Relatedness, body size and paternity in the alpine newt, *Triturus alpestris*

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Sexual selection has traditionally been investigated assuming that male quality is as skewed as patterns of male reproductive success can sometimes be. Recently, female choice has been investigated under the model of genetic compatibility, which assumes that each individual female has her own 'best' mate and there is no overall optimal choice for all females. We investigated female mate choice in the newt species *Triturus alpestris*, a member of a genus where female choice has been investigated only within the context of the optimal male (female choice for condition-dependent traits). We provided females with two males that differed in one condition-dependent trait (body size) and overall genetic composition. Both male body size and female body size did not influence paternity, but the degree of genetic relatedness between females and potential mates did. Two components of fitness (fecundity and hatching success) did not differ between singly and multiply sired clutches, indicating that females do not employ polyandry as a means of increasing offspring fitness through genetic bet-hedging. Instead, we hypothesize that females may mate initially for fertility assurance, but prefer less-related males as the most genetically compatible mates.

Keywords: *Triturus alpestris*; genetic compatibility; fecundity; multiple paternity; body size

1. INTRODUCTION

Traditional sexual-selection theory based on optimality models states that in species where females choose mates and males provide no direct benefits male reproductive success should be skewed towards a few high-quality males. Females judge male quality via a trait or traits, such as body size, physical vigour, or elaborate and costly ornaments, and a male that possesses the trait optimum represents the best choice for every female (Andersson 1994). Although evidence exists that supports this theoretical framework (Houle 1992; Andersson 1994), selection for a single optimum should rapidly exhaust any adaptive variation in a population (i.e. the paradox of the lek; Pomiankowski & Møller 1995). Several explanations have been put forward that might explain how adaptive genetic variation can be maintained in a population where strong sexual selection favours a certain phenotype (Hamilton & Zuk 1982; Pomiankowski & Møller 1995; Rowe & Houle 1996; Houle & Kondrashov 2002), but none of these explanations avoid the basic assumption that, at a single point in time, an optimum exists in a population that is the preferred choice for females. However, the prevalence of polyandry suggests otherwise (Birkhead 2000). Although convenience polyandry can drive multiple paternity, as can fertility assurance or the subsequent availability of better quality males after an initial insemination (Thornhill & Alcock 1983; Yasui 1998; Garner *et al.* 2002), the almost ubiquitous occurrence of polyandry indicates that male quality cannot be ranked on a single invariant scale. Instead, the suitability of a potential male may result primarily from the epistatic interactions of the individual male and female genotypes, referred to as their

genetic compatibility (but referred to as genetic incompatibility in Zeh & Zeh (1996, 1997)). Under genetic compatibility, the combination of male and female genotypes determines the mate-choice optimum, and adaptive genetic variation is maintained because each individual has its own best mate (Tregenza & Wedell 2000).

Sexual selection in newts (genus *Triturus*) has been explored within the context of the traditional model (Halliday 1977). All species use some combination of olfactory, vibrational and visual cues in courtship. Males of the two newt species commonly used to investigate sexual selection (*Triturus vulgaris* and *T. cristatus*) develop large dorsal crests during the breeding season (Halliday 1977). Crest height is condition dependent (Green 1991a; Baker 1992), and large-crested males are able to signal quality both through the height of the crest and the vigour of their courtship (Green 1991b). Large crests are favoured by females (Green 1991a; Gabor & Halliday 1997). Thus, in these two species, female choice should favour an optimal phenotype (i.e. large crest height). By contrast, males of some newt species located basally on the *Triturus* phylogeny (e.g. *T. alpestris*; Zajc & Arntzen 1999) develop no or relatively small and invariant crests during the breeding season and exhibit courtship behaviours that lack many of the strong vigour signals used by *T. vulgaris* and *T. cristatus* (Arntzen & Sparreboom 1989). Males of all *Triturus* species deliver chemical signals to females during courtship (Arnold & Houck 1982; Malacarne & Giacoma 1986), which include pheromones that attract females (Malacarne & Vellano 1987; Houck & Reagan 1990). Such chemical communication systems in vertebrates have been shown to carry signals of genetic composition (Penn & Potts 1999). It is conceivable, then, that males of *Triturus* species that do not rely heavily on strong phenotypic cues signal their suitability as mates more strongly through chemical signals that enable mate choice

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for genetic compatibility. Mate choice based on compatibility may in fact not be restricted to these species; species with large crests also use courtship pheromones. Currently, there is no evidence for a role of genetic compatibility in *Triturus* newts because it has yet to be investigated.

We investigated the roles of phenotype and genotype in the mating system of *T. alpestris*, an ancestral newt species where males do not exhibit an exaggerated crest and where courtship is comparatively simple (Halliday 1977; Arntzen & Sparreboom 1989). We assessed the effect that phenotype had on paternity by providing females with two potential mates who differed in body size, a condition-dependent trait that is sexually selected in caudate amphibians (Howard *et al.* 1997). We investigated the effect of genotype by estimating the genetic relatedness of potential and actual mates to females *post hoc* using allelic scores at seven microsatellite loci (Garner *et al.* 2003). We determined the paternity of offspring using microsatellites and included female body size as a covariate in the analysis, as female body size can be strongly linked to fecundity and can therefore influence sexual selection (Garner *et al.* 2002). Specifically, we asked the following questions.

- (i) Does relatedness influence paternity?
- (ii) Does male body size influence paternity?
- (iii) Does female body size influence paternity?

Finally, as polyandry has been detected in this species (Halliday 1998; Rafinski & Osikowski 2002) and polyandry *per se* can influence offspring fitness (Madsen *et al.* 1992; Byrne & Roberts 1999, 2000), we also assessed whether polyandry affected two components of fitness: fecundity and hatching success.

2. MATERIAL AND METHODS

(a) *Experimental protocol*

Adult *T. alpestris* were collected at the beginning of May 2000 at the Räubrichseen ponds located 20 km north of Zürich, Switzerland. All animals were collected after migrating to the pond verge but before entering the water. This ensured that females were unmated before the onset of the experiment (as in Pecio 1992). Animals were taken to the University of Zürich where they were housed terrestrially in single-sex groups until measurements were taken 2 days after capture. All newts were weighed to the nearest 0.01 g. We divided males into two groups (large (L) and small (S)) such that all L males had a greater mass than the median value, and all S males had a smaller mass. Males were paired, one L and one S, so that the largest L male was paired with the largest S male, the second largest males from each category were paired, and so on, until a total of 13 sets of paired males were available. Using this criterion, the smallest difference in male body size was greater than 10% of the mass of the largest male in the pairing. The smallest difference in size between paired males was 0.296 g, while the largest was 0.764 g (mean $\pm$ s.e. 0.472 ± 0.04 g). Each male pair was allocated haphazardly to one of 13 females, and the subsequent trios were placed in 90 l plastic tubs filled to within several centimetres of the rim with aged water. We allowed animals to interact unobserved and undisturbed for a 3 day period, commencing on 4 May 2000. At the end of the 3 day period, all males were removed, and we sampled a small piece of tail tip from each

male for genetic analysis. Tail tips were clipped after the mating period rather than before, as the tail is used during courtship (this means that we did not know genotypes before the experiment). Tail tips were frozen at -80°C for genetic analysis.

We placed anchored plastic strips into each tub. Females wrap their eggs in these strips, which serve as substitutes for the submerged vegetation used in nature (Miaud 1993). Starting on 6 May, we checked the strips in the tubs every 1–3 days for eggs. In addition, several *Rana* sp. larvae and zooplankton were added at regular intervals to each tub as food for females. Once eggs were detected (8 May) all strips were changed in each of the tubs so as to standardize the amount of disturbance each female experienced. We continued checking strips for eggs until 25 May. At this time, only six out of the 13 females were still depositing small numbers of eggs, while the other females had stopped depositing eggs and, in many cases, had left the water and were resting on floating wood. We removed all females, weighed and measured them, and sampled a small amount of tail tip for genetic analysis. All adults were returned to the site of collection after a suitable recovery period.

Eggs were kept indoors, separated by experimental replicate, in 0.5 l tubs containing aged tap water. We recorded the total number of eggs in each clutch and the total number of eggs hatching for each clutch. Once all the eggs that were developing in a given container had either hatched or stopped development, and hatched larvae showed no obvious signs of a yolk sac, larvae were sacrificed and stored individually at -80°C for genetic analysis. A subset ($n = 20$) of offspring from eight of the crosses were kept alive and maintained in a cool room for use in a related experiment. Upon completion of this experiment, tail tissue from these larvae was sampled. Mortality in the latter experiment was negligible, as only one or two larvae per cross did not survive (B. R. Schmidt and T. W. J. Garner, unpublished data).

(b) *Molecular analysis*

DNA was extracted from tail tips and sacrificed larvae using the QIAamp DNA mini kit (Qiagen). Initially, DNA samples from adult trios were amplified via the polymerase chain reaction (PCR) using all primers from Garner *et al.* (2003). The alleles scored from these amplifications were used for the analysis of relatedness described in § 2c. PCRs were performed using the protocol of Garner *et al.* (2003) and subsequently electrophoresed for 90–140 min at 100 V using Spreadex EL-300 S-100, EL-600 S-50 or EL-800 S-50 gel and the SEA 2000 advanced submerged gel electrophoresis apparatus (Elchrom Scientific AG, Switzerland). Primers specific for two complex microsatellite loci, Ta3Caga1 and Ta1Caga4, proved to be sufficient for differentiating parental alleles. Amplification products for Ta1Caga4 enabled unambiguous paternity assignment for 11 out of 13 crosses (no shared paternal alleles and no more than one maternal allele shared with any one potential father). Locus Ta3Caga1 performed similarly in eight out of 13 crosses, including the two crosses where paternity could not be clearly determined using locus Ta1Caga4 alone. We then amplified the offspring samples from each cross using these two primer sets and scored paternity accordingly. Results from amplification using one primer set did not contradict the results obtained using the second primer set in any of the clutches analysed.

Table 1. Clutch characteristics and the relative paternity (proportion of offspring fathered) of each male for all 13 crosses. (Abbreviations: pat. L male, the proportion of offspring fathered by the larger male; pat. S male, the same for the smaller male. *r* values are the relatedness category for each male in a given cross.)

cross	total eggs	total hatch	pat. L male	pat. S male	L <i>r</i>	S <i>r</i>
1	71	63	1.000	0.000	less	more
2	111	110	0.354	0.646	more	less
3	145	89	0.067	0.933	more	less
4	29	27	1.000	0.000	less	more
5	1	1	1.000	0.000	more	less
6	58	27	0.074	0.926	more	less
7	47	44	0.659	0.341	less	more
8	93	79	1.000	0.000	less	more
9	49	30	0.000	1.000	more	less
10	101	92	0.000	1.000	less	more
11	123	110	0.155	0.845	less	more
12	55	55	0.491	0.509	less	more
13	119	108	0.028	0.972	more	less

(c) Data analysis

Measures of genetic similarity (*r*-values) between adults within trios were estimated using the program KINSHIP v. 1.3.1 (Queller & Goodnight 1989), with cross (1–13) as the group variable and results displayed by group. We used a generalized linear model with binomial error (McCullagh & Nelder 1989; Krackow & Tkadlec 2001) to test the statistical hypotheses that female mass, difference in mass among paired males and male relatedness to females affect the proportion of offspring sired. The model was implemented with the procedure GENMOD of SAS v. 8.1 (SAS Institute Inc. 2000) using an EVENTS/TRIALS model statement and the logit link function. We used model option PSCALE to account for overdispersion. We analysed the proportion of offspring sired by the larger male (i.e. number of hatchlings sired by larger male/total number of hatchlings). Relatedness of males to females was entered into the model as a categorical variable (more versus less related). We tested whether multiply sired clutches are primarily fathered by a given relatedness category using a Fisher's exact test. We tested for differences in fitness components (clutch size and proportion hatching) between singly and multiply sired clutches using a two-sample *t*-test and a variance ratio test, respectively.

3. RESULTS

In one out of 13 crosses (cross 5, see table 1), only a single egg was deposited and genotyped. This cross was not included in the analyses below. The relatedness of females to males had a statistically significant effect on the proportion of offspring sired, whereas female mass and difference in mass between the males did not significantly affect the proportion of offspring sired. The relatedness of females to males had a statistically significant effect when it was entered into the model as a categorical variable, but had no statistically significant effect when it was entered as a continuous variable. We believe that using relatedness as a categorical variable matches the experimental design more closely because females were provided with a discontinuous choice of only two males. Overall, there were five singly sired clutches, and the less-related male sired four of them (table 1). Seven out of the 12 crosses were the result of polyandrous mating (table 1), and the majority of paternity in these crosses could be ascribed to the less-

related male (Fisher's exact test, $p = 0.015$). Both mean number of eggs and mean proportion hatched did not differ significantly between multiply and singly sired clutches (*t*-test, d.f. = 11, $t = 1.687$, $p = 0.120$ and *t*-test, d.f. = 11, $t = 0.359$, $p = 0.726$, respectively). Finally, the variances for both fitness components were not significantly different between multiply and singly sired clutches (total eggs, $p = 0.390$; proportion hatched, $p = 0.289$).

4. DISCUSSION

Our results show that paternity in the alpine newt is significantly influenced by parental genetic similarity but not body size (table 2). Our findings are similar to the findings of Olsson *et al.* (1996) in their studies of sand lizards. Olsson *et al.* (1996) interpreted their result as cryptic female choice for inbreeding avoidance, and inbreeding avoidance has been implicated in a variety of vertebrate mating systems (Keller 1998; Coltman *et al.* 1998, 1999; Coulson *et al.* 1998, 1999; Bull & Cooper 1999; Amos *et al.* 2001), including amphibians (Waldman *et al.* 1992). Inbreeding negatively affects reproductive output and survival (Lacy *et al.* 1993; Jiménez *et al.* 1994; Keller *et al.* 1994), and individual fitness is often negatively correlated with increased homozygosity, which results from matings between genetically similar individuals (Newman & Pilson 1997; Saccheri *et al.* 1998; Tregenza & Wedell 2000; Nieminen *et al.* 2001). All but one of the singly sired clutches were sired by the less-related male, therefore no meaningful comparison of the fitness consequences of mating with a less-related versus more-related mate could be made. We could compare components of fitness between singly and multiply sired clutches, as polyandry has been shown to be an important mechanism for inbreeding avoidance (Madsen *et al.* 1992; Tregenza & Wedell 2002). Our comparison of multiply and singly sired clutches revealed no differences in fecundity or fertility, indicating that polyandry *per se* affords no fitness benefits to newts.

Male body size often covaries with testis size, and testis size influences the amount of sperm transferred in a single ejaculate (Olsson *et al.* 1996; Hosken *et al.* 2001). Therefore, under sperm competition and all else being equal,

Table 2. The relationships between relatedness, male body size, female body size and paternity.

parameter	d.f.	estimate	Wald 95% confidence limits	χ^2	<i>p</i>
intercept	1	−10.76	(−29.69, 8.16)	1.24	0.2650
female mass	1	1.40	(−2.88, 5.68)	0.41	0.5223
difference in mass between males	1	3.99	(−5.31, 13.29)	0.71	0.4007
relatedness (categorical)	1	3.01 ^a	(0.03, 6.02)	3.85	0.0498
scale ^b		6.41			

^a This is the difference between the category levels ‘less’ and ‘more’. The latter is arbitrarily set to zero.

^b The scale parameter was estimated as the square root of Pearson’s χ^2 /d.f.

large-bodied males should achieve numerical sperm superiority and father more offspring (Parker 1998). Male body size has also been shown to function as a condition-dependent signal of male quality in salamander mating systems (Howard *et al.* 1997). Our study did not control for the number of spermatophores transferred to the female, so we do not know whether sperm load itself influenced paternity. We have shown, though, that paternity was not significantly affected by male body size, as was also the case with sand lizards (Olsson *et al.* 1996), indicating that there is no sexual selection on male body size in these newts. Other phenotypic characters, such as male coloration and courtship vigour, need to be investigated before the overall importance of phenotypic traits can be determined.

Generally, female body size correlates with clutch volume in ectothermic vertebrates, presumably owing to constraints on reproductive investment imposed by the size of the female body cavity (Olsson & Shine 1997). Furthermore, female body size and an increased reproductive investment have been shown to correlate positively with mating rate and number of sires (Greene & Brown 1991; Olsson 1993; Garner *et al.* 2002). In the absence of any detectable indirect benefits and in species where males provide only sperm, this effect has been ascribed to either increased male interest in high-investment females or increased sperm requirements for larger clutches (Olsson 1993; Osikowski & Rafinski 2001). In our study, though, larger females did not exhibit more multiple paternity than smaller ones, and female body size did not significantly influence paternity patterns irrespective of how many fathers sired a clutch. It is unlikely that increased male interest and harassment affect paternity patterns in *T. alpestris*, as female body size does not correlate significantly with the number of eggs deposited (T. W. J. Garner, unpublished data). Furthermore, in all *Triturus* species males court females without physical restraint, an unusual situation in amphibian species (Halliday 1977; Halliday & Tejedo 1995). Females must show interest and actively participate before sperm transfer is achieved, and a female may prevent either continued courtship or sperm transfer simply by walking away.

Biased paternity in sand lizards was ascribed to post-copulatory selection of genetically dissimilar sperm, but this is probably not the mechanism employed by *Triturus* females. Sperm are stored in sets of tubules and released in a relatively uncontrolled manner (Verrell & Sever 1988; Sever *et al.* 1999). More importantly, a recent study by Rafinski & Osikowski (2002) showed that sperm from controlled two-male matings are mixed and that paternity

over both the short and long term do not reflect preferential sperm usage. Neither first- nor second-male sperm precedence was detected. Therefore, our results are best interpreted in the context of a pre-insemination female preference for genetically dissimilar males rather than any post-insemination mechanism.

If less-related males are preferred, or, at least, more successful fathers, then why should females mate with more-related males? In a study of a related species, Osikowski & Rafinski (2001) showed that female multiple mating, but not necessarily polyandry, resulted in increased fecundity and fertility, and concluded that multiple mating is probably driven by fertility assurance. Jones *et al.* (2002) interpreted patterns of first-male sperm precedence in the rough-skinned newt, *Taricha granulosa*, as polyandry for fertility assurance. Although we did not detect any fitness benefits in clutches exhibiting mixed paternity, it is interesting to note that Osikowski & Rafinski (2001) detected a difference in fitness components between females that had accepted one to three versus four to twelve spermatophores in mating trials that ran for up to 10 days. In *T. alpestris*, clutch size averages 150 eggs (Griffiths 1996), and wild caught and inseminated female *T. alpestris* can continue to lay fertilized eggs after five weeks in captivity (T. W. J. Garner, unpublished data). The majority of clutches in our study contained fewer than 100 eggs (table 1), and females, for the most part, deposited eggs for two weeks or less. Benefits from multiple spermatophores may be detectable only over a longer mating and subsequent oviposition period than we provided, but this would not prevent females from seeking fertility assurance and genetic benefits over the short term. It is also possible that all of our study females did mate multiply, some repeatedly with the same male and some with more than one male. If so, then all clutches would exhibit the benefits of mating for fertility assurance. Our study thus provides no evidence against the fertility-assurance hypothesis because of the experimental procedures.

Unlike the mating systems of *T. cristatus* and *T. vulgaris*, female preference in *T. alpestris* does not seem to be driven by condition-dependent traits, and traditional sexual-selection theory based on a population optimum does not seem to apply. Instead, female mate choice in this species can most easily be explained as a form of pre-insemination mate choice for genetic compatibility: relatively simple male courtship that relies primarily on chemical communication and a female preference for genetically dissimilar males fit this theoretical framework best. This raises the question: what is the phylogenetic distribution of female choice for genetic compatibility versus condition-depen-

dent traits in the genus *Triturus*? Although data to date are limited, evidence exists that enables the formulation of a working hypothesis. Chemotactic responses of *T. carnifex* to courting pairs are muted with respect to both *T. alpestris* and *T. italicus* (Belvedere *et al.* 1988), and *T. carnifex* is a member of one of the two derived *Triturus* clades containing large-crested newts (Zajc & Arntzen 1999). Males from these clades exhibit complicated courtship that includes strong vigour signals and enlarged crests, and in both clades condition-dependent female mate choice has been described (Green 1991a; Gabor & Halliday 1997). *Triturus alpestris* and *T. italicus* instead belong to a more basal lineage that contains species that exhibit less highly developed male secondary sex characteristics (Zajc & Arntzen 1999). In this lineage males court females with continued bouts of tail fanning and flicking, but do not use strong vigour signals such as whips and whiplashes (Giacoma & Sparreboom 1987; Arntzen & Sparreboom 1989; Faria 1993). Across species, increasing crest height seems to correlate with an increase in the frequency of vigour signals such as the whip or whiplash (Halliday 1977; Pecio & Rafinski 1985; Green 1989). Therefore, female choice for genetic compatibility may be an ancestral condition for the genus, and condition-dependent mate choice may be a derived condition arrived at independently in the representatives of the two crown *Triturus* clades. Of course, further studies incorporating a phylogenetic approach focusing on the occurrence of female choice for genetic compatibility are required, and the potentially confounding effects of fertility assurance need to be accounted for. The genus *Triturus*, though, seems eminently suited to comparative investigations of the evolution of female choice for genetic dissimilarity and condition-dependent mate choice and the relative importance of the two processes.

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