

## Temporal Patterns of Mating Activity in Alpine Newts, *Ichthyosaura alpestris*

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**ABSTRACT.**—Many amphibians in temperate zones need to time their reproduction in spring carefully, as they need to find a partner, mate, and oviposit in good time to ensure that their offspring metamorphose before the terrestrial phase starts. Therefore, embracing as many mating opportunities as possible, especially early in the mating period, would be advantageous. Here we examine the temporal pattern of mating activity in Alpine Newts, *Ichthyosaura alpestris*. Males exhibit a highly complex courtship ritual, during which both olfactory and visual signals play a prominent role. The aim of this ritual is a close coordination between the movements of the male and the courted female, a prerequisite for successful spermatophore transfer. We observed mating activity in a large, group aquarium with a natural photoperiod. We recorded the number of mating pairs at every full hour over the course of 4 d, during light and dark conditions. We observed most courtship behavior during light conditions when males were able to guide females properly with visual cues to the spermatophore. Although greatly reduced, we also found some mating activity in dark conditions on the first experimental day; however, mating activity decreased drastically during the following dark phases. Our findings demonstrate that Alpine Newt males that have access to females over several days use mating opportunities predominantly during light conditions, when visual cues can be utilized optimally. Although olfactory cues might be important during mating, our data suggest that visual cues may confer an adaptive advantage during mate choice.

**ZUSAMMENFASSUNG.**—Viele Amphibien in gemäßigten Klimazonen müssen ihre Paarungsaktivitäten im Frühling zeitlich sorgfältig einteilen, da sie rechtzeitig einen Partner finden, sich verpaaren und Eier legen müssen, um sicherzustellen, dass ihre Nachkommen vor Beginn der Landphase die Metamorphose abgeschlossen haben. Aufgrund dessen wäre es vorteilhaft vor allem am Anfang der Paarungszeit so viele Paarungsmöglichkeiten wie möglich wahrzunehmen. Hier untersuchen wir das zeitliche Muster der Paarungsaktivität von Bergmolchen, *Ichthyosaura alpestris*. Die Männchen zeigen ein höchst komplexes Balzritual, bei dem sowohl olfaktorische als auch visuelle Signale eine wichtige Rolle spielen. Der Zweck dieses Rituals ist eine enge Koordination zwischen den Bewegungen des Männchens und des umworbenen Weibchens, was die Voraussetzung für einen erfolgreichen Spermatophorentransfer ist. Wir beobachteten die Paarungsaktivität in einem großen Gruppenaquarium, welches naturnahe Bedingungen und eine natürliche Photoperiode aufwies. Wir dokumentierten die Anzahl der Paare zu jeder vollen Stunde über vier Tage, bei hellen und dunklen Lichtbedingungen. Das meiste Balzverhalten beobachteten wir unter hellen Lichtbedingungen, währenddessen es den Männchen möglich war, die Weibchen mit visuellen Signalen zur Spermatophore zu führen. Obwohl stark reduziert, beobachteten wir auch eine Paarungsaktivität unter dunklen Lichtbedingungen am ersten Versuchstag. Die Paarungsaktivität nahm jedoch während der darauffolgenden Dunkelphasen drastisch ab. Unsere Ergebnisse zeigen, dass Bergmolchmännchen, die über mehrere Tage Zugang zu Weibchen haben, Paarungsmöglichkeiten hauptsächlich unter hellen Lichtbedingungen nutzen, wenn visuelle Signale optimal genutzt werden können. Obwohl olfaktorische Signale während der Paarung wichtig sein könnten, legen unsere Ergebnisse nahe, dass visuelle Signale wahrscheinlich einen adaptiven Vorteil bei der Partnerwahl bieten.

Finding the optimal time to mate is crucial for an individual to ensure the successful production of progeny, especially in heterogeneous environments. Heterogeneity in environmental conditions, such as light intensity and temperature, is common. Temperate-zone amphibians experience a distinct annual rhythm with a biphasic lifestyle (Taylor and Guttman, 1977). European newt species, for example, spend most of the year in a terrestrial phase. They switch to an aquatic phase during breeding, which lasts between 2 and 4 mo (Halliday, 1977). Therefore, during the breeding period, specific activities such as finding a partner, mating, and egg deposition have to be timed optimally to ensure that the larvae have enough time to metamorphose and leave the water before the next terrestrial phase starts. European newt species enter water bodies for mating in spring. Albeit males normally arrive earlier than females (e.g., Arntzen, 2002: Alpine Newts *Ichthyosaura alpestris*, Palmate Newts *Lissotriton helveticus*, Smooth Newts *Lissotriton vulgaris*, and Marbled Newts *Triturus marmoratus*; Verrell and McCabe, 1988: Smooth Newts), all reproductive newts should be present shortly after the beginning of migration (e.g., Blab and Blab, 1981: Alpine Newt, Crested Newts *Triturus cristatus*, Palmate Newt, and Smooth Newt). Adults then leave the water

in summer again. As a corollary, the newts form agglomerations of many males and females in suitable water bodies where they face scramble competition for mating partners and interference of courtship rituals from other conspecifics. During this time, males have continuous access to receptive females, but no chance to monopolize them. Because newts mate polygynandrously (Halliday, 1977; Thiesmeier and Schulte, 2010), strong sperm competition is present and males are expected to invest all energy and time into mating whenever gravid females are available. Although adults seem to be in the water for several weeks to a few months, observations on Smooth Newts suggest that they spend only a short period on intense sexual behavior (Verrell and McCabe, 1988). The rest of the time, females oviposit and become unresponsive to male courtship attempts. This suggests that despite a potentially longer presence in the water, males are under a time constraint to participate successfully in mate and sperm competition. Therefore, we would expect to observe activity patterns that allow individuals to embrace as many mating opportunities as possible, particularly early in the mating season. On the other hand, amphibians frequently adjust activity patterns including mating behavior to various biotic and abiotic factors. For example, the presence of fish affected the behavior of two species of newts in laboratory experiments (Winandy and Denoël, 2013; Winandy et

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al. 2016). Alpine Newts reduced mating behavior in the presence of fish and sought shelter more frequently than without fish. Palmate Newts also used shelters more often when fish were present; however, these newts left the shelter more often at dark conditions during the night. Lighting conditions also affect other activities of newts. In the aquatic phase, Smooth and Alpine Newts showed feeding behavior mainly during night (Griffiths, 1985; Martin et al., 1989), whereas mating activity occurred mainly in the morning in Alpine Newts or entirely crepuscular in Smooth Newts (e.g., Griffiths, 1985; Martin et al., 1989). In contrast to this, Alpine Newts courted during both day and night in a study where males had access to females for only one single courtship event (Denoël and Doellen, 2010). Taken together, observations on the temporal patterns of the mating activity in newts are not consistent.

To shed more light on this question, we examined the temporal pattern of the mating activity of Alpine Newts (*Ichthyosaura alpestris* = *Triturus alpestris* = *Mesotriton alpestris*). Alpine Newts exhibit a long and complex courtship ritual (see Halliday, 1977 for a detailed graphical ethogram; Arntzen and Sparreboom, 1989). The courtship starts when the male encounters a female and places himself in front of her to make initial overtures. Then the display begins as a complex, but stereotypic, suite of behavioral patterns. If the female follows the movements of the male and approaches him, the male turns around to deposit a spermatophore on the substrate and tries to guide the female over the spermatophore. The female may, in return, pick up that spermatophore with her cloaca. The spermatophore deposition may be repeated several times during one courtship event. In laboratory observations, males deposited up to nine spermatophores during one undisturbed mating event (Maag, 2013). The male uses a combination of visual and olfactory cues during this display (Himstedt, 1979; Belvedere et al., 1988), whereby visual signals were more frequent during light conditions, and olfactory signals were more frequent during dark conditions (Denoël and Doellen, 2010). Nevertheless, mating in dark conditions might be less favorable because the close coordination of the movements of both mating partners is essential for a successful uptake of the spermatophore, and this coordination may improve with visual cues. Observations in light conditions revealed that females picked up only 31% of all offered spermatophores (Halliday, 1977, 1990). In one of our own pilot studies where one male was allowed to court one female without conspecific interference, the spermatophore uptake was even lower (6.8%; Lüdtke, pers. obs.). If a female does not directly walk over the deposited spermatophore, or if she touches the spermatophore with any body part other than the cloaca, the spermatophore is lost. Females that do not closely follow the tail tip of the male during the final part of the courtship ritual never take up the spermatophore (Lüdtke, pers. obs.). We presume that it is risky for Alpine Newts to invest into courtship during dark conditions, as visual cues will be less reliable.

Loosing spermatophores during courtship is probably costly for Alpine Newt males. Males complete spermiogenesis during autumn and store mature sperm for the entire breeding season (Verrell et al., 1986). Consequently, sperm storage may be limited and may deplete over the course of the breeding season. In addition, Maag (2013) found that sperm numbers diminish in successively deposited spermatophores during a single courtship event in this species, as they did in the related Eastern Newts (*Notophthalmus viridescens*; Takahashi and Parris, 2009).

Therefore, courtship in daylight seems more advantageous for male Alpine Newts, as they can properly guide females over their spermatophores.

We previously found that Alpine Newt males choose their mates based on the color intensity of the female's belly, and on their body size (Lüdtke and Foerster, 2018). Males generally courted more colorful females for longer than less colorful ones, even if the colorful female was not responsive. On the other hand, males invested as much time courting a less colorful female only if that female was responsive. Therefore, an additional advantage of courtship activity during daylight hours is the increased potential of assessing differences in female coloration. Griffiths (1985) drew similar conclusions about the visibility of color signals during sexual behavior in Smooth Newts, as they elicited most sexual behavior under a narrow range of optimum illuminances. The conclusion in this study was that a poor visual acuity during dark conditions would be less appropriate for courtship.

Therefore, in a setting where males have continuous access to females over several days, we expected most mating behavior to occur during light conditions where males are able to inspect females visually and guide them during the courtship ritual with visual signals. To test this hypothesis, we quantified mating behavior in a group of newts in a large, group aquarium, by counting the number of mating pairs at defined time points over the course of multiple days. By doing so we were further able to explore whether Alpine Newts show a particular temporal pattern of courtship frequency.

#### MATERIALS AND METHODS

**Study Animals.**—We observed Alpine Newts from the lab population at the University of Tübingen, Germany. This population consists of wild individuals from the surrounding of the city of Tübingen caught in 2011 and 2012 ( $n = 25$ ) and their descendants, all born between 2011 and 2014 ( $n = 80$ ).

**Maintenance.**—We separated males ( $n_{\text{male}} = 51$ ) and females ( $n_{\text{female}} = 54$ ) after hibernation on 28 February 2017, before they entered the water. We separated males from females and kept each at a maximum density of 15 individuals per aquarium ( $\approx 40$  L each;  $60 \times 60 \times 38$  cm length  $\times$  width  $\times$  height). We regulated ambient air temperature between 12 and 16°C, and established a photoperiod of 12 : 12 LD (light from 8:00 to 20:00). We fed the newts with Red Mosquito larvae (Chironomidae) and Water Fleas (*Daphnia* sp.) ad libitum three times a week. We provided shelters (brick stones with many holes and water plants) in each aquarium. After the experiment, we transferred the males into their home aquaria and the females into special oviposition aquaria.

**Experimental Design.**—We conducted the experiments 1 wk after the newts came out of hibernation, between 6 and 21 March 2017, during the start of this species' main breeding period (Blab and Blab, 1981; von Lindeiner, 1992). We performed the observations in a large, group aquarium ( $\approx 300$  L;  $250 \times 60 \times 60$  cm; see Suppl. Fig. 1). The bottom of the aquarium was covered with black gravel and some foliage to offer a more natural environment. We provided the newts with four brick shelters (same as described before) and additional hiding places (PVC tubes), water plants, and swimming tree bark. Water temperature was set between 14 and 15°C. The observations took place in the same room in which the newts were maintained. We measured the illumination intensity using a SpectraScan PR 670 spectroradiometer (Photo Research, Inc., Syracuse, New York,

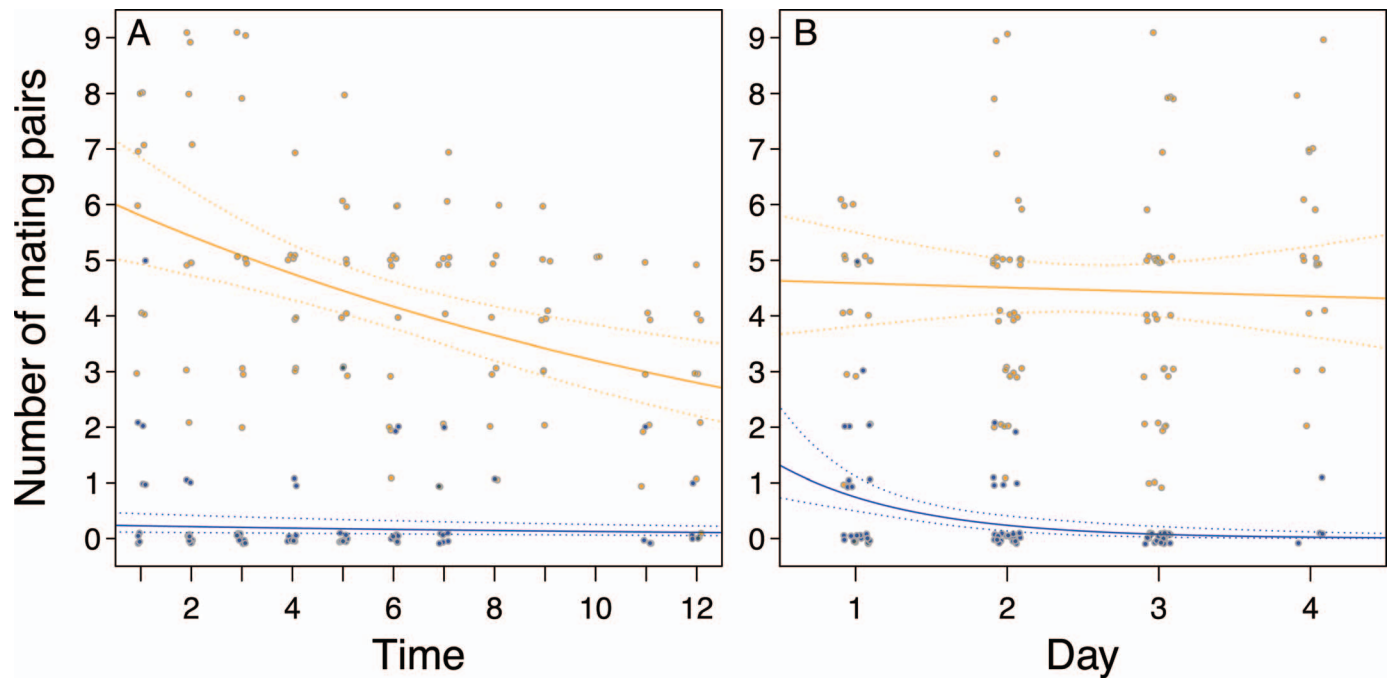


FIG. 1. Number of mating pairs in relation to (A) the time and (B) the day, with 95% credible intervals (dotted lines). The yellow lines show the model prediction for the number of mating pairs while the lights were on; blue lines show the model prediction for lights off. Points show the raw data (yellow: lights on; blue: lights off) and are jittered for better visibility.  $n = 172$  observations on 51 males and 54 females.

USA). The mean intensity was  $8.69 \times 10^{20}$  photon radiance (photons/sr/m<sup>2</sup>/sec) for daylight. For the recordings during the night, we used dim red light (40 W) to detect mating pairs. The light intensity of the red light during the night was  $2.02 \times 10^{20}$  photon radiance.

We randomly divided the newts into three groups with a 50 : 50 sex ratio. Three males were used twice to maintain an equal sex ratio. Hence, each group consisted of 36 newts. We introduced the three groups sequentially into the experimental aquarium creating a block design with three different levels. Each group was observed for 4 d. Newts were allowed to acclimatize for half a day in the test aquarium. We started the observations at 1300 h on day 1 and ended the observations at 1300 h on day 4. Because of a technical problem, we had a gap of 4 d between the first and the second group. We used a scan-sampling method in which we recorded the total number of mating pairs every full hour with the exception of 0300–0500 h and 1700. Eleven other observation points are missing because of technical problems. We counted the mating pairs by moving slowly around the aquarium (usually within 2 min) without disturbing the newts. Males and females can easily be identified, as the sexual dimorphism is distinctive in Alpine Newts: males tend to be smaller than females; they develop a large cloacal protuberance, a small dorsal crest, and a blue dorsal coloration (see Suppl. Fig. 2). Mating pairs can easily be detected in this species because males engage in a conspicuous stereotypic courtship display (Halliday, 1977; Arntzen and Sparreboom, 1989). A pair was considered a mating pair when the male exhibited any of the specific behaviors towards the female, such as tail fanning during the static phase (see Halliday, 1977) or spermatophore deposition. The maximal possible number of mating pairs would be 18 for one specific time point, if every male was associated with one female. We did not distinguish between individuals in this study. All observations were made by two teams of two persons each.

**Statistical Analyses.**—We tested the effect of light (on/off) and time on the number of mating pairs using generalized linear mixed models (GLMM) with log-link function and Poisson error distribution using the package ‘lme4’ (Bates et al., 2015). Time was coded as consecutive numbers from 1 to 12, where 1 was the observation when the light was just turned on or off, and 12 was the observation 1 h before the light condition was switched again. We included the continuous and Z-transformed variable day (consecutive day of the observation of each group ranging from 1 to 4) and the interaction between light and day. We then checked whether the inclusion of the three other variables date (also continuous and Z-transformed), observer (factor), or the interaction between light and time would improve the second-order Akaike information criterion for small sample size (AICc). We tested all combinations of the first-described model with those additional three terms and ranked them based on the AICc value and the Akaike weight (see Table 1).

Group was included as a random factor in all models to account for random variance because of the experimental block.

TABLE 1. Comparison of generalized linear mixed models for number of mating pairs. Models are ranked based on the AICc values (corrected AIC for small sample size) and Akaike weights;  $\Delta$ AICc is the difference from the AICc of the best model. The best model is given in the first line and is represented by the three dots in all other lines.  $n = 172$  observations on 51 males and 54 females.

Fixed terms included in model	AICc	$\Delta$ AICc	Weight
Light + Time + Day + (Light * Day)	525.76	0.00	0.367
+ (Light * Time)	527.39	1.63	0.163
+ Date	527.51	1.75	0.153
+ Observer	527.85	2.09	0.129
+ Date + (Light * Time)	529.16	3.40	0.067
+ Observer + Date	529.68	3.92	0.052
+ Observer + (Light * Time)	529.80	4.04	0.049
+ Observer + Date + (Light * Time)	531.63	5.87	0.020

Although the factor group never explained any of the variance, we still included it based on the design of the experiment (Zuur and Ieno, 2016). We used Bayesian statistics to calculate 95% credible intervals (CrI) for the parameter estimates (presented in the tables) and model predictions (presented in the figure) from the best model (Bolker et al., 2008). With Bayesian statistics we were able to calculate the exact uncertainty of model predictions, whereas frequentist methods can only approximate these measurements (Bolker et al., 2008). We obtained a sample of 10,000 simulated values from the joint posterior distribution of the model parameters using the function *sim* from the R package 'arm' (Gelman and Su, 2016). We concluded that a parameter was different from zero if the 95% credible interval of its estimate did not include zero (Bolker et al., 2008).

To cross-check our results, we used model averaging based on the models with a  $\Delta\text{AICc} \leq 2$  (R package 'MuMIn'; Barton, 2018). Instead of interpreting the results from a single best model, the model averaging method allows one to draw conclusions from multiple candidate models (Burnham et al., 2011). We present the importance of each predictor variable (i.e., the sum of Akaike weights) and the mean parameter estimates with their 95% confidence intervals (CI) in the supplements (Suppl. Table 1). Again, we concluded that a parameter was different from zero if the 95% confidence interval of its estimate did not include zero (Mazerolle, 2006).

One important difference between the averaged model and the best model is the inclusion of the interaction between light and time in the averaged model. The reason for this is that the second best model, as judged by the AICc, included this interaction (which never explained any significant variance; see results). Interaction terms change the meaning of main effects in the model, as these main effects become conditional. Because of this and our general reluctance to maintain nonsignificant interactions in linear models, we base our main conclusions on the best model.

All analyses were performed in R v.3.4.3 (R Core Team, 2017). We applied the function *dispersion\_glm* from the R package 'blmeco' (Korner-Nievergelt et al., 2015) and found no over- or underdispersion in either of the candidate models. All data summaries are reported as means  $\pm$  SD.

## RESULTS

Variance in the number of mating pairs was explained best by the variables light, time, day, and the interaction between light and day (based on the lowest AICc value; see Table 1). However, two alternative models had AICc values that exceeded the lowest value by  $<2$ . These models included the interaction between light and time and the variable date as predictor variables. The AICc of all other models exceeded the lowest AICc by  $>2$  (see Table 1) and these models were not considered for inferences. Any model that was simpler than the presented ones exceeded the here-presented lowest AICc by at least 14.7 (data not shown).

We detected mating pairs at 67% of all observed time points (115/172; mating pairs:  $2.73 \pm 2.57$ , range 0–9). Mating activity was notably high when the light was on, but low when the light was off (Table 2; light on:  $4.410 \pm 2.016$  mating pairs; light off:  $0.403 \pm 0.883$  mating pairs). We observed mating activity in 98% of the observations when the light was on. When the light was off, we observed mating pairs in only 24% of the observations.

TABLE 2. Estimates and their 95% credible intervals from the best generalized linear mixed model for number of mating pairs. For the fixed factor light (on/off) the effect is given for lights on compared to lights off. Significant estimates are shown in bold.  $n = 172$  observations on 51 males and 54 females.

Fixed factors and covariates	Estimate	SE	95% credible intervals
Intercept	−1.886	0.365	−2.602 to −1.167
Light (on)	3.330	0.368	2.610 to 4.057
Time	−0.221	0.055	−0.332 to −0.113
Day	−1.106	0.286	−1.673 to −0.545
Light * Day	1.089	0.291	0.515 to 1.660

In addition, the time (consecutive numbers from 1 to 12 within each light or dark phase; see methods) had an influence on the number of mating pairs in the best model (Table 2): we observed most pairs at the beginning of the light on phase with  $5.875 \pm 1.959$  mating pairs at the first time point (Fig. 1). Mating activity then decreased over time with only  $2.444 \pm 1.810$  mating pairs at the last time point of the light-on phase. This effect was less pronounced during the light-off phase (first time point:  $1.222 \pm 1.641$ , last time point:  $0.250 \pm 0.500$ ). In the averaged model, on the other hand, the effect of time did not seem to have a significant effect on the number of mating pairs (based on the 95% confidence interval; see Suppl. Table 1); however, the importance of this main effect was still 0.99. In addition, among the three candidate models, only the one including the nonsignificant interaction between light and time suggest no significant main effect of time (data not shown).

The number of mating pairs was also influenced by day (consecutive numbers from 1 to 4 as the observation days of each experimental group) and by the interaction between light and day (Table 2). Overall, fewer newts engaged in courtship later during the experiment. This effect was only very small during the light-on phase (Fig. 1). During the light-off phase, we generally observed fewer matings, but mating activity was somewhat higher during the first experimental day (first day:  $0.909 \pm 1.306$ ; last day:  $0.250 \pm 0.500$ ; Fig. 1). In the averaged model (see Suppl. Table 1), the confidence intervals for the parameter estimates of date and the interaction between light and time overlapped zero. We hence conclude that those variables did not affect the number of mating pairs.

## DISCUSSION

Our study demonstrates that mating activity in Alpine Newts was influenced by light, the number of successive days, and the time of the day in the experiment. We showed that if newts had continuous access to mating partners, mating occurred mainly in light conditions as opposed to darkness. Denoël and Doellen (2010) reported equally high mating activity (defined as males that displayed) during light and dark conditions, although this observation was based exclusively on data from the first courtship event started after the first contact between a single male and a female. One male and one female newt were put together in an aquarium in either a dark or a bright treatment and mating behavior was recorded immediately thereafter. The observations ended after a pair did not exhibit any further mating behavior for 1 min. In our experiment, we observed mating activity over four consecutive days and found overall strongly reduced numbers of mating pairs during dark conditions, with some mating activity in the dark on the first experimental day only. We conclude that Alpine Newts will

start to court as soon as they encounter potential mates for the first time in the season (and this situation was simulated in the experiment from Denoël and Doellen, 2010). After a few successful matings, however, the newts showed less courting behavior during dark conditions (as observed in our experiment). Similar observations were reported by Martin et al. (1989), where sexual behaviors in Alpine Newts occurred only during light conditions. These field observations were made in May, approximately midway through the breeding season, when most of the newts had already encountered several mates.

Although olfactory cues are important signals for mating (Johansson and Jones, 2007), and can induce typical courtship behaviors in females (Treer et al., 2013), our results suggest that visual cues also play a critical role in initiating courtship behaviors in males. Salamanders emit specific courtship pheromones only after the initial contact of potential mates (Houck, 1986). Accordingly, we observed tail fanning (a behavior that disseminates the male courtship pheromone towards the female; Halliday, 1977) only after the first contact with a female, and only in stereotype angle position to the female's body. Conversely, species-specific color characteristics of the female have been shown to be predominantly important for Alpine Newt males to initiate courtship behaviors (Himstedt, 1979). This visual discrimination before the onset of courtship might be especially important, as Alpine Newts occur sympatrically with all other European newt species (Smooth Newts, Palmate Newts, and Crested Newts; Thiesmeier and Schulte, 2010). Although males can easily be identified, females of these four species look relatively similar on the dorsal side. On the ventral side, however, females express species-specific color patterns. Alpine Newt females, as well as their male conspecifics, differ from other newt species in their more intensely orange colored belly. Observations with differently colored female models presented to a male showed that a red ventral side in combination with a differently colored dorsum is the sign stimulus to elicit male courtship behavior (Himstedt, 1979). In addition, we recently showed that males exhibited mate choice based on the intensity of the females' belly coloration and on their size (Lüdtke and Foerster, 2018), whereby males spent more time courting colorful and large females. This suggests that an influence of female visual cues during mating is not negligible. The importance of species recognition and the benefit of adaptive mate choice may explain the pattern of mating activity found in this study.

Alpine Newts exhibit a complex courtship dance in which the close coordination with the female is essential (Halliday, 1977; Arntzen and Sparreboom, 1989). As a successful spermatophore transfer requires such a close coordination with the courted female, males might preferably use light conditions to evaluate the female's responsiveness better. Alternatively, males may rely on pheromone information during dark conditions. Not unless the female is attentive will the male turn in front of her and deposit a spermatophore on the surface. The female then needs to remain attentive and closely follow the male's movements, so that the male might be able to guide the female over the spermatophore directly. However, two studies report that also in light conditions only few spermatophores were successfully transferred (33% in Denoël and Doellen, 2010; 31% in Halliday, 1977, 1990). In a pilot study where one male was allowed to court one female without interference, we also observed a small rate of spermatophore uptake (6.8% [Lüdtke, pers. obs.]). This follows from the fact that the female has to touch the

spermatophore with no other body part than the cloaca to allow successful sperm transfer.

The production and offering of spermatophores constitute a significant investment for males, and any situation where the spermatophore does not attach to the courted female's cloaca results in a loss of energy and opportunity. Many temperate urodele species complete spermiogenesis in autumn and store mature sperm until the mating period during spring. This was also observed in Smooth Newts, a species that is closely related to Alpine Newts (Verrell et al., 1986). Mechanisms in Alpine Newts might not be different, and stored sperm numbers might therefore diminish over time. Indeed, in uninterrupted courtship trials, sperm numbers decreased in consecutively deposited spermatophores of male Alpine Newts (Maag, 2013). This effect was already known for Eastern Newts (*Notophthalmus viridescens*; Takahashi and Parris, 2009). We conclude that male Alpine Newts should budget their spermatophore deposition because the number of spermatozoa might be limited within one mating season. Such a limitation in sperm numbers might be one reason why mating attempts by males decreased in successive nights during the experiment. In the first night of our experiment, male newts still had a full storage of spermatozoa, and they might hence take every opportunity to court females. This was the time when we observed elevated mating activity also during dark conditions. In successive nights, however, the sperm storage probably diminished and males might have become more cautious as to whom to offer spermatophores, and when to engage in courtship to ensure successful sperm transfer. Consequently, males courted almost exclusively under light conditions during the following days.

Additionally, female responsiveness might also influence the frequency of courtship during dark conditions. Females might also base their mate choice on visual cues (e.g., Andersson, 1994; Rosenthal, 2017). Male Alpine Newts develop conspicuous phenotypic characteristics (enlarged cloaca, dorsal crest, dorsally blue coloration, and a more conspicuous white-back-spotted stripe on the flank, see Suppl. Fig. 2) in the beginning of the mating season, which might function as quality indicators. Therefore, females might also be responsive predominantly during light conditions when they are able to assess males visually.

Studies on Smooth Newts (Gabor and Halliday, 1997) and other taxa (e.g., fish; Pitcher et al., 2003; or insects; Fedina and Lewis, 2007) reported that females become choosier with the progression of the breeding season. Although females showed no clear preference in the beginning of the breeding season, they clearly chose higher-quality males later during the season. Although one insemination event might be sufficient to fertilize all eggs, female newts mate polyandrously, as is common in many urodele species (Halliday, 1998; Osikowski and Rafinski, 2001). While the first mating events might function as an assurance of fertilization, females seem to focus on choosing high-quality partners through subsequent matings (Gabor and Halliday, 1996; Halliday, 1983). In our experiment, we found a decrease in the number of mating pairs with day, that is, with increasing time since the first mating. Our data, however, are insufficient to support or reject the hypothesis that females chose higher-quality males later during the observations.

The strongest decrease in mating activity over time occurred during the light-off phase. Females might have used the first night to ensure insemination by mating indiscriminately. Because eggs can be fertilized after one insemination, females could start laying eggs soon after. Interestingly, female Alpine



Newts seem to prefer laying their eggs during the night (dark conditions), rather than during the day (Morgenthaler, 1955; Lüdtke, pers. obs. in this and in previous experiments). The process of depositing an egg and securing it to a support (e.g., a water plant leaf) can last a few minutes (Díaz-Paniagua, 1989; Miaud, 1993; Thiesmeier and Schulte, 2010) in which the female might be especially vulnerable to predation. Ovipositing in dark conditions might transfer a selective advantage through better camouflage among the water plants. Therefore, females may use dark conditions preferably to lay their eggs safely, making themselves less available for mating during night. Female availability, rather than female responsiveness in general, may consequently have been another factor responsible for the lower mating activity during the dark phase in our experiment.

In summary, Alpine Newts expressed a pronounced temporal pattern in courtship and mating activities. Despite the fact that most amphibians mate during the night (Hartel et al., 2007), Alpine Newts mainly mated during light conditions when males and females were able to see and assess each other, and males were able to guide and coordinate the females with visual signals. As predicted, males were found to use the time that is most favorable for them, namely light conditions, almost continuously for mating. In conditions not optimal for mating (during dark conditions), males were found to court less. Males might use that time for other activities, such as resting and feeding. These results also indicate that behaviors must be observed over a longer period of time to identify specific patterns that might otherwise remain undetected.

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