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Non-random mating patterns and consistency of behaviour of amphibians

DISSERTATION

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Declaration of originality

I declare that this thesis has not been submitted for the purpose of obtaining the same or any other academic degree earlier or at another institution. My involvement in the research presented in this thesis is specified in the statement of contributions and it is also expressed through the authorship order of the included publications and manuscript. All relevant literature sources used while writing chapters in this thesis have been properly cited.

Petr Chajma

Prague, Czech Republic, August 2020

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Statement of contribution

This thesis is the cumulative work consisting of the five contributions (three published papers and two unpublished results). Except for the first publication, I am the first author in all contributions. Details on my contribution to the each of the chapters included in thesis are specified in detail below.

Chapter 1: The effect of sex ratio on size-assortative mating in two explosively breeding anurans

Jiří Vojar, Petr Chajma, Oldřich Kopecký, Vladimír Puš, Miroslav Šálek

My contribution: *I participated on the concept of the manuscript, statistical analysis, interpretation of the results and writing of the manuscript.*

Chapter 2: Unpublished results: First come, first served. The story of mating constraints of common toad.

Petr Chajma, Jiří Vojar

My contribution: *Together with my co-author, we designed the experiment, carried it out, I evaluated the results and interpreted them for the purpose of this thesis.*

Chapter 3: The effect of size-assortative mating on fertilization success of the common toad (Bufo bufo)

Petr Chajma, Jiří Vojar

My contribution: *With the help of my co-author, I designed the experiment, carried it out, evaluated and interpreted the results and wrote the manuscript.*

Chapter 4: Individual consistency of newt’s exploration and shyness, but not activity: The effect of habituation?

Petr Chajma, Oldřich Kopecký, Jiří Vojar

My contribution: *Together with both co-authors, we designed the experiment. I carried it out with the help of O. Kopecký, evaluated and interpreted the results and wrote the manuscript together with J. Vojar.*

Chapter 5: Unpublished results: Population density, sibship and the changes in behavioural traits

Petr Chajma, Jiří Vojar

My contribution: *Together with my co-author, we designed the experiment. I carried it out, evaluated the results and interpreted them for the purpose of this thesis.*

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Summary

Non-random mating patterns are frequently studied topic. However, the studies suffer from many misconceptions, built upon poor understanding of statistical methods. Most attention was given to the patterns based on size, such as size-assortative mating, size-disproportionate mating and size-dependent mating. In this thesis, I present the results of our study, describing the presence (and absence) of such non-random mating patterns in two explosively breeding anurans – the common frog (*Rana temporaria*) and the common toad (*Bufo bufo*) (**Chapter 1**), and examined the mechanics of physical mating constraints in pair formation, as one of the causes of these patterns (**Chapter 2**). Our findings showed that size may not be the most important trait in mate acquisition, as more active males had higher chances of acquiring mate, even if they were not exactly a match in terms of body size. Intrigued by these results, we tried to see, if the assortment by size can at least enhance individual fitness by improving egg fertilization success (**Chapter 3**), as expected by other authors. Fertilisation rate of common toads did not depend on the size difference of mates, giving another crack to the commonly accepted theory.

Next, we tried to build on the notion that male activity is important part of their mating pattern and tested, whether the activity and other behavioural traits are consistent, and can therefore be subjected to the selective pressure (**Chapters 4 and 5**). Although activity was not consistent in smooth newt (*Lissotriton vulgaris*), exploration and boldness were consistent in this species, showing the potential of behavioural traits in mate acquisition (**Chapter 4**). However, the question of heritability of such traits was still unanswered. We tried to shed some light on it by comparing behavioural traits and their consistency between siblings of common frog tadpoles, while addressing the potential changes in consistency caused by different population densities in early development of common frog tadpoles (**Chapter 5**). Although population density had a significant effect on tadpole behaviour, individuals showed consistency only in their boldness, not in activity and exploration. Furthermore, there was no consistency in the behaviour of siblings, which does not speak well about heritability of those traits.

Reviewing studies of other authors, I conclude that non-random mating patterns based on size are rare in amphibians and their influence is overestimated due to the incorrect interpretation of published results. The role of behaviour traits in

amphibian mating patterns is still yet to be examined, but the proof of their heritability should be presented, before considering their evolutionary meaning.

General introduction

Reproduction is a foundation of life. For me, the most fascinating part of reproduction are the events that lead to the mating itself. There are plenty of studies and books (e.g. Taylor & Guttman, 1977; Bateson, 1983; Duellman & Trueb, 1994; Jamieson, 2003; Wells, 2007), aiming to describe the courtship behaviour, pair formation and mating preferences of amphibians, yet after doing some research myself, I feel that there is still a considerable number of phenomena that need to be explained. Because of the diversity of amphibian morphology, life histories, reproductive strategies and behaviour, this thesis manages to only scratch the surface of this vast topic, revealing even more questions to be studied.

Probably due to vast adaptive radiation of amphibians, they acquired many different life strategies, still more or less dependent on water (Duellman & Trueb, 1994; Wells, 2007). This also includes their reproduction, which features many interesting behaviours that appear to enhance individual success rate in acquiring one's mate. Amphibians differ in form of communication (acoustic, visual, chemical) and therefore in a form of courtship, in form of fertilization (internal, external) and in reproductive modes, which are a combination of ovipositional and developmental factors, including oviposition site, ovum and clutch characteristics, rate and duration of development, stage and size of hatchling, and type of parental care, if any (Duellman & Trueb, 1994; Wells, 2007). Reproductive cycles in amphibians are subject to hormonal controls, which within genetic limitations respond to environmental variables and produce certain patterns, ranging from multiennial to continuous (Duellman & Trueb, 1994). However, despite these differences, it is believed that some common morphological and behavioural criteria exist, upon which individuals choose their mates, resulting in common mating strategies and mating patterns, that are the main focus of this thesis.

During my studies, I slowly moved from purely mechanistic approach to the mate choice, towards evaluation of individual differences in behaviour. The consistency of one's behaviour (sometimes labelled as personality, see Gosling, 2001) may play a significant role not only in the survival (Dingemanse & de Goede, 2004; Koprivnikar, Gibson & Redfern, 2011; Carlson & Langkilde, 2014a) and dispersal (Cote *et al.*, 2010, 2013; Gruber *et al.*, 2017a, 2017b; reviewed in Cayuela *et al.*, 2018), but also in reproductive ecology (Martin *et* *al.*, 1989; Jaquiéry *et al.*, 2010) of amphibians. This area of research seems, unfortunately, very new to the researchers specializing in amphibians and is still at relatively basic level. The description of behavioural traits and their consistency may thus prove invaluable to progress in reproductive ecology.

Mating patterns

My journey into the world of reproductive ecology of amphibians started with the study of non-random mating patterns. Such patterns can depend on reproductive mode, extrinsic factors (e.g. temperature and rainfall), which affect the length of the breeding period, and on mate traits (differences in fecundity, duration of development, reproductive effort, age at first reproduction, etc.) (Duellman & Trueb, 1994). Authors usually use the length of the breeding period (not season, as it can comprise of several periods) to divide breeders into two distinct groups and classify them as explosive (breeding period shorter than one month) or prolonged (breeding periods are longer than one month) (Wells, 1977).

Explosive breeders typically have asynchronous arrival at the breeding site (Wells, 1977). Males usually arrive earlier and in much higher quantities than females (Lodé se al., 2005). They use vocalisation mostly to attract females to the breeding site, not as a mean of mate choice (Mccauley *et al.*, 2000). Female choice is usually supressed by the sheer number of competing males. There is a frequent occurrence of multiple amplexus, which can sometimes result in drowning of the female (Licht, 1976; Davies & Halliday, 1979; Howard, 1980). Explosively breeding males are more prone to physical combat, trying to dislodge amplectant rivals. It seems, however, that the dislodgement rate is usually low (Dole & Durant, 1974; Davies & Halliday, 1978; Howard, 1980; Vaira, 2001, **Chapter 2**). In these cases, it may be beneficial to actively search for the mate, prior to entering the breeding pool. Explosively breeding species can also benefit from higher sexual dimorphism (e.g. colour change in moor frog, *Rana arvalis*; Hettyey *et al.*, 2009), as such will reduce energy expenditure on incorrectly aimed attempts of amplexus or dislodgement. If the distinction fails, anuran males produce a release call, that can even serve to compare sizes of competing males (Marco *et al.*, 1998; Castellano *et al.*, 2002). Some tropical anuran species use a mixture of gestures and poses instead (Hödl & Amézquita, 2001).

Prolonged anuran breeders depend heavily on vocalisation, including lekking behaviour (Castellano *et al.*, 2009). Females can thus judge the most appropriate male, based on the properties of males' call, mainly it's dominant (fundamental) frequency and call rate (Bee *et al.*, 1999). Some females prefer lower call frequences, which are negatively correlated with male size (Sullivan *et al.*, 1995; Murphy & Gerhardt, 1996; Bee *et al.*, 1999), other favor higher call rates, that show energy high expenditure and thus show current condition of the male (Cherry, 1993; Sullivan *et al.*, 1995). Vocalisation based female choice gave birth to alternative mating strategies. Sattelite males reside near the calling mates and wait for the approaching female. When the opportunity presents itself, they leave the cover and try to clasp the female for themselves, before they even arrive to the calling male (Arak, 1988; Lucas *et al.*, 1996). As opposed to explosive breeders, there is no need for direct male physical competition and lower benefit of active mate searching(Wells, 2007).

Urodeles show different mating patterns and do not rely on vocalisation for reproductive purpouses as anurans do, but mostly use gestures and chemical cues (Wells, 2007). Their breeding periods, however, can be divided to explosive and prolonged as well, even though highly explosive periods are quite rare(Wells, 1977). Nevertheless, they can exhibit patterns, similar to the scramble competition of anurans (Janzen & Brodie, 1989). Some species clasp the cluth instead of the female, while releasing the sperm and fighting their rivals (Hasumi, 1994). When the fertilization is iternal, explosively breeding urodeles can compete by laying spermatophores close to, or even on top of each other(Wells, 2007). Compared to anurans, multiple paternity seems to occurr more often, giving birth to different mating strategies (Park *et al.*, 1996; Jones *et al.*, 2002). E.g. the common newt females (*Lissotriton vulgaris*), which have prolonged mating period, become more choosy with increasing number of matings, as sperm that was deposited last, has higher fertilization rate (Gabor & Halliday, 1997).

Most-commonly studied non-random mating patterns of amphibians are based on size, but mating based on body colour (Hettyey *et al.*, 2009), fin size (Able, 1999; Jones *et al.*, 2002) or age (which is a function of body size) (Emlen, 1976; Eggert & Guyétant, 2003) were described as well. Usually, you can find two main size-based patterns – size dependent (SDM) and size assortative mating (SAM).

Size dependent mating

Size dependent mating hypothesizes increased mating success of larger (or smaller) individuals. E.g., larger size can imply physical superiority in a battle between males and it can show fecundity of females, but smaller size can put lower strain on the mate, if it must carry the other sex. The size is usually measured as snout to vent length (SVL) or mass (e.g. Hoglund & Saterberg, 1989; Bastos & Haddad, 1996; Giasson & Haddad, 2007; Gramapurohit & Radder, 2012), but even as tibia length (e.g. Hoglund & Saterberg, 1989; Yu & Lu, 2010) or fin length (Able, 1999; Jones *et al.*, 2002).

The advantage in larger size was reported for several species (Green, 2019). Some species, however, tend to show different results, based on the properties of each population. E.g. in the American toad (*Anaxyrus americanus*), SDM was found by Gatz (1981), but not by Wilbur *et al.* (1978), nor Kruse (1981). Particularly Gatz (1981) and Kruse (1981) measured toads in the same year and in states with very similar climatic properties. The population from Gatz (1981) had smaller males than the other one, which could have resulted in a higher selective pressure on body size. Eggert & Guyetant (2003), for example, report that age (and therefore size) of the common spadefoot toad (*Pelobates fuscus*) can alter male mating strategy. The difference in the results, could however have been caused by number of other factors that were not measured, such as operational sex ratio (OSR), population density, or something completely different. In fact, not only the size advantage, but the body-size itself is highly plastic at the population level (Yom-Tov & Geffen, 2011; Forsman, 2015) and influenced by density and resource availability (Huston & Wolverton, 2011; Gillespie *et al.*, 2017). Green (2019) also states, that size heritability is partial at best, with numerous studies reporting it as negligible or not demonstrable. Coupled with the strong variation in response to environmental influences (Green & Middleton, 2013; Băncilă *et al.*, 2016), large-bodied individuals may not necessarily produce large-bodied adult offspring. Green (2019) even goes as far as questioning the sufficiency of the body-size's selective value.

Size assortative mating

Size assortative mating expects a positive correlation of body sizes of mates (Lewontin *et al.*, 1968) – big individuals have big mates, while smaller individuals have smaller mates. This pattern is a tad more complicated than the previously mentioned one, as it occurs as result of a combination of multiple different processes. Crespi (1989) created three main hypotheses of the cause of SAM – mate choice, mate availability and mating constraints.

Mate choice hypothesis describes an interaction of male and female choices. It can occur if there is a benefit to choosing larger (or smaller) males and at the same time a benefit to choosing larger (or smaller) females. This can result in both positive and negative correlation of size. Positive correlation would appear if the size-based attractiveness were the same for both genders – more attractive males (regardless of which size is preferred) will mate with more attractive females. Negative correlation would appear if the preferred size of one sex is the opposite of the preferred size of the other sex – e.g. larger females would be more attractive due to a larger fecundity (Krupa, 1994) and smaller males would be more attractive due to lower detectability and higher chances of survival. As mentioned earlier, the influence of mate choice is positively affected by the length of the breeding period and lower OSR (Janzen & Brodie, 1989; Park *et al.*, 1996; Gopurenko *et al.*, 2006).

The mate availability hypothesis states, that SAM arises in conditions, where there is higher probability of similarly sized individuals appearing at the same time in the same space. In some frog species, bigger individuals arrive to the breeding site faster and are more visible to the other sex, raising the probability of SAM (Bateson, 1983; Andersson, 1994). This, however, works only in species with a gradual (prolonged) migration to the breeding site, contrary to the explosive breeders that move practically at the same time (Eggert & Guyétant, 2003). Uneven temporal and spatial availability of mates of different sizes can also be caused by size-specific habitat preference, when individuals of the same size prefer similar habitats, or are unable to reach farther or less accessible habitats (Ferrer & Penteriani, 2003).

The mating constraints hypothesis is built around the idea of physical constraints that limit the mating success of mismatched individuals. If amplexus is present, some size combinations of paired individuals will benefit from higher clasping force then other. If male competition is present and amplexus dislodgements are frequent, matching pairs will have higher chances of successful mating (Davies & Halliday, 1979; Lu *et al.*, 2010). The advantage might become more pronounced, with raising energy expenditure, e.g. if the pair must move a lot in places with overgrown flora, endure more attacks, or remain amplexed for longer periods of time. Mating constraints probably do not act symmetrical on differently sized individuals and can lead to asymmetric and heteroscedastic relationship of mate body-sizes (Fig. 1, **Chapter 2**). This is also supported by Green (2019), who states, that the assortment of the mates by this hypothesis is mostly secondary via male-male competition and proposes to call is “size-disproportionate mating” and emphasizes that it’s evolutionary implications are still unresolved and may differ from those of the “true” size-assortative mating.

These mechanisms most certainly do not occur exclusively, and their combination might be the most common culprit behind SAM. Moreover, with the population size fluctuations, that are so common in amphibians (Pechmann *et al.*, 1991; Marsh, 2001; Marsh & Trenham, 2001), and subsequent changes in OSR, the magnitude of these processes and SAM can also change on yearly basis (Takahashi *et al.*, 2010; Bierbach *et al.*, 2011).

In **Chapter 1**, I tested if non-random mating pattens occur in two explosively breeding anurans – common frog (*Rana temporaria*) and common toad (*Bufo bufo*). Although hypothesis of mate availability was also of the focus of this study, its' results, sadly, had to be removed from the paper, as the lack of positive results was underwhelming to the reviewers. The relationship between the individual size and arrival date to the reproductive site was very weak, as date explained only 1-4 % of variation in SVL. We found SAM in common frogs, but not common toads. The strength of the correlation of body sizes in frog pairs was positively affected by the male biased (OSR) (**Chapter 1**).

Higher OSR indirectly led to the stronger SAM, possibly through the male to male competition and dislodgements from amplexus, as we thought at the time. So, the mating constraints hypothesis and its relationship with OSR was tested in a follow-up experiment (**Chapter 2**). Unfortunately, only common toads from a completely different location were available for this experiment. We divided toads into 20 groups with male-biased OSR of 3 in first half and male-biased OSR of 6 in second. Each group had equal number of large males (smaller than female by 0-10 mm), medium males (smaller than female by 10-20) and small males (smaller than female by 20-30 mm). The resulting pairing (and subsequent mating) was not assortative by size (*Spearman's correlation*: $r = 0.12$, $p = 0.62$), mass (*Spearman's correlation*: $r = 0.18$, $p = 0.47$), nor body-mass index, although coming closest out of all three (*Spearman's correlation*: $r = 0.26$, $p = 0.3$). More incoming attacks meant more danger of dislodgement for amplectant male ($\chi^2 = 16.8$, $p < 0.001$), with highest probability of dislodgement coming from well matched males (medium size category). From the perspective of attacking male, the size was not so important – if he attacked more, his success rate rose regardless of opponents size ($\chi^2 = 13.3$, $p < 0.001$). This would mean, that what theory predicts is true and physical constraints can, indeed, create advantage of size-assorted pairs, and thus promote the occurrence of SAM. But because the overall dislodgement success rate was low ($\mu = 0.38$, $\sigma = 1$). The number of dislodgements caused by female was twice that ($\mu = 0.76$, $\sigma = 1.8$), but the advantage of size compatibility was still outshined by activity of the males. Active males were not only more successful in dislodging of their rivals, but tended to clasp the female first (pers. obs.). The saying "first come, first served" is applicable even to common toad mating and size may thus not be the most important indicator of reproductive success.

Consequences of SAM

Size assortative mating alone is an interesting phenomenon. However, does it have any benefit, or is it just an intriguing consequence of some processes? This is probably the most important question, but only a few researchers have tried to answer it and with very conflicting results. The most notable short-term consequence, proposed by some researchers is the effect on individual fitness (Davies & Halliday, 1977; Burley, 1983; Crespi, 1989). Long term effects may include the reduced probability of outbreeding, hybridisation or the role in speciation through creation or enhancement of reproductive barriers (Coyne, 1992).

Fertilization success

It is hypothesized, that SAM can increase individual fitness, because pairs of well-matched partners should also have a higher proportion of fertilized eggs (Davies & Halliday, 1977; Crespi, 1989). Higher fertilization success is achieved through smaller distance between cloacae of mates, increasing the precision of sperm deployment. The timing of sperm deployment might be improved when the mates are well matched, as the signal of egg deposition (usually a leg bump) might not be noticed when the male is too small or too big (Wells, 2007). Despite common acceptance of this theory, reviewed studies do not show positive effect of SAM on fitness.

No relationship between mate size ratio and fertilization success was reported for American toad ($r = -0.3$; $n = 19$; $p > 0.2$) (Kruse, 1981), American green tree frog (*Dryophytes cinereus*) (Gerhardt *et al.*, 1987), red-eyed tree frog (*Agalychnis callidryas*) ($r = 0.2$; $n = 56$; $p = 0.15$), nor Morelet's tree frog (*Agalychnis moreletii*) ($r = 0.19$; $n = 46$, $p = 0.21$) (Briggs, 2008). Fan *et al.* (2013) did not find a difference in fertilization success between groups with male and female biased size difference in Asiatic toad (*Bufo gargarizans*) ($F = 1.98$; $n = 65$, $p = 0.16$), nor in Asian common toad (*Duttaphrynus melanostictus*) ($F = 1.05$; $n = 149$; $p = 0.31$). Hettyey *et al.* (2011) did not find any effect of male size on the fertilization success in common toad ($F = 0.11$; $n = 9$; $p = 0.75$), but they did find a significant effect of male condition ($F = 13.65$; $n = 10$; $p = 0.006$). Howard (1983) reported that fertilization success in American bullfrog (*Lithobates catesbeianus*) correlated with male size ($r = 0.66$; $p = 0.0008$), but only during one of the six surveyed years, and did not correlate with female size during the whole period.

Bastos & Haddad (1996) reported dependency of egg fertilization success on mate size ratio and second power of the mate size ratio in *Dendropsophus elegans*, but judging from provided figure, their result is obviously incorrect (see Discusssion). Ryan (1983) found a weak relationship between number of unfertilized eggs and absolute value of mate size difference of túngara frog (*Engystomops pustulosus*) ($r = 0.30$; $n = 68$; $p < 0.05$ and $F = 15.41$; $n = 68$; $p < 0.05$). Number of fertilized eggs depended on clutch size ($F = 5811.4$; $n = 68$; $p < 0.05$), female size (highly correlated with clutch size) and absolute value of mate size difference ($F = 5.9$; $n = 68$, $p < 0.05$). The only strong evidence of the relationship of fertilization success and a measure of body size was provided graphically (with no test results) by Robertson (1990)for the smooth toadlet (*Uperoleia laevigata*), which showed a dependency of fertilization success on a difference from expected ideal mate mass ratio and it's second power.

My next step, naturally, was to assess the benefits of SAM on our recent study species, a common toad. In **Chapter 3**, we examined the presence of SAM in the common toad, and the effect of SAM on the proportion of fertilized eggs. Specifically, we evaluated the effect of male and female body size difference and ratio within paired toads on clutch fertilization success. The results were in line with previously published studies, as they showed no effect of mate size difference, nor mate size proportion on the proportion of fertilized eggs (**Chapter 3**).

Amphibian personality

It appears that non-random mating strategies, that are thought to be based on size, do not necessarily have expected fitness benefit (more on the topic in Discussion). This further promotes the thought that other traits are factored in the mate choice and judging from my previous experience, they could very well be of behavioural nature. Furthermore, morphological traits such as body size, are generally less closely associated with individual fitness than higher-level traits (such as life-history and behavioural traits) (Careau & Garland, 2012). Because of the realisation, that active males were more successful in mate acquisition (**Chapter 2**), I started to investigate amphibian behavioural traits. However, if activity, or any other trait, was selected upon, it would be very important, that individuals showed consistency and heritability in such trait, which is generally expected to be lower than the heritability of morphological traits (Mousseau & Roff, 1987; Kruuk *et al.*, 2000). Consistency in the expression of behavioural traits over time and in different situations is often labelled as personality and has already been studied in many taxa (e.g. Gosling, 2001; Sih *et al.*, 2004; Réale *et al.*, 2007; Garamszegi *et al.*, 2013). Across those taxa, behaviour consistency is being linked to survival in predator-prey interactions (Dingemanse & de Goede, 2004; Carlson & Langkilde, 2014a; but see Carlson & Langkilde, 2014b), disease risk (Koprivnikar *et al.*, 2011), dispersal tendencies (Cote *et al.*, 2010, 2013; Gruber *et al.*, 2017b, 2017a; reviewed in Cayuela *et al.*, 2018), and of course reproductive success (Dingemanse & Réale, 2005; Cole & Quinn, 2014).

An interesting example of the role of personality in mating pattern can be seen in great tits (*Parus major*). Their exploratory behaviour was found to be heritable (Drent *et al.*, 2003; Dingemanse *et al.*, 2004). Both *et al.* (2005) found a clear benefit to mate assortatively, based on exploratory behaviour, as assortative pairs at both ends of the behavioural spectrum produced fledglings in best condition and had most recruits in some years of the experiment. Nevertheless, older (but not 1-year old) individuals' mate disassortatively (Dingemanse *et al.*, 2004). Why? Selection over the entire life span may favour disassortative mating, because this apparently allows birds at the extremes of the behavioural distribution to increase their fitness by producing medium-exploring offspring, which is the most stable fenotype, due to the changing survival selection across years and sexes (Both *et al.*, 2005). Thus, adult males of the extreme phenotype may have maximized their fitness by means of adaptive mate choice to mate disassortatively with respect to personality type.

In contrast to significant advances in animal personality, this topic is still new to amphibian research. Generally, animal personality traits are divided into five different, and theoretically independent axes – (i) activity (general level of activity in a non-risky and a non-novel environment), (ii) exploration (reaction to a new situation – habitat, food, novel object, etc.), (iii) boldness or risk taking (reaction to a risky, but not new situation), (iv) aggressiveness (agonistic reaction towards conspecifics) and (v) sociability (non-aggressive reaction to the presence or absence of conspecifics) (Réale *et al.*, 2007). The broad definition of personality and personality traits can include many different types of behaviour and is the cause of a great variance in the choice of experimental methods. The consistency of at least one behaviour trait was reported for eight anuran and six urodele species, with seven studies performed on larval and 10 on post-metamorphic individuals (see Supplementary information of **Chapter 4** for details). Eleven studies focused on temporal consistency, with time gaps between repeated measurements ranging from four hours to nine weeks. Three studies quantified behavioural consistency across different treatments.

To quantify consistency, at least two sets of measurements on an adequate number of subjects should be taken. Repeatability is then estimated through standard or multiple (intraclass) correlation, and as in the detection of SAM, the most important outcome is the strength of the correlation. Keep in mind, that correlation coefficient ranges from −1 to 1, while intraclass correlation coefficient ranges from 0 to 1 and could be, just for comparison, viewed as a second power of correlation coefficient. Unfortunately, some authors still report only significance (p-value), so it is impossible to verify their results completely. Moreover, only two studies shared the same methodology for the measurement and quantification of behavioural traits and their consistency (Videlier *et al.*, 2014, 2015).

For amphibians that generally show notable differences in both physical and behavioural traits between larval state and post metamorphosis, it is also crucial to prove the consistency between life stages. To my knowledge, only one study has managed to do so, finding consistency of combined measure of activity and exploration (an axis of principal component analysis, performed in the study) between the life stages of marsh frogs (*Pelophylax ridibundus*) (Wilson & Krause, 2012). As opposed to that, Brodin *et al.* (2013) did not find a significant correlation between life stages of common frog for exploration ($r = 0.25$), nor boldness ($r = 0.25$).

Activity

Some form of consistency in activity was reported in moor frog tadpoles (*Rana dalmatina*) (Urszán, 2015a,b), marsh frog tadpoles and juveniles (Wilson & Krause, 2012), American bullfrog tadpoles (Smith & Doupnik, 2005; but not in Carlson & Langkilde, 2013), wood frog (*Lithobates sylvaticus*) tadpoles (Koprivnikar *et al.*, 2011), natterjack toad (*Epidalea calamita*) juveniles (Maes *et al.*, 2012), corroboree frog adults (*Pseudophryne corroboree*) (Kelleher *et al.*, 2017), wester clawed frog adults (*Xenopus tropicalis*) (Videlier *et al.*, 2014, 2015) and Bosca's newt adults (*Lissotriton boscai*) (Aragón, 2011). The repeatability of the activity in published studies differed between odour treatments (Urszán *et al.*, 2015a), arena structures (Smith & Doupnik, 2005), between breeding origins – wild or captive (Maes *et al.*, 2012), between sexes (Aragón, 2011), between differently experienced individuals (Urszán *et al.*, 2015b), between methods of measurement (Videlier *et al.*, 2014; Kelleher *et al.*, 2017) and slightly with age (Urszán *et al.*, 2015b) (see Supplementary information of **Chapter 4** for details). Repeatability did not differ with the size of a time gap between repeated measurements (Maes *et al.*, 2012). Higher activity levels were beneficial in mate searching in Alpine newts (*Ichthyosaura alpestris*) (Martin *et al.*, 1989) and as seen in **Chapter 2**, could positively affect amplexus displodgement rate in common toad. Activite European treefrog males (i.e. males with higher participation in chorus) had higher mating success (Jaquiéry *et al.*, 2010). Activity of wood frog tadpoles resulted in lower parasitic infection levels (Koprivnikar *et al.*, 2011), possibly raising individual body condition and survivability, and in long term, making them better prepared and possibly more attractive in future mating.

Similarly, to SAM, it seems that consistency of activity will vary a lot in different populations with different histories. But even within those populations, there will be differences in age and experience. This could present an opportunity for the change of individual mate choice throughout individuals' lifetime, in order to maximize its' reproductive success. Resulting mating patterns, might therefore not be as simple, as one would suspect.

Exploration

Explorative behaviour was consistent in moor frog tadpoles (Urszán, 2015b), American bullfrog tadpoles (Carlson & Langkilde, 2013), corroboree frog adults (*Pseudophryne corroboree*) (Kelleher *et al.*, 2017), wester clawed frog adults (*Xenopus tropicalis*) (Videlier *et al.*, 2014, 2015), and Ouachita dusky salamander adults (*Desmognatus brimleyorum*) (Gifford *et al.*, 2014). Repeatability estimates differed with age and experience, being slightly higher in older and experienced individuals (Urszán, 2015b). Interestingly, the consistency of exploration in Ouachita dusky salamanders lowered with increasing time between repeated measurements (Gifford *et al.*, 2014). This increasing difference in behaviour expression was connected to the decrease in amount of food income and body condition of the animals throughout their captivity, suggesting a link between body condition and exploration. There was, however, no correlation between exploration and metabolic rate and the amount and direction of change in exploration was highly individual (Gifford *et al.*, 2014). Exploration in cane toad (*Rhinella marina*) is related to dispersal patterns (Gruber *et al.*, 2017a) and may be used as for the prediction of individual movement patterns within and between habitats in a diversity of species (Kelleher *et al.*, 2018), which can transfer even to mating behaviour, especially in species, where active mate searching is common.

Boldness

Boldness seems to be consistent in common frog tadpoles (Brodin *et al.*, 2013), moor frog tadpoles (Urszán, 2015a), marsh frog tadpoles and juveniles (Wilson & Krause, 2012), natterjack toad juveniles (Maes *et al.*, 2012), cane toad adults (González-Bernal *et al.*, 2014; Gruber *et al.*, 2017a), corroboree frog adults (Kelleher *et al.*, 2017), western clawed frog adults (Videlier *et al.*, 2014, 2015) and streamside salamander (*Ambystoma barbouri*) larvae (Sih et al., 2003). As with exploration, the consistency of boldness was slightly higher in previously tested (experienced) individuals (Urszán, 2015b). Urzsán (2015a) found out that consistency of boldness depended on supplied odour. If absent or belonging to conspecific, expressed behaviour was inconsistent. Only in the presence of predator cues, individuals started to behave consistently bold (or shy). Maes *et al.* (2012) reported that only individuals caught from wild displayed consistency in boldness, as opposed to individuals raised in captivity, and that the consistency decreased with time. This may suggest, that to show consistently bold (or shy) behaviour, predatory pressure (or presence) is needed. Sih *et al.*, (2003), however, reported consistent boldness between different odour treatments, so some other reason for these discrepancies may be at play.

Aggressiveness

There is no information about the consistency of aggressive behaviour in amphibians. Aggression towards conspecifics might the mating pattern in more ways than one. One way may be through the male-male competition over females. Haubrich (1961) also showed, that more aggressive African clawed frog females (*Xenopus laevis*) eat more and have higher mass than less aggressive females (but Tornick (2010) reports no relationship of aggressiveness and size for female eastern red-backed salamanders, *Plethodon cinereus*). Although he did not test specifically for the consistency in this trait, he did have multiple measurements of aggressiveness for each individual and claimed that there was distinguishable individuality in number of aggressive encounters. However, one should be careful when assessing aggression, as it can vary due to several factors, such as genetic relatedness (Markman *et al.*, 2009), age of the clutch during clutch guarding (Tornick, 2010), aggressiveness of surrounding individuals (Brenovitz & Rose, 1994), or season (Shepard, 2004).

Sociability

As with aggressiveness, there is still no information about the consistency of social behaviour in amphibians. Sociable cane toads are, however, consistently less bold than non-sociable cane toads and their personality influences their reliance on social cues when choosing foraging sites in the field (González-Bernal *et al.*, 2014). That could be the case for mating behaviour as well and sociability may thus play an important role in the choice of mating strategy.

Behavioural syndromes

Correlated suites of behaviour belonging to different personality traits are often called behavioural syndromes and selection probably acts on these whole suites, rather than individual traits, as individuals have limited level of overall behavioural plasticity (Sih *et al.*, 2004). Interpretation of this correlation, however, might be difficult, as a single observed behaviour can fall into multiple personality axes and therefore be inherently correlated with other behaviour. Indeed, some behaviours were classified as different traits by different people, e.g. Activity level in presence of chemical cues was classified as both boldness (Carlson & Langkilde, 2014a) and activity (Sih *et al.*, 2003). Time spent active can be classified as activity and exploration, depending on the novelty of experimental arena. But how long does it stay novel? How do you quantify and compare novelty, if experiments have different-sized arenas, different trial duration and different number of trials?

Activity has been found to correlate with exploration (Koprivnikar *et al.*, 2011; Maes *et al.*, 2012; Wilson & Krause, 2012) and with boldness (Maes *et al.*, 2012; Wilson & Krause, 2012; Urszán *et al.*, 2015a). Boldness has also been found to correlate with exploration (Maes *et al.*, 2012) (but see Brodin *et al.*, 2013), and also with sociability (González-Bernal *et al.*, 2014). No positive correlation of activity, exploration and boldness has, however, ever been found to occur at the same time, mostly because it has not been thoroughly tested (but see Maes *et al.*, 2012). Recent studies show that not only personalities, but behavioural syndromes as well vary as a function of ecology and the events that affect personalities and syndromes can cause either temporary, or permanent change in personality / syndrome structure (Sih *et al.*, 2015). They also propagate the idea of state-dependent personalities, emphasizing that positive feedbacks between state variables and behaviour can link the co-evolution or co-development of state and behaviour, resulting in consistent among-individual variation in personality and state (Sih *et al.*, 2015).

I wanted to add to the growing body of evidence on amphibian personalities with the long-term aim to pursue their connection to non-random mating patterns. Because we had available group (n = 42) of smooth newts, I started to evaluate the consistency of their behaviour (**Chapter 4**). I found that smooth newts exhibited consistency in exploration and boldness, but not activity. The choice of the type of locomotion (walking vs. swimming) was individually consistent throughout three experimental trials. Based on exploration, individuals showed two distinct groups – fast and slow explorers. Fast explorers swam more and did not stay in each part of the arena for long time, while slow explorers mostly walked and took more time to truly "explore" the surroundings. Newt activity, shyness and exploration was correlated throughout the experiment, suggesting the possible existence of a common selective pressure. Active individuals tended to explore more and were less bold, spending more time trying to escape the arena.

My second behavioural experiment examined the consistency of activity, exploration and boldness in common frog tadpoles, that belonged to six different clutches and were raised in three different (low to high) densities (**Chapter 5**). Only boldness was individually consistent throughout three repeated measurements of tadpoles of Gosner stage 26 – 40 (Gosner, 1960). Activity, exploration and boldness were not repeatable for tadpoles from the same clutch, raising questions on heritability of these traits in common frogs. Activity raised with the raising density throughout tadpole development, lowered with increasing daytime and did not depend on the size of the tadpole. Exploration was highest if the tadpole was raised in medium density, followed by large density. Exploration decreased with daytime and was unaffected by tadpole size. Boldness was unaffected by density during tadpole development and by tadpole size and increased with increasing daytime.

Chapter 1

The effect of sex ratio on size-assortative mating in two explosively breeding anurans

Jiří Vojar, Petr Chajma, Oldřich Kopecký, Vladimír Puš, Miroslav Šálek

Size-assortative mating (SAM) is a widespread phenomenon related to individual fitness. In our study, we examined: (i) the appearance of SAM, and (ii) the effect of sex ratio on intensity of SAM in wild populations of two explosively breeding anurans, common frogs, *Rana temporaria*, and common toads, *Bufo bufo*. Despite a higher male-biased operational sex ratio (OSR) in toads, the body lengths of the paired males and females were significantly correlated only in frogs. Increasing male-male competition, assessed via the OSR, resulted in a stronger correlation also in frogs only. Thus, great variability in the presence and intensity of SAM has been observed within both studied species.

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The effect of sex ratio on size-assortative mating in two explosively breeding anurans

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Abstract. Size-assortative mating (SAM) is a widespread phenomenon related to individual fitness. In our study, we examined: (i) the appearance of SAM, and (ii) the effect of sex ratio on intensity of SAM in wild populations of two explosively breeding anurans, common frogs, *Rana temporaria*, and common toads, *Bufo bufo*. Despite a higher male-biased operational sex ratio (OSR) in toads, the body lengths of the paired males and females were significantly correlated only in frogs. Increasing male-male competition, assessed via the OSR, resulted in a stronger correlation also in frogs only. Thus, great variability in the presence and intensity of SAM has been observed within both studied species.

Keywords: *Bufo bufo*, correlation, explosive breeders, male-male competition, nonrandom mating, OSR, *Rana temporaria*, sexual selection.

Introduction

Nonrandom mating patterns have been found in many populations of amphibian species (e.g., Arak, 1983; Halliday and Tejedo, 1995; Wells, 2007) as a consequence of mate choice or competition for mates (Andersson, 1994). Nevertheless, different causes of nonrandom mating play a role in prolonged and explosively breeding anurans. In prolonged breeders, whose reproduction period can extend over several months, females often choose larger males with better territories (Howard, 1978). This leads to a size-dependent mating pattern and males larger than average usually achieve higher reproductive success (Wells, 1977; Howard, 1978; Arak, 1983).

On the other hand, pairing in explosive breeders has been considered to result from male-male competition rather than female choice, because females have limited opportunities to choose potential mates among competitive males (Wells, 1977; Davies and Halliday, 1979; Arak, 1983; Sullivan, Ryan and Verrel, 1995). In these species, mating and spawning takes only a few hours or days, as males struggle for females and often attack paired males, attempting to dislodge rivals from females and seize their positions (e.g., Davies and Halliday, 1978; Gittins, Parker and Slater, 1980a; Lu et al., 2008; Greene and Funk, 2009). Only a size-corresponding male (in relation to the female) can resist takeovers by other males under intense male-male competition (Wells, 1979; Arak, 1983). Such a mating pattern, wherein the body sizes of males and females correlate in pairs, is well known as size-assortative mating (SAM), the most documented nonrandom mating pattern in animals (Andersson, 1994). SAM can increase individual fitness (Burley, 1983), because pairs consisting of partners well matched for size should also have a higher proportion of fertilized eggs (Davies and Halliday, 1977; Bastos and Haddad, 1996).

The intensity of competition for mates in explosive breeders varies in time and space (Andersson, 1994; Wells, 2007), particularly due to changes in operational sex ratio (OSR, the ratio of sexually competing males to fertilizable females) (Emlen and Oring, 1977; Kvarnemo and Ahnesjö, 1996; Reynolds, 1996). It has been shown that an increasing male-biased OSR probably leads to more-intensive male-male

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competition (Tejedo, 1988; Höglund, 1989; Boll and Linsenmair, 1998; Lee and Park, 2009). If only pairs consisting of males and females well matched for size can resist takeovers by other males, then the increasing OSR and male-male competition should enhance the intensity of SAM within a population, expressed as the tightness of correlation between body size of males and females in pairs (Wells, 1979; Arak, 1983). Only a few studies have investigated the effect of OSR on the intensity of SAM in explosively breeding anurans (Arak, 1982; Elmberg, 1991; Lee and Park, 2009; Yu and Lu, 2010), and none of these have analyzed the statistical differences among the correlation coefficients obtained from samples with different OSRs.

Therefore, we examined the effect of different OSRs on SAM in wild populations of the most common explosively breeding European anurans, common frogs (*Rana temporaria*) and common toads (*Bufo bufo*).

Materials and methods

Study area

The study was carried out at Nový Tuchoraz pond (1.37 ha), near Český Brod, Central Bohemia, Czech Republic (50°03′N, 14°51′E, 210 m a.s.l.). The pond had a permanent water column during the field work, with a maximum water depth of 1.1 m. Emergent vegetation covering 25% of the water surface was concentrated on shallow (mostly 0.6 m deep) parts of the pond, which also attracted most of the individuals in the two anuran populations under study here.

Sampling of anurans

Both amplectant and single anurans were captured daily throughout the reproduction season from March to April 2004. Netting was performed using modified circle landing-nets until all observed frogs were caught. Pairs and singles were placed separately into plastic tanks with shallow water for a short period before measuring and marking. In total, we sampled 1013 adult frogs (460 single males, 83 single females and 235 pairs) and 2330 adult toads (1560 single males, 16 single females and 377 pairs). Snout-vent length (SVL) was measured to the nearest mm using a plastic ruler. All first-time captured anurans were uniformly marked, in order to prevent measuring of recaptured individuals. Marking was done by toe-clipping of only the last third of the outer phalange and then the place of marking was wiped with alcohol. Marked anurans were kept for several minutes in plastic tanks and then released at the site of capture. All marked anurans behaved normally after release, so we assume that toe-clipping had no harmful impact on their breeding activity.

The breeding season (i.e., the time between appearance of the first and last individuals in the reproduction pond) of common frogs lasted 15 d (from 20 March to 3 April). However, 93% of paired individuals and 86% of singles were captured during a short period of 4 successive days between 30 March and 2 April (the main reproduction season, MRS). The breeding season of common toads was much longer (33 d, between 20 March and 21 April) with a 6-day period (from 3 to 8 April) representing the MRS, when 82% of pairs and 75% of singles were sampled. The OSR was calculated in both species for each sampling day of the MRS.

Data analysis

The differences in the ratios of adult males and females (OSR) among sampling days of the MRS were analyzed using a chi-squared test of homogeneity. To compare OSRs during MRS between frogs and toads, we used the non-parametric Wilcoxon-Mann-Whitney U test. Because of the normal SVL distribution in all samples of both species, we examined the existence of SAM by Pearson's correlation coefficient. For each sampling day of the MRS, we calculated the correlation coefficient between the SVL of males and females in pairs, r, and the corresponding 95% confidence intervals for the population correlation coefficient, ρ, using the Fisher z-transformation (Zar, 1984). The Fisher z-transformation was also used to compare sample correlation coefficients within the MRS of each particular species.

All statistical analyses were performed in R statistical software, version 2.15.0 (R Development Core Team, 2009).

Results

The OSR was always skewed in favor of males and varied significantly during the MRS from 1.41 to 3.44 in frogs (table 1; Chi-squared test, $\chi^2_3 = 19.56$, $P < 0.001$) and from 3.31 to 12.22 in toads (table 1; $\chi^2_5 = 32.32$, $P < 0.001$). The OSRs in common toads were significantly greater than in common frogs during the MRS (Wilcoxon-Mann-Whitney U test, $W = 1$, $P < 0.05$). In the case of SAM detection, the sample correlation coefficients, calculated for each sampling day of the MRS, were positive and significant in 3 of the 4 d with the higher OSR in common frogs, but these were not significant in any of the 6 d in common toads (ta-

Table 1. Examination of size-assortative mating in common frogs and common toads during the main part of the reproduction season (MRS). n_{m} = total number of males, n_{f} = total number of females, $\mathrm{SVL}_{\mathrm{m\_p}}$ = mean of snout-vent length of paired males, $\mathrm{SVL}_{\mathrm{m\_up}}$ = mean of SVL of unpaired males, $\mathrm{SVL}_{\mathrm{f\_p}}$ = mean of SVL of paired females, $\mathrm{SVL}_{\mathrm{f\_up}}$ = mean of SVL of unpaired females, OSR = operational sex ratio measured as total number of males to total number of females on each sampling day during MRS, n_{p} = number of pairs, r = correlation coefficients, 95% CI for ρ = 95% confidence limits for population correlation coefficient ρ, R^2 = coefficients of determination, t = test statistic, P = significance of correlation.

Date	n_{m}	n_{f}	$\mathrm{SVL}_{\mathrm{m\_p}}$	$\mathrm{SVL}_{\mathrm{m\_up}}$	$\mathrm{SVL}_{\mathrm{f\_p}}$	$\mathrm{SVL}_{\mathrm{f\_up}}$	OSR	n_{p}	r	95% CI for ρ	R^2	t	P
						Common frogs							
30 March	109	58	7.56	7.29	7.14	7.83	1.88	41	0.34	0.04-0.59	0.12	2.27	0.029
31 March	111	79	7.30	7.21	6.89	7.76	1.41	62	0.11	−0.14-0.35	0.01	0.86	0.39
1 April	212	96	7.24	7.16	7.04	7.56	2.21	75	0.36	0.15-0.54	0.13	3.31	0.001
2 April	189	55	7.22	7.08	6.88	7.49	3.44	39	0.50	0.22-0.70	0.25	3.54	0.001
						Common toads							
3 April	220	18	6.75	7.22	8.63	8.25	12.22	16	0.22	−0.31-0.65	0.05	0.85	0.41
4 April	298	90	6.71	6.70	8.48	8.53	3.31	87	0.12	−0.09-0.32	0.01	−1.08	0.28
5 April	192	50	6.74	6.76	8.55	8.35	3.84	48	0.17	−0.12-0.43	0.03	1.18	0.24
6 April	285	60	6.79	6.68	8.56	9.55	4.75	58	0.21	−0.05-0.44	0.04	1.63	0.11
7 April	259	69	6.79	6.71	8.56	8.45	3.75	67	0.14	−0.10-0.37	0.02	1.12	0.27
8 April	225	34	6.68	6.60	8.37	7.90	6.62	33	0.23	−0.12-0.53	0.05	1.29	0.21

ble 1). Furthermore, we found no significant differences among sample correlation coefficients either in frogs or in toads (frogs: $P = 0.19$; toads: $P = 0.99$).

Discussion

The variability in presence of SAM

We found evidence of SAM in common frogs (statistically significant correlation on most of the sampling days within the MRS) but not in common toads (no significant correlation). Furthermore, the presence of SAM varied also within the population of common frogs and occurred in three of the four day-samples with the higher OSR.

In explosive breeders, great variability in mating patterns has been observed among species (Duellman and Trueb, 1994; Sullivan, Ryan and Verrel, 1995; Wells, 2007), among populations of the same species (Davies and Halliday, 1977; Gittins, Parker and Slater, 1980a), and even over time within the same population (Olson, Blaustein and O'Hara, 1986; Wagner and Sullivan, 1995; Briggs, 2008). Apart from the effect of species-specific ecology, variability within a species or population in the intensity of SAM is probably affected by differences in population densities and especially in OSRs (e.g., Emlen and Oring, 1977; Tejedo, 1988; Boll and Linsenmair, 1998; Lee and Park, 2009), and different results on the presence and intensity of SAM have been found within our both model species (e.g. Arak, 1983; Reading and Clarke, 1983; Elmberg, 1987; Höglund and Robertson, 1987; Ryser, 1989; Marco and Lizana, 2002).

Despite the significant positive correlation in most of the days in frogs, the coefficients of determination, R^2, were generally low here. Thus, relatively little of the (co)variance of male-female body size was explained (from 1% to 25%, table 1). Shine et al. (2001) have already pointed out that there is a great deal of "noise" in such relationships. Because the significance of the correlation is not a measure of the tightness of that correlation and depends not only on the value of the correlation coefficient but also on the sample size, we should not be satisfied merely with a significant correlation. For instance, a very weak correlation can provide a significant result if the sample size is large enough and, conversely, a relatively high correlation might not be revealed due to small sample size (Fisher, 1921; Zar, 1984). This implies that we must interpret the results with care and consider the amount of explained variation. It follows from the previous discussion that not only variability in the demographic parameters but also a misleading interpretation of correlation results may be the reason for ambiguous findings on the existence of SAM.

The effect of OSR on size-assortative mating

Although the OSRs were higher and varied greatly in toads, we detected positive correlation between the body length of males and females in pairs in frogs only. Furthermore, the sample correlation coefficients reflected increasing OSR only in frogs, but not in toads. Nevertheless, multiple comparisons among the correlation coefficients within the MRS showed non-significant differences in both frog and toad samples.

Although both common frogs and common toads are typical representatives of explosively breeding anurans (Arak, 1983), the differences in the existence and intensity of SAM related to OSR probably reflect their species-specific ecologies. For instance, the length of the MRS in frogs was shorter than in toads (4 vs. 6 d, respectively) in our study. The difference was even greater for the total length of breeding season (15 vs. 33 d). This is consistent with the findings of other authors (Arak, 1983; Elmberg, 1990). The length of the breeding season affects the population density and OSR at a breeding site. During the shorter reproduction period of common frog, the occurrence of reproductively active females is concentrated at a breeding site for only a few days. This could lead to relatively high daily proportions of females and a decline in OSR. During the longer reproduction period of common toads, on the other hand, there were probably fewer females at the breeding site on any one day. This is reflected in the heavily male-biased OSR (Emlen and Oring, 1977; Arak, 1983), and the OSR is usually higher in common toads, at between 2 and 8 (Davies and Halliday, 1979; Reading, 2001; Brede and Beebee, 2006), than in common frogs (in range 1-7, Elmberg, 1990, 1991). The intensity of competition could also be affected by species-specific locomotion abilities and overall activity (Eshel, 1979; Crowley et al., 1991; Jirotkul, 1999).

It has been found that the higher the OSR within a population the more intensive is the sexual selection occurring through male-male competition (Arak, 1983; Tejedo, 1988; Höglund, 1989; Lee and Park, 2009). However, few studies have analyzed the effect of different OSRs on the intensity of correlation or on the presence of SAM in amphibians (Arak, 1982; Elmberg, 1991; Yu and Lu, 2010). Within those studies cited, moreover, the authors compared only the significances of correlations among different OSRs. As we point out above, the significance of a correlation is also affected by the sample size and only the correlation coefficient is a measure of the tightness of that correlation. Therefore, the comparison solely of significances is improper and yields size-biased conclusions. We conclude that a better way to analyze the effect of different OSRs on the presence of SAM is to compare correlation coefficients, as in our study.

Conclusions

Within both studied species, great variability in the presence and intensity of SAM has been observed. Despite prevailing significant correlations in frogs, the coefficients of determination, R^2, were generally low here, and explained relatively little of the (co)variance of male-female body size in pairs. We conclude that not only variability in the demographic parameters but also a misleading interpretation of correlation results may be the reason for ambiguous findings of SAM. To examine the existence of SAM, we should not be satisfied merely with a significant correlation, because its significance is also affected by the sample size. Only the correlation coefficient, r, is a measure of the tightness of that correlation. This is especially true for the comparison of existence of SAM among studies, and also for the analysis of the effect of different OSRs on the presence of SAM. The best way is to compare correlation coefficients, as in our study. To avoid mistakes within studies on SAM, we should put emphasis on correct choice and interpretation of statistical methods.

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Chapter 2

Unpublished results: First come, first served. The story of mating constraints of common toad.

Petr Chajma, Jiří Vojar

The hypothesis of mating constraints states that physically well-matched mates may better endure the physical constraints of being in amplexus, which can lead to size-assortative mating (SAM). To test these constraints on the common toads, we divided 110 individuals into 20 groups with equal number of small, medium and large males, relative to the female, and with two male-biased operational sex ratios (OSR) of 3 and 6. While the physical constraints did favour medium sized males when being under opponents attack, overall number of successful dislodgements by an opponent was low ($\mu = 0.4$, $\sigma = 0.92$). More than by competing male, amplexus was at risk of braking by itself, or by dislodgement from the female. Higher OSR did not have any effect on number of dislodgements, nor time spent in amplexus, but unexpectedly lowered the number of dislodgements by female. Resulting mating pattern was not size-assortative.

Methods

All of studied toads (20 females and 90 males) were collected in a single day during their migration to breeding pools, using pitfall traps and barrier fencing as part of a rescue transfer of amphibians near Hradec Králové. The toads were divided into 20 experimental pools of the size of 70 x 70 x 50 cm, with 10 pools having male biased OSR of 3 and the rest having male biased OSR of 6. Reproductive activity was stimulated by adding 10 cm of water and all toad interactions were carefully noted for 45 minutes. Male sizes were carefully chosen for each pool, so that they can be divided as large (smaller than female by 0-10 mm), medium (smaller than female by 10-20) and small (smaller than female by 20-30 mm).

Presence of SAM was evaluated using Spearman’s correlation, as toad sizes, masses and body-mass index were not normally distributed. The dependency of number of times, the male was dislodged from amplexus and the number of times, the male dislodged someone from amplexus, on number of attacks, size category and OSR were tested by Generalized linear model with Poisson distribution, using type III tests. The dependency of total time, spent in amplexus on size category and OSR was tested by Linear model, using type III tests. All analyses were performed in R 3.5.3 (R Core Team, 2019), using "car" package (Fox & Weisberg, 2011).

Results

The resulting pairing (and subsequent mating) was not assortative by size (*Spearman's correlation*: $r = 0.12$, $p = 0.62$), mass (*Spearman's correlation*: $r = 0.18$, $p = 0.47$), nor body-mass index, although coming closest out of all three (*Spearman's correlation*: $r = 0.26$, $p = 0.3$). The number of times, the male was dislodged from amplexus by different male was fairly low ($\mu = 0.4$, $\sigma = 0.92$) and depended mostly on number of attacks ($\chi^2 = 16.8$, $p < 0.001$) and size category ($\chi^2 = 11.4$, $p = 0.003$), with OSR being insignificant ($\chi^2 = 2.36$, $p = 0.125$). The number of times, the male managed to dislodge other male was also low ($\mu = 0.38$, $\sigma = 1$) and depended only on the number of attacks he issued ($\chi^2 = 13.3$, $p < 0.001$), with size ($\chi^2 = 1.8$, $p = 0.414$) and OSR ($\chi^2 = 0.9$, $p = 0.332$) being insignificant (Fig. 1B). The number of times, male was dislodged by female herself, was twice the number of dislodgements by other males ($\mu = 0.76$, $\sigma = 1.8$) and grew with the amount of time spent in amplexus ($\chi^2 = 11.2$, $p < 0.001$) and size category ($\chi^2 = 18.4$, $p < 0.001$), while lowering in higher OSR ($\chi^2 = 8.04$, $p = 0.005$). The amount of time spent in amplexus was highest for medium-sized males ($\chi^2 = 0.71$, $p = 0.046$), unaffected by OSR ($\chi^2 = 0.04$, $p = 0.524$).

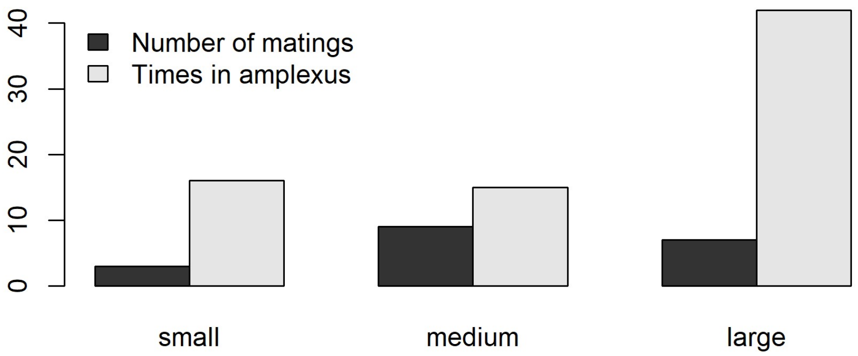

Figure 1. Influence of physical constraints, shown through the difference in number of times in amplexus (light grey) and number of successful matings (dark grey).

Unpublished.

Chapter 3

The effect of size-assortative mating on fertilization success of the common toad (*Bufo bufo*)

Petr Chajma, Jiří Vojar

Although size-assortative mating (SAM) is a frequently studied phenomenon in anurans, its effect on fitness rarely has been evaluated. Using a controlled experiment, we assessed the presence of SAM in the common toad, *Bufo bufo*, and evaluated the effect on fertilization success of male-female size (snout-vent length, SVL) difference and ratio in mated pairs. Even though the toads paired non-randomly with respect to size, the difference and ratio of female and male SVL in pairs had no significant effect on fertilization success. Our findings and the majority of other studies suggest that the commonly accepted idea that SAM serves to maximize fitness may not be completely accurate. The statistical power and effect size in these studies are often poor, however, and the methods used are inconsistent. We conclude that more manipulative experiments with appropriate sample sizes are needed to fully understand this phenomenon.

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The effect of size-assortative mating on fertilization success of the common toad (*Bufo bufo*)

Petr Chajma*, Jiří Vojar

Abstract. Although size-assortative mating (SAM) is a frequently studied phenomenon in anurans, its effect on fitness rarely has been evaluated. Using a controlled experiment, we assessed the presence of SAM in the common toad, *Bufo bufo*, and evaluated the effect on fertilization success of male-female size (snout-vent length, SVL) difference and ratio in mated pairs. Even though the toads paired non-randomly with respect to size, the difference and ratio of female and male SVL in pairs had no significant effect on fertilization success. Our findings and the majority of other studies suggest that the commonly accepted idea that SAM serves to maximize fitness may not be completely accurate. The statistical power and effect size in these studies are often poor, however, and the methods used are inconsistent. We conclude that more manipulative experiments with appropriate sample sizes are needed to fully understand this phenomenon.

Keywords: clutch size, explosive breeders, fertilization rate, fitness, non-random mating, sexual selection, size-dependent mating.

Introduction

Non-random or assortative mating is defined as a correlation of certain characteristics of males and females in pairs (Lewontin, Kirk and Crow, 1968). This phenomenon has been documented in a wide spectrum of invertebrates (Brown, 1993; Hegde and Krishna, 1997; Hargeby and Erlandsson, 2006) and vertebrates (Arak, 1983; Mckaye, 1986; Rintamäki et al., 1998; Shine et al., 2001). Characteristics of paired individuals commonly examined by correlation analysis include body mass (e.g., Márquez and Tejedo, 1990), age (Ferrer and Penteriani, 2003), physical condition (Bortolotti and Iko, 1992), and social experience (Freeberg, 1996). The most studied, however, is size-assortative mating (SAM). This is probably due to the fact that size is commonly measured throughout the animal kingdom (Crespi, 1989; Andersson, 1994), including for amphibians (Arak, 1983; Wells, 2007).

Among amphibians, SAM has been found in only anurans and the attention has been focused mainly upon the ranid and bufonid families (e.g., Davies and Halliday, 1977; Elmberg, 1987; Marco et al., 1998; Lu et al., 2009). The reports of its occurrence differ not only among species, but also within populations of the same species and even between breeding seasons. Some studies might report false positive or negative results as a result of incorrectly evaluating the correlation tests used in examining SAM's occurrence. False positive results are often the consequence of relying solely on the test of significance and not taking into account the effect size. Small sample size might, on the on the other hand, can cause false negative results (Fisher, 1921; Zar, 1984; Vojar et al., 2015).

Moreover, despite the considerable attention that has been given to SAM, only a few studies have moved beyond examining the presence or absence of SAM and undertaken to explain the causes and effects of this phenomenon. It is believed that SAM can increase fitness (Burley, 1983), because pairs consisting of partners well matched for size should also have a higher proportion of fertilized eggs (Davies and Halliday, 1977; Crespi, 1989). This is achieved due to a

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smaller distance between cloacae and a lesser chance of not noticing the signal for the start of egg deposition, which is usually provided by the female in the form of a leg bump (Wells, 2007). Furthermore, size-assortatively formed pairs could have better chances of withstanding the attacks of single males (Davies and Halliday, 1977; Lu et al., 2010).

The only studies which have found a positive effect of SAM on fitness have been those of Ryan (1983) in the tungara frog (*Engystomops pustulosus*) and Bastos and Haddad (1996) in the elegant forest treefrog (*Dendropsophus elegans*). Nevertheless, some studies accept it as a fact (e.g., Davies and Halliday, 1977; Yu and Lu, 2010). Using a controlled experiment, we therefore examined the presence of SAM in the common toad, *Bufo bufo*, and the effect of SAM on one component of fitness, i.e., the proportion of fertilized eggs. Specifically, we evaluated the effect of male and female body size difference and ratio within paired toads on clutch fertilization success.

The common toad is a temperate European toad with a breeding period approximately one month long (Wells, 1977; Davies and Halliday, 1979). The majority of mating occurs, however, in the short period of the main reproduction season, which can last for about a week (Vojar et al., 2015). The toad is therefore regarded as a typical explosive breeder (Arak, 1983). Within such species, males fight for the possession of females, and the mating pattern depends upon male density at the breeding site (Arak, 1983; Wells, 2007), because increasing male-male competition should enhance the intensity of both size-dependent (Davies and Halliday, 1979) and size-assortative (Vojar et al., 2015) mating within a population. The presence of size-dependent mating has been frequently documented within the species (e.g., Davies and Halliday, 1979; Höglund and Säterberg, 1989; Arntzen, 1999; Reading, 2001), as has been the occurrence of SAM (reviewed in Vojar et al., 2015). With an explosive breeder, it is easier to set up natural breeding conditions and control the experiment. A well-documented explosive breeder such as the common toad is therefore a good model species for this type of experiment.

Materials and methods

Experimental design

The experiment was carried out at artificial ponds on the campus of the Czech University of Life Sciences in Prague. The toads had been captured using pitfall traps and barrier fencing as part of a rescue transfer of amphibians which were attempting to migrate across roads near Hradec Králové, a city in eastern Bohemia, during the spring of 2013. Due to the strong migration, all the amphibians were collected in a single day. The collected toads were first examined for presence of the pathogen *Batrachochytrium dendrobatidis* according to a well-regarded methodology (Hyatt et al., 2007). Sampling was performed by a non-destructive method of skin swabbing using the sterile Dryswab® product (MW100, Medical Wire & Equipment Co, UK). Swab samples were processed using DNA isolation by PrepMan Ultra, and *Bd* detection was performed by real-time quantitative PCR (Boyle et al., 2004) with the addition of bovine serum albumin (BSA) to limit PCR inhibition (Garland et al., 2010). All captured toads were free of the pathogen.

To screen captured individuals, 120 males and 79 females were measured using a plastic calliper to obtain snout to vent length (SVL) to the nearest millimetre and weighed using a digital scale to the nearest 0.1 g. These measurements were then used to compare SVL and mass of males and females. In order to simulate a naturally male-biased operational sex ratio of toads at approximately three males to one female (Davies and Halliday, 1979; Reading, 2001; Brede and Beebee, 2006; Vojar et al., 2015), 120 males and 43 females were chosen for the experiment and moved into a single outdoor breeding pool of size 400 × 400 × 100 cm [length × width × height]. There, they were permitted to interact freely. The breeding pool was designed to be as close to a natural pond as possible, i.e. natural vegetation, size of the water body and a mild slope of the banks. Pairs were considered final if there was no successful male displacement for 15 minutes. After approximately one hour, all of the females had paired with a final male. Pairs were moved into separate containers of dimensions 37 × 24 × 26 cm, filled with aged tap water. The pairs were then permitted such time as they needed to reproduce.

After clutch deposition and fertilization, all paired males and females were measured in order to determine the presence of SAM. Thirty randomly chosen clutches were then moved into a specially designed permeable container made out of a polystyrene foam ring and nylon stockings (see Vojar, Doležalová and Solský, 2012) and into hatching pools of size 70 × 150 × 50 cm, filled with the same aged tap water. All the eggs therefore developed under the same environmental conditions in terms of water quality, temperature, shading, etc. In order to distinguish unfertilized and developing eggs, and to determine the proportion of fertilized eggs, clutches were transferred to white plastic trays with a small amount of water after reaching Gosner stage 16-20 (Gosner, 1960). All eggs were photographed in the trays and then carefully counted from the photographs.

To determine whether mating success of males was affected by their size (Arak, 1983), 28 randomly chosen single males and the same number of mated males were measured. To test the hypothesis that pairs consisting of partners well matched for size should also have a higher proportion of fertilized eggs (Davies and Halliday, 1977; Crespi, 1989), we used both the difference between and ratio of male and female SVL in pairs. Both of these obviously correlated parameters were used because both have been used in similar studies and we wanted to compare our results with the others. Furthermore, we tested if fertilization success depended on male SVL directly, which is highly correlated with testis size (Emerson, 1997).

Data analysis

Mean SVL of mated and unmated males were compared using a Student's t-test. Presence of SAM was tested using the non-parametric Spearman's correlation index, because the assumption of normal distribution of both male and female SVL was not met. The possible effect of difference and ratio of male and female body sizes in pairs, as well as of male SVL, on fertilization success could not be tested using a multiple linear regression model due to strong correlation among explanatory variables. To compensate for that, three simple linear regression models were constructed separately. Although this approach is also not entirely correct, because the effects of explanatory variables are overlapping, it did provide valuable insight. Due to heteroscedasticity of errors, dependence of clutch size on female SVL was tested by simple linear regression using heteroscedasticity consistent standard errors, or HC3, which is suggested for small samples (Long and Ervin, 2000). Statistical power and the effect sizes (Cohen's d, r and f^2) were calculated according to Cohen (1988). All statistical analyses were performed in R software, version 3.2.3 (R Development Core Team, 2015).

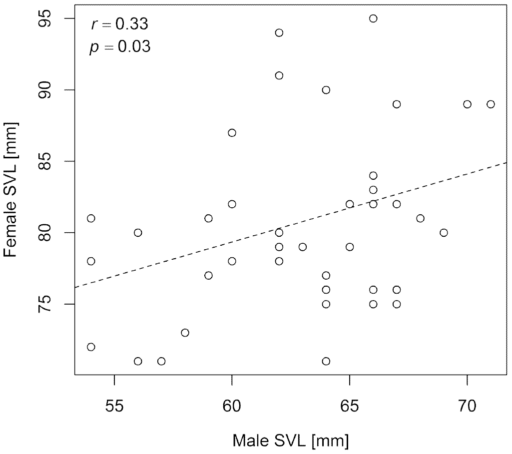

Figure 1. Correlation of body sizes (snout-vent length in mm, SVL) between the common toad males and females in pairs.

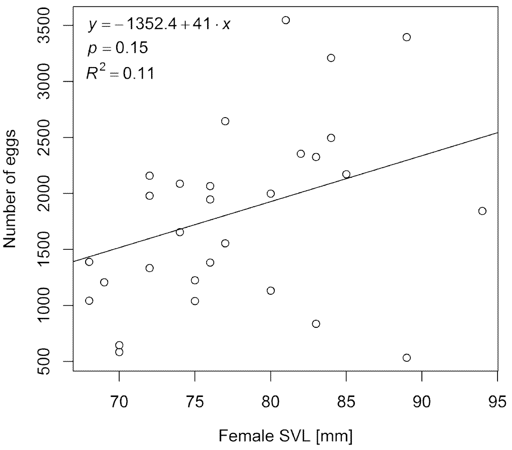

Figure 2. Relationship between clutch size (number of eggs) and female body size (female snout-vent length in mm, SVL) of the common toad.

Results

Body size of paired males (mean = 62.11, SD = 4.93) was significantly larger than that of single males by approximately 4.32% ($t = 2.21$, $df = 54$, $p = 0.02$; Student's t-test) with the mean difference of 2.57 mm (0.24-4.9, 95% CI, two-tailed test) and a medium-large effect size (Cohen's $d = 0.59$), thus indicating size-dependent mating. There was a significant positive correlation between male and female SVL in pairs, indicating the presence of weak SAM ($r = 0.33$, $n = 43$, $p = 0.03$; Spearman's rank correlation, fig. 1), with a relatively wide 95% CI (0.04-0.58).

Clutch size (the sum of developed and undeveloped eggs) was marginally independent of female body size ($t = 1.9$, $p = 0.07$). After accounting for an obvious heteroscedasticity issue (fig. 2), the effect appeared even less significant ($t = 1.47$, $p = 0.15$, $R^2 = 0.11$). Although the non-significant relationship could be caused by the lack of statistical power, the effect size (Cohen's $f^2 = 0.12$) was still low. With the given sample size and proper power (0.8), we would

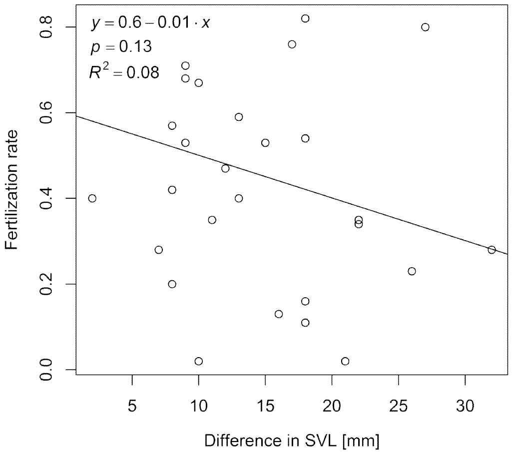

Figure 3. Relationship between fertilization success and difference in female and male body sizes (difference in snout-vent length in mm, SVL) of the common toad.

be able to detect only a medium to large effect ($R^2 \geqslant 22\%$; Cohen, 1988). Both the total number of eggs (534-3548) and fertilization rate (2-89%) varied significantly among pairs.

None of the tested variables was responsible for variation in fertilization rate (difference in SVL: $t = -1.56$, $p = 0.13$; ratio of SVL: $t = 1.52$, $p = 0.14$; male SVL: $t = 0.01$, $p = 0.93$). The best fit was achieved using the difference in SVL of paired toads and it only explained 8% of the variation (fig. 3). As with the previous analysis, the lowest statistically significant effect converted to R^2 would be 0.22.

Discussion

Occurrence of size-dependent and size-assortative mating

The common toad is a well-studied explosively breeding amphibian. In our experiment, as well as in few other studies (e.g., Davies and Halliday, 1977; Reading, 2001), the toads paired non-randomly with respect to size. Their mating was both size-dependent and size-assortative. Correlation of male and female SVL in pairs was significant, but the strength of this relationship was rather weak ($r = 0.33$). This level of correlation seems, however, to be common among other studies on SAM in amphibians that have reported both significant (e.g., Howard, 1978; Berven, 1981; Gramapurohit and Radder, 2012; Vojar et al., 2015) and non-significant (Davies and Halliday, 1977; Berven, 1981; Gatz, 1981; Lee and Park, 2009) relationships. This apparent inconsistency could have resulted from small sample sizes common in studies of SAM (Vojar et al., 2015). In order to properly compare the results of correlation analyses, we strongly advise the use of power analysis (see Cohen, 1988) prior to the start of an experiment and to focus on the effect size represented by correlation coefficient. We believe that the effect size should be at least “medium”, which Cohen (1988) defines using the cut-off value of 0.3. For example, if the correlation coefficient between female and male SVL in pairs is 0.3, the difference between female SVL of 1 SD (in our case 6.08 mm) would result in corresponding change in male SVL of 0.3 SD (in our case 1.83 mm). In our opinion any significant results having correlation coefficients with lower value should be treated with care.

Effect of SAM on fertilization rate

The greatest advantage of SAM is believed to be its potential effect on fitness (Burley, 1983). Unfortunately, only few studies actually tried to measure it. This effect was not found in our study, however, and neither the difference between nor ratio of SVL in pairs resulted in a corresponding change in proportion of fertilized eggs. Even if considered as a consequence of insufficient statistical power, its effect would be very low. These findings are the opposite of those described by Bastos and Haddad (1996) in *Dendropsophus elegans*, where the ratio of male and female SVL in pairs was responsible for 85% of variability in fertilization rate. Although Ryan (1983) had found a weak correlation between the difference in body sizes of males and females of paired *Engystomops pustulosus* and fertilization success ($r = 0.30$; $n = 68$, $p < 0.05$), the effect of body size difference in pairs had in that case explained only 9% of variability. That level was similar to that in our study (8%). The difference in significance is probably a consequence of different sample sizes between the studies (68 vs. 30 pairs), rather than different effect sizes.

Of the studies that revealed no significant relationship between SAM and fertilization success, Kruse (1981) found a negative correlation ($r = -0.30$, $n = 19$, $p > 0.2$) of fertilization success and the ratio of male and female SVL in pairs of the American toad (*Anaxyrus americanus*). In this case, the estimated power for $\alpha = 0.05$ would be 0.24. Gerhardt et al. (1987) found no significant relationship between the relative size of mates and fertilization success in their study of green treefrogs (*Hyla cinerea*), but they do not provide the precise results of their statistical analyses. Their study, like that of Kruse (1981), had a small sample size ($n = 21$). Briggs (2008) revealed no significant relationship between the ratio of SVL in pairs and fertilization success in the red-eyed leaf frog (*Agalychnis callidryas*) ($r = 0.20$, $n = 56$, $p = 0.15$) or in the black-eyed leaf frog (*Agalychnis moreletii*) ($r = 0.19$, $n = 46$, $p = 0.21$), and there was virtually no variation in fertilization success. There were only two clutches with $<100\%$ of fertilized eggs in *A. callidryas* and just one in *A. moreletii*. Fan, Lin and Ji (2013) found no significant relationship between fertilization success and ratio of mate SVL in either Asiatic toads (*Bufo gargarizans*) ($n = 65$, $p = 0.16$) or Asian common toads (*Duttaphrynus melanostictus*) ($n = 149$, $p = 0.16$).

The reports of SAM often share the weak magnitude of correlation between SVL in pairs. Considering that the effect of the size difference in pairs on fertilization success may be also very weak, there seems to be almost no benefit of SAM to this component of fitness in amphibians. Other than that it could affect fitness through mechanical constraints of amplexus (i.e. assortatively mating animals would have a higher probability of clutch deposition), there might be a benefit in a smaller chance for displacement by a rival male. The frequency of successful displacements, however, remains unknown for most species.

Conclusions

The possible effect of SAM on fertilization success in anurans has not been studied nearly enough to draw any major inferences about its existence. The majority of existing studies, however, do not support the commonly accepted idea of maximizing fertilization success through SAM. The statistical power and the effect size in these studies are often poor and the methods used among these studies are inconsistent. We advise the use of power analysis prior to conducting an experiment and, when it is not possible to ensure an appropriate sample size, it would be more appropriate to interpret the results based upon the effect size rather than upon the test of significance. In this light, manipulative experiments involving further taxa will be needed if non-random mating in amphibians is to be properly described.

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Chapter 4

Individual consistency of newt's exploration and shyness, but not activity: The effect of habituation?

Petr Chajma, Oldřich Kopecký, Jiří Vojar

Behavioural consistency (i.e. personality) is an important aspect of behavioural ecology that has yet to be thoroughly examined in amphibians. Currently published studies often address only one or two aspects of personality, using many different methods for evaluating behavioural consistency. This is the first study focusing on all relevant behavioural traits and their relationships in urodele amphibians. Based on three trials within the experiment, we examined the consistency of activity (time spent moving), the boldness (latency of the first movement and time spent escaping) and the exploration (number of visited segments of the test arena) of 42 smooth newts (Lissotriton vulgaris). Individual consistency, calculated through the intraclass correlation coefficient (ICC), was low in newt activity (ICC = 0.192) and was moderate in boldness (0.476) and in exploration (0.403). Activity was moderately consistent for each trial (0.425), indicating possible habituation, supported by a decrease in mean activity throughout the trials. Newt activity, shyness and exploration were correlated throughout the experiment, suggesting the possible existence of a common selective pressure. With a summary of existing studies and their effect sizes, we aim to highlight the inconsistencies in the methods and in the results of these studies, to emphasize the need for a complex approach to the study of amphibian personality and the need for a standardized methodology, which would solve the current difficulties in comparing published results.

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Individual consistency in exploration and shyness but not activity in smooth newts (Lissotriton vulgaris): the effect of habituation?

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Abstract

Behavioural consistency (i.e. personality) is an important aspect of behavioural ecology that has yet to be thoroughly examined in amphibians. Currently published studies often address only one or two aspects of personality, using many different methods for evaluating behavioural consistency. This is the first study focusing on all relevant behavioural traits and their relationships in urodele amphibians. Based on three trials within the experiment, we examined the consistency of activity (time spent moving), the boldness (latency of the first movement and time spent escaping) and the exploration (number of visited segments of the test arena) of 42 smooth newts (*Lissotriton vulgaris*). Individual consistency, calculated through the intraclass correlation coefficient (ICC), was low in newt activity (ICC = 0.192) and was moderate in boldness (0.476) and in exploration (0.403). Activity was moderately consistent for each trial (0.425), indicating possible habituation, supported by a decrease in mean activity throughout the trials. Newt activity, shyness and exploration were correlated throughout the experiment, suggesting the possible existence of a common selective pressure. With a summary of existing studies and their effect sizes, we aim to highlight the inconsistencies in the methods and in the results of these studies, to emphasize the need for a complex approach to the study of amphibian personality and the need for a standardized methodology, which would solve the current difficulties in comparing published results.

Introduction

Behavioural consistency (i.e. personality) is a well-known phenomenon that was studied in many taxa (e.g. Gosling, 2001; Sih *et al.*, 2004; Réale *et al.*, 2007; Garamszegi, Marko & Herczeg, 2013) including amphibians (e.g. Aragón, 2011; Koprivnikar, Gibson & Redfern, 2011; Maes, Van Damme & Matthysen, 2012; Wilson & Krause, 2012; Brodin *et al.*, 2013; Carlson & Langkilde, 2013, 2014a). Consistency in the expression of behavioural traits over time and in different situations, as well as the correlation of these traits, that is behavioural syndrome (Sih *et al.*, 2004), is often linked to survival in predator–prey situations (Dingemanse & de Goede, 2004; Carlson & Langkilde, 2014b) (but see Carlson & Langkilde, 2014a), reproductive success (Dingemanse & Réale, 2005; Cole & Quinn, 2014), disease risk (Koprivnikar *et al.*, 2011) and dispersal tendencies (Cote *et al.*, 2010, 2013; Gruber *et al.*, 2017a, 2017b; reviewed in Cayuela *et al.*, 2018). Consistent behaviour leads to consistent (dis)advantages in certain situations and, if heritable (if genetically determined), is subjected to selective pressure. Behaviour correlations can imply trade-offs that can result in maladaptive behaviour in some contexts and can potentially maintain individual variation in behaviour in a variable environment (Sih *et al.*, 2004). Thus, animal personality plays an important part in individual life histories and should be inspected and carefully considered when dealing with most aspects of animal ecology.

Amphibian personality research, focused mostly on anurans (for a recent review, see Kelleher, Silla & Byrne, 2018), has various approaches to behavioural consistency. Consistency of the same behaviour across time (e.g. Maes *et al.*, 2012; Wilson & Krause, 2012; Brodin *et al.*, 2013; Carlson & Langkilde, 2013) is measured in a time frame ranging from four hours to nine weeks. Different contexts in which the consistency of the behaviour is being measured can mean many different things, starting from the introduction of different odours to the introduction of a novel object or parasite infestation (e.g. Sih *et al.*, 2003; Aragón, 2011; Koprivnikar *et al.*, 2011). Even the behavioural traits representing certain types of behaviour (axes of personality) and their level of consistency (effect sizes) vary considerably.

The aim of our study was to measure the temporal consistency of the most commonly examined behavioural traits—activity, exploration and boldness—in the smooth newt, a common but surprisingly understudied urodele species. In addition, we wanted to assess the correlations between these behaviour traits and to see which behaviour traits, if any, might be shaped together. In particular, we were interested to find whether active individuals explored more, and how boldness/shyness interacted with these two traits. Additionally, to clearly show how the level of behavioural consistency in our study compares to existing amphibian personality research, without neglecting the differences in experimental approach mentioned above, we have summarized the most important findings in the Supplementary information section (Tables S1).

Materials and methods

Experimental design

The experiment was carried out in laboratory conditions at the Czech University of Life Sciences in Prague. We chose the urodele that was most abundant locally, the smooth newt (*Lissotriton vulgaris*). At the start of the reproductive season at the beginning of May 2017, 21 males and 21 females were captured using nets in a single pond in the village of Stará Lysá in the Central Bohemia region. The net catching was carried out manually, using a rapid, torrent-creating movement, and a sudden change of direction, to capture individuals that were swept by this torrent. This way of capturing, in our opinion, minimizes the advantages of certain personality types and reduces the risk of bias.

The newts were housed separately in plastic containers with dimensions of 18 × 12 × 14 cm that were filled with aged tap water, and the newts were fed *Daphnia* and Chironomidae larvae *ad libitum*. The air temperature in the laboratory was constant and was set to 17°C. Sufficient light intensity in a diurnal cycle was provided by the translucent roof of the laboratory.

The experiment itself was conducted between May 13th and 27th in two experimental arenas made of non-transparent round green water barrels with bottom diameters of 80 cm. Using a non-toxic waterproof marker, a square grid of 7-cm segments was drawn at the bottom to better assess the position of each newt. The arena was filled with 5 cm of cold tap water (10.8–11.2°C). After each recording, the water was changed, and the arena was thoroughly cleaned with a clean sponge and pressurized water, and was then left to dry to eliminate any potential chemical cues that remained from the previous tested individual.

Each trial within the experiment was 12 min long. Behaviour was recorded at 25 frames per second with a full HD camera, positioned approximately 150 cm above the water level. Newts were separately inserted under the transparent glass dome (10 cm diameter) into the centre of the arena and were left to acclimatize for the first two minutes. Then, the dome was carefully removed in a motion perpendicular to the ground, and the recording was initiated. To measure the temporal repeatability of the behaviour, each individual was recorded three times with a six-day gap between each recording. This was the longest gap possible before the newts started to shift to the terrestrial phase of the season, substantially changing their behaviour, and becoming unwilling to stay in the water for long periods of time. In total, we tested 39 newts in the behavioural assay three times and 3 newts twice (due to a loss of data as a result of technical difficulties).

Three types of behaviour (personality traits) were tracked: activity, exploration and boldness. Activity was measured as the amount of time [s] during which the individual moved. In addition, the movement activity was divided into walking and swimming, in order to distinguish the role of each in the total activity and the consistency of each activity, and also to determine the consistency of the choice of locomotion (i.e. the proportion of the activity that consisted of walking). Exploration was recorded as the number of grid blocks that an individual entered, not including blocks that had already been visited. For the sake of better comparison with other studies, boldness/shyness was measured as the latency of the first movement [s] (the most common but imprecise measure of boldness, see Discussion) and also the time [s] spent at the outermost edge of the arena (our preferred measure). Staying in the vicinity of the edge of the arena (thigmotaxis) can be interpreted as an escape response and can therefore be a valuable measure of shyness (Burns, 2008; Harris, D'Eath & Healy, 2009; Carlson & Langkilde, 2013). Behaviour was scored manually by the same person, using Observer XT v. 10 software (Noldus, 2010). The study was carried out in accordance with permit SZ-092744/2012KUSK/3, issued by the Regional Office of the Central Bohemian Region of the Czech Republic and approved by an institutional committee based on institutional accreditation No. 63479/2016-MZE-17214 of the Ministry of Agriculture of the Czech Republic.

Data analysis

To test the differences in activity, time spent walking, swimming, number of visited squares (exploration), latency of the first movement (boldness) and time spent near the outermost edge of the arena (shyness) between trials and sexes (independent variables), we created separate linear mixed effects models (LMM) for each of the characteristics (dependent variable) fitted by the restricted maximum likelihood (REML), with the individual (1–42) as a random intercept. Apart from the variables mentioned above, we also tested the dependency of the proportion of walking activity (i.e. time spent walking divided by time spent active) of each newt on the same fixed (trial and sex) and random effects (individual). This was done to assess whether the preferred type of locomotion differed between sexes and between trials. Although proportions were being modelled, this model reasonably met the assumptions for LMM.

Each model was also tested for the effect of the time of day at which the experimental trial took place. Because the dependency on time is rarely linear, we decomposed this variable to the sine and cosine of the time in radians, in order to take into account the periodic nature of the variable. When all underlying assumptions had been considered, the models were evaluated using Type III F-tests. Neither the time of day nor the sex of the newts affected any of the tested variables (see

Supplementary information Tables S1-S9 for details), and they were therefore not included in the repeatability analyses.

Individual consistency (repeatability) in the measured traits (dependent variables from previous models) was calculated using the intraclass correlation coefficient (ICC), computed from the variance components (available in Supplementary information section Tables S1-S9) of models like those discussed above, but with no fixed effects and with the trial number as a second random variable. Note that adding the trial number as a random intercept allowed us to estimate its consistency, that is the between-subject similarity in the expression of measured traits during each trial within the experiment and to estimate the individual repeatability while accounting for the effect of trial order.

Confidence intervals (CI) for the ICC were estimated by parametric bootstrapping with 1000 iterations (for details, see Nakagawa & Schielzeth, 2010). The confidence interval for the proportion of walking activity was estimated from the fixed intercept of the linear mixed effects model, with the individual and the trial as random intercepts, using the profile likelihood method.

The correlation of behavioural traits was tested using Kendall's coefficient of concordance, because unlike ICC, it relies on the number of concordant groups of measurements and is not influenced by the differences in the means of the selected behavioural responses. In order to reduce the potential dependence caused by the simultaneous scoring of all behavioural responses in the same assay, each trial was split into three equally long sections, and one of the traits (activity, shyness, exploration) was randomly assigned to each section. This was performed 500 times, and much like in non-parametric bootstrapping, the 95% confidence interval of the coefficient of concordance was constructed from the 2.5% and 97.5% quantile of its empirical distribution. To avoid pseudoreplication, repeated measurements of individuals were averaged. Pairwise similarities were analysed using Pearson's correlation coefficient. All statistical analyses were performed in R 3.5.1 (R Core Team, 2018) using *lme4* (Bates *et al.*, 2015), *car* (Fox & Weisberg, 2011) and *rptR* (Stoffel, Nakagawa & Schielzeth, 2017) packages at the level of significance $\alpha = 0.05$.

Results

Activity

The individual repeatability of activity was significant, but lower than the individual repeatability of both of its parts – walking and swimming (Table 1A). Walking was the most frequent type of locomotion, taking up 71% of the time spent moving (CI = [0.583, 0.840]). The individual choice of locomotion (i.e. the proportion of activity classified as walking) was also moderately consistent (Table 1A).

The mean activity differed significantly between trials (for details, see Supplementary information Tables S1-S9). The initial mean activity of 309.9 s decreased by 32% in the second trial and by 20% in the third trial, and was consistent throughout each trial (Table 1B), meaning that the change in activity was similar for each newt (see Figure 1).

Both the amount of walking ($P < 0.01$) and the amount of swimming ($P < 0.001$) fell significantly between the trials within the experiment. The proportion of activity classified as walking, however, increased only slightly during the second and third trials ($P = 0.05$). Unlike general activity, however, walking, swimming and also the proportion of walking activity were not repeatable for each trial (i.e. the trial number did not contribute to the differences in these measurements, for details, see Table 1B).

Exploration

We observed two different exploration patterns. Most of the newts started the trial with a quick escape response and then began by exploring the outer ring of the arena, rarely exploring the inner parts. The squares were usually visited only briefly and in rapid succession. A smaller group was startled at first and then explored the inner parts of the arena, eventually reaching the outer ring, and they stayed in the same square for a longer period of time. Individual exploration was significantly repeatable with a moderate ICC (0.403). As in the case of activity, there was a significant difference in mean exploration between each of the trials within the experiment ($P = 0.01$, see Supplementary information Tables S1-S9). The initial mean of 27.8 explored squares decreased by 2.5% in the second trial and by 18% in the third trial. Unlike in the case of activity, the general decrease in exploration was not consistent among individuals from the same trial (see Table 1B).

Boldness

Boldness was measured as the latency to move and the time spent with an escape response. These two measures differed in individual repeatability (latency to move: ICC = 0.121; time spent escaping: ICC = 0.476), which seems to be a relatively common finding for other studies as well (see Tables S1). There was no difference in the boldness values between the trials within the experiment. However, it should be noted that the time spent escaping was only marginally insignificant ($P = 0.07$, see Supplementary information Tables S1-S9). The initial mean time spent with an escape response of 226.9 s decreased by 5.5% in the second trial and then rose by 30% in the third trial. The repeatability of movement latency and time spent escaping was not significant for the trials within the experiment (see Table 1B).

Correlated behaviour

The similarity in mean activity, exploration and time spent escaping (shyness) of individuals was relatively high (*Kendall's* $W = 0.647$, CI = [0.55, 0.72]). Pairwise correlations showed a moderate positive relationship between activity and time spent escaping ($r = 0.542$, CI = [0.39, 0.69]), and no correlation between activity and exploration ($r = 0.259$, CI = [−0.03, 0.50]) and between time spent escaping and exploration ($r = 0.240$, CI = [−0.02, 0.48]).

Table 1 Intraclass correlation coefficients (ICC) and confidence intervals (CI) for repeatability of behavioural traits in the smooth newt

A. Repeatability of individual behaviour between the trials of experiment			B. Repeatability of behaviour demonstrated by different individuals in the same trial of the experiment		
Variable	ICC	CI	Variable	ICC	CI
Activity	0.192[a]	[0.06, 0.42]	Activity	0.416[a]	[0.01, 0.73]
Walking	0.254[a]	[0.05, 0.45]	Walking	0.058	[0, 0.22]
Swimming	0.385[a]	[0.18, 0.57]	Swimming	0.156	[0, 0.42]
Walking proportion	0.373[a]	[0.17, 0.55]	Walking proportion	0.046	[0, 0.18]
Squares explored	0.403[a]	[0.2, 0.59]	Squares explored	0.051	[0, 0.18]
Latency of the first movement	0.121	[0, 0.32]	Latency of the first movement	0	[0, 0.05]
Time spent escaping	0.476[a]	[0.27, 0.64]	Time spent escaping	0.022	[0, 0.12]

Activity – time spent moving.
Walking – time spent walking.
Swimming – time spent swimming.
Walking proportion – proportion of activity spent walking.
Squares explored – number of visited squares.
Latency of the first movement – time spent idle, before the first movement.
Time spent escaping – time spent in the outermost part of the test arena.
ICC – intraclass correlation coefficient.
CI – 95% confidence intervals.
[a] The estimate is significantly different from 0.

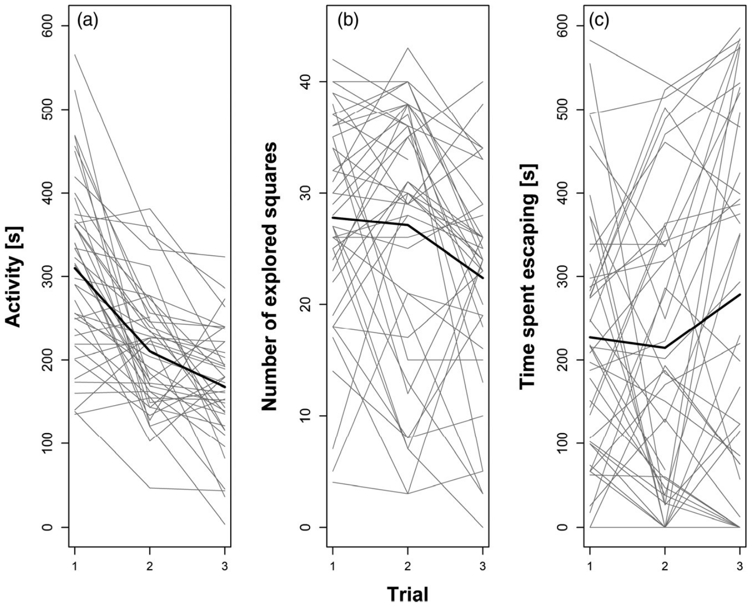

Figure 1 Individual (grey lines) and mean (bold line) responses of activity (a), exploration (b) and shyness (c) of smooth newts during three consecutive trials of the experiment. (a) – activity between trials, (b) – exploration between trials, (c) – escape response between trials.

Discussion

The observed behavioural traits of the newts in our study were moderately individually consistent for swimming activity, proportion of walking activity, exploration and escape response (thigmotaxis). They were weakly consistent for general activity and walking activity, and inconsistent for the latency of the first movement. Activity was moderately consistent for each trial, meaning that it decreased for all newts similarly between each of the trials within the experiment. The behaviour responses also did not differ between the sexes and were unaffected by the time of day when the experiment started.

Individual consistency in activity

The measures of activity presented in our study were all individually consistent. Slight differences in the repeatability estimates of walking and swimming might be more prominent with a larger sample, but in our case, they were well within the estimated confidence intervals. The division of activity into walking and swimming might be important for species where swimming is closely related to the escape response. Thus, the substitution of general activity with walking activity might separate it from a possible inherent correlation with shyness. The choice of the type of locomotion was also individually consistent, as shown by the individual consistency of the proportion of activity spent walking (walking proportion). Therefore, the consistency of the choice of locomotion might be a valuable indicator of individuality in smooth newts.

Consistency of activity did not depend on the sex of the newts, as opposed to the only other study that has dealt with the same question in urodele amphibians. Aragón (2011) reported stronger consistency for male newts, but weaker consistency for females. He tested this consistency in the context of different odours, so his results may reflect a sex-specific response to odours (his treatments) that does not translate into the temporal consistency of the behaviour when no odours are presented.

Taking into account all of the reviewed studies for both anuran amphibians and urodele amphibians, the repeatability of the activity was affected by odour treatments (Urszán *et al.*, 2015a), arena structure (Smith & Doupnik, 2005), by breeding origin – wild or captive (Maes *et al.*, 2012), by sex (Aragón, 2011, but not in our study), by previous experience (Urszán *et al.*, 2015b), by methods of measurement (Videlier *et al.*, 2014; Kelleher *et al.*, 2017) and slightly by age (Urszán *et al.*, 2015b) (see Tables S1). These are all factors that should be kept constant or should be taken into account when comparing multiple studies, populations or species. A less important factor (i.e. one that did not seem to affect the repeatability of the activity) was the time gap between repeated measurements (Maes *et al.*, 2012). In addition, the overall repeatability of the activity of larval and post-metamorphic amphibians did not differ much (Tables S1). However, none of the studies compared multiple life stages of the same individuals, probably due to the vast differences in the locomotor abilities of larval and post-metamorphic anurans.

Individual consistency in exploration

The number of squares explored by newts in our study was individually consistent. Consistency in exploration is often linked to all three stages of animal dispersal (departure, transience and settlements; for details see Cote *et al.*, 2010 or Cayuela *et al.*, 2018). For example, natal dispersal distance was found to be positively related to exploratory behaviour (Dingemanse *et al.*, 2003). Dispersal distance in natural streams was found to be positively related to the exploration score in an unfamiliar habitat (Fraser *et al.*, 2001). Immigrants were found to be faster explorers than locally born individuals (Dingemanse *et al.*, 2003), and individuals from island populations were found to have higher exploration than individuals from mainland populations (Brodin *et al.*, 2013). Consistent explorative behaviour in parents was also found to positively affect the explorative behaviour in offspring (Dingemanse *et al.*, 2003). While all these findings suggest that consistency of exploration might play an important role in smooth newt's ecology, they have yet to be tested on this species.

The only other study that describes consistency of exploration (but not other personality traits) in urodele amphibians shows a similar level of correlation (Gifford *et al.*, 2014). The consistency, however, decreased with the time between repeated measurements (see Tables S1). Other factors that can potentially affect the repeatability of exploration are age and experience (Urszán *et al.*, 2015b). However, the differences in consistency between the stages of development are not conclusive (Table S1).

The greatest drawback to a comparison of the published results is the difference in the definition of exploration. Exploration has been defined as a buffer around the trajectory of the individual (Brodin *et al.*, 2013), the number of visited squares (our study; Carlson & Langkilde, 2013; Gifford *et al.*, 2014) or the percentage of visited squares (Urszán *et al.*, 2015b). Kelleher *et al.* (2017) even used three definitions – distance travelled, time spent mobile and number of mobile events – each of which showed different repeatability (Tables S1). The strength of the correlation (repeatability) is usually used as the main indicator of the suitability of a certain measure as the best representative of exploration behaviour. However, the research would benefit above all from standardized approaches that would enable the studies to be compared effectively (Roche, Careau & Binning, 2016).

Individual consistency in boldness

In our study, repeatability of boldness was observed only for the escape response, which is a similar measure to that of Carlson & Langkilde (2013), who counted the number of square sides on the inner portion of the test arena crossed by an individual. This is also a measure of thigmotaxis. The accuracy of the latency of the first movement as a measure of boldness was impaired for our study by the inability to raise the glass dome in the centre of the arena consistently at the start of the experiment. We suspect that this has also been an issue in other studies, which makes the method impractical. This problem can be mitigated by changing the first movement to a movement longer than one body length (Wilson & Krause, 2012). However, in our opinion, it is better to choose a completely different option, for example shelter use, escape initiation distance or thigmotaxis.

Boldness has often been linked to predator-induced selection. Contrary to the common belief that more exposed (bolder) individuals have higher probability of being depredated (e.g. Brydges *et al.*, 2008; Smee & Weissburg, 2008), Carlson & Langkilde (2014a) found no dependence of tadpole survival on boldness, and Sih *et al.* (2003) even found a negative relationship between boldness and predator pressure for urodele amphibians. They attribute their results to the existence of behavioural syndromes that dilute the significance of predator pressure by other selective pressures.

Sih *et al.,* (2003) reported consistency of boldness for *Ambystoma barbouri* and *A. texanum* similar to our study, using shelter use as the preferred measure. Our investigation of other studies has shown that the consistency of boldness varied greatly (Tables S1). The variation was caused by previous experience (Urszán *et al.*, 2015a, 2015b) and by the breeding origin (Maes *et al.*, 2012) of the study subjects, and probably by differences in the definition of boldness (supported by Kelleher *et al.*, 2017). There does not seem to be any difference between larval and post-metamorphic amphibians, or between different time gaps, in the repeatability measurements (see Tables S1).

Trial repeatability

Activity and exploration decreased significantly with each trial in the experiment, which suggests that habituation may have taken place (but see Carlson & Langkilde, 2013). Habituation involves learning to disregard stimuli that are without significance to an animal, that is neutral stimuli that are not associated either with punishment or with reward (Cloninger, 1994). That being said, the difference in the mean, by itself, does not necessarily indicate that habituation took place. For this reason, it is important to show that the difference happened overall, that is to report the consistency of the expression of behavioural traits for each trial within the experiment (Roche *et al.*, 2016).

The decrease in the expression of behaviour traits could have been caused by insufficient time between the trials within the experiment, allowing the newts to remember the previous trial. The multisensory orientation system (see Sinsch, 2006) could then have allowed the newts to recognize the experimental arena and to adjust their behaviour across the trials. The speed of habituation and the habituation recovery time are unknown for the studied species. For the common toad, however, Ewert & Kehl (1978) stated that 6–24 h is long enough for recovery from habituation to an artificial rectangular-shaped prey dummy. Unfortunately, it was not possible to allow more time between the trials, because we feared that the newts might switch to the terrestrial phase and change their behaviour. The repeatability of behaviour traits does not seem to have changed with the amount of time between the trials in other studies (Tables S1), but it would be beneficial to test this relationship specifically through a single manipulative experiment.

With the exception of activity, no behavioural trait was repeatable for each trial within the experiment. This might mean that the extent of habituation varied individually, that is individuality was stronger than habituation (see Fig. 1), or that habituation was not present for the behavioural traits in question.

Correlated behaviour

Activity, shyness and exploration were positively correlated for our study. Unfortunately, the interpretation of the correlation of behavioural traits that were measured in the same behavioural assay is complicated, because the same movement can be classified at the same time both as general activity and as exploration or boldness. This causes an inherent correlation of unknown magnitude that we weakened by using randomly chosen non-overlapping parts of the assay.

A positive correlation of some of the measured traits has, however, already been found in a couple of previously published studies. Activity has been found to correlate with exploration (Koprivnikar *et al.*, 2011; Maes *et al.*, 2012; Wilson & Krause, 2012) and with boldness (Maes *et al.*, 2012; Wilson & Krause, 2012; Urszán *et al.*, 2015a). Boldness has also been found to correlate with exploration (Maes *et al.*, 2012) (but see Brodin *et al.*, 2013), and also with sociability (González-Bernal, Brown & Shine, 2014). No positive correlation of activity, exploration and boldness has, however, ever been found to occur at the same time, mostly because it has not been thoroughly tested (but see Maes *et al.*, 2012).

Active individuals from our study tended to explore more and were less bold, spending more time escaping. Although counter-intuitive at first sight, the negative correlation of boldness and exploration can be explained by differentiation of the exploratory behaviour. Verbeek, Drent & Wiepkema (1994) described two consistent types of explorers in juvenile great tits – slow explorers, which tended to explore slowly and to stay longer in each visited place, and fast explorers, which explored faster and did not spend much time in one place. In total, fast explorers were able to make more visits within the 10-min trial time. In our case, we discovered a similar pattern in exploring, too. As most of the tested newts could be classified as fast explorers, the positive correlation between time spent with an escape response (shyness) and exploration may be an artefact of this situation.

More time spent near the edge of the arena could also be caused by a size constraint of the arena, that is by the tendency of fast explorers to explore more, but to be restricted by the wall in front of them. This would also render the measure unsuitable for representing boldness, as it would automatically also contain a lot of information about exploration. Another cause of the correlation between shyness and exploration might be a common selective pressure that favours fast explorers, caused by increased predation or reduced mate availability – an increase in locomotor activity has been found to provide a benefit in mate searching (Martin, Joly & Bovet, 1989). Furthermore, an increase in mate searching ability might also result in a positive correlation between exploration and sociability, which was unfortunately not measured in the study. The absence of behavioural differences between the sexes might indicate that this pressure is beneficial for both males and females, or is at least not harmful for either. Whatever the cause, correlated behaviours should not be studied in isolation, because they develop as a group (Sih *et al.*, 2003, 2004). Additionally, they should be tested in a different study design, to see whether the correlations persist in different ecological contexts.

In conclusion, amphibian (especially urodele) personality research is still sparse, and the findings differ considerably both in approach and in results. Behavioural consistency is often studied for one or two types of behaviour only, in study-specific conditions, and behavioural correlations are sometimes neglected. The differences in the means of the expressed traits are never accompanied by consistency of the behaviour for each experimental trial and are therefore unsuitable as indicators of the habituation process. We believe that there is a need for a more complex approach (measuring more types of behaviour) and a standardized methodology (i.e. definition of behaviour types, correlation in time and different contexts, a standard time gap between repeated measurements, number of repeated measurements, duration of the experiment and sampling effort, and the shape and size of the test arena). It would only then be possible to make general assumptions on the global nature and consequences of the phenomena investigated here.

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Conflict of interest

The author(s) declare no competing interests.

Author contributions

O. Kopecký captured and cared for the newts, and together with P. Chajma participated in carrying out the experiment. P. Chajma analysed the data, prepared all figures and tables and, together with J. Vojar, wrote the main manuscript. All authors have reviewed the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary of amphibian personality studies' effect sizes.

Table S2. The influence of sex, time and trial order on general newt activity.

Table S3. The influence of sex, time and trial order on newt walking activity.

Table S4. The influence of sex, time and trial order on newt swimming activity.

Table S5. The influence of sex, time and trial order on the proportion of walking.The influence of sex, time and trial order on the proportion of walking.

Table S6. The influence of sex, time and trial order on newt exploration.

Table S7. The influence of sex, time and trial order on the latency of the first movement.

Table S8. The influence of sex, time and trial order on thigmotaxis.

Table S9. Variance components of repeatability estimates.

Chapter 5

Unpublished results: Population density, sibship and the changes in behavioural traits

Petr Chajma, Jiří Vojar

There is increasing number of reports of consistent behaviour in amphibians. In order to be subjected to sexual selection, it needs to show some level of heritability. Reports of its' heritability are, however, sparse. Furthermore, individual consistency in several behavioural traits was shown to be affected by previous experience, which suggests that individual personality can be shaped using specific cues at a specific time. To test behavioural similarity between relatives and the role of population density in the development of personality, we collected six different clutches of common frog (*Rana temporaria*) from one breeding pond. Upon hatching, tadpoles were raised in three different densities (estimated to be low, medium and high). After reaching Gosner stage 26, we tested the consistency of their behaviour in three repeated trials. Only boldness was individually consistent (ICC = 0.55) and no trait was consistent for individuals from the same clutch, raising questions about behavioural trait heritability in amphibians. Tadpoles lowered the expression of activity and exploration and increased their level of boldness with the increasing time of the day. Activity and exploration were higher in tadpoles, that developed in higher population densities, showing importance of this factor in their ecology. The size of the tadpole did not cause any change in its' behaviour.

Methods

Six common frog clutches were collected by hand from a single pond near Kozly, Central Bohemia. After tadpoles hatched and began swimming, a randomly chosen subset from each clutch was redistributed into smaller containers (18 x 12 x 13 cm) in three densities – small (two tadpoles), medium (eight tadpoles) and high (fourteen tadpoles), so that each clutch has two of each kind. In total, 36 of these containers were kept, feeding tadpoles spirulina and fish flakes ad libitum, keeping natural light cycle and aerating water to prevent hypoxia. After tadpoles reached Gosner stage 26, two out of each container were randomly selected and transferred to their own containers, in order not to use any marking techniques on them. Each individual participated in three open-field trials with the time gap of six days between repeated measurements. Testing arena was thoroughly cleaned after each trial and filled with 5 cm of water. Each tadpole was placed in the centre of the arena under a transparent dome and left for two minutes to acclimate. Then the dome was lifted and tadpoles' behaviour was recorded for ten minutes. General activity, number of visited squares and time spent near the outermost edge of the arena (thigmotaxis) were scored from recorded videos using BORIS software (Friard & Gamba, 2016).

Changes in activity (number of seconds active, out of total length of experiment), exploration (number of squares explored, out of total number of squares) and shyness (number of seconds, spent near the edge of arena, out of total length of experiment) were explained using Generalized mixed-effects models with binomial distribution and logit link function, fitted by maximum likelihood. Population density, individual length, and day-time, converted to sin and cos of radians, were used as fixed effects, while individual ID, clutch ID, and trial order were used as random intercepts, with length as a random slope for each individual. P-values were obtained using Wald's type II Chi-square tests. Repeatability of individuals, trials and clutches was estimated using intraclass correlation coefficient (ICC) from random effects of the model.

All analyses were done in R 3.5.3. (R Core Team, 2019) using lme4 (Bates *et al.*, 2015), lmerTest (Kuznetsova *et al.*, 2017) and rptR (Stoffel *et al.*, 2017) packages.

Results

Tadpole activity grew with the density of the population ($\chi^2 = 7.91$, $p = 0.019$), and lowered with day-time (sine: $\chi^2 = 32.0$, $p < 0.001$; cosine: $\chi^2 = 25.5$, $p < 0.001$) and was not affected by tadpole size ($\chi^2 = 0.09$, $p = 0.77$). Activity was not repeatable for individual (ICC = 0.12), clutch (ICC = 0.01), nor trial (ICC = 0.003). Tadpole exploration was highest in the middle density, followed by high and low densities ($\chi^2 = 7.41$, $p = 0.02$), lowered with day-time (sine: $\chi^2 = 1.76$, $p = 0.18$; cosine: $\chi^2 = 13.27$, $p < 0.001$) and was not affected by tadpole size ($\chi^2 = 1.46$, $p = 0.23$). Exploration was not repeatable for individual (ICC = 0.2), clutch (ICC = 0), nor trial (ICC = 0.01).Tadpole shyness was not affected by population density ($\chi^2 = 0.002$, $p = 0.99$), lowered with day-time (sine: $\chi^2 = 44.85$, $p < 0.001$; cosine: $\chi^2 = 80.09$, $p < 0.001$) and was not affected by tadpole size ($\chi^2 = 0.3$, $p = 0.58$). Shyness was repeatable for individual (ICC = 0.55), but not clutch (ICC = 0), nor trial (ICC = 0).

Unpublished.

Discussion

Non-random mating patterns are a function of ecology of individual, population and species. They are probably state-dependent and can change in time. Based on current knowledge, is it possible to shed some light into the processes, that form them? Yes and no. The studies that are available rarely measure and report all ecological data about sampled individuals and even if they did, they would not have enough statistical power to analyse them, as the sample sizes are more likely to be in tens, not hundreds nor thousands. Furthermore, these variables are likely to be correlated, which proves finding the real culprit difficult to impossible. Apart from that, there is a serious accusation that publication bias might be quite common, especially a "file drawer problem" (Green, 2019), where the significant results are more likely to be reported than non-significant results, which remain in a drawer (Rosenthal, 1979). When reading the studies, I found that there are huge differences in methods of data sampling and evaluation, especially in behavioural studies. In their analyses, authors use a plethora of different variable transformations, that are hardly suitable for their data and are not immune to common practices, that raise the probability of type I error (Forstmeier & Schielzeth, 2011). But despite all that, some studies still manage to find important processes, causing studied behaviour and the knowledge keeps steadily being accumulated, one time to be tested on a larger scale. Recently, more summarizing and meta-analytical studies have emerged (e.g. Sih *et al.*, 2015; Kelleher *et al.*, 2018; Green, 2019), that may show the future direction of the studies of mating patterns, which have long since moved away from basic comparison of morphological characteristics of animals.

Most commonly studied non-random mating pattern – size-assortative mating has many positive reports, but the strength of the assortment is mostly low. Is it as common as it seems? And what degree of mate size correlation is to be called assortative? In statistical analysis, researchers create null hypotheses, that they test and may or may not reject. The rejection depends on the set level of significance, statistical power and effect size. Even now, when statistical knowledge grows fast in biological sciences, most of the authors do not interpret their results based on the effect size (e.g. value of correlation coefficient or difference in mean), but solely based on p-value, that does not contain the information needed and depends heavily on the number of measurements. If the researcher studies a large population and has a large enough sample size, even small effects will be statistically significant. When citing other people's research, it is very easy to trust the reports that support our own results. This way, plausible theories can be built on false ground, or the importance of the phenomenon might get highly overestimated. In the case of size-assortative mating (SAM), Green (2019) reports as much as 282 studies coming from 101 publications on 68 species. Of these, 82 studies reported significant correlation between body sizes of mates, but the average effect size was $r = 0.227 \pm 0.018$, which is fairly low, considering that big portion of studies that reported non-significant effects, did not provide effect sizes, which considerably inflates this estimate.

But what effect should be considered high and what low? Different topics can have different recommendations, as you should always consider biological consequences of each effect (i.e. "biological significance"). Nevertheless, the most frequently used guidelines for general interpretation of the effect sizes come from social sciences and were published by Cohen (1988). He described the effect size (correlation coefficient) of $|r| = 0.1$ as small, $|r| = 0.3$ as medium, and $|r| = 0.5$ as large. I think the best way to interpret the correlation coefficient, which is regularly used for measuring the strength of assortment, is to square it and treat is as a coefficient of determination (r^2; shows how much variance is explained). That way, you get 1 % out of small effect, 9 % out of medium effect and 25 % out of large effect, which seems like a slight overestimation. The average effect size of studies on SAM, is therefore considered small, as it explains less than 5 % of the variance in mate sizes.

Studies that reported significant correlation were mostly pooled from several samples and the mate body-size correlation rose with the increasing number of samples pooled. While attractive at first sight, pooling of multiple independent samples for correlation can lead to false estimation of correlation coefficient, known as Simpson's paradox (Blyth, 1972; Wagner, 1982). If authors pool heterogeneous data, depending on the differences in sizes of males and females in multiple populations or in different time, they can get an increase, decrease or even directional change in correlation coefficient. Let me illustrate the problem on the mate availability hypothesis. In case of amphibians it usually means that SAM is caused by the decrease in size of mates with time, as larger individuals arrive first and mate earlier (Eggert & Guyétant, 2003). I randomly generated sizes of 30 pairs of hypothetical anuran amphibian for 10 days of the breeding season. Male snout-vent lengths (SVL) on the first day of breeding season were generated from normal distribution with the mean of $\mu = 80$ mm and standard deviation of $\sigma = 5$ mm. Female SVLs on the first day were generated from normal distribution with the mean of $\mu = 100$ mm and standard deviation of $\sigma = 7$ mm. Each day mean SVL dropped by 1 for both males and females. While the correlation coefficients for each day ranged between −0.09

and 0.1, showing no evidence for assortative mating, if pooled, coefficient rose to 0.26 and significantly differed from 0 ($p = 3.7*10^{-6}$) (Fig. 2).

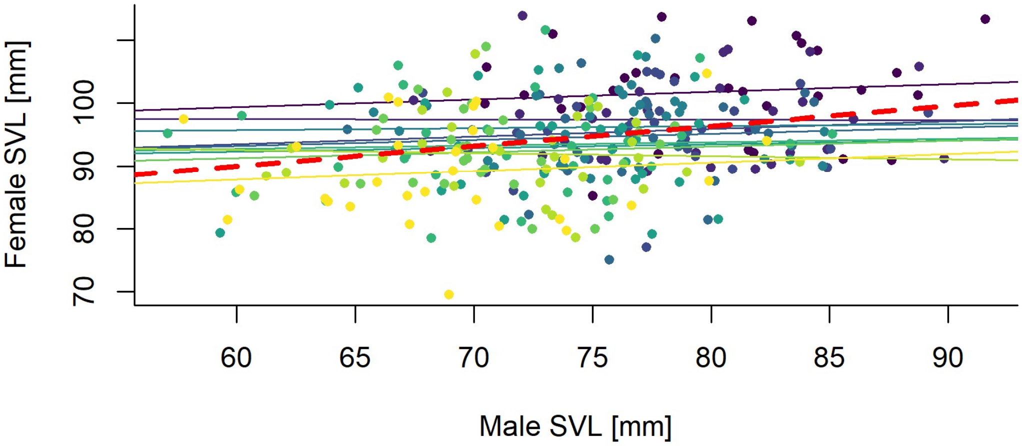

Figure 2. Simpson's paradox in mate availability hypothesis. Yellow to blue colours represent measurements of SVL in pairs (points) and their (in)dependency (lines). Red line represents the dependency of sizes in pairs for pooled data (r = 0.26).

This change of scale results in a change of correlation and is not limited to temporal scale but can happen even if authors measure two different populations of the same species (geographical scale). In fact, SAM that is caused by solely mate availability hypothesis might have different implications for individual fitness than assortative mating based on mate choice. This is also true when assortment is achieved through physical male-male competition (displacements from amplexus), as both are, in fact, a secondary sorting (Green, 2019). It is said, that primary pairing using mate choice is more stable, and depends less on population density, operational sex ratio, or length of the reproductive season, than secondary sorting does and may be subjected to a higher selection pressure, which can in turn more likely cause some larger evolutionary effect, such as sympatric speciation (Kopp *et al.*, 2018).

Contrary to the common belief, Green's (2019) meta-analysis showed that mate size correlation is present in explosive breeders only, while prolonged breeders, even though having time for mate choice, ended up pairing disassortatively. It is still not clear, what is the reason of the lack of assortment in prolonged breeders. Males of prolonged breeders usually attract females using their calls. Call's attributes can reveal both size (fundamental frequency of the call; Sullivan *et al.*, 1995; Murphy & Gerhardt, 1996; Bee *et al.*, 1999) and condition (call rate, or in other words activity; Cherry, 1993; Sullivan *et al.*, 1995), which can both be preferred through the mate choice (Sullivan *et al.*, 1995; Bee *et al.*, 1999). Some males might choose an alternative strategy and become satellites (waiting for the female silently in the vicinity of calling male; Emlen, 1976) or actively search for females by themselves. The choice of the strategy might depend on age (Emlen, 1976), body condition (Bee *et al.*, 1999) or might be based on behavioural traits (mainly aggressiveness, exploration, activity and socialisation). In order maximize their fitness, individuals can also change the preferred mating strategy throughout their lifetime (Dingemanse *et al.*, 2004). Even the changes within the same season might be possible, depending on the state of animal and conditions that are around him. Furthermore, I expect that some personalities may be more prone to strategy switching than other, creating additional means for sexual selection based on personality.

The main problem of size assortative and size disproportionate mating in amphibians is the combination of low strength of assortment, and lack of proofs of their positive effect on individual fitness (**Chapter 3**), thus questioning their evolutionary value. While the mate choice still clearly occurs, the reason of low assortment might be that (1) the mate choice results in the mating advantage of large individuals (SDM), (2) the mate choice is based on different criteria, e.g. behavioural traits, Major Histocompatibility Complex (MHC; Cortázar-Chinarro *et al.*, 2017) or (3) the heritability of these traits is too low to permanently establish these patterns. SDM and SAM / disproportionate mating do not have to occur exclusively and there are even reports, that they can appear and disappear with the effect sizes ranging from small to large (according to Cohen, 1988) in the matter of days, with no apparent reason (Olson *et al.*, 1986). It is probable, that this phenomenon is linked to some ecological processes, happening in each population, but they still need to be identified and described properly. Behavioural traits of amphibians have significant effect on the choice of mating strategy and on mating success. Higher activity levels are associated with increased reproductive output through mate searching (Martin *et al.*, 1989), scramble competition (**Chapter 2)**, and chorus attendance (Jaquiéry *et al.*, 2009) and with a greater body condition through the decrease in parasitic infection (Koprivnikar *et al.*, 2011). Tendency to explore influences the individual choice of movement pattern (Kelleher *et al.*, 2018), which can potentially raise encounter rates of the opposite sex. Aggressive individuals eat more and have higher mass than less aggressive ones (Haubrich, 1961). At the same time, higher activity, exploration and aggressiveness can lead to higher detectability and subsequent predation. And because all traits form single personality, some effects, might be promoted and some demoted, depending on particular phenotype.

Now if behavioural traits are consistent, the “shape” of the personality and its’ effect on mating patterns will stay the same, but even that seems to be very difficult to judge. There are considerable discrepancies in the type of variables measured in published studies and their assignment to one of the personality axes. Sometimes, authors themselves are not sure, what is the best measure of certain trait, so they choose multiple, to see “what suits better”, judging by the repeatability of the behaviour (to see what measures are used as proxies for different personality axes, please see Appendix of **Chapter 4**). This behaviour, although innocent at first sight, raises the probability of type I error, as each test has a 5 % chance to find a false positive result and when multiple measures of the same behaviour are tested, the chance to get at least one false positive result grows with each added measure ($1 - 0.95^3 = 0.14$ for three measures).

The time gap between repeated measurements of published studies ranged from one hour to one year. Increasing time gap lowered repeatability in some (Gifford *et al.*, 2014; **Chapter 4**), but not in other (*Maes et al.*, 2012; **Chapter 4**) behaviour traits, which makes it an interesting predictor for future research. It is important to find the source of the decrease, as it can lead to errors in judgment. E.g. the decrease in repeatability might be caused by habituation, which would show that the time gap is probably set too short, as the animal still remembers last trial and lowered its’ response, as the stimulus was neutral and was not associated with any negative (or positive) consequence (**Chapter 4**). The decrease in repeatability can also happen due to the change in animal condition, when kept in sub-optimal enclosure (Gifford *et al.*, 2014).

Studies on consistency of behaviour often employ similar statistical methods to the studies of SAM (correlation and repeatability), so they have similar drawbacks (low statistical power, incorrect interpretation of the effect size, etc; See above). And because there are usually more than two sets of repeated measurements (especially if authors assess temporal consistency), and it is more difficult to estimate adequate sample size for set number of repeats, authors seem more willing to just take a risk and set the sample size without considering all possible drawbacks. To show minimal recommended sample sizes with statistical power of 0.8 (recommended by Cohen, 1988), I ran 10000 simulations of randomly generated datasets, coming from multivariate normal distribution, with sample sizes ranging from 2 to 100, with 2 to 15 repeated measurements, and with effects sizes (repeatability) set to 0.1, 0.2, 0.3, 0.4 and 0.5. This way, I can show the trade-off between measuring many individuals, small number of times and measure small number of individuals, many times, which will help researchers, that are time or resource limited (Fig. 3).

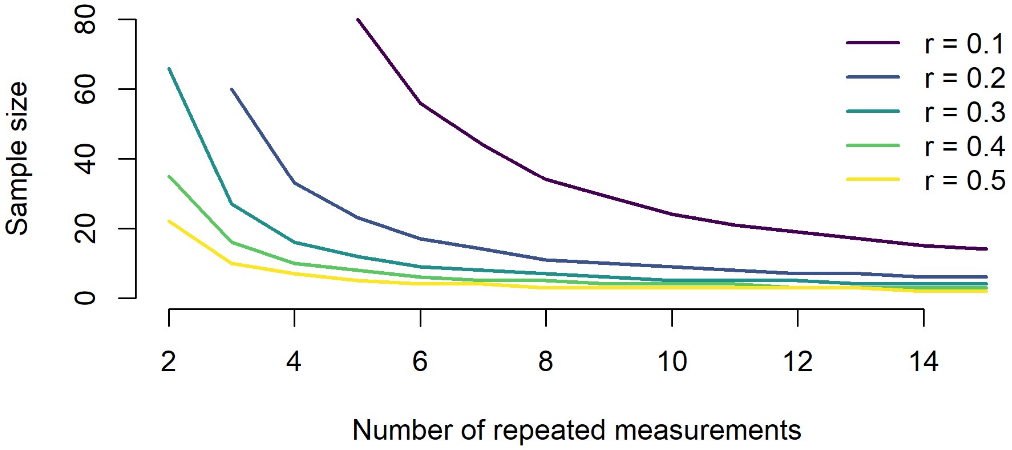

Figure 3. Minimal sample size, required for the set number of repeated measurements (trials), to achieve statistical power of 0.8, with repeatability of 0.1, 0.2, 0.3, 0.4 and 0.5. If the line does not start at x = 2, minimal sample size exceeded 100.

If we want to detect minimal repeatability of 30 %, while being able to find significant effect in 80% of times, for example, we would need at least 66 individuals if we repeat the measurement twice, 27 individuals if we repeat the measurement three times, and only 16 individuals if we measure them four times. It is therefore adviseable, to raise the number of repeated measurements as much as possible, if studying small populations.

Similarly, to the strength of assortment, the amount of consistency in expressed behaviour varies in different populations with different histories and depending on current conditions. Mean repeatability of activity in published studies was 0.31 ± 0.05, mean repeatability of exploration was 0.27 ± 0.08 and mean repeatability of boldness was 0.26 ± 0.04. As repeatability is, in fact, a second power of correlation coefficient, this would translate to mean correlation of 0.56, 0.52 and 0.51, respectively. According Cohen's (1988) classification of effect sizes, these would all be considered large. The variance in behavioural traits was caused mostly by age (Urszán *et al.*, 2015b), experience (Maes *et al.*, 2012; Urszán *et al.*, 2015b) and predator presence (Urszán *et al.*, 2015a). Certain situations, such as when amphibians are in immediate danger of being depredated, might therefore trigger a different state of behaviour, in which they act more instinctively and have lower variance of behavioural responses, thus behaving more consistently. It would be interesting to see, if there are similar "triggers" of behavioural change during reproductive season, leading to the choice of different mating strategy. If the behaviour consistently changes with age and upon a certain experience, individual mating strategy might change during individuals' lifetime as well, in order to maximize its' reproductive success. Resulting mating patterns, might therefore not be as simple, as one would suspect.

One serious question, however, remains and it is a question of heritability. While heritability of size, for amphibians, is partial at best (Green, 2019), the heritability of behavioural traits is expected to be even lower than the heritability of morphological traits (Mousseau & Roff, 1987; Kruuk *et al.*, 2000). Even though there are reports of heritability of behaviour in different classes (e.g. birds, Drent *et al.*, 2003; Dingemanse *et al.*, 2004), my study reported no consistency of activity, boldness, nor exploration between common frog tadpoles from the same clutch (**Chapter 5**). While it is possible that the consistency will appear later in their life, there are some reports of the consistency between life stages (Wilson & Krause, 2012; but not in Brodin *et al.*, 2013), so it is questionable to say the least.

Conclusions and further research

Despite many “proofs” of the existence of size-based non-random mating patterns in amphibians, it is unclear how widespread they are and if they provide direct or indirects benefits to their wearers. It appears that mechanisms of mating patterns are more flexible than previously thought and that they can be (de)activated by different situations and ecological contexts. Recent comparative studies reveal that the results of most studies reporting size-assortative mating are false positive and that this pattern is virtually non-existent as a rule in amphibians. Other problems lie in a weak or non-existent heritability of body size, which questions the legitimacy of size-dependent mating (large male advantage), as well as the effect of size-assortative mating on fertilization success.

Apart from size, behavioural traits are also thought to be the object of assortment. Some traits were proven to be consistent in time and different situations (activity, exploration and boldness), but some do not appear to be studied at all (sociability and aggressiveness). Amphibian personalities are already linked to dispersal syndromes and predatory prey situations, but their role in reproductive ecology is still understudied. Published studies are scarce and difficult to compare, due to different methods of measurement of behavioural traits and different contexts, in which consistency is examined.

Almost all the studies reviewed the behaviour in laboratory conditions, which does not reflect real-life situations. Because of the advances in electronics miniaturisation, the future in behavioural consistency essays lies in deployment of miniature microchips in natural conditions for longer periods of time, while logging all environmental and individual characteristics. Moreover, advances in machine learning algorithms can help with automatic classification of behaviour. It can then be tested on consistency and the influence on logged environmental variables, bringing better understanding of their biological and evolutionary meaning. We are working toward this goal, as we developed and tested on a group of common toads a small (approx. 1 g) multi-sensoric datalogging device, capable of measuring acceleration in 3 axes, magnetism, light intensity, temperature and humidity. We plan to compare the experimental results to open-field tests, that were executed parallel to the experiment, and judge the relevance of behaviour expressed in this common experimental setup.

The matter of heritability of behaviour needs to be thoroughly addressed as well, as so far, the results are controversial, to say the least. Apart from these plans, I would like to place more emphasis on research of the consistency of sociability and aggressiveness in amphibians, as these topics are missing.

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Curriculum vitae
&
List of publications

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Education

2014 – present:	Ph.D. studies Ecology Faculty of Environmental Sciences **Czech University of Life Sciences Prague** Thesis: *Non-random mating patterns and consistency of behaviour of amphibians*
2012 – 2014:	Master programme Nature conservation Faculty of Environmental Sciences **Czech University of Life Sciences Prague** Thesis: *Size-assortative mating in common toad (Bufo bufo)*
2009 – 2012:	Bachelor programme Ecology Faculty of Environmental Sciences **Czech University of Life Sciences Prague** Thesis: *Causes of size-assortative mating in amphibians*

Skills

- Programming in R (advanced), R Shiny (beginner), Arduino IDE (beginner), C++ (beginner), php (beginner), html (beginner), SQL (beginner)

- 3D modelling and design in Fusion 360, 3D printing

Publications

Vojar, J., **Chajma, P**, Kopecký, O., Puš, V. & Šálek, M. (2015). The effect of sex ratio on size-assortative mating in two explosively breeding anurans. *Amphibia-Reptilia* **36**, 149-154.

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Chajma, P, Kopecký, O. & Vojar, J. (2020). Individual consistency of newt's exploration and shyness, but not activity: The effect of habituation? *J. Zool.* **311**, 269-276.

Grants

IGA FŽP 20154233: The effect of SAM on fitness of common toad (*Bufo bufo*) and agile frog (*Rana dalmatina*), main researcher.

IGA FŽP 20164234: Personality of agile frog (*Rana dalmatina*) and smooth newt (*Lissotriton vulgaris*), main researcher.

IGA FŽP 20174216: Development and consistency of common frog (*Rana temporaria*) behaviour, main researcher.

TAČR TJ02000199: Modular system for complex monitoring of breeding behaviour and nest success in birds, key researcher.

TAČR SS01020383: Multisensory datalogging as a tool for assessment of the impact of environmental changes on wildlife circadian activity, key researcher

Teaching activities

- Computational methods II
- Computational methods III
- Ecological data processing
- Environmental data collection and processing
- Statistical methods I
- Statistical methods II
- Zoology II
- Processing of biological data
- Programming in R
- Methods of studying of ecosystems

- Practice management in CHKO Pálava
- Supervision of 6 bachelor and 1 diploma theses