

Warm vegetarians? Heat waves and diet shifts in tadpoles

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Abstract. Temperature can play an important role in determining the feeding preferences of ectotherms. In light of the warmer temperatures arising with the current climatic changes, omnivorous ectotherms may perform diet shifts toward higher herbivory to optimize energetic intake. Such diet shifts may also occur during heat waves, which are projected to become more frequent, intense, and longer lasting in the future. Here, we investigated how heat waves of different duration affect feeding preferences in omnivorous anuran tadpoles and how these choices affect larval life history. In laboratory experiments, we fed tadpoles of three species on animal, plant, or mixed diet and exposed them to short heat waves (similar to the heat waves these species experience currently) or long heat waves (predicted to increase under climate change). We estimated the dietary choices of tadpoles fed on the mixed diet using stable isotopes and recorded tadpole survival and growth, larval period, and mass at metamorphosis. Tadpole feeding preferences were associated with their thermal background, with herbivory increasing with breeding temperature in nature. Patterns in survival, growth, and development generally support decreased efficiency of carnivorous diets and increased efficiency or higher relative quality of herbivorous diets at higher temperatures. All three species increased herbivory in at least one of the heat wave treatments, but the responses varied among species. Diet shifts toward higher herbivory were maladaptive in one species, but beneficial in the other two. Higher herbivory in omnivorous ectotherms under warmer temperatures may impact species differently and further contribute to changes in the structure and function of freshwater environments.

Key words: *Amphibian; carnivory; climate change; diet regulation; Discoglossus galganoi; herbivory; Hyla arborea; Hyla meridionalis; omnivory; stable isotope mixing models.*

INTRODUCTION

Studies on the influence of temperature on nutrient acquisition by organisms are now in the forefront of climate change biology (Cross et al. 2015). In ectotherms, higher temperatures promote a greater increase in feeding and gut passage rates than in assimilation rates (Myrick and Cech 2000, Seifert et al. 2014). Consequently, the overall assimilation efficiency of nutrients with contrasting size and complexity may vary with temperature. For instance, in crayfish, which are omnivorous ectotherms, temperature changes the protein to carbohydrate assimilation ratio, which shifts to a greater assimilation of carbohydrates at higher temperatures (Croll and Watts 2004). Furthermore, higher temperatures have a stronger effect on catabolic than on anabolic processes, promoting a greater increase in respiration than in growth (Karl and Fischer 2008, Forster et al. 2011), and therefore increasing the demand for carbon over nitrogen. These imbalanced effects of temperature on different aspects of metabolism, together with the enhanced digestion of plant material at high temperatures (Floeter et al. 2005), suggest that, in order to maximize energy intake and sustain higher

metabolic rates, omnivorous ectotherms should perform diet shifts toward increased herbivory. In support of this hypothesis, a recent study found a seasonal decrease in the trophic level from winter to summer in copepods and showed experimentally that herbivory increased at higher temperatures (Boersma et al. 2016). The higher prevalence of herbivory found in fish communities of warmer waters at lower latitudes (Floeter et al. 2005, Behrens and Lafferty 2007, Jeppesen et al. 2010, Gonzalez-Bergonzoni et al. 2012) also suggests that optimization of energy intake at warmer temperatures may result in avoidance of protein-rich diets.

Freshwater ecosystems are particularly sensitive to climate change (Thompson et al. 2013), as approximately 99% of the species composing freshwater communities are ectotherms (Dudgeon et al. 2006). Yet, despite the strong influence of temperature in the physiological processes of ectotherms, there is only one recent study addressing the role of temperature in determining their feeding preferences (Boersma et al. 2016). Amphibian larvae are key components of freshwater communities and strongly affect nutrient recycling, zooplankton diversity, and macrophyte biomass (Arribas et al. 2015). Most anuran tadpoles feed on multiple resources: detritus, periphyton, phytoplankton, zooplankton, macrophytes, and macroinvertebrates (Petranka and

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Kennedy 1999, Altig et al. 2007, Arribas et al. 2015). Trophic plasticity can help amphibian larvae to mitigate the negative effects of different stressors, including higher temperatures, which, while accelerating tadpole growth and differentiation, sometimes have costs in survival, size at metamorphosis, and post-metamorphic fitness (Wilbur and Collins 1973, Tejedo et al. 2010, but see Earl and Whiteman 2015). Tadpoles can shift diet in response to, e.g., intraspecific competition and pond drying (Pfenning 1990, Caut et al. 2013), but no studies have examined diet shifts in response to changes in temperature.

While climate change can have severe effects on individual fitness, organisms may acclimate through phenotypic plasticity or evolve genetic adaptations (Hoffmann and Sgrò 2011). However, the increased climatic variability poses a more serious challenge, as species may be unable to show an immediate response, even if they have the potential for genetic adaptation (Reusch et al. 2005). Until recently, most experimental research neglected the importance of discrete weather events as components of climate change (Chapman et al. 2014, Vasseur et al. 2014). However, short-term climatic stress, comprising increased climate variability and extreme climatic events, is predicted to severely affect species' fitness and life-history traits (Clusella-Trullas et al. 2011, Kingsolver et al. 2013, Paaijmans et al. 2013). Specifically, extreme events such as heat waves may cause mass mortality (Garrahou et al. 2009), decrease in condition, immune function, and storage reserves of the organisms (Fischer et al. 2014), loss of genetic diversity and range shifts (Reusch et al. 2005), changes in species interactions (Eggers et al. 2012) and community structure (Ma et al. 2015), and can affect ecosystem function (Wernberg et al. 2012).

In this study, we investigated feeding preferences and diet shifts in tadpoles of three anuran species breeding along a seasonal climatic gradient. The tadpoles were fed animal-based, plant-based, or mixed diets and exposed to temperatures simulating either a currently commonly occurring short heat wave or a long heat wave expected to become more frequent in the near future (Meehl and Tebaldi 2004, Schär et al. 2004). These responses were compared to those of tadpoles reared under present spring conditions, or under an exceptionally cold spring (CS; see *Materials and methods*). We reconstructed the dietary choices of the mixed diet tadpoles with stable isotope mixing models to investigate diet shifts, and recorded treatment responses in terms of survival, growth rate, duration of the larval period, and mass at metamorphosis. We predicted that (1) the proportion of the animal diet should decrease from the earliest breeding species to the latest breeding species, following the seasonal climatic gradient. In the heat wave treatments, we predicted that (2) tadpole performance on the animal diet should decrease, (3) tadpole performance on the plant diet should improve, (4) tadpoles on the mixed diet should increase herbivory, and (5) diet shifts should improve tadpole performance.

MATERIALS AND METHODS

Study area and species

The Iberian Peninsula, Western Europe, is a climate change “hot-spot” that has experienced temperature increase during the latter half of 20th century, especially in early summer (Giorgi 2006, Dasari et al. 2014). In the 21st century, the mean temperature of this region is expected to increase up to 6°C and heat waves are expected to become longer, more frequent and intense (Jerez et al. 2013). Air temperatures in our study area in southwestern Portugal (Grândola, 38°05' N, 8°33' W) are monitored since 2002, and we used a 10-year data set (2002–2012) to characterize the most common spring heat waves occurring in the area. We followed the heat wave definition of Frich et al. (2002): a heat wave occurs when the daily maximum temperature exceeds the average maximum temperature by 5°C for more than five consecutive days, the reference period being 1961–1990. We found that heat waves occurred frequently in the end of April and lasted on average 7.8 ± 1.3 d (mean \pm SD). In some years they were followed by a second heat wave in mid-May lasting on average 7.5 ± 1.3 d (B. M. Carreira, *unpublished data*). According to a five-year survey carried out in 42 temporary ponds located in the study area during April and May (2010–2014), water temperatures ranged from 17 to 25°C with an average temperature of 20°C (R. Rebelo, *unpublished data*).

We focused our study on three common anuran species with larval development during spring. These species have similar thermal performance limits (Katzenberger 2015), but differ in the timing of breeding, so that tadpoles of the earliest species meet lower water temperatures than tadpoles of the later breeding species. The Iberian painted frog *Discoglossus galganoi* (Capula et al. 1985) is a Mediterranean species, which reproduces in temporary and ephemeral ponds between October and July (Rebelo and Crespo 1999). Its optimal thermal performance is between 20 and 34°C (Katzenberger 2015), and tadpoles face an average temperature of 10.3 ± 1.5 °C during the coldest month of their development (R. Rebelo, *unpublished data*). The European tree frog *Hyla arborea* (Linnaeus, 1758) is a Eurasian species, which, in southern Portugal, reproduces in temporary and semi-permanent ponds along the coast (Atlantic climate) between March and June (Rebelo and Crespo 1999). Its optimal thermal performance occurs between 23 and 36°C (Katzenberger 2015), and tadpoles face an average temperature of 12.6 ± 0.7 °C in the coldest month of their development (R. Rebelo, *unpublished data*). The Mediterranean tree frog *Hyla meridionalis* (Boettger, 1874) breeds in inland temporary ponds (Mediterranean climate) between April and June (Rebelo and Crespo 1999). Its optimal thermal performance is between 20 and 37°C (Katzenberger 2015), and tadpoles face an average temperature of 13.9 ± 1.6 °C in the coldest month of their development (R. Rebelo, *unpublished data*).

Experimental setup and procedures

We collected clutches of *D. galganoi* and *H. arborea* and brought them to the facilities at the University of Lisbon, where the eggs were kept at 20°C in 1.5-L aquaria (~30 eggs/aquarium) under 12L:12D photoperiod until hatching (see Table 1). Because of the near-threatened conservation status of *D. galganoi*, we collected only three large clutches (~600 eggs/clutch), which were later equally divided in the experimental treatments. In *H. arborea*, we collected 60 small clutches (~10 eggs/clutch), pooled the hatchlings and randomized tadpoles across the treatments. We found no egg clutches from *H. meridionalis* and collected newly hatched tadpoles (Gosner stages 21–24) from different parts of a pond with a large population with hundreds of adult frogs (see Table 1 for details).

At the start of the experiments, room temperature was lowered to 17°C to achieve the lowest experimental temperature, while temperatures in the treatments where warming was applied were obtained with aquarium heaters placed in the water baths. Tadpoles (stage 25; Gosner 1960) were photographed to determine their initial body size using ImageJ software and transferred to individual 0.5-L plastic vials placed in water baths. In each of the three species, we followed a fully factorial experimental design with diet (three levels) and temperature (four or five levels, see Temperature) as factors. In *D. galganoi* and *H. arborea*, we used 28 randomly assigned tadpoles individually reared per treatment combination, while, in *H. meridionalis*, we used 14 individually reared tadpoles per treatment combination. Tadpoles were fed ad libitum every other day along with water renewal, and the position of each vial in the water bath was randomized. Water bath temperatures were checked daily and adjusted when necessary. At the end of the early spring heat wave (see Temperature), one-half of the tadpoles in each treatment were weighed to determine growth rate at a fixed time and removed from the experiment for another study. The remaining tadpoles were allowed to complete metamorphosis. After the first

tadpole reached Gosner stage 42 (emergence of forelimbs), all vials were checked daily for metamorphs and the length of each individual's larval period was recorded. The metamorphs were transferred to plastic containers (12.5 × 12.5 × 9.0 cm) with moist paper towel in the bottom and allowed to absorb their tails (Gosner stage 46) at 20°C. After that, froglets were euthanized, photographed, and weighed (fresh mass). We euthanized froglets by rapid freezing at –18°C to prevent contamination of their isotopic signature with euthanizing agents.

Diet

The three diet treatments were A, animal diet, defrosted insect larvae collected in the study area or obtained commercially; P, plant diet, defrosted macrophyte stalks collected in the same ponds as tadpoles; and M, mixed diet, both food items in diets A and P offered ad libitum in similar proportions of fresh mass. The experimental diets were selected based on the results of preliminary experiments with different macrophyte and invertebrate species abundant in temporary ponds. As the plant diet, we selected the floating macrophyte *Ranunculus peltatus* for both hylids, and the emergent macrophyte *Juncus heterophyllus* for *D. galganoi*, because the latter did not consume *R. peltatus*. As the animal diet for *H. meridionalis*, we selected Ephemeroptera (mayfly) larvae, collected in the same pond as the tadpoles and killed by rapid freezing. Ephemeropterans occur at high densities in temporary ponds, but capturing the amount necessary to maintain tadpoles on this diet was extremely challenging. Consequently, we used commercially obtained frozen chironomid larvae for *D. galganoi* and *H. arborea*, as chironomids are also common in temporary ponds. The diet treatments complied with the basic assumptions that the animal diet was protein rich, the plant diet was protein poor, and the diets had very distinct C:N ratios (Table 2). Because of the use of different invertebrates and macrophytes in the diet treatments, we do not conduct life history comparisons among the tadpoles of the three species.

TABLE 1. Species origin and experimental procedures details.

Species	Collection				Experiment		Heat wave	
	No. clutches	Site	Coordinates	Date	Start	End	Early Spring (d)	Late Spring (d)
<i>Discoglossus galganoi</i>	3	Grândola (rain pool)	38°06' N, 8°34' W	6 January 2014	4 February 2014	17 July 2014	28–35	
<i>Hyla arborea</i>	60	Verdzela (temporary pond)	38°34' N, 9°08' W	20 March–20 May 2014	25 June 2014	16 December 2014	21–28	42–49
<i>Hyla meridionalis</i>	–	Grândola (temporary pond)	38°06' N, 8°34' W	26 April 2012	20 June 2012	9 October 2012	21–28	42–49

Note: Number of clutches collected for the study species, their collection site (type of water body), coordinates and dates of collection, dates of the start and end of each experiment, and experimental day of start and end of each heat wave.

TABLE 2. Isotopic and elemental composition of the food sources.

Diet	<i>Discoglossus galganoi</i>			<i>Hyla arborea</i>			<i>Hyla meridionalis</i>		
	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	C:N	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	C:N	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	C:N
Animal	-24.61 ± 0.18	5.50 ± 0.81	3.76 ± 0.04	-24.61 ± 0.13	5.09 ± 0.92	3.79 ± 0.05	-29.30 ± 1.08	14.54 ± 1.02	7.84 ± 2.01
Plant	-28.63 ± 0.19	4.41 ± 0.44	23.69 ± 11.52	-32.44 ± 0.55	4.39 ± 0.38	15.43 ± 0.02	-28.09 ± 0.05	3.36 ± 0.12	27.11 ± 0.05

Note: Isotopic signatures and C:N ratios of the food items provided to the tadpoles of each of the study species (mean \pm SD).

Temperature

The experiment included four temperature treatments (Fig. 1): Cold Spring (CS), constant temperature of 17°C; Normal Spring (NS), temperature was gradually increased from 17 to 25°C at the rate of 1°C/week (average of 21°C); Early Spring Heat Wave (ESHW), similar to NS, but in the fourth week of the experiment tadpoles were exposed to a 1-week heat wave of 25°C, after which temperature was decreased to 21°C (treatment average temperature of 21.4°C; and Long Heat Wave (LoHW), constant temperature of 25°C. Additionally, because *H. arborea* and *H. meridionalis* are spring breeders and more likely to face heat waves later in the season, we included a fifth temperature regime for these two species: Late Spring Heat Wave (LSHW), similar to NS, but, in the sixth week, tadpoles were exposed to 25°C after which temperature was decreased to 23°C (treatment average temperature of 21.4°C; Fig. 1). The 1 week long heat wave of the ESHW and LSHW treatments matches the duration of current spring heat waves at the study area. The 2 month long heat wave (LoHW treatment) simulates an extremely long heat wave, such as the one

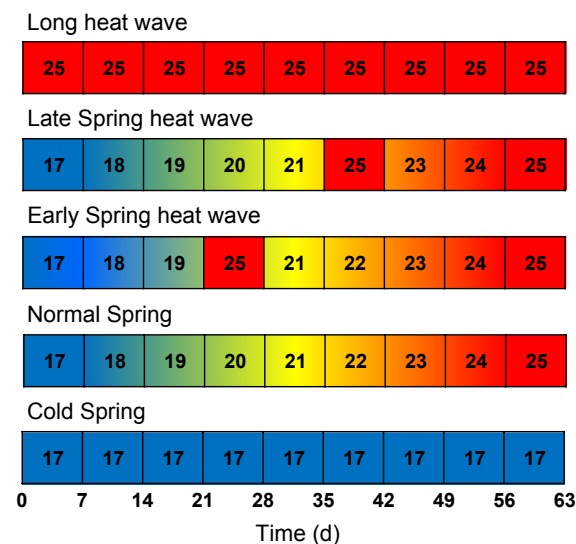


FIG. 1. Schematic representation of temperature variation over time in each experimental treatment. Numbers in the colored boxes are temperature (°C). [Color figure can be viewed at wileyonlinelibrary.com]

that afflicted Europe in 2003, which are expected to become more frequent in the future (Jerez et al. 2013). The three average temperatures (17, \approx 21, and 25°C) were chosen to match the temperature range measured in the pond survey.

Isotope analysis

We used the whole froglet body to attain the minimum mass required per isotopic sample (0.8–1.2 mg of dry tissue). For *D. galganoi* and *H. arborea*, nine randomly chosen froglets per treatment were degutted, dried at 60°C for 24 h, and ground to a fine powder with mortar and pestle. For *H. meridionalis* we used between three and seven froglets for each treatment. We extracted storage lipids with chloroform-methanol (2:1; Dobush et al. 1985), and re-dried the samples at 60°C for 24 h. Stable isotope ratios ($^{13}\text{C}/^{12}\text{C}$; $^{15}\text{N}/^{14}\text{N}$) and elemental analyses (C:N) were determined by continuous flow isotope mass spectrometry (Preston and Owens 1983), on a Hydra 20-22 (Sercon, Crewe, UK) stable isotope ratio mass spectrometer, coupled to a EuroEA (EuroVector, Milan, Italy) elemental analyzer for online sample preparation by Dumas combustion. The standards used were IAEA-CH6 and IAEA-CH7 for carbon isotope ratio, and IAEA-N1 and USGS-35 for nitrogen isotope ratio; $\delta^{13}\text{C}$ results were referred to PeeDee Belemnite and $\delta^{15}\text{N}$ to air. Precision of the isotope ratio analysis was $\leq 0.2\%$ (six to nine replicates of laboratory standards in every batch). Although clutches and hatchlings were collected in the wild and we could not control for maternal effects in the isotopic signatures, tadpole mass at early Gosner stage 25 represented only 10–20% of the froglet mass, and potential initial differences should be negligible.

Statistical analyses

To estimate the relative contributions of the animal and the plant diets to the isotopic signature of tadpoles on the mixed diet, we used stable isotope mixing models, which offer reliable and time-integrated results. The Stable Isotope Analysis in R package (Parnell et al. 2013) fits a Bayesian model to the proportions of the food sources contributing to the isotopic signature of the consumers, incorporating variability in the sources, trophic enrichment factors (TEF) and elemental composition (Parnell et al.

2010). We specified isotopic signatures and C:N ratios of the sources in each species model (Table 2), and accounted for the variability in the TEFs across species, sources and temperature conditions by using the isotopic signatures of the tadpoles fed on the pure diets (animal or plant) at each temperature. The food items differed considerably in their C:N ratio and more than 2‰ in their isotopic signatures at least in one of the isotopes. Half-life values for ^{13}C and ^{15}N turnover rates, determined in non-experimental tadpoles maintained at 20°C and fed on commercial fish food, were found to be of ~1 month, in agreement with values obtained by Thomas and Crowther (2015) on 10 mg–1 g organisms at 10–30°C.

We fitted a Cox proportional hazards regression model to test the treatment effects on tadpole survival (survival package of the R software). In the statistical analyses of larval period and mass at metamorphosis, we excluded the plant diet in *D. galganoi* and the animal diet in *H. meridionalis*, because mortality on these diets was close to 100% in several temperature treatments. We used general linear models (GLMs) to test the effects of temperature and diet (fixed effects) and their interaction on tadpole growth rate (mg/d; mass divided by the experimental day at the end of the ESHW), on larval period (days from stage 25–42); and on froglet mass at metamorphosis (mg; mass at Gosner stage 46). Tadpole body size on day 1 was included as a covariate in all models, as it had a significant effect on tadpole growth rate in all species and on larval period in *H. arborea*. Post hoc pairwise comparisons were corrected for multiple comparisons (Bonferroni's test). The GLMs were performed using the software STATISTICA 12.6.255.0 (Dell Inc., Tulsa, Oklahoma, USA).

RESULTS

Diet choice

The proportions of animal and plant diet assimilated by the tadpoles on the mixed diet differed among the species. *Discoglossus galganoi* was closer to the carnivorous end of the diet gradient, as the median proportion of plant material averaged 0.22 across the temperature treatments (Fig. 2). *Hyla arborea* assimilated a rather balanced proportion of the two food items as the median proportion of plant material averaged 0.57 across all treatments (Fig. 2). *Hyla meridionalis* was closer to the herbivorous end of the diet gradient, as the median proportion of plant material averaged 0.83 across the temperature treatments (Fig. 2).

Although still primarily carnivorous, in the early spring heat wave, *D. galganoi* increased herbivory and assimilated almost two times more plant material (0.27; Bayesian credible interval [BCI] = 0.22–0.31) than in the NS (0.15; BCI = 0.07–0.25). However, this increase was not found in the long heat wave (0.23; BCI = 0.14–0.30), where herbivory was similar to the CS (0.21; BCI = 0.13–0.30). In *H. arborea* the proportion of plant material assimilated was similar across four temperature treatments, ranging

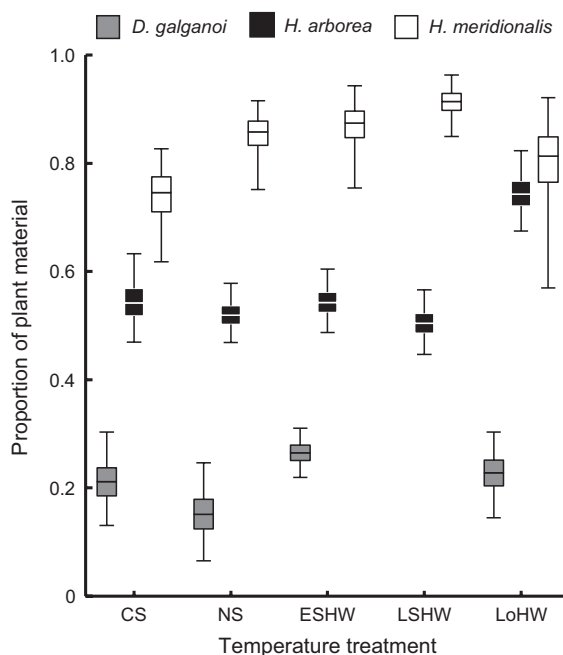


FIG. 2. Proportion of plant material assimilated by the mixed-diet tadpoles of each species in the temperature treatments: Cold Spring (CS), Normal Spring (NS), Early Spring Heat Wave (ESHW), Late Spring Heat Wave (LSHW), and Long Heat Wave (LoHW). Boxes show the median and the 25th–75th percentiles; whiskers indicate the 2.5th–97.5th percentiles. Species are *Discoglossus galganoi*, *Hyla arborea*, and *Hyla meridionalis*.

from 0.50 (BCI = 0.45–0.57) to 0.54 (BCI = 0.47–0.63), but in the long heat wave tadpoles increased herbivory and the proportion of plant material rose to 0.74 (BCI = 0.67–0.82; Fig. 2). Mainly herbivorous, *H. meridionalis* also increased herbivory, although to a lesser extent. In the CS the proportion of plant material was 0.75 (BCI = 0.62–0.83), but in the other temperature treatments was consistently higher and varied between 0.81 (BCI = 0.57–0.92) in the long heat wave (the second lowest value) and 0.91 (BCI = 0.85–0.96) in the late spring heat wave (Fig. 2).

Survival

In *D. galganoi*, survival on the animal and mixed diets was over 90%, but dropped to 45% on the plant diet (Table 3, Fig. 3). The highest survival for *D. galganoi* in the temperature treatments was in the CS (95% survival), and lowest in the long heat wave (61%; Table 3, Fig. 3). In *H. meridionalis*, survival on the plant and mixed diets was over 86%, but dropped to 33% on the animal diet (Table 3, Fig. 3). Survival for *H. meridionalis* in the temperature treatments was 76%, with the exception of the long heat wave (55%), where complete mortality the animal diet treatment caused a marginally significant diet × temperature interaction (Table 3, Fig. 3). In *H. arborea* diet and temperature had no significant effect on survival (Table 3, Fig. 3).

TABLE 3. Cox proportional hazards survival models and general linear models for growth rate, larval period, and mass at metamorphosis.

Factor	Survival			Growth rate			Larval period			Mass at metamorphosis		
	df	χ^2	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>
<i>Discoglossus galganoi</i>												
Diet	2	57.25	<0.001	2, 122	182.39	<0.001	1, 100	41.50	<0.001	1, 77	2.10	0.151
Temperature	3	78.09	<0.001	3, 122	53.48	<0.001	3, 100	48.62	<0.001	3, 77	65.92	<0.001
Diet × temperature	6	4.39	0.625	6, 122	7.08	<0.001	3, 100	3.20	<0.05	3, 77	2.02	0.118
<i>Hyla arborea</i>												
Diet	2	1.21	0.548	2, 121	6.42	<0.001	2, 179	60.96	<0.001	2, 168	65.11	<0.001
Temperature	4	5.52	0.238	3, 121	15.99	<0.001	4, 179	225.60	<0.001	4, 168	24.31	<0.001
Diet × temperature	8	5.20	0.736	6, 121	1.35	0.241	8, 179	21.55	<0.001	8, 168	7.22	<0.001
<i>Hyla meridionalis</i>												
Diet	2	74.95	<0.001	2, 51	50.51	<0.001	1, 50	5.13	<0.05	1, 50	15.82	<0.001
Temperature	4	43.28	<0.001	3, 51	10.88	<0.001	4, 50	70.19	<0.001	4, 50	10.39	<0.001
Diet × temperature	8	15.37	0.052	6, 51	5.54	<0.001	4, 50	0.78	0.541	4, 50	1.23	0.310

Note: Values with *P* < 0.05 are shown in boldface type.

Life history

Discoglossus galganoi.—Growth on the plant diet was lower than on the animal and mixed diets, ~63% and 71%, respectively, while on the mixed diet growth was 23% greater than on the animal diet (Table 3, Fig. 4a). Growth was two times greater in the cold and NS than in the heat waves (Table 3, Fig. 4a). A temperature × diet interaction indicated that while in the cold and NS growth was similar on the animal and the mixed diets, in the long heat wave growth on the animal diet was lower than on the mixed diet (Table 3, Fig. 4a).

Larval period was 28% longer on the mixed diet than on the animal diet (Table 3, Fig. 4a). In the early spring and long heat waves, larval period was almost 50% shorter than in the CS, and also 18% shorter than in the NS (Table 3, Fig. 4a). A diet × temperature interaction indicated that, in the long heat wave and NS, larval period on the mixed diet was longer than on the animal diet (*P* < 0.01), but, in the CS and in the early spring heat wave, there was no difference (*P* = 1.0; Table 3, Fig. 4a).

Mass at metamorphosis was not affected by diet, but in the CS it was almost two times higher than in the other temperature treatments (Table 3, Fig. 4a).

Hyla arborea.—Growth on the plant and mixed diets was 24% greater than on the animal diet (Table 3, Fig. 4b). Growth in the long heat wave was 20% greater than in the normal and 41% greater than in the CS, for which growth was 28% lower than in the NS and 35% lower than in the early spring heat wave (Table 3, Fig. 4b).

Larval period on the mixed diet was 13% shorter than on the animal and 25% shorter than on the plant diet (Table 3, Fig. 4b). Larval period in the long heat wave was shorter than in the other treatments and, in the early

spring heat wave, it was 14% shorter than in the NS and in the late spring heat wave (Table 3, Fig. 4b). A diet × temperature interaction indicated that in the CS larval period on the plant diet was much longer than in the other diets (*P* < 0.001), while, in the long heat wave, larval period on the animal diet was longer than on the other diets (*P* < 0.05; Table 3, Fig. 4b).

Mass at metamorphosis on the mixed diet was 9% higher than on the animal and 59% higher than on the plant diet (Table 3, Fig. 4b). Mass at metamorphosis in the CS was almost two times higher than in the long heat wave, in which mass at metamorphosis was 20% lower than in the NS, early, and late spring heat waves (Table 3, Fig. 4b). A diet × temperature interaction showed that mass at metamorphosis on the plant diet was lower than on the mixed diet in all temperature treatments, except in the long heat wave (*P* = 1.0; Table 3, Fig. 4b); and lower than on the animal diet in all temperature treatments, except in the late spring (*P* = 0.61) and in the long heat wave (*P* = 1.0; Table 3, Fig. 4b). Furthermore, in the NS and in the late spring heat wave mass at metamorphosis on animal diet was lower than on the mixed diet (*P* < 0.05; Table 3, Fig. 4b), but not in the other temperature treatments.

Hyla meridionalis.—Growth on the mixed diet was 33% higher than on the plant and 200% higher than on the animal diet, while growth on the plant diet was 66% higher than on the animal diet (Table 3, Fig. 4c). Growth in the heat waves was 46% higher than in the cold and NS (Table 3, Fig. 4c). A diet × temperature interaction showed that while in the CS diet had no effect on growth, in the heat waves growth increased on the mixed and plant diets, but not the animal diet (Table 3, Fig. 4c).

Larval period on the mixed diet was 7% shorter than on the plant diet (Table 3, Fig. 4c). Larval period in the

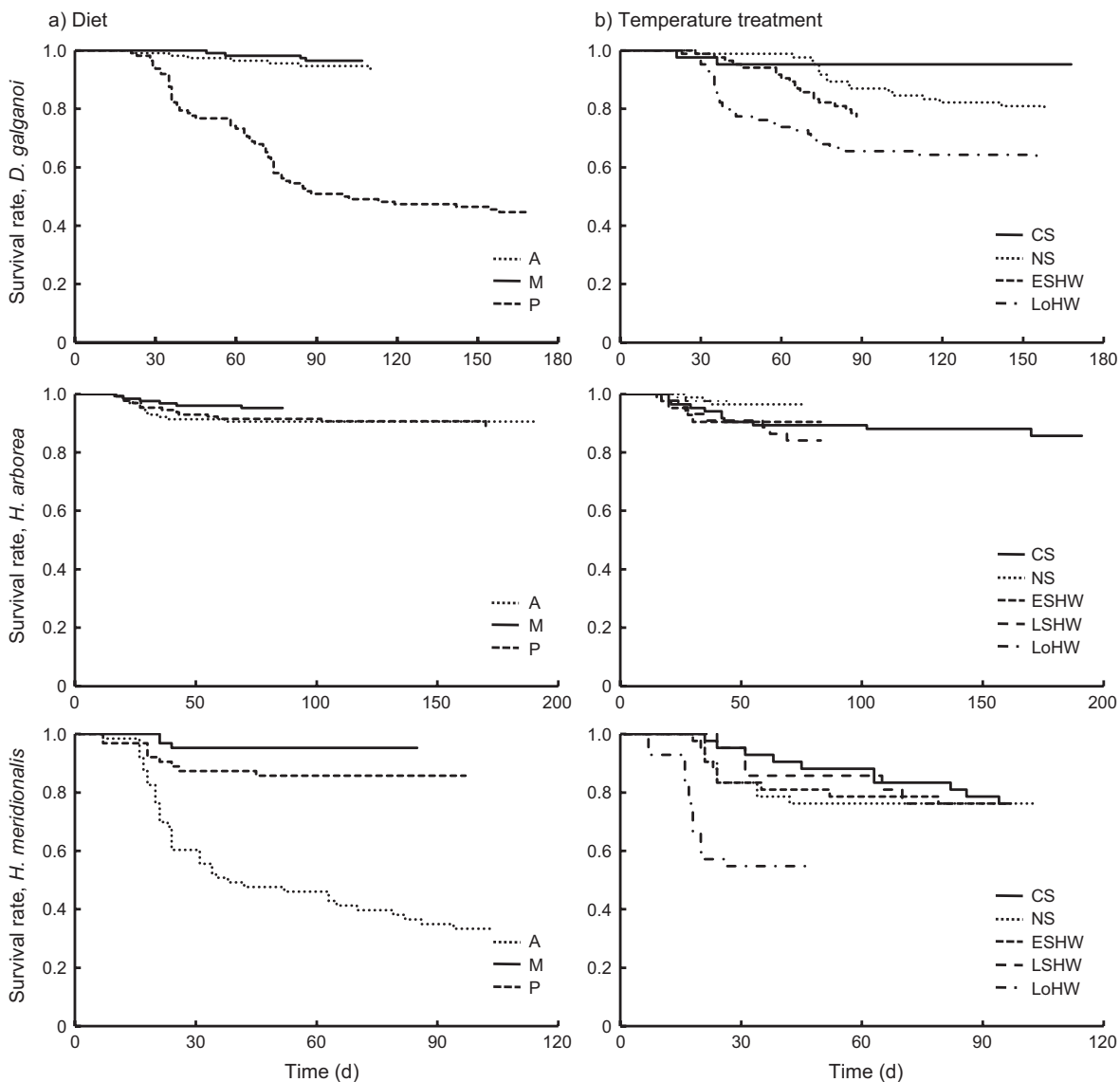


FIG. 3. Cox proportional hazards regression for the survival rate of the study species on the different (a) diet treatments (A, animal; M, mixed; P, plant) and (b) temperature treatments.

long heat wave was shorter than in all the other treatments, while larval period in the CS was longer than in all the other treatments (Table 3, Fig. 4c).

Mass at metamorphosis on the mixed diet was 15% higher than on the plant diet (Table 3, Fig. 4c). Mass at metamorphosis in the CS was higher than in all the other treatments (Table 3, Fig. 4c).

DISCUSSION

We found evidence for dietary specialization in larvae of three anuran species occurring along a seasonal temperature gradient, the degree of herbivory increasing with minimum temperature experienced in nature. Furthermore, we found that tadpoles shifted their diet in

response to heat waves. However, these responses were not uniform as species responded differently to the various heat waves and none of the species responded to all types of heat wave. Nevertheless, whenever tadpoles performed diet shifts they always increased herbivory in response to higher temperature. This response was clearly adaptive in one species and potentially adaptive in another, but maladaptive in the most carnivorous species, indicating that the adaptive value of increasing herbivory at higher temperatures was species-specific. To our knowledge, this is one of the first studies to present hard evidence that ectotherms may maximize energy intake at higher temperatures by increasing herbivory, and the first study to investigate temperature-induced diet shifts in vertebrates (see Boersma et al. [2016] for a previous study on invertebrates).

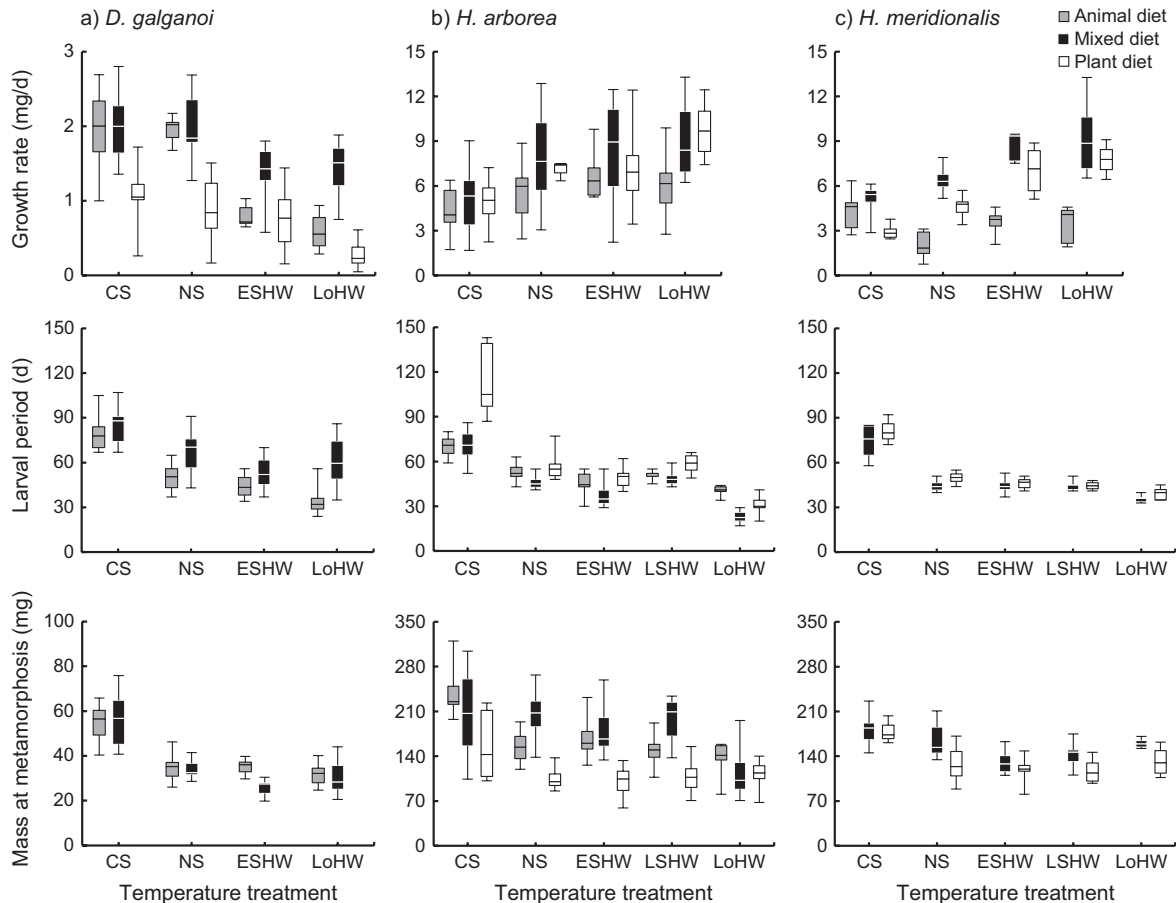


FIG. 4. Growth rate (mg/d), larval period (d), and mass at metamorphosis (mg) for (a) *Discoglossus galganoi*, (b) *Hyla arborea*, and (c) *Hyla meridionalis* tadpoles fed on animal, plant, or mixed diet in the temperature treatments: Cold Spring (CS), Normal Spring (NS), Early Spring Heat Wave (ESHW), Late Spring Heat Wave (LSHW), and Long Heat Wave (LoHW). Boxes show the median and the 25th–75th percentiles; whiskers indicate the minimum and the maximum values.

Our results support the omnivorous character of anuran larvae (Petranka and Kennedy 1999, Caut et al. 2013, Arribas et al. 2015), since tadpoles of the three species assimilated nutrients from both animal and plant material. However, the species' trophic positions were clearly different, suggesting different degrees of specialization along the trophic gradient. As predicted, proportion of animal-based diet was higher in *Discoglossus galganoi*, the species that starts breeding earliest in the season (i.e., at the lowest temperature), while the proportion of the plant-based diet was higher in *Hyla meridionalis*, the species breeding at the highest temperature. The low survival rates of these species under the less-preferred diets (plant diet for *D. galganoi*, animal diet for *H. meridionalis*) further reinforced their relative specialist character. *Hyla arborea*, reproducing at an intermediate thermal environment, occupied a central position along the diet gradient, showing less diet specialization. Although the number of species in our study is limited, the present results suggest a general effect of temperature on the feeding preferences and the extent of herbivory in

tadpoles. While previous studies have shown higher abundance and richness of herbivorous fishes in tropical latitudes (Floeter et al. 2005, Behrens and Lafferty 2007, Jeppesen et al. 2010, Gonzalez-Bergonzoni et al. 2012), this is the first study to experimentally demonstrate a relationship between the trophic niche of vertebrate omnivores and the temperatures to which they normally experience in natural environments.

Our study found support for lower benefits of the animal diet in the heat wave treatments in all species. When compared with the mixed diet, the animal diet markedly decreased tadpole growth in the early spring and long heat wave treatments in *D. galganoi*. In *H. arborea*, the animal diet hampered growth and extended the larval period in the long heat wave without an increase in mass at metamorphosis, the latter decreasing markedly on both the animal and mixed diets. In *H. meridionalis*, the animal diet caused high mortality in the long heat wave. We also found support for increased benefits of the plant diet in the heat wave treatments in two species. In both hylids, growth on the plant diet

increased in the heat waves, especially in the long heat wave, in which the growth on the plant diet equaled growth on the mixed diet in *H. arborea*. Furthermore, in *H. arborea* the plant diet extended the larval period in the CS, but in the long heat wave larval period on this same diet was shorter than on the animal diet. These findings suggest that animal diet has a lower nutritional value than plant diet at higher temperatures, and support the adaptive value of increasing herbivory under these conditions, but also indicate that the temperature effect on animal diets may be stronger than on plant diets. While growth on the plant diet decreased with temperature in *D. galganoi*, the relative quality of the plant diet increased because in the heat waves growth on the plant diet was no longer lower than on the animal diet. However, in the long heat wave, the mixed diet extended the larval period more than the animal diet, suggesting even a low proportion of plant diet was sufficient to hamper development in the late pre-metamorphic stages.

Although the study species shifted their diet toward increased herbivory, these responses were not uniform, as the heat wave treatments inducing diet shifts varied among species, as did the magnitude of the diet shifts when they occurred. While *D. galganoi* and *H. meridionalis* appear to have performed modest diet shifts in the short heat wave treatments, the true extent of the diet shifts may have been greater, being lessened by a partial reversion in the isotopic signatures during the ~2 weeks the tadpoles took to reach metamorphosis. The essentially carnivorous *D. galganoi* (Richter-Boix et al. 2012, Arribas et al. 2015) performed a potentially maladaptive shift toward higher herbivory in the early spring heat wave, as suggested by the low mass at metamorphosis when compared to strictly carnivorous tadpoles. *Hyla meridionalis* increased herbivory in the late spring heat wave, suggesting a higher sensitivity in the late pre-metamorphic stages. The lack of notable benefits or costs in *H. meridionalis* may result from its already high herbivory in the experiments and from the small extent of the diet shift. The more specialized diets of *D. galganoi* (carnivory) and *H. meridionalis* (herbivory) may have prevented tadpoles from sustaining long-term diet shifts in the long heat wave. We found that both *D. galganoi* and *H. meridionalis* shortened larval period without detectable costs in mass at metamorphosis. In fact, each of these two species metamorphosed with a similar mass in all treatments (except the CS), suggesting a general response where, above a certain temperature threshold, tadpoles initiate metamorphosis at a minimum size required for its successful completion (Kuparinen et al. 2010). Since *D. galganoi* breeds in ephemeral and *H. meridionalis* in temporary ponds (Richter-Boix et al. 2006, Escoriza and Boix 2014), such sensitivity to heat is not surprising as they need to complete metamorphosis before pond desiccation.

In the long heat wave, *H. arborea*, unlike *H. meridionalis*, sustained a rather large increase in herbivory. In this treatment, *H. arborea* shortened larval period without increasing growth rate, so this diet shift came at

the cost of lower mass at metamorphosis. However, the benefits may outweigh the costs because a short larval period is adaptive in Mediterranean temporary ponds, given the mortality risk due to pond desiccation (Richter-Boix et al. 2011), and early metamorphosis may compensate for the negative effects of small body size in hylids (Smith 1987). *Hyla arborea* did not perform diet shifts in the early spring heat wave, but slightly decreased the larval period, which allowed tadpoles to complete metamorphosis earlier and without detectable costs in mass. *Hyla arborea* experienced the late spring heat wave earlier than *H. meridionalis* (Gosner stages 35–36 vs. 37–38; data not shown). Therefore, despite the lack of response in this study, this species might still be sensitive to heat waves in later pre-metamorphic stages.

This study allows two important inferences regarding the mechanisms underlying diet shifts and their potential effects in freshwater communities. First, short-term climatic events such as 1-week heat waves increased the contribution of plants to the diet of tadpoles (*D. galganoi* and *H. meridionalis*), either through an active change in the feeding preference, or through a temperature-mediated passive change in the digestion and/or assimilation efficiency of each diet. Our study suggests that beyond possible temperature mediated passive mechanisms, which may have increased the assimilation of the plant diet over the animal diet at higher temperatures, tadpoles actively regulated nutrient acquisition and changed feeding preferences according to the temperature. This is because a purely passive mechanism would have resulted in higher herbivory in the long heat wave than in the other heat waves, which did not occur in both *D. galganoi* and *H. meridionalis*. Second, the adaptive value of the temperature-induced diet shifts varied among species, suggesting the challenges imposed by heat waves may affect community structure, as diet shifts toward herbivory may favor some species and impair others, especially the species close to strict carnivory.

Although unavoidable, the use of different food items in the diet treatments may have affected our results. However, we maintain that this variation should have only a reduced effect when considering the overall difference between animal and plant diets and should have little impact on our conclusions. Unfortunately, logistic constraints did not allow cyclical diel variation in our experimental setup, which could have buffered the effects of high daytime temperatures and as such our results may overestimate responses in nature. Furthermore, the temperature range tested prevents generalizations to extreme temperatures, because under acute heat stress demand for nitrogen may increase (for e.g., synthesis of heat shock proteins; Podrabsky and Somero 2004). However, we focused on spring heat waves with mild temperatures (25°C), because our study species inhabit Mediterranean temporary ponds which dry by the end of spring and the tadpoles are never exposed to the summer heat waves with temperatures above 35°C. Although the study species have similar thermal performance ranges and

upper critical thermal limits above 38°C (Duarte et al. 2011, Katzenberger 2015), the temperature range in the experiments may have induced different levels of stress among the species, especially in *D. galganoi*, in which overall growth decreased in the heat waves.

Climate change projections show heat waves are likely to become more frequent, intense, and longer lasting over the century. Although diet regulation may help omnivore ectotherms to cope with increased heat stress, the potential to perform diet shifts as well their adaptive value are likely to differ across species. More importantly, generalized diet shifts toward higher herbivory may trigger changes in freshwater ecosystems and disturb the structure and functioning of freshwater communities and food webs. Awareness of temperature effects on diet regulation may contribute to a better understanding and reassessment of the ecological effects of the ongoing climate change.

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