Selection on Female Body Colouration through Male Mate Choice in Alpine Newts, *Ichthyosaura alpestris*

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DEIKE U. LÜDTKE
aus HAMBURG

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Erklärung

Die Zulassung als Doktorandin im Sinne von §7 der Promotionsordnung vom 24. April 2015 erfolgte am 24. November 2014. Diese Dissertation wurde im Sinne von §6 von Prof. Dr. Katharina Foerster betreut.

Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass diese Dissertation von mir selbstständig – abgesehen von der Beratung und Hilfe meiner Betreuerin – und ohne unerlaubte Hilfsmittel erarbeitet wurde.
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Diese Dissertation wurde an keiner anderen Prüfungsbehörde vorgelegt.

Tübingen, 10. Oktober 2019

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(Deike Lüdtke)

Dissertation eingereicht am:

1. Gutachter: Prof. Dr. Katharina Foerster

2. Gutachter: PD Dr. Katja Heubel

"In nature, nothing exists alone."
— Nichts existiert in der Natur allein. —
Rachel Carson

Abstract

Colourful animal ornaments have been a core research focus of the field of sexual selection in evolutionary biology. Traditionally, females are considered to be the choosing sex, while males compete for access to females and advertise themselves through extravagant colourations and decorations. However, in many species not only males but also females show bright body colourations, giving rise to the potential of quality differentiation much in the same way as in males. Male mate choice and the signalling content of bright female colourations remains a contentious issue, and empirical evidence for male mate preferences and honesty in female ornamentation in conventional sex-role species is scarce for vertebrate species other than birds and fish.

In this thesis, I investigated a link between female belly colouration, male preference and female fecundity in the Alpine newt, *Ichthyosaura alpestris*, a medium-sized, aquatic salamander. Both sexes of this species have colourful bellies that vary naturally from pale yellow to bright red or dark orange among individuals. Because male Alpine newts are likely to be limited in their mating capacity, males would benefit when they mate with females of particular high quality. I hypothesised that the belly colouration of females may act as an indicator of individual quality and that males therefore respond to variation in this colouration.

The first paper describes the mate preferences of male Alpine newts, in an experimental setup. Males had a choice between females that varied in belly redness (red versus yellow). Additionally, I tested the preference of males in different light conditions (control and blue filter) to determine whether male choosiness solely depends on the female's colour signal. I found that males generally spent more time courting red females but increased the time when a yellow female was particularly interested. This verifies the existence of male mate choice in Alpine newts; a species that had previously been thought of as being a traditional model of female choice only.

The second paper examines the temporal pattern of mating activity in Alpine newts. I observed mating behaviour in undisturbed groups of males and females during light and dark conditions over several days. I recorded most courtship behaviour during light conditions, which demonstrates that males use mating opportunities when visual cues can be utilised optimally and when they can properly assess females by their colour.

The last paper provides first evidence for the signal content of the female belly colouration. By counting the number of eggs of females that varied in belly colouration, I showed that more orange-bellied females were more fecund and could lay their eggs faster than yellower ones. This enabled more orange-bellied females to cease egg laying at a similar time as yellow females in spite of a higher number of eggs. This result presents an explanation for the preference patterns described before because it suggests a direct benefit for males that mate with ornamented females.

Taken together, the results of this thesis indicate that female belly colour is subject to sexual selection through male mate choice in Alpine newts. My results are further consistent with the general idea of mate choice based on colourful ornaments and give additional insight into the function of female colouration in diverse taxa.

Zusammenfassung

In der Evolutionsbiologie sind farbenfrohe Ornamente bei Tieren seit geraumer Zeit ein zentraler Forschungsschwerpunkt auf dem Gebiet der sexuellen Selektion. In der Regel gelten Weibchen als das wählerische Geschlecht, während Männchen um den Zugang zu Weibchen konkurrieren und sich durch extravagante Färbungen und Dekorationen anpreisen. In vielen Arten zeigen jedoch nicht nur Männchen, sondern auch Weibchen leuchtende Körperfärbungen, was das Potenzial einer Qualitätsdifferenzierung in ähnlicher Weise wie bei Männchen ermöglicht. Männchenwahl und der Signalgehalt leuchtender Färbungen bei Weibchen bleibt ein umstrittenes Thema, und empirische Belege für männliche Paarungspräferenzen und Ehrlichkeit in der weiblichen Ornamentik bei Arten mit konventionellen Geschlechterrollen sind für Wirbeltiere mit Ausnahme von Vögeln und Fischen selten.

In dieser Arbeit untersuchte ich einen Zusammenhang zwischen weiblicher Bauchfärbung, männlicher Präferenz und weiblicher Fruchtbarkeit beim Bergmolch, *Ichthyosaura alpestris*, einem mittelgroßen, aquatischen Salamander. Beide Geschlechter dieser Art haben farbenprächtige Bäuche, die bei den Individuen auf natürliche Weise von hellgelb bis knallrot oder dunkelorange variieren. Da männliche Bergmolche wahrscheinlich in ihrer Paarungsfähigkeit eingeschränkt sind, würden Männchen davon profitieren, wenn sie sich mit Weibchen von besonders hoher Qualität paaren. Ich vermutete, dass die Bauchfärbung von Weibchen als Indikator für die individuelle Qualität dienen könnte und dass die Männchen daher auf Schwankungen dieser Färbung reagieren.

Das erste Paper beschreibt die Paarungspräferenzen männlicher Bergmolche in einem experimentellen Versuchsaufbau. Männchen hatten die Wahl zwischen Weibchen, die in ihrer Bauchröte variierten (rot gegenüber gelb). Zusätzlich habe ich die Präferenz von Männchen unter verschiedenen Lichtverhältnissen (Kontroll- und Blaufilter) getestet, um festzustellen, ob die männliche Wahlentscheidung ausschließlich vom Farbsignal der Weibchen abhängt. Ich fand heraus, dass Männchen im Allgemeinen mehr Zeit damit verbrachten, rote Weibchen zu umwerben, aber die Balzzeit verlängerten, wenn ein gelbes Weibchen besonders interessiert war. Dies bestätigt die Existenz von Männchenwahl bei Bergmolchen, einer Art, die bisher nur als traditionelles Beispiel der Weibchenwahl angesehen wurde.

Das zweite Paper untersucht das zeitliche Muster der Paarungsaktivität bei Bergmolchen. Ich beobachtete das Paarungsverhalten bei ungestörten Gruppen von Männchen und Weibchen bei hellen und dunklen Lichtbedingungen über mehrere Tage. Ich registrierte das meiste Balzverhalten bei hellen Bedingungen, was zeigt, dass Männchen Paarungsmöglichkeiten nutzen, bei denen visuelle Signale optimal genutzt werden können und Weibchen anhand ihrer Farbe richtig beurteilt werden können.

Das letzte Paper liefert erste Hinweise auf den Signalgehalt der weiblichen Bauchfärbung. Indem ich die Anzahl der Eier von Weibchen, die in ihrer Bauchfärbung variierten, zählte, konnte ich zeigen, dass Weibchen mit orangeneren Bäuchen fruchtbarer waren und ihre Eier schneller legen konnten als jene mit gelberen Bäuchen. Dies ermöglichte es orangeneren Weibchen, trotz einer höheren Anzahl von Eiern die Eiablage zu einem ähnlichen Zeitpunkt wie gelbe Weibchen zu beenden. Dieses

Ergebnis liefert eine Erklärung für die zuvor beschriebenen Präferenzmuster, da es einen direkten Nutzen für Männchen, die sich mit ornamentierten Weibchen paaren, darlegt.

Zusammenfassend zeigen die Ergebnisse dieser Arbeit, dass bei Bergmolchen die weibliche Bauchfarbe der sexuellen Selektion durch Männchenwahl unterliegt. Meine Ergebnisse stimmen zudem mit der allgemeinen Annahme von Paarungswahl auf der Grundlage bunter Ornamente überein und geben zusätzliche Einblicke in die Funktionalität der weiblichen Körperfärbung verschiedenster Taxen.

List of Papers

The results described in this thesis are published or have been accepted in the following peer-reviewed journal articles, which are referred to in the text by their Roman numerals:

I. Lüdtke, Deike U. and Foerster, Katharina (2018). **Choosy males court both large, colourful females and less colourful but responsive females for longer**. *Animal Behaviour* 146: 1-11. doi: 10.1016/j.anbehav.2018.09.018

II. Lüdtke, Deike U. and Foerster, Katharina (2019). **Temporal patterns of mating activity in Alpine Newts, *Ichthyosaura alpestris***. *Journal of Herpetology* 53(3): 245-251. doi: 10.1670/18-129

III. Lüdtke, Deike U. and Foerster, Katharina (2019). **A female color ornament honestly signals fecundity**. *Frontiers in Ecology and Evolution* 7(432): 1-9. doi: 10.3389/fevo.2019.00432

Declaration of contribution (Faculty of Science PhD regulations § 5 Abs. 2 No. 8)

I performed the data collection, laboratory work, and data analyses myself, and written and revised the manuscripts and this thesis.
Katharina Foerster, the co-author of all three papers, supervised this thesis and helped to conceive and design the experiments. She also commented on all manuscripts and provided scientific input whenever needed.

Contents

Introduction

This thesis focuses on colouration as a signal of female quality in mate choice and its influence on male investment during sexual encounters. I begin this introduction with a short, general overview to familiarise the reader with a few diverse topics, including sexual selection, signalling theory and the concept of male mate choice and female ornaments. As it has the most relevance to this thesis, I focus primarily on secondary sexual signals in species with no sex role reversal.

Sexual selection and signalling

Sexual selection theory

Exaggerated ornaments, typically seen in males, were once one of the greatest mysteries in nature. These traits seemed too costly to benefit survival and to withstand under the theory of evolution by natural selection until Darwin (1871) presented his theory of sexual selection. He argued that selection will favour traits that increase male mating success and allow a male to sire more offspring. Differences in the trait expressions of ornament bearers would not lead to death of the unsuccessful competitor but would lead to fitness differences due to variations in obtaining a mate and the ability to reproduce. Such differences can be obtained either through competition within a sex (intrasexual selection) or through mate choice between the sexes (intersexual selection). Darwin's initial interpretation was, however, limited to the so-called secondary sexual traits, elaborate weapons or decorations that might be energetically wasteful and potentially harmful to their bearer. Today, we have a broader comprehension of which additional patterns in nature can be explained through sexual selection, for example sexual divergence, sperm competition, or sexual conflict to name a few (reviewed in Jennions and Kokko, 2010). A more modern definition would therefore be: "*sexual selection favours investment in traits that improve the likelihood of fertilization given limited access to opposite sex gametes due to competition with members of the same sex* (cited from Jennions and Kokko, 2010; p. 345).

This dissertation, however, mainly addresses mate choice on the basis of ornamental traits, and I will therefore focus on the previously mentioned secondary sexual traits, especially on colourful ornaments and intersexual selection.

Several theories have been put forward to explain the presence of secondary sexual ornaments. For one of the mechanisms that have been investigated most on how secondary sexual ornaments might evolve two prerequisites are required: first, the signal must be perceived by the receiver; second, the ornament must communicate a benefit to both the signaller and the receiver. In the case of intersexual selection, the benefit for the signaller is to be chosen by the other sex despite the cost of carrying that ornament (Zahavi, 1975). For the receiver, on the other hand, the benefit is to gain information on the signaller's quality that may help to assess future actions, for example to evaluate whether mating with that particular signaller would be adaptive. When choosing high-quality partners to mate with, an individual can benefit through direct (e.g., Iwasa and Pomiankowski, 1999; Price et al., 1993) or indirect benefits (e.g., Kirkpatrick and Barton, 1997). Direct benefits, in this context, might implicate the provision of food resources, protection, parental care or even the health status such as parasite load of a mate (e.g., Andersson, 1994; Hoelzer, 1989). Indirect benefits are associated with 'good-gene' effects that provide attractive (Fisher, 1915) or more viable offspring (Hamilton and Zuk, 1982). These benefits might potentially increase the individual fitness of the receiver. An exact assessment of the quality of a sexual partner would hence be substantially important for the choosing individual (reviewed in Kokko et al., 2003). Further, the sex that invests more resources into each reproductive event and is less available for mating (in many cases the female) is generally expected to be choosy (Bateman, 1948; Jennions and Petrie, 1997; Trivers, 1972). That sex consequently does not need to advertise itself as much as the other sex and may sample potential mates. To enable correct decision by a choosing individual, however, any secondary sexual ornament must reliably signal its bearer's quality.

Signal reliability

Behavioural ecologists distinguish between a signal and a cue. A signal, in this context, is defined as "*any act of structure which alters the behaviour of other organisms, which evolved because of that effect, and which is effective because the receiver's response has also evolved*" (cited from Maynard-Smith and Harper, 2003; p. 3). A cue, on the other hand, has not evolved to convey a specific information; it is merely a "*feature of the world [...] that can be used by an animal as a guide to future action*" (cited from Maynard-Smith and Harper, 2003; p. 3). In this sense, the secondary sexual traits investigated by Darwin can be defined as signals, that evolved through sexual selection. As previously mentioned, a signal of quality must be reliable or "honest" to be effective and evolutionarily stable (Grafen, 1990; Johnstone, 1995). In other words, there must be a consistent correlation between ornament expression and some unobservable quality parameters of the sender that are of interest to a receiver. Signal honesty in ornamental traits can be maintained either through associated costs entailed in the production or maintenance of these signals (Handicap hypothesis; Zahavi, 1975), or through an inevitable link between signal expression and a highly conserved mechanism indicative of an individual's condition (Index hypothesis; Biernaskie et al., 2014). A large number of empirical studies on a variety of taxa suggest that colouration can indeed be an honest signal for quality (e.g., Garratt and Brooks, 2012; Svensson and Wong, 2011).

Colouration as an indicator of quality

In many animals, colourful traits can be found as signals that are used during sexual display (e.g., Andersson, 1994; Maynard-Smith and Harper, 2003). Especially yellow, orange and red colours are striking examples of how these ornaments might signal quality advantages. Body colourations that appear in such colours are caused by carotenoids or pteridines, or a combination of both, that accumulate in feathers or skin cells (Griffith et al., 2006; Olson and Owens, 1998; Weiss et al., 2012). The intensity of these colours is positively correlated with pigment concentration (e.g., Barber et al., 2000; Weiss et al., 2012). Suitable for honest signalling is the fact that both of these pigment classes are relevant, powerful antioxidants and important boosters of immune response (Britton, 2008; Chew and Park, 2004; Hõrak et al., 2004; Huber et al., 1984; Oettl and Reibnegger, 2002). Therefore, allocating carotenoids or pteridines towards colouration comes at the cost of those pigments not being available for other functions (Aguilera and Amat, 2007; Baeta et al., 2008). Colourful individuals could therefore either have better access to resources that grant them the capacity to obtain more compounds through their diet or they might have an advantage in coping with oxidative stress and are hence better able to allocate their resources into colouration and potentially be of better quality also in other areas. Similarly, other colours such as melanin-based or structural colours may be used for ornamentation indicating quality through other mechanisms (e.g., Doucet, 2002; Siefferman and Hill, 2003). The assumption of a connection between colours and individual quality have made colouration popular among behavioural ecologists and made them a favoured subject in studies on animal signals (e.g., Liu et al., 2014; Mautz et al., 2013; Rodrigo et al., 2016).

Male choosiness and female ornamentation

Although in many cases females invest more in each reproductive event, male investment into reproduction can also be costly and mating can be constrained (Bonduriansky, 2001; Kasumovic et al., 2007; Wedell et al., 2002). In species where males mate multiply, studies have shown that male fertilisation rates may decrease and sperm stores become depleted with increasing mating frequency (e.g., Nakatsuru and Kramer, 1982; Preston et al., 2001). Therefore, if the number of receptive females exceeds the number a male is capable of inseminating, then a male should mate with females that can maximise his reproductive success (Etienne et al., 2014; Johnstone et al., 1996; Kokko and Monaghan, 2001). If females vary in reproductive value, males are expected to base their choice on a more or less direct proxy of female quality (Cunningham and Birkhead, 1998; Servedio and Lande, 2006). In species without maternal care, any trait that affects a female's fecundity and the quality of her eggs would be relevant for choosy males. While little is known about potential male choice for female traits that may affect egg quality (e.g., female age, female health or oviposition site), traits that signal female fecundity have been shown to be under sexual selection through male choice (Clutton-Brock, 2009; Jones et al., 2001).

Oftentimes, female body size is positively correlated with female fecundity (Honěk, 1993) and males were found to prefer larger females (e.g., Bonduriansky, 2001; Eddy et al., 2016; Wong and Svensson, 2009). However, also colourful signals might indicate female fecundity as observed in birds and fish. In blue tits, *Cyabistes caeruleus*, for example, female colouration was positively correlated with clutch size, fledgling success and recruitment (Doutrelant et al., 2008), and the size of the yellow belly patch of female lagoon gobies, *Knipowitschia panizzae*, was an indication of quantity of eggs relative to that female's own body size as well as absolutely (Massironi et al., 2005).

In many species, especially in amphibians, also females can carry very conspicuous colourations. Despite that these traits in females have long been regarded as non-adaptive, genetic correlations to male ornaments (reviewed in Kraaijeveld et al., 2007; Lande, 1980; Nordeide, 2002), an increasing number of studies suggest that female ornamentation can actually be important in male mate choice, and might have evolved through such (Clutton-Brock, 2009). Assuming female colouration can be an honest indicator of fecundity, different studies demonstrated male preferences for female ornamentation (e.g., Amundsen et al., 1997; Baldauf et al., 2011; Griggio et al., 2005). Females of the two-spotted goby, *Gobiusculus flavescens*, for example, have yellow-orange bellies during the breeding season and males prefer colourful over drab females (Amundsen and Forsgren, 2001).

The aforementioned examples demonstrate that mate choice is not necessarily unilateral even in species with otherwise conventional sex roles. As a consequence, choosers might not solely make decisions about which courters to mate with and can be the object of choice themselves, leading to mutual mate choice. The strength of preference might, however, be context dependent and can take on dynamics that differ quite strongly from one another (Rosenthal, 2017). One determinant of the degree of choosiness can be mate availability (Kokko and Monaghan, 2001); the choosy sex might, hence, be sensitive to sex-ratio. For example, females of pot-bellied seahorses, *Hippocampus abdominalis*, normally choose males. In populations with a female biased sex-ratio, however, males were found to be choosy (Mattle and Wilson, 2009). Such a sex role variation, however, might also occur temporal. The two spotted goby, *Gobiusculus flavescens*, was found to shift sex roles during breeding season (Forsgren et al., 2004). In the beginning of the breeding season, mature females are rarer and males actively court females. Later in the season, males become unavailable and female courtship as well as female-female agonism increases drastically.

The simplest form of mutual mate choice is, however, reciprocal preference. Reciprocal mating can take on the form of mutual rejection where males for example will reject non receptive females who would, in turn, reject the male anyway and preferentially respond to females displaying receptivity (e.g., Appelt and Sorensen, 2007; Clutton-Brock, 1989), or they respond context-dependent to a female's behaviour, terminating courtship when females respond aggressively (e.g., Stuart-Fox and Whiting, 2005). Lastly, reciprocal preference can, however, also be expressed in the form of assortative or disassortative mating (Rosenthal, 2017).

Since mutual mate choice is more difficult to analyse than one-way choice and can take on such diverse forms, detecting and investigating these various types of choice systems is therefore a crucial task for empiricists to better understand evolutionary patterns.

Aims of the Study

Although mate choice research has focused on male ornamentation, there is a growing number of studies investigating the role of female ornamentation even in systems with otherwise conventional sex roles (e.g., Amundsen, 2000; Svensson, 2006). Previous work on female ornamentation focused on birds (Amundsen et al., 1997), fish (Baldauf et al., 2011) and insects (Cotton et al., 2010), occasionally reporting a male preference for particularly ornamented females (e.g., Amundsen and Forsgren, 2001; Cotton et al., 2015). However, the field lacks empirical investigations of male mate choice and the quality of female ornaments in other taxa, especially amphibians.

Both sexes of the Alpine newt, *Ichthyosaura alpestris*, have bright orange ventral sides. The intensity of this colouration varies naturally between individuals within a population (Kopecký and Šichtař, 2014). Previous studies have shown that this visual characteristic is important for partner recognition and the initiation of courtship behaviour by the male (Himstedt, 1979). Studies also suggested that newts enter the breeding season with a rather fixed number of spermatozoa since they complete spermiogenesis before hibernation and store mature sperm until the next breeding season (Verrell, 1986; Verrell et al., 1986). These preconditions made the Alpine newt a great system to test hypotheses regarding female ornamentation and male choice.

The main aim of this dissertation is to analyse the variation in female belly colouration in relation to male mating decisions, and to determine a possible signalling content of that colouration. This dissertation is divided into three chapters. The first two provide data on male mating behaviour. The first one particularly investigates male mating preferences based on visual indicators, while the second one determines whether males predominantly mate when they can visually assess their mates. The final (third) chapter investigates the effect of female belly colouration on fecundity.

Finally, by studying a relatively uncommon model system, I wanted to broaden the horizon of our knowledge in this field. Specific species information will help to increase the generality of theories on the function of female colouration and male mate choice in species with traditional sex roles.

"*One might ask where the generality lies in studying a peculiar system. In fact, systems like these can help test the generality of the theory. Unusual species shed light on those universal principles governing evolution in general, and the relationships between the signalling and the physiological benefits [...].*" (cited from Svensson, 2006; p. 16)

Study Species – The Alpine Newt

Ecology

The Alpine newt, *Ichthyosaura alpestris* (Salamandridae), is a medium-sized (approx. 7–11 cm) aquatic salamander inhabiting Central Europe with Germany at the centre of its distribution (Berger and Günther, 1996; Thiesmeier and Schulte, 2010). Despite its common name, it can also be found in lowlands, where it can be quite abundant, as well as in higher altitudes. The highest place of discovery lies at 2500 m above sea level (Grossenbacher, 1988, cited in Thiesmeier and Schulte, 2010). It occurs in or close to bodies of water of both natural and anthropogenic origin. Preferred by the newts are, however, stagnant, cooler, and 'fish-empty' waters that are close to or within broadleaf and mixed forests. Temporary puddles or water-filled tracks made by heavy vehicles are also frequently used for breeding (Feldmann, 2007).

Although the Alpine newt is regarded as an aquatic salamander, it spends most of the year in a terrestrial phase on land (9–10 months). The active terrestrial phase is, however, only 4 to 5 months, whereas 5–6 months are spent hibernating. Therefore, the aquatic stage only lasts for 2–3 months (Blab and Blab, 1981; von Lindeiner, 1992). Nevertheless, this is the most active phase of the newt's year, during which they breed and lay eggs.

The age structure of the Alpine newt in natural populations can be quite variable depending on the habitat (reviewed in Thiesmeier and Schulte, 2010). In higher altitudes, the average age of a population is higher than in lowlands. The same applies to the life expectancy. Whereas newts in lowlands were found to be on average 2–5 years old and reach a maximum age between 6–10 years (Perret et al., 2003), observations from higher altitudes suggest that they were on average about 14 years old and can reach a maximum age of up to 29 years (cited in Grabherr et al., 2009).

Alpine newts will not reach maturity with a specific age but rather with a certain developmental stage that strongly correlates with a newt's size and body mass (Miaud et al., 2000). However, in optimal conditions, they can become sexually active with 2–3 years of age.

Ornamentation

The Alpine newt is sexually dimorphic (see Fig. 1 A). Not only do males tend to be smaller than females, they are also characterised by a high proportion of blue colouration on the dorsal side, especially during the breeding period. In addition,

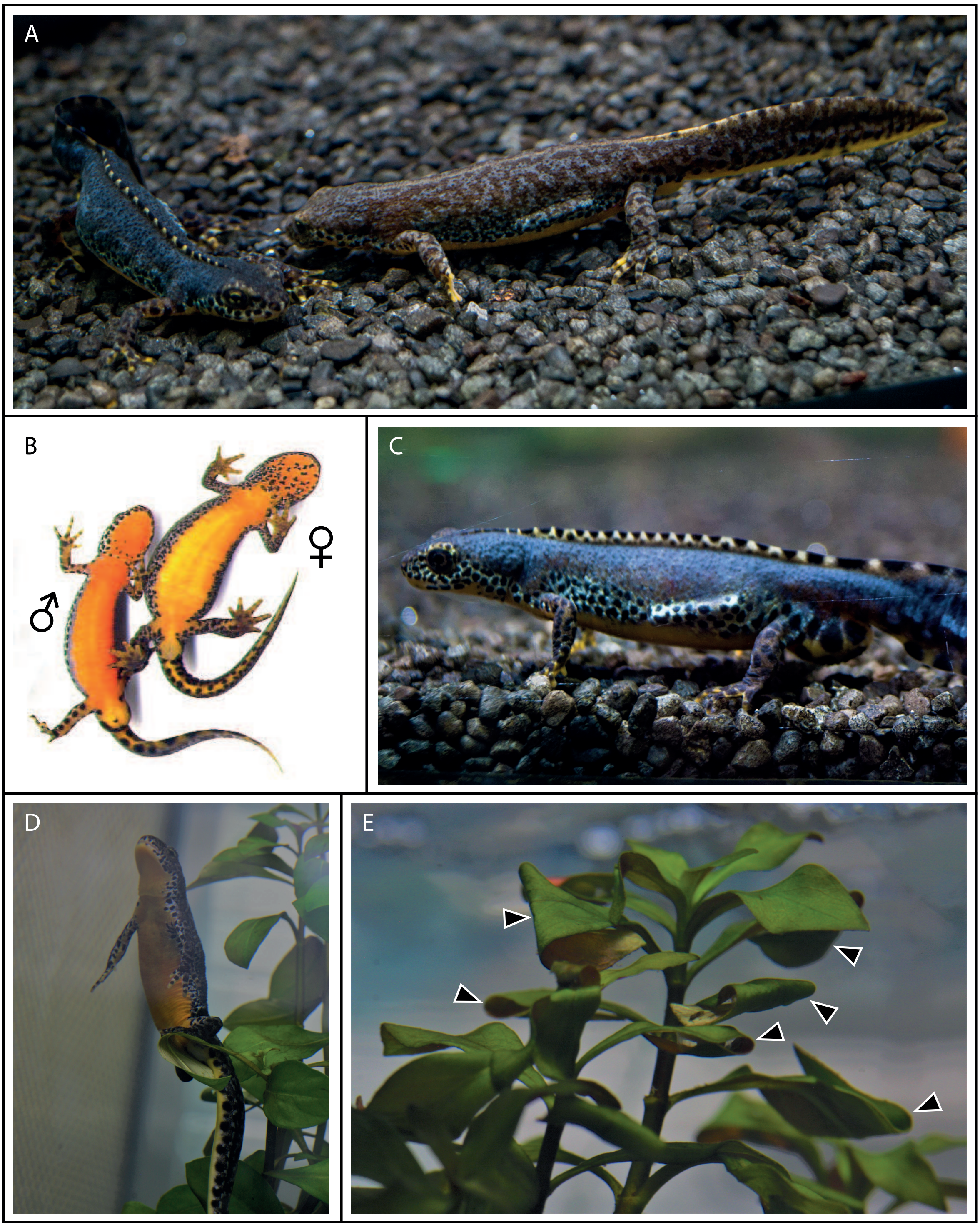

Fig. 1 A) Alpine newt pair during courtship. The male (left) has a small, yellow and black striped dorsal crest and a higher proportion of blue colouration; the primary colour of the female (right) is brown to grey. B) Bright orange ventral sides of a male (left) and a female (right). C) Alpine newt male with its distinct breeding colouration and a transponder inserted subdermally in the lateral part of the abdomen for individual recognition. D) Female Alpine newt during oviposition with a leaf folded between her hind legs. E) Water plant with deposited eggs, individually wrapped into leaves. Arrows indicate folded leaves with eggs inside. Picture B) by Uwe Manzen.

males develop a large cloacal protuberance and a small yellow-black striped dorsal crest. Females, on the other hand, are more inconspicuous with a light brown to grey dorsal side that can be slightly marbled.

The ventral side of both males and females is distinct from the dorsal side, with a bright colour ranging from pale yellow to bright red or dark orange (see Fig. 1 B). However, colour intensity of the belly varies naturally between individuals within a population (Kopecký and Šichtař, 2014). Although more pronounced in males, the belly is bounded by a white to silvery coloured band spotted with many black dots (Fig. 1 C).

Vision

Because this dissertation investigates visual characteristics as sexual signals, it is important to consider the current knowledge about the visual system of the species under study.

Like humans, the retinas of urodele species contain rod cells that are especially sensitive in low lights, and cone cells, responsible for colour vision (Möller, 1951, as cited in Himstedt, 1972). One of the first investigations of colour vision in Alpine newts was carried out by Himstedt (1972). With a well-designed experiment based on the reaction towards a moving prey dummy projected on a surface in different colours and contrasts, he found that Alpine newts can recognise and distinguish all investigated colours between 457 and 635 nm (human colour vision is approximately between 400−700 nm). The reactions were, however, strongest towards red and blue dummies.

Despite the lack of detailed investigations on the spectral sensitivity in Alpine newts, inferences can be drawn from investigations on a close, sympatric relative, the Smooth newt, *Lissotriton vulgaris*. The eyes of Smooth newts contain "red" (rhodopsin/porphyropsin) rods (Rh1) with an absorption maximum of 499/523 nm, and several types of cones: small single cones, large single cones and double cones (Korenyak and Govardovskii, 2013). Double cones and large single cones are red-sensitive (LWS) with an absorption peak at 609 nm. Single cones can either be blue-sensitive (SWS2) with an absorption maximum of 470 nm, or UV-sensitive (SWS1) with an absorption maximum of 340 nm. Correspondingly, newts are able to perceive UV light as well as light that is visible to the human eye.

In addition, another study found that the specific colour pattern of female Alpine newts is important for the male to recognise a female and to initiate courtship behaviour (Himstedt, 1979). With the aid of differently coloured dummy models, the study found that the key stimulus for partner recognition was indeed a red belly, confirming the importance of visual stimuli.

Sex roles and mate preference

Male Alpine newts search and court females actively, and females are the ones that determine the progress of the courtship and the transfer of spermatophores (Halliday, 1977). Studies suggest that females select their mates by appearance, display and odour. Since crest height is condition dependant in some newt species (e.g., Baker, 1992; Green, 1991), females were hypothesised to prefer males with larger crests. Additionally, olfaction takes a high priority in newts. During courtship, males secrete courtship pheromones into the water that they convey towards the courted female. These pheromones have been suggested to enhance female receptiveness, and additionally to be important for species recognition and function as sophisticated reproductive isolating mechanisms (Malacarne and Giacoma, 1986). Another study indicated that females base their mating decisions on the degree of genetic relatedness investigated through such pheromones (Garner and Schmidt, 2003). In a mate choice experiment with two males and one female, and subsequent genetic analyses, paternity was influenced by parental genetic similarity and eggs were more likely to be sired by the less-related male. The authors concluded that female choice is based on a genetic dissimilarity rather than on condition-dependent traits. On the other hand, both males and females were observed to orientate themselves towards another mating pair in selection trials (Belvedere et al., 1988). This observation illustrates that both sexes might search for potential mates.

Further, males were also found to be guided by visual aspects and discriminate between sexes. As mentioned before, preference trials with female-dummies revealed that a key stimulus for the initiation of male courtship behaviour was a red belly (Himstedt, 1979). If the red colour was missing or present on the dorsal side of the dummy, males did not react. If the dummy also showed male attributes such as yellow, white and black spots typical for males together with the red belly, males did not respond either. In accordance with the aforementioned reports on the orientation towards mating pairs, males were also found to interfere in the ongoing courtship of such pairs (Verrell, 1988). Especially in smaller bodies of water, with a higher density of individuals, multiple males might court a female at the same time. During an interference, a male rival would approach a pair and would start to display typical courtship behaviour either towards the female or the first male. Whether males actively choose to interfere in the courtship of certain females or if they always interfere once given the chance, is unclear. Since females play the decisive role in whether a spermatophore is picked up, the Alpine newt has been previously considered to be a conventional sex role species only (Thiesmeier and Schulte, 2010). To my knowledge, this dissertation presents some of the first research investigating whether males choose to court certain females over others or if they allocate differential effort towards various females based on phenotypic characteristics of those females.

Courtship and oviposition

Alpine newts mate polygynandrously in scramble competition. At the beginning of the breeding period, all reproductive newts enter bodies of water and males have continuous access to receptive females. As it is in all European newt species, the mating behaviour of the Alpine newt is stereotypic and can easily be detected. During this time consuming ritual, three main phases can be distinguished (see Fig. 2): the orientation phase, the display phase and the spermatophore transfer phase. However, during the whole period of time, a close and coordinated interaction between the male and the female is essential.

The courtship starts when a male encounters and approaches a female. He places himself in front of her to claim the full attentiveness of the female. Then the display starts in the form of a fanning movement of the male's tail (for a detailed description, see also Halliday, 1977 and Arntzen and Sparreboom, 1989; and for a video, the supporting information by Treer et al., 2013). This movement creates a water current to fan courtship pheromones towards the female's nose (Halliday, 1977; Houck, 1986; Sever, 2003). Occasionally, the male performs a so-called catbuckle and lean-in movement, where he raises his body by extending his legs, creating a catbuckle-like arched back, and leaning in towards the female. During this movement, the male might touch the female's snout with his flanks or tail. The effect of this movement was proposed to be an accentuation of the male's crest (Arntzen and Sparreboom, 1989). The female, in turn, might prod the male on the flank or even fan back at him. However, only if the female stays attentive will the male start to creep away. When the female follows and touches the male's tail, he deposits a spermatophore, a gelatinous package containing sperm, on the substrate. The male then guides the female directly over the spermatophore, blocks her way and occasionally pushes her back. The spermatophore then attaches to the female's cloaca. For a successful insemination, however, the female has to touch the spermatophore with no other body parts than the cloaca.

At any time during this whole process, the male might retreat and terminate when the female is not interested or he can backtrack to any stage of the ritual. Nevertheless, the whole process may also be repeated several times, resulting in the deposition of multiple spermatophores. In laboratory observations, males deposited up to nine spermatophores during one undisturbed mating event (Maag, 2013).

On the other hand, since the male can only vaguely guide the female over the spermatophore, many spermatophores are not successfully attached to the female's cloaca. Under laboratory conditions, only few spermatophores are normally successfully transferred (33 %: Denoël and Doellen, 2010; 31 %: Halliday, 1990). In nature, females might actually pick up even fewer spermatophores, because the substrate might be uneven, which makes the guidance of the female even more difficult, and disturbances by conspecifics or other organisms are more likely.

The loss of spermatophores, however, represents a substantial cost for the Alpine newt male, because it is likely that he enters the breeding period with a limited number of sperm. The Smooth newt, *Lissotriton vulgaris*, a close, sympatric relative of the Alpine newt, for example, completes spermiogenesis in autumn during the terrestrial phase (Verrell et al., 1986). Mature sperm is then stored until the next breeding period in spring. Additionally, other studies also report that sperm numbers might decrease in consecutively deposited spermatophores (Eastern newt, *Notophthalmus viridescens*: Takahashi and Parris, 2009; Alpine newt: Maag, 2013). On top of that, strong sperm competition is also present since females mate with multiple males and store the received sperm of these males in the spermatheca (*receptaculum seminis*), a special storage organ in females, after a successful insemination.

Soon after the first few matings, females start to lay eggs and become less responsive to male courtship attempts (Verrell and McCabe, 1988). In nature, a female can lay between 100−500 eggs (Miaud, 1994), but even so, females lay their eggs singly on supports such as aquatic plants (Berger and Günther, 1996; Thiesmeier and Schulte, 2010). To do that, a female grasps a support (e.g., a water plant leaf) with her hind legs and bends it in a fold (Fig. 1 D). She then deposits an egg and tightens the fold. Adhesive substances at the surface of the egg secure it to the support (see Fig. 1 E). This process can last a few minutes (Díaz-Paniagua, 1989). Apart from that, there is no further post-oviposition parental care.

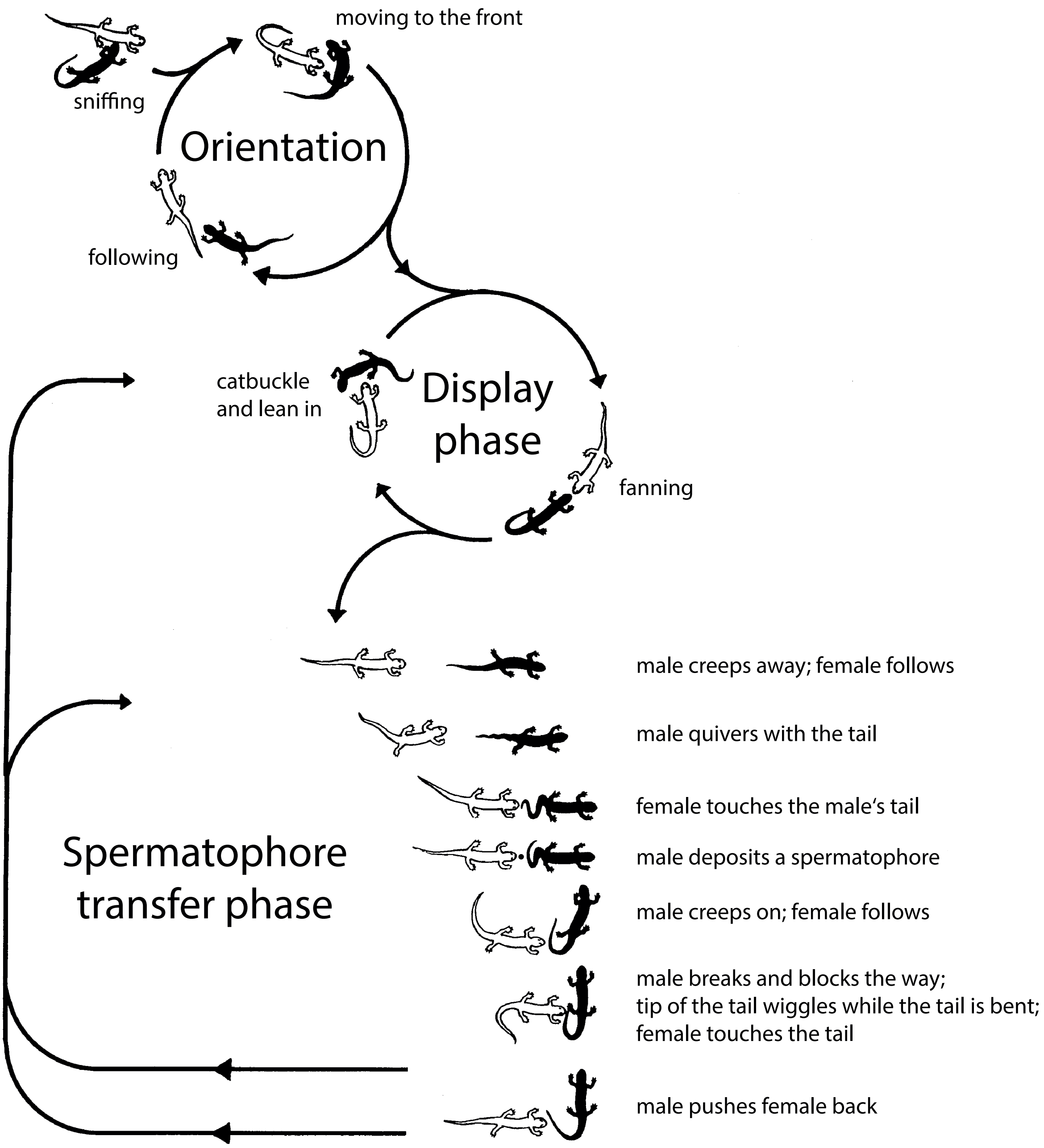

Fig. 2 Schematic courtship sequence of the Alpine newt. The male is depicted in black; the female in white. (after Arntzen and Sparreboom, 1989; and Thiesmeier and Schulte, 2010). For a detailed description of the ritual, see the text.

Methods

This chapter includes a general overview of the husbandry conditions I used during this project and a summary of the methods I applied to conduct the experiments for the three different investigations.
All work involving newts was carried out in the Comparative Zoology group of the Institute of Evolution and Ecology at the University of Tübingen, Germany (48°32′11.6″ N, 9°02′11.3″ E).

Laboratory newt population

All newts that were used for the different behavioural observations were part of the established lab population from the Comparative Zoology group at the University of Tübingen. The founder population consisted of 243 wild individuals (125 females and 118 males) that were caught between 2011 and 2013 in two forests from the surrounding area of Tübingen; the Schönbuch forest (48°34′55.1″ N, 9°02′01.4″ E) and the Rammert forest (48°26′25.19″ N, 8°59′44.48″ E). The remaining individuals all hatched in the lab between 2011 and 2016 and are descendants of the founders and the lab-reared individuals.
For most of my investigations, however, I used only adult individuals that were ready to mate. For **Paper I**, I used individuals from the founder population ($N = 21$) and descendants that were born between 2012 and 2014 ($N = 71$). For **Paper II**, I used founder individuals ($N = 20$) and their descendants born between 2011 and 2014 ($N = 85$). For **Paper III**, I used only adult females ($N = 54$) that again included founder individuals ($N = 9$) and their descendants born between 2011 and 2014 ($N = 45$).

Husbandry and types of aquaria

Each phase in the life cycle of a newt demands different types of aquaria and terraria. In addition, every investigation also had its own demands on husbandry, the size of tanks, tank equipment and group constellation.

Terrestrial phase

During their terrestrial phase, newts were held in large polycarbonate (PC; Makrolon) tanks (61 × 43.5 × 21.5 cm). The tanks were filled with a small layer of pebbles which were then covered with a fleece liner. This allowed for a bit of ventilation and prevented ponding water. The tanks were then equipped with soil to cover the ground, and with bigger lumps of soil, moss, foliage and tree bark to create hiding places. The tanks were regularly sprayed with water to keep them moist. Newts were held at a maximum of 15 individuals per tank in mixed groups of males and females. Photoperiod was set to a light:dark cycle of 12:12 h.

Before and after hibernation, the temperature was regulated to 14−16 °C. To simulate the winter, the room temperature was first gradually reduced to 5 °C in intervals of 1 °C approximately every three to four days in the beginning of December. The newts then hibernated at 2−5 °C for approximately 6−8 weeks. In February, the temperature was gradually increased to the original temperature in intervals of 1 °C every 2 days to simulate the start of spring.

Transitional phase

For the first transitional phase (from terrestrial to aquatic), newts were sex separated and transferred to aquaria (60 × 60 × 38 cm) after hibernation. The aquaria were equipped with black gravel, a brick and a large onshore platform (34.5 × 23 cm) just above water level. The platform was equipped with soil, moss and tree bark.

The water level started at a few centimetres and was increased gradually by 2 cm every day until it reached approximately 20 cm. Room temperature was regulated between 12−18 °C, depending on the outside temperature, and the light:dark cycle was constant at 12:12 h. After the final water level was reached, a water plant, *Ludwigia repens*, was added to the aquarium.

The procedure for the second transitional phase (from aquatic to terrestrial) was similar to the first one in reversed order. During this time, newts were held in mixed groups of males and females. The aquaria and onshore platforms were equipped in the same manner as previously described. The water plant was removed and the water level was reduced every day by 2 cm. When the newts stayed permanently on land, they were transferred to the terrariums for the terrestrial phase.

Aquatic phase

After the newts had fully transitioned into the aquatic phase, home aquaria (60 × 60 × 38 cm) were filled with approximately 72 litres, i.e. 20 cm in depth, of clear filtered water. The aquaria were equipped with black gravel, a brick with many holes and water plants. Before each investigation, I kept males and females in separate aquaria. However, after each of the investigations, I transferred them back into their home aquaria and held them in mixed groups of males and females. I kept the newts at 15−18 °C room temperature and a light:dark cycle of 12:12 h.

For the investigations for **papers I** and **III**, I used custom designed aquaria (60 × 60 × 38 cm) with three compartments (see Fig. 3). Each compartment was approximately 60 × 20 cm. The compartments were separated by narrow strips of Plexiglas dividers at the bottom, and perforated metal dividers on top. While the strips of Plexiglas enabled visual contact between neighbouring compartments, the metal grids allowed the exchange of water and especially olfactory cues. For **Paper I**, I made use of the ability to remove those dividers and to thus bring two newts together. Water height was approximately at 20 cm. I equipped each compartment of the aquaria with black gravel, a hiding place (PVC tube; 10 cm length, 2.5 cm ⌀) and a platform above the water level (Fig. 3 A). I also added a 5 cm strip of green tape in the back of each compartment to have a reference distance for the behavioural test (see below). For **Paper III**, I additionally added a water plant (*Ludwigia repens*) to each compartment (Fig. 3 B).

For **Paper II**, I used a large group aquarium (250 × 60 × 60 cm) filled with approximately 300 litres of clear-filtered water (Fig. 3 C). The bottom of the aquarium was again covered with black gravel. To provide a more natural environment, I also added some foliage. I additionally equipped the aquarium with four bricks with many holes and PVC tubes, which the newts both used for shelter, ten water plants and swimming tree bark.

Types of food

In nature, the diet of the Alpine newt is quite diverse and depends on the respective availability of food resources and the season of the year (reviewed in Thiesmeier and Schulte, 2010). In their aquatic phase, newts feed mostly on chironomid larvae and pupae (Chironomidae) but also on small copepods, Trichoptera and other Coleoptera larvae, along with amphibian eggs and tadpoles. Even cannibalism of newt eggs and larvae is not uncommon (Denoël and Andreone, 2003). The diet during their terrestrial phase is not as thoroughly investigated. However, single observations provide evidence for the consumption of mainly insects and their larvae, worms, maggots, arachnids and isopods (Thiesmeier and Schulte, 2010).

General diet in the lab

During their aquatic phase in the lab, newts received red mosquito larvae (Chironomidae; Poseidon – Aquakultur, Ruppichteroth, Germany) and water fleas (*Daphnia sp.*; own breeding) *ad libitum* three times a week. In their terrestrial habitat during both the transitional and the terrestrial phases, they also received additional sludge worms

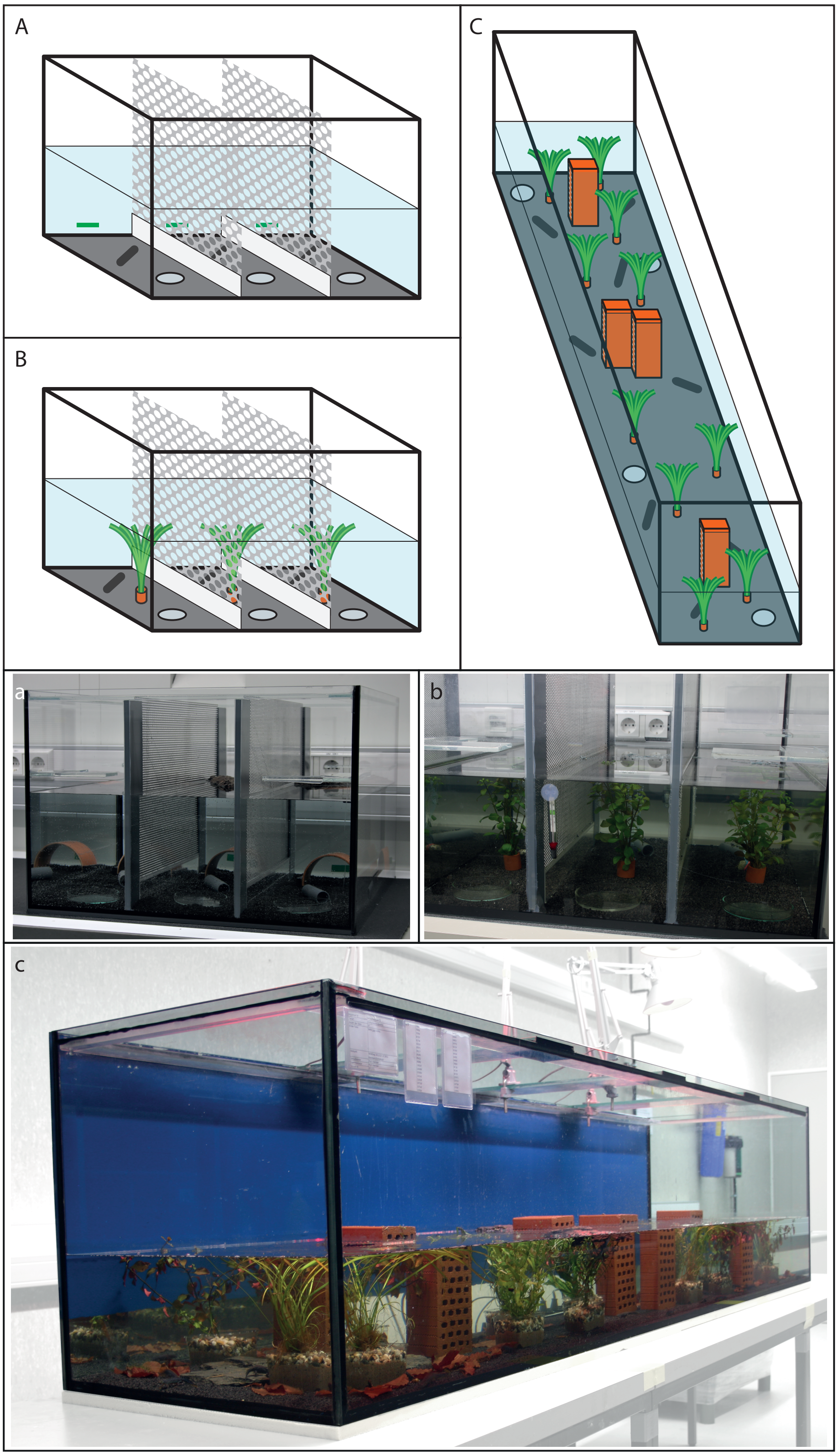

Fig. 3 Types of experimental aquaria. A) Aquarium for the mate choice experiment (Paper I) with removable dividers. B) Aquarium for the egg laying experiment (Paper III) with an additional water plant, *Ludwigia repens*. C) Large, group aquarium for the observation of mating activity (Paper II). Capital letters show the schematic structure; lowercases show the actual setup. For more detail, see the text. Schematic graphics by Tobias Haas.

(*Tubifex tubifex*) three times a week. Once the newts fully transitioned to the terrestrial phase, they received micro crickets (b.t.b.e Insektenzucht GmbH, Schnürpflingen, Germany) once a week, in addition to the sludge worms three times a week.

Carotenoid supplementation

Preliminary analyses on related newt species, the Japanese newt (*Cynops pyrrhogaster*) and the red-spotted newt (*Notophthalmus viridescens*), showed that the red ventral colouration might be caused by the presence of a relatively high carotenoid content (Forbes et al., 1973; Matsui et al., 2002). Additionally, carotenoid supplementations were shown to increase colouration in fish (e.g. Liu et al., 2014), birds (reviewed in Hill, 2006) and other amphibians (Steinicke, 1976).

Therefore, I also aimed to enhance the red ventral colouration of the Alpine newts through supplemental feeding. Prior to the first experiment (**Paper I**), female newts from that experiment were randomly divided into two groups. I assigned females from the first group to receive colour-enhancement treatment and used the other females from the second group as controls. For the colour-enhancement, female newts received carotenoid-enriched water fleas. To obtain the carotenoid enrichment, water fleas were raised in a solution containing lutein and β-carotene *ad libitum*. The control females received untreated water fleas raised in a clear solution.

This treatment, however, did not have an effect on the intensity of the ventral colouration.

Measurements and individual recognition

All newts that were part of the lab population received a passive RFID transponder (1.41×9 mm) with a weight of 0.035 g, which corresponds to approximately $0.6-1.5\%$ of a newt's mass. Usually in the second year of each newt's life, the transponder was inserted subdermally in the lateral part of the abdomen (see Fig. 1 C). To do that, the newts were anaesthetised using 2-phenoxyethanol. They were bathed in the solution for approximately $5-10$ minutes or until they fell asleep. In addition, when the newts were under anaesthesia, snout-vent length (SVL) and snout-tail length (STL) were measured to the nearest millimetre.

The determination of the weight always happened directly before the start of the respective investigation with a scale. Lastly, to obtain a standardised measurement of standardised body condition for **Paper I**, I divided each newt's weight by its SVL or STL, respectively.

Spectrophotometry of belly colour

A large part of this dissertation relies on an accurate quantification of colouration. Therefore, I used a method to objectively quantify the colouration of the ventral side of each newt. In all investigations including colour, I measured the reflectance from 380 to 700 nm (**Paper I & III**). I used a spectroradiometer (SpectraScan PR 670, Photo Research Inc., Syracuse, NY, USA) in combination with two repro lamps (R95, OSRAM) as the sole light source. To reduce the stress for the newts caused by handling, I used a table with a hole specially designed to measure the newts from underneath. I then placed one newt at a time into a petri dish that was placed directly over the hole so that the belly faced downwards. I secured the newt with a sponge cut into shape and applied light pressure from above. The spectroradiometer and the repro lamps were mounted underneath the table with the lens 45 cm from, and facing, the hole and the newts' belly (Fig. 4).

I measured reflectance in the middle of a newt's belly and in the middle of its throat. To compare results, I also measured the reflectance of a polytetrafluoroethylene (PTFE 98) white reflectance standard (Berghof Fluoroplastic Technology GmbH, Eningen, Germany). I then calculated the reflectance of the two regions of the ventral side relative to the standard with the program Spectrawin 5.0 (Avantes, Apeldoorn, The Netherlands).

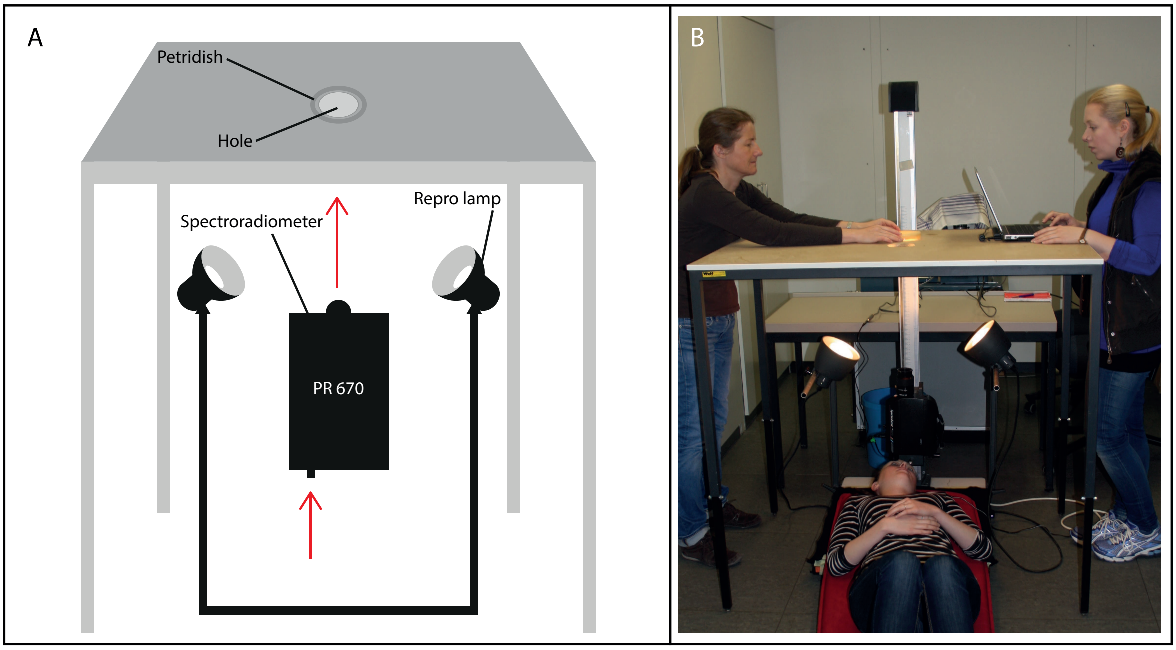

Fig. 4 Setup for the spectroradiometric measurements A) schematic and B) in practice. Schematic by Beck, 2013; modified by Tobias Haas.

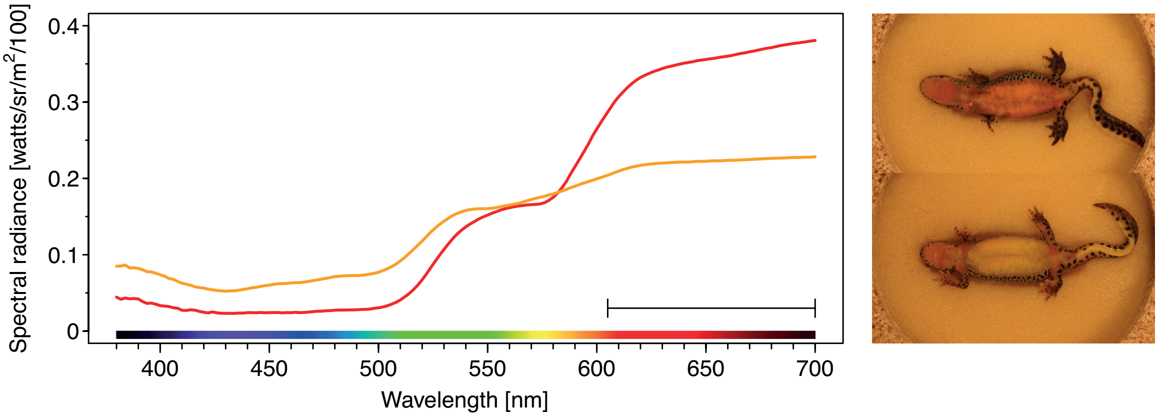

Fig. 5 Example of the difference between a yellow and a red female. Corrected spectral curve of two bellies (left). Red curve shows the spectral radiance of a "red" or dark orange individual (top picture); yellow curve shows the radiance of a yellow individual (bottom picture). Bar shows the red region from 605 to 700 nm for the calculation of the red chroma value.

Since the ventral side of the newts ranges from yellow to dark orange (see Fig. 5), I was interested in how red a newt's belly was. To obtain a comparable value, I calculated chroma (spectral purity) in the red region of each reflectance curve/spectrum from 605 nm to the maximally measured wavelength (700 nm; indicated by the bar in Fig. 5) using the R package *pavo* (Maia et al., 2013). Chroma describes the relative colour saturation and is calculated as the reflectance sum over a wavelength range of interest (in my case the red range) divided by the total reflectance sum (Montgomerie, 2006). In **Paper III**, I additionally calculated hue (peak wavelength) as the wavelength where reflectance is at its maximum.

To obtain a measurement that describes the belly in its entirety, I averaged the two red chroma values and the two hue values obtained for each newt. In the following, I refer to the mean as the chroma or the hue of a newt's belly, respectively. For the experiment in **Paper I**, I divided the females into two different colour groups: a red colour group with higher red chroma values and a yellow colour group with lower values. In **Paper III**, I used chroma and hue as a continuous variable.

Experimental procedure

Mate choice experiment (Paper I)

For the preference trials, I placed one male in the central compartment and one female from the red group and one from the yellow group into the neighbouring compartments (see experimental aquaria Fig. 3 A). To start a trial, I removed one of the dividers. The male and only one of the females at a time were then able to come into contact. I defined a male coming into contact with a female when his snout was within 5 cm of the female's body and he continued to approach her. If he then started to fan towards her, I recorded the trial as executed mating behaviour and observed for 45 min. If the male did not start fanning 15 min after the first contact, I recorded the male as disinterested and terminated the trial. In addition to the parameters indicative of male mate preferences, I also recorded female reactions to control for any behavioural differences.

To test whether males react to female colour rather than to other signals, I also performed preference trials under manipulated light conditions. I covered all light sources with colour-effect filters (colour 115 "Peacock Blue", LEE Filters) to exclude longer (red) wavelengths from 600 to 750 nm. Red objects (and belly skins) cannot reflect red light and thus appear grey.

Temporal mating activity (Paper II)

To investigate the mating activity in a more natural situation throughout the day, I observed mixed groups of males and females. I performed the observations in a large group aquarium (see experimental aquaria Fig. 3 C). I applied a scan-sampling method in which I recorded the total number of associated mating pairs at specific time points throughout the entire day. I considered a male and a female as a mating pair only when the male showed clear stereotypic courtship behaviour and the female was attentive towards that male.

Counting eggs (Paper III)

Several studies have investigated oviposition in the lab using artificial supports (e.g., Miaud, 1993, 1994; Orizaola and Braña, 2003; Thomas et al., 2002). However, in a pilot investigation, I found that, when given the choice between natural plants and artificial plastic supports, newts preferred to oviposit on the natural plants (pers. obs.). Hence, I provided females with natural plant supports (*Ludwigia repens*; see experimental aquaria Fig. 3 B) to investigate fecundity. Each female obtained one plant at a time. Every four to seven days, I removed the previous plant and provided a new one. I counted the number of eggs a female laid by carefully removing each egg from the plant with forceps. I scanned the plant a second time on the next day in case I missed an egg before.

Results and Discussion

This thesis investigates the courtship behaviour and mating activity of males and one potential signalling content of the females' belly colouration in Alpine newts, *Ichthyosaura alpestris*. The first two papers address different aspects of male mating. In the first paper (**Paper I**), I examined, in an experimental setup, the courtship behaviour of males towards females that vary in belly colouration and responsiveness, while the second paper (**Paper II**) focuses on mating activity throughout the entire day, including in light and dark conditions. For the final paper (**Paper III**), I investigated fecundity in females that vary in belly colouration. I will first summarise and discuss the main results of all investigations and show supplementary graphics in addition to the graphs in the different papers. I will then further discuss overall implications and potential future directions.

Variation in female belly colour

Animal ornaments that seem most striking and are clearly visible in our surroundings are of yellow, orange and red colours. Such colours in animals normally contain carotenoid or pteridine pigments (e.g., Griffith et al., 2006; Weiss et al., 2012). The intensity of these colours has been shown to be positively correlated with pigment concentration in the tissue (e.g., Barber et al., 2000; Weiss et al., 2012). In previous investigations, both pigment classes (carotenoids and pteridines) were found in the belly skin of Alpine newts (Günder, 1954) and other newt species (e.g., Matsui et al., 2002; Obika, 1963). To investigate potential individual quality variation in female Alpine newts related to colour, I measured the reflectance of the orange belly, a potential nuptial ornament. Although redness is not as pronounced as it is in males, females also tend to vary from yellowish to dark orange in their belly colouration (see Fig 5). To objectively quantify variation in the redness of individuals, I calculated the red chroma value. In all investigations, I found a rather pronounced variation in belly chroma (see **papers I** and **III**). This suggests that females allocate different concentrations of colour pigments to their skin. Since both compounds (carotenoids and pteridines) enhance immune responses in animals (Chew and Park, 2004; Hõrak et al., 2004; Huber et al., 1984), this could indicate that more colourful individuals might have an advantage in coping with oxidative stress and are hence better able to allocate their resources to traits not related to immune functions.

In the studies included in this dissertation, female red chroma was not correlated with weight, body condition or age in either of the experiments. However, there was a relationship between red chroma and snout-vent length in both investigations that included colour (**papers I** and **III**). This might suggest the possibility that redder females are generally larger in this population.

Male mate choice experiment (Paper I)

Male Alpine newts are most likely limited in the amount of sperm they can expend in one mating season. They complete spermiogenesis in autumn and store mature sperm until the next breeding season as described in previous investigations for the closely related Smooth newt, *Lissotriton vulgaris* (Verrell et al., 1986). When courting a single female, males may sequentially deposit several spermatophores (Halliday, 1977; Maag, 2013; pers. obs.), and each male courts a large number of females if they are available to him (pers. obs.). However, the amount of sperm in male Alpine newts has also been found to decrease in successive spermatophores (Maag, 2013; pers. obs.). This limited mating capacity might have promoted the evolution of male mate choice in this species. In **Paper I**, I therefore investigated whether male Alpine newts exert mate choice for female colouration.

For the experiments, I divided females into two experimental groups according to their belly chroma (yellow and red). These two groups did not differ in body condition (Lüdtke and Foerster, 2018; Fig. 6 A), but were significantly different in the chroma of the belly (Lüdtke and Foerster, 2018; Fig. 6 B). During the experiments, a male was able to court a female from the yellow group and one from the red group in succession under natural and manipulated, colour-deprived light conditions. As hypothesised, males courted red females over a longer period, and hence invested more time, than when courting females from the yellow group (Lüdtke and Foerster, 2018). If females in this species reflect individual quality through the colour of the belly, this could have favoured the evolution of male choosiness. An adaptive response would hence be that males invest a greater proportion of time in courting a red female to increase the chances of securing her as a mate.

Male preference in this study was, however, also dependent on the female's reaction. Yellow females that indicated their interest by tail fanning received similar courtship investment from a male as red-bellied females. If a female from the yellow group did not signal particular interest by fanning, males courted over a shorter period of time. In contrast, female interest signalled through tail-fanning was not decisive for the time a male spent courting females from the red group. This suggests that selection favoured males that express a more persistent courtship for colourful females with

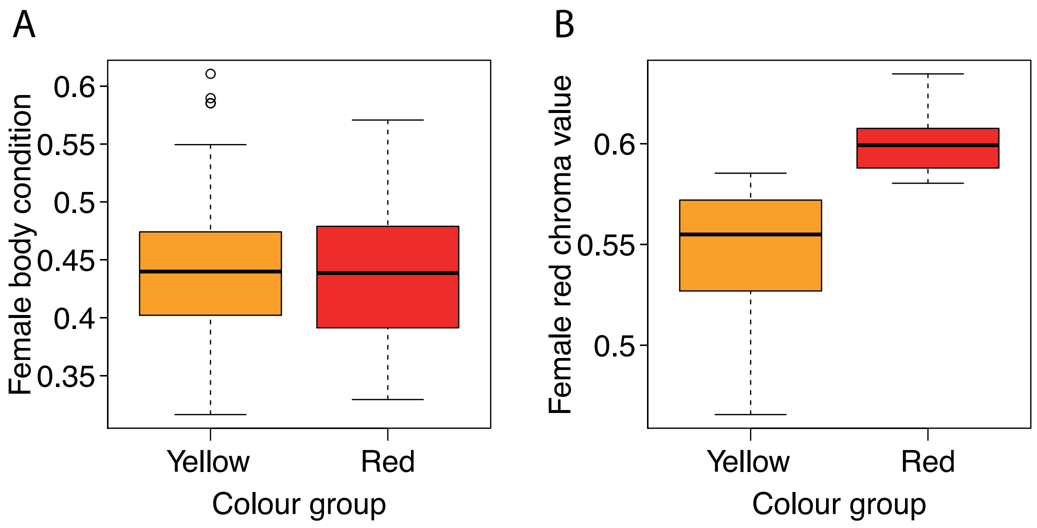

Fig. 6 Differences between the two female colour groups for A) body condition, measured as weight divided by snout-vent length; and B) red chroma. The top and bottom of the boxes represent the 25th and 75th percentiles with the median represent as the thick lines; the whiskers extend to the most extreme data points $\leq$1.5 times the interquartile range from the box. Outliers are shown as separate data points. $N = 23$ in each group.

their putatively high reproductive value. On the other hand, less colourful females were able to compensate for their lack of colouration by being more responsive to the males. In addition, I further observed that males generally reduced their courtship investment and number of spermatophores when females showed more disinterest by turning away from the male irrespective of the colour group. Because of the necessity of a close coordination of both partners and the costs of losing spermatophores, it seems adaptive for a male to reduce mating efforts for disinterested females.

Finally, male mate preference did not differ under the manipulated, blue-light treatment in the absence of chromatic indicators, where females were indistinguishable based on colour alone. Female snout-vent length also explained differential courtship time investment by males. Since belly colouration correlated with body size in our test population, the males' decision to invest courting time might also have been based on female size. Nevertheless, not all information on female quality was transferred through size since snout-vent length could not explain a differential investment of spermatophores in this study. This suggests that females potentially indicate their individual quality through multiple indicators and males might additionally base their choice on other cues that correlate with female belly colour, such as pheromones.

Temporal mating activity (Paper II)

During courtship, male Alpine newts use a combination of visual and olfactory signals (Belvedere et al., 1988; Himstedt, 1979). Previous studies on the mating behaviour have shown that visual signals were more frequent during light conditions, and olfactory signals were more frequent during dark conditions (Denoël and Doellen, 2010). These studies reported equally high mating activity during light and dark conditions. Nevertheless, mating in dark conditions might be less favourable because the close coordination of the movements of both mating partners is essential for a successful spermatophore uptake, and this coordination may improve with visual signals. In addition, males have an increased potential to assess differences in female colouration during daylight hours. To investigate whether males predominantly mate during conditions that are optimal to assess visual indicators, I describe in **Paper II** the temporal pattern of mating activity in a large group aquarium with a 12:12 photoperiod over multiple days. In this experiment, males had continuous access to mating partners throughout the entire day during light and dark conditions.

Although most amphibians normally mate at night (Hartel et al., 2007), I observed a diurnal pattern in Alpine newts (Lüdtke and Foerster, 2019a). In accordance with my expectation, mating mainly occurred in light conditions as opposed to dark conditions, and the animals almost continuously used that time for mating. Light conditions, therefore, seemed to be most favourable for them because both sexes are able to see and assess each other, and males are able to guide and coordinate the females with visual signals during their courtship ritual. In less optimal (dark) conditions, males courted less and might use that time for other activities, such as resting and feeding.

Another factor influencing the lower mating activity during dark conditions might be that females preferably lay their eggs at night (Morgenthaler, 1955; pers. obs.), which makes them less available for mating during that time. Females lay their eggs by individually wrapping them into submersed aquatic plant supports (Miaud, 1994, 1995; pers. obs.). This process can last a few minutes for each egg (Díaz-Paniagua, 1989; Miaud, 1993) during which the female might be especially vulnerable to predation. Dark condition might provide them with better camouflage among the plants and therefore transfer a selective advantage.

Additionally, despite less matings in dark conditions, I found that mating activity was somewhat higher during the first night compared to the following days. During the first dark phase, males might still have been more eager to find mating partners than in the following dark phases. This result also highlights the importance of observing behaviours over a longer period of time in order to identify specific patterns. A previous finding claiming that mating activity is equally high during both light and dark phases was exclusively based on observations of the first encounter of a male and a female in either dark or bright treatment (Denoël and Doellen, 2010). I concluded that this would be an indication that Alpine newts will start to court as soon as they encounter potential mates for the first time, but reduce efforts during unfavourable conditions after a few courtships.

In summary, I confirmed in this study that males predominantly mated during light conditions when they are potentially able to exert the preference on visual indicators.

Investigation of female fecundity (Paper III)

Traditionally, conspicuous female ornaments were considered to merely genetically correspond to the male ornaments (Lande, 1980). However, a growing number of investigations report that specific female colourations can also function as a signal to advertise a female's quality (e.g., Doutrelant et al., 2008; Massironi et al., 2005) and thus indeed have a selective advantage (reviewed in Kraaijeveld et al., 2007).

In **Paper III**, I investigated the relationship between belly colouration and fecundity (number of laid eggs). I first compared the chroma values of the females in the lab population to 36 wild females that were captured in the population where our founder individuals originated from. Although captive amphibians are known to be less colourful as conspecifics found in the wild (Frost and Robinson, 1984; Matsui et al., 2002; Steinicke, 1976), I did not want to include individuals in these investigations that were more than 5 % outside the natural occurring range and possibly show an unnatural colouration. I suspected that females outside the lower end of this range were possibly unable to store certain pigments in their skin. I found three females that were unusually faint (chroma < 0.525) and excluded them from the analyses (Fig. 7). I also excluded females that laid less than 20 eggs.

Among the females that were included in the study, belly chroma as well as hue was positively correlated with number of eggs (Lüdtke and Foerster, 2019b). More orange females laid more eggs than less orange ones, suggesting a direct benefit for males that mate with more orange females. By choosing to court more orange females, males gain an adaptive advantage through a potentially higher number of fertilised eggs. There was no relationship between belly chroma or hue and the duration of egg laying. In other words, although more orange females laid more eggs, they did not need more time to deposit them.

The investigation of the daily cumulative egg numbers could confirm that more

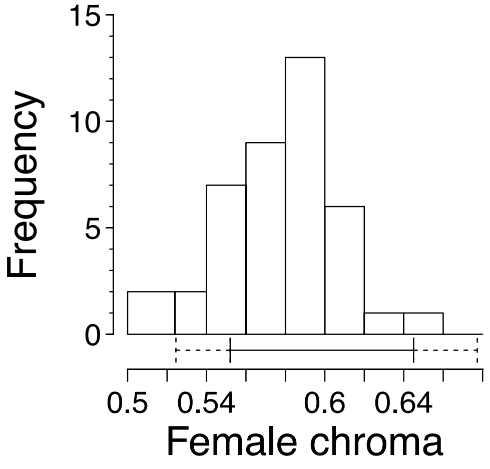

Fig. 7 Distribution of red chroma values in laboratory females from Paper III. The bars show the range of a natural population (solid line; chroma: 0.552−0.645) and the 5 % buffer zone (dashed line). Two females outside the buffer zone were excluded from the analyses.

orange females laid their eggs faster than less orange ones. As a result, more orange females ceased egg laying at a similar time as less orange females. Female Alpine newts may be selected to lay their eggs rather quickly for two reasons. First, European newts spend most of their time on land (Taylor and Guttman, 1977) and they enter bodies of water only during the breeding period. To ensure that the larvae have enough time to metamorphose and leave the water before the next terrestrial phase starts, it would be advantageous to lay eggs quickly after insemination. By doing so, they are enabling a high number of hatchlings to be born early enough to get a head start in their development compared to other competing larvae in the pond. Second, female newts compete for access to laying substrate (small leafed water plants), and newt larvae compete for food in crowded breeding ponds. In both cases, it is probably advantageous to be among the first competitors present. For the same reasons, males would profit when they mate with a female that lays her eggs quickly.

Lastly, I did not find any correlation between female weight and number of eggs, nor did larger females with a higher snout-vent length lay more eggs. There was also no indication that heavier nor larger females laid their eggs faster.

In conclusion, this study provides experimental evidence that the belly colouration of female Alpine newts can be an important signal to determine individual quality. This result can thus explain the previous finding that males preferred more orange females.

A sexually selected female ornament?

Adult Alpine newts, *Ichthyosaura alpestris*, like many other newt species, show a distinct anti-predator or defence behaviour known as the "Unkenreflex" (Kupfer and Teunis, 2001). During this behaviour, newts arch their bodies so that their forelegs and the front of their body are lifted from the ground, displaying their orange ventral side as an aposematic signal to the attacker. This can be an indication that the orange belly is influenced by natural selection.

On the other hand, the fact that the ventral colouration might act as an aposematic signal and the assumption that it plays a function in sexual selection are not mutually exclusive (reviewed in Briolat et al., 2019). Many aspects indicate that the female belly colouration is still subject to sexual selection. Firstly, I found a rather strong variation in female belly colouration, as already reported by previous studies (Kopecký and Šichtař, 2014). An explanation for such a variation of a presumable warning signal can be that alternative selection pressures act upon the same signal. If that is the case, the signal actually serves more than one function (Briolat et al., 2019) and selection pressures may act at different strengths at the same time. Here, sexual selection would maintain ornament variation. In contrast, natural selection alone would disfavour variation in that specific ornament and favour monomorphism, because large variation would decrease learning speed of a predator and, at the same time, increase mistaken attacks by such predators (Mallet and Joron, 1999).

Secondly, I could show that males preferred females with more orange ("red" colour group) bellies. I assume that males are able to evaluate this signal and do so, since previous studies have shown that specifically the red belly of female Alpine newts is a key stimulus for partner recognition (Himstedt, 1979). When females swim or relax in vegetation, males can see both the orange belly and the throat from below. In addition, males approach females very closely during courtship and occasionally prod the female's flanks and cloaca, which enables them to investigate the colour. Further, I could confirm that males predominantly use conditions in which they are potentially able to see and assess colourations.

Lastly, to substantiate the assumption that female belly colouration is subject to sexual selection, my results revealed that female colouration is related to individual fecundity and males might indeed gain fitness advantages when they base their choice on a female's colouration.

Males that mate with a more orange-bellied female have the opportunity to fertilise more eggs. This can explain why males spent more time courting those females. In addition to the number of eggs, female belly colour could potentially also advertise other benefits such as the quality of those eggs. For example, colourful females could produce eggs of higher quality by incorporating specific contents into the eggs, as shown for the striped plateau lizard, *Sceloporus virgatus*. In that species, concentration and total amount of yolk antioxidants was positively correlated with the female's ornament colour (Weiss et al., 2011). This suggests that colourful females might also produce eggs of higher quality, provide better yolk content for the early growth phase of embryos, and potentially give rise to healthier offspring. Whether similar mechanisms apply to Alpine newts is not known and remains to be tested.

However, belly colour was positively correlated with female body size, and males also seemed to prefer larger females. Therefore, the males' decision to invest courting time might also have been based on female size or potentially on other cues that also correlate with colour. Despite this correlation, there was no indication that larger females produce more eggs, suggesting that female size does not indicate fecundity in this species. Female size could nevertheless correlate with other aspects of reproductive value, such as the size of the eggs. Larger eggs would presumably also produce larger larvae, as shown for spadefoot toads, *Spea multiplicata*. Such a relationship could explain the male preference seen in this species, because larger body size would greatly affect the larvae's survival and hence their parents' fitness. Whether female size advertises such components of reproductive value in Alpine newts remains to be tested.

Future directions

While the aim of this thesis was to investigate the role of the female's belly colouration in a context of male mate choice, there are still many open questions. Apart from fecundity, colouration could potentially also signal additional aspects of individual quality. In addition to the already mentioned relationship between female colouration and yolk antioxidants found in striped plateau lizards, a study on two-spotted gobies, *Gobiusculus flavescens*, found similar results. The authors observed that orange belly colouration of females positively correlated with carotenoid concentrations of the eggs (Svensson et al., 2008, 2006). Investigating egg content of differently ornamented Alpine newt females could therefore provide further insight into the adaptive role of choosing to mate with a more orange female. Although carotenoids and pteridines are suggested to have a positive effect on immunocompetence (e.g., Britton, 2008; Oettl and Reibnegger, 2002), the question whether the offspring of Alpine newts will also be of better quality is not yet known and could be determined by observing larval mortality and developmental rate. In addition, further empirical investigation is needed concerning how costly the female's belly colouration is to produce and to maintain.

Another interesting factor indicating mate quality is body size. In many species, males prefer to mate with larger females (e.g., Bonduriansky, 2001; Eddy et al., 2016; Wong and Svensson, 2009). As my investigations unveiled no relationship between female size and number of eggs, concentrating on egg and larval sizes might reveal further reasons as to why males might prefer larger females in Alpine newts. Especially because males also exerted choice under manipulated light when colour differences were putatively indistinguishable, there might be the possibility that multiple signals can act as indicators for mate choice (Møller and Pomiankowski, 1993). This can either take the form of multiple signals indicating different quality aspects or multiple signals indicating the same quality aspect (reviewed in Candolin, 2003). Therefore, if one of these indicators cannot be evaluated by the choosing individual, multiple indicators might function as back-up signals that step in and reduce mate choice errors.

Due to the unique mating biology of newts in general, a very close coordination between both partners is necessary for a successful spermatophore transfer. Hence, females have the decisive role during courtship and females are also expected to choose based on male quality indicators (e.g., Andersson, 1994; Rosenthal, 2017). My results further confirmed that the female's responsiveness explained male choosiness. However, to date, data on assortative mating in newts is absent from the literature. Future work on the mating biology should therefore include detailed observations on female responses in male mate choice as well as observations of mating partners in conditions that are more similar to natural situations (i.e. in large groups of males and females) over extended time periods.

Finally, the question remains as to whether male choice has an impact on the females' fitness, a topic that needs further empirical consideration.

Conclusion

This thesis provides evidence for female ornamentation selected through male mate choice in a non-model species. I provided detailed experimental evidence that female belly colouration can function as an honest signal for individual quality in Alpine newts. This can explain the evolution of the male preference for redder females, because males would benefit when they choose to mate with such females. I described the advantage to predominantly mate during light conditions when males can see and assess females according to their visual indicators.

I further discussed the possibility that females potentially signal their reproductive value through body size and other, yet unknown cues, which offer males substitutional routes for evaluating a female. I also showed that the responsiveness of females could further explain male choosiness. Although female responses and correlations with colouration suggest further traits on which males could base their choice, my results nevertheless provide solid evidence that male mating effort responded to variation in female belly colouration.

I therefore conclude that the female's belly colouration is an honest female ornament used in male mate choice in this species.

The results of my thesis, together with previous work on ornamentation and male choice, endorses the generality of colourful ornaments that signal benefits and that are therefore used in mate choice.

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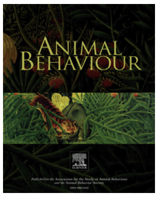

Choosy males court both large, colourful females and less colourful but responsive females for longer

Deike U. Lüdtke*, Katharina Foerster

Department of Comparative Zoology, Institute for Evolution and Ecology, University of Tübingen, Tübingen, Germany

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Male mate choice based on female secondary sexual traits is increasingly reported, even in species with otherwise conventional sex roles. However, empirical evidence of male mate preference remains scarce for vertebrate species other than birds and fish. Here we examined male mating investment in response to female coloration in the Alpine newt, *Ichthyosaura alpestris*. In our experiment, males had a choice between females that varied in the redness of the belly (red versus yellow). We recorded five parameters indicative of male preference, and the female's response. Additionally, we performed these preference trials in different light conditions (control and blue filter) to test whether male choosiness depends on the female's colour signal. We found that males adjusted their mating investment according to the encountered female. Males courted red females longer than yellow females, regardless of the red female's response. Nevertheless, if yellow females were responsive, males invested as much courtship time as for red females. Males might have a guaranteed insemination with responsive females regardless of belly colour. In addition, redder females might provide quality advantages when an insemination is successful. We further detected that less colourful males tended to invest more spermatophores for red females than males that were colourful themselves. Both effects, however, were evident in both light conditions. Since larger females had redder bellies, body size and belly colour may confer similar information about female quality. Taken together, our results provide evidence that males choose a specific female coloration and size, but also reveal potential behavioural compensation mechanisms by less colourful females to obtain similar male effort. Our findings are thus consistent with the idea of mate choice based on colourful ornaments but highlight that interactions may be complex and multiple cues as well as female responses should be considered when investigating male mate choice.

The sex that invests more in each reproductive event and is less available for mating (because of the higher investment) is expected to be choosy (Bateman, 1948; Trivers, 1972). In many cases, this is the female, while males typically compete over access to those females (Jennions & Petrie, 1997; Trivers, 1972). However, male investment in reproduction has often been underestimated and mating can also be constrained or costly for males (Bonduriansky, 2001; Kasumovic, Bruce, Herberstein, & Andrade, 2007; Wedell, Gage, & Parker, 2002). Although the costs of producing a single sperm cell can indeed be neglected, the costs of producing unlimited sperm, full-size ejaculates or spermatophores cannot (Dewsbury, 1982; Wedell et al., 2002). It has been shown that male fertilization rates decrease and sperm stores become depleted with an increase in mating frequency (Nakatsuru & Kramer, 1982; Preston, Stevenson, Pemberton, & Wilson, 2001). In other words, if the number of receptive females exceeds the number a male is capable of inseminating, then that male should choose particularly fertile females to maximize reproductive success (Bonduriansky, 2001). Taking that to the extreme can lead to a complete reversal of sex roles in courtship and mate choice. Male choosiness is also expected to evolve to some extent even in systems with conventional sex roles, when males are limited in their mating capacity and females vary in quality (Gwynne, 1991; Kokko & Monaghan, 2001). If females exert mate choice too, this results in mutual mate choice. Mutual mate choice seems to be the rule even in highly polygynous species (Rosenthal, 2017). The strength of preference might, however, be context dependent and can take on very differing dynamics. Under a high availability of fertile females, male choosiness is likely. On the other hand, if the encounter rate of females is low and mate choice is sequential, male choosiness might fail to evolve because the advantages of choice might not

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* Correspondence: D. U. Lüdtke, Department of Comparative Zoology, Institute for Evolution and Ecology, University of Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany.

E-mail address: deike.luedtke@uni-tuebingen.de (D. U. Lüdtke).

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compensate for the search costs (Barry & Kokko, 2010; Etienne, Rousset, Godelle, & Courtiol, 2014).

If females vary in quality, males are expected to base their choice on a more, or less, direct proxy of female quality (Cunningham & Birkhead, 1998; Servedio & Lande, 2006). In species without maternal care, any trait that affects a female's fecundity and the quality of her eggs would be relevant for choosy males. While little is known about potential male choice for female traits that may affect egg quality (e.g. female age, female health or oviposition site), traits that signal female fecundity have been shown to be under sexual selection through male choice (Clutton-Brock, 2009; Jones, Monaghan, & Nager, 2001). Body size could serve as a proxy for fecundity, since female body size and fecundity tend to be positively correlated (Honěk, 1993). Several studies have indeed confirmed a male preference for large female size (Bonduriansky, 2001). Male Australian desert gobies, *Chlamydogobius eremius*, for example, spend more time courting large females when offered a choice between a large and a small one (or adjust their mating effort towards a second female according to the size of a previously encountered one in sequential choice experiments; Wong & Svensson, 2009). Male red-legged salamanders, *Plethodon shermani*, also court more gravid or larger females for longer (Eddy et al., 2016).

Just as in female choice, males may base their choice not only on the size of females but also on female secondary sexual ornaments assuming they honestly signal reproductive value. Such relationships between female ornaments and reproductive value have been confirmed for birds (e.g. Doutrelant et al., 2008; Pilastro, Griggio, & Matessi, 2003), some fish (e.g. Kekäläinen, Huuskonen, Tuomaala, & Kortet, 2010; Massironi, Rasotto, & Mazzoldi, 2005) and insects (e.g. Cotton, Small, Hashim, & Pomiankowski, 2010). In many polygynous species, females show colourful traits that might reflect their quality and thus represent easy-to-detect targets for male choice (Amundsen, 2000; Kraaijeveld, Kraaijeveld-Smit, & Komdeur, 2007). A few studies have demonstrated male preference for female ornamentation in species with otherwise conventional sex roles (Amundsen, Forsgren, & Hansen, 1997; Baldauf, Bakker, Kullmann, & Thünken, 2011; Griggio, Valera, Casas, & Pilastro, 2005). Females of the two-spotted goby, *Gobiusculus flavescens*, for example, have yellow-orange bellies during the breeding season and males prefer colourful over drab females (Amundsen & Forsgren, 2001).

Despite a good theoretical foundation of when to expect male mate choice (e.g. Clutton-Brock, 2009; Courtiol, Etienne, Feron, Godelle, & Rousset, 2016; Kokko & Monaghan, 2001), empirical evidence for male mate choice based on female secondary sexual traits remains scarce in vertebrate species with conventional sex roles, especially in taxa other than birds and fish.

In the present study, we tested whether the Alpine newt, *Ichthyosaura alpestris*, exerts male choice for female coloration. Males and females mate polygynandrously in a scramble competition. Males have comparatively high mating costs (Thiesmeier & Schulte, 2010): they invest in a long courtship ritual (Arntzen & Sparreboom, 1989; Halliday, 1977), during which close and coordinated interaction with the female is essential. Only when the female remains fully attentive and closely follows the movements of the male's tail tip does the male place a spermatophore on the ground. The male then guides the female directly over the spermatophore, which then attaches to the female's cloaca. For a successful insemination, the female has to closely follow the male and touch the spermatophore with no other body parts than the cloaca. Therefore, many spermatophores are not successfully attached to the female's cloaca and do not find their way to her reproductive organs, even in the undisturbed situation of staged mating experiments in an aquarium (Halliday, 1977; Thiesmeier & Schulte, 2010; Lüdtke & Foerster, personal observation). Males often have to repeat the entire courtship ritual and deposit several spermatophores to obtain a single successful mating. Even if a spermatophore is successfully transferred, sufficiently interested males continue courting, as there does not seem to be any reliable feedback for successful spermatophore transfer (Lüdtke & Foerster, personal observation).

We consider it likely that the production of spermatophores and their supply with sperm constitute a significant investment by Alpine newt males. Like many other temperate urodele species, the smooth newt, *Triturus vulgaris*, a close, sympatric, relative, completes spermiogenesis in autumn during the terrestrial phase (Verrell, Halliday, & Griffiths, 1986). Males then store mature sperm until the next mating period during spring. This suggests that sperm numbers are limited during the mating season. Indeed, spermatophore production has been found to be limited by intrinsic male factors in this species (Verrell, 1986). Finally, sperm numbers decrease in consecutively deposited spermatophores in the eastern newt, *Notophthalmus viridescens* (Takahashi & Parris, 2009), as well as the Alpine newt (Lüdtke, 2016; Maag, 2013). It is therefore likely that the Alpine newt experiences a constraint regarding spermatophore and spermatozoa availability. This constraint might hence have promoted the evolution of male mate choice in the Alpine newt.

Male mate choice, however, requires the male to discriminate between potential partners of varying reproductive value. Alpine newts of both sexes have colourful bellies ranging from pale yellow to bright red or dark orange (Thiesmeier & Schulte, 2010). Belly colour intensity varies naturally between individuals within a population. Red, yellow or orange coloration in animals is often based on carotenoids (Griffith, Parker, & Olson, 2006; Olson & Owens, 1998) or pteridines (Weiss, Foerster, & Hudon, 2012). Both carotenoids and pteridines are known to enhance immune responses and act as free radical scavengers in animals (e.g. Chew & Park, 2004; Huber et al., 1984). In addition, carotenoid supplementation increases fecundity in female red-eyed tree frogs, *Agalychnis callidryas* (Ogilvy, Preziosi, & Fidgett, 2012) and the pterin-based ornament of female striped plateau lizards, *Sceloporus virgatus*, is positively correlated with concentrations of yolk antioxidants (Weiss, Kennedy, Safran, & McGraw, 2011). Therefore, we predicted that the orange belly colour of the Alpine newt may act as an indicator of individual quality and thus as a putative secondary sexual trait. We can, however, only speculate in which way the orange belly signals a higher reproductive value of a certain female. It is possible that the above-mentioned mechanisms affect egg quality and consequently also offspring performance in this species.

Alpine newt males can see both the orange belly and throat when females swim. In addition, males approach females very closely during courtship and occasionally prod the female's flanks and cloaca. We thus assumed that males receive this colour signal and hypothesized that male mating effort responds to variation in female belly coloration. Specifically, we expected males to invest more resources in redder bellied females. In contrast, if a potential colour difference between females is removed, we expected no differential male investment in courting. Further, we hypothesized that the female's response to male mating effort modulates the male's investment, as the success of a courtship ritual depends critically on the behavioural coordination between male and female.

To test these hypotheses, we used mate preference trials to evaluate male mating investment. We presented males with one colourful (red) and one less colourful (yellow) female and recorded male courtship and mating effort towards each female and her responses. To test whether male choice still exists when the colour signal is removed, we conducted the same experiment under manipulated light conditions.

METHODS

Study Animals and Housing Conditions

We used adult newts from the established laboratory population at the University of Tübingen. This population included individuals that were caught from wild populations close to Tübingen, Germany, in 2011 and 2012, as well as their descendants born between 2012 and 2014. After they had emerged from hibernation and prior to the experiments, we kept males and females in separate aquaria (60 × 60 cm and 38 cm high) with ca. 40 litres of clear filtered water at 15–18 °C room temperature and a light:dark cycle of 12:12 h. Each aquarium contained a maximum of 15 newts and was equipped with black gravel, hiding spots, water plants and an onshore platform. We fed red mosquito larvae (Chironomidae) and water fleas (*Daphnia* sp.) ad libitum three times a week. We regularly exchanged some water between the male and the female groups, to simulate the presence of individuals of the opposite sex and stimulate mating behaviour.

Supplemental Feeding and Colour Measurements

Prior to the experiments, we randomly selected half of the females and assigned them to a colour-enhancement treatment; the other half of the females were used as controls. We aimed at enhancing the red ventral colour through supplemental feeding. First, we raised water fleas in a carotenoid solution containing lutein and β-carotene ad libitum. These water fleas were then fed to the females in the colour enhancement group. The control group received untreated water fleas. Since the carotenoid treatment showed no effect on the red colour in females, we assigned females to the experimental groups (see below) based on their natural belly colour within each feeding group. Hence, we always kept the feeding groups separate.

We measured reflectance of the ventral sides of the newts from 380 to 700 nm using a spectroradiometer (SpectraScan PR 670, Photo Research Inc., Syracuse, NY, U.S.A.) with two repro lamps (R95, OSRAM) as the sole light source. We designed a table with a hole to measure the bellies from below. The spectroradiometer and the repro lamps were then mounted underneath the table with the lens 45 cm from, and facing, the hole. For each measurement, we put a newt in a petri dish with light pressure from above using a sponge cut into shape. We then placed the petri dish above the hole so that the belly faced towards the spectroradiometer.

We measured the reflectance of each newt in two different regions of the ventral side: the middle of the belly and the middle of the throat. Reflectance was calculated relative to a polytetrafluoroethylene (PTFE 98) white reflectance standard (Berghof Fluoroplastic Technology GmbH, Eningen, Germany) with the program Spectrawin 5.0 (Avantes, Apeldoorn, The Netherlands). We calculated the chroma (spectral purity) in the red region from 605 nm to the maximally measured wavelength (700 nm) of each reflectance spectrum using the R package pavo (Maia, Eliason, Bitton, Doucet, & Shawkey, 2013; Montgomerie, 2006). Chroma describes the relative colour saturation and is calculated as the reflectance sum over a wavelength range of interest (in our case the red range) divided by the total reflectance sum. The two red chroma values obtained for each newt were averaged and we refer to the mean as the chroma of that newt's belly.

General Observational Procedure: Colour Variation

We conducted all experiments from 12 April to 21 May 2016 on 45 males and 47 females. This was within the main mating period in the local wild Alpine newt population, which lasts from mid-March to early June (von Lindeiner, 1992; Thiesmeier & Schulte, 2010; Lüdtke & Foerster, personal observation). We performed 162 mate preference trials in aquaria (60 × 60 cm and 38 cm high, ca. 40 litres) with three compartments, with a male placed in the centre and two females placed on either side of him. The compartments were separated by narrow strips of Plexiglas dividers at the bottom to allow visual contact, and perforated metal dividers on top of that to allow water and especially olfactory cue exchange. We supplied all three compartments with black gravel, a hiding place and a platform above the water level. We always ensured that the hiding place and the platform were at the same distance to the neighbouring compartments.

We divided the females into two experimental groups according to their belly chroma. We ranked all females according to chroma value within each of the feeding groups (carotenoid-enriched or control). For each feeding group separately, we assigned half of the females with the highest chroma values to the red-bellied group and the other half with the lower chroma values to the yellow-bellied group. The range of chroma was 0.580–0.635 (control: 0.587–0.627; carotenoid: 0.580–0.635) in the red group and 0.466–0.585 (control: 0.466–0.585; carotenoid: 0.506–0.576) in the yellow group. We then paired females from the red and yellow groups within each feeding group so that pairs were approximately from the same rank in each colour group (difference between female partners: 0.051 ± 0.025).

Alpine newts do not reach maturity at a specific age but at a certain developmental stage, which is closely linked to their size and mass (reviewed in Thiesmeier & Schulte, 2010). To account for this and for potential fecundity, we weighed the females just before they entered their individual experiments. The two female groups did not differ in weight (red: 4.12 ± 0.60 g; yellow: 4.01 ± 0.62 g; $t_{157.98} = -1.152$, $P = 0.251$). We divided the weight by the respective snout–vent length to obtain a standardized measure of body condition. We measured snout–vent length when the animals were under anaesthesia. The two female groups were similar in average body condition (body mass/snout–vent length; red: 0.78 ± 0.09; yellow: 0.79 ± 0.09; $t_{43.00} = -0.790$, $P = 0.434$) but differed distinctively in the chroma of the belly (red: 0.600 ± 0.014; yellow: 0.549 ± 0.030; $t_{30.97} = 7.540$, $P < 0.001$). Additionally, the two groups also differed in snout–vent length (red: 5.27 ± 0.23 cm; yellow: 4.99 ± 0.25 cm; $t_{43.87} = 3.977$, $P < 0.001$). We provide all morphometrics of males and females in the appendix (Tables A1 and A2).

We first placed the male in the central compartment. Thereafter, we introduced the females (a pair with one female from the red group and one from the yellow group) into the neighbouring compartments. We randomized the positions of the red and yellow females in the experimental aquarium. Then we allowed the three newts to acclimatize for 36–46 h. In each trial, we opened one of the two female compartments by removing the Plexiglas and metal divider, while an opaque screen covered the other Plexiglas divider between the male and the second female (which remained separated from the other animals). Before each trial, we removed all objects from the male's and from the experimental female's compartments. We started the trial when the divider was removed, and we recorded the time until the male stepped into contact with the female. We then recorded the behaviour of the interacting newts (see below). Each trial lasted 45 min. We recorded five parameters indicative of male mate preferences: (1) latency to courtship was the time from trial start until the male's snout was within 5 cm of the female and the male stepped into contact with her; (2) latency to the first spermatophore was measured as the time from the beginning of courtship until the male placed the first spermatophore on the ground; (3) the number of spermatophores a male offered to a female during one trial; (4) courtship investment was measured as the proportion of time during one trial where the male showed interest in the female, i.e. stayed within 5 cm of her; (5) fanning duration was measured as the proportion of time a male fanned his tail from the time he showed courtship investment.

To control for any differences in female behaviour, we recorded two female reactions. We measured female disinterest as how many times a female turned away from the courting male. Some females occasionally fanned their tail during courtship, but this behaviour was much less frequent than male tail fanning. We thus recorded female interest as a binomial variable set to 0 if the female never fanned her tail during the trial and 1 if she fanned at least once.

After completion of the trial, we gently returned both newts to their initial compartment, slid the dividers back into place, replaced all objects in the compartments and removed the opaque cover from the other female's compartment. We then allowed the male to rest for ca. 48 h (range 36–57 h) before we started the second trial with the other female. We randomized the order in which males could court the females of the red and the yellow group. All observations were done by three teams of two observers each.

Light Reflectance Manipulation

To test whether males react to female colour rather than to other signals that may correlate with colour we performed an experiment in which we manipulated the ambient light ($N = 82$ trials). We covered the light sources in the two experimental rooms with colour-effect filters (colour 115 ‘Peacock Blue’, LEE Filters) to exclude longer (red) wavelengths from 600 to 750 nm. Red objects (and belly skins) cannot reflect red light and thus appear grey. The variation among females with differing ventral chroma was thus not visible to the males. As a control, we used two different neutral density (ND) filters (209 ‘0.3ND’ and 210 ‘0.6ND’, LEE Filters). ND filters reduce the light intensity without changing the colour spectrum. This reduction was slightly smaller than the light intensity reduction of the blue filter in one case (0.3ND), and slightly larger in the other (0.6ND).

Each male underwent four trials: two under blue light with one set of females (one each from the red and yellow group) and two under one of the control light conditions (0.3ND or 0.6ND) with a different set of females (one each from the red and the yellow group). The order of the light treatment for a specific male was randomized.

Every female was tested only once with the same male. However, all females participated in four trials, with four different males. After the experiments, we released all newts into their home aquarium.

Statistics and Data Analysis

We investigated the relationships between female red chroma and age, snout–vent length and female disinterest (mean over all trials) through Pearson correlations. We applied a generalized linear mixed model (GLMM, function glmer, package lme4; Bates, Mächler, Bolker, & Walker, 2015) with logit link function and binomial error distribution to test for the relationship between female chroma and female fanning. We included female ID as a random factor in the analysis to account for repeated measures. We also tested the relationship between whether a male started mating behaviour or not and the standardized date with a GLMM and included male ID as a random factor.

For the experimental data set, we excluded trials where males did not start to show any mating behaviour (32 cases). Male behaviours were measured in 130 trials. To analyse the latency to the first spermatophore we only included data in which a male deposited at least one spermatophore ($N = 109$ trials). We used arcsine-square root transformation for the courtship investment and fanning duration and a square root transformation for the latency to courtship to meet the criteria for a normal distribution.

We tested the effect of female group (yellow/red) and light manipulation on the different parameters of male courtship investment. For the dependent variables latency to courtship, courtship investment and fanning duration we applied linear mixed models (LMM, function lmer, package lme4; Bates et al., 2015). For the dependent variables latency to the first spermatophore and number of spermatophores we applied GLMMs (function glmer, package lme4; Bates et al., 2015) with log link function and Poisson error distribution.

We conducted repeated measures for males and females. To account for that, we included male ID, female ID and the other female's ID (partner ID) as random factors in all models; we likewise included the room to account for random variance between the two rooms. However, in all analyses the room explained either none or very little of the variance. Since the model of latency to the first spermatophore showed overdispersion, we included an observation level random factor in the model (Korner-Nievergelt et al., 2015).

We standardized all continuous variables so that their means were zero and their standard deviations one. To account for confounding conditional effects of the male, we included measures of the male's standardized body condition and the number of the trial in all models. Along with the light condition (filter), we also included date as a dependent variable and the interaction between filter and colour group. In addition to the female's colour group, we included her disinterest, her fanning behaviour and the interactions between the colour group and the two female behaviours in all models except for latency to courtship. Lastly, we included the number of spermatophores that a male had deposited before in the model for number of spermatophores (see also Table 1). Since we detected a correlation between female body size and coloration in our data set (see Results), we compared the Akaike information criterion (AIC) of models with either colour group or snout–vent length (keeping all other explanatory variables constant) to investigate which of these best explained variation in the data.

Before we fitted the models, we checked for collinearity between all covariates by inspecting the correlation coefficient and the variance inflation factors (VIF; Zuur, Hilbe, & Ieno, 2013). We used the function corvif in the HighstatLib.R file from the supporting information of Zuur, Ieno, and Elphick (2010). Since we did not find strong collinearity and all VIFs were below the cutoff of 3 (Zuur, Ieno, & Smith, 2007), we used all covariates in the same model.

We tested whether the two control filters had different effects on male behaviour. Since we did not find any differences between them (data not shown), we pooled the data and obtained a factor light condition with two levels for blue and control light. We also tested whether individual observers or the feeding group (whether females received carotenoid-enriched water fleas or not before the study) had an influence on the males' or females' behaviour. Since these two factors never explained significant variance (data not shown), we removed them from the models. Finally, we added male belly chroma and the interaction between male belly chroma and the female's colour group as explanatory variables to all models, to investigate any patterns of assortative or disassortative mating. This variable was only kept in the final model if it explained significant variance.

Otherwise, we decided to include all predictor variables in the models that we considered potentially important. We did not reduce the model to the most parsimonious ‘best’ model as this may lead to a bias in the parameter estimates (Whittingham,

Table 1
Mixed model analysis of male mate preference parameters

Dependent variable	Model	No. of trials	AIC	Fixed factors and covariates	2.5% quantile	Estimated effect	97.5% quantile
Latency to courtship	LMM	130	871.9	Intercept	16.352	18.558	20.650
				Date	**1.126**	**2.426**	**3.678**
				Filter (neutral density)	−3.769	−0.716	2.436
				Colour group (yellow)	−2.685	0.328	3.511
				Male standardized body condition	−1.341	−0.102	1.122
				Male trial	−1.965	−0.777	0.383
				Filter∗colour group	−5.836	−1.180	3.256
Latency to first spermatophore	GLMM	109	1268.7	Intercept	3.830	4.515	5.203
				Date	−0.238	−0.008	0.222
				Filter (neutral density)	−0.260	0.262	0.810
				Colour group (yellow)	−0.210	0.714	1.606
				Disinterest	−0.147	0.225	0.597
				Female fanning (yes)	−0.682	0.034	0.720
				Male standardized body condition	−0.154	0.056	0.265
				Male trial	−0.158	0.111	0.369
				Spermatophores before	−0.339	−0.093	0.149
				Filter∗colour group	−1.436	−0.636	0.171
				Colour group∗disinterest	−0.823	−0.256	0.306
				Colour group∗female fanning	−1.583	−0.679	0.260
Number of spermatophores	GLMM	130	388.8	Intercept	0.079	0.545	1.026
				Date	−0.337	−0.168	0.006
				Filter (neutral density)	−0.508	−0.108	0.276
				Colour group (yellow)	−0.868	−0.229	0.404
				Disinterest	**−0.541**	**−0.274**	**−0.009**
				Female fanning (yes)	−0.501	−0.010	0.488
				Male standardized body condition	−0.208	−0.054	0.097
				Male chroma value	−0.342	−0.153	0.036
				Male trial	**−0.444**	**−0.229**	**−0.023**
				Spermatophores before	**0.010**	**0.198**	**0.389**
				Filter∗colour group	−0.339	0.210	0.773
				Colour group∗disinterest	−0.410	−0.004	0.404
				Colour group∗female fanning	−0.561	0.088	0.759
				Male chroma value∗colour group	−0.002	0.285	0.573
Courtship investment	LMM	130	42.4	Intercept	0.854	1.023	1.193
				Date	**−0.152**	**−0.083**	**−0.013**
				Filter (neutral density)	−0.217	−0.102	0.011
				Colour group (yellow)	**−0.544**	**−0.334**	**−0.125**
				Disinterest	**−0.163**	**−0.090**	**−0.017**
				Female fanning (yes)	−0.164	0.002	0.167
				Male standardized body condition	−0.050	0.014	0.077
				Male trial	−0.055	−0.012	0.033
				Filter∗colour group	−0.011	0.150	0.312
				Colour group∗disinterest	−0.076	0.028	0.132
				Colour group∗female fanning	**0.055**	**0.265**	**0.485**
Courtship investment	LMM	130	41.2	Intercept	0.740	0.861	0.977
				Date	**−0.146**	**−0.076**	**−0.006**
				Filter (neutral density)	−0.113	−0.029	0.053
				Female snout-vent length	**0.031**	**0.122**	**0.214**
				Disinterest	**−0.131**	**−0.075**	**−0.020**
				Female fanning (yes)	**0.025**	**0.135**	**0.244**
				Male standardized body condition	−0.041	0.020	0.080
				Male trial	−0.049	−0.005	0.038
				Filter∗female snout-vent length	−0.144	−0.067	0.013
				Female snout-vent length∗disinterest	−0.038	0.012	0.063
				Female snout-vent length∗female fanning	−0.180	−0.076	0.025
Fanning duration	LMM	130	−150.0	Intercept	0.319	0.402	0.482
				Date	**−0.061**	**−0.030**	**0.000**
				Filter (neutral density)	−0.041	0.019	0.078
				Colour group (yellow)	−0.168	−0.071	0.026
				Disinterest	−0.033	0.002	0.038
				Female fanning (yes)	−0.087	−0.009	0.068
				Male standardized body condition	−0.023	0.006	0.035
				Male trial	−0.041	−0.019	0.003
				Filter∗colour group	−0.085	−0.001	0.083
				Colour group∗disinterest	−0.043	0.007	0.057
				Colour group∗female fanning	−0.011	0.089	0.192

AIC: Akaike information criterion. For the fixed factors filter (neutral density, blue), colour group (yellow, red) and female fanning (yes, no), the estimated effect is given for the indicated factor level compared to the other factor level. The presented quantiles limit the 95% credible interval. Significant effects are shown in bold and trends are underlined.

Stephens, Bradbury, & Freckleton, 2006). However, we compared all full models presented here with reduced models that did not contain any nonsignificant effects except colour group and light condition (not presented). We found that this model reduction approach did not lead to different conclusions, except for the model on number of spermatophores, and we refer to this in the Results.

We graphically assessed the goodness-of-fit of each model. We inspected residual distributions and their relationship with the fitted values and all the explanatory variables. We further checked model assumptions by plotting the quantiles of the residuals and of all random effects against the quantiles of a normal distribution.

We applied Bayesian methods to obtain 95% credible intervals (CrI) of the parameter estimates and model predictions (Bolker et al., 2008). By using 10 000 simulated random values obtained from the joint posterior distribution of the model parameter (function sim, r package arm; Gelman & Su, 2016) we were able to calculate the exact uncertainty of model predictions (Gelman & Su, 2016). We present estimated effects along with the 2.5% and 97.5% quantiles as the lower and upper limit of the 95% CrI of the parameter estimate. A parameter was judged to have a significant effect on the response variable if its 95% CrI did not cross zero. We made all calculations using R 3.3.2 (R Core Team, 2016).

Ethical Note

Maintenance and handling of the newts were conducted in accordance with German animal welfare law (Tierschutzgesetz) and FELASA guidelines. All experiments were approved by the local veterinary office 'Veterinäramt, Regierungspräsidium Tübingen', under the licence number ZO 5/15.

All newts in our laboratory population are individually marked with a passive RFID transponder (1.41 × 9 mm). Prior to this study (usually in the second year of each newt's life) the transponder was inserted subdermally in the lateral part of the abdomen. This was done under anaesthesia using 2-phenoxyethanol. The transponder weighs 0.035 g, which is 0.6–1.5% of the newt's mass.

After the experiments, we released all newts in mixed-sex groups into their home aquarium and provided extra water plants for egg deposition.

RESULTS

We found a correlation between female red chroma value and snout–vent length ($N = 46$, $r_P = 0.465$, 95% confidence interval (CI) = 0.202–0.666, $P = 0.001$). However, there was no relationship between red chroma values and age for individuals of known age ($N = 37$, $r_P = 0.054$, 95% CI = -0.275–0.371, $P = 0.753$). In addition, we found a correlation between female chroma and disinterest ($N = 46$, $r_P = 0.326$, 95% CI = 0.039–0.563, $P = 0.027$), but not between female chroma and female fanning (GLMM: $b = -0.241 \pm 0.261$, $z = -0.923$, $P = 0.356$).

In 80% of the trials, the male courted the experimental female (130/162; latency to courtship: mean ± SD = 5.98 ± 4.11 min, range 0.03–16.43 min; courtship duration: mean ± SD = 28.60 ± 11.51 min, range 0.78–43.13 min). In about 84% of these trials (109/130), males deposited at least one spermatophore (mean ± SD = 2.03 ± 0.96, range 1–5). In addition, males refused courtship behaviour more frequently later in the season (GLMM: $b = -1.068 \pm 0.247$, $z = -4.321$, $P < 0.001$).

Males showed a similar latency to start courting yellow or red females (red: 6.23 ± 4.32 min; yellow: 5.70 ± 3.87 min; Table 1). This behaviour was also not influenced by the light condition, the male's trial or his body condition. However, males started to court later as the mating season progressed (Table 1). In the first 10 days of the experiment, for example, males started courting on average within 4.48 ± 3.50 min. During the last 10 days, they started only on average after 8.22 ± 3.65 min. Using female snout–vent length instead of female colour group as the explanatory variable did not improve the model fit (data not shown) nor did it result in different conclusions.

In line with this, males also tended to deposit fewer spermatophores later in the mating season and they deposited fewer spermatophores the more trials they had passed (Table 1). During the first 10 days of our experiment, males invested on average 1.73 ± 1.14 spermatophores; during the last 10 days the average investment was only 1.17 ± 0.81 spermatophores. Males offered on average one spermatophore more to the female in the first than the last trial (first trial: 2.30 ± 1.33; last trial: 1.35 ± 0.94). On the other hand, males deposited even more spermatophores the more they had deposited in the previous trials. Additionally, the more disinterest females showed, the fewer spermatophores were offered to them. The males' own belly coloration tended to influence the number of spermatophores offered to females of the two groups (Table 1). Less colourful males offered more spermatophores to red-bellied females whereas colourful males offered similar numbers of spermatophores to yellow- and red-bellied females (Fig. 1). This weak indication of a preference for red females, at least among less colourful males, appeared irrespective of the light environment. The male's body condition and the fanning behaviour of the female did not affect the number of deposited spermatophores. Using female snout–vent length instead of female colour group as the explanatory variable reduced the model fit (data not shown) and there was no interaction between male chroma value and female snout–vent length.

Males exhibited a more pronounced preference for redder-bellied females through their courtship investment (Table 1). Males courted red females for longer, and hence invested more time, than when courting females from the yellow group. A significant interaction showed that this effect was more pronounced if the female did not signal any interest by tail fanning (Fig. 2). On

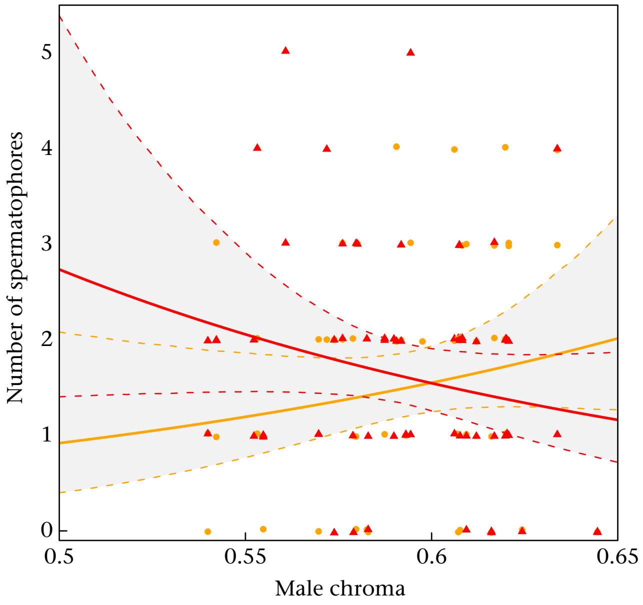

Figure 1. Number of spermatophores offered to yellow (yellow line) and red-bellied (red line) females in response to the male's own belly chroma (low values: less colourful; high values: more colourful). Yellow dots: raw data of yellow females; red triangles: raw data of red females. Fitted values (solid lines), predicted from a GLMM with log link function and Poisson error distribution, with 95% credible intervals (dashed lines) are given assuming average values for all other independent variables. $N = 130$ trials (63 control and 67 blue light), 62 yellow females and 68 red females.

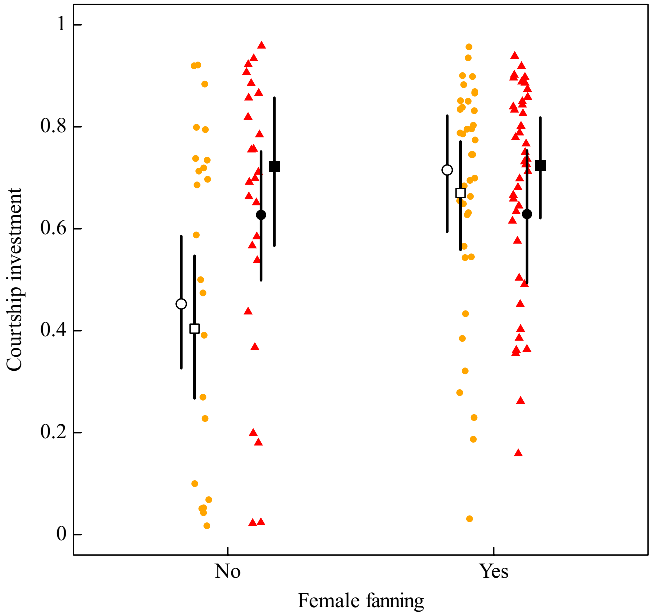

Figure 2. Courtship investment of males in yellow- (open symbols) and red-bellied (closed symbols) females that did or did not fan in response to the male's courtship. Courtship investment was measured as the proportion of time a male was interested in the female during one trial. Yellow dots: raw data of yellow females; red triangles: raw data of red females. Fitted values, predicted from an LMM, with 95% credible intervals are given assuming average values for all other independent variables, and for neutral density (control; circles) or blue (squares) light filters. $N = 130$ trials (63 control and 67 blue light), 62 yellow females and 68 red females.

average, males courted for 29.95 ± 10.44 min if they were in a trial with a female from the red group regardless of that female's response. For an interested female from the yellow group, a male invested on average 30.28 ± 10.26 min. In contrast, when the yellow female did not show interest by tail fanning, males courted, on average, for 22.12 ± 14.23 min. We observed this effect in both light environments, under the blue and control filters.

Irrespective of the colour group, males invested less time courting a female that showed more disinterest by turning away from the male. Males spent on average 31.31 ± 12.24 min courting the 10 least disinterested females. In contrast, males' courtship investment was on average only 19.62 ± 13.83 min for the 10 most disinterested females.

As already reported for the former two male behaviours, we found that males generally invested less time in courtship during the later stages of the mating season (Table 1; first 10 days: 29.64 ± 12.28 min; last 10 days: 26.65 ± 11.68 min). Male body condition and how many trials the male had been involved in before had no effect on the total time a male invested in courtship. In this case, a model using female snout–vent length instead of female colour group as the explanatory variable explained the data equally well (Table 1). Males courted on average for 20.56 ± 14.70 min for the four smallest females and 33.53 ± 6.22 min for the four largest ones. The interaction between female snout–vent length and their fanning behaviour, however, had no effect on courtship investment.

Males fanned equally long for red and yellow females, but they decreased their fanning duration over the breeding period (Table 1). For the first 10 days, males fanned on average almost twice as long for a female than for the last 10 days (first 10 days: 6.58 ± 4.84 min; last 10 days: 3.60 ± 2.33). The fanning duration also tended to decrease with successive trials (Table 1). Using female snout–vent length instead of female colour group as the explanatory variable did not improve the model fit (data not shown).

We found no effect of any of the tested explanatory variables on the latency to the first spermatophore (Table 1).

DISCUSSION

Male Alpine newts clearly tailored their courtship investment in response to variation in female belly coloration. Our experiments show that when males were offered the chance to court a red-bellied and a yellow-bellied female sequentially, they spent a greater proportion of time courting the red one. In addition, males tended to exert differential mate choice when investing in spermatophores. Here, males with less colourful bellies tended to invest more spermatophores in red-bellied females whereas males with colourful bellies tended to invest similar amounts of spermatophores in red- and yellow-bellied females. These findings indicate a male preference for female belly colour and that males can exert sequential mate choice. Alpine newts thus show mutual mate choice, since females of this species were found to exhibit choice as well (e.g. Garner & Schmidt, 2003; Hoeck & Garner, 2007). Alpine newts mate in scramble competition during a long mating season: courtship behaviour can be observed between March and early June in the local wild population (reviewed in Thiesmeier & Schulte, 2010). Both males and females have easy access to multiple mating partners and can express choice simultaneously and sequentially. Thus, potential partners can be compared without high searching costs or costs of lost mating opportunities. Along with usually high annual survival, these circumstances meet the major requirements for the evolution of mutual mate choice (Courtiol et al., 2016; Etienne et al., 2014; Rosenthal, 2017).

Colourful traits in many species reflect individual quality (e.g. Hill, 1991) and males are expected to benefit by mating with colourful rather than drab females (Pilastro et al., 2003). We do not know yet whether and in which way red Alpine newt females are of higher reproductive value. If redder females are generally larger in this species, as our data suggest, they could be more fertile and hence lay more eggs. However, redder females could also produce more viable eggs or provide better yolk contents for the early growth phase of embryos. In the striped plateau lizard, for example, the pterin-based ornament of females is positively correlated with concentration and total amount of yolk antioxidants, suggesting that the ornament advertises egg quality (Weiss et al., 2011). Whether a similar mechanism also applies to the Alpine newt remains to be tested. However, any of these effects could have favoured the evolution of male choosiness. An adaptive response would hence be that males invest a greater proportion of time in courting a red female to increase the chances of securing her as a mate. This may be especially true for males of lower intrinsic quality as might be indicated by the stronger preference for red females by less red males.

However, we also observed the same male response in a colour-deprived, blue-light treatment in the absence of chromatic cues. In addition to the colour signal, females may indicate their reproductive value to males in other ways such as subtle behavioural cues, olfactory signals (Johansson & Jones, 2007) or size. Since belly coloration correlated with body size in our test population, the males' decision to invest courting time might also have been based on female size. This is supported by our results, as female snout–vent length (but not female body condition) explained differential courtship time investment as well as female experimental colour group. Body size often correlates with fecundity and males in various taxa have been found to prefer larger females (e.g. insects: Bonduriansky, 2001; frogs: Dittrich et al., 2018; Liao & Lu, 2009; fish: Schlupp, 2018). Even among salamanders, male red-legged salamanders display their dancing courtship more than twice as much to larger females than to smaller ones (Eddy et al., 2016). Nevertheless, female body size could not explain the putative differential investment of spermatophores in our study. Here, a tendency under both natural and blue light conditions suggested that less red males invest more spermatophores when courting red females, but not when courting larger females. This implies that not all information on female quality was transferred through size, and that other cues that correlate with female belly colour may influence male mate choice. It has long been suggested that multiple signals can act as cues for mate choice (Møller & Pomiankowski, 1993). If one of these cues cannot be evaluated by the choosing individual, there might be back-up signals that step in and therefore reduce mate choice errors (Candolin, 2003). However, high cross-correlations of such traits can lead to problems for study design and data analysis (Hedrick & Temeles, 1989). This is one reason why we still lack further knowledge on the impact of multiple signals on mate choice. In our study, we consider it likely that Alpine newt males based their decision on both size and other cues (e.g. pheromones) that correlate with female belly colour and reflect the same quality, especially in the blue-light treatment when colour could not be evaluated.

Our results further suggest that female responsiveness modulates male mating decisions. If males also base their decision on behavioural cues from females, they should increase their mating effort towards responsive females. This is particularly important if a successful mating depends critically on the behavioural coordination between the male and the female, as is the case in our study species. Yellow females that indicated their interest by tail fanning received similar courtship investment by a male as red-bellied females. However, an unresponsive yellow female elicited significantly less courtship investment from the male. This suggests that the putative reproductive value of yellow females did not warrant maximum mating effort from the male. In contrast, males did not reduce their courtship investment when a red female was unresponsive. We suspect that selection favoured males that express a more persistent courtship for colourful females with their putative high reproductive value. Notwithstanding, more colourful females showed more disinterest then less colourful ones. This behaviour could be a mechanism of defence for particularly colourful females, as they might receive most mating attempts (as our results suggest). If attractive females of a polygynous species are frequently harassed by males, they might even suffer from a reduction in lifetime fitness (Long, Pischedda, Stewart, & Rice, 2009; Rosenthal, 2017). Whether this is true in newts is not yet known. On the other hand, less disinterest expressed by yellow females could just reflect a compensation mechanism among low-quality females. Our results suggest that yellow females can receive similar male courtship investment as red females by being more responsive. It thus seems adaptive for such females to not turn away too often from a courting male.

Overall, males should reduce their mating effort for disinterested females, which frequently turn away from the courting male. We observed that males reduced their courtship investment and number of spermatophores when females showed more disinterest. In this species, the successful transfer of a spermatophore depends critically on a close and highly coordinated interaction between the male and the female. The spermatophore will only attach to the female's cloaca if she closely follows the movements of the male (reviewed in Thiesmeier & Schulte, 2010; Lüdtke & Foerster, personal observation). Successful transfer of a spermatophore often happens only after a number of unsuccessful attempts (Lüdtke & Foerster, personal observation). Females that interrupt the ritual by frequently turning away from the male thus critically reduce the chance of a successful mating. It seems adaptive for males to minimize the time wasted in a long and finally unsuccessful courtship, as alternative mating opportunities are usually available due to the intensive scramble competition during the mating season.

Although, to our knowledge, the exact costs of a spermatophore for an Alpine newt male have not been investigated, it is likely that spermatophore production is not unlimited. Most urodele species of the temperate zones decouple spermiogenesis from the breeding season: sperm mature in autumn and are then stored until the next spring (Verrell, 1989). In the smooth newt, males deplete their sperm reserves at the end of a breeding season (Verrell et al., 1986). This suggests that male newts may run short of sperm later in the breeding season if they deposit too many spermatophores early on. There also seems to be a limit on the number of sperm available during one courtship event. In eastern newts, for example, sperm quantity and spermatophore volume decrease with successive spermatophores within a mating trial (Takahashi & Parris, 2009). We observed in previous mating trials with Alpine newts that consecutive spermatophores contained decreasing sperm numbers (Lüdtke, 2016; Maag, 2013). Since only one of the later deposited spermatophores of a mating event may enter the female's reproductive tract (if any), it seems unlikely that reducing the sperm content of spermatophores as courtship duration increases is adaptive for the male. More likely, sperm resources for immediate use are depleted. We observed here that males deposited fewer spermatophores in later than in earlier trials, and they tended to deposit fewer spermatophores over time. This suggests that males adjust their courtship and mating investment not only to female colour and responsiveness, but also to their individual sperm reserves. A tendency for less colourful males to invest more spermatophores for red females suggests that individual male quality or health (if reflected in male coloration) may additionally influence a male's decision to invest spermatophores. To maximize reproductive success, males should therefore reduce their time and sperm investment for unresponsive females, to enhance their potential for further matings with more responsive partners. Unfortunately, we know very little about variation in individual quality and sperm availability among Alpine newt males, and we acknowledge that this should be further addressed in upcoming studies on male mate choice in this species.

The timing of courtship also appeared to be an important aspect of male mating choices. We observed a decrease in almost all parameters over time (date). One argument might be that courtship is energetically costly, as mentioned before, and males become energy and sperm depleted towards the end of the breeding season. However, owing to our experimental design, some males started mating much later than others and should thus still have had equal energy and sperm reserves as males that entered the experiment earlier. Independent of the effect of each male's number of conducted mating trials, the date effect might rather be due to an endogenous seasonal rhythm that defines the start of physiological changes as preparation for the terrestrial phase rather than the depletion of supplies. Under natural conditions, most females have laid most of their eggs towards the end of the breeding season, and few eggs would be available for fertilization by sperm transferred at such a late stage. Although females remain longer in the water on average than males (Arntzen, 2002; Blab & Blab, 1981), the operational sex ratio thus appears to change to fewer fertile females later in the season. However, in our experiment, males reduced their mating effort towards the end of the breeding season, being thus also less available for reproduction, and this may also be adaptive in the wild. Male Alpine newts lose weight during the aquatic phase (von Lindeiner, 1992; Lüdtke, 2015), and it seems that the costs of prolonging this phase are higher than the benefits from any additional successful mating. This hypothesis is also supported by our finding that more males withheld courtship behaviour altogether later in the season than at the start of the experimental period.

In conclusion, male mate choice can be an important source of variation in the expression of sexual display in the Alpine newt. It can hence favour the evolution of a female ornament in a conventional sex role species and our results partly suggest that the females' colourful belly is subject to sexual selection acting directly on them. In many amphibian species, females show colourful traits. Whether these traits are subject to direct selection through male choice or to female competition needs further investigation. Further, we saw that females potentially signal their reproductive value through body size and other, yet unknown cues, which offer males substitutional routes to evaluating a female. Our results also confirmed the expectation that the responsiveness of females further explained male choosiness. We therefore suggest researchers should pay more attention to female responses in future studies on male mate choice. The question remains whether male choice has an impact on the females' fitness, a topic that needs further empirical consideration.

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Appendix

Table A1
Morphometric data of all male individuals used in the experiment

ID	Weight	SVL	Belly chroma	Throat chroma	Origin	Age
1	2.88	4.5	0.598	0.636	Lab	3
2	2.83	4.4	0.516	0.568	Lab	3
3	2.29	4.4	0.591	0.625	Lab	3
4	3.37	4.6	0.603	0.629	Wild	Unknown
5	3.77	4.4	0.616	0.598	Wild	Unknown
6	3.18	4.4	0.569	0.612	Lab	2
7	2.49	4.3	0.551	0.609	Lab	3
8	3.23	4.5	0.577	0.580	Lab	3
9	2.79	4.5	0.550	0.608	Lab	3
10	3.00	4.6	0.597	0.563	Wild	Unknown
11	3.11	4.7	0.572	0.643	Lab	3
12	3.04	4.6	0.599	0.626	Lab	3
13	2.95	4.8	0.581	0.562	Wild	Unknown
14	3.59	5.0	0.609	0.640	Wild	Unknown
15	2.73	4.4	0.547	0.560	Lab	3
16	2.40	4.2	0.535	0.574	Lab	3
17	2.61	4.4	0.552	0.643	Lab	3
18	2.59	4.5	0.576	0.599	Lab	3
19	3.29	4.4	0.640	0.649	Wild	Unknown
20	2.94	4.6	0.553	0.634	Lab	2
21	3.01	4.7	0.596	0.636	Lab	3
22	3.28	4.6	0.587	0.654	Lab	2
23	3.29	4.7	0.544	0.577	Lab	3
24	2.93	4.4	0.599	0.581	Wild	Unknown
25	3.08	4.5	0.595	0.619	Wild	Unknown
26	3.04	4.9	0.572	0.667	Wild	Unknown
27	3.09	4.6	0.654	0.586	Wild	Unknown
28	2.88	4.5	0.561	0.627	Lab	3
29	2.70	4.5	0.574	0.644	Lab	3
30	2.93	4.3	0.612	0.630	Lab	3
31	3.31	4.8	0.589	0.626	Lab	3
32	3.48	5.0	0.624	0.644	Wild	Unknown
33	3.11	4.7	0.517	0.622	Lab	3
34	3.00	4.7	0.544	0.561	Lab	3
35	3.98	4.9	0.555	0.593	Lab	3
36	3.11	4.4	0.599	0.616	Lab	3
37	3.82	4.8	0.622	0.544	Wild	Unknown
38	2.60	4.5	0.534	0.545	Lab	3
39	2.62	4.5	0.560	0.637	Lab	3
40	2.77	4.4	0.561	0.591	Lab	3
41	2.77	4.5	0.571	0.641	Lab	3
42	3.10	4.5	0.527	0.562	Lab	3
43	2.84	4.5	0.585	0.580	Lab	3
44	2.68	4.4	0.580	0.660	Lab	3
45	3.05	4.3	0.537	0.647	Lab	3

Males were tested with four different females (two from each colour group) consecutively in different light conditions (control and blue filter). Weight was measured just before the four trials of that male. Snout–vent length (SVL) and chroma were measured before the experiment started.

Table A2
Morphometric data of all female individuals used in the experiment

ID	Weight 1	Weight 2	Weight 3	Weight 4	SVL	Belly chroma	Throat chroma	Colour group	Origin	Age	Feeding group
1	5.80	5.56	5.56	5.6	5.4	0.496	0.614	Y	Lab	2	Control
2	4.61	4.41	4.02	3.95	5.2	0.517	0.614	Y	Lab	2	Carotenoid
3	3.41	3.77	3.28	3.53	4.7	0.516	0.582	Y	Lab	2	Carotenoid
4	3.50	3.33	3.33	3.16	4.2	0.519	0.631	Y	Lab	2	Carotenoid
5	4.21	3.62	3.54	3.34	4.7	0.531	0.620	Y	Lab	2	Carotenoid
6	3.75	3.23	3.16	3.13	5.3	0.592	0.630	R	Wild	Unknown	Control
7	3.70	3.99	4.09	4.47	4.9	0.454	0.571	Y	Lab	2	Carotenoid
8	3.39	3.51	3.22	3.15	5.0	0.536	0.595	Y	Lab	3	Control
9	5.21	4.12	4.03	3.96	5.4	0.580	0.642	R	Wild	Unknown	Carotenoid
10	3.90	3.84	4.04	4.64	4.9	0.514	0.660	R	Lab	2	Carotenoid
11	4.42	4.38	4.80	4.29	5.0	0.521	0.649	Y	Lab	2	Control
12	4.40	4.54	4.41	4.97	5.0	0.517	0.607	Y	Lab	2	Control
13	3.81	3.80	4.38	4.41	5.4	0.574	0.612	R	Wild	Unknown	Carotenoid
14	4.91	3.75	3.73	3.34	5.3	0.598	0.628	R	Wild	Unknown	Carotenoid
15	4.12	3.63	3.86	3.48	5.7	0.580	0.650	R	Wild	Unknown	Control
16	5.09	4.38	3.92	3.69	5.3	0.572	0.628	R	Wild	Unknown	Carotenoid
17	4.68	4.64	3.99	3.92	5.0	0.467	0.580	Y	Lab	2	Carotenoid
18	3.81	3.66	4.21	4.29	5.2	0.576	0.602	R	Lab	3	Carotenoid
19	3.49	3.30	2.99	3.76	5.0	0.473	0.538	Y	Lab	3	Carotenoid
20	4.54	3.96	4.16	4.64	5.0	0.501	0.629	Y	Lab	3	Control
21	4.67	4.53	4.01	3.78	5.2	0.520	0.652	R	Lab	3	Carotenoid
22	4.66	3.68	3.63	3.40	5.3	0.547	0.626	R	Lab	2	Control
23	3.70	3.66	4.22	4.26	5.0	0.526	0.527	Y	Wild	Unknown	Carotenoid
24	4.51	4.10	3.70	3.42	5.1	0.521	0.623	Y	Lab	3	Control
25	3.54	3.85	3.34	3.90	4.8	0.531	0.630	R	Lab	2	Carotenoid
26	3.62	3.98	3.56	3.18	5.8	0.596	0.673	R	Wild	Unknown	Carotenoid
27	4.77	4.48	4.31	4.31	5.3	0.539	0.637	R	Lab	3	Control
28	4.10	4.16	4.35	3.59	5.3	0.525	0.625	Y	Lab	3	Control
29	4.29	4.46	4.36	4.62	5.1	0.552	0.643	R	Lab	3	Control
30	5.49	5.65	4.76	5.53	5.5	0.580	0.674	R	Wild	Unknown	Control
31	5.22	4.21	4.24	3.84	5.2	0.501	0.610	Y	Lab	3	Carotenoid
32	5.10	4.98	4.81	5.24	5.5	0.569	0.633	R	Lab	3	Control
33	3.12	3.31	3.01	3.74	5.0	0.524	0.649	R	Lab	3	Carotenoid
34	4.01	3.80	3.17	4.16	5.2	0.545	0.526	Y	Lab	5	Control
35	4.96	4.87	4.33	4.10	5.2	0.558	0.638	R	Lab	3	Carotenoid
36	4.10	3.22	3.22	2.88	5.1	0.578	0.458	Y	Lab	5	Control
37	4.99	4.69	3.96	3.71	5.3	0.557	0.642	R	Lab	3	Control
38	4.34	3.54	NA	NA	5.2	0.536	0.597	Y	Lab	3	Carotenoid
39	4.15	3.72	3.73	3.29	5.2	0.557	0.650	R	Lab	3	Control
40	4.33	3.61	3.43	3.32	5.4	0.535	0.642	R	Lab	3	Control
41	4.21	3.81	3.11	2.95	4.8	0.535	0.636	Y	Lab	3	Control
42	4.86	4.16	4.38	4.11	5.1	0.545	0.663	R	Lab	3	Carotenoid
43	4.05	3.61	3.53	3.34	5.0	0.485	0.446	Y	Lab	2	Control
44	3.94	4.02	3.84	3.51	5.0	0.537	0.668	R	Lab	3	Control
45	4.66	4.10	4.01	3.74	4.8	0.469	0.584	Y	Lab	2	Carotenoid
46	4.02	3.73	4.13	4.02	4.9	0.475	0.629	Y	Lab	2	Carotenoid

Females were tested with four different males consecutively in different light conditions. Weight was measured just before each trial; hence there are four different weight measurements. Snout–vent length (SVL) and chroma were measured before the experiment started. According to the mean chroma, females were assigned to either the red (R) or the yellow (Y) colour group.

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

DEIKE U. LÜDTKE[1,2] AND KATHARINA FOERSTER[1]

[1]*Department of Comparative Zoology, Institute for Evolution and Ecology, University of Tübingen, Tübingen, Germany*

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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[2]Corresponding Author. E-mail: deike.luedtke@uni-tuebingen.de
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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 ($n_{\mathrm{male}} = 51$) and females ($n_{\mathrm{female}} = 54$) 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 (≈40 L each; 60 × 60 × 38 cm length × width × 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 (≈300 L; 250 × 60 × 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,

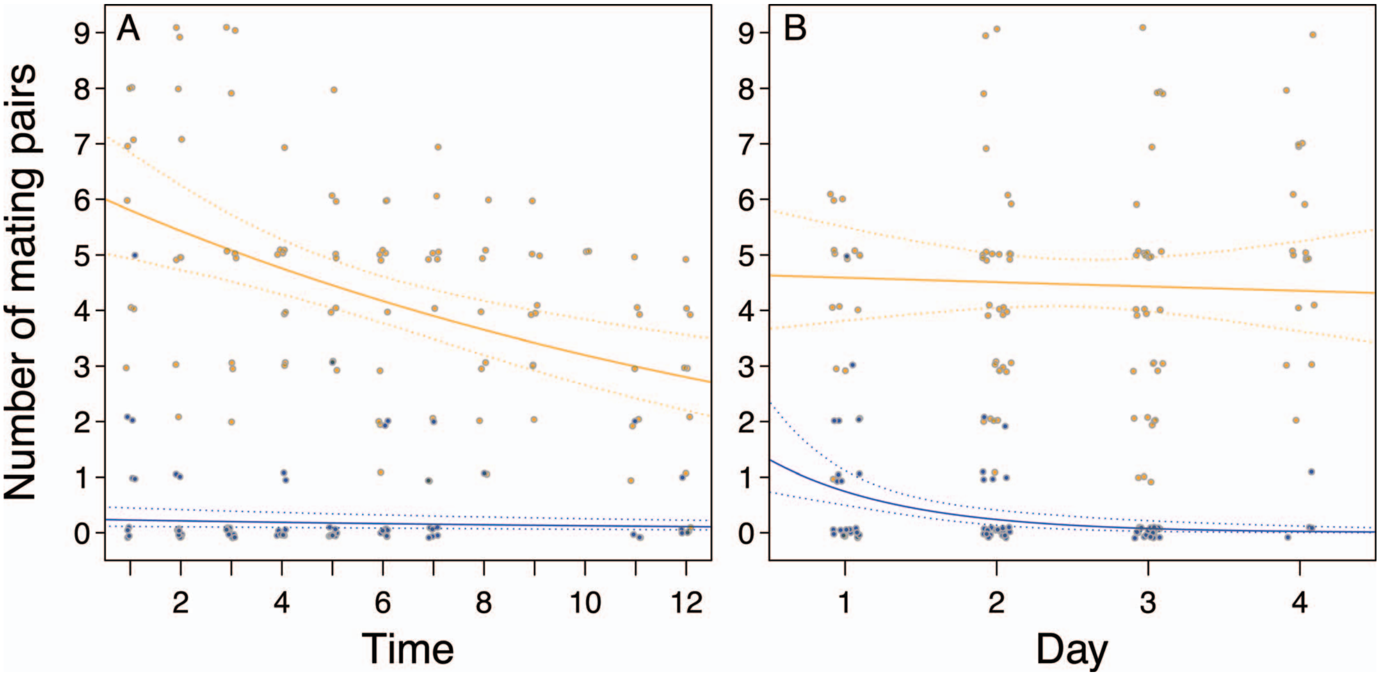

FIG. 1. Number of mating pairs in relation to (A) the time and (B) the day, with 95% credible intervals (dotted lines). The yellow lines show the model prediction for the number of mating pairs while the lights were on; blue lines show the model prediction for lights off. Points show the raw data (yellow: 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 8.69×10^{20} photon radiance (photons/sr/m^2/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 2.02×10^{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; ΔAICc 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	ΔAICc	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 ΔAICc ≤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_glmer* 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 ± 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 ± 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 ± 2.016 mating pairs; light off: 0.403 ± 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 ± 1.959 mating pairs at the first time point (Fig. 1). Mating activity then decreased over time with only 2.444 ± 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 ± 1.641, last time point: 0.250 ± 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 ± 1.306; last day: 0.250 ± 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 (Diaz-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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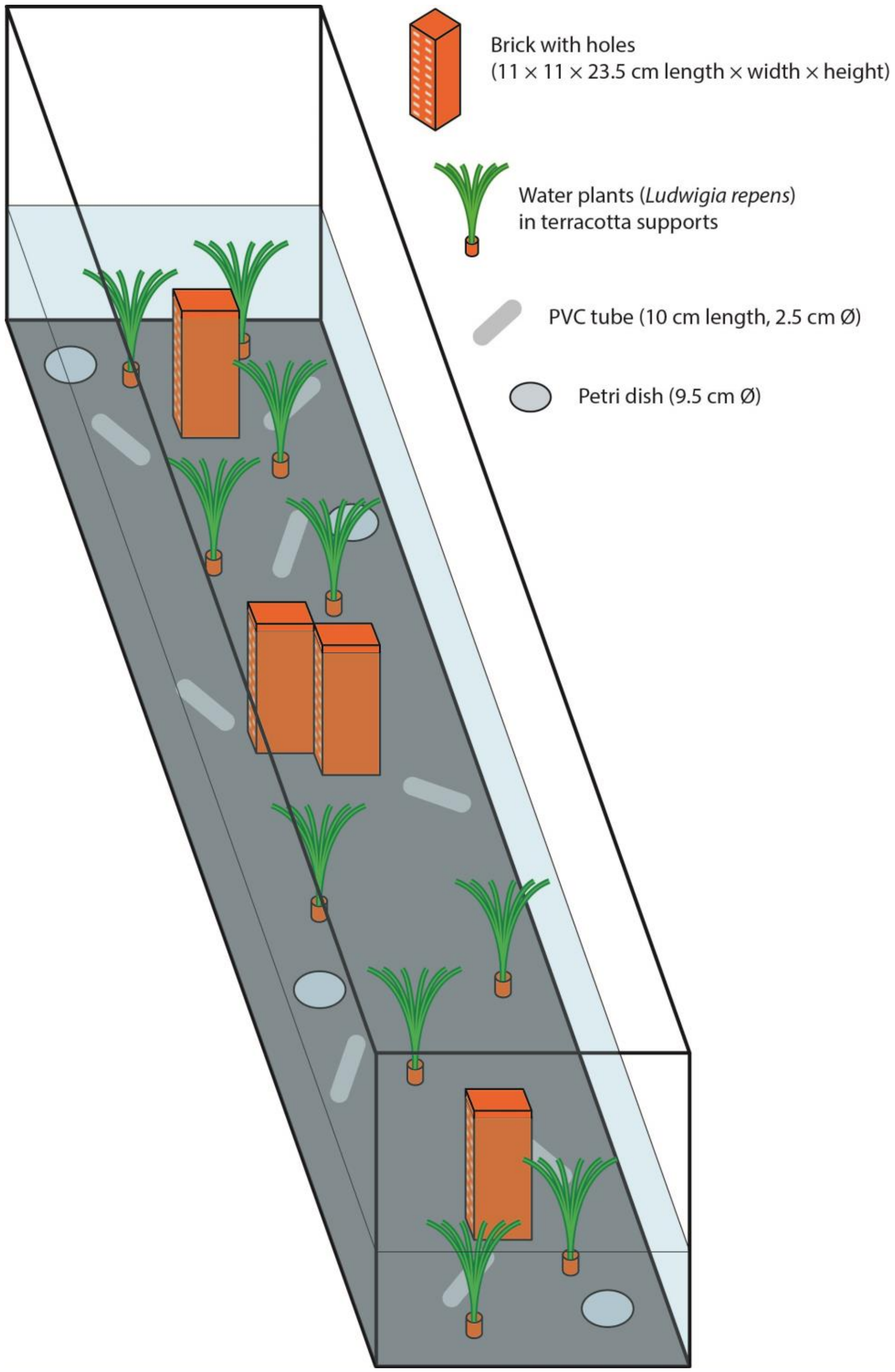
Supplemental Material

Supplemental Table 1. Summary results of the average generalized linear mixed models (based on the best models with ΔAICc ≤ 2) for number of mating pairs. The importance (i.e., the sum of Akaike weights), mean estimates and 95% confidence intervals of all fixed factors and covariates are reported. 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	Importance	Average estimate	SE	95% confidence intervals
Intercept		**-1.861**	0.376	-2.598 to -1.123
Light (on)	**> 0.99**	**3.307**	0.379	2.564 to 4.050
Time	> 0.99	-0.175	0.164	-0.497 to 0.148
Day	**> 0.99**	**-1.110**	0.288	-1.675 to -0.545
Light * Day	**> 0.99**	**1.093**	0.293	0.518 to 1.668
Light * Time	0.24	-0.206	0.278	-0.750 to 0.338
Date	0.22	-0.031	0.048	-0.124 to 0.063



Supplemental Fig. 1. Overview of the experimental aquarium (250 × 60 × 60 cm length × width × height). Water depth was approximately 21 cm. The bottom was covered with black gravel and foliage (not shown in the graphic); shelters were provided by four bricks with many holes, small PVC tubes, and water plants. To provide onshore platforms and shelter, the first row of holes of the four bricks were above the water line and floating tree bark was provided (not shown in the graphic).

Supplemental Fig. 2. Male (left) and female (right) Alpine Newts, *Ichthyosaura alpestris*, during courtship.

III

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A Female Color Ornament Honestly Signals Fecundity

Deike U. Lüdtke* and Katharina Foerster

Comparative Zoology, Institute for Evolution and Ecology, University of Tübingen, Tübingen, Germany

In many species, particularly in ectothermic vertebrates, not only males but also females show bright body colorations. In Alpine newts, *Ichthyosaura alpestris*, both sexes have colorful orange bellies. This coloration varies in redness (yellowish to dark orange) among individuals, and previous work has shown that courting males preferred females with more orange bellies. Because males in this species are likely to be limited in their mating capacity, selection would favor this preference if this coloration honestly signals female quality. In this study, we investigated whether female belly coloration can predict individual fecundity in which case males might have the chance to fertilize more eggs when they choose to mate with a more ornamented female. We found that more orange females were more fertile than less orange ones. Additionally, we saw that more ornamented females could lay their eggs faster, which means that they ceased egg laying at a similar time as less ornamented females despite the greater number of eggs. This suggests that female color can convey quality advantages in a species with no sex-role reversal much in the same way as male colors can, making female ornaments subject to sexual selection acting directly on them.

Keywords: alpine newt, fecundity, female ornament, female quality, *Ichthyosaura alpestris*, nuptial coloration, sexual selection

Edited by:
Renoult P. Julien,
UMR5175 Centre d'Ecologie
Fonctionnelle et Evolutive
(CEFE), France

Reviewed by:
Rebecca E. Adrian (Koch),
Monash University, Australia
Nuno Miguel Monteiro,
University of Porto, Portugal

****Correspondence:***
Deike U. Lüdtke
deike.luedtke@uni-tuebingen.de

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INTRODUCTION

Several theories have been put forward to explain the presence of secondary sexual ornaments. One well-documented mechanism for the evolution of such ornaments acts when that ornament functions as a quality indicator that provides a benefit during mate choice to both, the signaler, and the receiver. Since an individual may benefit by choosing a high-quality partner to mate with through direct (e.g., Price et al., 1993; Iwasa and Pomiankowski, 1999) or indirect benefits (e.g., Kirkpatrick and Barton, 1997), an exact assessment of the quality of a sexual partner may be substantially important for the choosing individual (reviewed in Kokko et al., 2003).

Many studies have demonstrated female preferences for male ornaments and have investigated different benefits that females might gain from choosing a highly ornamented male, suggesting that the ornaments can visualize quality in males. These benefits can include higher fertility of males (Pitcher and Evans, 2001), better paternal care (Hoelzer, 1989), or they could be associated with less pathogen infections (e.g., Ressel and Schall, 1989). However, benefits are not always as obvious. Females could also obtain offspring in better condition (health: Barber et al., 2001; or size: Parker, 2003) or highly ornamented males can provide their offspring with genes that make them more attractive as mating partners (e.g., Gwinner and Schwabl, 2005).

Female ornamentation has long been regarded as non-adaptive correlations to male ornaments (reviewed in Nordeide, 2002; Kraaijeveld et al., 2007). They were thought to appear through a genetic link between the male and the female ornament, and the selection acting on the male ornaments (Lande, 1980). However, a growing number of investigations prove these assumptions not to be universally applicable.

Although studies on female ornamentation are not as numerous, we now know that in some species, males may also base their choice on female secondary sexual ornaments. This was predominantly documented for sex-role reversed species (e.g., Berglund and Rosenqvist, 2001), but an increasing number of studies also demonstrate a male preference for ornamented females in otherwise conventional sex-roles (e.g., Torres and Velando, 2005; Baldauf et al., 2011; LaPlante, 2015; Belliure et al., 2018; Lüdtke and Foerster, 2018). Females of the rock sparrow, *Petronia petronia*, for example, have a yellow breast patch and males were found to court females with a smaller or experimentally reduced patch size less intensely and less frequently (Griggio et al., 2005).

This evidence, together with reports on male choosiness even in polygynous species (Servedio and Lande, 2006) suggests that the intensity of male choosiness is more dynamic than previously thought (Clutton-Brock, 2009; Clutton-Brock and Huchard, 2013). In fact, mutual mate choice, that is when both sexes exert mate choice, is rather common (Clutton-Brock, 2009; Rosenqvist and Berglund, 2011; Rosenthal, 2017) and the intensity of male choosiness is expected to vary with the availability of partners relative to its mating capacity (Edward and Chapman, 2011).

Consequently, female nuptial signals can well have a selective advantage (reviewed in Kraaijeveld et al., 2007). On the one hand, females may use them as an aggressive signal predominantly in intrasexual competition (Beeching et al., 1998), as it is the case in convict cichlids, *Cichlasoma nigrofasciatum*. Only females of this species typically develop an orange ventral coloration and only females seemed to use this color as a visual signal. Females were more aggressive toward more brightly colored individuals than intermediate ones. On the other hand, nuptial signals can also function as a signal to advertise females' reproductive state or individual quality. When this is the case, males benefit by mating with highly ornamented females. In species without maternal care, female quality is characterized by fecundity, the quality of the eggs or offspring performance after birth or hatching. For choosy males, any trait that reflects such quality aspects would be relevant. For example, the plumage color of blue tit females, *Cyanistes caeruleus*, correlated positively with clutch size, fledgling success, and recruitment (Doutrelant et al., 2008). Such a correlation between colorful ornaments and individual quality has also been shown for other birds (e.g., Pilastro et al., 2003) and some fish (e.g., Massironi et al., 2005; Kekäläinen et al., 2010). Further studies on other taxa that demonstrate a correlation between a colorful ornament and individual quality in females are currently lacking.

With this study, we want to extend the knowledge about the function of female coloration and the potential for selection on female nuptial signals in a Eurasian amphibian, the Alpine newt (*Ichthyosaura alpestris*). Alpine newt males and females have colorful orange bellies that vary naturally in their color hue (Kopecký and Šichtar, 2014; own obs.). Body colorations that appear red, orange, or yellow are caused by carotenoids or pteridines that accumulate in feathers or skin cells (Olson and Owens, 1998; Griffith et al., 2006; Weiss et al., 2012). Ornaments composed of such pigments have often been reported to communicate quality advantages of the bearer (reviewed in Weiss et al., 2011). For example, pigment content has been suggested to correlate with immune defense especially in mammals (Huber et al., 1984; Chew and Park, 2004). Whether these compounds directly enhance immune responses or provide important precursors for other compounds is, however, still debated (e.g., Pérez-Rodríguez, 2009; Koch et al., 2018). Ornamental pigment content has also been suggested to relate to individual reproductive potential. Carotenoid-based coloration was reported to correlate positively with fecundity in a bird (Doutrelant et al., 2008) and a fish (Svensson, 2006), and the pteridine-based ornament of female striped plateau lizards, *Sceloporus virgatus*, was positively related to the total amount and concentration of yolk antioxidants (Weiss et al., 2011). These findings suggest that colorful individuals cope better with immune challenges and are better able to allocate their resources to reproduction.

Both carotenoid and pteridine pigments were found in the belly skin of Alpine newts (Günder, 1954) and other newt species (e.g., Obika, 1963; Matsui et al., 2002). We therefore expect that the belly pigmentation might function as nuptial ornamentation in this species. We recently found that male Alpine newts exhibited preference toward certain females (Lüdtke and Foerster, 2018). Males spent more time courting larger females and females that had more orange bellies. However, for male choosiness to evolve, males are expected to be limited in their mating capacity and females are expected to vary in quality (Gwynne, 1991; Kokko and Monaghan, 2001). Male Alpine newts have comparatively high mating costs (reviewed in Thiesmeier and Schulte, 2010). They engage in a time consuming mating ritual (see Arntzen and Sparreboom, 1989) and are most likely limited in their mating capacity in terms of sperm expenditure (see below). Males would gain fitness advantages if they mate with a female that lays more eggs. If females vary in the amount of eggs they lay, males should be choosy and invest more into females that are more fecund. Therefore, males should choose more orange females if this color is an honest signal for fecundity.

In this study, we investigated whether Alpine newt females with more orange bellies lay more eggs. We hypothesize that the color intensity is a signal for the number of eggs a female lays and hence a signal for individual quality. We also tested whether more orange females laid their eggs faster compared to less orange ones.

MATERIALS AND METHODS

Study Species

Ichthyosaura alpestris is a medium-sized aquatic salamander with its main distribution in Central Europe (Thiesmeier and Schulte, 2010). They mate and oviposit in water bodies during spring to early summer but spend the rest of the year in a terrestrial phase. Alpine newts are sexually dimorphic in size and body shape, as

FIGURE 1 | Male (left) and female (right) Alpine newt, *Ichthyosaura alpestris*.

well as in their dorsal and lateral coloration. Yet, both sexes show strikingly orange bellies (**Figure 1**).

During courtship males attract females with a complex but stereotypic suite of behaviors (Halliday, 1977; Arntzen and Sparreboom, 1989). They transfer sperm in form of spermatophores that they deposit on the substrate and try to guide the females over them. Unfortunately, many of those spermatophores are lost, because females frequently miss to take them up with their cloaca. Observations show that females pick up only 31% (or less) of deposited spermatophores (Halliday, 1977, 1990). Males probably enter the breeding season with a limited number of sperm because spermiogenesis is most likely completed during autumn and spermatozoa are stored until the next breeding season as described for smooth newts, *Lissotriton vulgaris* (see Verrell et al., 1986). During the mating season in early spring, Alpine newts gather in often crowded ponds, where high mating activity and scramble competition is observable at any time of the day (e.g., Blab and Blab, 1981; Lüdtke and Foerster, 2019). When courting a single female, males may sequentially deposit several spermatophores (Halliday, 1977; Maag, 2013; pers. obs.), and each male courts a large number of females if they are available to him (own observation in large group aquarium). Given the long duration of the Alpine newt breeding season (several weeks to a few months, Blab and Blab, 1981), we have thus reason to assume that a male's sperm capacity is limited and that any loss of spermatophores is costly for males.

After mating, females lay their eggs by individually wrapping them into submersed aquatic plant supports (Miaud, 1994, 1995; own obs.). Individual females can lay between 100 and 500 eggs (Miaud, 1994). The average number of counted oocytes from wild caught individuals was 258 ($N = 8$; Thomas et al., 2002), and 293 ($N = 7$; von Lindeiner, 1992). In laboratory experiments, female fecundity was observed to fluctuate between 20 and 271 eggs (reviewed in Thiesmeier and Schulte, 2010). Although female body size may be a proxy for fecundity in many species (e.g. Honěk, 1993; Bonduriansky, 2001; Wong and Svensson, 2009; Eddy et al., 2016), reports about Alpine newts on that matter are not consistent. Where Miaud (unpublished data, cited in Miaud and Merilä, 2001) reported a positive correlation between body size and egg number, such a relationship could not be confirmed when Thomas et al. (2002) investigated fecundity by counting the number of oocytes.

Lab Population and Husbandry

The study was carried out at the University of Tübingen, Germany, from 10 March to 20 July 2017. The lab population consisted of wild individuals caught in 2011 and 2012 from the surrounding of the city of Tübingen, and their descendants, all born in the lab between 2011 and 2014. The founder population included 243 individuals (125 females and 118 males), collected from two separate wild subpopulations. After hibernation, we separated males and females and kept them in aquaria at a maximum density of 15 individuals per aquarium (≈ 72 l each; 60 × 60 × 38 cm lwh). We regulated ambient air temperature to be between 12 and 16°C and set the photoperiod to 12:12 LD (light from 8:00 to 20:00). Home aquaria were equipped with black gravel, shelters (brick stones with many holes), water plants, and an above-water platform. We fed the newts with red mosquito larvae and water fleas (*Daphnia* sp.) *ad libitum* three times a week. Before we transferred the females into special oviposition aquaria, we placed males and females together into a large-group aquarium for four days to ensure insemination as described in Lüdtke and Foerster (2019). We randomly divided the newts into three groups with 36 individuals and a 50:50 sex ratio. We introduced the groups into the mating aquarium in succession.

Oviposition and Egg Handling

After 4 days in the mating aquarium, where females had free access to several males, we transferred the females ($N = 54$) into oviposition aquariums (≈ 40 l each; 60 × 60 × 38 cm lwh) that were divided into three same-sized compartments (20 × 60 × 38 cm lwh). Each female was placed in one of these compartments. We supplied each compartment with black gravel, a small PVC tube (10 cm length, 2.5 cm Ø) as a hiding place, a platform above the water level and a water plant (*Ludwigia repens* in terracotta supports) to lay eggs on. The first group of 18 females was introduced into the oviposition aquariums on 10 March, the second group on 18 March, and the last one on 22 March. One female escaped from the oviposition aquarium during the experiment and was excluded from the analysis. Every 4–7 days, we replaced plants, scanned the compartments for eggs that were laid outside of the provided plant and counted the total number of eggs a female had laid. To accurately define the number of eggs we carefully removed each counted egg with forceps and scanned the plant the next day for any remaining eggs. If a female did not lay a single new egg in 4 weeks, we assumed that it ceased laying and released it into the home aquarium in the husbandry.

Color and Size Measurements

Prior to the experiment, we measured reflectance of the ventral sides to quantify the color of the females' bellies. We measured reflectance in two different regions (belly and throat) from 380 to 700 nm using a spectroradiometer (SpectraScan PR 670, Photo Research Inc., Syracuse, NY, U.S.A.) (see also Lüdtke and Foerster, 2018). Each reflectance spectrum was calculated relative to a polytetrafluoroethylene (PTFE 98) white reflectance standard (Berghof Fluoroplastic Technology GmbH, Eningen, Germany) with the program Spectrawin 5.0 (Avantes, Apeldoorn, The Netherlands). We used two indices to describe color.

We calculated hue (peak wavelength) as the wavelength where reflectance is at its maximum, and chroma (spectral purity) which describes the relative color saturation in a wavelength range of interest. We calculated chroma as the reflectance sum over the red range from 605 nm to the maximally measured wavelength (700 nm), divided by the total reflectance sum (R package *pavo*, Maia et al., 2013). For both, hue and chroma, we averaged the two measured regions (belly and throat) and refer to the mean as that female's belly hue or chroma, respectively. We measured snout-vent length (SVL) (5.2 ± 0.3 cm) when the animals were under anesthesia and weighed them (5.00 ± 0.88 g) just before they entered their individual oviposition aquaria. Additionally, we weighed the females again after they finished the experiment.

Statistics

We performed all statistical analyses using R version 3.5.1 (R Core Team, 2018) and all data summaries are reported as means ± SD. We excluded females from the statistical analyses that laid <20 eggs (12 individuals). We considered those females not to be fully ready to lay, either because they were still waiting for additional copulations or because their reproductive rhythm was delayed with respect to the other females. Although reports suggest that lab-reared amphibians are not as colorful as conspecifics found in the wild (e.g., Steinicke, 1976; Frost and Robinson, 1984; Matsui et al., 2002), we did not want to include individuals in our investigations that had an unnatural color scheme (more than 5% outside the range of the natural occurring range). As a reference, we compared the chroma values of newts in our experiment to 36 wild females that were captured in the population where our founder individuals originated from, measured in the same way as described before, and released into the wild in 2013 (chroma: 0.606 ± 0.023, range: 0.552–0.645; unpublished raw data). We then identified three experimental females that were unusually faint (chroma: 0.500, 0.514, and 0.521) and suspected that they were unable to store certain pigments in their skin. We therefore excluded two more females from the analyses (as the third was already excluded because of low egg numbers, see above). We finally used 39 females for all analyses.

The data for the total number of eggs did not show a normal distribution, nor did it fit a Poisson distribution. We thus applied non-parametric statistics: we tested the effect of hue, chroma, size, and weight on the total number of laid eggs with Kendall rank correlations. To test whether the daily laying activity was individually different, we also investigated the individual curves of the cumulative number of eggs. We performed linear mixed models (function *lmer*, package *lme4*; Bates et al., 2015) and included the day since introduction to the laying-compartment, either hue or chroma, and their interactions as fixed factors. We also included either SVL or weight, and their interaction with the day in the models. In all models, we log-transformed day (decadic logarithm) to fit the curve of the data. Apart from day, we standardized all continuous variables so that their means were zero and their standard deviations one. As random factors, we included female ID to account for repeated measures on each female, and mating group to account for random variance between the three groups that were mating in succession. We also included a random slope for the covariate day dependent on ID to account for unsystematic variance between the females (Korner-Nievergelt et al., 2015). For the parameter estimates (presented in the table) and model predictions (presented in the figure), we obtained 95% credible intervals (CrI) by using Bayesian statistics (Bolker et al., 2008). We calculated the exact uncertainty of model predictions (Gelman and Su, 2016) by using 10 000 simulated random values obtained from the joint posterior distribution of the model parameter. 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). We present the Akaike Information Criterium for small sample size ($\mathrm{AIC_c}$) for each model and compare models based on the difference in $\mathrm{AIC_c}$ ($\Delta\ \mathrm{AIC_c}$).

Ethical Note

Maintenance and handling of the newts were conducted in accordance with German animal welfare law (Tierschutzgesetz) and FELASA guidelines. All experiments were approved by the local veterinary office "Veterinäramt, Regierungspräsidium Tübingen," under the license number ZO 5/15.

RESULTS

Total Number of Eggs and Laying Duration

Female weight was not correlated to hue or chroma, and SVL was not correlated to hue (all $p > 0.05$). However, females with larger SVL were more orange as measured by chroma ($r_p = 0.446$, $p = 0.001$). Three females did not lay a single egg and were excluded from the analyses. Additionally, we excluded the ones that laid <20 eggs from the analyses ($N = 9$), as well as the three females that were more than 5% less orange than females in the natural population (see **Figure S1**). Among all other females, the range of chroma was 0.535–0.642 (0.581 ± 0.025) and the total number of laid eggs varied between 23 and 451 (158.54 ± 106.60).

We found no relationship between a female's weight and the number of eggs it laid ($r_\tau = 0.041$, $p = 0.717$; **Figure 2A**); nor did we find a relationship between a female's SVL and the number of eggs ($r_\tau = 0.051$, $p = 0.660$; **Figure 2B**). However, females lost 0.72 ± 0.55 g, on average, during oviposition, although this weight loss was not correlated to number of eggs ($r_\tau = 0.020$, $p = 0.856$).

When we analyzed the effect of the two measurements of belly color (hue and chroma) on the total number of eggs, we found that more orange females (females with a higher hue and higher chroma value) laid significantly more eggs than less orange females (hue: $r_\tau = 0.248$, $p = 0.031$; chroma: $r_\tau = 0.220$, $p = 0.049$; **Figures 2C,D**). The five females with the lowest hue in our study laid on average 92.20 ± 69.52 eggs, while the four females with the highest hue produced on average 278.25 ± 181.44. For chroma, the five least orange females (lowest chroma value) laid on average 44.8 ± 27.29 eggs, while the five most orange females (highest chroma value) produced on average 159.29 ± 106.61 eggs.

We observed variation in the laying duration of the females. They laid eggs between 6 and 120 days (62.38 ± 21.47), but there was no correlation between belly color and the number of days a

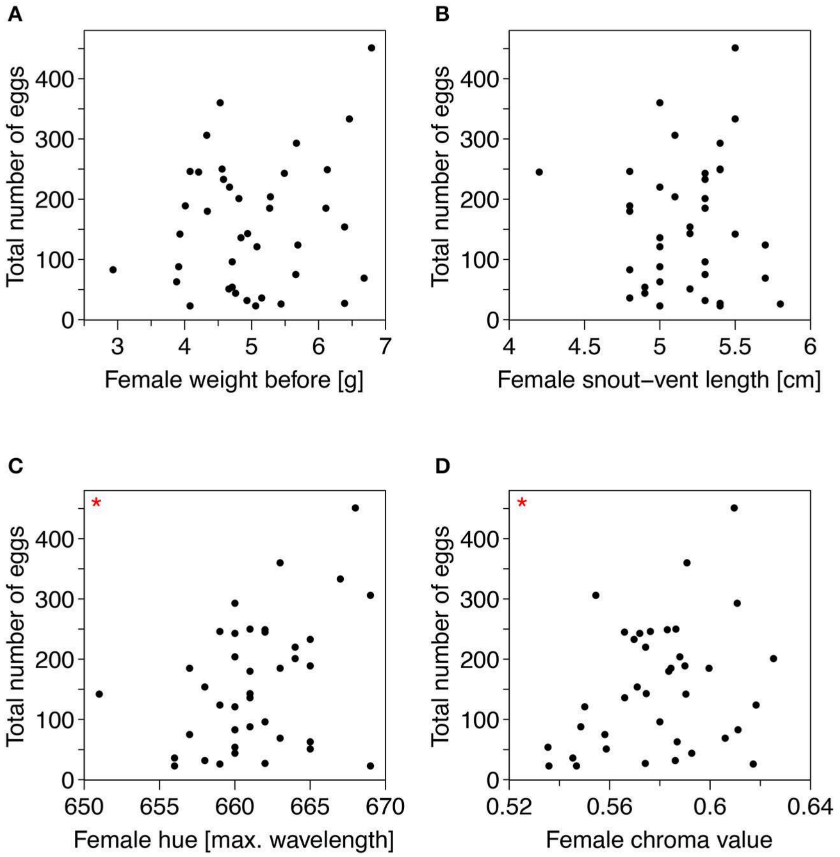

FIGURE 2 | Scatterplots illustrating the relationships between number of laid eggs and **(A)** female weight before the experiment started, **(B)** female snout-vent length (SVL), **(C)** female belly hue, and **(D)** female belly chroma. The red asterisks indicate significant correlations ($p < 0.05$). $N = 39$.

female laid eggs (hue: $r_p = 0.251$, $p = 0.123$; chroma: $r_p = 0.277$, $p = 0.088$).

Daily Cumulative Egg Numbers

Females with varying belly color showed differences in the daily cumulative egg numbers. The slopes of the interactions between day (log-transformed) and hue or chroma indicate how fast females with different belly color laid their eggs. When we included the weight of the female as covariate in both models, we found a significant effect of the interaction between day and hue as well as between day and chroma on the cumulative egg numbers (**Table 1**). Females with more orange bellies laid their eggs faster than less orange females (**Figure 3A**). In the other two models, we used SVL as a covariate, instead of weight (**Table 1**). Here, we found the same significant interaction effect (**Figure 3B**). For hue, the first model with weight explained the data better than the one with SVL (Δ AIC$_c > 2.0$), but since both models with chroma explained the data similarly well (Δ AIC$_c < 2.0$; see **Table S1**), we computed an averaged model. This averaged model suggested again that females with a higher chroma value laid their eggs faster, than females with lower value (see **Table S2**). This difference was particularly obvious when a female started to lay eggs: in the first 19 to 21 days, the four most orange females (according to hue) laid on average 176.25 ± 111.40, while the five least orange females laid only 66.4 ± 51.24 eggs on average. When we compare the females with the highest and lowest chroma values we found that the five most orange females laid on average 99.8 ± 71.21 eggs, while the five least orange females produced only 39.8 ± 25.15 eggs on average. Body size and weight did not affect the speed of egg laying: both, the interaction between day and SVL and the interaction between day and weight, were not significant in any of the tested models.

DISCUSSION

We showed that the intensity of a female's nuptial ornament had an influence on some indicators of reproductive quality. Therefore, the belly coloration of female Alpine newts might indeed function as an honest signal for fecundity. Less orange females laid their eggs more slowly, compared to more orange females. Further, we found that less orange females laid considerably fewer eggs than females with a more orange belly.

In many species, yellow, orange, or red colors in the skin or plumage are based on carotenoids (Blount and McGraw, 2008) or pteridines (e.g., amphibians: Ziegler-Günder, 1956; birds: McGraw, 2006). The intensity of these colors is positively

TABLE 1 | Estimates, standard errors, and their 95% credible intervals from the different linear mixed models for daily cumulative egg numbers **(A)** with weight and **(B)** with SVL as a covariate.

Model	Fixed factors and covariates	Estimate	SE	2.5% quantile	97.5% quantile
A1	**Intercept**	**−27.793**	**3.250**	**−34.253**	**−21.317**
	log (Day)	**43.547**	**3.684**	**36.334**	**51.001**
	Hue	**−6.946**	**3.102**	**−13.120**	**−0.939**
	Weight	−5.760	3.212	−12.093	0.555
	log(Day) *Hue	**9.518**	**3.502**	**2.648**	**16.309**
	log(Day) *Weight	5.623	3.669	−1.531	12.702
A2	**Intercept**	**−26.449**	**3.331**	**−33.098**	**−19.915**
	log(Day)	**41.644**	**3.651**	**34.239**	**48.932**
	Chroma	−4.618	4.105	−12.795	3.505
	Weight	−5.294	3.416	−11.993	1.435
	log(Day) *Chroma	**10.913**	**4.533**	**2.358**	**19.785**
	log(Day) *Weight	3.709	3.793	−3.804	11.138
B1	**Intercept**	**−27.683**	**3.373**	**−34.366**	**−20.992**
	log(Day)	**43.329**	**3.785**	**35.841**	**50.955**
	Hue	**−7.395**	**3.214**	**−13.808**	**−1.092**
	SVL	0.392	3.310	−6.140	7.128
	log(Day) *Hue	**9.889**	**3.596**	**2.736**	**17.176**
	log(Day) *SVL	1.417	3.726	−5.920	8.718
B2	**Intercept**	**−26.020**	**3.375**	**−32.630**	**−19.361**
	log(Day)	**41.231**	**3.631**	**34.144**	**48.260**
	Chroma	−8.349	4.466	−17.218	0.204
	SVL	3.609	3.688	−3.680	10.844
	log(Day) *Chroma	**14.724**	**4.863**	**5.040**	**24.278**
	log(Day) *SVL	4.656	4.016	−12.575	3.258

The presented quantiles limit the 95% credible interval. Significant effects are shown in bold and trends are underlined. N = 39.

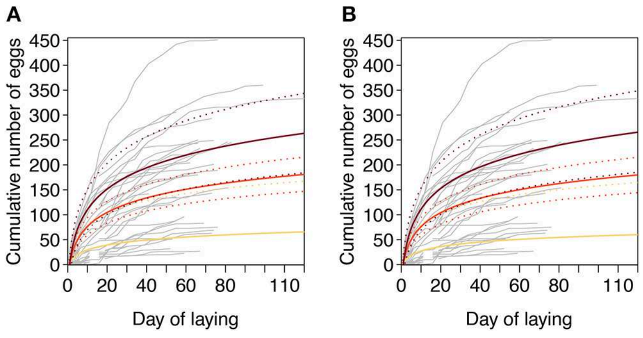

FIGURE 3 | Model predictions of the cumulative number of eggs for minimum (yellow line), mean (red line), or maximum (dark red line) hue values. Gray lines represent the raw data from 39 females. Each curve represents the cumulative number of eggs laid up to each day by each individual female. Fitted values (solid lines), predicted from an LMM, with 95% credible intervals (dashed lines) are given assuming average values for **(A)** weight and **(B)** SVL as independent variables in the model. N = 39.

correlated with pigment concentration. In striped plateau lizards, *Sceloporus virgatus*, for example, more colorful individuals had a higher concentration of drosopterin in their skin, a pterine that causes the orange color patches on the throat of this species (Weiss et al., 2012). In male three-spined sticklebacks, *Gasterosteus aculeatus,* the intensity of the red belly coloration correlated positively with astaxanthin concentrations in the tissue, a carotenoid that fish take up through their diet (Barber et al., 2000).

Both of these pigment classes were suggested to enhance immune system functions (e.g., Huber et al., 1984; Chew and Park, 2004; Hõrak et al., 2004). Carotenoids, for example, are well-known as effective antioxidants (Britton, 2008; Fiedor and Burda, 2014) and pteridines can also act antioxidatively under certain conditions (Oettl and Reibnegger, 2002). Therefore, colorful individuals might have an advantage in coping with oxidative stress and are hence better able to allocate their resources into the production of viable eggs. This was shown for blue tits, *Cyabistes caeruleus*, where female coloration was positively correlated to number of eggs when females were forced to produce a replacement clutch (Doutrelant et al., 2008), and for the two-spotted goby, *Gobiusculus flavescens*, where more colorful females produced larger clutches (Svensson, 2006). Our result show that Alpine newt females with more orange bellies also laid more eggs. Not only the number of eggs can be advertised by the color of the ornament but also the quality of those eggs. In striped plateau lizards, the color of the ornament was positively correlated with the concentration and total amount of yolk antioxidants (Weiss et al., 2011). This suggests that colorful females might also produce eggs of higher quality and provide better yolk content for the early growth phase of embryos. Whether more orange Alpine newt females produce higher quality eggs remains to be tested.

In addition to a higher number of eggs, we also found that more orange females laid their eggs faster than less orange females. As a result, more orange females ceased egg laying at a similar time as less orange females. Female Alpine newts may be selected to lay their eggs speedily, for two reasons: First, European newts spend most of their time on land (Taylor and Guttman, 1977), and they enter water bodies only during the breeding period. To ensure that the larvae have enough time to metamorphose and leave the water before the next terrestrial phase starts, it would be advantageous to lay eggs fast after insemination. Second, female newts compete for access to laying substrate (small leafed water plants), and newt larvae compete for food in crowded breeding ponds. In both cases, it is probably advantageous to be among the first competitors present.

Our results suggest that belly coloration can be an important signal to determine the individual quality of Alpine newt females. Because males complete spermiogenesis during autumn and store mature sperm until the next breeding season, they are likely to be limited in the total sperm numbers that are available during one mating season (Verrell, 1986). Sperm stores may hence deplete over the course of the breeding season. During a single courtship ritual, males deposit often more than one spermatophore if they are not interrupted by conspecifics (Halliday, 1977; own observation). Within successively deposited spermatophores, the number of spermatozoa decreased continuously (Maag, 2013). This suggests that males are sperm limited even within a particular day during the mating season, as multiple courtships per day (often with various partners) are common in this species (own observation). To invest the available sperm optimally, male Alpine newts should decide to court very fecund females or females whose eggs have a high reproductive value. Our findings suggest that by choosing to court more orange females (Lüdtke and Foerster, 2018), Alpine newt males gain adaptive advantages through a potentially higher number of fertilized eggs. Further, through the speedy laying pattern of orange females, there is a better chance that a high number of these eggs will be deposited on suitable substrate, and that a high number of hatchlings will be born early enough to get a head start in their development compared to other competing larvae in the pond. Since female belly color correlated with fecundity and with laying speed, it can function as an easy-to-detect target for male choice.

In a previous study, we found indeed that male Alpine newts courted more orange bellied females for longer than less orange ones (Lüdtke and Foerster, 2018). In that study, however, we also found that males courted longer for larger females. Despite anecdotal evidence that body size positively correlates with egg numbers in this species (Miaud and Merilä, 2001), other studies could not confirm such a relationship (Thomas et al., 2002) nor could we find a correlation between female size or female body weight and number of eggs laid in this experiment. This suggests that female size does not indicate fecundity in Alpine newts. Female size could nevertheless correlate to other aspects of reproductive value, such as the size of the eggs. In spadefoot toads, *Spea multiplicata*, for example, larger females laid larger eggs and larger eggs produced larger tadpoles (Martin and Pfennig, 2010). Larger body size, in turn, would greatly affect the tadpoles' survival and hence the fitness of their parents. It remains to be shown whether female body size or body weight advertise any component of female reproductive value in Alpine newts.

In conclusion, our results provide experimental evidence that female belly coloration of Alpine newts can function as an honest signal for individual quality. Males could consequently benefit when they choose to mate with a more orange female. The relation between female belly color and fecundity can further explain the evolution of male preference for a female ornament. The results from this study, together with our previous finding that males did indeed prefer more orange females in mate choice experiments, lead us to conclude that in Alpine newts, female belly color is subject to sexual selection acting directly on females.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Regierungspräsidium Tübingen, Referat 35.

AUTHOR CONTRIBUTIONS

All authors conceived and designed the experiments. DL performed the experiments and the data collection, analyzed the data and wrote the manuscript. KF revised and edited the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00432/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

Table S1. Comparison of the linear mixed models with either hue or chroma as independent variables for daily cumulative egg numbers. Models are ranked based on the AICc values (corrected AIC for small sample size) and Akaike weights; Δ AICc is the difference from the AICc of the best model. *N* = 39.

Model	Fixed terms included in model	AIC_c	Δ AIC_c	Weight
A1	log(day) + hue + weight + (log(day) * hue) + (log(day) * weight)	4499.19	0.00	0.91
B1	log(day) + hue + SVL + (log(day) * hue) + (log(day) * SVL)	4503.83	4.64	0.09
A2	log(day) + chroma + weight + (log(day) * chroma) + (log(day) * weight)	4581.12	0.00	0.61
B2	log(day) + chroma + SVL + (log(day) * chroma) + (log(day) * SVL)	4582.01	0.89	0.39

Table S2. Summary results of the averaged linear mixed model for daily cumulative egg numbers, with chroma as independent variable. The model was averaged across the models with weight and with snout-vent length. The importance (i.e., the sum of Akaike weights), mean estimates and 95% confidence intervals of all covariates and interactions are reported. Significant estimates are shown in bold. *N* = 39.

Fixed factors and covariates	Importance	Average estimate	SE	95% confidence intervals
Intercept		**-26.307**	**3.364**	**-32.900 to -19.715**
log(Day)	**> 0.99**	**41.447**	**3.655**	**34.283 to 48.611**
Chroma	> 0.99	-6.139	4.633	-15.220 to 2.943
Weight	0.61	-5.286	3.424	-11.998 to 1.425
SVL	0.39	3.617	3.697	-3.629 to 10.863
log(Day) * Chroma	**> 0.99**	**12.419**	**5.026**	**2.568 to 22.269**
log(Day) * Weight	0.61	3.631	3.803	-3.822 to 11.084
log(Day) * SVL	0.39	-4.675	4.026	-12.566 to 3.216

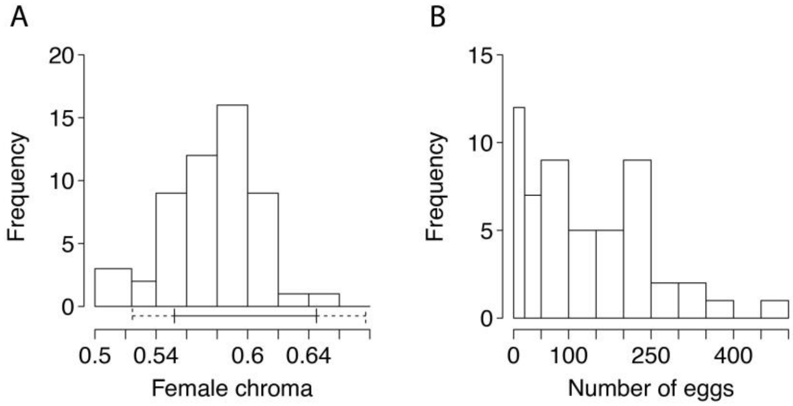

Figure S1. Histogram illustrating the frequency of **(A)** female chroma, and **(B)** number of eggs. **(A)** The line indicates the range of the natural population (solid line; chroma: 0.552-0.645) and the 5 % buffer zone (dashed line). **(B)** First bar represents the females that laid between 0 and 20 eggs. *N* = 53.