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

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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 heat wave (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 heat wave. The heat wave may have triggered an increase in the abundance of *Klebsiella*, together with an 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 microbiome 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.

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 heat waves, with responses varying according to the intensity and duration of thermal stress (Carreira et al., 2016). The increasing occurrence of heat waves 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 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 heat wave (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 heat waves affect gut bacterial diversity and composition, even when accounting for clutch effects (host genetic background); (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 (pro-metamorphic stages; digit development in the hind limbs; *sensu* Gosner 1960), approximately half of the surviving individuals from each treatment were exposed to a heat-wave 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.

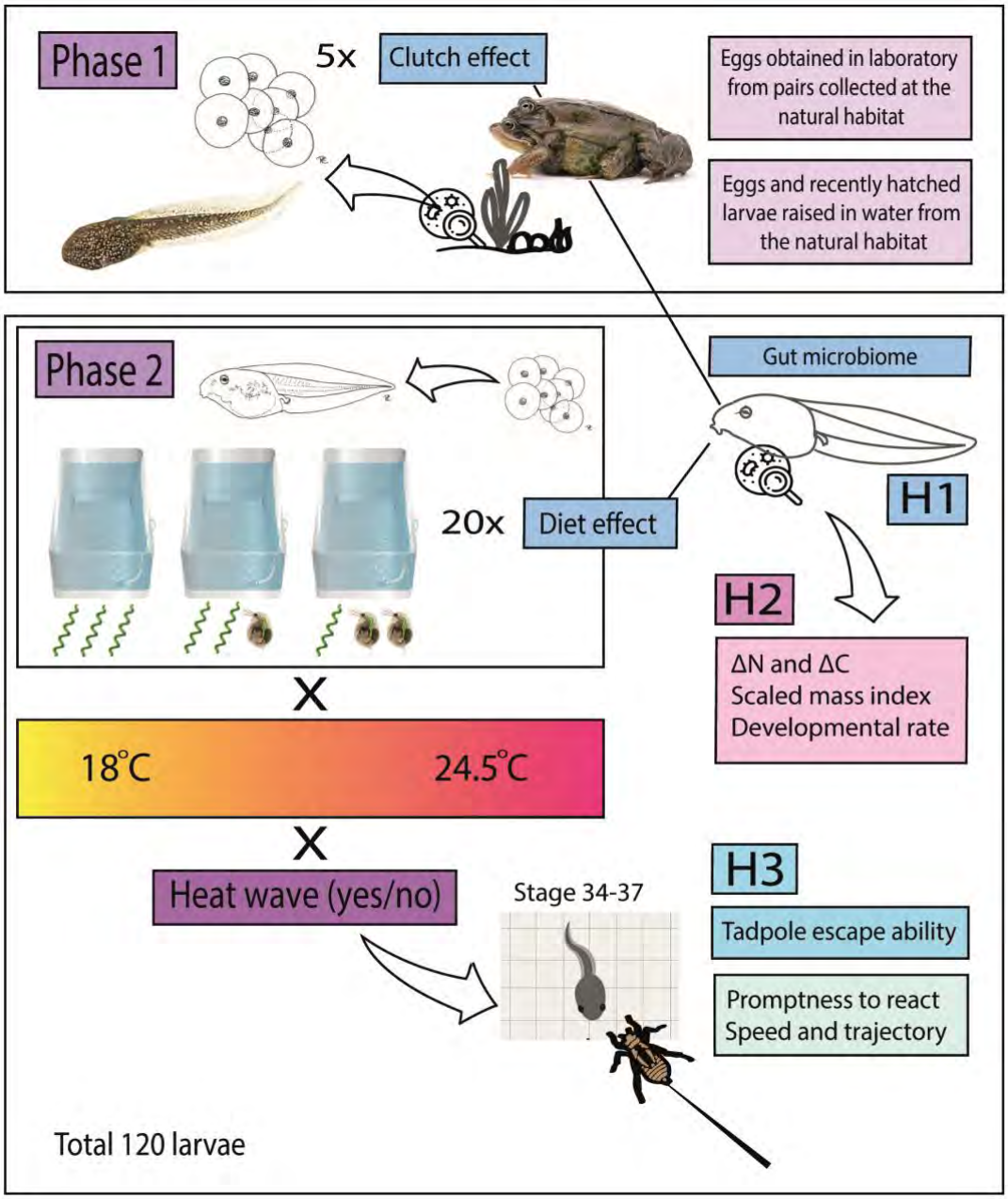

Fig. 1. Graphical summary of the experimental design representing acquisition of offspring (larvae) from five different egg clutches from *Rana temporaria* and the experiment itself. The experiment structure is shown based on three main hypotheses to be tested: whether diet and temperatures experienced 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 heat wave had returned to their original rearing temperature, behavioral trials were conducted with both heat-wave 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.

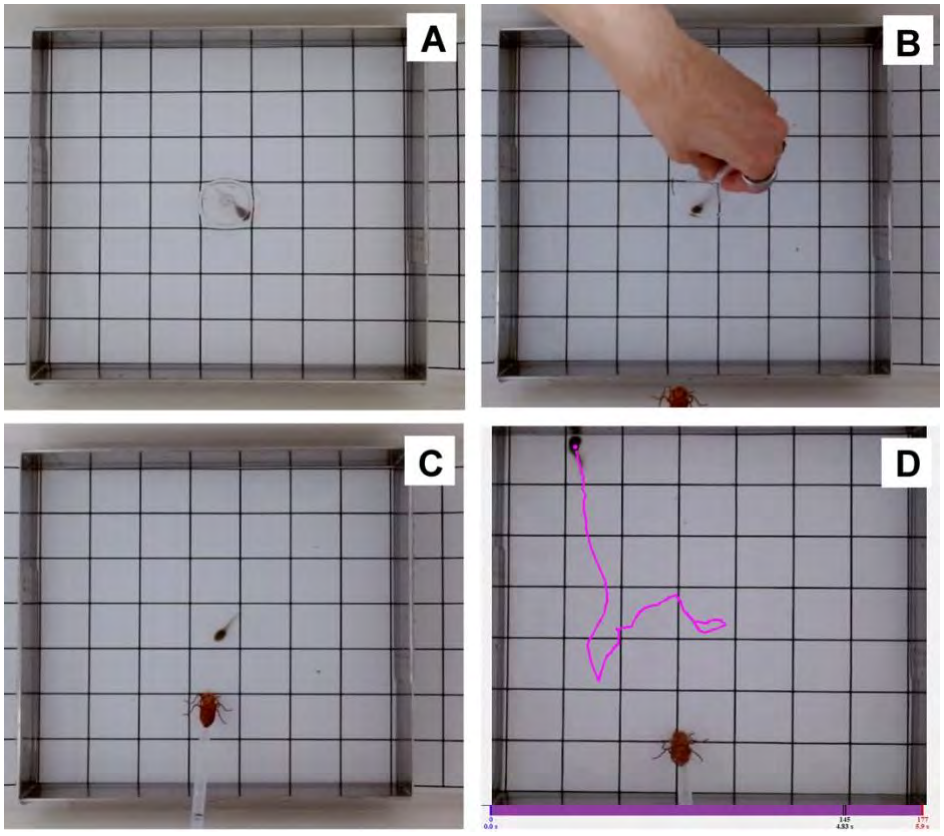

Fig. 2. Experimental setup for behavioral tests. Plastic trays filled up to 1 cm with rested tap water at larvae rearing temperature were lined with a grid of 5 x 5 cm squares. A space of 35 x 35 cm was delimited with a metal frame and the larva to be tested was placed at the central square, where it 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 heat-wave 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 heat-wave exposure on larval kinematics. We expected larvae fed higher-quality diets, reared at 18 °C, and not exposed to the heat wave 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 $g \times L^{-1}$ 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 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 alpha and beta diversity analyses were performed in QIIME2 (Bolyen et al., 2019). Details on sequence quality filtering, sample depth and taxonomic assignment are provided as supplementary material.

Beta diversity was assessed using unweighted UniFrac distances and compared among treatments using PERMANOVA with pairwise post hoc tests. Metagenomic functional predictions of the gut microbiota were generated using PICRUSt2 (Douglas et al., 2020).

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.

The effects of food treatment, rearing temperature, and heat-wave exposure - including all two- and three-way interactions - on larval 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 heat-wave 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 α-diversity (Shannon entropy) was analyzed with GLMMs in afex, using food treatment, rearing temperature, and heat-wave exposure (and all interactions) as fixed effects and clutch identity as a random effect.

To assess microbiome composition, we constructed a phyloseq object (McMurdie & Holmes, 2013) normalized via Total Sum Scaling (TSS) and tested for differential microbial markers across the 12 treatment combinations (3 diets × 2 rearing temperatures × heat-wave vs. no heat-wave). 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).

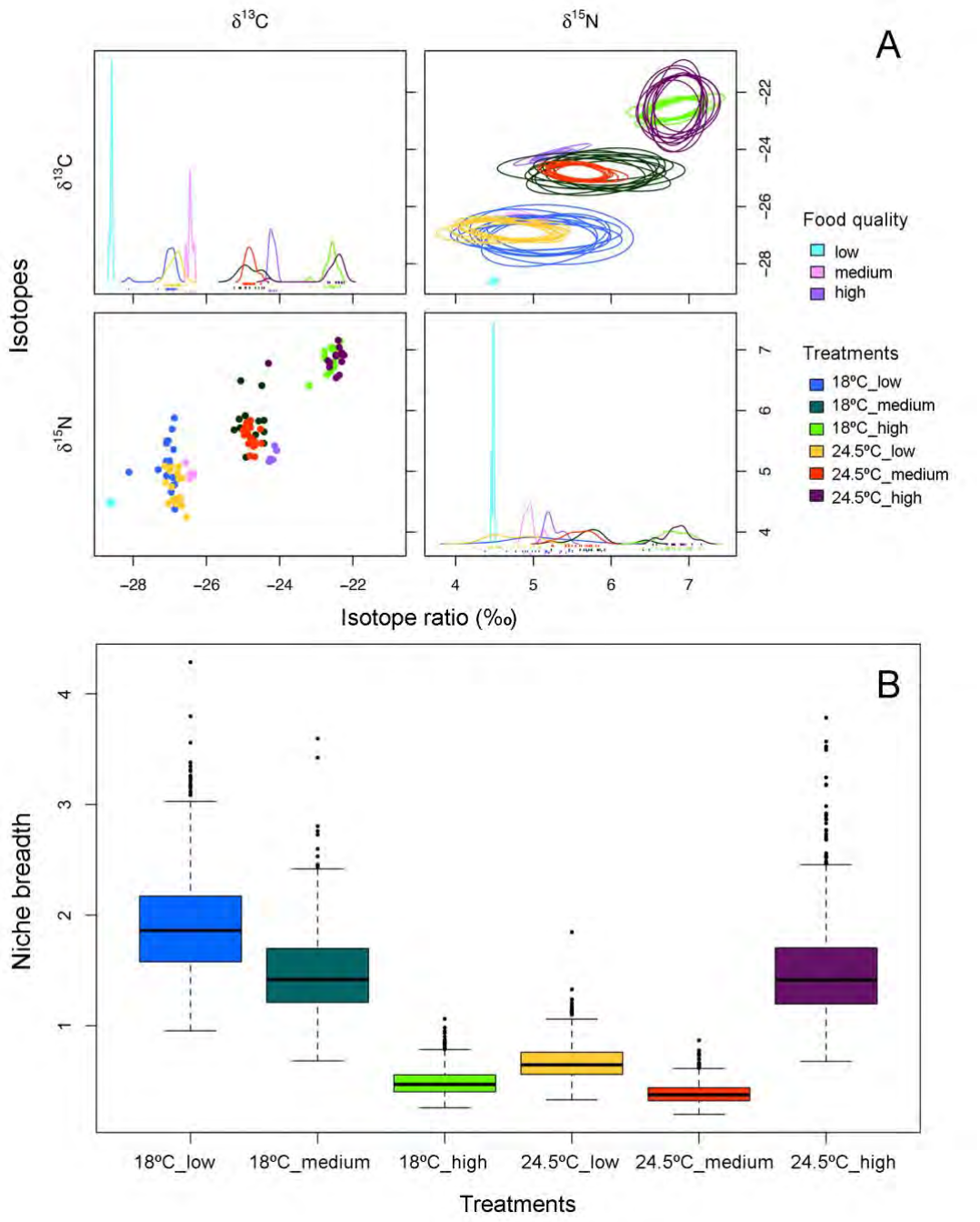

Fig. 3. Isotopic signatures (A) and niche breadth (B) of *Rana temporaria* larvae reared with three food treatments and two temperatures in a crossed experimental design. Food treatments correspond to diets with increasing levels of protein, fat, and components of animal origin (their isotopic signatures 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

heat-wave phase (three heat-wave larvae and two controls). One larva developed hydrops and was excluded.

Larval body condition (SMI) did not differ among food treatments, rearing temperatures, or heat-wave 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 *lmer* function (Table 2). Simpler models including only individual predictors and single interactions yielded consistent results using the mixed function.

Larvae reared on high-quality food were more likely to react than those fed intermediate-quality food at 24.5 °C (free-method post-hoc: estimate = –0.379, SE = 0.124, df = 86.8, t = –3.059, p = 0.013) and not exposed to the heat wave (estimate = –0.379, SE = 0.126, df = 80.9, t = –3.011, p = 0.015; Fig. 4B). Heat-wave exposure increased reaction likelihood only at 24.5 °C, whereas at 18 °C it reduced the likelihood of reacting (Fig. 4B, Table 2; see Fig. S10 for residual diagnostics).

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) 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 (Shannon entropy)				
mixed (diversity ~ diet*temperature*HW + (1|Clutch))	diet	2; 77.10	3.297	**0.042***
	temperature	1; 78.21	8.716	**0.004***
	HW	1; 79.97	0.034	0.854
	diet:temperature	2; 78.08	4.763	**0.011***
	diet:HW	2; 79.08	1.647	0.199
	temperature:HW	1; 77.66	0.161	0.689
	diet:temperature:HW	2; 73.15	3.677	**0.030***

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) 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))	diet	2; 87.05	0.889	0.419	102
	temperature	1; 86.93	0.186	0.667	
	HW	1; 89.42	0.035	0.851	
	diet:temperature	2; 88.09	5.627	**0.005***	
	diet:HW	2; 89.44	5.748	**0.004***	
	temperature:HW	1; 89.06	18.327	**<0.001***	
	diet:temperature:HW	2; 83.36	1.346	0.266	
Reaction time					
mixed (reaction_time ~ diet*temperature*HW + (diet||day_filmed+clutch))	diet	2; 0.45	0.015	0.985	81
	temperature	1; 1.17	0.307	0.667	
	HW	1; 53.64	1.014	0.319	
	diet:temperature	2; 1.07	0.081	0.927	
	diet:HW	2; 42.53	1.503	0.234	
	temperature:HW	1; 61.17	0.789	0.378	
	diet:temperature:HW	2; 60.96	1.166	0.319	

Touch by the predator model before reaction (binary)					
mixed (touch ~ diet*temperature*HW + (1|day_filmed) + (1|Clutch))	diet	2; 12	6.897	**0.032***	81
	temperature	1; 13	0.000	1.000	
	HW	1; 13	0.000	1.000	
	diet:temperature	2; 12	0.598	0.741	
	diet:HW	2; 12	6.701	**0.035***	
	temperature:HW	1; 13	0.000	1.000	
	diet:temperature:HW	2; 12	7.838	**0.020***	
Speed while fleeing (log)					
mixed (logspeed ~ diet*temperature*HW + (diet||day_filmed+clutch))	diet	2; 67.19	1.084	0.344	81
	temperature	1; 0.94	0.037	0.881	
	HW	1; 66.91	0.018	0.892	
	diet:temperature	2; 67.02	1.097	0.340	
	diet:HW	2; 65.12	1.624	0.205	
	temperature:HW	1; 66.08	0.001	0.976	
	diet:temperature:HW	2; 66.71	1.481	0.235	
Trajectory non-linearity while fleeing or "meander" (log)					
mixed (logmeander ~ diet*temperature*HW + (diet||day_filmed+clutch))	diet	2; 1.06	0.212	0.836	81
	temperature	1; 0.89	1.288	0.478	
	HW	1; 58.33	0.420	0.520	
	diet:temperature	2; 1.29	0.037	0.965	
	diet:HW	2; 51.95	0.661	0.520	
	temperature:HW	1; 61.90	1.561	0.216	
	diet:temperature:HW	2; 62.24	1.391	0.256	

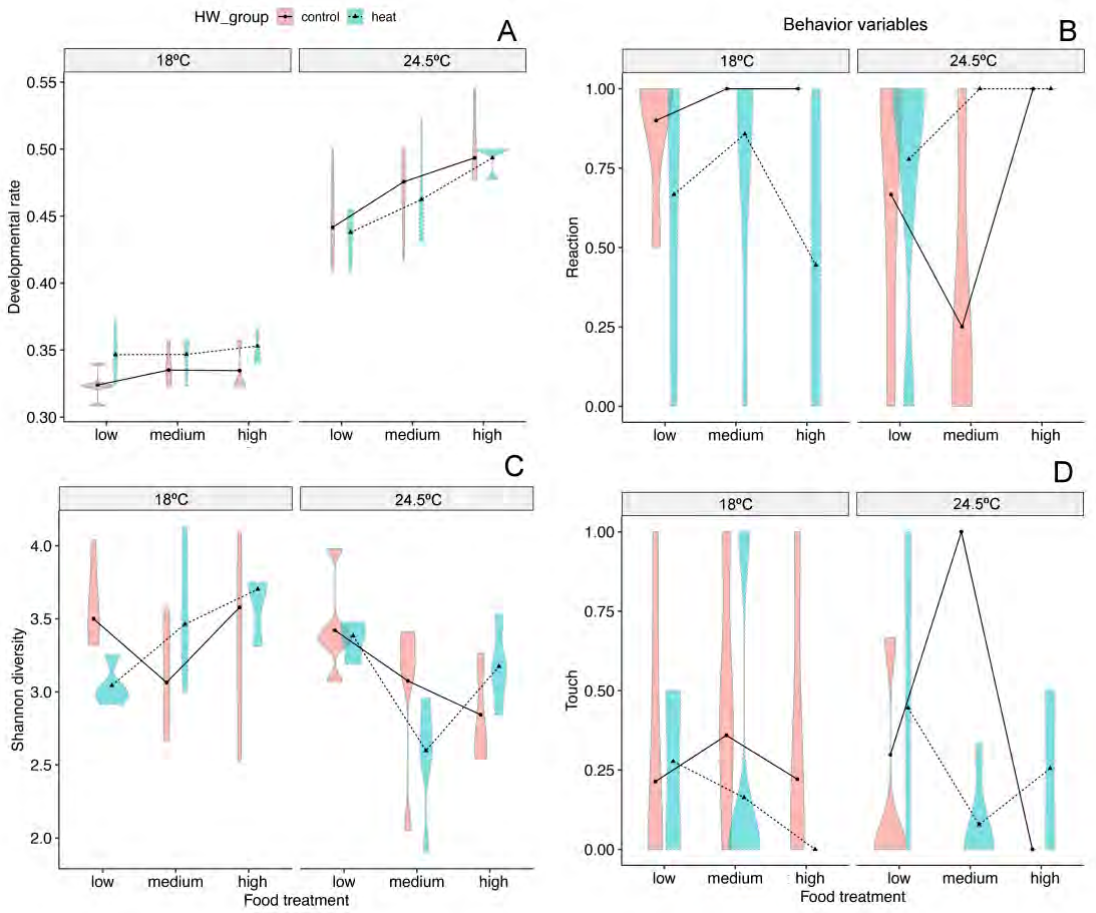

Fig. 4. Interactive effects among food quality, rearing temperature, and exposure to a heat wave in *Rana temporaria* larvae developmental rate (A), variables describing behavior (B, D) and gut bacteria diversity (C). Food quality refers to increasing levels of protein, fat, and components of animal origin. Rearing temperatures were 18 ºC and 24.5 ºC. The heat wave 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 corresponding model (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. S11), body condition (Adjusted $R^2 = –0.013$, $F_{79} = 0.005$, $p = 0.946$; Fig. S12), or the number of positioning attempts before the trial ($\rho = –0.125$, $p = 0.263$; Fig. S13). Reaction time was also unaffected by any experimental factor - food treatment, rearing temperature, heat-wave 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. S14), SMI ($W = 697$, $p = 0.343$; Fig. S15), or the number of attempts needed to position them ($W = 533$, $p = 0.366$; Fig. S16). In contrast,

reaction depended on food treatment, its interaction with heat-wave exposure, and the three-way interaction among food treatment, rearing temperature, and heat-wave exposure (Table 1; Fig. S17). At 24.5 °C, larvae fed intermediate-quality food were more likely to be touched before fleeing than those fed high-quality food (free method post-hoc: estimate = 1.021, SE = 0.334, z = 3.059, p = 0.025; Fig. 4D), although this pattern did not occur in larvae exposed to the heat wave.

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. S18; Adjusted $R^2 = –0.013$, $F_{79} = 0.009$, $p = 0.923$; Fig. S19; $\rho = –0.104$, $p = 0.354$; Fig. S20; meander: Adjusted $R^2 = 0.003$, $F_{79} = 1.271$, $p = 0.263$; Fig. S21; Adjusted $R^2 = –0.009$, $F_{79} = 0.247$, $p = 0.620$; Fig. S22; $\rho = 0.050$, $p = 0.657$; Fig. S23). Food treatment, rearing temperature, heat-wave 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 food treatment, rearing temperature, their interaction, and the three-way interaction with heat-wave exposure (Table 1). Larvae fed medium-quality food exhibited reduced gut bacterial diversity compared to larvae fed low-quality food, but only when reared at 24.5 °C and exposed to a heat wave (Fig. 4C).

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. S24). The two extraction controls yielded consistent results (Fig. S24), indicating that any deviations in relative abundances were systematic rather than random.

In total, 207 Operational Taxonomic Units (OTUs) were recovered from the gut microbiomes of 92 *R. temporaria* larvae. The dominant phyla across treatments were *Pseudomonadota* and *Bacteroidota* (Fig. S25). Most treatment pairs differed significantly in gut bacterial community composition, with a few exceptions. No differences were detected between medium-quality food with heat-wave exposure and high-quality food without exposure at 18 °C. At 24.5 °C, larvae fed low-quality food with heat-wave exposure did not differ from those fed medium-quality food (with or without heat-wave exposure) or high-quality food (with or without heat-wave exposure) (Fig. 5A; Table S1).

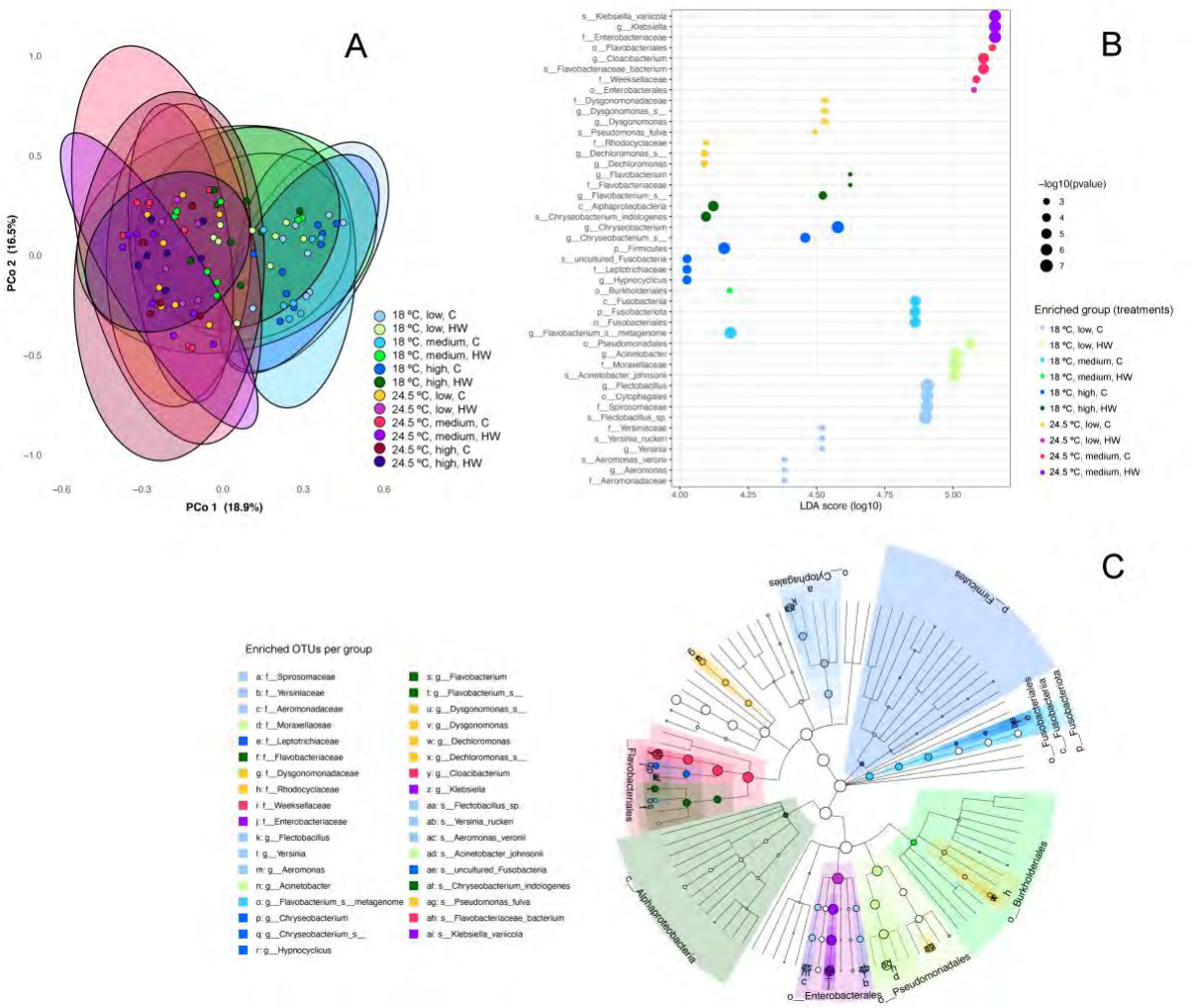

Fig. 5. Gut bacteria community composition (A) and enriched Operational Taxonomic Units (OTUs; B) according to treatments imposed to larvae of *Rana temporaria*, corresponding to three diets with increasing levels of protein, fat, and components of animal origin (low-, medium-, and high-quality), two rearing temperatures (18 ºC and 24.5 ºC) and exposure or not to a heat wave (HW vs. C = control). Clustering of taxa with differences in abundance among treatments is also shown (C). Colors of OTUs 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 heat-wave exposure) had OTUs identified as biomarkers, totaling 45 OTUs (Fig. 5). At low food quality, the main biomarkers at 18 °C without heat-wave exposure were *Flectobacillus* (*Spirosomaceae*, *Cytophagales*), *Yersinia ruckeri* (*Yersinaceae*), and *Aeromonas veronii* (*Aeromonadaceae*). When exposed to a heat wave, *Acinetobacter johnsonii* (*Moraxellaceae*) was predominant. At 24.5 °C, *Dysgonomonas* (*Dysgonomonadaceae*), *Pseudomonas fulva*, and *Dechloromonas* (*Rhodocyclaceae*) dominated without heat-wave exposure, whereas *Enterobacterales* predominated under heat-wave exposure.

At intermediate food quality, *Fusobacteriales* (*Fusobacteriia*, *Fusobacteriota*) and *Flavobacterium* were characteristic at 18 °C without heat-wave exposure, while *Burkholderiales* dominated with heat-wave exposure. At 24.5 °C, *Cloacibacterium* (*Weeksellaceae*, *Flavobacteriales*) predominated without heat-wave exposure, whereas *Klebsiella variicola* (*Enterobacteriacae*) was selected as a biomarker under heat-wave exposure.

At high food quality and 18 °C, *Chryseobacterium*, *Bacillota*, and *Hypnocyclicus* (*Leptotrichiaceae*) were biomarkers without heat-wave exposure, and *Cryseobacterium indologenes*, *Flavobacterium* (*Flavobacteriaceae*), and *Alphaproteobacteria* predominated under heat-wave 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), as well as synthesis of L-tryptophan and aerobactin, and conversion of amino acids into putrescine (Fig. 6A).

Larvae clustered into four major groups based on the top 15 significantly differing pathways (Fig. 6B). One group showed under-expression across all pathways and consisted mostly of larvae reared at 18 °C without heat-wave exposure, although individuals from other treatment categories were also included. A second group showed intermediate expression and was highly heterogeneous across food treatments, temperatures, and heat-wave exposure. The two groups with the highest predicted pathway expression were composed predominantly of larvae reared at 24.5 °C, exposed to a heat wave, or both (Fig. 6B).

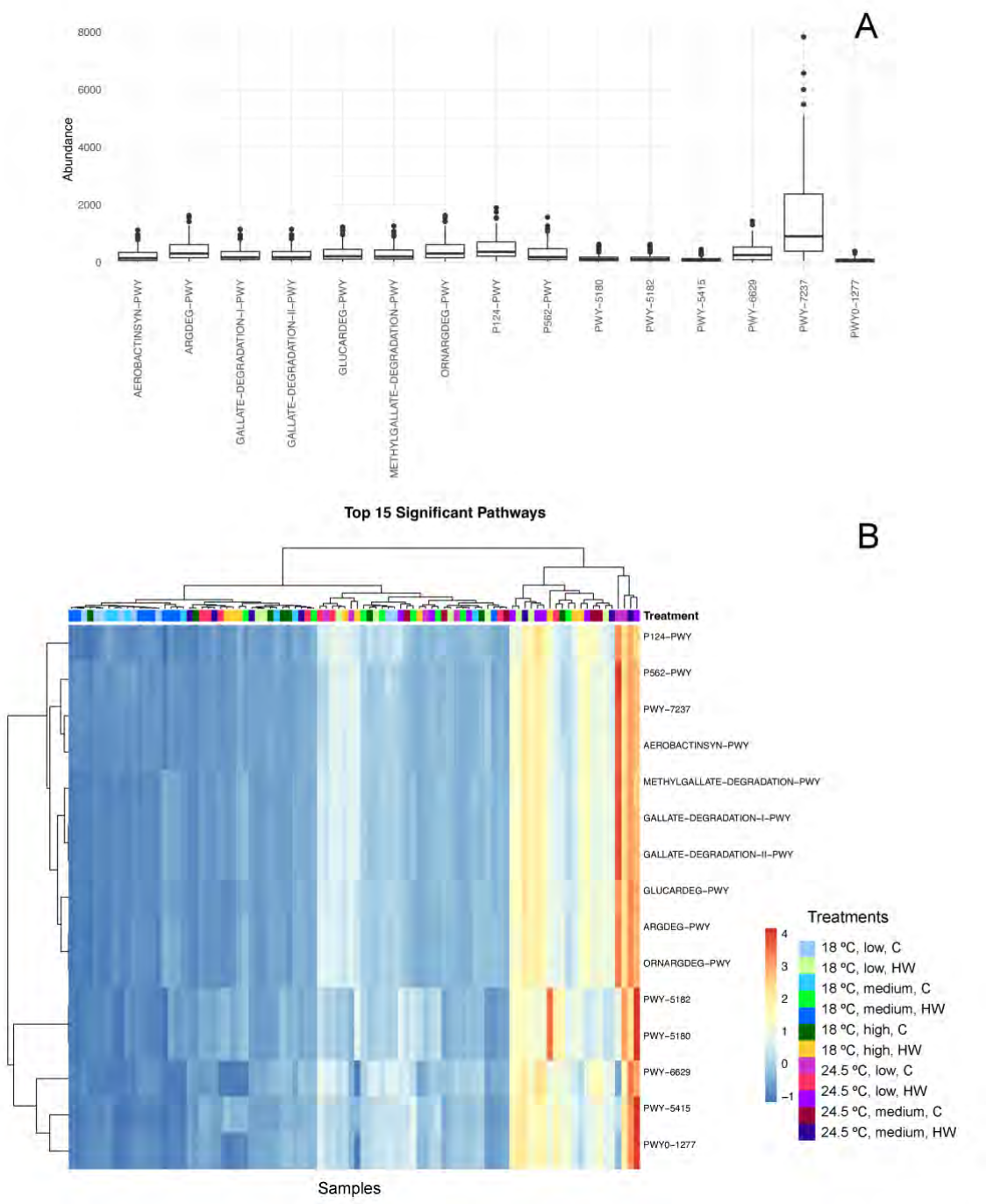

Fig. 6. The predicted 15 most significant pathways (A) influenced by gut bacteria from larvae of *Rana temporaria* and their expression among treatments (B) corresponding to a multifactorial experimental design of three diets with increasing levels of protein, fat, and components of animal origin (low-, medium-, and high-quality), two rearing temperatures (18 ºC and 24.5 ºC) and exposure or not to a heat wave (HW vs. C = control). Treatment colors are as in Fig. 5.

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. Moreover, both long-term warming and short heat waves appeared to induce changes in the microbiome that, in turn, influenced the larvae's ability to react.

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, based on stable isotope analyses, was higher at 18 ºC with low to intermediate food quality and at 24.5 ºC with high food quality. Because food within treatments was uniform, niche breadth reflects individual variability in the assimilation of food components, which may indicate microbiome-mediated modulation (discussed below). Niche breadth was markedly lower at 18 ºC with high food quality and at 24.5 ºC with intermediate food quality. Low-quality food resulted in intermediate niche breadths at 24.5 ºC. The microbiome is modulated by diet and host genetics and, in turn, can influence nutrient

absorption and host metabolism (Huda et al., 2022; Corbin et al., 2023). Thus, broader niches within treatments may reflect greater plasticity of the holobiont (i.e., microbiome–host association) in adjusting nutrient absorption at the individual level.

More diverse microbial communities are likely to possess higher functional redundancy, allowing metabolic functions to be maintained despite changes in the abundance of specific taxa (Louca et al., 2018). In humans, distinct well-balanced host–microbial symbiotic states have been identified, and these states respond differently to diet (Arumugam et al., 2011). Such plasticity may allow the holobiont to meet the nutritional demands of the host, depending on the interaction between the microbiome and host genetic background. In our study, host genetic variability was unlikely to differ among treatments, which contained equal numbers of larvae from five clutches. Therefore, the larger niche breadths observed in some treatments may indicate a higher adaptive capacity of the microbiome to interact with host genetics and enhance host performance, which could be beneficial.

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, is therefore 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 - evaluated as both the likelihood to react and whether the reaction occurred before or after being touched - 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 heat wave 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. However, exposure to a heat wave improved the reactivity of larvae reared at 24.5 ºC with intermediate-quality food, potentially due to shifts in gut bacterial abundance and activation of metabolic pathways that enhance performance (discussed below).

The use of a complex stimulus combining visual, tactile, and chemical cues may have masked differences in reaction time, as perception and response can vary depending on the cue (Melo et al., 2021). Although testing each cue separately would be informative, we combined them to increase the likelihood that all larvae would perceive and respond to the aversive stimulus. Non-reacting larvae were interpreted as less able to respond to threats, and larvae that waited until being touched were considered less responsive, as contact with a predator in nature would likely result in capture.

Rana temporaria larvae develop in small ponds in the Kleiwiesen, where they are exposed to dragonfly naiads but not predatory fish. Higher escape speed is adaptive for larvae facing active predators like fish but not for ambush predators such as Odonata, as phenotypes associated with increased speed are induced by co-occurrence with the former but not the latter (Teplitsky et al., 2005). In this context, the ability to flee promptly upon perceiving a threat likely has a greater impact on survival than escape speed or trajectory in Kleiwiesen larvae (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 heat wave. 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 heat wave under the same dietary and rearing temperature conditions showed improved escape performance and a higher abundance of *Klebsiella* (*Enterobacteriaceae*, *Enterobacterales*). This suggests that the heat wave may have

triggered proliferation of *Klebsiella*, which in turn could have contributed to enhanced performance. However, this shift in microbial composition came with a reduction in gut microbiome diversity, which 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 heat wave 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 fastest and exhibited effective escape responses, likely due to functional redundancy in a diverse 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-heat wave 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 heat wave 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 heat wave. 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 PWY-5182, 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 heat waves - 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, the gut bacterial community may have adjusted to variations in food quality and exposure to short-term heat stress, contributing to the maintenance of 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 heat waves, shaping 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 a herbivorous diet - low in protein, fat, and component diversity - also exhibited effective escape responses. These comparable outcomes suggest that alternative bacterial communities, triggered by environmental conditions, may provide functional redundancy, supporting host performance despite differences in diet.

Larvae receiving intermediate-quality diets, with moderate inclusion of animal components, showed variable outcomes depending on heat wave 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.

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Ethics approval

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Consent for publication

Not applicable.

Data availability

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

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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 heat-wave 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 heat wave) 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 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. Samples with fewer than 556 reads (15 samples, one to three per treatment) were excluded from further analyses.

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Supplementary figures

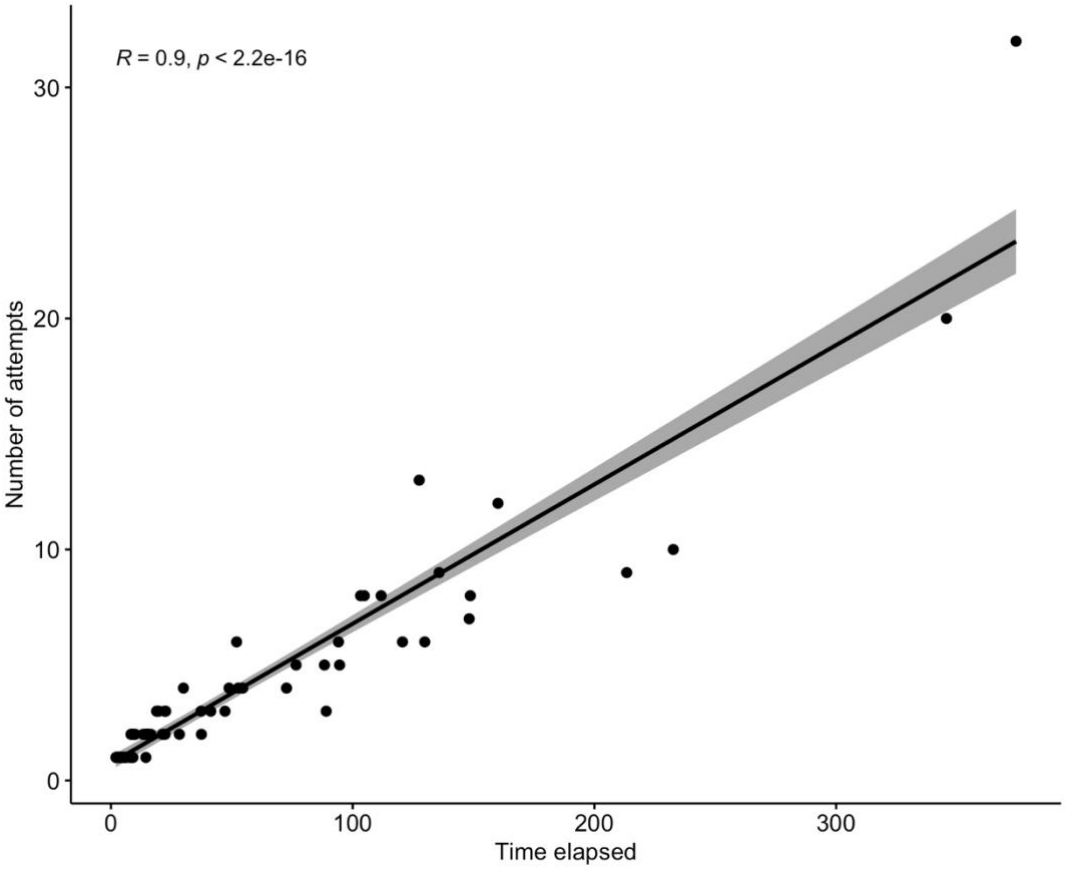


Fig. S1. Correlation between time elapsed from the end of the 3 minutes larvae remained under the funnel and the actual start of the behavioral trial (when the dragonfly naiad model touched the water) and number of attempts (number of times the larva had to be repositioned on the center of the tray).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

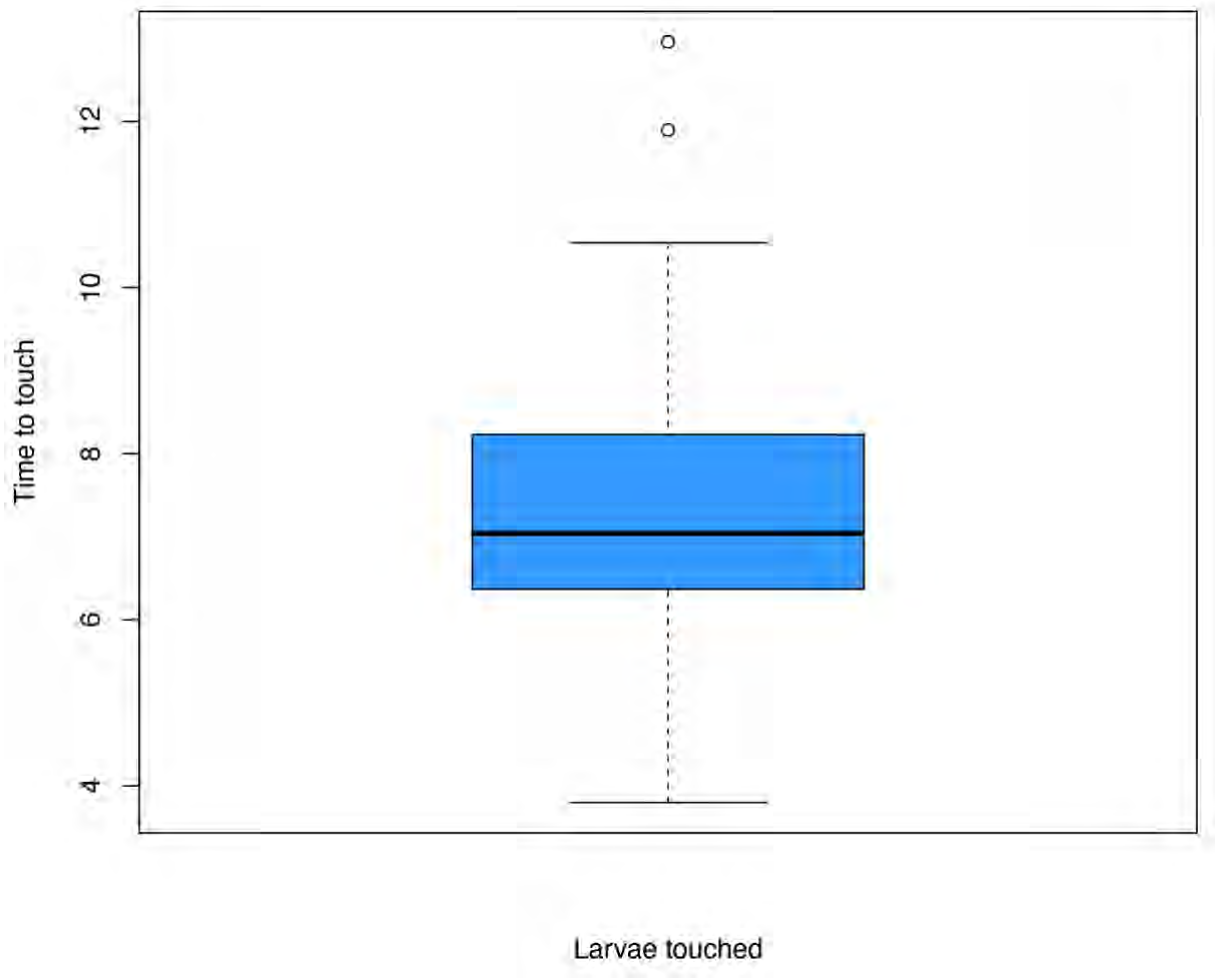


Fig. S2. *Rana temporaria* larvae were tested for escape behavior to an aversive stimulus represented by an approaching transparent plastic pipette filled with 4 ml of water containing chemical predator cues to be released and a predator model glued to the top of the pipette. The graph shows the time 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.

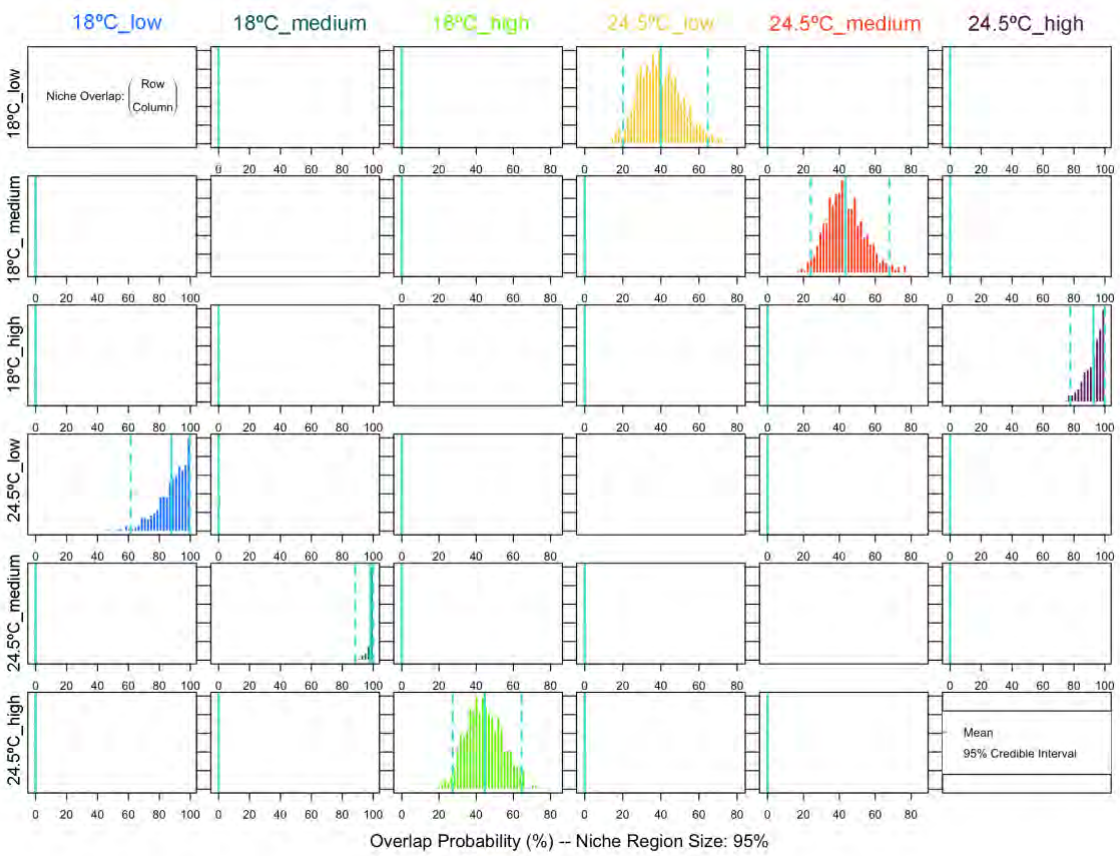


Fig. S3. Posterior distribution of the probabilistic niche overlap of *Rana temporaria* larvae reared with three different diets varying in nutritional quality and two temperatures in a crossed experimental design (colors correspond to treatments on the columns). Niche overlap metrics were generated 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).

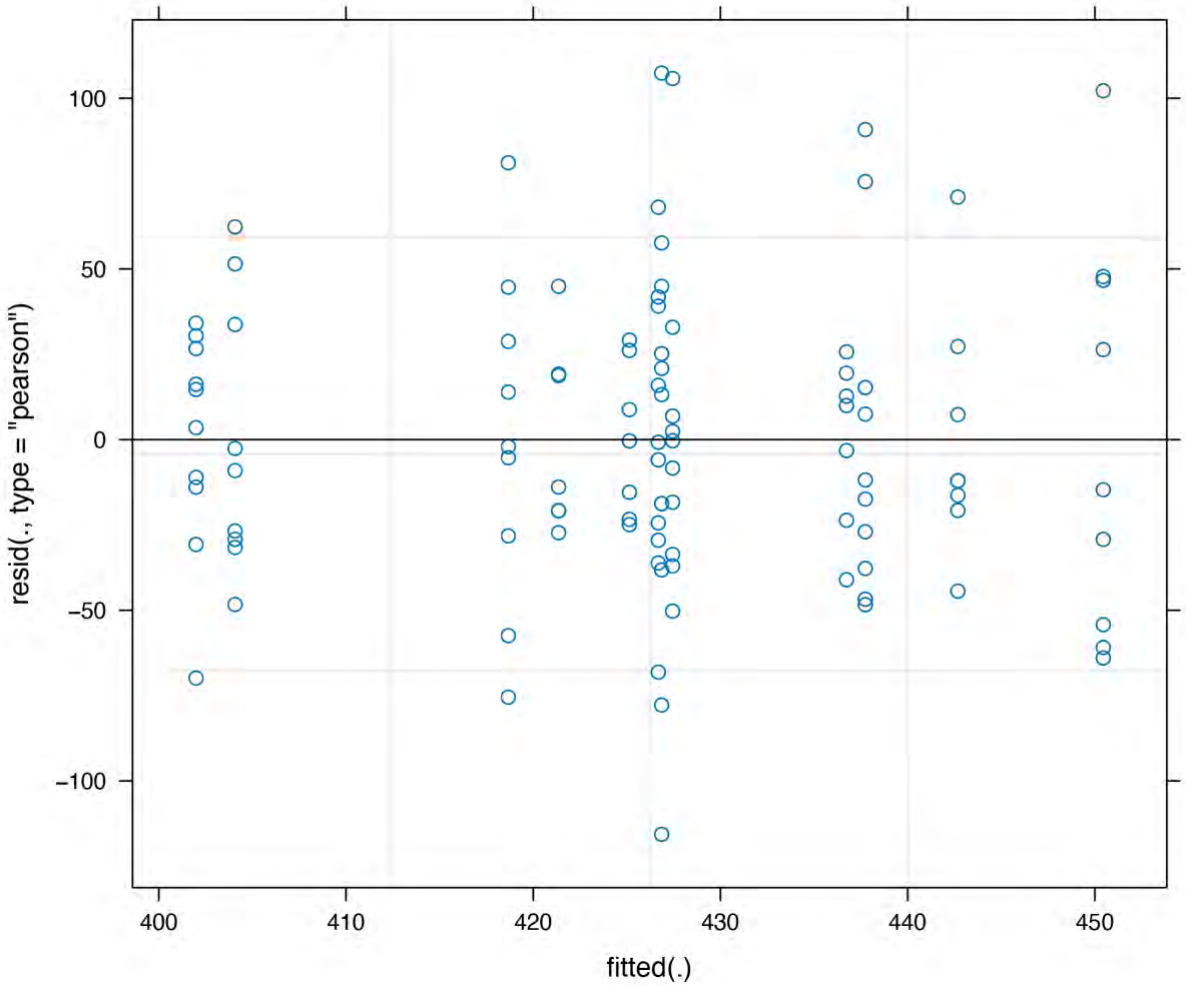


Fig. S4. Residual distribution of the model testing the effects of food treatment, rearing temperature, and exposure or not to a heat wave on body condition (SMI) of *Rana temporaria* larvae (see Table 1 for model description).

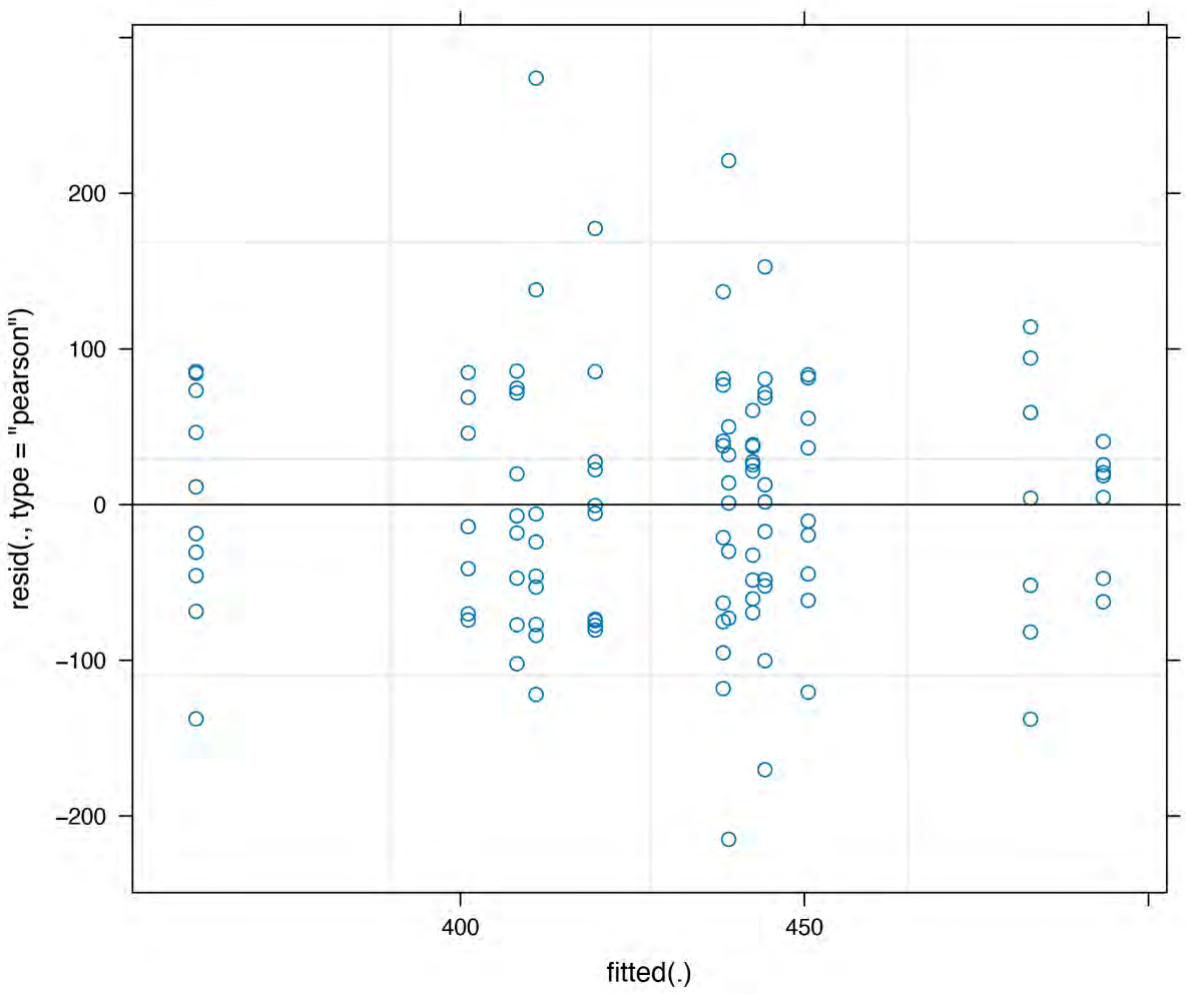
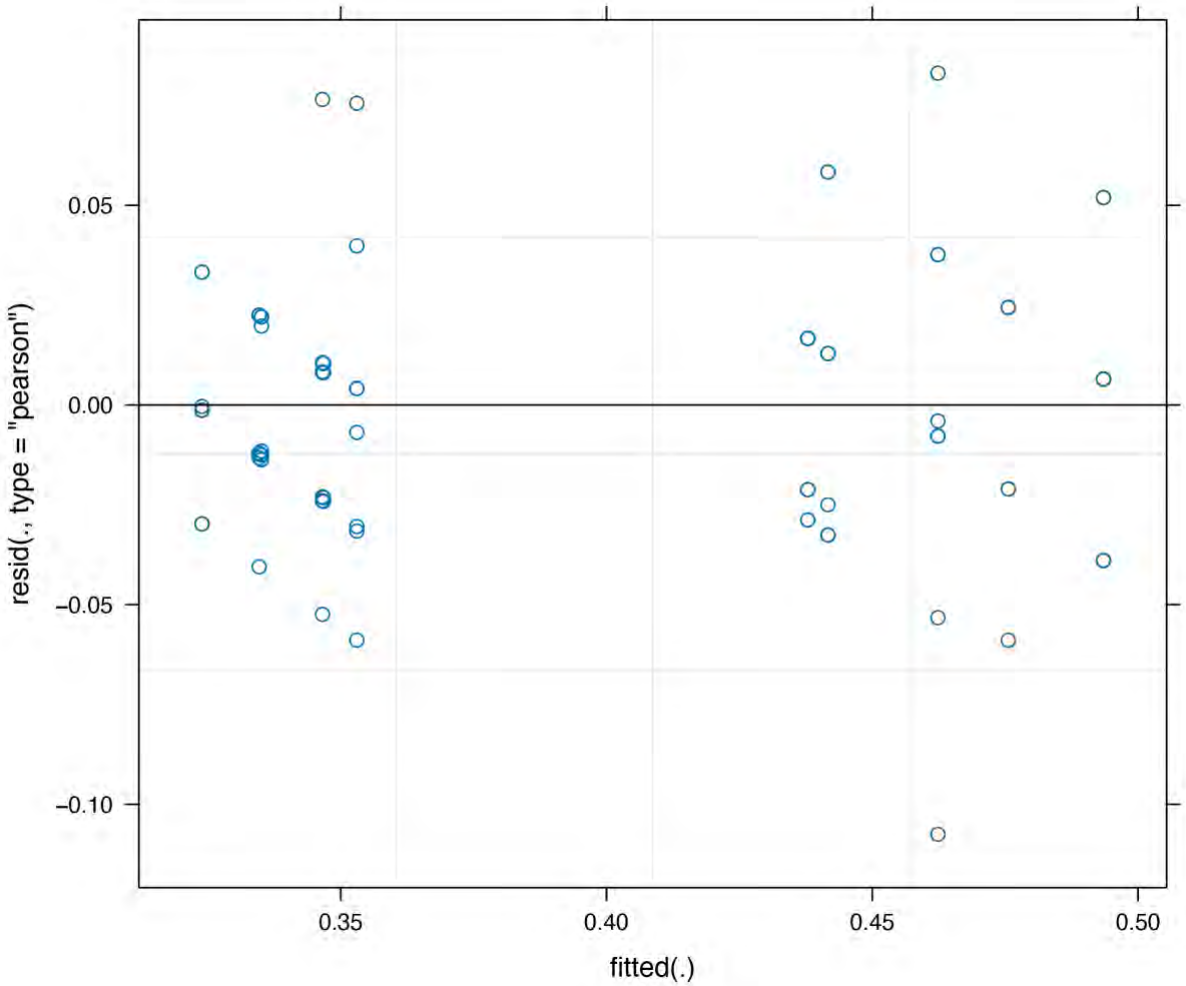


Fig. S5. Residual distribution of the model testing the effects of food treatment, rearing temperature, and exposure or not to a heat wave on mass of *Rana temporaria* larvae (see Table 1 for model description).



1341

1342 Fig. S6. Residual distribution of the model testing the effects of food treatment, rearing
1343 temperature, and exposure or not to a heat wave on developmental rate of *Rana temporaria*
1344 larvae (see Table 1 for model description).

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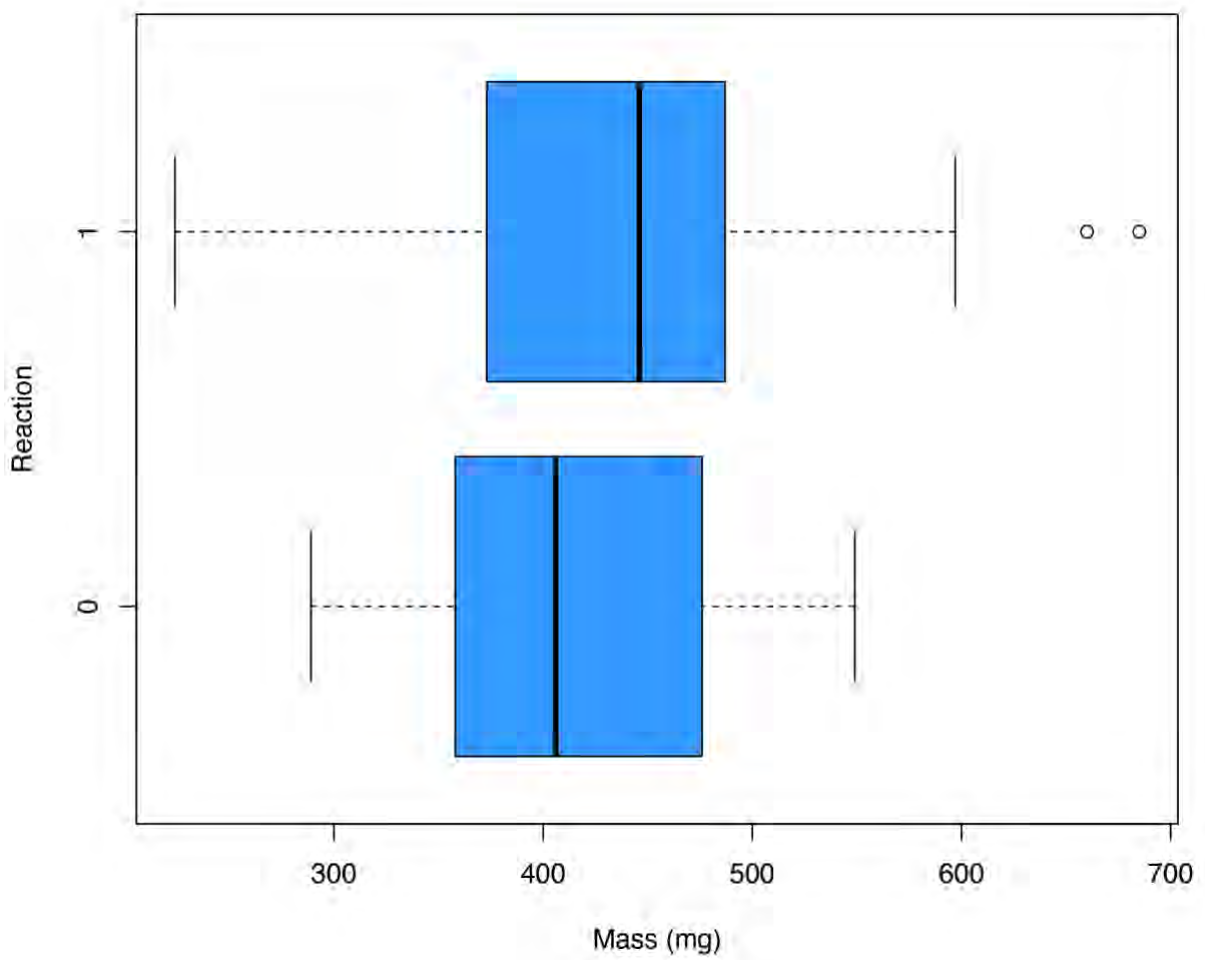


Fig. S7. Mass (mg) of *Rana temporaria* larvae that either reacted to the aversive stimulus presented in behavioral trials (1) or not (0). Wilcoxon-test: W = 711.5, p = 0.252.

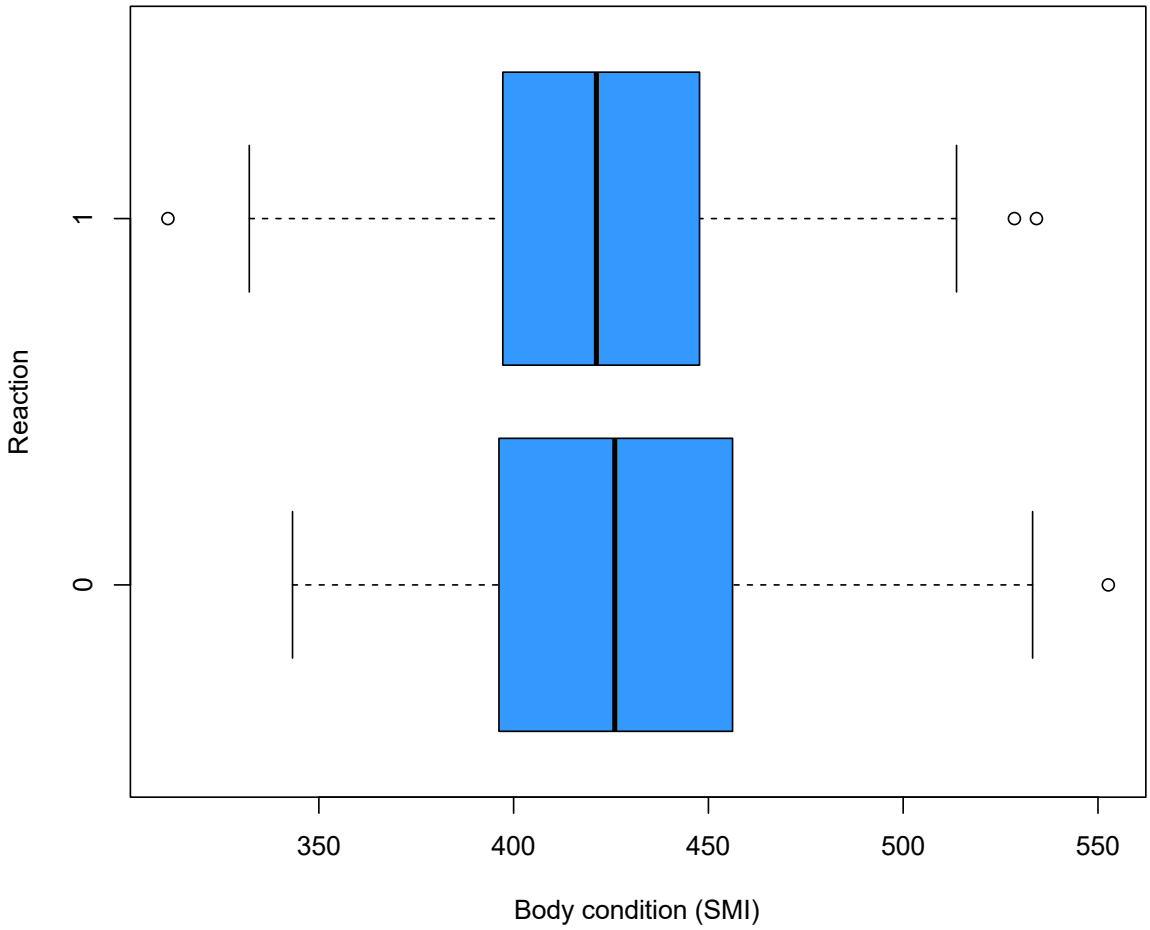


Fig. S8. Body condition (SMI) of *Rana temporaria* larvae that either reacted to the aversive stimulus presented in behavioral trials (1) or not (0). Wilcoxon-test: W = 936, p = 0.482.

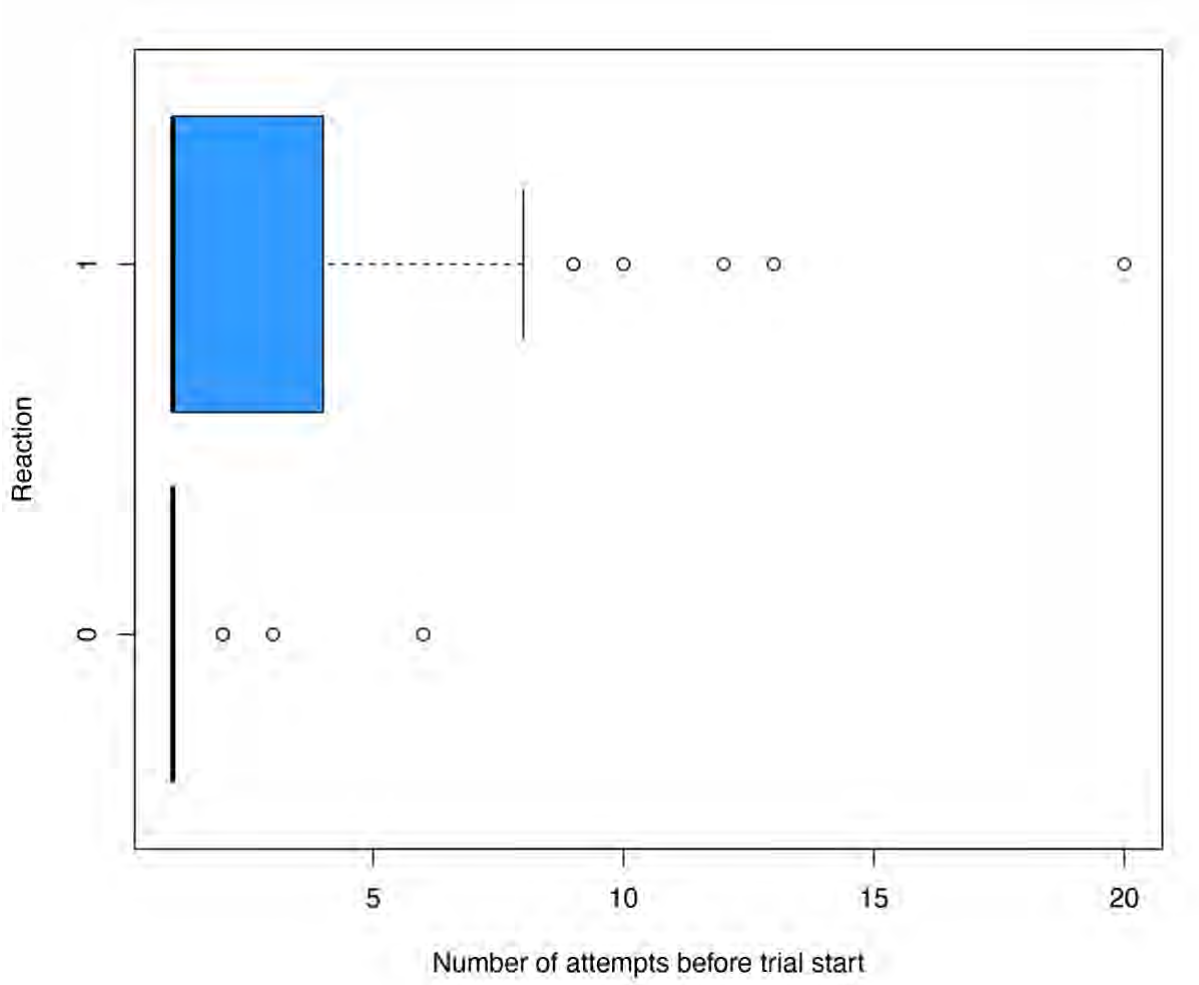


Fig. S9. Number of attempts to position *Rana temporaria* larvae before the start of the behavioral trials compared between larvae that either reacted to the aversive stimulus presented in behavioral trials (1) or not (0). Wilcoxon-test: W = 601, p = 0.022.

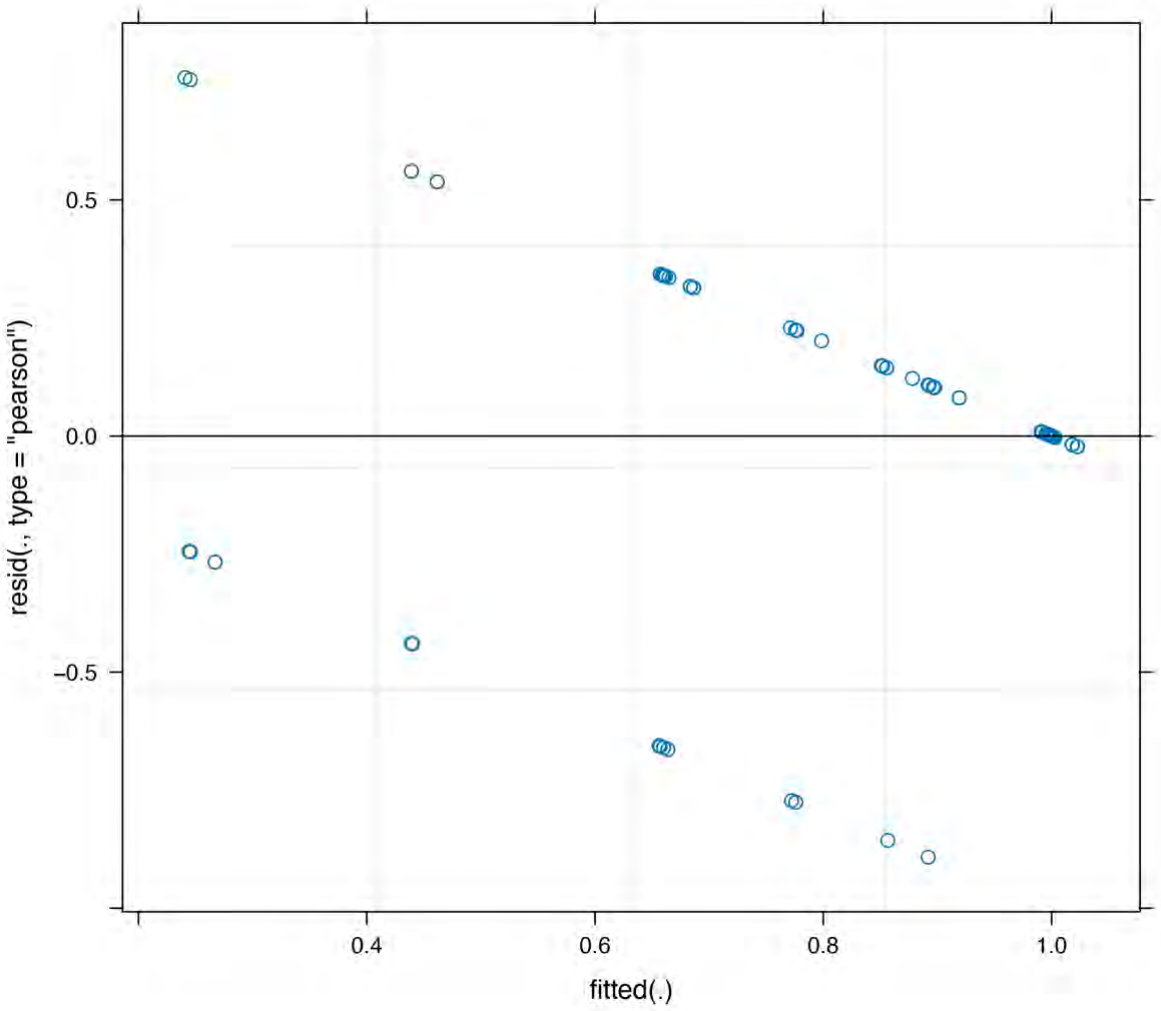


Fig. S10. Residual distribution of the model testing the effects of food treatment, rearing temperature, and exposure or not to a heat wave on likeliness to escape from an aversive stimulus of *Rana temporaria* larvae (see Table 2 for model description).

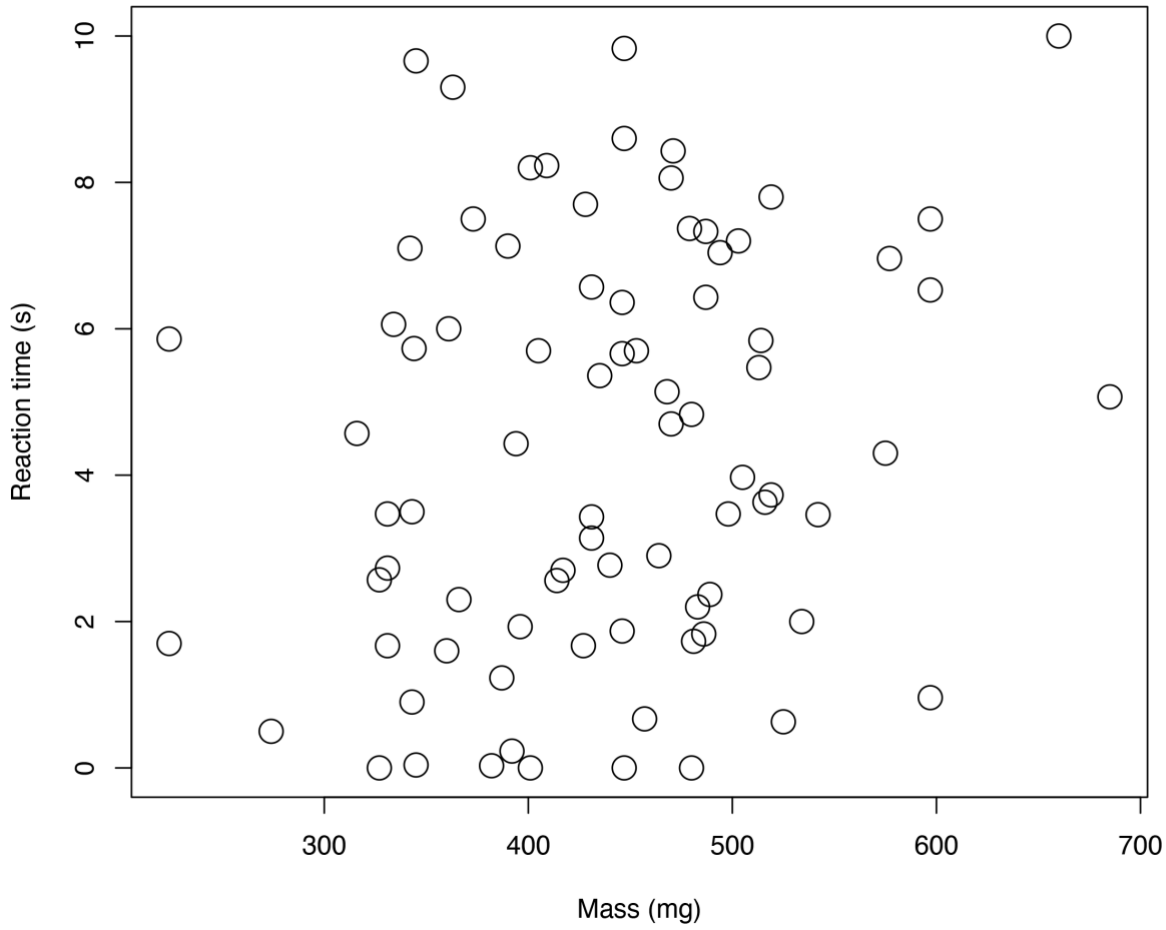


Fig. S11. Relationship between mass (mg) of *Rana temporaria* larvae and time to react to the aversive stimulus presented in behavioral trials. Adjusted R-squared = 0.030, F = 3.487, df = 79, p = 0.066.

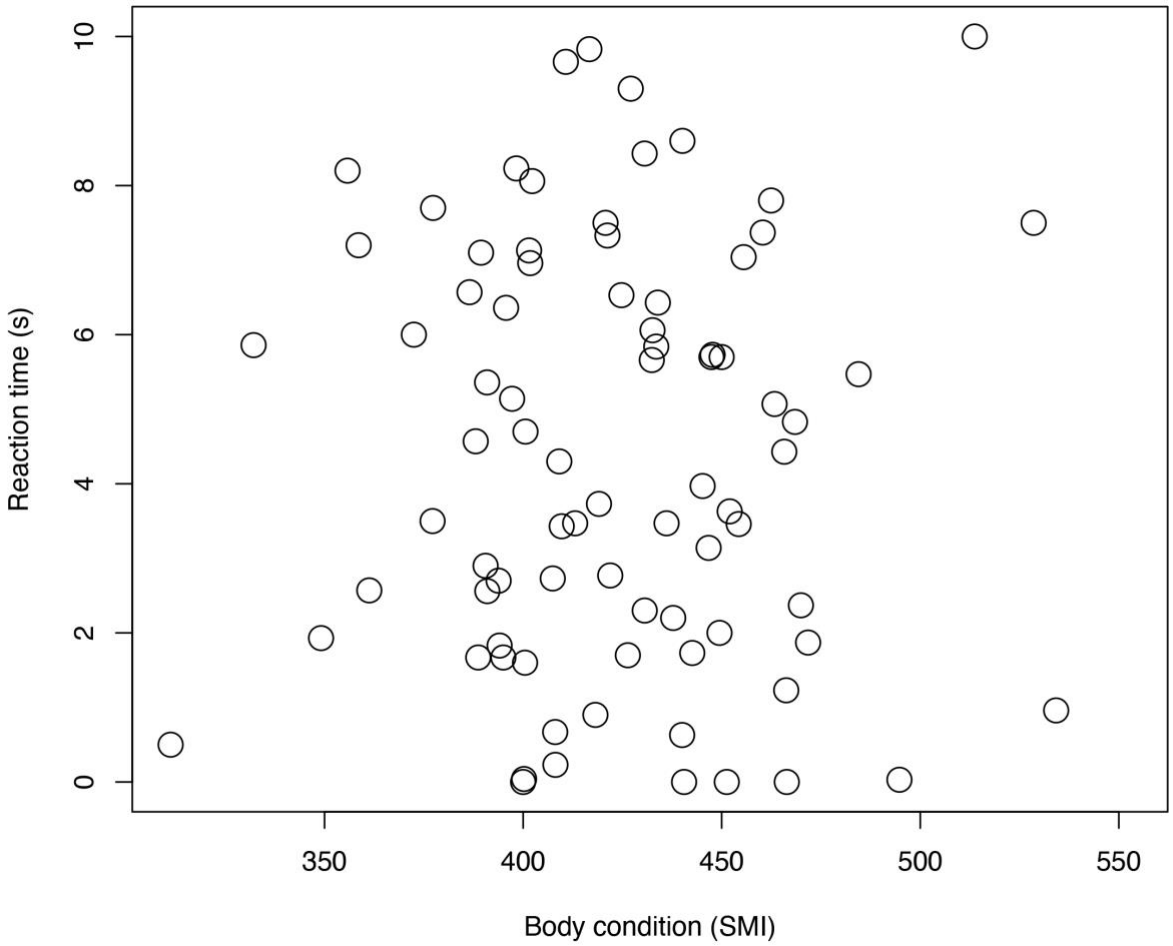


Fig. S12. Relationship between body condition (SMI) of *Rana temporaria* larvae and time to react to the aversive stimulus presented in behavioral trials. Adjusted R-squared = -0.013, F = 0.005, df = 79, p = 0.946.

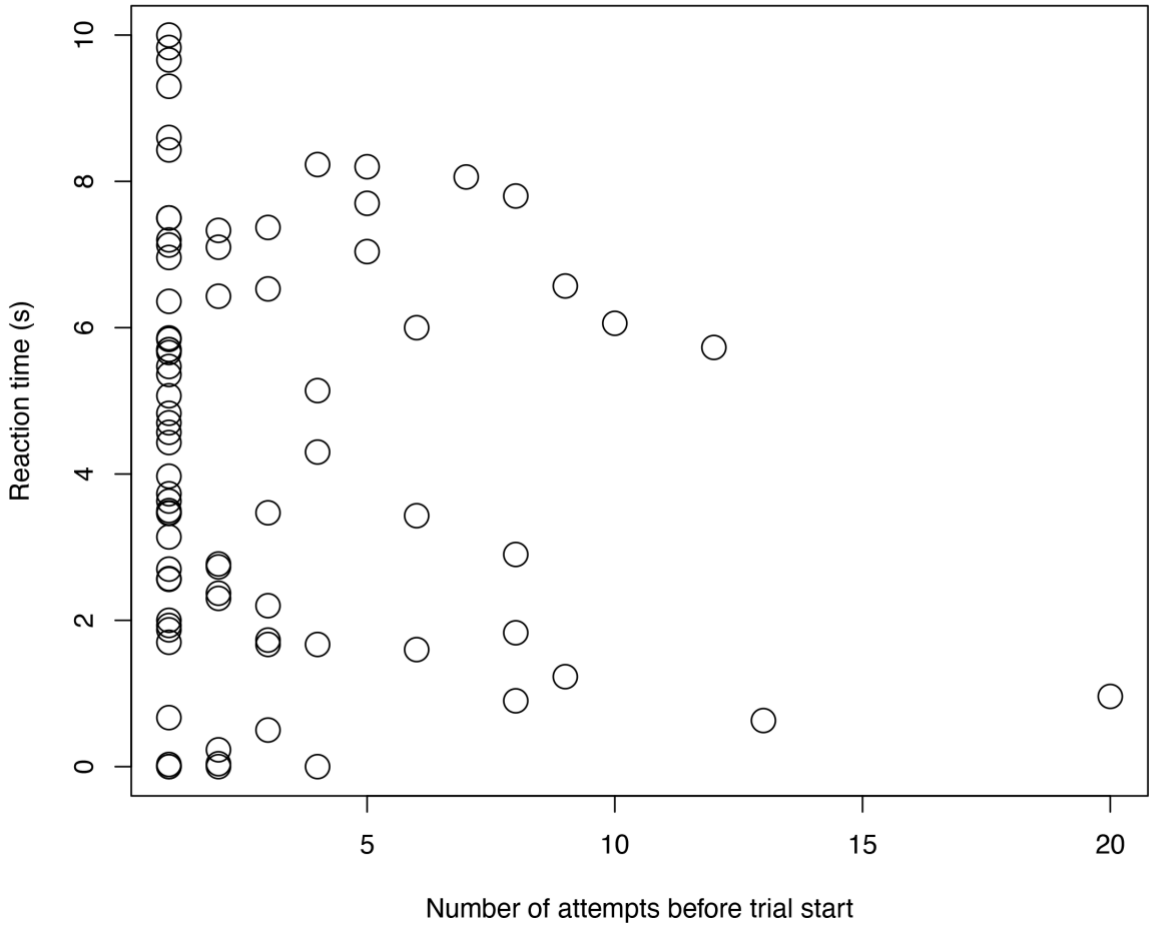


Fig. S13. Relationship between number of attempts to position *Rana temporaria* larvae before the start of the behavioral trials and time (s) the larvae took to react to the aversive stimulus presented. rho = -0.125, p = 0.263.

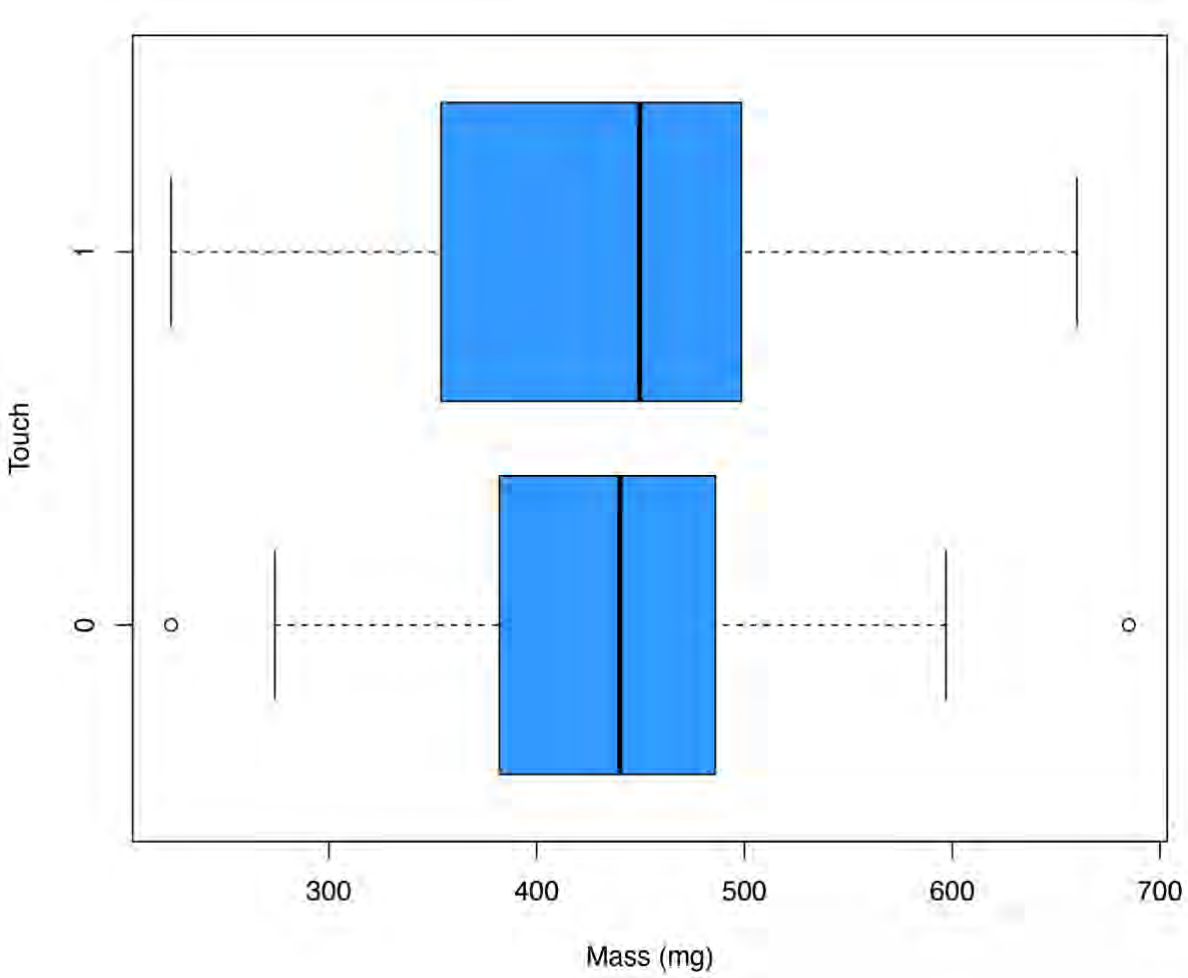


Fig. S14. Mass (mg) of reacting *Rana temporaria* larvae that either were touched by the predator model approached to them in behavioral trials (1) or not (0) before fleeing. Wilcoxon-test: W = 577, p = 0.722.

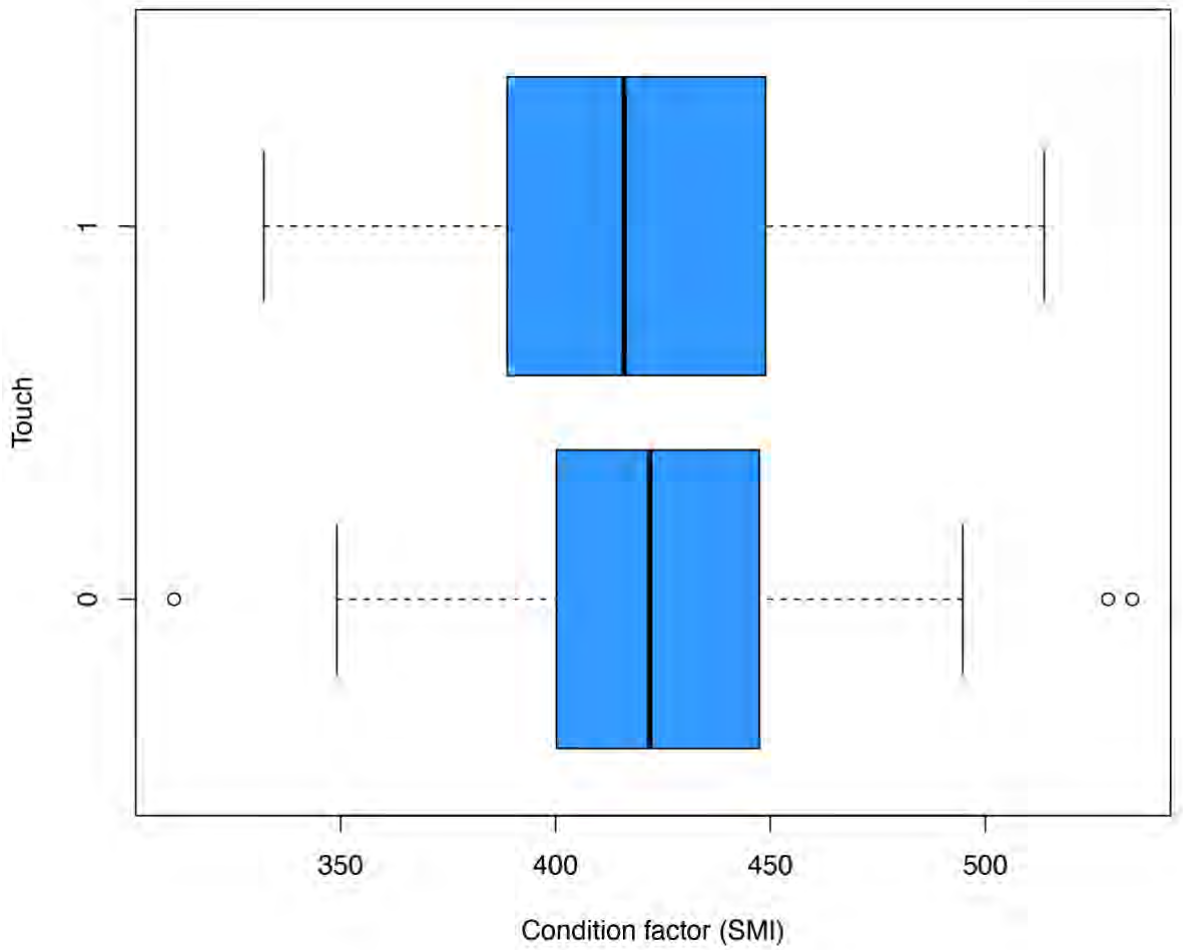


Fig. S15. Condition factor (SMI) of reacting *Rana temporaria* larvae that either were touched by the predator model approached to them in behavioral trials (1) or not (0) before fleeing. Wilcoxon-test: W = 697, p = 0.343.

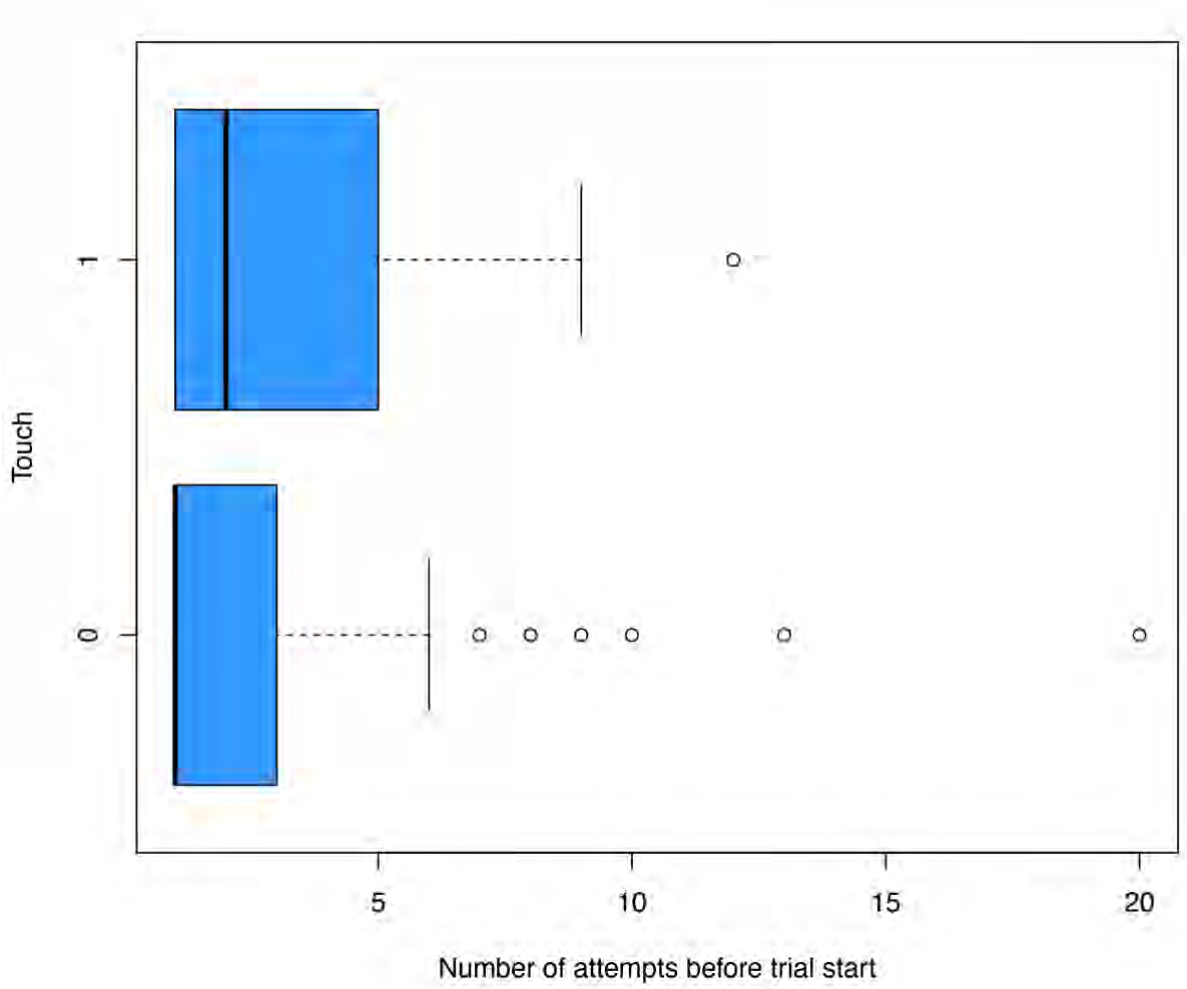


Fig. S16. Number of attempts to position *Rana temporaria* larvae before the start of the behavioral trials compared between larvae that either were touched by the predator model approached to them in the behavioral trials (1) or not (0) before fleeing. Wilcoxon-test: W = 533, p = 0.366.

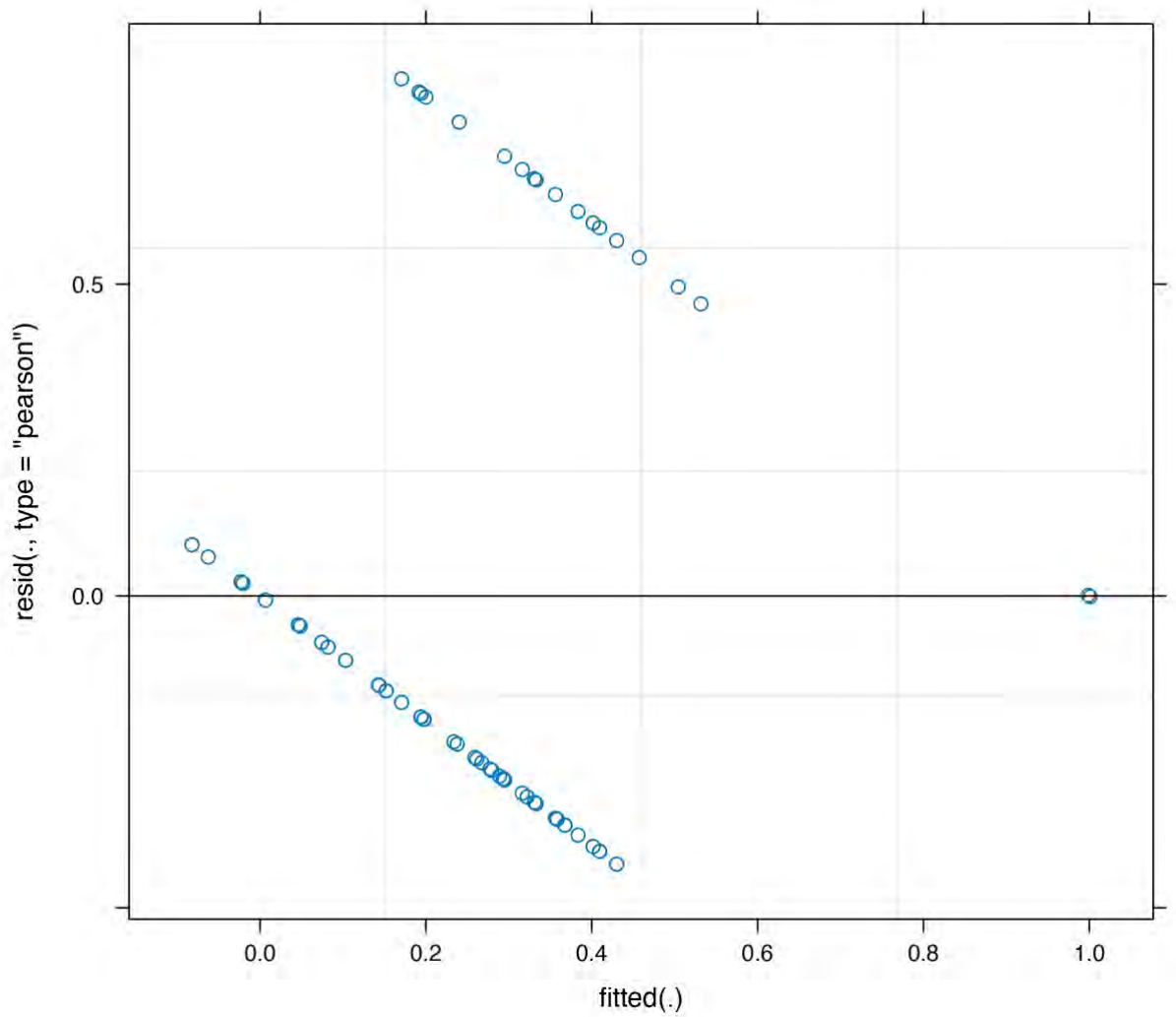


Fig. S17. Residual distribution of the model testing the effects of food treatment, rearing temperature, and exposure or not to a heat wave on likeliness to be touched by a predator model during an aversive stimulus of *Rana temporaria* larvae (see Table 2 for model description).

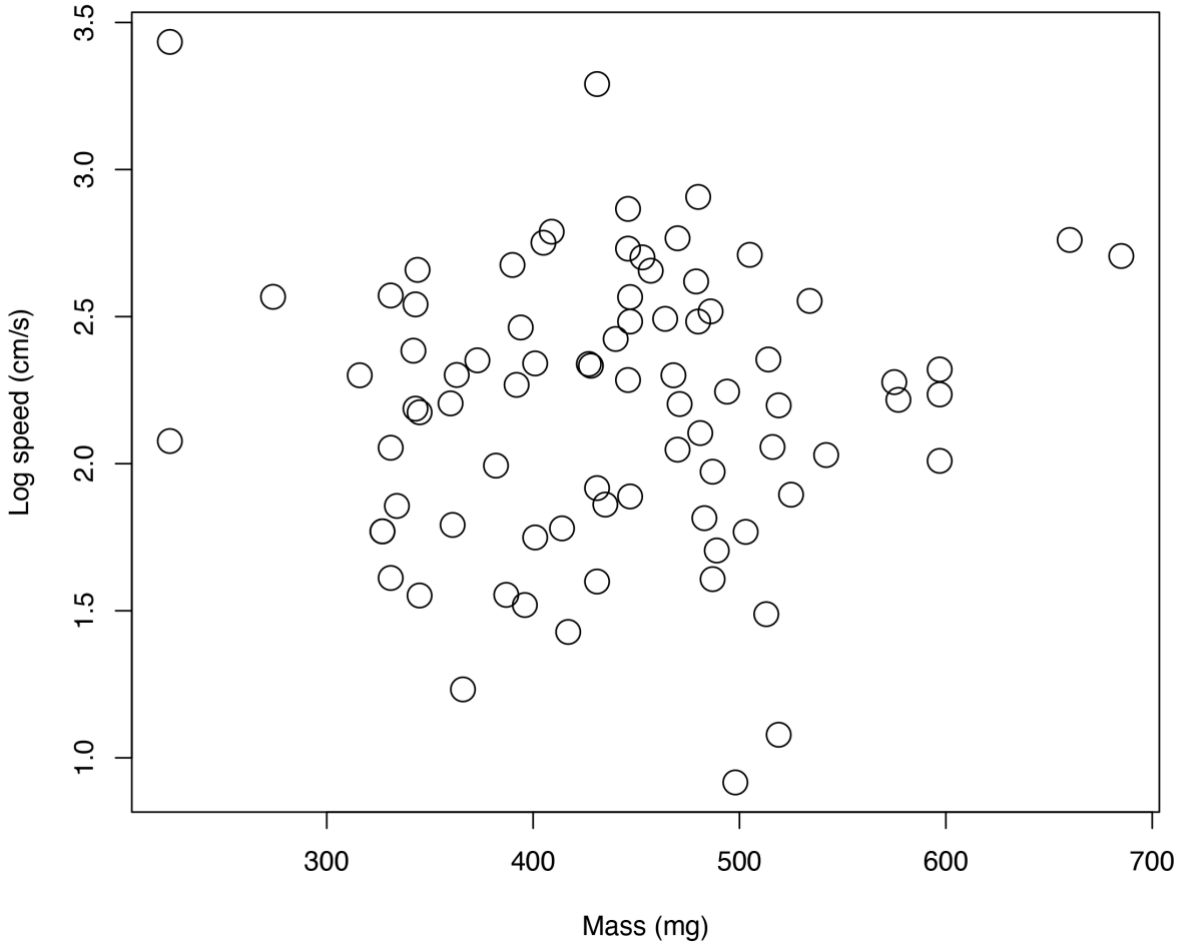


Fig. S18. Relationship between mass (mg) of *Rana temporaria* larvae and average speed (in cm/s, log transformed) while fleeing from the aversive stimulus presented in behavioral trials. Adjusted R-squared = -0.013, F = 0.004, df = 79, p = 0.949.

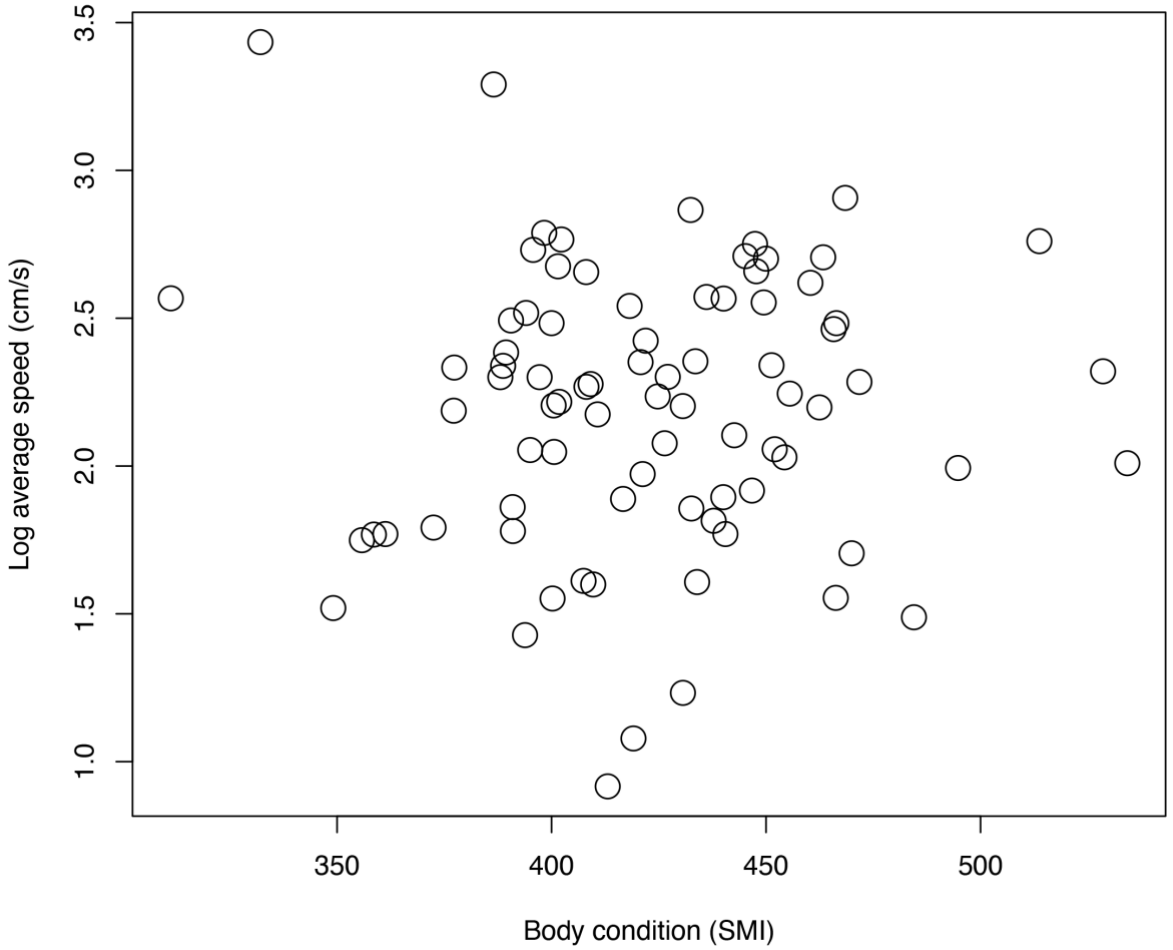
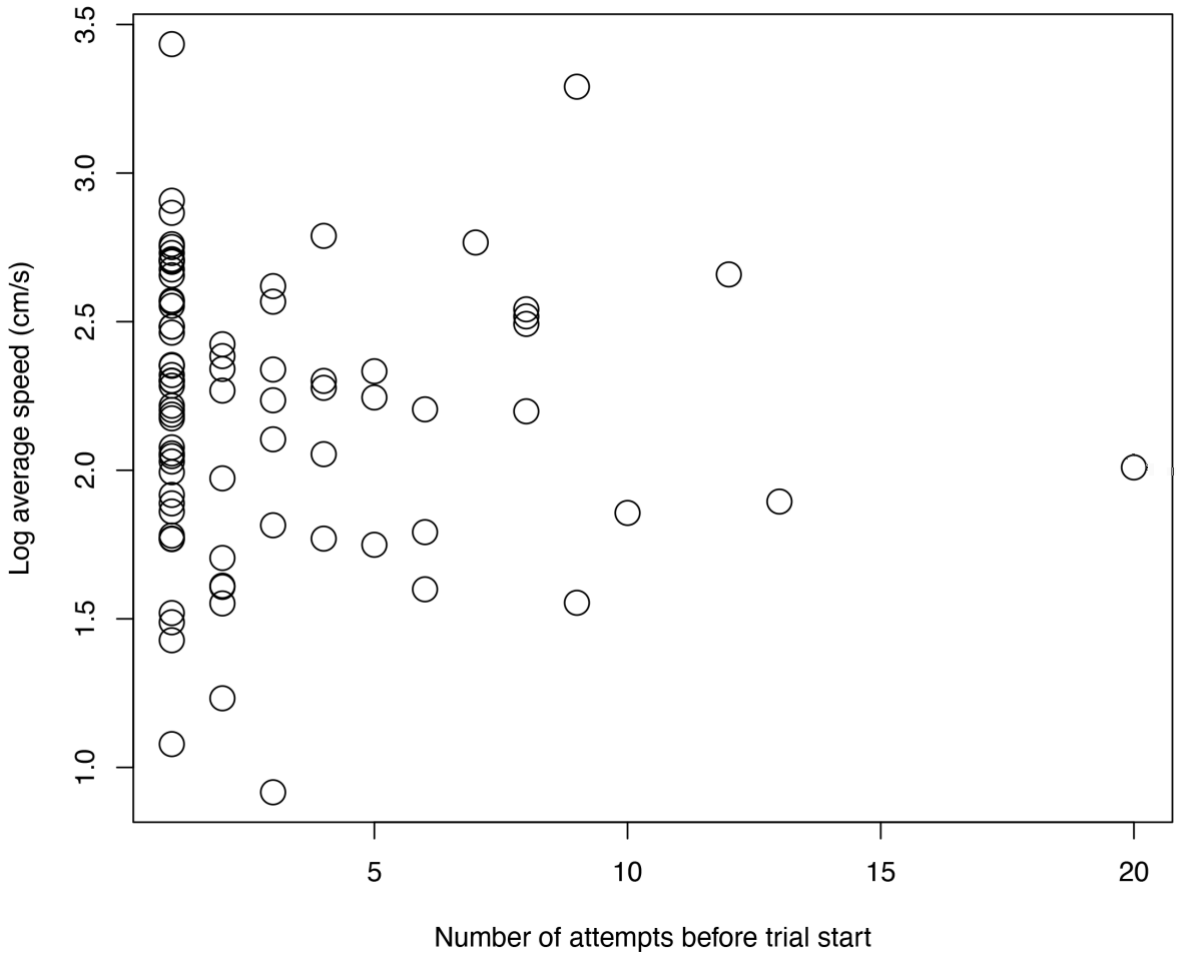


Fig. S19. Relationship between body condition (SMI) of *Rana temporaria* larvae and average speed (in cm/s, log transformed) while fleeing from the aversive stimulus presented in behavioral trials. Adjusted R-squared = -0.013, F = 0.009, df = 79, p = 0.923.



1410

1411 Fig. S20. Relationship between number of attempts to position *Rana temporaria* larvae before
1412 the start of the behavioral trials and average speed (in cm/s, log transformed) of the larvae
1413 while fleeing from the aversive stimulus presented. rho = -0.104, p = 0.354.

1414

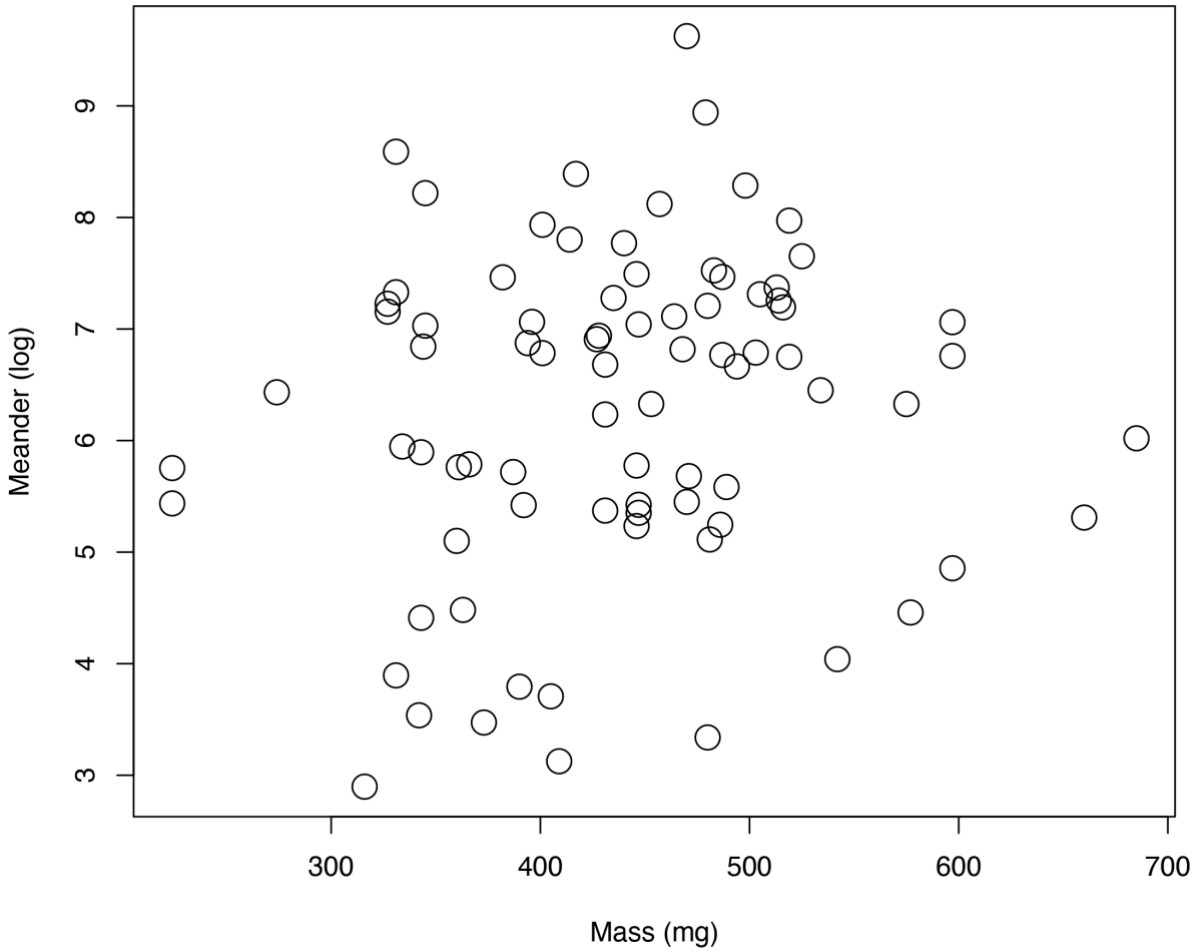


Fig. S21. Relationship between mass (mg) of *Rana temporaria* larvae and trajectory non-linearity ("meander", log transformed) while fleeing from the aversive stimulus presented in behavioral trials. Adjusted R-squared = 0.003, F = 1.271, df = 79, p = 0.263.

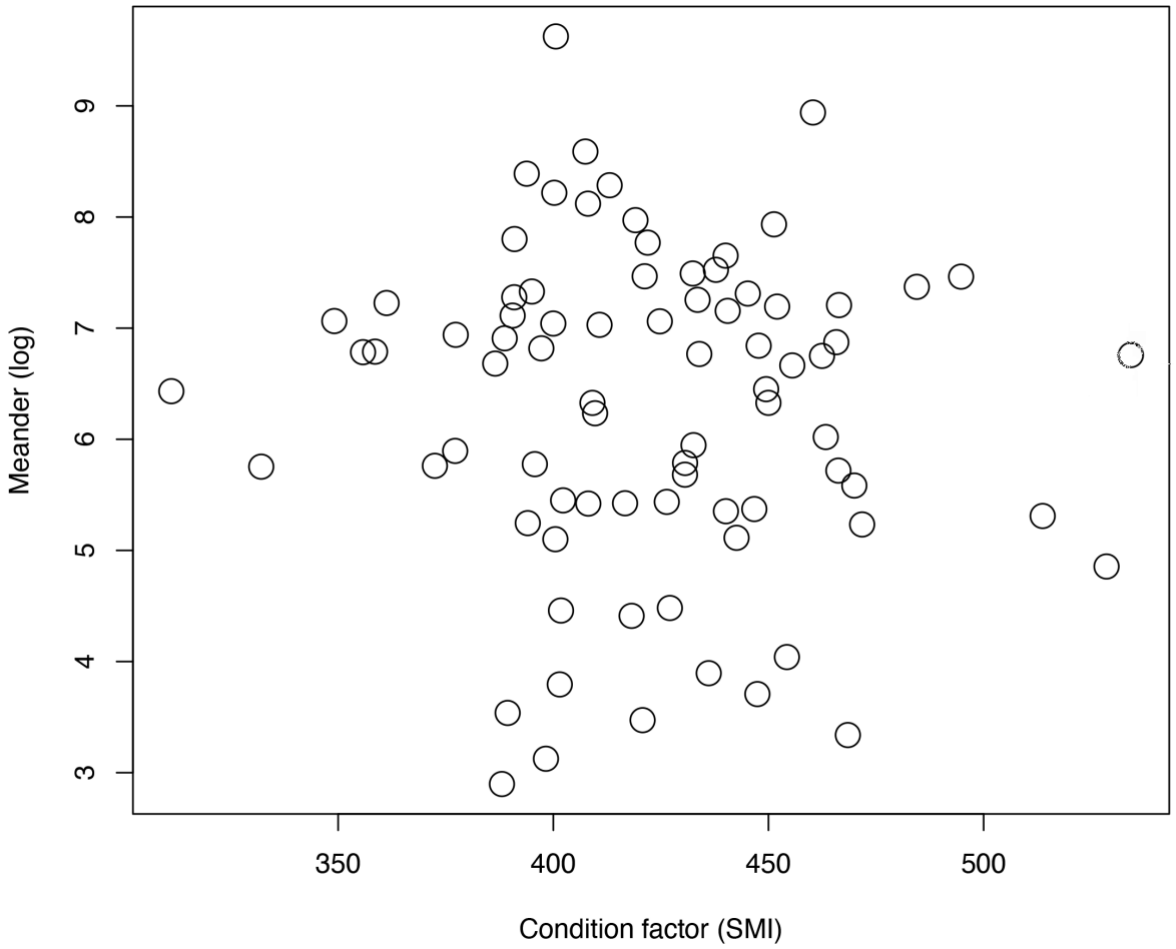


Fig. S22. Relationship between body condition (SMI) of *Rana temporaria* larvae and trajectory non-linearity ("meander", log transformed) while fleeing from the aversive stimulus presented in behavioral trials. Adjusted R-squared = -0.009, F = 0.247, df = 79, p = 0.620.

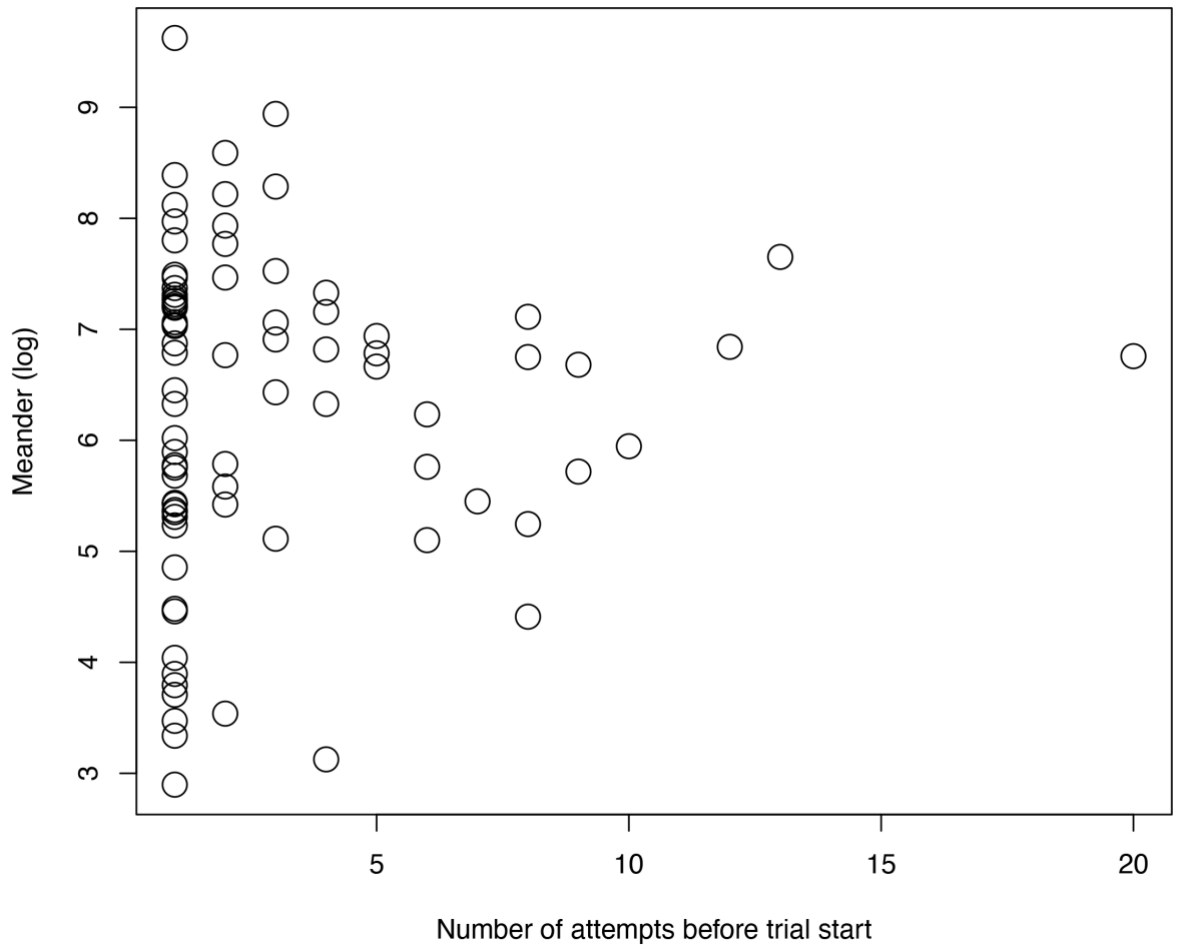


Fig. S23. Relationship between number of attempts to position *Rana temporaria* larvae before the start of the behavioral trials and trajectory non-linearity ("meander", log transformed) of the larvae while fleeing from the aversive stimulus presented. rho = 0.050, p = 0.657.

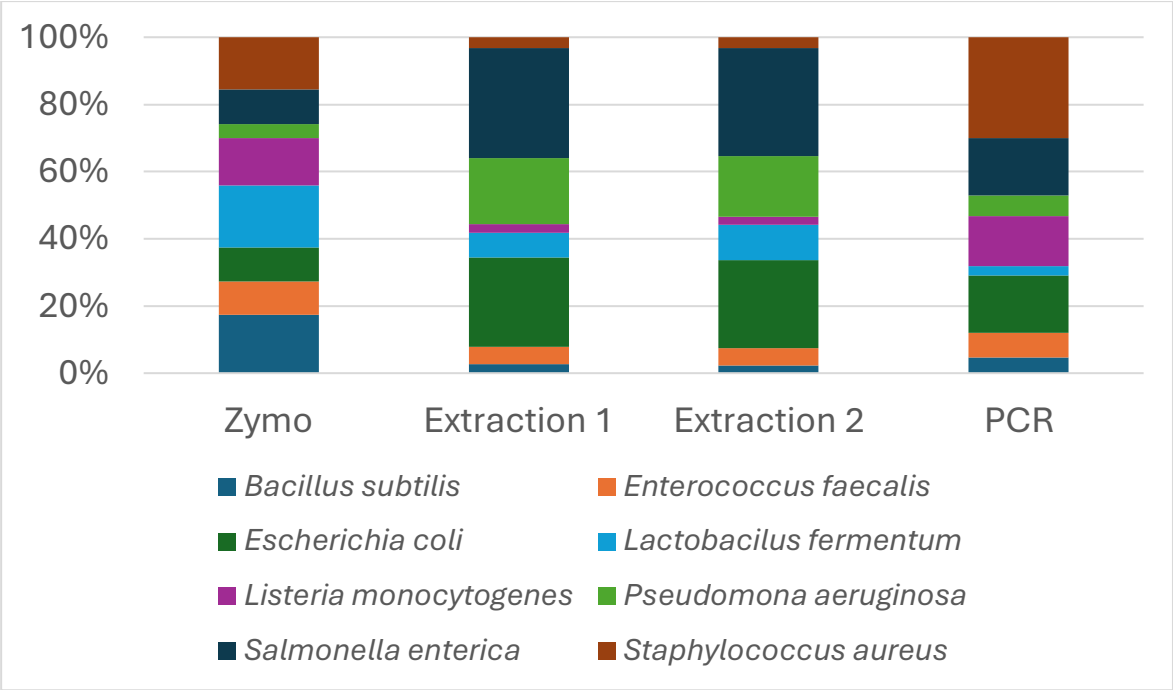


Fig. S24. Results of two positive controls for DNA extractions (ZymoBIOMICS™ microbial community standard, Zymo Research Europe GmbH) and one positive PCR control (ZymoBIOMICS™ microbial community DNA standard, Zymo Research Europe GmbH) in comparison with the expected community profile (Zymo), showing 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.

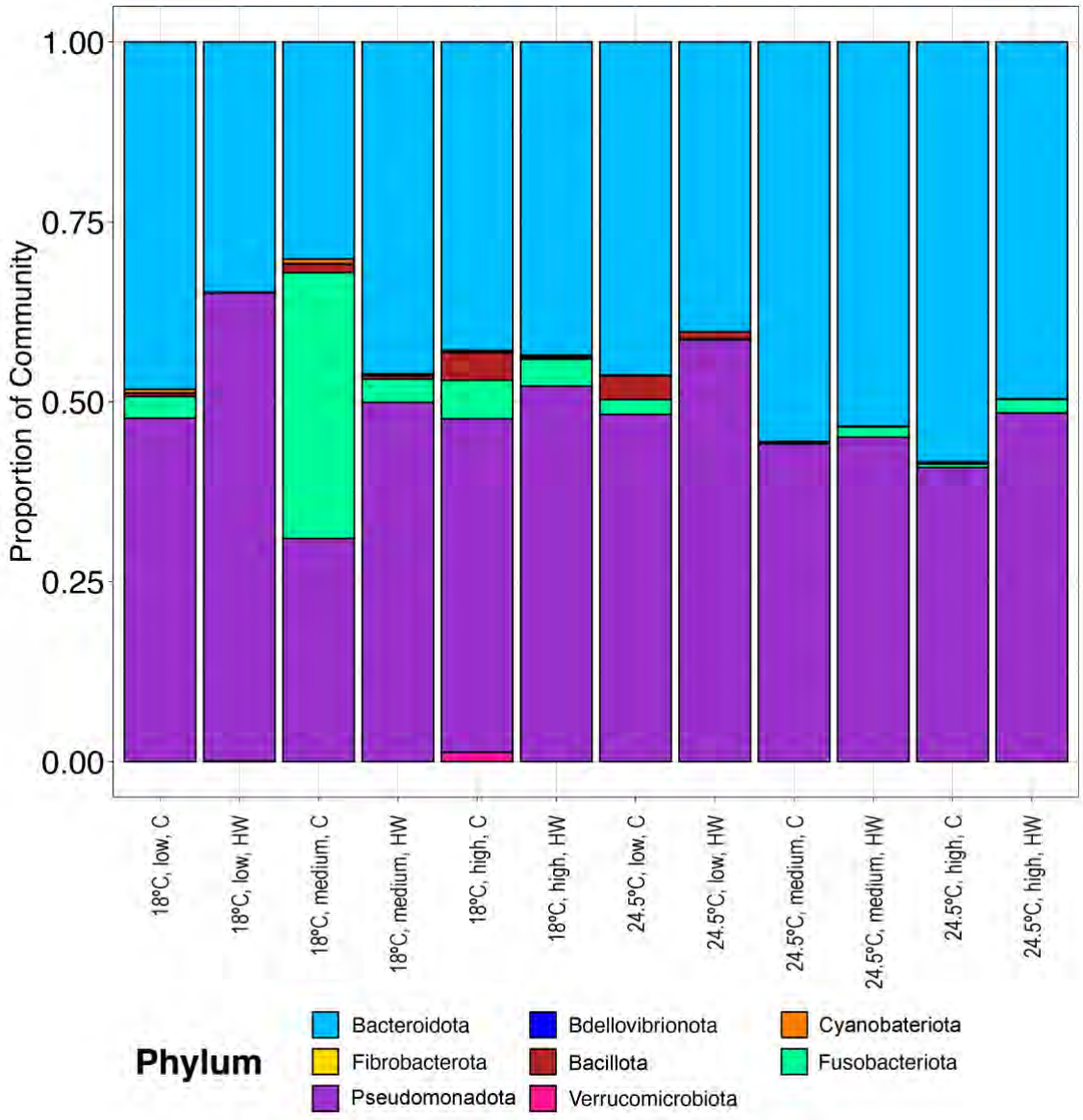


Fig. S25. Community composition of gut bacteria based on phylum for *Rana temporaria* larvae fed three diets with increasing levels of protein, fat, and animal components (considered as low-. medium- and high-quality), reared at either 18 ºC or 24.5 ºC. and exposed or not to a heat wave, in a crossed experimental design. The heat wave 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 heat wave (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

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