Research Paper Environment

Aquaculture of First Larval Stages of the North African Green Water Frog

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ABSTRACT

Frog rearing is becoming a common activity in aquaculture since demand on frog legs is increasing. We evaluated the relationship between starting body size and multiple measures of larval growth in the North African green water frog Pelophylax saharicus; we analysed within-clutch variation, to remove co-variation between provisioning and genetic maternal effect. We reared tadpoles from multiple clutches in a common environment under two food treatments (high- and low-protein content), and measured the mortality, tadpole size during development and development rate. Tadpoles with a large starting size remained the largest ones through the entire larval development, and attained metamorphosis earlier. Food with high-protein content reduced mortality and increased the growth and development rate; the choice of food may be important in captive-breeding/headstarting programs. We did not detect effects of the interaction between provisioning and type of food on tadpole growth. Our study confirms the importance of egg provisioning in amphibians, showing that it can affect multiple traits, and that their effects can last through the entire larval development.

KEYWORDS North African green water frog; tadpole; metamorphosis; development rate.

Introduction

The efficient and reliable rearing of healthy larvae and metamorphs is important in the ex situ conservation breeding programs for anurans. This is particularly important in the production of large numbers of amphibians for release during rehabitation programmes. In rearing of aquatic species, egg provisioning is a major pathway of maternal investment (Kaplan, 1998). Females laying bigger eggs can confer important advantages to their offspring, including larger size (Kaplan, 1992), increased survival and stress tolerance (Rasanen et al., 2003; Kaplan & Phillips, 2006), earlier metamorphosis (Dziminski & Alford, 2005; Rasanen et al., 2005) and the development of phenotype polymorphism (Martin & Pfennig, 2010); some studies suggest that egg provisioning can ultimately affect the fitness of amphibians in nature (Dziminski et al., 2009). There is therefore a growing interest in the importance of egg provisioning on amphibian growth traits, as it can provide important insights to our understanding of amphibian life history and evolutionary biology (Kaplan, 1998). Nevertheless, until now, studies have focused on a limited array of species and growth traits, compared with other vertebrates such as birds.

However, egg provisioning can co-vary with the genotype of females, and with maternal effects that are different from egg provisioning (Pakkasmaa et al., 2003; Lesbarreres et al., 2007), for example, if egg size is correlated to the heterozygosity of mothers or alleles at specific genes (Krist & Remes, 2004; Brouwer et al., 2007). Analysing the importance of egg provisioning in amphibian requires approaches that remove co-variation with other maternal (either genetic or non-genetic) effects (Krist & Remes, 2004). As chromosomes segregate at random at meiosis, Krist & Remes (2004) proposed that the co-variation between egg provisioning and maternal genetic effect is expected to be zero among sibs; therefore, analysing withinclutch variation would allow removal of such a co-variation. Only a subset of studies on amphibians used protocols enabling the removal of the co-variation between egg provisioning and other maternal effects (e.g. Pakkasmaa et al., 2003; Rasanen et al., 2005; Dziminski et al., 2008). In some cases, the relationship between provisioning and amphibian growth was weaker when taking into account such a co-variation (Pakkasmaa et al., 2003).

Here, we assessed how food type and tadpole starting size (a surrogate of egg provisioning; Kaplan, 1985) affect multiple measures of larval growth (mortality, growth and development rate); we used the Krist & Remes (2004) approach in order to remove the covariation between egg provisioning and genetic maternal effect.

Methods

North African green water frog Pelophylax saharicus is a green frog belonging to P. ridibundus complex; adults are usually associated with lowland forests and breeding occurs in small wetlands (Barbieri & Mazzotti, 2006). Because of the small distribution range, habitat loss, fragmentation and limited genetic diversity, the North African green water frog is included in the Annexes II and IV of the 'Habitat directive' of the European Union (EC 43/1992), and is considered globally vulnerable by the IUCN (Andreone et al., 2008). In early spring, each female lays a single egg mass (c. 1300 eggs surrounded by gelatinous capsules); clutches are usually deposited onto submerged woods and tree roots within 50 cm of the water surface (Barbieri & Mazzotti, 2006).

Multiple paternities in a North African green water frog clutch have never been recorded; therefore, each clutch was

assumed to be a full-sib family. Some studies have described multiple paternity in some brown frogs (Rana dalmatina and R. temporaria), but the proportion of offspring attributable to polyandrous mating was small (≤ 5%) in both these species (Lodé & Lesbarréres, 2004; Vieites et al., 2004). Thus, it is unlikely that multiple paternities would affect our study significantly. The biology of North African green water frog makes it an ideal model species for the study of evolutionary ecology of threatened amphibians; studies can also be useful for conservation of populations. On 31 March 2005, we removed a small portion (about 50 eggs) from each of nine recently laid (within 24 h) clutches. Eggs were transferred into plastic containers and returned immediately to the laboratory.

All clutch samples were reared under standard laboratory conditions (constant 20 °C with natural light/dark cycles). On reaching Gosner's (1960) stage 25, we randomly chose eight healthy tadpoles per clutch (total: 72 tadpoles). At this stage, larvae have absorbed their external gills, are free swimming and start feeding. Up to stage 25, larvae have not been fed; therefore, assuming that environmental conditions were equivalent among tadpoles, body size at stage 25 (starting size) is mostly determined by the amount of yolk in the egg. Under constant environmental conditions, previous studies showed a strong correlation between egg size and starting size in frogs belonging to the genus Rana (Laugen, Laurila & Merila, 2003); starting size was therefore used as a proxy of egg size (e.g. Zeisset & Beebee, 2003; Rasanen et al., 2005). In the present study, we used starting size as a surrogate of egg size because in North African green water frog (as in other anurans), eggs are strictly clumped in masses; attempts to isolate the eggs individually would result in the risk of damaging embryos. Therefore, we used a method enabling us to individually measure larvae, and we assumed that differences in starting size indicate differences in egg size. Tadpoles at stage 25 were blotted, dried and weighed using a Sartorius balance (accuracy: 0.1 mg) to measure starting weight, and individually reared in plastic containers filled with 200mL of aged tap water. All containers were randomly sorted over the same bench of the laboratory. Water was changed weekly; tadpoles were fed ad libitum depending on the diet treatment and monitored daily to record the occurrence of death. For each clutch, half tadpoles were randomly allocated to each of the following diet treatments (i.e. 36 tadpoles per food treatment) (Alvarez & Nicieza, 2002): (1) low-protein diet: tadpoles were fed with commercial rabbit food (vegetable food: protein 17%, lipids 3%); (2) high-protein diet: tadpoles were fed with an integrated died, composed by a mix of 50% rabbit food and 50% commercial fish food (composed mostly by dry fish and molluscs: protein 43%, lipids 11%). At the beginning of the study, weight was similar in tadpoles assigned to the two diet treatments [low-protein diet (mean ± SD) 9.6 ± 2.9 mg; high-protein diet: 8.9 ± 2.3 mg; $F_{1,62}=1.42$, $P=0.24$ in a model controlling for differences among clutches]. To measure larval growth, tadpoles were weighed 33 and 44 days after reaching stage 25. Furthermore, we recorded mortality and age at metamorphosis [defined as the emergence of forelimbs; Gosner's (1960) stage 42; see also (Walsh, 2010)]. After metamorphosis, all froglets were released into their wetland of origin.

Data analysis Individuals originated from nine clutches; therefore, mixed models are required for within-clutch analysis. However, standard mixed models can have problems in analyzing multivariate or non-Gaussian-dependent variables (Bolker et al., 2009). Bayesian methods constitute a straightforward and flexible framework to the analysis of hierarchical data (Browne & Draper, 2006; Zhao et al., 2006; Bolker et al., 2009). We therefore used Markov chain Monte Carlo generalized linear mixed models (MCMCglmm) (Hadfield, 2010) to evaluate the effect of starting weight and diet on tadpole mortality and growth, considering clutch of origin as a random factor. For the analysis of mortality, we ran a univariate MCMCglmm over all the individuals, assuming a binomial distribution of the dependent variable. For the analysis of tadpole growth, we ran a multivariate model with three dependent variables (weight at 33 and 44 days, age at metamorphosis) and Gaussian distribution of variables. This analysis was run over all the individuals surviving until metamorphosis. For each MCMCglmm analysis, we performed 5000 runs of burn-in, followed by 250 000 runs with a thinning interval of 25. These parameters were analogous to the ones suggested by Hadfield (2010); we also inspected output plots, and found that MCMC always reached stability with these parameters. We used deviance information criterion (DIC) to compare the validity of different models. DIC is an information criterion particularly useful to assess Bayesian models; models explaining more deviance with a lower number of parameters have lower DIC and are considered to have a better cogency (Hadfield, 2010). We performed analyses in R 2.9 (http://www.r-project.org) with the MCMCglmm package (Hadfield, 2010).

Results

The starting weight was significantly different among clutches ($F_{8,63}=3.46$, $P=0.002$). Nevertheless, clutch identity explained only 31% of variation in the starting weight, indicating strong variability among siblings. Mortality from stage 25 to metamorphosis was significantly lower for tadpoles on the high-protein diet; mortality was 42% for tadpoles on the low-protein diet, and 17% for tadpoles on the high-protein diet. There was no relationship between mortality and starting weight (Table 1a). Preliminary models showed that the interaction between starting weight and diet did not have a significant effect on within-clutch variation of tadpole growth (dependent: weight 33 days, $P=0.62$; dependent: weight 44 days, $P=0.91$; dependent: age at metamorphosis, $P=0.56$) (supporting information Fig. S1). Furthermore, preliminary univariate models showed that the interaction was highly collinear to the other independent variables (variance inflation factor410), and therefore, may bias the analysis estimates. For these reasons, we built multivariate MCMCglmm without the interaction between independent variables.

Seventy-two tadpoles from nine different clutches were reared under two diet treatments (low- and high-protein content).

Table 1 Results of Markov chain Monte Carlo (MCMC) generalized linear mixed models, relating tadpole mortality and tadpole growth to diet and starting weight

Dependent	Independent	P
(a) Univariate model, analysing mortality		
	Diet	0.025
	Staring weight	0.918
(b) Multivariate model analysing larval growth		
Weight 33 days	Diet	0.111
	Starting weight	<0.0001
Weight 44 days	Diet	<0.0001
	Starting weight	<0.0001
Age metamorphosis	Diet	0.0002
	Starting weight	0.0002

Both diet and starting weight had significant effects on tadpole growth (MCMCglmm; Table 1b; Fig. 1). Tadpoles with a higher starting weight and on a high-protein diet metamorphosed significantly earlier

(Table 1b; Fig. 1). Furthermore, after taking into account differences in starting size, growth was different among clutches, as the DIC of a model not including clutch of origin as a random factor (DIC=1603) was higher than the DIC of the complete model (DIC=1565).

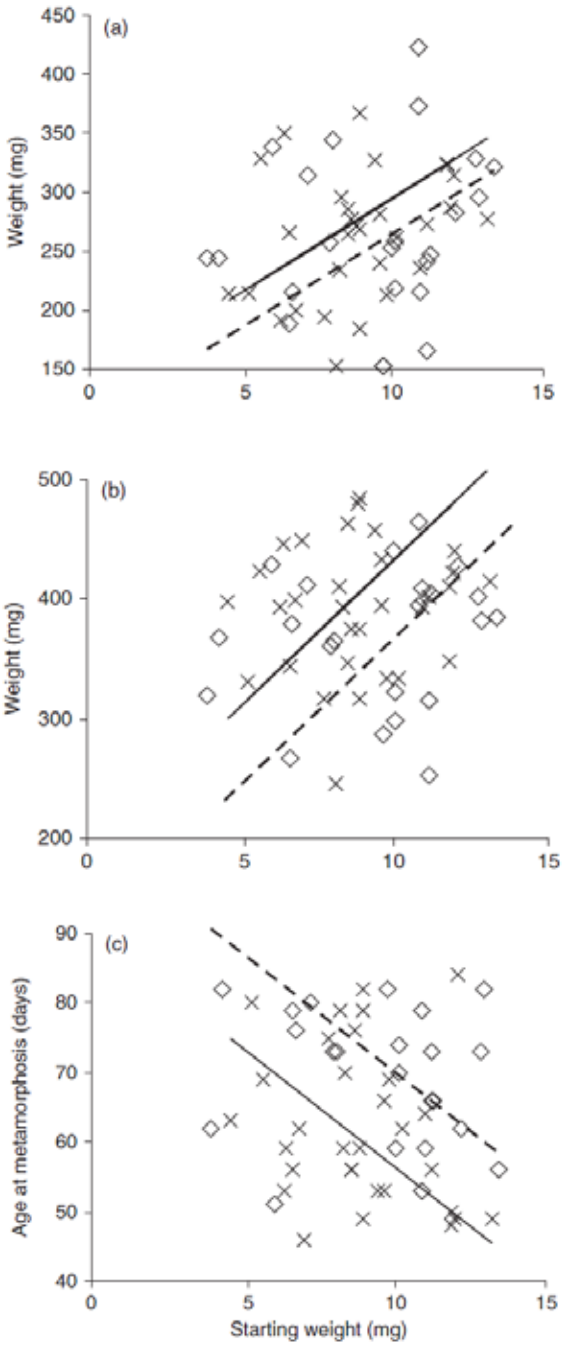

Figure 1 Relationship between starting weight, diet and three measures of tadpole growth: (a) weight at 33 days; (b) weight at 44 days; (c) age at metamorphosis. Diamonds, dashed lines: lowprotein diet; crosses, continuous lines: high-protein diet. Lines represent values fitted by the Markov chain Monte Carlo generalized linear mixed model (Table 1).

Discussion

Our study shows that starting body size, a proxy of egg provisioning, can have profound, long-lasting effects on some measures of tadpole growth: tadpoles that were larger at hatch remained the largest ones through the entire larval development, and attained metamorphosis earlier. Larval size and development rate can have important consequences for fitness in frogs. For example, larger tadpoles can have a lower predation rate, can have better foraging and locomotory ability and can store more resources, which can be important even after metamorphosis (Travis, Keen & Juilianna, 1985; Newman, 1988; Kaplan & Phillips, 2006; Martin & Pfennig, 2010). Furthermore, a fast larval development can help to avoid the risk of pond desiccation and increases the probability of escaping aquatic predators (Travis et al., 1985; Banks & Beebee, 1988; Newman, 1988); previous studies showed that development rate is probably a trait under selection in this population of North African green water frog. Therefore, the positive relationship between starting size and larval growth is in agreement with other studies showing that egg provisioning (and therefore maternal investment) may have profound consequences on offspring fitness in amphibians (Kaplan & Phillips, 2006; Dziminski et al., 2009). Egg size (which in turn depends on yolk content) is considered to be the major maternal investment in anuran amphibians (Kaplan, 1998; Pakkasmaa et al., 2003); therefore, most studies assumed that a relationship between egg size/starting size and growth indicates an effect of egg size. Our analysis supports the hypothesis that egg size is a major cause of variation in tadpole growth; nevertheless, the amount of yolk can be only a part of egg provisioning. Egg size can be positively related to other maternal effects such as hormones or antibodies in the egg, for example, because larger eggs can have a larger amount of these compounds (Krist & Remes, 2004). However, the difficulty of controlling the amount of other important compounds in amphibian eggs poses a challenge to the study of maternal effect. To our knowledge, only a few studies did not focus on egg size only, and considered the importance of gelatinous capsules for embryo survival (Rasanen et al., 2003; Marquis et al., 2006), while studies considering other compounds remain scarce (but see Johnson, Boucaut & Desimone, 1992 and references therein), particularly within a life history or evolutionary biology framework: more work is needed to clarify their role.

Type of food had important consequences on tadpole growth, affecting mortality, growth and development (Table 1; Fig. 1). Rana tadpoles are traditionally described to feed on detritus or periphyton, but are opportunistic and can occasionally scavenge on carcasses (e.g. Loman, 2001; Lanza et al., 2007); actually, some species like R. latastei tadpoles have sometimes been observed feeding on dead tadpoles and earthworms (Pozzi, 1980). The better growth of tadpoles fed with the integrated diet (Alvarez & Nicieza, 2002) confirms the importance of a diet integrating vegetable and animal matter. Experimental studies on Rana tadpoles often fed them with rabbit food or other vegetable foods only (e.g. Rasanen et al., 2005; Capellan & Nicieza, 2007). The results of our analysis suggest that an integrated diet might be more appropriate, and reduce the mortality rate. The North African green water frog and other frogs are threatened and are the focus of captive breeding and headstarting activities aimed at reintroduction or supplementation; an integrated diet may improve the effectiveness of such programmes.

Clutches were collected in a natural environment, and therefore, not certainly been laid exactly at the same time. Nevertheless, all clutches were laid the night before sampling, and therefore, differences in age were minor; actually, all clutches hatched within 24 h, confirming that all the embryos were of similar age. Therefore, differences in the starting size or in the measures of tadpole growth (tadpole size, age at metamorphosis) could not be due to differences in age among clutches. Indeed, even if small (i.e. o24 h) age differences among clutches may exist, they clearly cannot explain the strong differences in age at metamorphosis: the tadpoles with the largest starting size metamorphosed about 15 days before the smallest ones (Fig. 1c).

Previous analyses focusing on mortality suggested a lack of relationship between tadpole survival and egg provisioning in (Unpublished data). Our study is in agreement with these early findings (Table 1a), but observed significant relationships between provisioning and other measures of growth (Table 1b). In amphibians, multiple features (e.g. growth and development rate) can have long-lasting effects even after metamorphosis; therefore, it is important to take into account multi-

ple parameters when measuring larval growth. The Bayesian framework allows a straightforward integration of multiple response variables within multivariate hierarchical models, thereby increasing the power of analyses.

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