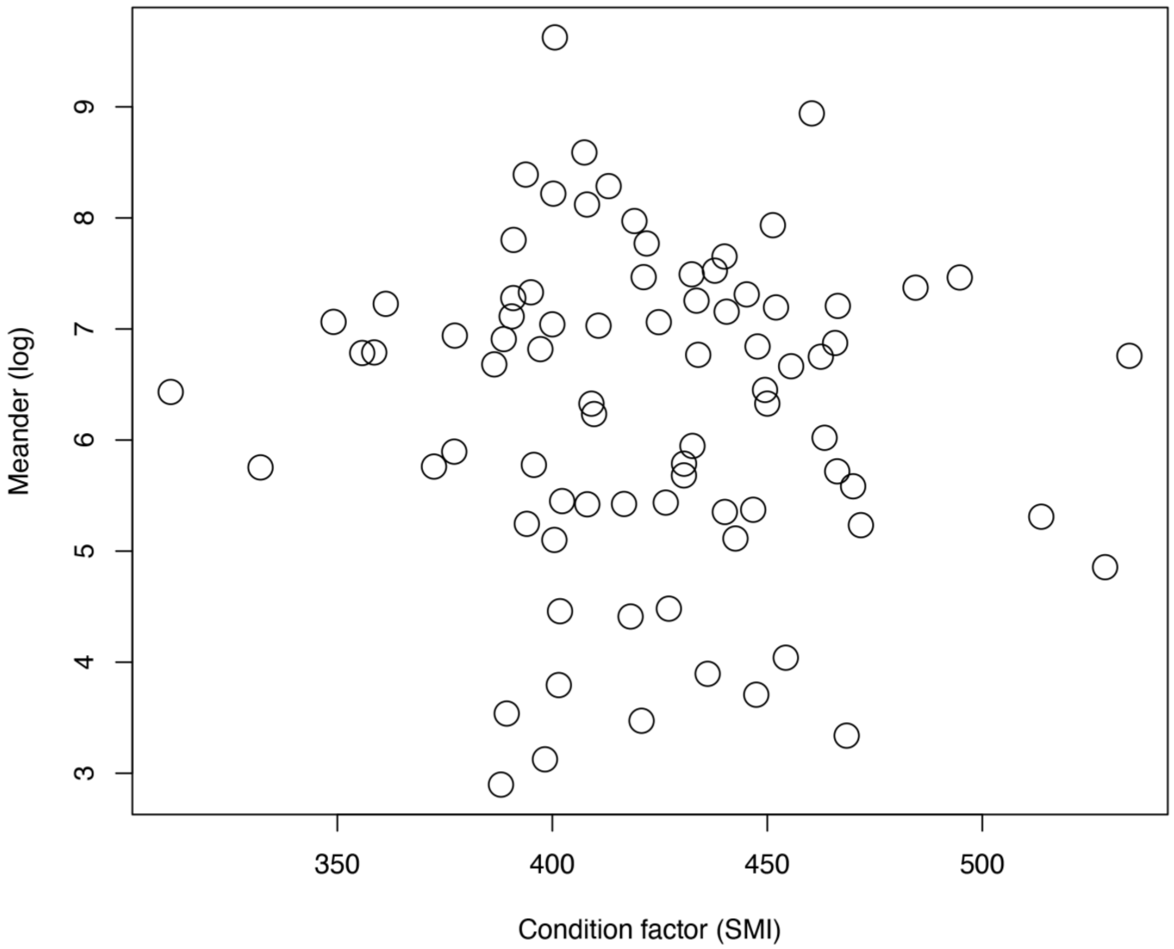

Fig. S20. Relationship between body condition (SMI) of *Rana temporaria* larvae and trajectory non-linearity (“meander”, log transformed) while fleeing from the aversive stimulus presented in behavioral trials. Adjusted R-squared = -0.009, F = 0.247, df = 79, p = 0.620.

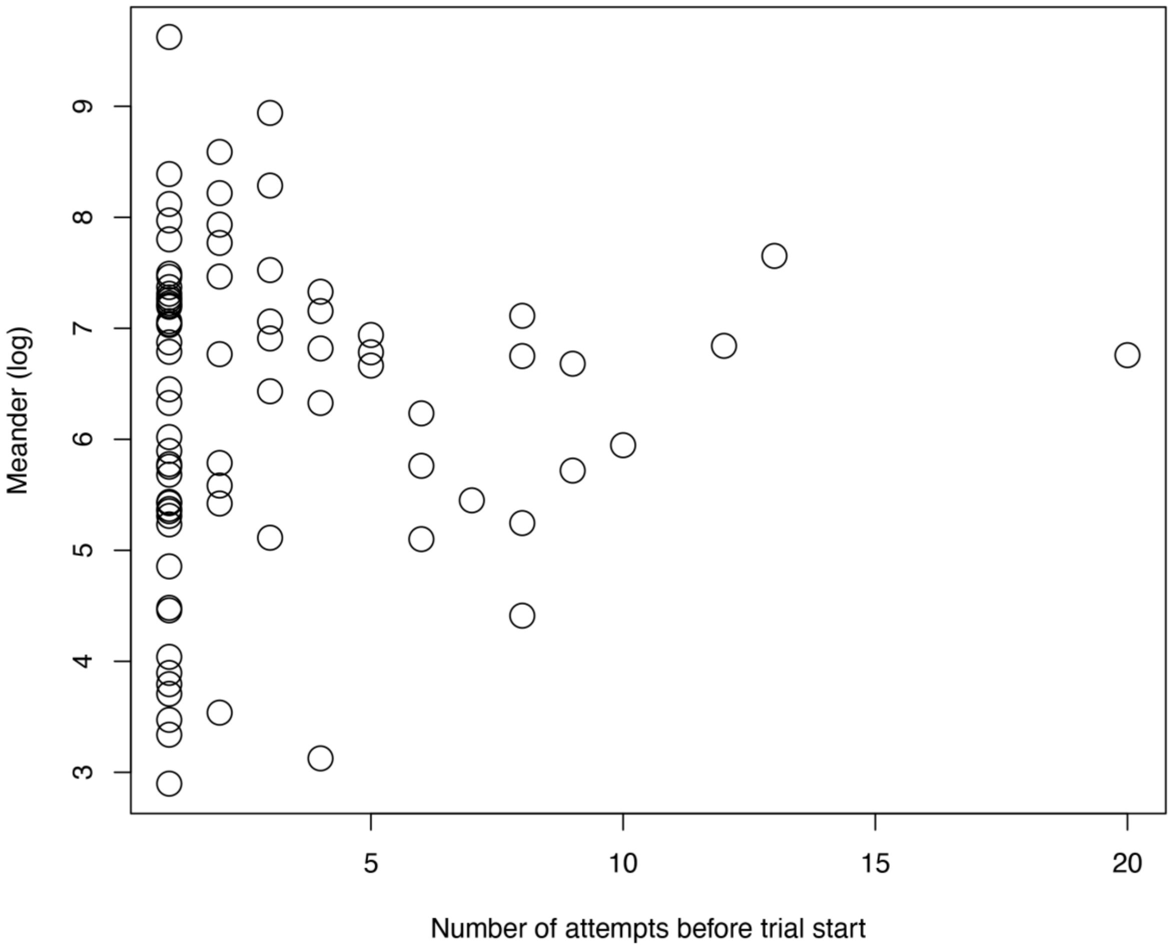

Fig. S21. Relationship between number of attempts to position *Rana temporaria* larvae before the start of the behavioral trials and trajectory non-linearity ("meander", log transformed) of the larvae while fleeing from the aversive stimulus presented. rho = 0.050, p = 0.657.

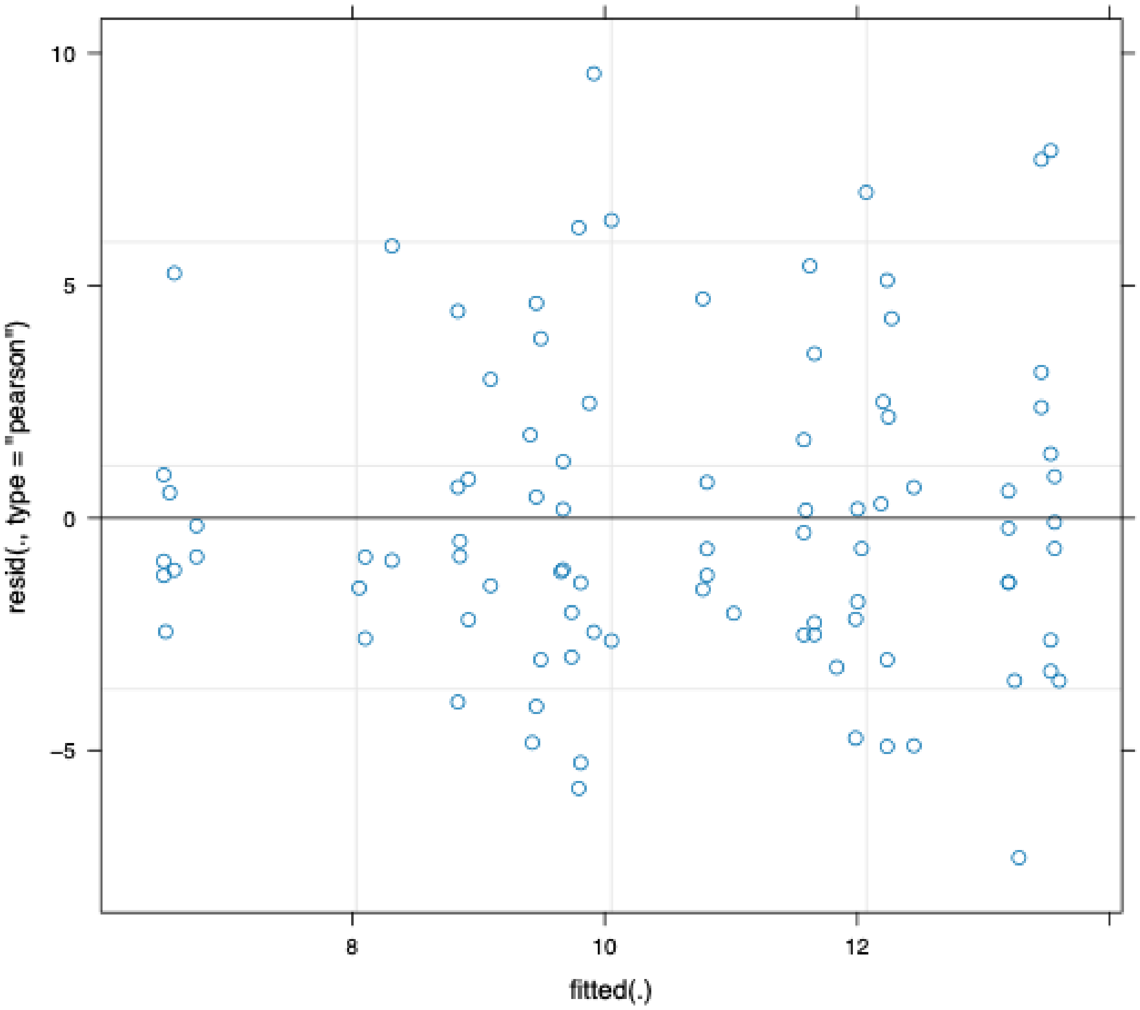

Fig. S22. Residual distribution of the model testing the effects of food treatment, rearing temperature, and exposure or not to a heatwave on bacteria alpha diversity (Hill numbers) in the gut microbiome of *Rana temporaria* larvae (see Table 1 for model description).

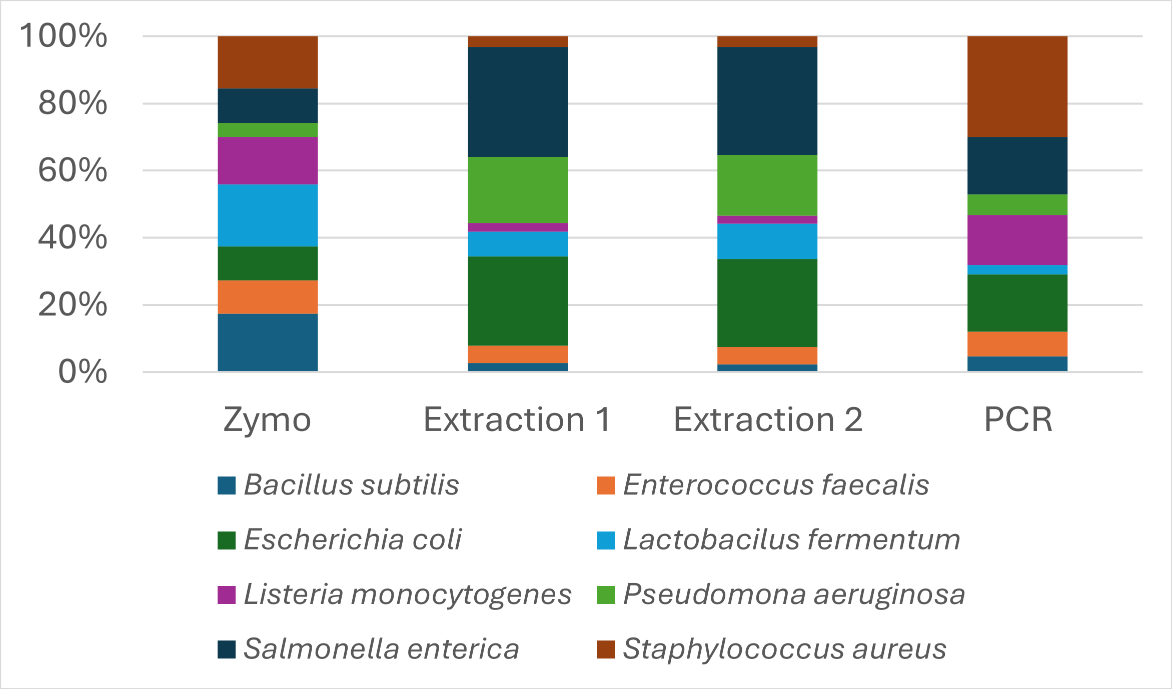

Fig. S23. Results of two positive controls for DNA extractions (ZymoBIOMICS™ microbial community standard, Zymo Research Europe GmbH) and one positive PCR control (ZymoBIOMICS™ microbial community DNA standard, Zymo Research Europe GmbH) in comparison with the expected community profile (Zymo), showing that taxonomic composition was precisely assessed, but not relative abundances. The similarity of the two extractions shows repeatability, meaning that bias in reflecting the real abundance of given taxa are consistent and, thus, comparable among samples.

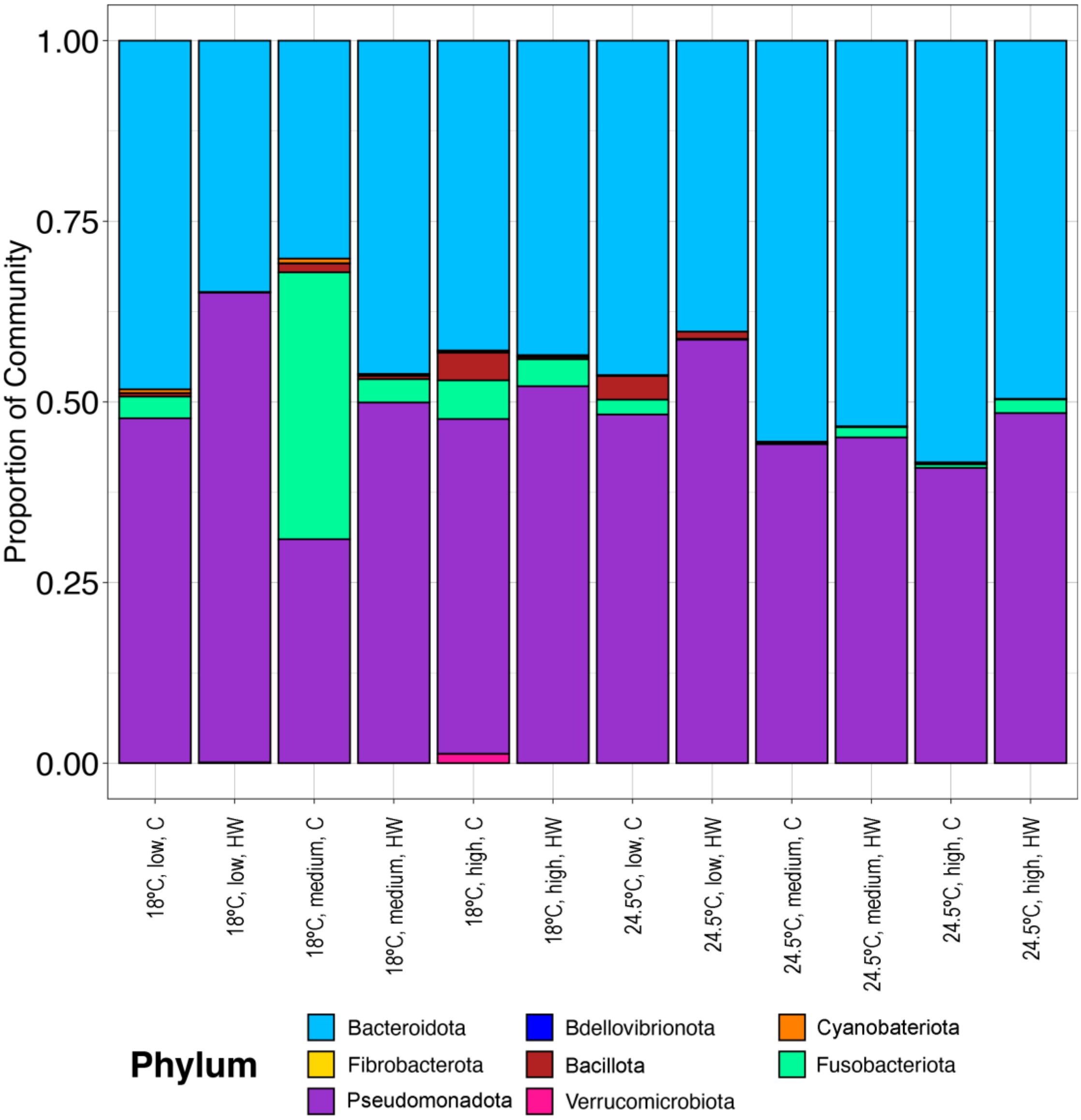

Fig. S24. Community composition of gut bacteria based on phylum for *Rana temporaria* larvae fed three diets with increasing levels of protein, fat, and animal components (considered as low-. medium- and high-quality), reared at either 18 ºC or 24.5 ºC. and exposed or not to a heatwave, in a crossed experimental design. The heatwave corresponded to increasing temperature at a ramping rate of 0.5 ºC per hour until 28 ºC, maintenance at 28 ºC for 48 h and subsequent temperature decrease of 0.5 ºC per hour until original rearing temperature.

Table S1. Permanova pairwise comparisons among treatments applied to *Rana temporaria* larvae based on unweighted unifrac distances. Treatments corresponded to three diets with increasing levels of protein, fat, and animal components (considered as low-. medium- and high-quality), two rearing temperatures (18 ºC or 24.5 ºC), and exposed or not (C = control) to a heatwave (HW).

		Sample size	**Permutations**	**pseudo-F**	**p-value**	**q-value**
Group 1	**Group 2**					
18C_highC	**18C_highHW**	17	999	3.930	0.002	0.003
18C_lowC		17	999	2.343	0.023	0.026
18C_lowHW		18	999	7.540	0.001	0.002
18C_mediumC		16	999	1.173	0.278	0.286
18C_mediumHW		17	999	3.086	0.001	0.002
24.5C_highC		14	999	4.626	0.001	0.002
24.5C_highHW		16	999	4.023	0.001	0.002
24.5C_lowC		18	999	6.196	0.001	0.002
24.5C_lowHW		15	999	4.923	0.001	0.002
24.5C_mediumC		17	999	4.745	0.001	0.002
24.5C_mediumHW		17	999	5.242	0.001	0.002
18C_highHW	**18C_lowC**	16	999	4.995	0.002	0.003
18C_lowHW		17	999	4.244	0.001	0.002
18C_mediumC		15	999	5.374	0.001	0.002
18C_mediumHW		16	999	2.238	0.021	0.025
24.5C_highC		13	999	3.895	0.007	0.009
24.5C_highHW		15	999	2.699	0.012	0.015
24.5C_lowC		17	999	6.442	0.001	0.002
24.5C_lowHW		14	999	3.416	0.003	0.004
24.5C_mediumC		16	999	2.896	0.003	0.004
24.5C_mediumHW		16	999	4.640	0.002	0.003
18C_lowC	**18C_lowHW**	17	999	6.007	0.001	0.002
18C_mediumC		15	999	2.496	0.031	0.034
18C_mediumHW		16	999	2.567	0.010	0.013
24.5C_highC		13	999	6.103	0.001	0.002
24.5C_highHW		15	999	4.878	0.001	0.002
24.5C_lowC		17	999	6.651	0.001	0.002
24.5C_lowHW		14	999	4.584	0.003	0.004
24.5C_mediumC		16	999	5.013	0.001	0.002
24.5C_mediumHW		16	999	5.295	0.001	0.002
18C_lowHW	**18C_mediumC**	16	999	8.262	0.001	0.002
18C_mediumHW		17	999	3.289	0.002	0.003
24.5C_highC		14	999	5.691	0.002	0.003
24.5C_highHW		16	999	3.821	0.001	0.002
24.5C_lowC		18	999	8.517	0.001	0.002
24.5C_lowHW		15	999	2.451	0.027	0.030
24.5C_mediumC		17	999	3.688	0.002	0.003
24.5C_mediumHW		17	999	4.716	0.001	0.002

18C_mediumC	**18C_mediumHW**	15	999	3.181	0.002	0.003
24.5C_highC		12	999	6.624	0.002	0.003
24.5C_highHW		14	999	4.186	0.001	0.002
24.5C_lowC		16	999	8.238	0.001	0.002
24.5C_lowHW		13	999	5.406	0.001	0.002
24.5C_mediumC		15	999	4.792	0.002	0.003
24.5C_mediumHW		15	999	4.985	0.003	0.004
18C_mediumHW	**24.5C_highC**	13	999	3.343	0.001	0.002
24.5C_highHW		15	999	2.502	0.003	0.004
24.5C_lowC		17	999	3.319	0.004	0.005
24.5C_lowHW		14	999	1.998	0.022	0.025
24.5C_mediumC		16	999	2.708	0.002	0.003
24.5C_mediumHW		16	999	3.227	0.001	0.002
24.5C_highC	**24.5C_highHW**	12	999	0.817	0.644	0.644
24.5C_lowC		14	999	4.226	0.001	0.002
24.5C_lowHW		11	999	3.547	0.005	0.006
24.5C_mediumC		13	999	2.029	0.037	0.040
24.5C_mediumHW		13	999	2.342	0.017	0.021
24.5C_highHW	**24.5C_lowC**	16	999	5.245	0.002	0.003
24.5C_lowHW		13	999	2.052	0.015	0.019
24.5C_mediumC		15	999	1.557	0.121	0.128
24.5C_mediumHW		15	999	1.003	0.423	0.429
24.5C_lowC	**24.5C_lowHW**	15	999	4.575	0.001	0.002
24.5C_mediumC		17	999	4.276	0.001	0.002
24.5C_mediumHW		17	999	6.036	0.001	0.002
24.5C_lowHW	**24.5C_mediumC**	14	999	1.933	0.037	0.040
24.5C_mediumHW		14	999	2.296	0.022	0.025
24.5C_mediumC	**24.5C_mediumHW**	16	999	1.612	0.144	0.150