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Physiology and acclimation potential are tuned with phenology in larvae of a prolonged breeder amphibian

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***Keywords*:** metabolic scope, *Alytes almogavarii*, breeding phenology, developmental rate, growth, metabolic rate, RMR, SMR.

1. Introduction

The Earth's climate is rapidly changing and the same trend is forecasted for the next decades (IPCC 2014, Seneviratne et al. 2014). Global temperature rises count among the most evident consequences of climate change and most organisms have already responded by shifting phenology (Beebee 1995, Both and Visser 2001, Menzel et al. 2006). Simultaneously, the frequency of extreme climatic events (e.g. droughts, heatwaves, sudden temperature drops) and their duration are also increasing (Montori et al. 2011, Morán-Ordóñez et al. 2018, Smale et al. 2019). Thus, phenological adjustments may be insufficient to prevent organisms from experiencing stressful environmental conditions. In order to avoid extinction, species could shift distribution ranges, change their behaviour (e.g. thermoregulatory behaviour), adapt through genetic evolution or adjust their phenotypes through phenotypic plasticity (Chevin et al. 2010, Moritz and Agudo 2013, Pecl et al. 2017). Due to the velocity of climate change, rapid response mechanisms such as phenotypic plasticity could be the most effective means with which to buffer the impacts of extreme climatic events (DuBois et al. 2020). However, we still need a deeper appreciation of whether (and how) phenology and phenotypic plasticity in key traits interact.

Phenotypic plasticity stands for the ability of organisms to express different phenotypes without changing their genotype (Pigliucci 2005) and contributes in keeping biological functions relatively constant despite environmental variation. Plasticity comprises a variety of phenomena that occur at different time-scales. Within-generation plasticity (WGP) includes irreversible plastic changes that occur during development (i.e. developmental plasticity; Beaman et al. 2016, Sgrò et al. 2016, but see Enriquez-Urzelai et al. 2019, Gunderson et al. 2020) and short-term reversible plasticity, also known as acclimation (Rogers et al. 2004, Huey et al. 2012). Besides, exposure to certain environmental conditions can also have delayed effects, sometimes referred to as *stress memory* (Bigot et al. 2018, Byrne et al. 2020), suggesting that previous experience can facilitate the response to later exposure. For instance, animals exposed to warm temperatures over a short-period of time have been shown to endure later heat-waves better than naïve animals (Loeschcke & Hoffmann, 2007). In addition to the plasticity expressed within a generation, the

influence of the environment on phenotypes can be transmitted to future generations: this is known as anticipatory parental effects (Uller et al. 2013) or transgenerational plasticity (TGP), and may involve epigenetic mechanisms (Fox and Mousseau 1998, Salinas and Munch 2012, Donelson et al. 2018). Although phenotypic plasticity is theoretically beneficial, the degree of induced phenotypic changes might fail to reach expected environmental changes (Gunderson and Stillman 2015, Gunderson et al. 2017, Enriquez-Urzelai et al. 2020).

Yet, plasticity in physiological traits, notably in metabolic rates, will be crucial for organisms to persist in changing environments (Norin and Metcalfe 2019, Alton et al. 2020, Jutfelt 2020). In ectotherms, standard metabolic rates (SMR) represent the minimum metabolic conversion of food into energy required to sustain life (Norin et al. 2016, Winterová and Gvoždík 2020). Routine metabolic rates (RMR), in turn, represent the metabolic rate of undisturbed but spontaneously active individuals (Lindgren and Laurila 2009, Nadler et al. 2020). The difference between these two rates reflects the amount of energy available for routine activities (metabolic scope hereafter; Naya and Bozinovic 2012, Naya et al. 2012, Bozinovic and Naya 2015), like spontaneous activity, growth, development and reproduction (Fry 1947, Claireaux and Lefrançois 2007). Because metabolic rates are temperature-dependent, climate change will impact upon them (Pörtner and Knust 2007, Pörtner and Farrell 2008, Dillon et al. 2010). In the absence of compensatory phenotypic plasticity (e.g. metabolic plasticity), this will very likely curtail net energy gain, ultimately reducing activity periods, growth and developmental rates, and fitness (Huey and Kingsolver 2019). However, because global warming can result in a shortening of the effective winter (e.g., shorter snow cover periods) and extended growing seasons, the opposite is expected at higher elevations and latitudes (Chen et al. 2005, Prislan et al. 2019).

In species with prolonged breeding seasons (hereafter prolonged breeders), offspring born early or late in the year encounter drastically different thermal conditions, impacting on their metabolic performance (Uller and Olsson 2010). Thus, to counteract this variation, breeding phenology could be coupled with physiological and plasticity differences between cohorts (e.g. in metabolic rates; Orizaola et al. 2010, 2013, Richter-Boix et al. 2014, Sun et al. 2018). The presence, absence or combination

of these mechanisms may determine the ability to respond to unexpected extreme events due to climate change: while short-term plasticity (i.e. acclimation) and stress memory could enhance metabolic performance (e.g. increasing survival, growth and development), TGP could turn out detrimental if parental pre-conditioning oppose the direction of extreme events (i.e., phenological mismatches).

Here, we explored whether phenotypic plasticity is linked to breeding phenology to enhance metabolic performance and key life-history traits in a prolonged breeder amphibian, the Catalonian midwife toad (*Alytes almogavarii*). Midwife toads are good candidates to tackle this question because, in addition to breeding throughout most of the year, they often breed in small and thermally homogeneous waterbodies (García-París et al. 2004, Richter-Boix et al. 2006). This spatial uniformity in the thermal conditions encountered by larvae, combined with high temporal variation, may promote differences in physiology and/or potential for plasticity (Wu et al. 2007). We analysed the effects of phenology and temperature on metabolic rates, survival, growth and development by comparing larval *A. almogavarii* entering the aquatic phase at different times; thus, these larvae expect distinct thermal conditions (Fig. S1). Specifically, we compared three groups of larvae: autumn pre-overwintering (Ap), autumn overwintering (Ao), and spring (S) tadpoles. Pre-overwintering and overwinter tadpoles belonged to the same (autumn) cohort, but they were tested either as young, small tadpoles (Ap), or by late winter as older, bigger larvae (Ao). We expect tadpoles expecting low temperatures (Ap) to have lower SMR and higher RMR at low temperatures –leading to a better performance in growth/development–, compared to tadpoles expecting high temperatures (Ao and S). Further, we hypothesize that overwintering animals should show stress memory of the plastic responses required to overwinter: specifically, we expect stress memory in the form of enhanced potential for future acclimation to low temperatures.

2. Materials and methods

2.1 Animals and experimental design

We collected three groups of 50 *A. almogavarii* tadpoles from the Garraf massif (41.272º N, 1.877º E) at 26-28 Gosner developmental stage (Gosner 1960) and ~9 mm

(S and Ap) or ~12 mm (Ao) of SVL. To evaluate the effect of reproductive phenology (i.e., tadpole cohort) on physiological and developmental responses, we collected the first group by November 2012 (group 'Ap', freshly laid, pre-overwintering autumn cohort; all tadpoles in this cohort would overwinter in the field), and two more groups by March 2013 (group 'Ao', overwintered autumn cohort; and group 'S', freshly laid spring cohort). Ao and S tadpoles were unmistakable due to the evident difference in size.

Tadpoles were transported to The University of the Basque Country's facilities and immediately placed at a constant temperature room (20ºC) for approximately 1 week, allowing them to acclimate to laboratory conditions. Tadpoles were kept individually in 150 ml crystal glasses filled with dechlorinated tap water and submerged in a water bath. After the pre-acclimation, half the tadpoles from each group were moved to a 10ºC constant temperature room (low temperature treatment; T10) and the other half was maintained at 20ºC (high temperature treatment; T20); these temperatures roughly match the average pond temperatures in autumn and spring in the Garraf massif (see Supporting Information). This resulted in a set of six combinations of temperature and tadpole cohort (Ap20, Ap10, Ao20, Ao10, S20, and S10). Tadpoles were fed *ad libitum* with slightly boiled spinach and we changed the water every second day. Photoperiod was set at 12L:12D throughout the experiment. We photographed all tadpoles every week, and recorded their Gosner developmental stage and size (snout-vent length, SVL; ± 10^{-8} mm) based on digital images using SigmaScan Pro 5.0. In addition, we checked tadpoles and recorded mortality events (day of death) every day.

2.2 Estimation of metabolic rates (SMR, RMR and metabolic scope)

We estimated the metabolic rates of the experimental animals from the oxygen consumptions (O_2 µl h^{-1}) at the rearing temperature (10 and 20ºC for tadpoles in T10 and T20 treatments, respectively). Tadpoles were individually placed in plastic tubes sealed with LDO oxygen probes connected to oxygen meters (HATCH HQ40d) and we observed the decrease in oxygen concentration over time (1-2 hours). We measured the oxygen consumption of eight tadpoles simultaneously in each trial. Two empty plastic tubes were used as controls in every trial to measure any potential bacterial

148 activity. To calculate tadpole metabolic rates, the (mean) oxygen consumption in the
149 control tubes during each measurement, when found to be significant, was subtracted
150 from tadpoles' consumption. All the set-up was kept submerged in a temperature-
151 controlled water bath to prevent temperature oscillations.

152 We measured routine metabolic rates (RMR) as the energetic maintenance cost
153 plus any other energetic cost due to spontaneous activity and stress. Metabolic rates
154 were estimated just before splitting of T10 and T20 treatments (day 0), the day after
155 exposing animals to 10ºC (day 1; measurements only at 10ºC), at days 2, 7, and once
156 every week thereafter until no sign of metabolic acclimation was observed. In addition,
157 from day 2 onwards, after the measurement of RMR, half of the tadpoles in each
158 treatment were deprived of food until their metabolic rates reached an asymptote (3
159 and 4 days for the T10 and T20 treatments, respectively) to obtain an estimate of SMR
160 (the energetic maintenance costs in absence of substantial movement and digestive
161 and absorptive activity). We used consistently the same set of food-deprived animals
162 because starvation is known to affect the response in front of environmental factors
163 (Enriquez-Urzelai et al. 2013).

164 A separate group of 25 animals was reared for three weeks in order to determine
165 the allometric scaling component between metabolic rate (RMR) and tadpole size
166 (SVL). We fitted a standardized major axis regression with the estimated variance
167 matrix of measurement error in order to account for repeated measurements using
168 the *smatr* R-package (Warton et al. 2012). The resulting scaling component was b =
169 2.513 (R^2 = 0.413, $F_{1,49}$ = 93.44, p < 0.0001; based on log-transformed values of RMR
170 and SVL). Accordingly, metabolic rates (SMR and RMR) were standardized to a
171 common size of 10 mm using the expression: $MR_{st} = MR_e \times (10/SVL)^{2.513}$, where MR_{st}
172 states for the standardized SMR or RMR, and MR_e represents the experimental SMR or
173 RMR. Using these standardized metabolic rates, we computed the metabolic scope
174 subtracting SMR values to RMR, and the thermal sensitivity (Q_{10}) of SMR ($Q_{10\text{-SMR}}$)
175 dividing SMR values of animals at 20 ºC by SMR values of animals at 10 ºC.

176 **2.3 Statistical analyses**

We analysed tadpoles' survival using the *survival* R-package (Therneau 2012) and non-parametric Cox proportional hazard (CoxPH) models. Hazard states for the instantaneous potential of an event to occur (here death). CoxPH models assume that hazards are proportional and that a baseline hazard exists. Then, the multiplicative effect of covariates over that baseline are estimated. Prior to adjusting non-parametric CoxPH models, we assessed the proportional hazard (PH) assumption for all the individual variables (size, Gosner stage of development, animal cohort and temperature treatment) fitting univariate CoxPH models. Temperature treatment did not meet the assumption of PH ($\rho = 0.509$, $\chi^2 = 7.14$, $p = 0.008$) and, consequently, we analysed the effects of size, developmental stage, and animal cohort in each temperature treatment separately. We fitted a CoxPH model with all the variables (*survival* ~ *size* + *stage* + *cohort*) within each temperature treatment, and then, we reduced the model following a backward stepwise algorithm based on Akaike's Information Criterion (AIC). All the models were constructed using the robust sandwich variance estimator to account for repeated measures. These models estimate a hazard ratio for each covariate. Hazard ratio values > 1 indicate that risk of death decrease and values < 1 indicate that risk of death increases with a unit increase of the corresponding covariate.

We assessed differences in standardized metabolic rates at the start (days 0, 1 and 2) and at the end of the experiment (day 42) with a factorial ANCOVA using Gosner stage of development as a covariate and cohort and temperature treatment as fixed factors. When ANCOVA indicated a significant effect of cohort, we used the Tukey's HSD post-hoc test to evaluate which groups differed. Besides, we tested for differences in RMR between days 1 and 2 in the T10 tadpoles using a factorial ANCOVA with developmental stage as covariate, day and cohort as fixed factors, and individual tadpoles (subjects) as random effect to avoid pseudoreplication. We used *t*-tests to check whether the thermal sensitivity of SMR ($Q_{10\text{-SMR}}$) at days 2 and 42 was significantly different from 2.5, which approximates the expected Q_{10} value of non-acclimated – and even post-acclimation – individuals (Clarke and Johnston 1999, Willmer et al. 2005, Jutfelt 2020). To report $Q_{10\text{-SMR}}$ values for each group, we computed mean SMR values at 20 and 10ºC, and we divided the mean value at 20ºC

by the mean value at 10ºC. To perform *t*-tests, however, we divided SMR values of tadpoles exposed to 20ºC by the SMR of tadpoles at 10ºC at a random fashion.

To evaluate acclimation responses in RMR, SMR and metabolic scope between days 7 and 42, we used generalized additive mixed models (GAMM) as implemented in the *mgcv* (Wood 2011) and *nlme* (Pinheiro et al. 2016) R-packages. We established developmental stage as a covariate, cohort, temperature treatment, and their interaction as fixed factors, and time (i.e. day) as the smooth term. To correct for temporal autocorrelation and pseudoreplication, we added a temporal correlation structure to the model (Zuur et al. 2009) and individual tadpoles (subjects) were included as a random intercept (Schielzeth and Nakagawa 2013). Further, we used the *VarIdent* variance structure to control for different spreads between treatments (Zuur et al. 2009). An important parameter estimated by this analysis is the "estimated degrees of freedom" (edf) of the examined covariate. Edf equal to 1 implies a linear effect and values greater than 1 indicate a nonlinear effect (Stenseth et al. 2006). Since mortality could potentially be different for individuals with different metabolic rates (e.g. higher mortality in tadpoles with higher metabolic rates), we repeated all analyses regarding metabolic rates only including the animals that survived until the end of the experiment. Results are identical to those obtained when including all animals (see Tables S1–S3). Thus, we only present results with all tadpoles in the main text.

To compare the patterns of development and growth between treatments, we used the analysis of phenotypic trajectories (Adams and Collyer 2009), only including constantly fed tadpoles. We constructed a matrix consisting of developmental stage and size over time for all treatments and calculated their corresponding trajectories. These trajectories are characterized by size (magnitude), orientation (direction) and shape. Pairwise comparisons of size, orientation and shape ($MD_{i,j}$, $\theta_{i,j}$ and $D_{Shape:i,j}$ respectively) were done using the residual randomization method with 10000 random permutations. We performed the analysis of phenotypic trajectories using the *geomorph* R-package (Adams et al. 2018).

3. Results

Our results showed that survival differed between cohorts at high but not low temperatures. Mortality ranged from 0% (Ao10 and S10) to 59% (Ap20). At high temperature (T20), the best-fit model for tadpole survival included all the terms (size, developmental stage and cohort). Mortality risk decreased along with developmental stage, but size did not have a significant effect (Table 1a). Taking S tadpoles as the baseline hazard, at high temperatures mortality was ~9 times higher for Ap tadpoles. We found no significant differences in the mortality between Ao and S tadpoles. The best-fit model at low temperatures (T10) only included size and developmental stage (Table 1b), but the model was not significant, indicating a failure to detect any pattern in mortality risk.

At the start of the experiment we found differences in RMR and the acute change of RMR, but not SMR between different cohorts. At day 0, tadpoles assigned to different temperature treatments did not differ in RMR ($F_{1, 132} = 1.193$, $p = 0.277$). However, we found a strong effect of animal cohort ($F_{2, 132} = 13.098$, $p < 0.0001$). Ap tadpoles showed higher RMR than the rest (Ap vs. Ao: $p < 0.0001$; Ap vs. S: $p < 0.0001$; Ao vs. S: $p = 0.958$; Fig. 1). At low temperatures, tadpoles maintained constant metabolic rates from day 1 to day 2 (T10: ANCOVA; $F_{1, 71} = 0.712$, $p = 0.402$). However, animal cohort had a significant effect on metabolic rate changes (i.e. different cohorts differed in the degree of plasticity; $F_{2, 71} = 4.961$, $p = 0.010$). The interaction between cohort and temperature had a significant effect on RMR of day 2 (Table 2). Consequently, we analysed the effect of cohort separately within each temperature treatment. We found no significant effect of cohort at the low temperature treatment; in the T20 treatment, however, cohort and developmental stage had significant effects (Table 2; Fig. 1). In contrast, differences in SMR between cohorts were negligible ($F_{2, 58} = 0.393$, $p = 0.677$), and only the effect of temperature was significant ($F_{1, 58} = 126.208$, $p < 0.0001$). We recorded $Q_{10\text{-SMR}}$ values of 1.91, 2.42 and 2.65 on pre-overwintering, overwintering and spring cohort tadpoles, respectively. None of these Q_{10} values were significantly different from the expected 2.5 value (Ap: $t_9 = -1.811$, $p = 0.104$; Ao: $t_{11} = 0.359$, $p = 0.727$; S: $t_{11} = 0.920$, $p = 0.377$).

In addition to the direct effects on initial metabolic rates, different cohorts showed differences in their potential for plasticity (i.e. the interaction between cohort

and temperature), which influenced the acclimation processes of RMR and SMR, and consequently the metabolic scope. Ao10 tadpoles showed an increase in RMR and a decrease in SMR. In contrast, tadpoles in the rest of the groups showed a decrease in both RMR and SMR (Table 3, Fig. S2-S3), with the exception of S10 tadpoles, which showed no significant trend. We found a significant effect of cohort (F = 15.377, p < 0.0001), temperature (F = 32.924, p < 0.0001), and their interaction (F = 25.433, p < 0.0001) on metabolic scope over time. The acclimation process affected metabolic scope only in Ao10 (edf = 1.00, F = 19.802, p = 0.0001), Ap20 (edf = 1.00, F = 9.136, p = 0.003) and S20 (edf = 1.00, F = 50.328, p < 0.0001) groups. While Ao10 tadpoles showed a significant increase, Ap20 and S20 tadpoles showed a significant decrease in metabolic scope over time (Figs. 2, S4).

At the end of the experiment we found differences in RMR and SMR between cohorts due to differences in plastic responses, but complete acclimation in SMR regardless of the cohort. At day 42 the interaction between developmental stage and cohort significantly affected RMR (Fig. S5). We also detected a significant effect of temperature and developmental stage (Table 4; Fig. 1). As in the case of RMR, the interaction between developmental stage and cohort affected SMR (Table 4; Fig. S5). Remarkably, the effect of temperature on SMR was nonsignificant. The $Q_{10\text{-}SMR}$ values were 1.00, 1.38 and 1.41 for Ap, Ao and S tadpoles respectively. These values were significantly different from 2.5 for Ao and S tadpoles (Ao: t_{10} = -5.075, p = 0.0004; S: t_{10} = -3.477, p = 0.006), but not for Ap tadpoles (t_5 = -2.007, p = 0.101), likely due to the low sample size derived from mortality.

The development-growth trajectories also differed between cohorts and temperature treatments (i.e. in size and orientation). Regarding size, we identified two sets of trajectories (Fig. 3): large (Ap20 and S20) and small (Ao20, Ao10, Ap10 and S10). Tadpoles in different treatments could be assigned to four distinct orientations: 1) Ap10, 2) Ao20, 3) S10, S20 and Ap20, and 4) Ao10 (Table 5). At low temperatures, S and Ap tadpoles showed different allocation strategies, with autumn tadpoles allocating more energy to development than spring tadpoles (Ap10 had a steeper slope than S10; Table 5; Fig. 3).

4. Discussion

As global temperatures rise and the frequency and duration of extreme events increase, the ability of organisms to sustain metabolic performance and adjust life-history transitions (e.g. the timing of metamorphosis) will be key to avoid extinction (Briscoe et al. 2012, Kielland et al. 2019, Alton et al. 2020). All these aspects may be modulated by breeding phenology (Richter-Boix et al. 2014, Sun et al. 2018). Thus, to understand the impacts of climate change on biodiversity, we need to establish the links between phenology and metabolic and life-history flexibility. A primary finding of our study was that different seasonal cohorts (autumn and spring) of a prolonged breeder (*A. almogavarii*) differ markedly in survival, physiological acclimation, and developmental trajectories. The offspring of prolonged breeders recruited at different times experience very different levels of environmental stress, thus generating marked within-population variation both in reproductive success and offspring survival. In this line, we demonstrate that breeding phenology is coupled with offspring anticipatory responses: metabolic acclimation and the growth-development trajectories of discrete seasonal cohorts were different and consistent with future, expected thermal regimes. This suggests that extreme events can yield different vulnerability to climate change within populations (e.g., associated to discrete within-year cohorts), and not only between species or populations (Seebacher and Franklin 2012).

Short term WGP (e.g. acclimation) could also modify thermal physiology and ameliorate the impacts of altered temperatures (Morley et al. 2019, Kielland et al. 2019). However, the potential for metabolic acclimation seems to vary between taxa (Marshall and Grigg 1980, Sandblom et al. 2014, Markle and Kozak 2018). Our results show that RMR differed among animal cohorts at the start of the experiment (days 0, 1 and 2, especially at high temperatures), and that the thermal sensitivity of SMR ($Q_{10\text{-}SMR}$) matched the expected value of ~2.5 (Willmer et al. 2005, Lardies et al. 2008). Despite these initial differences in RMR, the acute response to exposure to 10ºC was similar among animal cohorts and all reached similar metabolic rates (Fig. 1). Further, all the experimental groups (with the exception of S10) showed metabolic acclimation, and post-acclimation $Q_{10\text{-}SMR}$ approximated a value of 1, which indicates complete acclimation (Sandblom et al. 2014, Kielland et al. 2019, Jutfelt 2020). Metabolic

acclimation, however, varied among groups: overwintering tadpoles at 10ºC (Ao10) increased their RMR, while the rest of the groups decreased RMR and SMR (Fig. 1, S1, and S2). The observed differences in acclimation trends (both of RMR and SMR) between tadpole groups (Fig. 1; Table 3) had a direct impact on metabolic scope through time: while metabolic scope decreased in Ap20 and S20, it increased in Ao10 (Fig. 2 and S4). Thus, we show that in addition to differences between species (Markle and Kozak 2018) and individuals within a population (Norin et al. 2016), discrete cohorts within a population can also differ in the potential for metabolic acclimation. Further, the increase in metabolic scope of overwintering tadpoles at low temperatures suggests that undergoing overwintering increases the acclimation potential to later exposure to low temperatures (i.e. *stress memory*), as reported for other ectotherms (Angilletta 2009, Nyamukondiwa and Terblanche 2010).

Anticipatory TGP has been shown to increase the survival probability of offspring in a wide range of organisms, form invertebrate to vertebrate ectotherms (Rosa et al. 2012, Chirgwin et al. 2018, Diaz et al. 2020). Our results suggest that larvae of *A. almogavarii* may also benefit from TGP, which might act as a link between the environment experienced by parents and the expected thermal conditions of larvae to increase survival. However, our experimental design does not allow to discriminate between WGP and TGP. Yet, at high temperatures, survival was higher in larvae expecting an increase in temperatures (Ao and S) compared to larvae expecting decreasing temperatures through development (Ap). The higher mortality of pre-overwintering compared to overwintering tadpoles is not surprising because of the size differences between them (Reinke et al. 2020). However, differences in mortality between pre-overwintering (autumn) and spring tadpoles cannot be explained by size. In nature, the embryo and larval stages of autumn and spring cohorts expect opposite temperature trends: decreasing and increasing temperatures, in autumn and spring respectively (Fig. S1). Since in our study animals were kept in identical conditions, external cues such as photoperiod (Sanabria and Quiroga 2011) can be ruled out as triggers of anticipatory mechanisms. Then, the observed difference in survival between autumn (Ap) and spring (S) cohorts is compatible with a scenario of TGP (Fox and Mousseau 1998, Richter-Boix et al. 2014, Yin et al. 2019), in which the breeding

phenology of parents precondition their offspring thermal physiology and, thus, survival probability given their environment (Putnam and Gates 2015, Sun et al. 2018, Diaz et al. 2020).

From the energy available for aerobic activities, a considerable proportion should be allocated to growth and development at larval stages, since these may determine survival to the adult stage (Kingsolver et al. 2012). Our results revealed distinct allocation strategies between different cohorts (Fig. 3). Breeding phenology has been shown to alter the energy investment to either growth or development in amphibian tadpoles, due to the difference in length of favourable conditions between life-history transitions (Orizaola et al. 2010, Dahl et al. 2012, Burraco et al. 2020). Surprisingly, although metabolic scope decreased in spring and pre-overwintering tadpoles at high temperature, they grew and developed faster than overwintering tadpoles, in which metabolic scope did not change significantly. Taken together, these results suggest that overwintering tadpoles arrested developmental and growth (as shown for other amphibian tadpoles before the onset of winter; Walsh et al. 2008, 2016). On the contrary, pre-overwintering and spring tadpoles – which invested a similar amount of energy to growth and development – tried to exploit transient favourable conditions, plausibly, at expenses of other functions (e.g. fighting disease; Kirschman et al. 2018). However, at low temperatures spring and pre-overwintering tadpoles took different allocation strategies. Pre-overwintering tadpoles allocated more energy to development than spring cohort tadpoles, possibly to reach metamorphosis, or at least an advanced developmental stage, before the onset of winter (Walsh et al. 2008, 2016). In contrast, spring cohort tadpoles do not expect limiting thermal or food conditions. Thus, they might develop at a slower pace, leading to bigger sizes at metamorphosis (Enriquez-Urzelai et al. 2013, Benard 2015, Burraco et al. 2020).

In the face of climate change, unravelling the mechanisms that will effectively protect biodiversity (e.g. metabolic acclimation), or those that could render them more susceptible to extinction has become a priority (Seebacher and Franklin 2012, Alton et al. 2020). Many factors including phenology, however, could modulate the effectiveness of this mechanisms. Our results demonstrate that offspring survival, metabolic acclimation, and energy allocation strategies are linked to phenology (i.e.

breeding date) in *A. almogavarii*. Apparently, the date at which embryos become free-living, swimming larvae influences their physiological and life-history responses to thermal conditions. Newly hatched autumn and spring tadpoles show differential survival and energy allocation strategies (i.e. energy invested in growth or development) matching the thermal conditions they are likely to encounter. This is supported by the higher mortality and faster developmental trajectories of pre-overwintering, autumn tadpoles compared to spring tadpoles at high temperatures. Plausibly, this is mediated by a combination of TGP – e.g. parental influences on offspring phenotype to increase survival chances – and WGP – which allows tadpoles to adjust their phenotype to prevailing conditions –. However, our experimental design only allowed us to unequivocally determine WGP differences between cohorts. Further, we show that overwintered tadpoles were able to increase the energy available for aerobic activities by increasing RMR, as opposed to pre-overwintering tadpoles. Thus, previous thermal history might improve the response capacity to later exposures, conforming to stress memory. It is noteworthy that different cohorts could belong to separate genetic units, and that some of the reported variation in metabolism and life-history have a genetic basis (Sinsch 1992; but see Jourdan-Pineau et al., 2012). Since there is no data on the temporal structuring of midwife toad populations, further studies should investigate this aspect. Altogether, our results suggest that discrete cohorts across the breeding season could be sensitive to climate change, not only due to a differential exposure, but also due to limitations imposed by the temporal matching of phenology, larval physiology and energy allocation strategies.

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Figure 1: Change in standardized metabolic rates through time in **(a)** pre-overwintering (Ap), **(b)** overwintering (Ao) and **(c)** spring (S) cohort tadpoles. *Filled* symbols correspond to RMR and *empty* symbols SMR. *Circles* correspond to animals at high temperature and *triangles* animals reared at low temperature.

Figure 2: Change in metabolic scope (RMR - SMR) through time at **(a)** high and **(b)** low temperatures. *Circles* represent pre-overwintering (Ap), *triangles* overwintering (Ao) and *squares* spring (S) cohort tadpoles.

Figure 3: Bivariate trajectories of development (Gosner developmental stage; y axis) and growth (body size; x axis) for the experimental groups. These trajectories are characterized by size (magnitude), orientation (direction) and shape, which capture the amount of net energy allocated to growth/development (magnitude), the difference in allocation to growth and development (direction), and temporal changes in allocation (shape). *Circles* represent pre-overwintering (Ap), *triangles* overwintering (Ao) and *squares* spring (S) cohort tadpoles. *Filled* symbols correspond to high temperature and *empty* symbols to low temperature treatments.

Figure 1

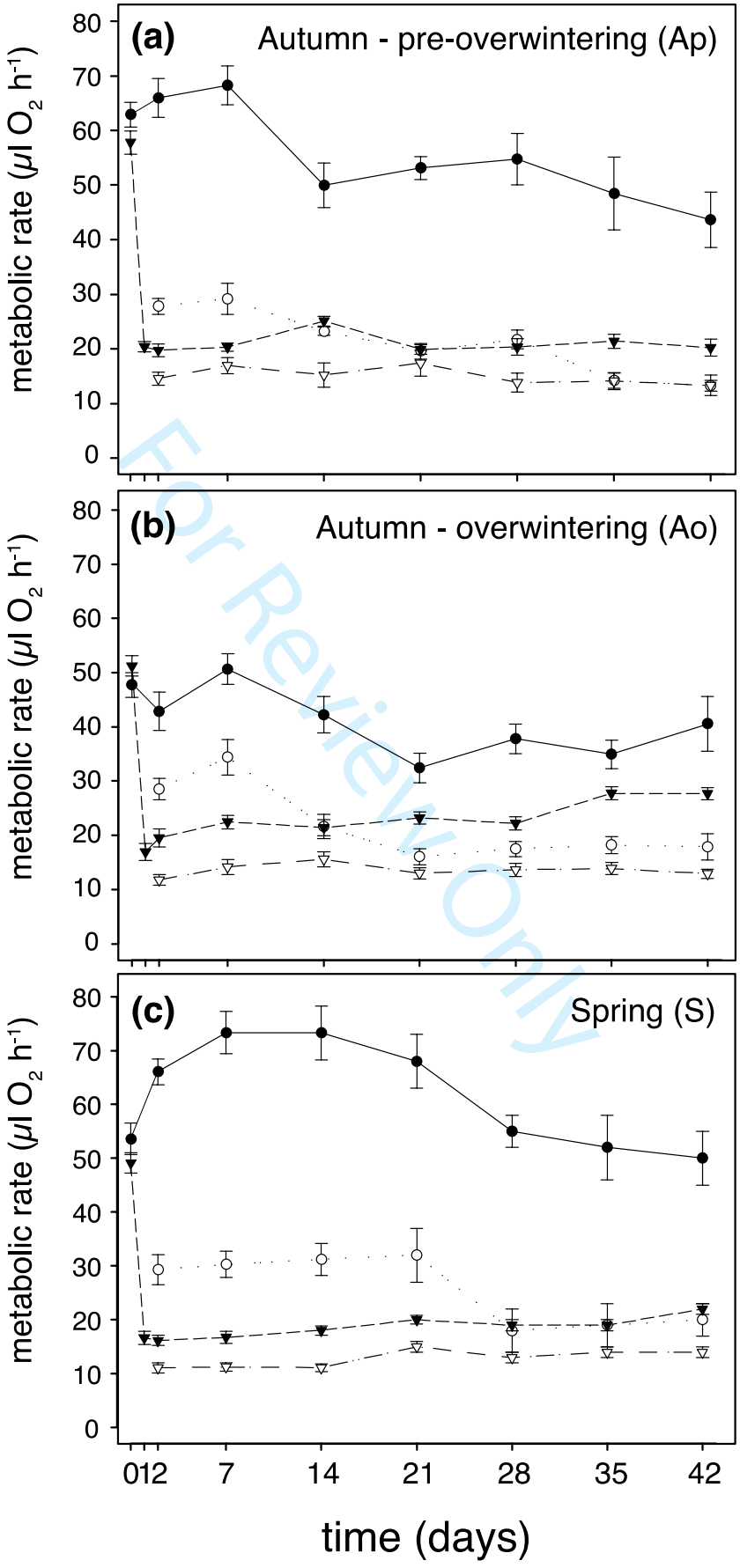

Figure 2

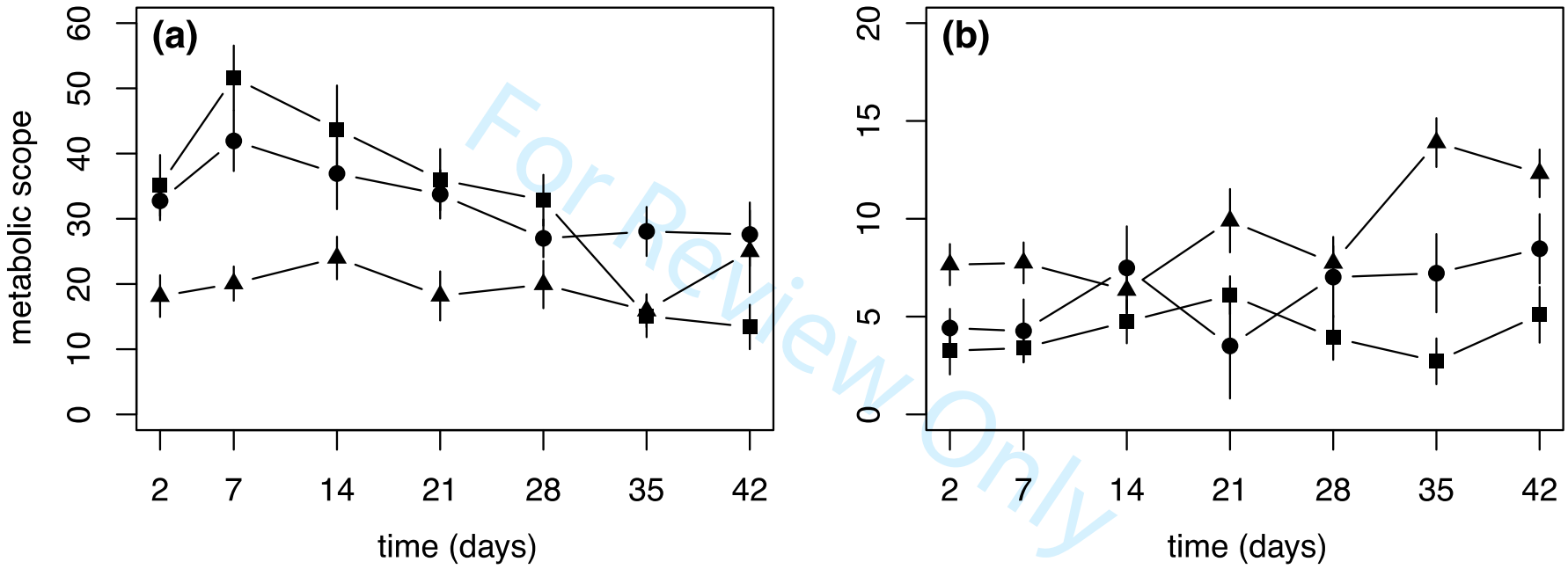

Figure 3

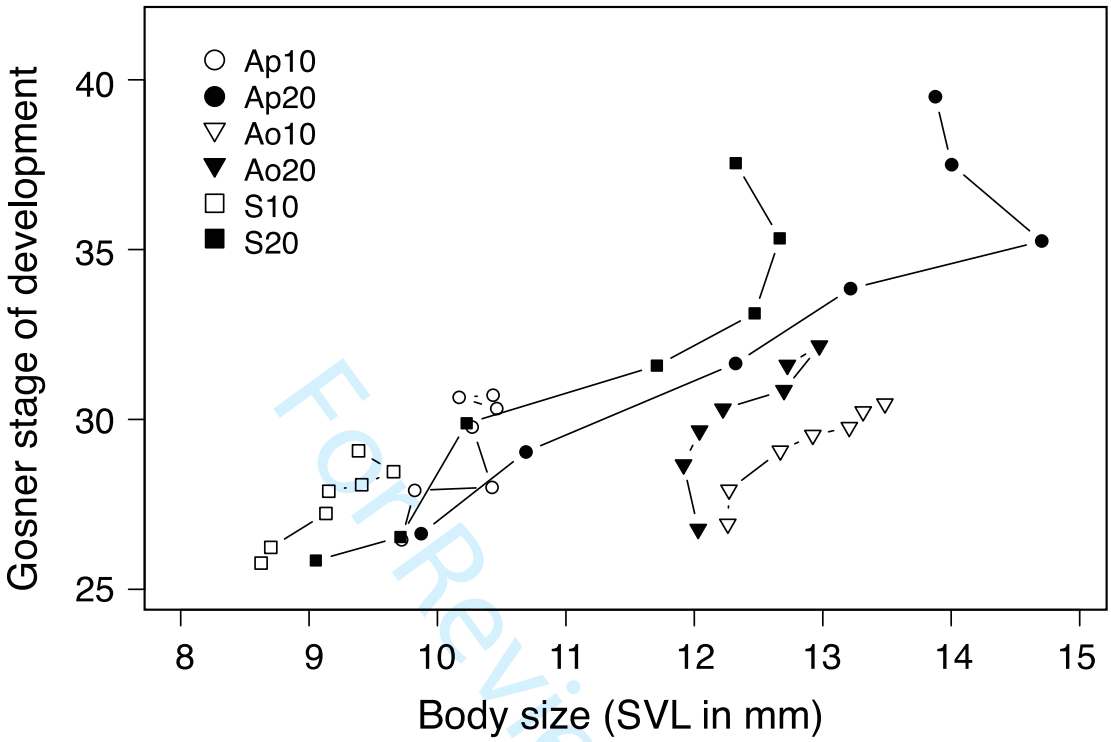

Table 1: Results of survival analyses using CoxPH models after model selection based on AIC at (a) high temperatures (20ºC) and (b) low temperatures (10ºC). We present estimated β coefficients (β), its associated standard (SE) and robust standard error (Robust SE), the exponentiated coefficient (also known as hazard ratio), lower and upper 95% confidence interals for the exponentiated coeficients, Wald statistic value (z), and its associated *P*-value. In addition, we report the *Robust* score value and the *P*-value for the whole model.

	β	SE (β)	Robust SE	Exp (β)	Lower 0.95	Upper 0.95	*z*	*P*-value	*Robust*	*P*-value
(a) T20 treatment									19.35	< 0.0001
Size	0.333	0.211	0.224	1.395	0.900	2.163	1.489	0.136		
Gosner	-0.498	0.153	0.165	0.608	0.440	0.845	-3.011	0.003		
Cohort: Ap	2.198	0.658	0.602	9.010	2.769	29.314	3.652	< 0.001		
Cohort: Ao	0.828	0.797	0.670	2.288	0.616	8.497	1.236	0.216		
(b) T10 treatment									3.95	0.139
Size	-1.280	0.671	0.373	0.278	0.134	0.578	-3.429	< 0.001		
Gosner	1.382	0.542	0.533	3.983	1.401	11.323	2.593	0.010		

Table 2: Analysis of covariance for routine metabolic rates (RMR) at day 2 of the experiment, including Gosner developmental stage (covariate; G), cohort (C), temperature (T) and their interactions for **(a)** all animal groups and **(b)** 20ºC and **(c)** 10 ºC temperature treatments separately. 'df': degrees of freedom; 'SS': sum of squares.

	df	SS	*F*-value	*P*-value
(a) Whole model				
Gosner (G)	1	140.4	1.959	0.167
Cohort (C)	2	1694.2	11.821	< 0.0001
Temperature (T)	1	28215.0	393.737	< 0.0001
G × C	2	117.6	0.821	0.445
G × T	1	590.5	8.240	0.006
C × T	2	2040.8	14.239	< 0.0001
G × C × T	2	131.8	0.920	0.404
Residuals	61	4371.2		
(b) 20ºC treatment				
Gosner (G)	1	797.6	6.479	0.016
Cohort (C)	2	3657.7	14.856	< 0.0001
G × C	2	254.7	1.035	0.368
Residuals	30	3693.2		
(c) 10ºC treatment				
Gosner (G)	1	19.9	0.912	0.347
Cohort (C)	2	81.3	1.858	0.173
G × C	2	7.5	0.172	0.843
Residuals	31	678.0		

Table 3: Results of generalized additive mixed models (GAMM) for RMR and SMR, including Gosner developmental stage (covariate; G), cohort (C), temperature (T) and their interactions. We used time (i.e. day) as the smooth term and we added a temporal correlation and individual tadpoles as a factor to account for temporal autocorrelation and repeated measurements (i.e. pseudoreplication). 'df': degrees of freedom; 'edf': estimated degrees of freedom.

	RMR			**SMR**		
	df	*F-value*	*P-value*	df	*F-value*	*P-value*
Gosner (G)	1	17.261	<0.0001	1	6.745	0.010
Cohort (C)	2	49.815	< 0.0001	2	5.478	0.005
Temperature (T)	1	63.775	< 0.0001	1	8.388	0.004
C × T	2	51.875	< 0.0001	2	4.527	0.011

Time effect	**RMR**				**SMR**			
	edf	*F-value*	*P-value*	Trend	edf	*F-value*	*P-value*	Trend
Ao10	2.45	13.144	< 0.0001	Increase	1.00	6.634	0.010	Decrease
Ao20	2.42	11.550	< 0.0001	Decrease	3.29	34.419	< 0.0001	Decrease
Ap10	1.00	6.185	0.013	Decrease	1.00	8.659	0.003	Decrease
Ap20	2.44	10.645	<0.0001	Decrease	1.00	61.022	< 0.0001	Decrease
S10	1.00	2.506	0.114	-	1.00	1.546	0.214	-
S20	1.00	45.665	< 0.0001	Decrease	1.00	29.383	< 0.0001	Decrease

Table 4: Analysis of covariance for **(a)** routine metabolic rates (RMR) and **(b)** standard metabolic rates (SMR) at day 42 of the experiment, including Gosner developmental stage (covariate; G), cohort (C), temperature (T) and their interactions. 'df': degrees of freedom; 'SS': sum of squares.

	df	SS	*F*-value	*P*-value
(a) RMR				
Gosner (G)	1	14362.0	96.491	< 0.0001
Cohort (C)	2	1959.6	6.583	0.002
Temperature (T)	1	2852.1	19.162	< 0.0001
G × C	2	2428.0	8.156	<0.0001
G × T	1	23.1	0.155	0.695
C × T	2	252.5	0.848	0.431
G × C × T	2	302.2	1.015	0.366
Residuals	103	15330.8		
(b) SMR				
Gosner (G)	1	313.7	10.521	0.002
Cohort (C)	2	391.0	6.557	0.003
Temperature (T)	1	1.18	0.040	0.843
G × C	2	254.2	4.262	0.019
G × T	1	15.2	0.511	0.478
C × T	2	6.5	0.109	0.897
G × C × T	2	186.2	3.122	0.052
Residuals	52	1550.7		

Table 5: Statistical assessment of differences in development-growth trajectory size (up the diagonal; $MD_{i,j}$) and orientation (down the diagonal; $\theta_{i,j}$) between experimental treatment pairs. Between parentheses the observed significance levels (*p*-values) empirically generated from 10,000 random permutations.

	Ao20	**Ao10**	**Ap20**	**Ap10**	**S20**	**S10**
Ao20	-	2.386 (0.311)	**8.137 (0.006)**	1.119 (0.628)	**6.510 (0.015)**	2.598 (0.271)
Ao10	**9.306 (0.047)**	-	**10.523 (0.001)**	1.267 (0.501)	**8.896 (0.0001)**	0.213 (0.900)
Ap20	9.149 (0.090)	0.157 (0.974)	-	**9.256 (0.002)**	1.627 (0.512)	**10.735 (0.001)**
Ap10	3.467 (0.478)	**12.773 (0.005)**	**12.616 (0.017)**	-	**7.629 (0.0003)**	1.479 (0.436)
S20	6.690 (0.153)	2.616 (0.528)	2.459 (0.614)	**10.157 (0.023)**	-	**9.108 (0.0001)**
S10	5.398 (0.253)	3.907 (0.333)	3.751 (0.450)	**8.866 (0.043)**	1.291 (0.761)	-

Supplementary Information for: **Physiology and acclimation potential are tuned with phenology in larvae of a prolonged breeder amphibian**

We obtained temperature data from two meteorological stations at 3.15 km (Begues) and 6.08 km (Sant Pere de Ribes) from the sampling pond (Fig. S1a) from September 2012 to May 2013. We chose this temporal window because we could analyse the temperatures experienced by their parents before spawning (brown area in Fig. S1b and c) and their expected temperature trend at the aquatic stage (blue area in Fig. S1b and c).

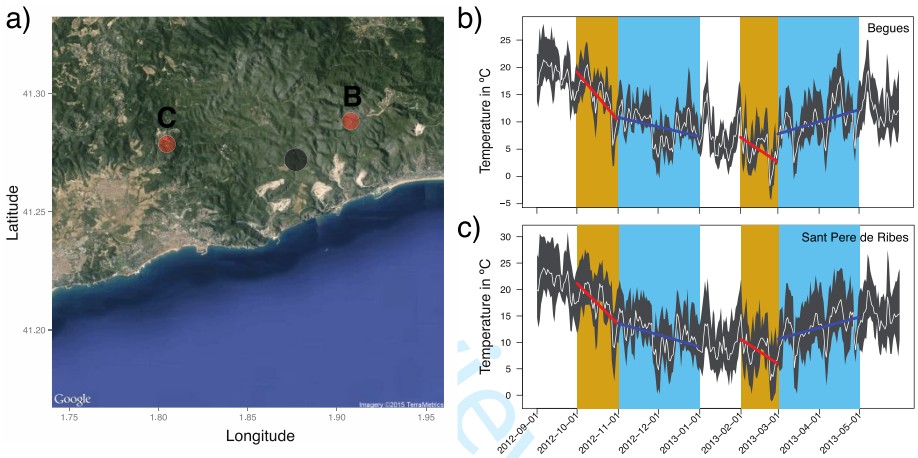

Figure S1: (a) Map showing the study population (grey circle) and the location of the two meteorological stations (red circles). **(b-c)** Temperature records for those two stations.

We compared mean, maximum and minimum temperatures of October (terrestrial phase for Ao and Ap groups) and February (terrestrial phase temperatures for S) using t-tests. Mean (Sant Pere de Ribes: $t_{56.39} = 11.370$, $p < 0.0001$; Begues: $t_{56.54} = 11.525$, $p < 0.0001$), maximum (Sant Pere de Ribes: $t_{56.5} = 11.709$, $p < 0.0001$; Begues: $t_{56.73} = 10.303$, $p < 0.0001$), and minimum temperatures (Sant Pere de Ribes: $t_{55.64} = 9.846$, $p < 0.0001$; Begues: $t_{55.85} = 10.101$, $p < 0.0001$) differed between October and February. We also compared the expected temperatures at the aquatic phase of Ao and Ap (November and December) and S (March and April) groups. We found differences for mean ($t_{120} = -2.491$, $p = 0.014$) and maximum temperatures ($t_{114.35} = -3.371$, $p = 0.001$) at Sant Pere de Ribes station and maximum temperatures at Begues ($t_{111.6} = -2.504$, $p = 0.014$). Further, the temperature trend during the aquatic phase of autumn and spring cohorts differed (blue regression lines in Fig. S1b and c).

Table S12: Analysis of covariance for routine metabolic rates (RMR; excluding animals that died during the course of the experiment) for routine metabolic rates (RMR) at day 2 of the experiment, including Gosner developmental stage (covariate; G), cohort (C), temperature (T) and their interactions for **(a)** all animal groups and **(b)** 20ºC and **(c)** 10 ºC temperature treatments separately. 'df': degrees of freedom; 'SS': sum of squares.

	df	SS	*F*-value	*P*-value
(a) Whole model				
Gosner (G)	1	482.0140.4	6.97251.959	0.01148940.167
Cohort (C)	2	1081.21694.2	7.819311.821	<0.0001
Temperature (T)	1	23039.928215.0	333.2596393.737	< 0.0001
G × C	2	125.3117.6	0.90600.821	0.41172700.445
G × T	1	192.4590.5	2.78268.240	0.10256150.006
C × T	2	1161.52040.8	8.400514.239	< 0.0001
G × C × T	2	448.2131.8	3.24130.920	0.04884810.404
Residuals	4361	2972.84371.2		
(b) 20ºC treatment				
Gosner (G)	1	221.66797.6	1.35176.479	0.264420.016
Cohort (C)	2	1501.023657.7	4.576514.856	0.02956< 0.0001
G × C	2	528.81254.7	1.61231.035	0.234350.368
Residuals	1430	2295.913693.2		
(c) 10ºC treatment				
Gosner (G)	1	22.1819.9	0.95010.912	0.33780.347
Cohort (C)	2	73.8081.3	1.58081.858	0.22300.173
G × C	2	8.267.5	0.17700.172	0.83870.843
Residuals	2931	676.90678.0		

Table S23: Results of generalized additive mixed models (GAMM; excluding animals that died during the course of the experiment) for RMR and SMR, including Gosner developmental stage (covariate; G), cohort (C), temperature (T) and their interactions. We used time (i.e. day) as the smooth term and we added a temporal correlation and individual tadpoles as a factor to account for temporal autocorrelation and repeated measurements (i.e. pseudoreplication). 'df': degrees of freedom; 'edf': estimated degrees of freedom.

	RMR			**SMR**		
	df	*F-value*	*P-value*	df	*F-value*	*P-value*
Gosner (G)	1	19.2917.261	<0.0001	1	8.2516.745	0.0040.010
Cohort (C)	2	48.8849.815	< 0.0001	2	4.9185.478	0.0080.005
Temperature (T)	1	50.3163.775	< 0.0001	1	8.4638.388	0.0044
C × T	2	50.3051.875	< 0.0001	2	3.7784.527	0.02411
Time effect	**RMR**				**SMR**			
	edf	*F-value*	*P-value*	Trend	edf	*F-value*	*P-value*	Trend
Ao10	2.3262.45	13.2761 3.144	< 0.0001	Increase	1.00	7.4056.6 34	0.0106	Decrease
Ao20	2.4072.42	12.7201 1.550	< 0.0001	Decrease	2.8443.29	36.71934 .419	< 0.0001	Decrease
Ap10	1.00	6.0616.1 85	0.014070. 013	Decrease	1.00	9.3628.6 59	0.0023	Decrease
Ap20	1.0002.44	8.90210. 645	<0.00301	Decrease	1.00	35.92661 .022	< 0.0001	Decrease
S10	1.00	1.7852.5 06	0.182010. 114	-	1.00	1.1391.5 46	0.28714	-
S20	1.00	55.9564 5.665	< 0.0001	Decrease	1.00	30.77729 .383	< 0.0001	Decrease

Table S34: Analysis of covariance excluding animals that died during the course of the experiment for **(a)** routine metabolic rates (RMR) and **(b)** standard metabolic rates (SMR) at day 42 of the experiment, including Gosner developmental stage (covariate; G), cohort (C), temperature (T) and their interactions. 'df': degrees of freedom; 'SS': sum of squares.

	df	SS	*F*-value	*P*-value
(a) RMR				
Gosner (G)	1	14171.514362.0	94.60919 6.491	< 0.0001
Cohort (C)	2	2158.41959.6	7.20486.583	0.0012
Temperature (T)	1	2655.22852.1	17.726119.162	< 0.0001
G × C	2	2436.52428.0	8.13308.156	<0.0001
G × T	1	17.723.1	0.11840.155	0.7315325 0.695
C × T	2	186.2252.5	0.62150.848	0.5391605 0.431
G × C × T	2	297.3302.2	0.99231.015	0.3742886 0.366
Residuals	102103	15278.615330.8		
(b) SMR				
Gosner (G)	1	324.62313.7	10.691910.521	0.002
Cohort (C)	2	381.80391.0	6.28776.557	0.003
Temperature (T)	1	1.631.18	0.05380.040	0.817427 0.843
G × C	2	252.94254.2	4.16554.262	0.021104 0.019
G × T	1	14.2815.2	0.47020.511	0.495979 0.478
C × T	2	6.616.5	0.10890.109	0.897029 0.897
G × C × T	2	187.58186.2	3.08913.122	0.054157 0.052
Residuals	512	1548.411550.7		

Figure S2: Change in routine metabolic rate (RMR) over time of **(a)** Autumn overwintering tadpoles at 10ºC, **(b)** Autumn overwintering at 20ºC, **(c)** Autumn pre-overwintering at 10ºC, **(d)** Autumn pre-overwintering at 20ºC, **(e)** Spring tadpoles at 10ºC and **(f)** Spring tadpoles at 20ºC. Note that the number in the 'y-axis' label denotes the estimated degrees of freedom.

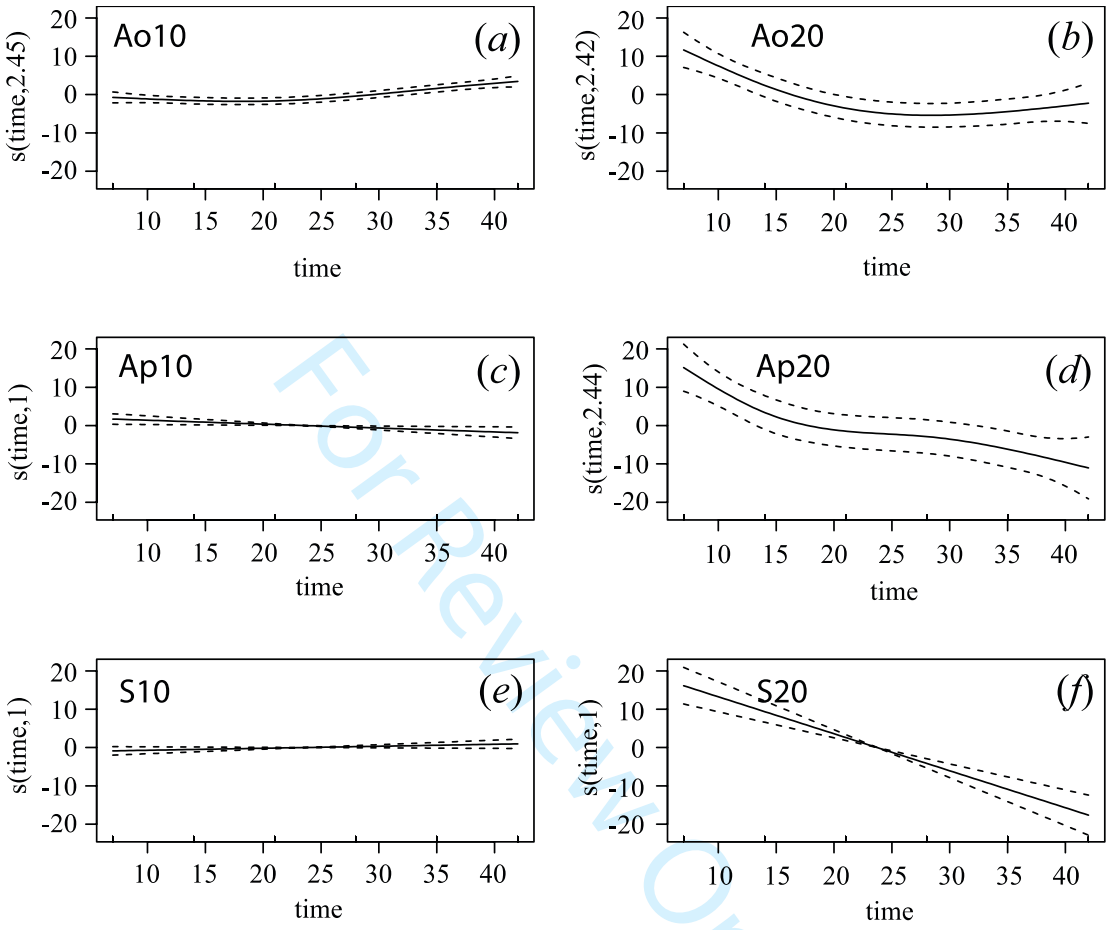

Figure S3: Change in standard metabolic rate (SMR) over time of **(a)** Autumn overwintering tadpoles at 10ºC, **(b)** Autumn overwintering at 20ºC, **(c)** Autumn pre-overwintering at 10ºC, **(d)** Autumn pre-overwintering at 20ºC, **(e)** Spring tadpoles at 10ºC and **(f)** Spring tadpoles at 20ºC. Note that the number in the 'y-axis' label denotes the estimated degrees of freedom.

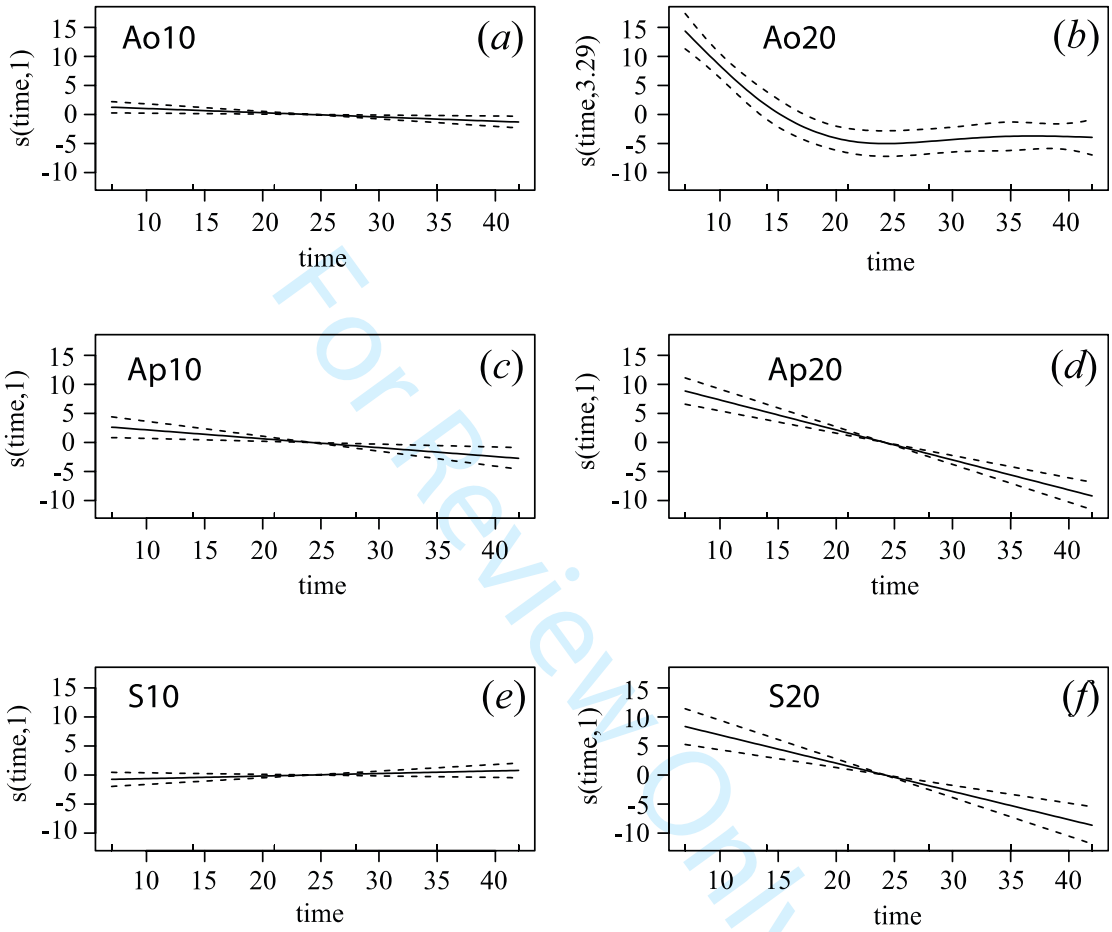

Figure S4: Change in aerobic scope for routine activity (ASRA) over time of **(a)** Autumn overwintering tadpoles at 10ºC, **(b)** Autumn overwintering at 20ºC, **(c)** Autumn pre-overwintering at 10ºC, **(d)** Autumn pre-overwintering at 20ºC, **(e)** Spring tadpoles at 10ºC and **(f)** Spring tadpoles at 20ºC. Note that the number in the 'y-axis' label denotes the estimated degrees of freedom.

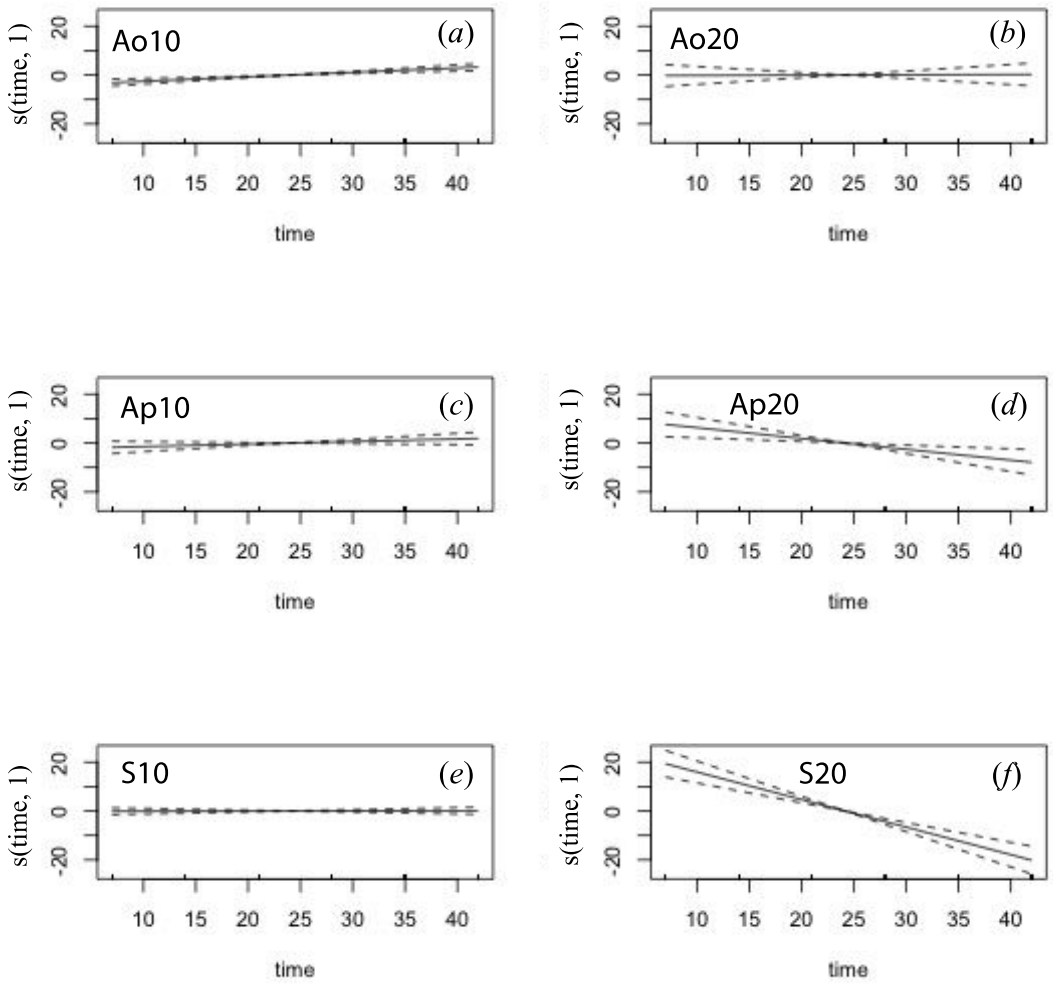

Figure S5: Relationship between developmental stage and (a) RMR or (b) SMR in overwintering (*blue*), pre-overwintering (*red*) and spring cohort (*green*) tadpoles, at the end of the experiment (day 42).