

The influence of a water current on the larval deposition pattern of females of a diverging fire salamander population (*Salamandra salamandra*)

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Manuscript received: 5 December 2013

Accepted: 24 June 2014 by STEFAN LÖTTERS

Abstract. Fire salamanders are amphibians that exhibit a highly specific reproductive mode termed ovo-viviparity. The eggs develop inside their mothers, and the females give birth to fully developed larvae. The larvae in our study area cluster in two distinct genetic groups that can be linked directly to the habitat (stream or pond) in which the larvae were deposited. Apart from genetic differences, larvae living in the two different habitat types differ morphologically, indicating that female fire salamanders exhibit some type of ecological adaptation to the different habitats. In this study, we investigated whether pregnant fire salamander females of the two habitat-specific genotypes (stream and pond) specifically prefer to deposit their larvae in water bodies with a continuous current (i.e., emulated stream habitats) or those without a current (i.e., emulated pond habitats). We assumed that the presence of a water current might be used by the females as a cue to deposit their larvae in the matching aquatic habitat (flowing/stagnant) according to their own habitat-specific genotype. However, the female fire salamanders of the two habitat-specific genotypes did not show predilections for depositing their larvae in the water body that would match their genotype cluster (stream/pond). Furthermore, the larval genotype did not necessarily match the water type in which the larvae were deposited. Overall, this study aimed to test whether fire salamander females of two different habitat-linked genotypes use the presence/absence of a water current as a criterion for choosing an aquatic habitat for larval deposition. Our data do not support this hypothesis, leading to the assumption that fire salamander females use other environmental cues to select water bodies for larval deposition.

Key words. Amphibia, Caudata, reproduction, habitat choice, speciation, population, sympatry, isolation barriers, mechanism.

Introduction

Fire salamanders are amphibians that exhibit a highly specific reproductive mode termed ovo-viviparity. The eggs develop inside their mothers, and females give birth to fully developed larvae (GREVEN 1998, THIESMEIER 2004, KRAUSE et al. 2013). The larvae produced by one mother have not necessary all been sired by a single father (STEINFARTZ et al. 2006, CASPERS et al. 2014) and larval deposition can be a prolonged process and be spread over different locations (THIESMEIER 2004). After birth, the larvae remain in their aquatic habitat for 2–3 months (KRAUSE et al. 2011a, THIESMEIER 2004) before they metamorphose and become fully terrestrial. Female fire salamanders usually deposit their larvae in small streams (THIESMEIER 2004). In the absence of adequate streams, however, larvae can also be found in ponds (e.g., WEITERE et al. 2004, THIESMEIER 2004). At our field study site, the Kottenforst close to the city of Bonn, Germany (50°41'09 N, 7°07'03 E), larvae can be found in both types of water body, in streams as well as

in ponds (STEINFARTZ et al. 2007). Larvae occurring in the study area cluster in two distinct genetic groups, which can be linked directly to the habitat (stream or pond) in which they have been deposited (STEINFARTZ et al. 2007). In addition to these genetic differences, the larvae of the two different habitat types also differ morphologically (REINHARDT et al. 2013), indicating that the resident female fire salamanders exhibit some type of ecological adaptation to the different habitats. However, such ecological differentiation would imply that larvae will do best if they are deposited in the water body specific to their adaptive characteristics, i.e., pond-type larvae should be deposited in ponds and stream-type larvae in streams. It follows from this hypothesis that we would expect females not to make use of habitat types randomly, but rather exhibit a specific predilection in accordance with the female's genotype (linked to stream or pond habitat). However, the hypothesis that females have a preference for a specific larval deposition habitat has not been previously tested. Streams and ponds differ in several respects, e.g., the risk of desiccation, oxy-

gen level, food resources, and water current (REINHARDT et al. 2013). All of these factors might play important roles in a female's choice of an appropriate deposition habitat. Most likely, the water current is the most obvious impact factor, as streams have a permanent current whereas ponds do not have a current at all.

In this study, we used a common-garden experimental design (KRAUSE et al. 2011b) to identify whether pregnant fire salamander females of both habitat-specific genotypes (stream and pond) have a specific predilection for depositing their larvae in water bodies with a continuous current (i.e., emulated stream habitats) or those without a permanent current (i.e., emulated pond habitats). We hypothesised that the females would deposit their larvae in water bodies that matched their own habitat-specific genotype, i.e., that females with the stream genotype should show a relative preference for emulated stream conditions for larval deposition, whereas pond females prefer emulated pond conditions for larval deposition.

Methods

We conducted our experiments with pregnant female fire salamanders that were captured in the Kottenforst near Bonn, Germany (50°41'09 N, 7°07'03 E) between 2009 and 2012. The collected females were transferred to the Department of Animal Behaviour of the Bielefeld University, Germany. Females were maintained during the course of the experiments with permission from the "Untere Landschaftsbehörde der Stadt Bonn". Immediately after having been captured, the females were weighed and their snout-vent lengths measured (KRAUSE et al. 2013). The females were released after our experiments had been concluded at the location where they had originally been captured.

During the experiment, the females were kept individually in experimental terraria (150 × 50 cm; Fig. 1). The cen-

tral part (150 × 30 cm) of each terrarium was filled with soil (7.5 cm deep) and outfitted with stones, bark and an earthenware pipe that was meant to serve as a shelter. Both lateral compartments were filled with water (each 150 cm long × 10 cm wide × 7.5 cm deep; Fig. 1). Green gardening hoses were connected to either end of both compartments. In one aquatic compartment, the hoses were not connected to a water pump. The water in this compartment was not moving ("stagnant"), i.e., it simulated the pond habitat. In the other aquatic compartment, two water pumps (Eheim classic 250, #2213010; pump power [50 Hz] of approximately 440 l per hour) were connected in series to create a constant water flow, simulating a stream ("flowing") situation. The quality of the water filling in both lateral compartments was kept stable with respect to ionisation, pH, and hardness (monitored with a conductivity meter, Bischof; "3-fach Tauchtest", Dennerle, Vinningen). The water in both compartments was replenished/exchanged at regular intervals. However, even though we checked for certain water parameters, it is highly likely that differences in water flow were accompanied by differences in oxygen content. In both aquatic compartments, no food was made available to adults and potential larvae. The terraria were all set up in one room without daylight and exposed to a 12/12 h regime of artificial illumination. The fire salamander females were fed once a week with crickets and earthworms. Every day, all aquatic compartments were checked for newly deposited larvae. If larvae had been deposited, they were captured and removed. The compartment (stagnant/flowing) in which a larva was deposited was recorded. A small tissue sample for DNA extraction and subsequent genotyping was taken from the tail fin and immediately stored in 70% ethanol. The proportion of larvae that each female deposited in the flowing-water compartment was tested against chance expectation (50%) to test whether the females had a predilection for a specific water current situation (flowing/stagnant). Approximately eight

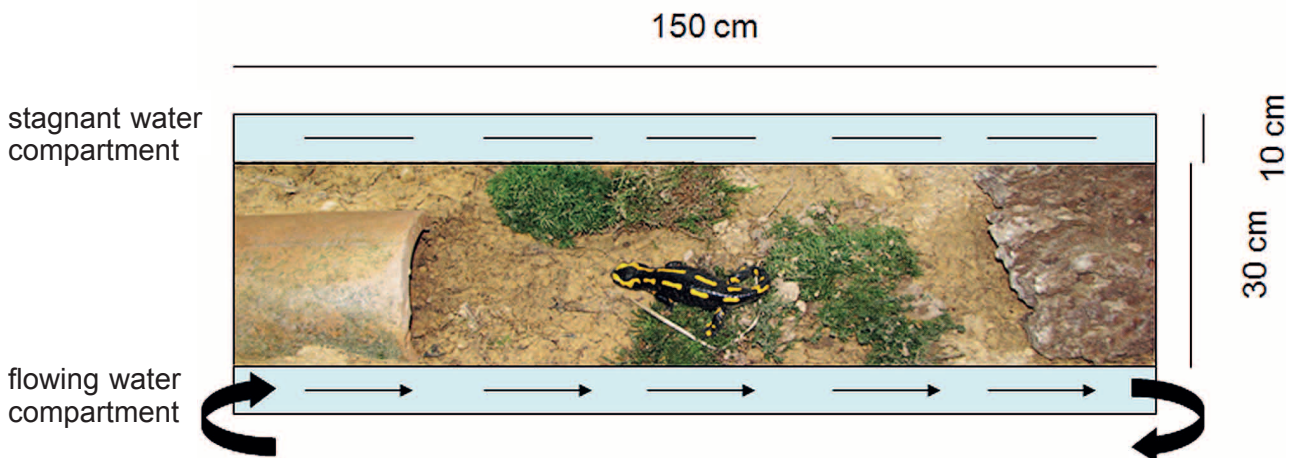


Figure 1. Schematic drawing of the experimental setup, i.e., the terraria in which individual females were kept. The central section was filled with soil and outfitted with stones, bark, and an earthenware pipe meant to serve as a shelter. Both lateral compartments were filled with water, but only one was animated by means of a water pump to create a constant current (indicated with arrows), emulating a stream situation; the opposite compartment was not animated and emulated a pond situation.

weeks after the last larvae had been deposited, the females were removed from the experimental setup. Immediately afterwards, we collected a small tissue sample from the toe of each female for subsequent DNA extraction and genotyping. Thus, the experiments were conducted blindly with respect to the maternal habitat-specific genotype (stream or pond). The experiments were conducted in accordance with the German laws for experimentation with animals.

Genotyping mother and larvae

Sixteen female fire salamanders deposited larvae in the experiment and produced a total of 478 individual larvae. DNA was successfully genotyped from 469 (98.11%) individuals. To genetically assign mothers and larvae to either of the two sub-populations (stream/pond), we extracted DNA from small tissue samples. DNA extraction was performed using DNA Extraction Kits (Quiagen®). Thereafter, the DNA from the females and larvae was genotyped using 18 highly polymorphic microsatellite loci (HENDRIX et al. 2010, STEINFARTZ et al. 2004). On the basis of the microsatellite data, all individuals were genetically assigned to either the stream or the pond cluster using GENEClass2. Groups consisting of 100 pond and 100 stream genotypes (STEINFARTZ et al. 2007) that unambiguously clustered in one of the two population types based on a STRUCTURE analysis served as reference groups. For reasons of simplicity, we refer to individuals assigned to the genetic pond cluster as pond-type and individuals assigned to the genetic stream cluster as stream-type.

Statistical Analysis

For each larva, we checked whether its genotype (stream/pond) matched the water compartment where it had been deposited (flowing/stagnant) (e.g., a match would be a stream-genotype larva that was deposited in the flowing-water compartment and a mismatch would be a pond-type larva that was deposited in the flowing-water compartment). From these match/mismatch data, we calculated the average matching rate for each of the 16 mothers and compared this average with chance expectation (50%). We tested the data for normal distribution with a Kolmogorov-Smirnov test. To compare stream-type with pond-type females, we used independent t-tests for normally distributed data. Non-normally distributed data were analysed using the non-parametric Mann-Whitney U-test. One-sample t-tests were used to test data against expected values. All the tests were performed using SPSS 20.

Results

Adult pond- and stream-type females did not differ in body mass or body length at the beginning of the experiment, i.e., prior to larval deposition ($N_{\text{Pond}} = 6$, $N_{\text{Stream}} = 10$;

mean body mass, stream-type females $32.96 \text{ g} \pm 2.51 \text{ SE}$; pond-type females $36.79 \text{ g} \pm 2.08 \text{ SE}$; t-test, $t_{14} = -1.05$, $p = 0.31$; mean body length, stream-type females $10.45 \text{ cm} \pm 0.21 \text{ SE}$; pond-type females, $10.70 \text{ cm} \pm 0.24 \text{ SE}$; t-test, $t_{14} = -0.75$, $p = 0.47$).

A total of 478 larvae were deposited in the experimental tanks. Pond- and stream-type females did not differ with regard to the total number of larvae deposited ($N_{\text{Pond}} = 6$, $N_{\text{Stream}} = 10$; total number of larvae deposited, stream-type females $29.7 \pm 5.4 \text{ SE}$; pond-type females $30.2 \pm 5.9 \text{ SE}$; t-test, $t_{14} = -0.06$, $p = 0.96$). The ratio of larvae deposited in the flowing-water compartment did not differ between stream- and pond-type females either ($N_{\text{Pond}} = 6$, $N_{\text{Stream}} = 10$; ratio of larvae in flowing water; t-test, $t_{14} = -0.94$, $p = 0.36$; Fig. 2). The ratio for each of the female groups did not differ from chance expectation (stream-type females: $N_{\text{Stream}} = 10$; one-sample t-test, $t_9 = 0.59$, $p = 0.57$; pond-type females: $N_{\text{Pond}} = 6$, one-sample t-test, $t_5 = 2.53$, $p = 0.053$; Fig. 2).

The proportion of larvae with a stream genotype differed significantly between stream- and pond-type females. The stream-type females deposited a significantly higher proportion of stream-type larvae than the pond-type females ($N_{\text{Pond}} = 6$, $N_{\text{Stream}} = 10$; stream-type larvae ratio; t-test, $t_{14} = -4.40$, $p = 0.001$; Fig. 3)

No significant difference was found between stream- and pond-type females in the mean matching of the larval genotype with the water compartment in which the larvae were deposited (flowing/stagnant) ($N_{\text{Pond}} = 6$, $N_{\text{Stream}} = 10$; mean matching ratio of larval genotype to water compartment type, stream-type females $0.47 \pm 0.09 \text{ SE}$; pond-type females 0.43 ± 0.07 ; t-test, $t_{14} = -0.33$, $p = 0.75$). Additionally, the matching ratio for stream-type females ($N_{\text{Stream}} = 10$; one-sample t-test, $t_9 = -0.31$, $p = 0.77$; Fig. 4) and pond-type

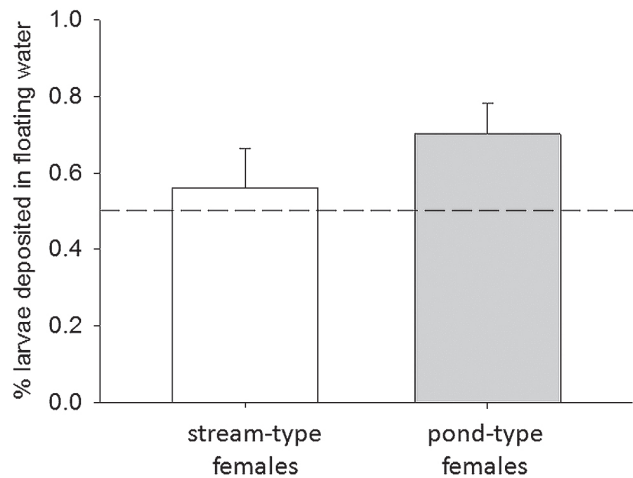


Figure 2. Percentage of larvae per female deposited in the flowing-water compartment (i.e., the emulated stream). The ratios of larvae deposited in the water compartments did not differ between stream- and pond-type females. Furthermore, the percentage of larvae in the flowing-water compartment for each of the two female groups (stream/pond) did not differ significantly from expectation. The dashed line indicates the chance level.

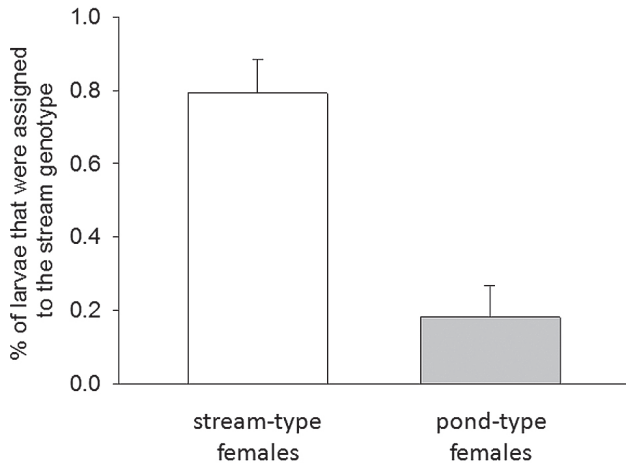


Figure 3. The percentage of larvae that were assigned genetically to the stream genotype differed significantly with respect to female genotypes, i.e., stream- and pond-type females. Stream-type females had a significantly higher proportion of stream-type larvae than pond-type females.

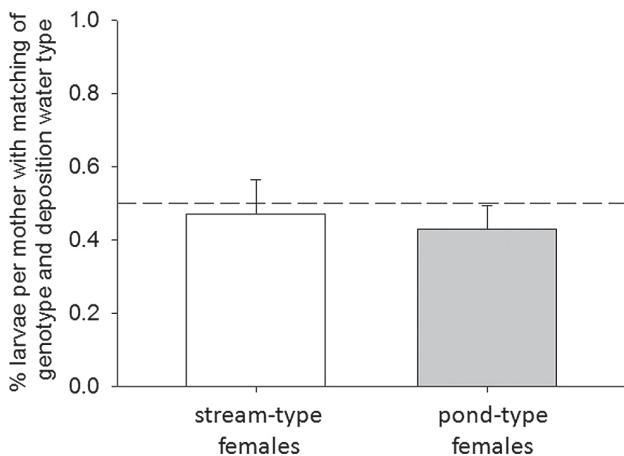


Figure 4. The percentage of larvae per mother for which the genotype and expected deposition compartment (either flowing or stagnant water) matched, calculated for mothers of both genotype clusters. The matching ratio for the larvae of both maternal groups did not differ from chance expectation. The dashed line indicates the chance level.

females ($N_{\text{Pond}} = 6$, one sample t-test, $t_5 = -1.08$, $p = 0.33$; Fig. 4) did not differ significantly from chance level, i.e., the females deposited their larvae randomly (Fig. 4).

Discussion

Female fire salamanders of different habitat-specific genotypes (i.e., pond- and stream-types) did not exhibit a predilection for depositing their larvae in a water body with the current characteristics of a specific habitat. Furthermore, we could not demonstrate that the females matched the larval

genotype with the specific water current characteristics, as the probability of matching did not differ significantly from chance level. Thus, we have to discard our initial hypothesis that the females used the water current as a proxy for selecting a certain habitat in which to deposit larvae. However, stream-type mothers primarily deposited larvae of their own habitat-linked genotype, as did pond-type mothers.

Contrary to our hypothesis, adult fire salamander females did not choose larval deposition habitats based on the water current. This finding could mean either that (i) females select a water body for larval deposition based on habitat characteristics other than water current, or that (ii) females in general do not have a predilection for specific water bodies for larval deposition, and the two habitat-specific genetic clusters are merely maintained by geographic isolation or female philopatry (e.g., pond-type females happen to live in the vicinity of ponds and by chance deposit more larvae in ponds); or that (iii) females deposit their larvae randomly in different water bodies and only well-adapted larvae survive, i.e., those whose genotype matches the aquatic habitat. The relative likelihood of these three alternatives differs, however.

(i) It may indeed be that females learn about the locations in which to deposit larvae, e.g., through experience or by learning the characteristics of their specific aquatic habitat type early in life, as is known from other vertebrate species (e.g., SCHOLZ et al. 1976, NEVITT et al. 1994, GERALACH et al. 2007). Alternatively, other characteristics, such as food abundance or water chemistry (which was kept stable for most of the parameters in the present study), are used by the females to select a suitable aquatic habitat for their larvae. The energetic value of potential food is lower in pond habitats than in stream habitats. Moreover, water temperatures and, consequently, other water parameters such as oxygen content are more variable and potentially more extreme in pond habitats (WEITERE et al. 2004, REINHARDT et al. 2013).

(ii) We can rule out the second scenario that the genetic clusters are maintained by geographic isolation, as adult fire salamanders in the study area are known to migrate over great distances (SCHMIDT et al. 2007, SCHULTE et al. 2007). However, we cannot rule out that females might be philopatric and deposit larvae only in their natal deposition habitats.

(iii) It might also be that under natural conditions, females deposit their larvae randomly in both streams and ponds, as in our study; however, due to strong selection pressure on larvae in nature, only those larvae whose genotype matches their deposition habitat will survive as a result of their adaptive predisposition. The outcome of facilitating this selective process would be that pond-type larvae are found more often in ponds and stream-type larvae more frequently in streams. However, such a mechanism would be relatively costly to females, as it is highly likely that a substantial proportion of larvae were lost due to having been deposited in the wrong habitat. This hypothesis is rendered quite unlikely by previous findings that stream- and pond-genotype larvae have certain adaptations (WEI-

TERE et al. 2004) that are specific to their genotype (= habitat) class. To date, there is little evidence that female fire salamanders would deposit their larvae in the “wrong” aquatic habitat type. We cannot therefore rule out this possibility at the moment, but it seems more likely to us that either other environmental characteristics (scenario I) that were not available or kept stable to the females in our experimental setup or female philopatry (scenario II) make females select a certain aquatic habitat for larval deposition in nature.

An interesting aspect of our study is that approximately 80% of the larvae of the stream-type females could be assigned to the stream genotype and approximately 81% of the pond females had larvae of the pond genotype. These high percentages are hints that non-random processes are involved in female reproduction, e.g., habitat-specific assortative mating (CASPER et al. 2009); these non-random processes might be involved in maintaining the two genetic clusters in the population.

In summary, we have tested whether the criterion “water current” is used by fire salamander females of two different habitat-linked genotypes, namely, the stream and the pond genotype, for choosing a certain aquatic habitat for larval deposition. Our data do not support this hypothesis and lead to the assumption that fire salamander females use other cues (most likely, environmental cues) to select a habitat for larval deposition. However, the exact cues still need to be identified in future studies.

Acknowledgements

This study was funded by the Wilhelm-Peters-Fonds of the Deutsche Gesellschaft für Herpetologie und Terrarienkunde e.V. (DGHT) to ETK, BAC and SEBASTIAN STEINFARTZ and a research grant from the Ethologische Gesellschaft e.V. to ETK. BAC was funded during the study by the DFG (Ca 889/1). We thank SEBASTIAN STEINFARTZ for supporting the study by providing facilities and access to animals, and for providing helpful and constructive comments during all stages of this study. We thank TIMM REINHARDT, RALF HENDRIX, TERRY MORLEY, DANIEL GELLER and ANNIKA KELLER for collecting the adult fire salamanders from the field. WERNER JAMIN provided technical help with the experimental setup, and ELKE HIPPAUF helped in the laboratory.

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