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Metabolic costs of altered growth trajectories across life transitions in amphibians

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Abstract

1. Climate change is causing increases in temperature and in the frequency of extreme weather events. Under this scenario, organisms should maintain or develop strategies to cope with environmental fluctuations, such as the capacity to modify growth trajectories. However, altering growth can have negative consequences for organisms' fitness.

2. Here, we investigated the metabolic alterations induced by compensatory growth during the larval development of the common frog (*Rana temporaria*), quantifying changes in oxidative stress, corticosterone levels, and telomere length. We induced compensatory growth responses by exposing frog embryos to cold conditions (i.e. a "false spring" scenario), which cause a delay in hatching. Once hatched, we reared larvae at two different photoperiods (24:0, representing the natural photoperiod of larvae, and 18:6) to test also for the interactive effects of light on growth responses.

3. Larvae experiencing delayed-hatching showed fast compensatory responses, and reached larger size at metamorphosis. Larvae shortened their developmental period in response to delayed-hatching. Non-permanent light conditions resulted in relaxed growth compared with larvae reared under permanent light conditions, which grew at their natural photoperiod and closer to their maximal rates.

4. Growth responses altered the redox status and corticosterone levels of larvae. These physiological changes were developmental-stage dependent, and mainly affected by photoperiod conditions. At catch-up, larvae reared at 18:6 light:dark cycles showed higher antioxidant activities and glucocorticoid secretion. On the contrary, larvae reared at 24:0 developed at higher rates without altering their oxidative status, likely an adaptation to grow under very restricting seasonal conditions at early life. At metamorphosis, compensatory responses induced higher cellular antioxidant activities probably caused by enhanced metabolism. Telomeres length remained unaltered by experimental treatments but apparently tended to elongate across larval ontogeny, which would be a first evidence of telomere lengthening across metamorphosis.

5. Under the forecasted increase in extreme climatic events, adjusting growth and developmental rates to the dynamics of environmental fluctuations may be essential for survival, but it can carry

metabolic costs and affect later performance. Understanding the implications of such costs will be essential to properly estimate the impact of climate change on wild animals.

Introduction

Climate change is defined not only by gradual warming, but also by a diverse range of other environmental effects, including shifts in seasonality and the increase in the likelihood of extreme climatic events (Bailey & van de Pol, 2016). Organisms can often detect such alterations and trigger responses in order to reduce their impact on fitness (Hoffmann & Sgrò, 2011). Some of these responses include changes in life histories, such as variations in phenology or behaviour under thermal stress (Hoffmann & Sgrò, 2011; Huey et al. 2012), which can alter growth trajectories and result in smaller individuals (Parry, Rosenzweig & Livermore, 2005; Sheridan & Bickford, 2011). However, individuals of many species can speed up growth once favourable conditions are restored (Dmitriew, 2011), trying to avoid negative size-dependent effects (Dmitriew & Rowe, 2005; Auer, Arendt, Chandramouli, & Reznick, 2010). The trade-off between costs and benefits of modifying growth trajectories determines the ecological relevance of growth plasticity. In the long-term, costs of compensatory growth can impact fitness by reducing reproductive investment or by shortening lifespan (Mangel & Munch, 2005; Inness & Metlcafe, 2008).

Conditions during early stages can affect growth, development, behaviour, and physiology later in life (Monaghan, 2007). Non-optimal growth conditions at early stages induce shifts in resource allocation, away from the development of new structures (Metcalfe & Monaghan, 2001). Early harsh conditions often have later consequences for juveniles and adults, particularly in species with indeterminate growth like fish, amphibians, or reptiles (Mangel & Munch, 2005; Dmitriew & Rowe, 2007; Murillo-Rincón, Laurila, & Orizaola, 2017). Such long-term consequences can be particularly intense in species with abrupt ontogenetic transitions (i.e. metamorphosis) like many arthropod, fish, and amphibian species (De Block & Stoks, 2005; Stoks & Córdoba-Aguilar, 2012; Schmidt, Hödl, & Schaub, 2012). In these species, adult fitness is strongly dependent on the timing to and size at metamorphosis (Pechenik, 2006), which can be severely affected by earlier suboptimal growth and developmental conditions.

Growth acceleration at early developmental stages can result in the accumulation of damage at different molecular levels. Growth in vertebrates is mainly enhanced by the activation of neuroendocrine pathways, which are regulated by the hypothalamic-pituitary-adrenal/interrenal (HPA/I) and by the hypothalamic-pituitary-somatotropic (HPS) axis (Wada, 2008; van den Beld et al. 2018). The insulin-like growth factors are stimulated by the growth hormone, secreted by the HPS-axis, and interact with hormones produced by the HPA/I axis (glucocorticoids and thyroid

hormones; Chrousos, 2009; Crespi, Williams, Jessop, & Delehanty, 2013), regulating the timing and the extent of morphogenesis. These hormones activate a cascade of catabolic routes that can unbalance the redox status of organisms and overproduce reactive oxygen species (ROS), damaging essential biomolecules like proteins or DNA (Monaghan, Metcalfe, & Torres, 2009). The alteration of these metabolic routes can reduce individual health and fitness (Crespi et al. 2013; Costantini, 2014, 2019). For instance, birds compensating losses in growth experience oxidative stress and reduced immunocompetence (Alonso-Alvarez, Bertrand, Faivre, & Sorci, 2007), and similar responses have been detected in insects (De Block & Stoks, 2008a, De Block & Stoks, 2008b) and fish (Kim, Noguera, & Velando, 2019). Compensatory responses can also affect organisms' physiology later in life, as in individuals growing faster after a period of poor nutritional conditions, which still maintain higher metabolic rates during adulthood (Criscuolo, Monaghan, Nasir, & Metcalfe, 2008).

Amphibians are great subjects for the study of the effects of compensatory growth responses. Most amphibians have an aquatic larval stage before metamorphosing into a terrestrial juvenile, and have retained high growth and developmental plasticity in order to adjust metamorphosis size and timing to the variation on environmental conditions (Scott, Casey, Donovan, & Lynch, 2007; Crespi & Warne, 2013). Poor initial growth conditions, such as low temperature or high salinity, induce compensatory responses in amphibians (Squires, Bailey, Reina, & Wong, 2010; Hector, Bishop, & Nakagawa, 2012; Orizaola, Dahl, & Laurila, 2010; Orizaola, Richter-Boix, & Laurila, 2016; Murillo-Rincón et al. 2017). Compensatory growth negatively affects fitness-related components of larvae by reducing, for instance, their swimming speed and immune status, or the locomotor performance of metamorphs (Hector et al. 2012; Dahl, Orizaola, Nicieza, & Laurila, 2012a; Murillo-Rincón et al. 2017).

Here, using the common frog (*Rana temporaria*) as a model species, we examined the metabolic changes induced by altering growth trajectories early in life, by measuring oxidative stress, corticosterone levels, and telomere length across the entire larval development. In a laboratory experiment, we exposed frog embryos to cold conditions that intended to mimic a period of cold weather shortly after laying, stopping development and inducing a delay in hatching (Orizaola et al. 2016; Murillo-Rincón et al. 2017). This cold period is representative of a "false spring" scenario, in which weather conditions fluctuate and winter temperatures come back after breeding, something expected to occur more and more often as a consequence of climate change (Chamberlain, Cook, García de Cortázar-Atauri, & Wolkovich, 2019). After hatching, we

examined compensatory growth responses and reared larvae under two different photoperiods to test for possible interactive effects of light on growth responses. Variations in photoperiod affect larval growth and development in amphibians (Wright et al. 1988; Laurila, Pakkasmaa, & Merilä, 2001; Kukita et al. 2015), and photoperiod is considered as a reliable phenological cue for many other organisms with complex life cycles in temperate environments (e.g. Śniegula & Johansson, 2010; Śniegula, Nilsson-Örtman, & Johansson, 2012). We hypothesized that a) individuals experiencing delayed hatching would compensate their initial loss in development, and that b) interactive effects of photoperiod levels would shape the extent of such compensation. Since compensatory growth is expected to be physiologically costly, we predicted that c) larvae showing compensatory growth responses would experience changes in their redox and corticosterone levels, as well as telomere shortening.

Material and methods

Study system and sampling

The common frog (*Rana temporaria*) is a widespread species that breeds throughout Europe, from northern Spain to the coast of the Arctic Ocean (Sillero et al. 2014). We collected ca. 150-200 freshly laid eggs from each of seven clutches of *R. temporaria* at a breeding locality near Leipojärvi, Gällivare (Northern Sweden, 67º 03'N, 21º 13'E) on 24 May 2014, on the northernmost part of the species distribution. At this latitude, breeding starts once pond ice melts (ca. mid May-early June), and tadpoles develop within ca. 30 days, under permanent light conditions (i.e. 24:0 photoperiod).

Experimental design

After collection, we quickly transported the eggs to our laboratory at Uppsala University (Sweden) where we conducted the experiment. The experiment had a 2x2 design in which we crossed two phenological levels corresponding to different hatching timing (non-delayed hatching treatment and five-day delayed hatching caused by cold exposure during the embryonic stage) with two photoperiod levels (24:0 and 18:6 light:dark cycles). Early cold conditions mimic a "false spring" scenario (Chamberlain et al. 2019) in which temperature fluctuates and periods of low-temperature reappear shortly after laying (Richter-Boix, Orizaola, & Laurila, 2014). This type of instability of early spring conditions is expected to increase in the future as a consequence of climate change, with potential severe consequences for wildlife (see Chamberlain et al. 2019).

Regarding photoperiod, 24:0 light:dark cycles simulate natural light conditions at the sampling site for *R. temporaria* during the breeding season, whereas 18:6 cycles represent the conditions experienced by this species in southern Sweden. Additionally, 18:6 photoperiod conditions have been used in many previous studies with *R. temporaria* (e.g. Dahl et al. 2012a; Dahl, Orizaola, Winberg, & Laurila, 2012b, Richter-Boix et al. 2014; Orizaola et al. 2016), thus allowing easy comparisons. We split the seven collected clutches into two portions containing 75-100 eggs each and placed them in 3-L buckets. For five days, we maintained half of them on an incubator set at 4ºC, and the other half on an incubator set at 18 ºC (Termarks 6395 F7FL incubators; both on a 24:0 light:dark cycle, Figure 1). After this period, we kept all eggs at 18 ºC in the incubators until hatching. Once hatchlings reached Gosner developmental stage 25 (free swimming and feeding, reabsorption of gills, ca. three days after hatching; Gosner, 1960), we haphazardly selected nine larvae from each clutch and treatment combination (total of 252 larvae), placed them individually in 0.8 L buckets, and assigned the buckets to two different incubators set at 18 ºC. Each of these two incubators represented one photoperiod treatment, one initially set up with 24:0 and the other with 18:6 light:dark cycles (Figure 1).

We filled the experimental buckets with reconstituted soft water (see Richter-Boix et al. (2014) for details), and renewed the water twice a week. We fed larvae *ad libitum* with par-boiled chopped spinach every second day. Every five days, we reprogrammed the incubators and switched photoperiod conditions between them, before moving all the larvae from one incubator to the other, thereby avoiding unintended incubator effects in the study. During early embryonic development, we sampled a pool of five embryos per clutch for telomere analysis. We sampled individuals at two additional points during their development: at larval stage, when individuals caught-up in size with non-delayed larvae (thereafter catch-up point; five larvae per clutch and treatment for body mass, growth rate, oxidative stress, corticosterone, and telomere analysis), and at metamorphosis (four larvae per clutch and treatment for body mass, growth rate, oxidative stress and telomere analysis; see Figure 1 for a schematic representation of the experimental design and sampling points).

Starting on the first day of the larval part of the experiment, we took one dorsal picture of each individual every fifth day in order to determine the timing at which larvae caught-up in size with non-delayed ones. A ruler was included in the frame for scale calibration. We measured larvae body length using Image J software (version 1.47), and look for differences between treatment combinations after each checking (see 'statistical analysis' section). We determined the

catch-up point as the day at which there were no significant differences in body length among phenology treatments. At this point, we collected five individuals from each clutch and treatment for physiological measurements and let the remaining larvae to complete their development. Larvae at catch-up point were at similar developmental stages among treatments, Gosner stages 28-30; at these stages, tadpoles only differ in minimal changes in the development of the hind limb bud (Gosner, 1960). The duration of the larval period was estimated as the difference in days between the start of the larval part of the experiment and the date of metamorphosis, recorded when tadpoles reached Gosner stage 42 (i.e. emergence of fore-limbs). Growth rate was estimated at catch-up point and at metamorphosis by dividing the body mass of the measured individuals at these points by the time (in days) elapsed since hatching.

Tissue collection

Before tissue sampling, we weighed individuals to the nearest 0.001 g in a high precision balance and quickly immersed them into a buffered MS-222 solution. For oxidative stress assays, we first eviscerated individuals to avoid possible food interferences in the assays. These individuals were snap frozen in liquid nitrogen and stored at -80 °C until assayed. For corticosterone assays, we collected blood from larvae at catch-up point, following standardized procedures that lasted ca. 90 seconds in order to avoid changes in CORT levels due to tadpole manipulation (Romero & Reed, 2005). We collected blood from the ventricle of tadpoles with the help of heparinized capillaries, and immediately centrifuged blood at 4000 rpm at 4 ºC for 20 minutes to obtain plasma (Burraco & Gomez-Mestre, 2016). Plasma samples were snap frozen in liquid nitrogen and stored at -80 ºC in Eppendorf tubes until assayed. We decided to only sample blood at this point, and not at metamorphosis, to avoid possible confounding results due to high corticosterone variation over very short periods of time around metamorphosis (Jaudet & Hatey 1984). For telomere length measurements, we collected whole-embryo tissues during embryonic development (tissue from 5 pooled embryos per clutch and treatment, at Gosner stage 10-12), and a portion of tail muscle at catch-up point and at metamorphosis. Samples were snap frozen in liquid nitrogen and stored at -80 °C until assayed.

Oxidative stress assays

We quantified the activity of three antioxidant enzymes: catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR). Antioxidant enzymes are the main defence against ROS in

animals and they protect cells from oxidative injuries (Costantini, 2019). We also quantified lipid peroxidation by measuring malondialdehyde (MDA) and total reduced glutathione (GSH_t) levels. Increases in MDA levels are indicative of oxidative damage in lipids, which often takes place when antioxidant enzymes are not able to fully counteract ROS production, then disturbing the integrity of membranes and leading to rearrangements in the cell structure (Birben et al. 2012). Importantly, MDA measures can be also affected by total lipid concentration in organisms (Pérez-Rodriguez et al. 2015). On the other hand, GSH is a hydrogen donor present in almost every cell in the body, which plays key antioxidant functions, and also participates in the detoxification of xenobiotics (Weschawalit et al. 2017). Elevated GSH levels are associated with higher antioxidant capacity (Aquilano et al. 2014), which can be induced via a coordinated action with GR to induce oxidative attack (Hanna, 2012), and also by changes in organisms' feeding habits (Gao et al. 2011).

We individually homogenized eviscerated tadpoles with a Miccra homogenizer (Miccra D-1) at 35,000 rpm in a buffered solution that prevents proteolysis in a homogenized sample:buffer proportion of 1:4.5 in ice (Burraco, Duarte, & Gomez-Mestre, 2013). We centrifuged the homogenates at 14,000 rpm for 30 min at 4 ºC and the resulting supernatants were aliquoted and stored at -80 ºC. We followed standard protocols for determining enzyme activities, lipid peroxidation, and the glutathione levels (see Supplementary information S1 for details). We run all samples in duplicate. Intra-sample CV% were 3.74 for CAT, 3.06 for GR, 1.35 for GPx, 2.36 for MDA, and 4.05 for GSH, whereas inter-plate CV% were 10.50 for CAT, 12.90 for GR, 3.45 for GPx, 7.98 for MDA, and 4.07 for GSH.

Corticosterone assay

We determined plasma corticosterone content using a commercial enzymoimmunoassay kit (Arbor Assays, K014-H5), which has low cross-reactivity with related molecules such as progesterone (0.24%), cortisol (0.38%) or 11-desoxycorticosterone (12.3%). This is a reliable method for measuring corticosterone in amphibian larvae (Burraco, Arribas, Kulkarni, Buchholz, & Gomez-Mestre, 2015). We used a volume of 5 µL of plasma to perform the assay following the procedure as suggested by suppliers. All samples were run in duplicate. We run three different EIA trays, each one including its own standard curve with a lowest point of 2.29 pg/mL. Baseline corticosterone concentration was calculated from the $\%B/B_0$ curve, by using the 4PLC routine and following an online tool (https://www.myassays.com/corticosterone.assay; last accessed 10th

September 2019). The intra-assay variation was 14.52% whereas the inter-assay variation was 10.1%. Average R^2 for the 4PLC fitting curve was 0.94.

Relative telomere length quantification

We isolated genomic DNA from embryos, larvae, and metamorphs using a high salt extraction protocol. Relative telomere length (rTL) assay was conducted through quantitative PCR (qPCR). This method reports relative measurements of telomere length by comparing qPCR cycle threshold (C_t) of a control sequence non-sensible to shortening with the C_t of the telomere sequence (see S1 for details on qPCR protocols). We ran samples in duplicate and measured again those with coefficient of variation higher than 5%. Intra-plate CV% was 0.87 and 1.15 for GADPH and telomere gene, respectively, whereas inter-plate CV% was 1.64 and 3.05 for GAPDH and telomere gene, respectively. We used a total of seven plates.

We used mean values to calculate rTL by applying the following formula (Pfaffl, 2001):

$$rTL = [(E_{telomere})_t{}^{\Delta Ct\ telomere\ (control-sample)}] / [(E_{GAPDH})^{\Delta Ct\ GAPDH\ (control-sample)}]$$

where $E_{telomere}$ is the qPCR efficiency of the telomere fragment; E_{GAPDH} is the qPCR efficiency of the GAPDH fragment; ΔC_t telomere is the deviation in C_t of the standard compared with the telomere fragment for each sample; ΔC_t GAPDH is the deviation in C_t of the standard compared with the GAPDH fragment for each sample. We checked for the specificity of the qPCR reactions by performing a melting curve to detect possible primers dimers or secondary amplifications, without detecting undesirable amplified fragments. We also examined the suitability of GAPDH as a control gene by testing the stability of this fragment using linear mixed models with phenology and photoperiod as fixed factors, and clutch as random factor.

Statistical analyses

We checked for parametric assumptions on the variables by conducting Kolmogorov-Smirnov tests for normality ('lillie.test', nortest package version 1.0-4) and Breusch-Pagan tests for homoscedasticity ('bptest', lmtest package version 0.9-35). We fitted linear mixed models using the function 'lmer' for parametric data (tadpole length at each sampling point, mass, body condition, growth rate, duration of the larval period, CORT levels, oxidative stress parameters, and telomere length). We used generalized mixed models with the function 'glmer' (binomial family) survival data. Both functions are included in the lme4 package (version 1.1-14). In all models, we included 'photoperiod' and 'phenology' treatments, and their interaction, as fixed factors, and

'clutch' as random factor. We conducted post-hoc Tukey tests to check for differences among treatments by using the function 'emmeans' implemented in the package emmeans (version 0.9.1).

We used the function 'emmeans' to estimate marginal means (EMMs) for both linear and generalized linear mixed models. We modelled survival data using binomial distribution. We also run a non-linear mixed model using the 'nlme' function following a logistic distribution, and including 'individual' nested within 'clutch' as random factor, in order to compare growth curves. We checked for changes in the activity of antioxidant enzymes (catalase, glutathione peroxidase, and glutathione reductase) using principal component analysis (PCA) in order to avoid collinearity problems.

Larvae length, larval period, total reduced glutathione concentration, and telomere length data were log(10) transformed to meet parametric assumptions. We estimated body condition for each experimental factor at catch-up point and metamorphosis, using body mass and tadpole length, and following Peig & Green (2009). In order to examine possible confounding effects of lipids storage on MDA levels, we correlated body condition with MDA data (Pérez-Rodriguez et al. 2015). Since body mass and GSHt showed a significant positive correlation we included mass as covariate in the GSH analysis. We conducted all statistical analyses in R software (version 3.5.2, R Core Team 2017).

Results

Life-history traits

Survival was very high during the experiment (95.86%) and did not differ among treatments (all *P-values* > 0.227).

We used larval body length to estimate the timing of catch-up growth. At day five of the experiment, larvae at the delayed treatment had just hatched (Figure 1), and were smaller than larvae in the non-delayed treatment that had been already developing for five days (23.0% smaller on average; df = 1,250; $\chi^2 = 496.98$, $P < 0.001$; Figure 2). Larvae from the delayed hatching treatment were smaller than the non-delayed ones until day 20 (all $P < 0.001$; see Table S2), when size differences between treatments disappeared (df = 1,106; $\chi^2 = 0.18$; $P = 0.668$; Figure 2), defining the catch-up point. Compensation occurred regardless of photoperiod conditions (Tukey post-hoc $P = 0.324$ and $P = 0.704$, for 24:0 and 18:0 cycles, respectively). The lack of differences in size continued until metamorphosis (Figure 2; Table S2), when individuals from the delayed hatching treatment were larger than non-delayed ones (7.42% on average; df =

1,106; $\chi^2 = 17.11$; $P < 0.001$; Figure 2). Larvae exposed to 24:0 cycles metamorphosed with larger size than those reared under 18:6 cycles (8.40% larger on average; df = 1,106; $\chi^2 = 21.70$; $P < 0.001$; Figure 2).

Larvae from the different phenology treatments did not differ in mass at catch-up point (day 20; df = 1,137; $\chi^2 = 0.33$; $P = 0.564$; Figure 3A). At this developmental point, photoperiod conditions significantly affected body mass, with tadpoles reared under 24:0 cycles being heavier than those under 18:6 cycles (10.58% heavier on average; df = 1,137; $\chi^2 = 9.28$; $P = 0.002$; Figure 3A). At metamorphosis, both delayed-hatching and 24:0 light cycle induced heavier individuals (15.76% and 20.22% heavier on average, df = 1,103; $\chi^2 = 17.08$; $P < 0.001$, and df = 1,103; $\chi^2 = 25.62$; $P < 0.001$, respectively; Figure 3B).

Growth rate at catch-up point was significantly higher in larvae from delayed-hatching treatments (19.88% higher on average; df = 1,137; $\chi^2 = 34.92$; $P < 0.001$; Figure 3C). Larvae exposed to 24:0 cycles also had higher growth rates than those exposed to 18:6 cycles (11.63% higher on average; df = 1,137; $\chi^2 = 11.48$; $P = 0.001$). Growth rates at metamorphosis (i.e. covering the entire larval period) were still higher in larvae from delayed-hatching treatment, and in those exposed to 24:0 cycles (24.53% and 12.54% higher on average, respectively; df = 1,103; $\chi^2 = 41.50$; $P < 0.001$, and df = 1,103; $\chi^2 = 41.50$; $P < 0.001$; Figure 3D). Non-linear mixed model following a logistic distribution revealed general differences in growth trajectories among treatments (all P-values < 0.001; Fig. 2).

Larvae from delayed-hatching treatment had shorter larval periods than larvae from non-delayed hatching (10.95% shorter on average; df = 1,103; $\chi^2 = 76.70$; $P < 0.001$; Figure S3). Photoperiod treatment did not affect the duration of the larval period (df = 1,103; $\chi^2 = 0.05$; $P = 0.735$; Table S2 and Figure S3).

Oxidative stress

Principal component analysis of the antioxidant enzyme activity yielded a first axis (PC1) that explained 73.4% of the antioxidant variance at catch-up point. Enzyme loading values for PC1 were: 0.61 (CAT), 0.64 (GR), and 0.47 (GPx). The interaction between phenology and photoperiod factors was significant (df = 1,102; $\chi^2 = 4.18$; $P = 0.041$; Figure 4A), revealing that larvae at 18:6 cycles had higher activities of CAT and GR when developed under non-delayed hatching conditions. PC2 explained 22.6% of the antioxidant variance. Enzyme loading values for PC2 were: 0.45 (CAT), 0.20 (GR), and -0.86 (GPx). The interaction between phenology and

photoperiod was significant (df = 1,102; $\chi^2 = 7.00$; $P = 0.008$; Figure 4A), indicating lower activity of GPx in tadpoles reared at 18:6 cycles and compensating for delayed hatching. Larvae exposed to delayed hatching had lower levels of MDA at catch-up point (27.74% lower on average; df = 1,109; $\chi^2 = 15.35$; $P < 0.001$; Figure 5A). Larvae reared under 18:6 cycles also had lower MDA levels than those under 24:0 cycles (15.75% lower on average; df = 1,109; $\chi^2 = 4.57$; $P = 0.032$; Figure 5A). Larvae from non-delayed hatching reared at 18:6 cycles had the highest GSH_t levels, as indicated by a significant hatching delay by photoperiod interaction (df = 1,83; $\chi^2 = 4.23$; $P = 0.040$; Figure 5C).

At metamorphosis, PC1 explained 41.2% of the variance in antioxidant enzyme activities. Enzyme loading values for PC1 were: 0.40 (CAT), 0.76 (GR), and 0.52 (GPx). A significant hatching delay by photoperiod interaction (df = 1,99; $\chi^2 = 11.16$; $P = 0.001$; Figure 4B) reveals higher GR activities in all treatments compared with antioxidant levels at 24:0 cycles and control hatching conditions. The second component (PC2) explained 36.0% of the variance. Enzyme loading values were: 0.76 (CAT), 0.04 (GR), and -0.65 (GPx). For PC2, a significant interaction between phenology and photoperiod indicated that larvae reared under 18:6 light conditions and non-delayed hatching had the highest CAT activity and lowest GPx activity (df = 1,99; $\chi^2 = 4.77$; $P = 0.029$; Figure 4B). Larvae from delayed hatching treatments showed lower MDA levels at metamorphosis (28.61% lower on average; df = 1,98; $\chi^2 = 16.33$; $P < 0.001$; Figure 5B). Contrary to the effects of photoperiod treatments at catch-up point, individuals reared at 18:6 cycles showed higher MDA levels at metamorphosis than larvae reared under permanent light conditions (20.79% on average; df = 1,98; $\chi^2 = 8.48$; $P = 0.004$). Coefficients of correlation for MDA and body condition were 0.20 and -0.33 at catch-up point and metamorphosis, respectively (see Figure S4). GSH_t values were higher in larvae reared under 24:0 cycles than under 18:6 cycles (42.63% higher on average; df = 1,98; $\chi^2 = 11.61$; $P = 0.001$; Figure 5D), whereas delayed-hatching did not affect GSH_t values at metamorphosis (df = 1,98; $\chi^2 = 0.49$; $P = 0.486$).

Corticosterone levels

A significant hatching delay by photoperiod interaction reveals that, at catch-up point, delayed hatching did not affect baseline corticosterone levels in larvae exposed to 18:6 cycles, whereas in larvae reared under 24:0 cycles corticosterone levels were lower in larvae from delayed-hatching treatment (15.60% lower on average; df = 1,77; $\chi^2 = 4.077$; $P = 0.043$; Figure 6).

Telomere length

GAPDH gene stability across treatments was high, and there were no differences in GAPDH levels between phenology and photoperiod treatments at the embryonic stage, catch-up point, or metamorphosis (all *P*-values > 0.489). Neither hatching treatments nor photoperiod affected telomere length, either at embryonic stage, catch-up point or metamorphosis (all *P*-values > 0.181; Figure 7A, B, and C, respectively). However, telomere length significantly increased across developmental stages, from embryo to metamorphosis (26.42% on average; df = 2,239; $\chi^2 = 6.93$; $P = 0.031$; Figure 7D and Figure S5). Interplate error was 1.64% for GAPDH gen and 3.05% for TEL gen. CV% for interplate RTL was 3.48.

Only significant interactions across treatments are reported above. See Table S2 for complete statistical details.

Discussion

Our study reveals that modifying growth trajectories in response shifting environments can alter metabolism across amphibian life stages. Compensating an early developmental delay generated by low temperatures, altered antioxidant activities and induced hormonal changes in *Rana temporaria* larvae, although most of these changes were photoperiod-dependent. Alterations in metabolism were not only detected at catch-up point, but also during the transition to the juvenile stage. Telomere length, on the contrary, was not affected by either delayed-hatching nor photoperiod, but we found signs of telomere elongation from embryonic to juvenile stages, which may represent the first evidence of telomere lengthening across metamorphosis.

Organisms often grow at sub-maximal rates, reducing the costs of intense cellular proliferation and catabolism (Dmitriew, 2011; Stoks & Córdoba-Aguilar, 2012). In our study, larvae increased their growth rates in response to delayed hatching after harsh conditions (i.e. cold temperature during the embryonic stage) disappeared. Larvae did not show a steady increase in growth rate during the entire larval period, but showed a fast response and caught-up in size with non-delayed larvae already at day 20, which represents half of their larval period. These results suggest that compensatory responses, when examined in detail, can reveal the existence of periods in which organisms maintain much faster growth than previously expected, which may affect the intensity and costs of the responses against increasing climatic instability.

During the amphibian larval stage, growth and development are often decoupled, thus individuals that shorten their larval period often metamorphose at smaller sizes (Rot-Nikcevic & Wasserug, 2004). However, we found that individuals compensating for a developmental delay can grow faster, shortening their larval period, and still metamorphose with the same (or larger) size than control individuals. This suggests that organisms following complex life cycles may be able to simultaneously activate the machineries involved in growth and development acceleration.

Climate instability, including spells of cold weather, is common at high latitudes even during the short growth season in summer. In these environments, selection should favour mechanisms to quickly increase growth rate, i.e. as soon as optimal conditions are restored. In our study, larvae not only caught-up in size with control individuals, but even reached a larger size at metamorphosis than controls. These results agree with growth overcompensation often found in fish or insects in response to early resource limitation (Nikki, Pirhonen, Jobling, & Karjalainen, 2004; Dmitriew & Rowe, 2007). Although the underlying mechanisms of overcompensation are not well defined, it is likely that individuals cannot easily shut down those pathways promoting growth once activated during compensation (Fuentes et al. 2013).

Photoperiod conditions can shape growth trajectories in many organisms (e.g. Boeuf & Le Bail, 1999; Laurila et al. 2001; Śniegula et al. 2012). In our study, daily fluctuations of light, i.e. non-permanent light conditions, resulted in lower larval growth rates than those observed in larvae reared under their natural (24:0) photoperiod. In contrast, photoperiod had no effect on larval development, suggesting that the developmental response to photoperiod is canalized on larvae of the *R. temporaria* population used in our study (Laurila et al. 2001).

Altering growth and development in response to phenology and mostly photoperiod treatments caused complex changes in the antioxidant machinery across amphibian larval development. At catch-up point, non-natural photoperiod conditions (i.e. 18:6 cycles) induced metabolic demands in larvae from non-delayed hatching conditions, whereas larvae from delayed-hatching treatments larvae maintained normal antioxidant activities. These results suggest that the *R. temporaria* population used in this study is likely adapted to grow at extremely fast rates and constant light conditions (i.e. in response to delayed-hatching and 24:0 cycles) during early larval development. This process might allow individuals to adaptively grow at constant high rates without incurring in ROS overproduction caused by intense mitochondrial activity. Indeed, plastic changes in the mitochondrial efficiency has been suggested as a mechanism to protect cells from damages caused by oxidation (e.g. Galli et al. 2016). At catch-up point, lipid peroxidation was

lower at 18:6 cycles, probably indicating the benefits (less ROS) of having activated the antioxidant machinery (Costantini, 2019). Alternatively, lower MDA levels may indicate a lower accumulation of fat reserves in larvae (Pérez-Rodríguez et al. 2015; Burraco, Iglesias-Carrasco, Cabido, & Gomez-Mestre, 2018), reflected also in the positive relationship between MDA levels and body mass at this point.

At metamorphosis, compensatory strategies appeared to activate to some extent the antioxidant machinery, as suggested by the higher antioxidant activity of larvae compensating for delayed-hatching at 24:0 light conditions compared to control individuals. This may indicate that accelerating growth close to maximum capacity throughout the entire development can imply a high catabolic demand at life transitions (Metcalfe & Alonso-Alvarez 2010). However, such increases in antioxidant enzyme activities may not incur in later performance costs for individuals since compensatory responses seemed not to generate lipid damages or lower antioxidant levels (Costantini, 2019). At metamorphosis, individuals compensating for delayed-hatching had lower MDA levels, which may indicate scarce lipid peroxidation favoured by antioxidant activities. Intriguingly, those individuals also had higher levels of GSH, which plays an essential role in counteracting free radical production in vertebrates (Birnie-Gauvin et al. 2017). Higher GR activity may have generated large amounts of GSH, which can reduce damages in lipids (Hanna, 2012). Elevated production of GSH molecules may have been orchestrated by differences in feeding habits, i.e. by a higher food consumption by larvae at permanent light conditions (Laurila et al. 2001; Gao et al. 2011). As observed at catch-up, 18:6 photoperiod conditions consistently induced higher enzymatic activities at metamorphosis, however such responses across development seemed to be insufficient to buffer oxidative damages at metamorphosis. Previous studies have detected alterations in the redox machinery across taxa, as for instances in zebra finches (Alonso-Álvarez et al. 2007), sticklebacks (Kim et al. 2018), or damselflies (De Block & Stoks, 2008b). Perturbations in the redox status at early life can have carry-over effects in vertebrates, such as reductions in reproduction investment or in life expectancy (Monaghan et al. 2009; Metcalfe & Alonso-Alvarez, 2010), although long-term studies are need to fully elucidated this question (Costantini, 2019).

Compensatory growth responses altered the levels of the glucocorticoid corticosterone in amphibian larvae, although this change was photoperiod-dependent. Corticosterone levels, at catch-up point, were higher in individuals compensating for a delayed hatching under 18:6 cycles than in those reared under permanent light conditions. Glucocorticoids play a key role during

stress events and are essential in regulating growth and the timing of ontogenetic transitions in vertebrates (Wada, 2008). In our study, we used *R. temporaria* from one of the northernmost populations of this species, and previous studies have shown that selection may have favoured low baseline corticosterone levels during larval development on populations of this species inhabiting high latitudes (Dahl et al. 2012b). Therefore, the lower corticosterone levels observed in larvae growing at close to their maximal rates (i.e. those responding to delayed-hatching under permanent light conditions) would be a consequence of a life-history strategy maximizing morphogenesis and growth (Crespi & Denver, 2005). These results, suggest that organisms adapted to the strong seasonal constraints of high latitude may have developed constitutive low corticosterone levels as a physiological adaptation to reduce the negative effects of high corticosterone, especially in early developmental stages.

Compensatory growth responses did not shorten telomeres across amphibian development. Intense cellular oxidative stress often shortens telomeres, however, the levels of redox changes detected in larvae showing compensating growth responses may not be enough to produce DNA damages, be tissue-dependent or even be only apparent later in life. Previous studies in amphibians found shorter telomeres in tadpoles surviving predators and maintaining high growth rates (Burraco, Díaz-Paniagua, & Gomez-Mestre, 2017), or in tadpoles developing faster against permanent desiccation risk (Burraco, Valdés, Johansson, & Gomez-Mestre, 2017). The lack of telomere shortening observed in our study can be also a consequence of the life-history of the *R. temporaria* population used in this experiment. Populations living at high latitudes, exposed to very short growth seasons, are selected to maintain extremely fast constitutive developmental rates (Laugen, Laurila, Räsänen, & Merilä, 2003), and may have evolved effective repair mechanisms in order to avoid cellular ageing during fast larval development. Interestingly, we found some signs of a possible telomere elongation during the aquatic stages of amphibian development. However, we should acknowledge that for practical reasons telomere analyses were conducted in embryonic cells, and in tail muscle later across larval development. Telomere elongation has been observed in a few ectotherms (fish: McLennan et al. 2018; reptiles: Ujvari et al. 2017) and under particular conditions in mammals (Hoelz, Cornils, Smith, Moodley, & Ruf, 2016), and birds (Haussmann & Mauck, 2007). Our study suggests that telomere elongation across early developmental stages may be common in ectotherms and, more remarkably, may represent the first evidence of telomere elongation across metamorphosis.

Conclusions

Our study indicates that amphibian larvae are able to compensate for adverse early-life conditions, mimicking a "false spring" scenario, and that under these circumstances their physiology is greatly altered by photoperiod conditions. Responses to these factors include alterations in the antioxidant machinery and at the endocrine level, but did not erode telomeres. Under the forecasted scenario of environmental instability, adjusting growth and developmental rates to the level of climatic fluctuations can be essential for the survival of many organisms. A good understanding of the long-term costs of physiological and metabolic responses to climatic variation will be critical for an accurate estimation of the impact of climate change on wildlife.

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Authors' contribution

P.B. and G.O. designed and performed the experiment, A.E.V. conducted the corticosterone assays, P.B. conducted the statistical analyses, and P.B. and G.O. wrote the manuscript with inputs from A.E.V.

Data accessibility

Data are accessible at https://figshare.com/articles/data_Burraco_et_al_xlsx/9992201

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Figure legends

Figure 1. Schematic framework of the experimental design and sampling points. Blue regions represent the five-days period of cold temperature exposure for inducing delayed-hatching in *Rana temporaria* embryos. Photoperiod conditions are represented by white (24:0 light:dark cycles) and grey (18:6 light:dark cycles) regions. Dashed lines indicate sampling points across development.

Figure 2. Effects of hatching timing and photoperiod on *Rana temporaria* body length until metamorphosis. Non-delayed hatching: solid line; Delayed hatching: dashed line. Permanent light (24:0 light:dark cycles): open symbol; 18:6 dark:light cycle: filled symbol. Values are estimated marginal means ± standard error.

Figure 3. Effects of hatching timing and photoperiod on *Rana temporaria* larvae body mass at: a) 20 days after the onset of the experiment (catch-up point); b) metamorphosis; and on larvae growth rate at c) day 20, and d) metamorphosis. Values are estimated marginal means ± standard error.

Figure 4. Effects of hatching timing and photoperiod at catch-up point (a) and metamorphosis (b) on *Rana temporaria* antioxidant enzymatic activities. At catch-up point, PC1 and PC2 axis explained 73.4% and 22.6% of the variance in antioxidant activities, respectively. Loadings values for CAT, GR, and GPx are 0.61, 0.64, and 0.47 for the PC1, and 0.45, 0.20, and -0.86 for the PC2. At metamorphosis, PC1 and PC2 axis explained 41.2% and 36.0% of the variance in antioxidant activities, respectively. Loadings values for CAT, GR, and GPx are 0.40, 0.76, and 0.52 for the PC1, and 0.76, 0.04, and -0.65 for the PC2. Values are estimated marginal means ± standard error.

Figure 5. Effects of hatching timing and photoperiod at catch-up point and metamorphosis on *Rana temporaria* malondialdehyde levels (MDA; a) and total reduced glutathione concentration (GSH_t; b). Values are estimated marginal means ± standard error.

Figure 6. Effects of hatching timing and photoperiod at catch-up point on *Rana temporaria* baseline corticosterone levels. Values are estimated marginal means ± standard error.

Figure 7. Effects of hatching timing and photoperiod on *Rana temporaria* relative telomere length (rTL) at, a) embryonic stage, b) catch-up point, and c) metamorphosis. d) Variation in rTL across ontogeny. We measured rTL in whole-embryos at the embryonic stage, and in tail muscle tissue at catch-up and metamorphosis. Values are estimated marginal means ± standard error.