

# Clutch quality is related to embryonic development duration, hatchling body size and telomere length in the spined toad (*Bufo spinosus*)

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Reproductive success is often related to parental quality, a parameter expressed through various traits, such as site selection, mate selection and energetic investment in the eggs or progeny. Owing to the complex interactions between environmental and parental characteristics occurring at various stages of the reproductive event, it is often complicated to tease apart the relative contributions of these different factors to reproductive success. Study systems where these complex interactions are simplified (e.g. absence of parental care) can help us to understand how metrics of parental quality (e.g. gamete and egg quality) influence reproductive success. Using such a study system in a common garden experiment, we investigated the relationships between clutch hatching success (a proxy of clutch quality) and offspring quality in an amphibian species lacking post-oviposition parental care. We found a relationship between clutch quality and embryonic development duration and hatchling phenotype. We found that hatchling telomere length was linked to hatching success. These results suggest that clutch quality is linked to early life traits in larval amphibians and that deciphering the influence of parental traits on the patterns we detected is a promising avenue of research.

ADDITIONAL KEYWORDS: amphibian – clutch quality – early life – embryonic development – hatching success – reproductive success.

## INTRODUCTION

Reproductive success is mediated, in part, by parental quality, expressed through complex interactions among ecology, physiology and behaviour during a reproductive event. The influence of parental quality on reproductive performance can occur at early stages of reproduction, such as during mate selection (Amos *et al.*, 2001), breeding site selection (Smiseth *et al.*, 2012), gamete production and energetic investment (Ratikainen *et al.*, 2018). Other determinants of reproductive success take place later during the reproductive event and are often mediated through the expression of parental care to the progeny (Trillmich, 2010; Smiseth *et al.*, 2012) or the quality of the reproductive environment (Trevail *et al.*, 2019). Owing to the complex interactions between environmental and parental characteristics occurring at various stages of the reproductive event, it is often

complicated to tease apart the relative contributions of these different factors to reproductive success (Ridley, 2007; Fox *et al.*, 2018).

In species that do not exhibit parental care, the influence that parents have on reproduction is often reduced to a few simple components of reproductive investment, namely the quality of the gamete, the energetic investment in the eggs (Ratikainen *et al.*, 2018) and the selection of optimal oviposition sites (Smiseth *et al.*, 2012). In situations where breeding site selection can be excluded as a parental effect (e.g. communal nesting), studies can focus simply on how metrics of parental quality (the quality of gametes and eggs; Ratikainen *et al.*, 2018) influence reproductive success.

Amphibians represent a taxonomic group particularly suitable for investigations of the relationships between parental investment in reproduction (egg quality) and indicators of offspring quality (hatchling phenotype). In amphibians lacking parental care, many species lay their eggs communally (i.e. in the same breeding pond),

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thereby largely eliminating the potential influence of oviposition site selection as a parental effect (Doody *et al.*, 2009; Refsnider & Janzen, 2010). In this study, we explore the relationships between clutch hatching success and offspring quality in an amphibian species that breeds communally and lacks post-oviposition parental care, the spined toad (*Bufo spinosus*).

Hatching success in toad species is known to be highly variable (Häkkinen *et al.*, 2001; Rowe *et al.*, 2001; Bishop *et al.*, 2010; Bókonyi *et al.*, 2018). Although environmental conditions can influence hatching success (Chivers *et al.*, 2001; Bishop *et al.*, 2010; Delia *et al.*, 2019), studies have shown that maternal transfer (i.e. glucocorticoid hormones: Saino *et al.*, 2005; contaminants: Unrine *et al.*, 2006, 2007; Bergeron *et al.*, 2010; Metts *et al.*, 2013) can also affect hatching success. This effect suggests that hatching success is under strong parental influence and might be used as a proxy of clutch quality.

In this study, we investigated the relationship between clutch quality (assessed as hatching success) and offspring quality in clutches originating from the same breeding site. Our laboratory setting (common garden in controlled conditions) allowed us to assess hatching success independently from natural factors affecting embryonic development (Chivers *et al.*, 2001; Gomez-Mestre *et al.*, 2013; Egea-Serrano *et al.*, 2014). Offspring quality was assessed by using two complementary sets of markers that are known to be linked to fitness. First, we used two traits of offspring, namely embryonic development duration and hatchling body size, because these traits can be used as markers of offspring quality in amphibians (Van Buskirk, 2002; Delia *et al.*, 2019). Second, we assessed the telomere length of hatchlings, because this marker has been suggested as a promising molecular tool to evaluate individual quality (Angelier *et al.*, 2019; Eastwood *et al.*, 2019; Bichet *et al.*, 2020).

Telomeres are non-coding, repetitive short sequences of DNA situated at the ends of eukaryotic chromosomes. Telomere attrition can lead to programmed cell death (Campisi & d'Adda di Fagagna, 2007). Telomere length can vary across environmental conditions (Blévin *et al.*, 2016; Angelier *et al.*, 2018; Chatelain *et al.*, 2020), ontogenetic stages (Burraco *et al.*, 2020) and species (Whittemore *et al.*, 2019). Thus, telomeres can provide insight into phenotypic and physiological responses to environmental conditions during early life stages (Heidinger *et al.*, 2012; Herborn *et al.*, 2014). As in most vertebrates, telomeres shorten through adult life in amphibians (Sánchez-Montes *et al.*, 2020), and the developmental period is thought to be a critical period for hatchling telomere length (Foote *et al.*, 2011; Eastwood *et al.*, 2019; Burraco *et al.*, 2020; Stier *et al.*, 2020). Importantly, hatchling telomere length is also linked to fitness parameters, such as

longevity, in multiple vertebrate species (Heidinger *et al.*, 2012; Boonekamp *et al.*, 2014; Wilbourn *et al.*, 2018), emphasizing the relevance of this measurement to assess hatchling quality. Accordingly, we predicted that offspring from lower-quality clutches (i.e. with greater embryonic mortality) would have reduced body sizes and telomere lengths.

## MATERIAL AND METHODS

### ETHICAL APPROVAL

All applicable institutional and/or national guidelines for the care and use of animals were followed. This work was approved by the French authorities (COMETHEA ethic committee and Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation) under permit #APAFIS#13477-2018032614077834 v7.

### EGG SAMPLING AND DEVELOPMENT

Spined toad (*B. spinosus*) egg strings ( $N = 10$ ) were collected by hand during a single day in January 2018 in a pond near the Centre d'Etudes Biologique de Chizé (46°8'48.624; 0°25'32.661W, France), brought back to the laboratory and kept in controlled conditions (see below). Eggs were collected immediately after laying (direct observations) in order to avoid development in the natural environment. A piece of each string containing 30 eggs was selected randomly and kept for our experiment, and the remaining eggs (i.e. 3000–5000) were released at their site of origin.

The 30 eggs from each clutch were placed in separate glass tanks containing 2 L of dechlorinated tap water (changed once a week) and monitored until hatching (total  $N = 300$  eggs). We determined the stage of development according to Gosner (1960). Hatching occurred at Gosner stage 22 after  $16.09 \pm 0.04$  days (see Results). All experiments took place in a thermally controlled room, with air and water temperature set at 17 °C and with a natural day–night photoperiod (9 h–15 h).

### MEASUREMENTS

We monitored egg segments and counted all the individuals that hatched (Gosner stage 22), which we considered a metric of hatching success. Undeveloped embryos were counted individually. Our observations showed that undeveloped embryos had stopped their development at early stages, usually within the first few hours, and that this number of undeveloped embryos remained steady during the remaining developmental period. Development duration was assessed as the time elapsed between the date of egg collection and the date

of hatching. We did not observe variation in embryonic development duration within clutch. We took photographs of all live hatchlings using a camera (Panasonic Lumix DC-TZ55) in order to assess the length of individuals ( $N = 285$ ). Morphological measurements were performed with the software IMAGEJ (Schneider *et al.*, 2012).

#### RELATIVE TELOMERE LENGTH

Immediately after hatching, we selected two hatchlings randomly from the embryos that hatched in each clutch, for telomere analysis ( $N = 20$  hatchlings from ten clutches). The remaining hatchlings were released in their pond of origin. Hatchlings were euthanized, and we used the whole individual for the assay. We deliberately kept small sample sizes for this subset of analyses for ethical reasons.

Telomere length was determined by quantitative PCR (qPCR; BioRad CFX 96; Bio-Rad, USA) using an established protocol (McLennan *et al.*, 2019), which was adapted for the spined toad. Whole hatchlings were digested with proteinase K, and DNA was extracted using the Nucleospin Tissue Kit (Macherey-Nagel), following the manufacturer's instructions. The DNA concentration and purity were assessed with a Nanodrop ND1000 spectrophotometer (Thermo Scientific). Universal telomere primers were used, and the control single-copy gene recombination activating gene 1 (*RAG1*) was selected and amplified using specific primers designed for the spined toad using the sequence alignment methodology: RAG1-F 5'-GGGTCCTCTGATAGCCGAAA-3' and RAG1-R 5'-CATCATAACCTGTACCCCGGA-3'. This single-copy gene has previously been used successfully in multiple species (birds: Sebastiano *et al.*, 2020; fish: Petitjean *et al.*, 2020; reptiles: McLennan *et al.*, 2019; Dupoué *et al.*, 2020), including amphibians (Canestrelli *et al.*, 2021). All qPCRs were performed on three plates for each gene (*RAG1* and telomere) using 7.5 ng of DNA per reaction. The telomere and single-copy gene primers were used at concentrations of 800 and 300 nM, respectively. To generate a six-point standard curve (from 50.0 to 1.5 ng) for controlling the amplification efficiency of the reactions, serial dilutions of DNA from a pooled sample of ten tadpoles were included on the plate (in triplicates). For both telomere and *RAG1* amplification, the melt curves displayed single sharp curves, validating amplification specificity of these primers. A reference toad sample was run in triplicate in all plates to account for inter-plate variation. All samples were run in duplicates and distributed randomly across the PCR plates. The cycle threshold (*Ct*) values determined for duplicates were averaged, and samples with a *Ct* SD > 0.2 between duplicates were repeated. Amplification efficiencies reached  $87.48 \pm 5.92$  (mean  $\pm$  SE, %) for *RAG1* and

$92.60 \pm 6.00$  for the telomere, and all  $R^2$  values were very high (> 0.99). The relative telomere length (expressed as the *T/S* ratio) was calculated as the number of telomere copies (*T*) relative to the single-copy gene (*S*; *RAG1*), according to Cawthon (2002). Inter-plate variations for telomere and *RAG1* were 2.07 and 1.89%, respectively. Inter-plate variation for the *T/S* ratio was 5.62% ( $N = 3$ ).

#### STATISTICAL ANALYSES

All statistical analyses were conducted with R.STUDIO v.1.2.5042 (R Core Team, 2020).

All data were tested for homogeneity of variance and normality using the Barlett's test, the Shapiro–Wilks test and diagnostics plots of residuals. All clutches were collected on the same date; hence, we did not include laying date as a predictor.

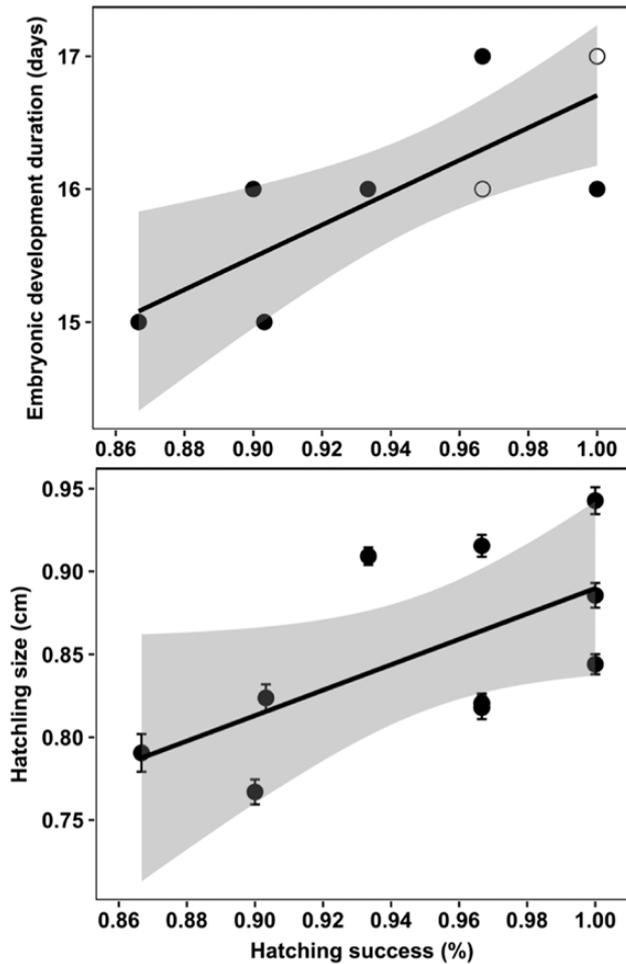
First, on the whole dataset, to test whether there were correlations between hatching success and development duration and hatchling size, we fitted two linear models (lm, 'stats' package). We used hatching success as the fixed effect in our models. Hatching success was expressed as the proportion of eggs that hatched from the total number of eggs. In these models, we did not include 'clutch identity' as a random effect because each clutch was represented by a single hatching success.

Second, on the subset of individuals for which we measured telomere length, we investigated whether there was a correlation between hatching success, embryonic development duration and hatchling size, and relative telomere length. When hatching success was used as a fixed effect in our models, we did not include 'clutch identity' as a random effect for the same reason as above, and we fitted linear models (lm, 'stats' package). When embryonic development duration or hatchling size was used as a fixed effect in our models, we added 'clutch identity' as a random effect and thus used a linear mixed model (LMER, 'lmerTest' package). Further information on model outputs is available in the Supporting Information (Table S1).

#### RESULTS

The Supporting Information (Table S2) provides the whole dataset.

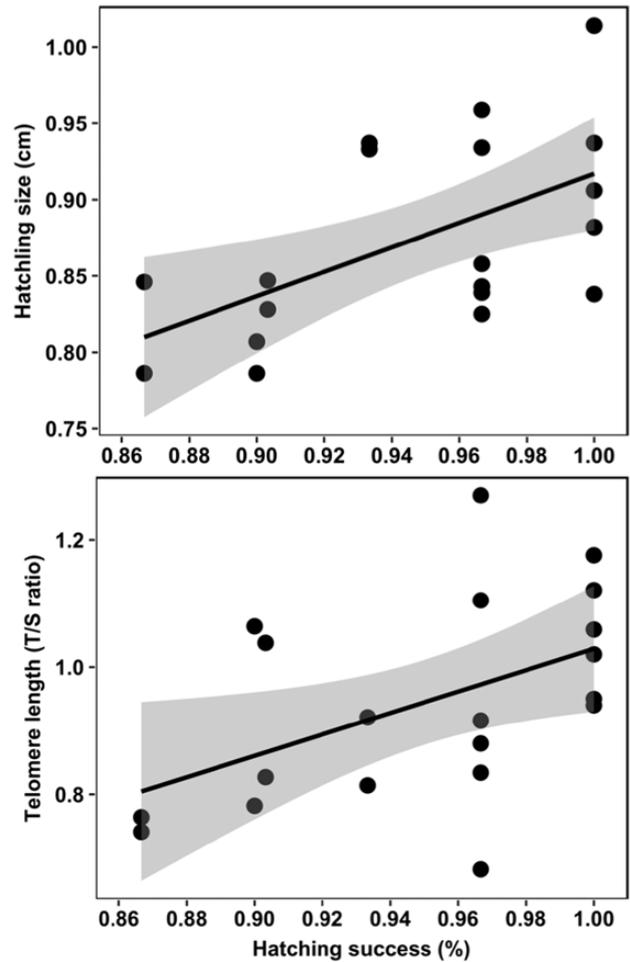
Hatching success was variable among clutches, ranging from 86 to 100% (coefficient of variation = 5%) and was positively related to embryonic development duration of the eggs that hatched ( $F_{1,283} = 432.9, P < 0.0001, R^2 = 0.60$ ; Fig. 1). Hatchling size was different among clutches ( $F_{9,275} = 61.08, P < 0.0001, R^2 = 0.67$ ) and positively related to hatching success ( $F_{1,283} = 95.96, P < 0.0001,$



**Figure 1.** Upper panel, development duration relative to hatching success. Filled circles represent all the individuals within one clutch. Given that two clutches displayed similar hatching success and development duration, we represented them with open circles; hence, the six filled circles represent six clutches and the two open circles represent four clutches. Lower panel, hatchling size (mean  $\pm$  SE) relative to hatching success. Grey shading indicates 95% confidence intervals.

$R^2 = 0.25$ ; Fig. 1). Hatchling size was positively correlated with development duration ( $F_{1,283} = 44.44$ ,  $P < 0.0001$ ).

Among individuals for which telomere length was assessed, telomere length was positively related to hatching success ( $F_{1,18} = 5.71$ ,  $P = 0.03$ ,  $R^2 = 0.20$ ; Fig. 2) but not related to embryonic development duration ( $F_{1,9} = 3.26$ ,  $P = 0.11$ ). Hatchling size was not correlated with telomere length ( $F_{1,9} = 2.25$ ,  $P = 0.164$ ). On this subset of individuals and like the whole dataset analysed above, hatchling size was different among clutches ( $F_{1,9} = 10.78$ ,  $P < 0.001$ ) and positively related to hatching success ( $F_{1,18} = 9.33$ ,  $P = 0.007$ ,  $R^2 = 0.30$ ; Fig. 2).



**Figure 2.** Hatchling size (upper panel) and relative telomere length (lower panel) relative to hatching success for the individuals for which telomere length was assessed. Grey shading indicates 95% confidence intervals.

## DISCUSSION

Overall, we found that hatching success was related to the duration of embryonic development and the size of hatchlings. Among the group of individuals for which telomere length was assessed, we found that telomere length was also linked to hatching success. These results suggest that clutch quality has a strong relationship with early life traits of larval amphibians. Using hatching success as a metric for clutch quality has the advantage that it is relatively straightforward to measure.

We found that clutches with lower hatching success were characterized by shorter embryonic development durations for the individuals that hatched. Such a relationship could be the result of an intrinsic characteristic of the clutch, whereby some clutches develop more rapidly than others (Magrath, 1990; Nager *et al.*, 2000). In turn, rapid embryonic

development might lead to an accumulation of cellular damage by overproduction of reactive oxygen species, resulting in lower hatching success (Lee *et al.*, 2013). Alternatively, such a relationship could be an adaptive response of embryos to environmental cues. Embryos can respond to chemical cues secreted by neighbouring dead embryos and accelerate their development in order to evade detrimental environmental conditions (Lee *et al.*, 2013; Delia *et al.*, 2019). Hatching plasticity is known to occur in amphibians and has been hypothesized as an adaptive response to predation risks (Sih & Moore, 1993; Warkentin, 1995; Lee *et al.*, 2013; Touchon *et al.*, 2013). No damage to the embryos occurred in our experimental system, and we suggest that dead and decaying embryos that might be used as a substrate for potential pathogens (e.g. bacteria and/or fungi) might secrete cues indicating a possible susceptibility to the spread of pathogens to neighbouring live embryos. Future studies are required to disentangle these hypotheses and to assess whether amphibian embryos can respond to predation risk and/or susceptibility to pathogens during embryonic development.

We also found that embryos that took less time to develop hatched as smaller hatchlings. This result was expected given the well-known negative relationship between the duration of embryonic development and size at hatching across ectothermic vertebrates (Gillooly & Dodson, 2000). For instance, ectotherms are known to develop faster and mature at smaller body size when exposed to warmer temperature (Zuo *et al.*, 2012). Thermal conditions were identical in our experimental study, and we suggest that the size at hatching is simply a consequence of embryonic development duration. Whether the duration of embryonic development is linked to maternal investment in eggs remains to be tested (Martin & Schwabl, 2008; Chen *et al.*, 2013).

In support of these results, we found that telomere length was linked to hatching success. Clutches with lower hatching success produced hatchlings with shorter telomeres. It is known that the rate of cell replication during embryonic development is negatively correlated with the size of telomeres at hatching (Jennings *et al.*, 2000; Foote *et al.*, 2011; Stier *et al.*, 2020). Given that shorter telomeres during early life are linked to lower fitness later in life (e.g. longevity: Heidinger *et al.*, 2012; Boonekamp *et al.*, 2014; Wilbourn *et al.*, 2018; Stier *et al.*, 2020), our results suggest that offspring from lower-quality clutches might experience overall relatively poorer performances later in life. Although we failed to detect the expected relationship between telomere length and body size (Scott *et al.*, 2006; Ringsby *et al.*, 2015), we believe that the relatively low number of hatchlings included in our telomere analysis might have obscured

such a pattern (but see Monteforte *et al.*, 2020), and future studies are required to test this hypothesis.

Whether smaller body sizes and shorter telomeres have long-term consequences for developing tadpoles remains to be assessed. Future studies are required to test whether shorter telomeres influence tadpole development until metamorphosis (Cabrera-Guzmán *et al.*, 2013; Burraco *et al.*, 2017; Semlitsch *et al.*, 2000) or, indeed, until adulthood (Sánchez-Montes *et al.*, 2020). Given the correlates of shorter telomere size on life-history traits (e.g. shorter life expectancy, lower quality and senescence: Heidinger *et al.*, 2012; Wilbourn *et al.*, 2018; Angelier *et al.*, 2019; Eastwood *et al.*, 2019; Bichet *et al.*, 2020), we believe that our study system might provide insights to assess the trade-offs between early life development and individual quality later in life. For instance, although fast development might allow embryos and tadpoles rapidly to evade the high levels of competition and predation occurring in breeding ponds (Burraco *et al.*, 2020), it might have consequences later in life on crucial attributes, such as individual quality and life expectancy (Burraco *et al.*, 2020).

Futures studies should investigate the effect of clutch size on hatching success and offspring quality. We did not record clutch size in our study, but given the well-known trade-off between clutch size and egg size (Gould *et al.*, 2020), we can hypothesize that clutch size has a direct effect on hatching success (Brown & Shine, 2009). In addition, multiple paternity is known to occur in explosive breeding amphibians (Laurila & Seppä, 1998; Liebgold *et al.*, 2006), and deciphering whether variation in hatching success is linked to a paternal genetic contribution is required. Finally, future studies should explore the implications that the positive relationship between hatching success and telomere length can have for ectothermic species, such as amphibians (e.g. interactions between clutch quality and suboptimal environments). Overall, deciphering the influence of parental traits (e.g. quality and fecundity) on the patterns we detected is a promising avenue of research.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1.** Linear model coefficient and estimates.

**Table S2.** Whole dataset.