

Operational sex ratio in newts: field responses and characterization of a constituent chemical cue

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Operational sex ratio (OSR) has been traditionally thought of as a force imposing competition for mates rather than also a cue used to regulate the intrasexual competition individuals encounter. To assess whether eastern red-spotted newts, *Notophthalmus viridescens*, could appropriately compare OSRs, we quantified field responses to traps containing four males, a sexually receptive female, four males plus a female, or nothing as a control. Early in the breeding season, males from two populations chose competitive mating opportunities over no mating opportunity at all, but generally preferred less competitive mating prospects. Later in the breeding season, as the OSR of newt populations becomes more male biased, males accordingly increased their acceptance of intrasexual competition. Females avoided groups of four males, and for both sexes, avoidance of male-biased courting groups increased their probability of amplexus courtship. We then isolated an approximately 33-kD protein from male cloacal glands that was used by males to compare OSRs. To our knowledge, this protein represents the first isolated and characterized component of an olfactory cue used to evaluate OSR. These results support two important principles regarding mating systems: (1) OSR can somewhat paradoxically be both the source imposing competition for mates and the source used to reduce it, and (2) analogous to the sex in short supply often being "choosy" selecting mates, the sex in excess (here, males) appears to be choosy about its acceptance of intrasexual competition. *Key words:* chemical cues, mating competition, mating system, *Notophthalmus viridescens*, olfaction, operational sex ratio, pheromone, salamander, sexual selection, spatiotemporal variation. [Behav Ecol]

Many species encounter spatial variation in operational sex ratio (OSR; i.e., the ratio of sexually active males to fertilizable females ready to mate; Emlen and Oring, 1977) when searching for mates (Quinn et al., 1996) and thus must repeatedly decide whether to approach a potential mate or search elsewhere. These decisions can affect mating success by influencing the intensity of intrasexual competition that individuals encounter (Andersson, 1994). Consequently, similar to optimal foraging, there should be selection for organisms to optimally mate search.

A variety of factors should influence the decision to approach a potential mate or search elsewhere. If groups of conspecifics, hereafter referred to as exhibiting local OSRs, are detected, an organism may use cues released from these groups to select the least competitive mating opportunity. For instance, in environments in which long distance visual cues are unreliable, such as in ponds (Dodson et al., 1994), animals may use chemicals released from conspecifics to appraise OSR. In addition to directly comparing intrasexual competition among conspecific groups, it may also be useful to estimate the OSR of the entire population (perhaps by

keeping a running average of local OSRs). That way, local OSRs could be compared to the OSR of the population (population OSR; i.e., the mean of all local OSRs) to facilitate distributing among mates in an ideal free manner (Fretwell and Lucas, 1970). For example, if a male detects a courting group with one male and one female in a population with an OSR of four males to every female, then all else being equal, the male should benefit from approaching this group because he is apt to encounter more competitive mating prospects if he continues to search for mates (assuming that local OSRs are normally distributed around the population OSR).

In addition to spatial variation in local OSRs, population OSRs often vary temporally (Kvarnemo and Ahnesjö, 1996), further complicating the decision to approach a courting group. In fact, temporal variation in population OSRs has been associated with shifts in competition for mates (Ahnesjö, 1995; Grant et al., 2000) and reversed sex roles in a variety of species (Gwynne and Simmons, 1990; Simmons, 1992; Vincent et al., 1994). In theory, as the population OSR varies temporally, so too should responses to local OSRs because the probability of finding less competitive mating prospects should parallel changes in the OSR of the population. However, at the extremes of the OSR continuum, it may be uneconomical to search for less competitive mating opportunities (Grant et al., 2000).

Despite the potential benefits of evaluating OSRs, OSR has been traditionally thought of as a generic force imposing levels of intrasexual competition on individuals (see Clutton-Brock and Parker, 1992; Grant et al., 2000; Kvarnemo and Ahnesjö, 1996) rather than both this force and a source of information used by individuals to regulate the level of

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intrasexual competition they encounter. Perhaps because of this conventional view, we know little about whether species generally compare OSRs, what cues other than visual are used to assess OSR, and whether the acceptance of local OSRs parallels changes in the population OSR. We addressed these gaps in the literature by using the mating system of the eastern red-spotted newt, *Notophthalmus viridescens*.

We focused on red-spotted newts because males of this species are strong candidates for using chemical cues to reduce intrasexual competition and because the OSR of newt populations varies temporally (Massey, 1988; Verrell, 1989). Adult newt sex ratios are typically two males to every female (see Gill, 1978; Massey, 1988), generating intense scramble competition among males for access to mates (Able, 1999). Further, there is no evidence of female mate choice that might promote the formation of male groups (Gabor et al., 2000). As a result, male-male sexual interference can account for nearly 40% of all insemination failures in the field (Massey, 1988). Male newts rely heavily on olfaction to locate conspecifics (Dawley, 1984; Rohr and Madison, 2001, 2003; Rohr et al., 2002a, 2003), and their response to male odors depends on the number of males in a courting group (Park and Propper, 2001). These characteristics of the newt mating system suggest that detecting chemical cues of OSR would be of great value to male newts.

The newt mating system also provides a natural experimental context to investigate how breeding season progression and changes in the population OSR might influence responses to spatial variation in local OSRs. Males provide no parental investment after zygote formation, but females take several weeks to scatter and wrap individual eggs in vegetation (Petranka, 1998). Therefore, as oviposition nears, female sexual receptivity declines (Massey, 1988), resulting in a temporal increase in the male skewness of the population OSR (Verrell, 1989).

We conducted a series of field and laboratory experiments to test the following four predictions regarding newt response to conspecific groups: (1) males prefer less competitive mating opportunities in ponds; (2) males increase their acceptance of intrasexual competition later in the breeding season as the likelihood of finding less competitive mating opportunities decreases; (3) these responses to conspecific groups increase male courtship probability; and (4) males use conspecific odors to assess local OSRs. Finally, we attempted to characterize the chemical cue released from males that provides information on local OSR.

METHODS

Field experiment 1

This first field experiment was designed (1) to assess whether male newts prefer less competitive reproductive opportunities, and (2) to determine how male-male competition affects the likelihood of amplexus, the act of a male clasping the neck of a female with his hindlimbs that is typically a prerequisite to mating in nature (Massey, 1988; Verrell, 1982, 1984). We used amplexus to estimate mating probability because we wanted to stop each trial before spermatophore deposition so that the OSR of our experimental animals remained relatively constant across trials.

Forty-four adult male and 11 adult female red-spotted newts were collected from a private pond in Chenango County, New York, in late May 2000. Groups of four males and individual females were housed separately in plastic containers filled with dechlorinated tap water, were maintained in a controlled environmental chamber at 18°C on a 14-h light/10-h dark cycle, and were fed a liberal diet of chopped earthworms and

freeze-dried bloodworms daily. The unique dorsal spot pattern of each newt was recorded to facilitate identification during trials (Gill, 1978).

Two days after collection, males and females were carried in Petri dishes containing moist paper towels to Nuthatch Hollow Pond, Broome County, New York. We chose to transfer newts to the field in moistened Petri dishes because it kept newts separated but was less cumbersome than carrying tubs of water. We used a completely randomized factorial design in which we crossed our male treatments (presence and absence of four males) by our female treatments (presence and absence of one female). Thus, we tested newt response to the following four treatments: four males alone (4M), a female alone (1F), four males and one female (4M+1F), or neither males nor females as a control. The ratio of four males to one female was chosen because this group sex ratio was found in this pond at all times of the breeding season (Rohr JR, personal observations), but was approximately two times the estimated population sex ratio of two males to one female obtained at this pond by dip-netting. Once at the pond, these treatments were randomly placed into 22 minnow traps (8-mm wire cylinder with funnel ends; 40 cm long and 23 cm diameter) 5 m apart and 1 m from shore. Twenty-two minnow traps was the maximum number of traps we could place around the circumference of this pond while maintaining the 5-m spacing.

In a field study conducted by Massey (1988), amplexus persisted, on average, for 204.3 ± 8.4 min (\pm SE) before the series of behaviors leading to spermatophore transfer began. Thus, traps in the present study were set for only 120 min so that it was improbable for any experimental animals to release or accept a spermatophore during trials. Newts placed into traps are referred to as "experimental" animals (they cannot escape the traps), and newts already in the pond that entered traps are referred to as "captured" animals.

Checking traps entailed three steps. First, we looked through the water into the trap to determine whether any male and female were amplexed, and then removed the trap from the water. Most pairs remained amplexed out of the water and had to be physically separated. Second, we identified the experimental newts by their dorsal spot pattern (Gill, 1978) and placed them back into their Petri dish. They were later returned to their laboratory holding containers. Finally, captured newts were counted, sexed, and returned to the pond where caught. Trials were conducted between 0830–1430 h at the peak of mating activity for this population (Rohr et al., 2003). Traps were left open between trials so that nonexperimental newts could swim freely through the traps.

This procedure was used in successive trials with one important stipulation. In each trial, we used the same experimental males and female at each trap location. For example, the first trap location always received female number one when it received a treatment containing a female, and always received male group number one when it received a treatment containing four males. Consequently, even though some females may have been inseminated before collection and some male groups may have differed in competitive abilities or size, these potential differences were controlled for by using the same organisms at each trap location. This ensured that differences in capture rates between treatments could not be attributed to male or female differences. After each trap location received each of the four treatments in random order, the entire protocol was repeated, providing a sample size of 44 for each of the four treatments. Newt densities are sufficiently high that the same individuals were unlikely to have been recaptured in traps containing the same treatment (Rohr et al., 2002b, 2003).

Field experiment 2

Our second field experiment was designed to examine (1) whether the results of the first experiment were consistent across populations, and (2) how male newts respond to reproductive opportunities through the breeding season. Animal care and experimental protocols were identical to those described for field experiment 1 with the following exceptions. Twenty-four minnow traps were placed at Harpur Pond, Broome County, New York (for a comparison of Nuthatch Hollow and Harpur ponds, see Rohr et al., 2003). Like at Nuthatch Hollow Pond, the ratio of four males to one female was found locally in Harpur Pond at all times during the breeding season (data not shown), but was approximately two times the estimated population sex ratio of two males to one female obtained at this pond by dip-netting. Newts placed into traps at Harpur Pond were collected from Nuthatch Hollow Pond, Broome County, New York, in 2002 for three successive trials conducted from 23–26 March, 12–15 April, and 16–19 May. Unlike field experiment 1, experimental females for this experiment were collected early in the breeding season (21 March 2002). The same 12 experimental females were used for each successive trial. Forty-eight experimental males were collected 2 d before each monthly trial and thus had opportunities to assess changes in photoperiod, temperature, and OSR within their pond before collection.

Similar to the first experiment, these collection regimes allowed us to control for mate and competitor quality by using the same female at each trap location within and across monthly trials, and the same four males at each trap location within each monthly trial. At the beginning of the breeding season, experimental females were expected to represent only a small portion of the sexually receptive females in the pond. However, because experimental females presumably did not mate during the experiment whereas the majority of females in the pond presumably did (and must shift time from mate search to oviposition), by the end of the breeding season, experimental females were expected to represent a substantial portion of those females that were sexually receptive. Males can complete two amplexus courtships and deposit two spermatophores in rapid succession before reaching satiety (Verrell, 1985), and there is little evidence that their receptivity declines at this site until they leave the pond in early summer (Rohr JR, personal observation). Thus, in contrast to females, both experimental males and males in the pond were presumed to have been receptive to mating throughout the breeding season.

Hence, this design allowed us to assess how males respond to a relatively constant local OSR (the OSR in traps) as conditions in the pond, such as the population OSR, presumably changed through the breeding season. We did not quantify the temporal change in the population OSR owing to the difficulty of doing this accurately (Ahnesjö et al., 2001; Clutton-Brock and Parker, 1992), but our previous work suggests that female receptivity declines and population OSR becomes more male-biased later in the breeding season (see Rohr et al., 2002b, 2003), as described for various newt populations and species (e.g., *Notophthalmus viridescens*: Massey, 1988; Verrell, 1989; *Taricha granulosa*: Propper, 1991; *Triturus vulgaris*: Verrell and McCabe, 1988).

Cue identification experiment

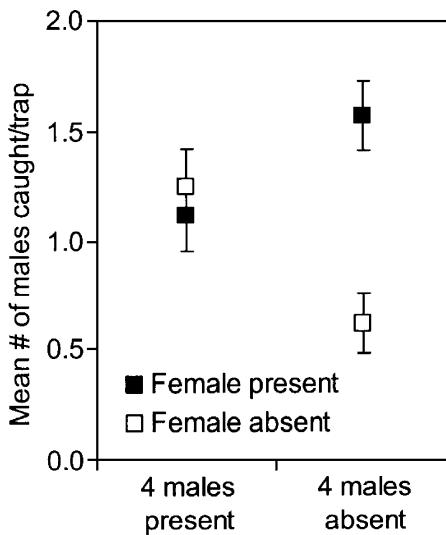
This experiment was designed to determine whether chemical cues from conspecifics were used by male newts to compare local OSRs to reduce intrasexual competition. To test this hypothesis, we excised the entire cloaca and genial (cheek) region of four anaesthetized (4% ether) males, presumably obtaining all the cloaca and genial glands. After gland

excision, males were immediately euthanized. The cloacal and genial glands were chosen because they release pheromones used in mate attraction or courtship (see Arnold and Houck, 1982; Kikuyama et al., 1995; Pool and Dent, 1977b; Sever, 1992; Verrell, 1988). We then used a standard Y-maze olfactometer to test male response to female odor, male cloacal gland extract, male genial gland extract, and combinations of these two extracts with female odor (for treatment combinations tested, see Table 2). Newts used for these tests were purchased from a supplier (Charles D. Sullivan Company, Sullivan County, Nashville, TN) on 15 February 2001, were in breeding condition, and were maintained as previously reported (Park and Propper, 2001). The excised cloacal and genial glands were incubated separately in 1 ml 0.8 mM acetylcholine chloride (AchCl; pH 8.4) for 30 min (Pool and Dent, 1977a; Rollmann et al., 1999), which induces pheromone release into solution (Pool and Dent, 1977a). The resulting supernatants were centrifuged at 10,000g for 10 min, frozen at -80°C, and thawed before use. In a control study, AchCl alone did not repel or attract males.

All Y-maze experiments were conducted between 17 February 2001 and 17 March 2001. In Y-maze olfactory preference tests (Park and Propper, 2001), 300 ml aged tap water was the source of continuous, laminar flow (60 ml/min) entering each arm (4.5 × 22 × 5 cm). Males were selected at random from a pool of 50 males, were held behind a start gate for 3 min before the gate was raised, and were not exposed to any treatment more than once. Treatment assignment to the arms was randomly stratified. When testing male response to female odor versus male cloacal or genial gland extracts plus female odor, we placed three females into the source reservoir and delivered (1.35 ml/min) either cloacal or genial gland extracts (dissolved in 300 ml distilled water) into one arm. When testing male response to male cloacal gland extract versus male cloacal gland extract plus female odor, we placed one visually concealed female in one arm and delivered (0.84 ml/min) male cloacal gland extract to both arms. We recorded the arm the male traveled more than half its length. Trials occurred between 1000 and 1430 h, and after each trial, the Y-maze was washed thoroughly.

Chemical cue characterization experiment

In this experiment, we used electrophoresis, gas chromatography, and spectral analysis to characterize the chemical cue released from the cloaca of males that provides information on local OSR. To screen for potential proteinaceous pheromones, approximately 20 µg cloacal extract was run on a 4–20% gradient prepak SDS-PAGE gel, stained with Coomassie brilliant blue, and destained with distilled water (Diezel et al., 1972; Jiang et al., 1990). To partially purify the pheromone, 2.2 mg lyophilized cloacal extract (pooled from 12 males) was separated by using a Bio-Gel P60M column standardized with the proteins Ferritin (440 kDa), Chicken Albumin (40 kDa), Carbonic Anhydrase (29 kDa), and Cytochrome C (2.5 kDa) and preequilibrated with 0.15 M NH₄HCO₃ (Kikuyama et al., 1995). In a control study, lyophilizing the extract did not alter male response to this odorant. A flow rate of 0.02 ml/min was maintained by an EP-1 Econo peristaltic pump (Bio-Rad), and 0.5 ml fractions of three molecular-weight groups (less than 30.6, 30.6–36.2, more than 36.2 kDa) were collected by using a FC 203B fraction collector (Gilson). The absorbance at 280 nm of each fraction was measured with a Shimadzu UV 160U dual beam spectrophotometer (Shimadzu Scientific Instruments Inc.). To determine the attractiveness of the fraction groups, we boiled each for 5 min to remove NH₄HCO₃ (a boiled 0.15 M solution of NH₄HCO₃ did not repel males), created aliquots

**Figure 1**

Effects of four males, one female, four males plus one female, and nothing on male newt capture rates during the peak of the breeding season (May) at Nuthatch Hollow Pond. Plotted values represent means (\pm SE) of 44 traps. See text for statistics males \times female interaction.

of one-third the fraction volume of each group (11, 19, 48 ml for less than 30.6, 30.6–36.2, more than 36.2 kDa, respectively), brought aliquots up to 300 ml with distilled water, and tested male response to each group in a Y-maze with female odor entering both arms.

Statistical analyses

All statistical analyses were conducted by using Statistica 5.5a (Statsoft, Inc.). For both field experiments, we tested for the effects of treatments (main effects and interactions) on male newts captures per trap by using Poisson regression (PR; McCullagh and Nelder, 1989). Traps that caught females were excluded from the analysis so that all comparisons were based on a single female in a trap. Binomial regression (BR) was used to compare the percentage of traps that contained an amplexed pair between 4M+1F and 1F treatments. Only 1F traps that caught at least one male were included in these analyses because only this subset of 1F traps had the potential of containing an amplexed male-female pair. We expected to observe amplexus more often earlier in the breeding season and in traps with more males; however, the probability of amplexus per male was expected to decrease with increasing numbers of males. For female data, we used a chi-square test to compare capture rates between 4M and empty traps because female smooth newts avoid multiple males (Verrell, 1984). In the cue identification and chemical cue characterization experiments, we tested for arm preference in Y-maze trials by using binomial tests, which were two-tailed with the exception of male response to female odor alone, because it is well established that males are attracted to female sex pheromones (Rohr and Madison, 2001; Rohr et al., 2002b).

RESULTS

Field experiment 1

PR revealed that males were attracted to traps with a single experimental female (female: $\chi^2 = 7.063$, df = 1,168, $p = .008$) and were indifferent to traps with four experimental males (four males: $\chi^2 = 0.621$, df = 1,168, $p = .431$) (Figure 1).

However, as expected, males were more attracted to 1F than 4M+1F traps, but preferred 4M+1F traps to traps that were initially empty (four males \times female: $\chi^2 = 13.082$, df = 1,168, $p < .001$) (Figure 1), indicating that males preferred less competitive mating prospects, but chose competitive mating opportunities more often than no mating opportunity at all. The difference in sex ratio, rather than total number of newts in traps, likely induced the lower capture rates in 4M+1F traps relative to 4M or 1F traps because male newt attraction increases in response to increasing numbers of males alone (Rohr et al., 2002b) and of females alone (Rowland et al., 1990; Rohr JR, unpublished data). Only four females were captured, one in each treatment.

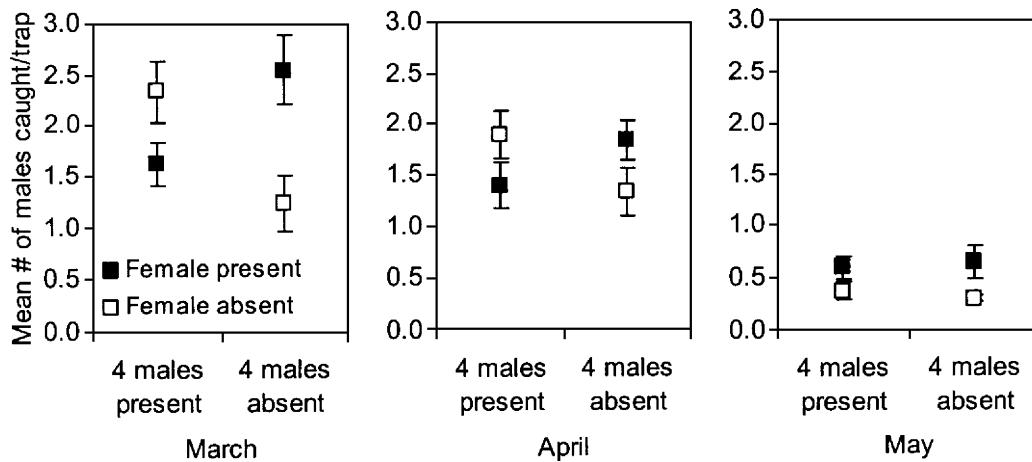
Amplexed pairs were found in 56% of the 1F traps (22 of 39) but in only 11% of the 4M+1F traps (five of 44), resulting in a significantly larger percentage of 1F traps with amplexed pairs ($\chi^2 = 19.11$, $p < .001$). This was despite 1F traps containing significantly fewer males (laboratory + captured) after the 2-h trapping period (mean \pm SE: 1F = 1.57 ± 0.16 , 4M+F = 5.11 ± 0.17 ; PR: df = 1,86, $\chi^2 = 87.18$, $p < .001$).

Field experiment 2

Early in the breeding season (March), when the population OSR should closely match the actual adult population sex ratio because most males and females should be receptive to mating (Verrell, 1989; Waights, 1996), males were more attracted to 1F than 4M+1F, but preferred 4M+1F to traps that were initially empty (PR: four males \times female $\chi^2 = 23.92$, df = 1,174, $p < .001$) (Figure 2). Thus, similar to at Nuthatch Hollow Pond, males at Harpur Pond preferred less competitive mating prospects but favored a competitive mating opportunity over no opportunity at all. Rohr et al. (2003) demonstrated that the breeding seasons at Nuthatch Hollow and Harpur ponds were not synchronous with one another owing to various factors. When comparing approximately corresponding times of their breeding seasons (late May at Nuthatch Hollow Pond and April at Harpur Pond), we detected no significant difference in male response between these populations (PR: site \times four male \times female $\chi^2 = 0.03$, $p = .859$).

In addition, in May males showed no preference for 1F over 4M+1F, and preferred both 1F and 4M+1F to empty traps (PR: four males \times female $\chi^2 = 0.46$, df = 1,174, $p = .498$). Hence, males accepted significantly greater intrasexual competition in May than in March (significant three-way interaction in Table 1 and Figure 2), becoming less “choosy” of mating opportunities later in the breeding season. Responses in May, the only month where oviposition was observed and newt eggs were found, appears to be driving this pattern because there was no significant increase in acceptance of intrasexual competition from March–April (PR: month \times four males \times female $\chi^2 = 1.93$, $p = .165$) (Figure 2).

The mean number of males an experimental female attracted declined through the breeding season (as did capture rates in general), but there was no evidence that their “relative attractiveness,” defined as the difference in male capture rates between traps containing a female alone and an empty trap, changed through the breeding season (month \times female $p = .380$, 95% confidence intervals: -0.21 to 0.10 , -0.05 to 0.25) (Table 1 and Figure 2). In fact, 1F traps caught significantly more males than did empty traps in March (PR: $\chi^2 = 20.66$, $p < .001$) and May (PR: $\chi^2 = 5.95$, $p = .015$) and nearly more males than did empty traps in April (PR: $\chi^2 = 3.19$, $p = .074$) (Figure 2). Thus, although it is possible that the receptivity and sexual pheromone production of experimental females declined through the breeding season, there was no detectable evidence of a significant change in

**Figure 2**

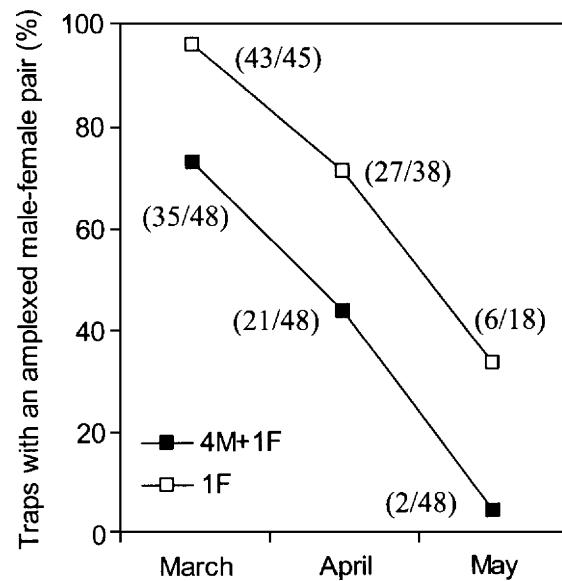
Effects of four males, one female, four males plus one female, and nothing on male newt capture rates during 3 months of the breeding season at Harpur Pond. Plotted values represent means (\pm SE) of 48 traps. See Table 1 for associated statistics.

the magnitude of their relative attractiveness. In addition, our experimental females did not oviposit in the laboratory, under conditions in which females from this population have readily oviposited in the past (Rohr JR, personal observations). These data suggest that the OSR in traps remained relatively constant through the study, implying that the OSR of a trap at the start of each trial was equivalent to the sex ratio of experimental newts in the trap.

Similar to at Nuthatch Hollow Pond, the percentage of traps with an amplexed pair at Harpur Pond was significantly lower when it contained 4M+1F than a female alone (BR: treatment df = 1,239, $\chi^2 = 30.29$, $p < .001$). In addition, the percentage of amplexed pairs steadily declined as the breeding season progressed (BR: month df = 1,239, $\chi^2 = 81.35$, $p < .001$). This rate of decline did not significantly differ between 4M+1F and 1F traps (BR: treatment \times month df = 1,239, $\chi^2 = 2.34$, $p = .311$) (Figure 3). Males were frequently observed attempting to displace amplexant males in traps, as previously described (Verrell, 1983, 1986). The greater percentage of 1F than 4M+1F traps with amplexed pairs, once again, occurred despite 1F traps having significantly fewer males after the 2-h trapping period (mean \pm SE: 1F = 1.60 ± 0.15 , 4M+1F = 5.35 ± 0.13 ; PR: df = 1,286, $\chi^2 = 308.61$, $p < .001$). We also compared the probability of amplexus per male (both experimental and captured) through time between 4M+1F and 1F treatments to control for the temporal increase in attractiveness to 4M+1F traps (relative to 1F traps). The results of this repeated-measures

ANOVA (on arcsine square-root transformed data) were not qualitatively different from the conservative analyses comparing the percentage of traps with an amplexed pair. The probability of amplexus per male was greater in 1F than 4M+1F traps (ANOVA: treatment df = 1,80, $F = 54.629$, $p < .001$). This probability declined through the breeding season (ANOVA: month df = 2,80, $F = 8.966$, $p < .001$), and the rate of decline did not significantly differ between treatments (ANOVA: treatment \times month df = 2,80, $F = 0.084$, $p = .919$).

In contrast to male capture rates, female capture rates were very low (4M = 5, 4M+1F = 8, F = 11, C = 13), providing little

**Figure 3**

Mean percentage of traps at Harpur Pond starting with either four males and one female (4M+1F) or one female alone (1F) that contained an amplexed male-female pair when the trap was checked. Data are provided for three times during the breeding season. Because female alone traps did not start with any males, only female alone traps that captured at least one male were included in the means. The denominator in the parentheses associated with each plotted value represents the number of traps that contained at least one male, and the numerator represents the number of these traps in which an amplexed pair was found.

Table 1

Poisson Regression (PR) on capture rates of males in traps with and without four males and with and without one female during three months of the newt breeding season

Factor	df	Log-likelihood ratio χ^2	p
Month	2	188.51	<.001
Males	1	0.27	.604
Female	1	8.35	.004
Month \times males	2	0.47	.789
Month \times female	2	1.94	.380
Males \times female	1	12.36	<.001
Month \times males \times female	2	18.41	<.001

Table 2

Male response to female odor (F), male cloacal extract (MCE), male genial extract (MGE), and each extract combined with female odor when paired against various treatments in Y-maze trials

Odor sources	No. of times chosen	p
F versus water	10 versus 2	.039
MCE versus water	12 versus 2	.007
MGE versus water	10 versus 6	.277
MCE + F versus water	16 versus 2	<.001
MCE + F versus F	4 versus 16	.006
MGE + F versus F	7 versus 6	.500
MCE + F versus MCE	11 versus 15	.278

p values are binomial calculations (an exact randomization test).

statistical power to detect differences in female preference among treatments or changes in female response through time. To increase our statistical power, we added female capture rate data from a preliminary experiment conducted from 30 April–3 May 2000 at the same site, using the same methods, and compared female capture rates for all months and years combined. These data ($4M = 7$, $4M+1F = 12$, $F = 16$, $C = 17$) suggest that females avoided 4M traps relative to empty traps ($\chi^2 = 4.17$, $p = .041$). No other significant differences were detected.

Cue identification experiment

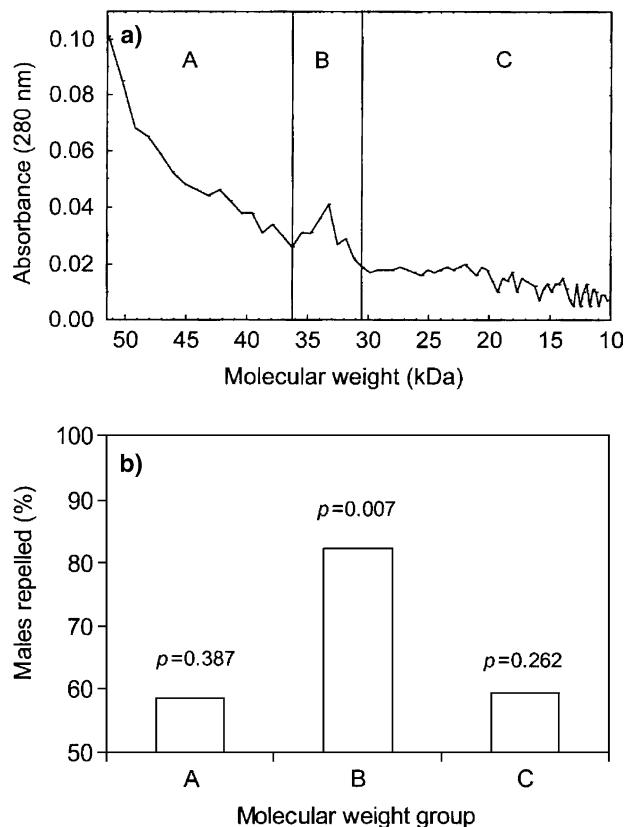
Our tests revealed that males were indifferent to male genial gland extract regardless of what it was paired against (Table 2). Conspecific males were attracted to male cloacal gland extract, female odor, and their combination, when these odorants were paired against aged tap water. Males were more attracted to female odor (alone) than to the combination of female odor plus male cloacal gland extract, suggesting that chemicals released from male cloacal glands are one of the cues (the other presumably being released from the female) used to avoid more competitive reproductive opportunities.

Chemical cue characterization experiment

An approximately 33-kDa protein band on the gel stained intensely, and the column chromatograph produced only one distinct peak, also at approximately 33 kDa (Figure 4a). In olfactory tests, only the 30.6- to 36.2-kDa group significantly repelled test males (Figure 4b).

DISCUSSION

In both the laboratory and field, male newts preferred less competitive mating prospects, but chose even highly competitive mating opportunities over no mating opportunity at all. This conditional response is not surprising because, in most cases, males should only avoid a mating opportunity if a less competitive opportunity is nearby. The one exception to this result was that males did not prefer four males plus a female (a mating opportunity) to four males alone (no mating opportunity). During the breeding season, dense patches of males are typically found near potential mates, and thus, it has been proposed that multiple males may be a reliable indirect cue to the vicinity of a female (Rohr et al., 2002b). Consequently, the relative responses to all treatments in our studies may improve male mating success by either directly or indirectly increasing encounters with females or by reducing intrasexual competition.

**Figure 4**

(a) Protein absorbance spectrum of male cloacal extract run through a column-chromatograph (Bio-Gel P60M column). Vertical lines divide the extract into three molecular-weight groups labeled with capital letters. (b) Percentage of male newts in Y-maze trials that were repelled by each molecular-weight group combined with female odor when female odor alone was provided in the alternative arm of the Y-maze. *p* values were calculated by using a two-tailed binomial test. Sample sizes for groups A, B, and C are 12, 17, and 22, respectively.

Although the OSR in our traps seemed to remain relatively constant throughout our study, male response to these local OSRs paralleled the temporal change in population OSR that has been documented for various newt populations and species (see Massey, 1988; Propper, 1991; Verrell, 1989; Verrell and McCabe, 1988). Early in the breeding season, when $4M+1F$ was approximately twice as male biased as the population OSR, this highly competitive group was less attractive to males than a female alone. However, after oviposition commenced, $4M+1F$ attracted similar numbers of males as did a female alone. Males should benefit from this greater tolerance of intrasexual competition later in the breeding season because the increase in population OSR would reduce the probability of encountering less competitive mating prospects. In fact, near the end of the breeding season, there may be so few sexually responsive females, that avoidance of any seemingly receptive female, regardless of the number of accompanying males, may be reproductively costly (see similar result for male-male aggression in Grant et al. 2000).

The present study, and a variety of others showing increased male aggression and decreased female aggression with increasing population OSR (for review, see Grant et al., 2000), suggest that many species integrate variation in OSR across levels of organization, such as from the population to

conspecific groups. Whether the population OSR itself is being quantified or whether organisms rely on other cues to estimate population OSR remains to be tested. For example, the response to local OSRs in the present study that paralleled presumed changes in the population OSR is consistent with males comparing the OSR of groups to that of the population to optimize mate search. However, it is also possible that factors that may covary with the OSR of the population, such as photoperiod and temperature, were used by males as proxies for the population OSR.

Selection for males to evaluate OSRs likely came from the apparent reduction in mating probability associated with increasing male competition. Amplexus probability was considerably lower with increasing numbers of males, and because amplexus is typically a prerequisite to mating in ponds (Massey, 1988; Verrell, 1982, 1984), a concomitant decline in mating probability would be expected. Near the end of the breeding season, 1F traps were eight times more likely to have an amplexed pair than were 4M+1F traps, despite 1F traps having more than four times fewer males to amplex the female. Furthermore, we are likely underestimating this difference in amplexus probability because amplexus duration is substantially longer in the presence than in the absence of potential competitors (Massey, 1988; Verrell, 1983, 1985), creating a bias toward observing amplexus in 4M+1F traps.

Why then is the probability of amplexus per courting group lower with greater numbers of males? Two defendable, but nonexclusive, hypotheses are male interference with amplexus attempts and female reluctance to amplex in the presence of multiple males. Frequent male-male and male-female chases during the breeding season (Rohr JR, personal observations) and positive selection on male tailfin depth that increases amplexus and mating probability (Able, 1999; Gabor et al., 2000) support the notion that intense pre-amplexus, male-male interference competition can reduce male mating prospects. From the female perspective, male groups appear to be reproductively costly, potentially providing females with a benefit for evading groups of males. In the present study, the probability of a female being amplexed early in the breeding season, when female newts should be motivated to mate (Wrights, 1996), was significantly lower with greater numbers of males. Similarly, the probability of insemination for female smooth newts, the Old World counterpart to the red-spotted newt, decreases with increasing numbers of males (Verrell, 1983). Male groups would also increase the opportunity costs of courtship for females because amplexus duration is longer with increasing numbers of males (Verrell, 1983, 1985).

The observed female avoidance of groups of four males provides further evidence that large male groups are costly to females. Although female avoidance of male groups in the present study was based on small sample sizes and combined data, this result is consistent with female smooth newts preferring single males to four males (Verrell, 1984) and being averse to male-male interactions (Verrell and McCabe, 1988). The infrequent capture of females unfortunately prevented us from thoroughly examining female response to spatiotemporal variation in OSRs, and thus, additional work is needed in this area.

Although our field experiments did not test for the cues used to assess and compare OSRs (visual cues were not controlled), results from previous studies and our laboratory experiments indicate that males were likely using chemicals released from conspecific groups. Park and Propper (2001) demonstrated that when intersexual interactions were permitted, male, but not female, red-spotted newts released a chemical that was avoided by approaching males when the odor of a lone female was provided as an alternative cue, corroborating our findings in the field. When intersexual

interactions were prevented (no visual, chemical, or tactile cues between sexes), males showed no preference for the odor of a female over that from males and a female, suggesting that the results of the present study cannot be explained simply by multiple males masking the odor of a female. Park and Propper (2001) also demonstrated that males were more likely to choose the odor of a lone female as the odor of an alternative courting group became more male-biased, providing support that males use chemicals released from males and females to compare OSRs. Our cue identification experiment revealed that a chemical released from male *N. viridescens* cloacal glands was the source initiating this conditional response to local OSRs.

The chemical cue from male cloaca glands that provides information on OSR appears to be a 33-kDa protein because only proteins in the 30.6- to 36.2-kDa size category repelled males, and the only distinct peak that stained intensively was at approximately 33 kDa. To our knowledge, this protein represents the first isolated and characterized constituent, olfactory cue of local OSR. A chemical released from females is presumably the other component of the OSR cue. Although insect pheromones are typically comprised of a precise combination of multiple compounds, a single protein that influences the reproductive behavior of newts is consistent with the structure and function of the only two isolated and reported pheromones in amphibians. Kikuyama et al. (1995) identified a lone peptide from male red-bellied newts, *Cynops pyrrhogaster*, that attracted females at short distances, and Rollmann et al. (1999) isolated a single protein from male Jordan's salamanders, *Plethodon jordani*, that increased female sexual receptivity. Given that selection in amphibians seems to favor male production of courtship pheromones targeting females, it is possible that the 33-kDa protein in male red-spotted newts evolved for this function and is secondarily used as a component cue of OSR.

Alternatively, rather than there only being selection for males to detect cues of local OSRs, there may have also been selection for males to release a signal of female attendance, and thus intrasexual competition. If males released a component signal of OSR when in a sufficiently male-biased courting group, then it would inform receiving males that there was little chance of mating success, which could reduce male-male competition for the senders. In addition to intrasexual selection for an OSR signaling pheromone, there may also be intersexual selection. Because large male groups appear to be reproductively costly to female red-spotted newts and a pheromonal signal of OSR would serve to reduce male group size, females may choose males that release an honest or dishonest (e.g., a male releasing pheromones representing more than one male) signal of male-male competition. Male moths (Bijpost et al., 1985; Hirai et al., 1978; Lecomte et al., 1998; Wago, 1978) and spiders (Rao Ayyagari and Tietjen, 1986) that are accompanied by a receptive female release chemicals that redirect the mate search of male conspecifics. However, as for the red-spotted newt, we do not know whether males are releasing these compounds to reduce intrasexual competition.

The results of the present study support two principles regarding mating systems that, to our knowledge, have yet to be stated explicitly in the literature. First, OSR can somewhat paradoxically be the source imposing intrasexual competition and the source used to reduce it. Second, analogous to the sex in short supply often being "choosy" with respect to potential mates, the sex in excess can be spatially and temporally choosy about its acceptance of intrasexual competition. Identifying the sources of information used to assess local and population OSRs and determining whether these sources are cues or honest or deceptive signals should be an intriguing new avenue for sexual selection research.

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