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Is Reproductive Effort Environmentally or Energetically Controlled? The Case of the Danube Crested Newt (*Triturus dobrogicus*)

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Reproductive strategies have evolved from a series of trade-offs between cost and timing of reproduction. We tested whether the reproductive effort of female Danube crested newts, *Triturus dobrogicus*, was environmentally or energetically constrained. We collected females migrating towards the water and kept them separately, with males. Deposited eggs were collected daily during the experiment. More eggs were deposited by older females and by females starting to reproduce earlier. Batches of eggs from females depositing more eggs had a lower hatching success, suggesting decreased viability. Oviposition lasted on average 22.7 days. Female newts showed no loss of weight during this period. At the end of the egg deposition period we injected a subset of females with hormones that triggered the deposition of additional eggs. This suggests that egg deposition in Danube crested newts is environmentally constrained, as females stopped oviposition despite having mature eggs in their ovaries.

Key words: oviposition, egg-wrapping, skeletochronology, reproductive trade-off, egg survival

INTRODUCTION

A topic of major interest in evolutionary studies is the trade-off between present and future reproduction. The reproductive effort, i.e., the proportion of the total energy budget that is devoted to reproductive processes (Angelini and Ghiara, 1984), at a given age within a species will be selected to maximize the reproductive success at that age. Each species has developed reproductive strategies that reflect the ways in which an individual or a population partitions its reproductive effort throughout their life cycle. Reproductive success depends on both the reproductive output (i.e., number of eggs) and the rate of egg and larval survival. Most animals develop some forms of parental care in order to improve the survival of eggs or offspring. For example, female newts protect their eggs by individually wrapping them in vegetation, a time-consuming process that limits the number of eggs that can be deposited daily. Egg wrapping protects eggs not only from predators and from mechanical damage but also from UV radiation, especially in shallow water (Marco et al., 2001). The threats the eggs are facing are multiple: inter and intra-specific oophagy (Avery, 1968), invertebrate and fish predators (Orizaola and Brana, 2003). The choice of female newts when and where to deposit their eggs is influenced by a variety of environmental factors, like temperature and the presence of egg-wrapping vegetation (Dvorak and Gvozdik, 2009).

Laying more eggs during the breeding season involves a prolonged oviposition period. Since female newts can store sperm from multiple matings (Sever and Brizzi, 1998), they have freedom of choice in regard to where and when to deposit eggs. Egg dispersal in space and time limits the environmental and predatory risks for the spawn, while egg wrapping behavior increases the survival of individual eggs (Miaud, 1994). Spreading oviposition over time periods lasting weeks raises two additional problems: larvae from eggs deposited later during the season face both a higher risk of desiccation before metamorphosis, and an increased risk of predation from larger siblings (Arntzen and Teunis, 1993). For example, larvae of *T. cristatus* were found to be more nectonic than adults, which was interpreted to be a mechanism limiting cannibalism (Dolmen, 1983). Early-hatching salamander larvae may not only prey upon the late-hatching ones but also reduce shared prey resources (Segev and Blaustein, 2007). Female newts face a trade-off between depositing more eggs and decreasing survival rates for later eggs. We hypothesize that, faced with the energetic costs of additional eggs being deposited and the decreasing survival rate, females must decide to halt oviposition at a certain moment when the costs exceed the benefits. Egg deposition is therefore limited to a certain time period.

The crested newts (*Triturus cristatus* superspecies) are parapatrically distributed across large parts of Europe and have radiated in a short temporal interval (Arntzen et al., 2007). The differentiation of *T. dobrogicus* from its sister species is thought to be related to multiple vicariant events dating back to the Miocene (Arntzen et al., 2007). Crested

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newts suffer from a chromosomal mutation, called the developmental arrest syndrome, that causes the mortality of 50% of embryos (Horner and Macgregor, 1985). Their large eggs also suffer a high predation rate, resulting in a hatching success as low as 3% (Miaud, 1994).

The Danube crested Newt (*Triturus dobrogicus*) is narrowly distributed in central and eastern Europe, in the lowlands of the Tisza and Danube River systems. Its Red List status is considered "near threatened" (Temple and Cox, 2009), while according to the European Union Habitats Directive (92/43/EEC), it is included in Annex 2, as an animal species of community interest whose conservation requires the designation of special areas of conservation. The Danube crested newt is in significant decline due to widespread habitat loss through much its range (Arntzen et al., 1997; Wilbur and Rudolf, 2006), thus the understanding of the reproductive trade-offs is vital for sound conservation measures.

A population of Danube crested newts from the lower Danube floodplain had a relative low lifespan of maximum five years, with sexual maturity being achieved by males at two years and females at three years (Cogălniceanu and Miaud, 2003), limiting the number of annual reproductive events. Danube crested newts are thus faced with a trade off between the need to invest more energy in reproduction within a single year, and the risks associated with a highly unpredictable aquatic environment (Cogălniceanu, 2012) that can cause massive larval mortality.

In the present study we tested if the number of eggs deposited by Danube crested newt females is environmentally or energetically constrained. We hypothesized that if the number of eggs deposited is limited by environmental constrains, at the end of the oviposition period the females would still have mature eggs in their ovaries. If only energy resources limited egg deposition, we hypothesize that at the end of the oviposition period there will be no mature eggs left in the ovaries.

MATERIALS AND METHODS

Site description

The animals were collected on a natural levee (Grindul Lupilor) separating two lakes, Zmeica and Sinoe (44°37′N, 28°48′E), located in the southern part of the Danube Delta Biosphere Reserve (eastern Romania). It is a flat sand dune of Holocene origin with a maximum height of 1.5 m a.s.l. The levee is covered by steppe vegetation, partly on salinized land, bordered by permanent and temporary wetlands covered with reed (*Phragmites australis*) and rushes (*Typha* sp.) that grow in shallow water.

Experimental design

We collected 15 females and 12 males on the 21st of March 2010, at the beginning of their spring migration. Each individual was measured at the beginning and the end of the experiment. Body weight was measured on an electronic balance (Kern, ABJ model) with a precision of 0.01 g. The snout-vent length (SVL) was measured using a digital caliper with a precision of 0.01 mm. The female newts were kept in individual plastic containers of 5-liter capacity, mixed at a 1:1 ratio of aged tap water and osmosis water that was changed every other day. The osmosis water was prepared with a reverse osmosis unit (GBL Osmose 120) to mitigate total water hardness of tap water. The newts were fed every other day with fresh earthworms *ad libidum*. After developing sexual secondary characters, the males were randomly added to each female container every other day. To provide support for egg deposition we added transparent plastic strips in each container (Cogălniceanu, 1999). The containers were checked daily and all the fresh eggs deposited were removed, counted and kept in plastic containers in batches of 20–30 eggs at room temperature until hatching. Dead eggs and embryos were removed. The first part of the experiment was considered complete when the egg deposition rate dropped and remained low for a week. Two females failed to oviposit and were discarded from the experiment.

At the end of the oviposition period, the females were again weighed. Seven females were randomly chosen and injected subcutaneously with 50 units of luteinizing hormone (LH) dissolved in 1 ml saline solution and the following day with 50 units of follicular stimulating hormone (FSH) (swine FSH-LH, pharmaceutical grade) also dissolved in 1 ml saline solution. The six other remaining females were kept as a control group and injected only with saline solutions following the same protocol. The hormonal treatment triggered a massive and rapid egg deposition. Oviposition was again observed for five days after the last hormone injections in the same conditions as the previous experiment. The females from the control group did not deposit any eggs.

Table 1. Parameters describing the experiments of egg deposition by female *Triturus dobrogicus*.

Parameter	Mean ± SD	Minimum	Maximum
Experiment 1, *n* = 13			
SVL (mm)	70.7 ± 3.2	65.1	76.4
Body mass (g)	6.6 ± 1.2	5.3	9.2
Age (years)	4.07 ± 0.9	3	6
Total number of eggs deposited	306.3 ± 119.8	160	489
Length of oviposition (days)	22.7 ± 5.1	13	31
Maximum number of eggs/day	36.5 ± 9.2	26	58
Day of peak deposition as a percentage of the oviposition period	36.7 ± 25.0	8	88.9
Average rate of deposition (number eggs/deposition period)	13.5 ± 4.3	8.1	23
Number of hatching larvae	118.8 ± 41.4	72	199
Percentage of hatching success	39.9 ± 6.0	29.2	51.9
Average number of eggs deposited after hormone treatment (4 days)	68.7 ± 41.5	7	110
Total number of eggs deposited	368 ± 119.6	230	554
Experiment 2, *n* = 7			
Maximum number of eggs deposited daily after hormone treatment (day 3)	53.1 ± 32.9	7	104
Percentage of oviposition after injection compared with total number of eggs deposited	22.2 ± 15.5	1.4	38

Age assessment

At the end of the experiment we toe clipped all the females to estimate age by skeletochronology. The skeletochronological method used was according to Castanet and Smirina (1990), slightly modified by Cogalniceanu and Miaud (2003). We decalcified the second phalange for 1 hr with 5% HNO_3, followed by inclusion in TissueTek, freezing and sectioning with a Tehsys CR 3000 Cryotome at 14 μm. The cross sections were stained with Ehrlich's Haematoxylin for 3 hrs. Age was estimated from the Lines of Arrested Growth (LAGs) using an Olympus CX 31 microscope and Quick Photo Micro 2.3 software. Age could not be assessed in one of the females.

RESULTS

The parameters characterizing egg deposition of Danube crested newt females are presented in Table 1. Oviposition started six days after capture and lasted on average 22.7 days. The rate of egg deposition increased rapidly, with a maximum in the sixth day, with 30% of the eggs deposited. Half the number of eggs was deposited after nine days, 75% after 14 days, and 90% after 20 days. The hormonal treatment triggered a second short burst of oviposition in all females that lasted on average four days (Fig. 1).

The body weight of females did not differ significantly between the start and end of the oviposition period (ANOVA, $F_{1,24} = 0.67$, $P = 0.41$). The body weight at the end of the egg deposition period, expressed as a percentage of the initial body weight, was 102.8 ± 15.8% (range 79.9–129%). The total number of eggs deposited at the end of the first experiment and the body weight changes were not significantly correlated ($n = 13$, $r = 0.36$, $P = 0.21$).

The animals used in the study were young, with five

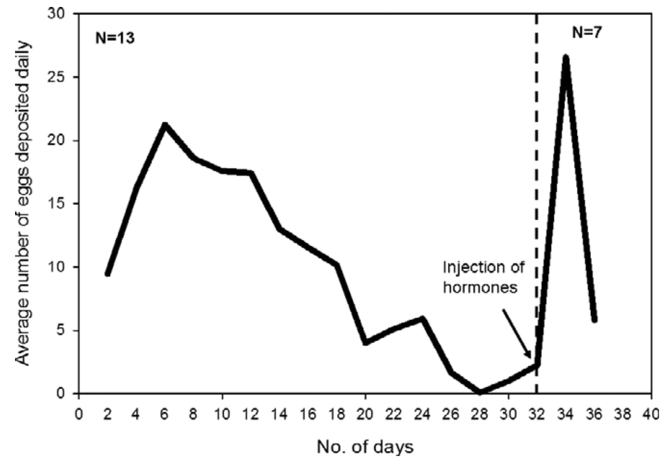

Fig. 1. The average number of eggs deposited daily by Danube crested newt females. The first part of the experiment lasted 31 days and involved 13 females, and the second part four days and involved seven females that were injected with hormones, while six females were kept as control. See text for details.

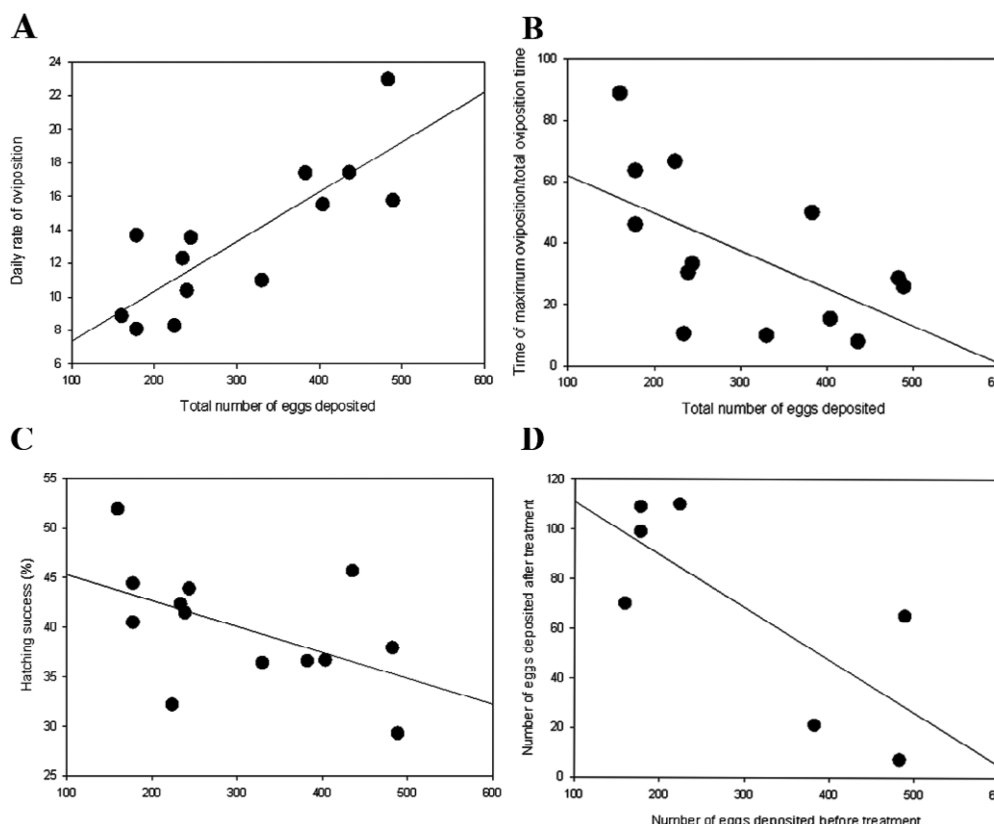

Fig. 2. **(A)** The correlation between the total number of eggs deposited by a female and the time of maximum deposition (%). **(B)** The negative relationship between the total number of eggs deposited and hatching success. **(C)** The positive relationship between the total number of eggs deposited and the daily rate of oviposition ($r = 0.820$, $P < 0.001$, $n = 13$). **(D)** The negative relationship between the total number of eggs deposited before and after the treatment ($r = 0.757$, $P < 0.05$, $n = 7$).

females aged 3 (age of sexual maturity), four aged 4, and the remaining five females with ages of 5–6 years. Body mass slightly increased with age (r = 0.49, P = 0.087, n = 13). The number of eggs deposited was not significantly correlated with body mass and SVL (r = 0.11 and r = 0.19, P > 0.05, n = 13), but the age of females was (r = 0.54, P < 0.05, n = 13). There was a significant positive correlation between the total number of eggs deposited and the daily rate of oviposition (r = 0.82, P < 0.001, n = 13) (Fig. 2A).

The dynamics of oviposition was a good predictor of the reproductive output; females that reached the peak of oviposition at the beginning of the period, deposited more eggs (Fig. 2B). We observed a pattern between the total number of eggs and hatching success, albeit non-significant. Thus, batches of eggs from females depositing more eggs tend to have a lower hatching success (Fig. 2C).

The hormonal treatment induced the deposition of an additional batch of eggs representing 22.2% of the total number of eggs deposited. The maximum daily number of eggs deposited was higher after treatment, with some eggs not wrapped but being deposited in small clusters (Table 1).

DISCUSSION

Our study has shown that egg deposition in Danube crested newts is mostly environmentally constrained. Egg deposition rate was limited since females provided protection for the eggs by individually wrapping them in vegetation. Since prolonged egg deposition involves increasing risks for the reproductive success, selective pressures will tend to limit the period of oviposition. Our results are similar to those of Cogălniceanu (1999), who found that smooth newt females (*Lissotriton vulgaris*) originating from similar unpredictable floodplain habitats did not deposit all their eggs. Thus the end of oviposition was not a consequence of the depletion of mature oocytes, but was triggered by environmental factors.

Studies of reproductive biology have traditionally focused on estimating size and age at sexual maturity and fecundity, whereas there is little focus on assessing reproductive traits at the individual level and how reproductive success is influenced (Cam et al., 2002). Age and size at maturity determine both the speed with which individuals can start to reproduce and also how much they can reproduce, since fecundity is frequently associated with body size (Roff, 1992). In the explosive breeding common frog (*Rana temporaria*), females experience significant weight loss during reproduction caused by a seasonally elevated metabolism in combination with a lack of feeding (Ryser, 1989). The spread of the oviposition effort over a period of weeks during which the females actively forage and feed diminish the costs of reproduction in newts. On average, female Danube crested newts ended the reproduction with a slight gain in body weight despite the costs of reproduction. This is contradictory to the usually high energetic costs of reproduction. Thus, smooth newt females' body weight decreased on average 24%, despite the intensive feeding during oviposition (Hartel and Cogălniceanu, 2005). Similar costs of reproduction were reported also in Anura: female body weight loss being between 33–37% in *Rana temporaria* (Ryser, 1989), depending on year, 27% in *Rhinella arenarum* (Bionda et al., 2011; Castellano et al., 2004), 23% in *Bufo viridis* (Castellano et al., 2004) or 27% in *Pelobates syriacus* (Cogălniceanu et al., submitted).

The different crested newt species differ in their ecological demands, and it was suggested that the changes in phenotype were doubled by an ecological switch (Cvijanovic et al., 2009). The Danube crested newt appears as an outlier species having (i) the smallest egg size, (ii) the smallest larval size at hatching, (iii) the longest larval period resulting in the largest metamorphosed juveniles and (iv) the most pronounced body elongation doubled by a reduction in body size (Furtula et al., 2009; Vukov et al., 2011). Since they inhabit wetlands usually associated with the Danube River and with its major tributaries, they experience high environmental uncertainty due to rapid changes in water level. Many studies have shown that environmental uncertainty can strongly influence the evolution of life histories, by developing specific adaptive strategies (Wilbur and Rudolf, 2006).

Our study has found a much higher reproductive output (306 eggs on average, with a range of 160–489) than the study of Furtula et al. (2008), that reported for Danube crested newts from two populations a total number of eggs of 23.5 and 147 respectively. This is most probably due to the fact that the females were gathered after the oviposition started. The oviposition period is nevertheless rather large ranging between 17–41 days and 4–19 days for females from the two populations. As in many ectotherms, larger crested newt females tend to produce larger eggs (Furtula et al., 2008), indicating that older and larger females can not only produce more offspring, but also invest more energy in each one. Our study has confirmed that older females produce more eggs.

Our study suggests that reproductive effort in Danube crested newts is mostly environmentally constrained, as females stopped oviposition despite having mature eggs in their ovaries. Nevertheless, the lower hatching success of eggs from females that deposited more eggs indicated a possible physiological limitation to egg deposition due to an increase of the fatal chromosomal mutation or a decrease in egg viability.

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