Male vocalization and female choice in the hybridogenetic Rana lessonae/Rana esculenta complex

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Abstract

In many species, females can improve their fitness by preferring particular males over others. In Palaeartic water frogs of the Rana lessonae/R. esculenta complex the consequences of such mate choice are particularly pronounced. To produce viable offspring, the hybrid R. esculenta (genotype RL) must mate with the parental species R. lessonae (LL); but R. lessonae should avoid mating with R. esculenta, because the resulting hybrid offspring will eliminate the L genome from the germline (hybridogenesis). Hence, there exists a conflict between the sexual parasite (RL) and its sexual host (LL) over the best mating partner. Previous studies have shown a preference for LL males in LL and RL females; but they have also shown that females cannot usually realize their choice when in close proximity to males, because the males forcefully and indiscriminately amplex them. We tested whether females use male vocalizations as a long-distance signal to increase their chances of mating with the preferred LL males. We exposed female R. lessonae and R. esculenta to playbacks of single LL and RL mating calls (experiment 1) and to choruses with a 3:1 excess of LL and RL calls, respectively (experiment 2). In experiment 1, both female types were attracted more by the LL than by the RL calls. In experiment 2, no discrimination between LL- and RL-dominated choruses was observed. The results suggest that females do not use distant male vocalization to approach preferentially ponds or arenas within a pond that hold an excess of LL males. But once they have arrived in a chorus, mating calls from nearby males can direct them to the preferred LL mates. We discuss possible reasons for the failure to discriminate between choruses and the chances for successful choice between individuals within choruses.
Male vocalization and female choice in the hybridogenetic *Rana lessonae* / *Rana esculenta* complex

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In many species, females can improve their fitness by preferring particular males over others. In Palearctic water frogs of the *Rana lessonae* / *R. esculenta* complex the consequences of such mate choice are particularly pronounced. To produce viable offspring, the hybrid *R. esculenta* (genotype RL) must mate with the parental species *R. lessonae* (LL); but *R. lessonae* should avoid mating with *R. esculenta*, because the resulting hybrid offspring will eliminate the L genome from the germ line (hybridogenesis). Hence, there exists a conflict between the sexual parasite (RL) and its sexual host (LL) over the best mating partner.

Previous studies have shown a preference for LL males in LL and RL females; but they have also shown that females cannot usually realize their choice when in close proximity to males, because the males forcefully and indiscriminately amplex them. We tested whether females use male vocalizations as a long-distance signal to increase their chances of mating with the preferred LL males. We exposed female *R. lessonae* and *R. esculenta* to playbacks of single LL and RL mating calls (experiment 1) and to choruses with a 3:1 excess of LL and RL calls, respectively (experiment 2). In experiment 1, both female types were attracted more by the LL than by the RL calls. In experiment 2, no discrimination between LL- and RL-dominated choruses was observed. The results suggest that females do not use distant male vocalization to approach preferentially ponds or arenas within a pond that hold an excess of LL males. But once they have arrived in a chorus, mating calls from nearby males can direct them to the preferred LL mates. We discuss possible reasons for the failure to discriminate between choruses and the chances for successful choice between individuals within choruses.
According to sexual selection theory and empirical studies of mating systems, reproductive success of males usually increases with their access to females, whereas female success is related to the quality of the male and/or his resources. As a result, typical mating strategies differ between the two sexes, with intrasexual competition dominating in males and intersexual mate choice dominating in females (reviewed by Trivers 1972; Emlen & Oring 1977; Davies 1991; Andersson 1994; Ryan 1997). This can create a sexual conflict, which is most obvious in those cases where competing males restrict a female’s opportunity for mate choice by forcefully copulating with her (Clutton-Brock & Parker 1995).

Palaeartic water frogs (Rana lessonae, R. ridibunda and R. esculenta) provide an excellent model system for studying how the conflict between the opposing strategies of male sexual coercion and female choice can be solved. In this group, males amplex females forcefully and fairly indiscriminately, which is typical for several frog and toad species (Wahl 1969; Heym 1974; Halliday 1983; Bourne 1993). Females, however, must be extremely selective, because mating with the wrong male will result in no reproductive success at all, rather than the small reduction in fecundity that has been reported for other anuran species (cf. Davies & Halliday 1977; Ryan 1985; Robertson 1990). This is due to a rare reproductive mode, known as hybridogenesis (Schultz 1969; Tunner 1974). Rana esculenta (genotype RL), originally produced by hybridization between the two genuine species R. ridibunda (RR) and R. lessonae (LL), eliminates one of the parental genomes (in western Europe L) premeiotically, duplicates the remaining genome (R) and transmits it clonally to eggs and sperm cells. As a result, matings between hybrids (RL female x RL male) lead to R. ridibunda tadpoles (RR), but these do not usually survive to metamorphosis, probably because lethal alleles accumulate on the clonally transmitted R genome (Berger 1977, 1983; Graf & Müller 1979; Uzzell et al. 1980; Semlitsch & Reyer 1992). Where R. ridibunda is absent, as in most parts of Western Europe, R. esculenta can persist only if it lives in mixed populations with R. lessonae and mates with them to regain the previously eliminated L genome. Both heterotypic matings (RL female x LL male and LL female x RL male) lead to R. esculenta offspring, whereas the homotypic mating between LL females and LL males produces new R. lessonae.

In this system, mate choice has pronounced consequences for both the fitness of individuals and the dynamics of populations. In terms of fitness, both female types should choose LL-males: hybrid females because they must rent the L genome for successful reproduction and parental females to avoid production of hybrid offspring that will later exclude the maternal L genome from the germline. In terms of the population, theoretical models have revealed that mate choice can contribute to stabilizing LL/RL ratios within ponds and to creating different ratios between ponds (Plötner & Grunwald 1991; Som et al. in press; Hellriegel & Reyer 2000).
In two-fold choice experiments, females of both species do, indeed, prefer LL over RL males (Abt & Reyer 1993; Engeler 1994), but under more natural conditions their opportunities for choice are severely restricted. Water frogs are prolonged breeders (Wells 1977b): males remain at ponds for several weeks or months and form choruses to which the females come asynchronously over the breeding period (Blankenhorn 1977; Abt 1992). This leads to strongly male-biased operational sex ratios (OSR), and the resulting competition and sexual coercion by males more or less overruns the overt preferences of females (Bergen et al. 1997). To some extent, females can counteract forceful copulations, for example by fleeing, adopting a vertical position in the water or giving a release call when not ready to spawn (Wahl 1969; Blankenhorn 1974; Abt & Reyer 1993). If this does not prevent amplexus, they can try to get rid of a male by provoking fights with other males (Abt & Reyer 1993; Bergen et al. 1997), or they can exert cryptic mate choice during copulation: when grabbed by the undesired *R. esculenta* males, females reduce clutch size by 30-40% and either release the remaining eggs in a second mating during the same season or resorb them which improves success in the next season (Reyer et al. 1999). Nevertheless, any forced copulation with RL males that leads to oviposition entails a fitness loss. Hence, females should strive to reduce the probability of encountering *R. esculenta* males at close range.

The most likely mechanism allowing discrimination over larger distances is vocalization. Anuran mating calls are known to contain information on species, size and spatial position of males, and females use them for finding breeding ponds and choosing males between and within species (Wells 1977a; Gerhardt 1982, 1994b; Ryan 1985; Fritzsch et al. 1988; Roy 1997; Castellano & Giacoma 1998). In the *R. lessonae*-*R. esculenta* complex, three conditions make vocalization a likely candidate for mate choice. First, the mating calls of the two male types differ in several characteristics (Günther 1969, 1990; Schneider et al. 1979; Radwan & Schneider 1988; Schneider & Joermann 1988). Second, although mating can be initiated throughout the day, the majority of pairs form during the night (personal observations), when visual cues at least are unlikely to operate. Third, ratios between LL and RL males differ both between ponds and between different chorus aggregations within ponds (Heym 1974; Berger 1977; Blankenhorn 1977; Günther 1990; Abt 1992; Balmelli 1995; Holenweg 1999).

Consequently, females could theoretically increase the probability of achieving amplexus with the preferred males by moving to ponds and/or arenas within ponds holding a high proportion of LL males and/or by approaching individual LL callers within a mixed chorus of both species. To test this hypothesis, we exposed *R. lessonae* and *R. esculenta* females to playbacks of male vocalizations and tested their response. Specifically, we asked the following questions: (1) Are females attracted by male vocalizations? (2) Do females prefer
calls of single nearby LL males over those of single nearby RL males? (3) Do females distinguish between distant choruses with an LL/RL male ratio of 3:1 over those with a ratio of 1:3?

METHODS

Male vocalization

Animals and housing conditions

In mid-October 1997, we caught 19 LL and RL males from a pond near Hellberg (Zürich, Switzerland), identified their species by albumin electrophoresis (Tunner 1973), and individually marked them with PIT tags (Trovan ID 100, Pameda AG, Basel, Switzerland). The albumin analysis was based on a lymph sample of 3-8 μl, drawn with a capillary tube from a small incision made into a toe web of one of the hind feet. The PIT tag, a capsule of 1.8 x 11.0 mm, was inserted into a lateral lymph sack through a tiny incision in the skin. Neither operation seemed to have adverse effects on the frogs. In most cases there was no indication that they even sensed the cut into the toe web; and PIT tags are considered to be a generally harmless marking technique for large anurans (Henle et al. 1997). After being marked and measured, the frogs were evenly distributed over three boxes (56x37x30 cm) and overwintered in a coolroom where the temperature was gradually lowered from 12°C (i.e. the ambient temperature at catching) to 5°C within a week, then kept constant until early February, and subsequently raised to 20-21°C within 10 days. Both the rate of temperature change and the absolute temperature minima and maxima are well within the range that frogs experience under natural conditions (cf. Holenweg & Reyer 2000). After warming them up, we transferred all the frogs into another room with an average temperature of 20-23°C and kept them in two containers (100x100x30 cm), where we recorded vocalizations (see below). Three to four times per week we added live crickets to the containers, which were all eaten within a few minutes. We fed with live crickets, because frogs only take moving prey. Wherever the animals were kept, the containers were filled with water to a depth of 4-12 cm and enriched with small pieces of foam or stones and pieces of floating wood to provide perches and shelter.

Recording

To stimulate reproductive condition, we increased daylength from 10 h at the end of hibernation in early February to 16 h in early March, and played tape-recordings of $R$.  


lessonae and R. esculenta choruses several times per day, starting in late February (cf. Abt & Reyer 1993). Once the males began calling themselves, their vocalizations were tape-recorded (Sony TC-D5M) via a directional microphone (Sony ECM-707), hanging 10cm above the water surface. To minimize differences in call structure resulting from temperature fluctuations (cf. Wahl 1969; Radwan & Schneider 1988; Schneider & Joermann 1988) we made all recordings at water temperatures of 20-21°C. After completing the recordings, we returned all the frogs to their pond of origin in mid-April.

Preparation of playback tapes

The mating calls of both R. lessonae and R. esculenta consist of groups of highly repetitive pulses which are separated from each other by short pauses. Typical examples of the structural and spectral call patterns have been published previously (e.g. Wahl 1969; Schneider et al. 1979; Brzoska 1980, 1982; Radwan & Schneider 1988; Günther 1990; Sinsch & Schneider 1996).

According to the experimental design (see below) we needed two mating calls from each of four R. lessonae and four R. esculenta males. Although this is a small sample, it suffices to represent the typical mating calls, because these differ markedly between R. lessonae and R. esculenta and permit reliable species identification, even when individuals come from geographically far apart areas (Wahl 1969; Schneider et al. 1979; Radwan & Schneider 1988; Sinsch & Schneider 1996). The call parameters we measured not only agree with those measured by these previous studies, but also show that there is almost no overlap in terms of call length and number of pulse groups (see Fig. 4 in the Results).

When selecting the calls from the recordings, we (1) eliminated interrupted calls and those produced during male-male amplexus, (2) took calls of about equal length and volume, (3) gave preference to consecutive calls from the same recording and (4) chose randomly from the ones remaining after applying criteria 1-3. These calls were digitized on a SoundBlaster Pro2 (Creative Labs, Inc., Singapore, Singapore) edited and multiplied with the public domain computer programs AviSoft Light 3.4, WAVmaker 2.5 and Midi Genie 1.06 for Windows and then transferred back to tapes.

Each tape for experiment 1 comprised calls of one male only ('solo calls'). Tapes S-LL1 to S-LL4 came from four different R. lessonae males and tapes S-RL1 to S-RL4 from four different R. esculenta males. A solo call consisted of two different mating calls, separated by the median interval length between calls of the particular male (Fig. 1). We prepared tapes for experiment 2 by randomly choosing one of the two mating calls and composing them to form choruses as shown in Fig. 2a. Four choruses (C-LL1 to C-LL4) consisted of three R. lessonae and one R. esculenta calls; in the other four choruses (C-RL1 to C-RL4) the
species ratio was reversed (Fig. 2a). The four mating calls forming a chorus were separated by intervals of less than 3.7s and sometimes even overlapped (Fig. 1). For each chorus (i.e. tape) we produced two versions, one with the call of the rarer male type in the first position and one with the rare call in a randomly chosen second, third or fourth position. This was meant to compensate for potential chorus leader effects (cf. Dyson & Passmore 1988). Solo calls and choruses, respectively, were followed by a longer pause, another solo call or chorus, again a longer pause, etc. Lengths of the pauses were chosen in such a way that they allowed an alternating playback with vocalizations from one tape falling into the pauses of the other.

Female choice

Experimental arena

For the choice experiments we used a circular basin with a diameter of 2.1m and a rim 15cm high. It was filled with water to a level of 7cm and structured with regularly arranged stones (Fig. 3). A grid, consisting of three rings and 24 equally sized sectors, divided the bottom of the basin into a central circle with a radius of 15cm and 72 fields, each with an opening of 15° and 25cm long. Two loudspeakers (Sony SRS-33), connected to tape recorders (Sony TC-D5M), were placed at the edge of the basin, 97cm from the centre of the arena and with an angle of 120° between them. The loudspeakers were hidden behind a black fabric curtain which ran around the arena, 15cm from its rim. A weak red bulb (25W), hanging 2.1m above the basin, provided just enough light to observe the frogs under otherwise night-time conditions.

The distance between the two loudspeakers (160cm) corresponded to the average distance between two males in a chorus (Wahl 1969; Heym 1974; Blankenhorn 1977; Brzoska 1982; Schuchardt & Klingel 1984; Radwan & Schneider 1988). To guarantee that females initially received equally loud calls from both speakers, we measured maximum sound pressure levels (SPL) in the centre of the arena (roline-RO-1350 sound level meter, Steinegger & Co, Schaffhausen, Switzerland) and adjusted the volume for all playback tapes to realistic SPLs. In experiment 1, testing for the choice between calls of nearby single males, we used 74±1dB. According to our own measurements and those of Brzoska (1982) and Engeler (1994), this is the SPL level received by a female 1-3 m away from the calling male. In experiment 2, testing for potential discrimination between distant choruses, we used an SPL of 51±1dB, which is typical for calls from a distance of 15-50 m. This was meant to simulate the situation where a female finds herself between two small adjacent ponds with different ratios of LL/RL males (Holenweg 1999; A.-K. Holenweg & H.-U. Reyer, unpublished...
data) or approaches a larger pond with calling LL and RL males concentrated in different parts (Heym 1974; Blankenhorn 1977; Balmelli 1995; H.-U. Reyer & G. Abt, unpublished data).

Measurements of SPL at 39 points in the arena revealed no evidence of any sound reflection, which might have masked the direction from which the loudspeakers sounded the vocalizations.

Choice experiments

We collected females at the beginning of the reproductive season (mid-May 1998 and 1999) in the same pond from where the males had come in 1997 (Hellberg). Some females were caught in amplexus, others not; but early in the season, when the majority of females can be assumed to carry eggs, mate choice of amplexant and nonamplexant females does not seem to differ (cf. Murphy & Gerhardt 1996). Females were transported to the University, identified via albumin electrophoresis (Tunner 1973), measured (snout-vent length, SVL), weighed and tested for their preference within 2-90 h of being caught (average 21 h).

Females were individually exposed to alternating vocalizations from the two loudspeakers, one sounding *R. lessonae* and the other *R. esculenta* calls. With four tapes for each of the two species, 16 pairwise combinations were possible, both in experiment 1 (solo calls) and experiment 2 (choruses) (Fig. 2b). In eight of the combinations, playback started with the LL tape, in the other eight with the RL tape. Also, in eight combinations the LL vocalization came from the left and the RL vocalization from the right loudspeaker; in the other eight combinations directions were reversed. As a result, we had a fully balanced factorial design, which controlled for potential attractiveness of individual males as well as for call leader and side effects (Fig. 2b). Females were assigned randomly to one of the 16 combinations.

At the beginning of an experiment, a female was placed under a cylindrical wire cage in the centre of the arena and kept there for 4 min, the first 2 min without hearing any vocalization and the following 2 min with LL and RL sounds alternating between the two loudspeakers. We then carefully pulled up the cage by a string, and noted the female’s position in the grid system every 5 s for 10 min. Then the female was removed from the basin, the water was thoroughly stirred to eliminate directional chemical cues possibly left by her choice, and a new female was introduced. After each experiment we measured the water temperature to include it as a covariable in the analyses (see below).

We made behavioural observations from an elevated dark room, separated from the arena by windows. According to the females’ behaviour, experiments were grouped into two categories. (1) Invalid experiments: The female remained within the two innermost grid circles 1 and 2 (Fig. 3) for the whole 10-min period or she left the two inner circles, but visited
only neutral sectors (white in Fig. 3), far from the loudspeakers. (2) Valid experiments: After leaving the two inner circles 1 and 2, the female visited at least one of the sectors surrounding the loudspeakers (grey areas in circles 3 and 4 in Fig. 3), thus indicating her interest in male vocalizations. Once she had fulfilled this criterion, preference was also noted when she subsequently returned to the grey area in circle 2. The appropriate size of the grey area had been determined in a pilot study, and results did not change when the choice area was reduced or enlarged.

We tested each of the 16 pairwise tape combinations from Fig. 2b with a different individual, thus requiring 16 *R. lessonae* and 16 *R. esculenta* females. However, because some experiments were invalid (see above), we had to repeat several tests and more than 16 females per species were needed. The procedure was the following: each female was first tested in experiment 1 for her preference for solo calls. If the experiment was invalid, we tested the same female once more the next day, but with a different pair of LL and RL calls. If, again, performance was classified as invalid, the female was no longer used. Females that performed valid experiments either in the first or the second run were given a chorus choice in experiment 2 within a period ranging from 1 to 24 h. If this trial was invalid, she was tested once more the next day with a different pair of choruses. Hence, each female was tested in the arena at least twice and no more than four times. With this procedure and criteria, only 49% of the females tested made a valid choice in both experiments 1 and 2. These are the females used for the analysis. Among those that did, 52% chose in the first runs of both experiments, 23% in the first run of experiment 1 and the second run of experiment 2, 21% in the second run of experiment 1 and the first run of experiment 2 and 4% in the second runs of both experiments.

After completing, or being excluded from, the choice experiments females were transferred to seminatural ponds (4 x 2 x 0.8m) enriched with natural vegetation. Here they were kept 1 more week, together with males (16 individuals per pond), for observations of the natural mating behaviour. Thereafter, all animals were returned to their pond of origin. All methods of housing, handling and experimenting used in this study fully conform to Swiss ethical regulations and were approved by the Kantonales Veterinäramt Zürich.

**Measures of mate choice and data analysis**

We measured the overall *interest* of females in male vocalizations by (1) the proportion of valid and invalid experiments and (2) the proportion of time females spent in either loudspeaker sector (grey areas in Fig. 3), compared to that expected under a random distribution. Preference for a particular male type was expressed by (3) the first chosen vocalization, measured by the first sojourn in the grey sectors of circles 3 and 4 and (4) the
proportion of time a female spent in the grey sectors of *R. lessonae* and *R. esculenta*, respectively. Both interest and preference measures were related to the following independent variables: species (LL, RL), experiment (solo calls, chorus), female identity, playback tapes (S-LL/C-LL 1-4, S-RL/C-RL 1-4), water temperature during the experiment and female body size. Water temperature and body size are known to affect aural sensitivity and preference of anuran females (Moffat & Capranica 1976; Hubl & Schneider 1979; Gerhardt & Mudry 1980; Wilczynski et al. 1984; Stiebler & Narins 1990; Castellano & Giacoma 1998) and the condition index is a rough indicator of the presence or absence of eggs and, hence, of reproductive motivation (H.-U. Reyer, unpublished data). For statistical analyses we used the programs SPSS 6.1.3 and SYSTAT 7.1 (SPSS Inc., Chicago, U.S.A.). The specific tests are mentioned with the results.

**RESULTS**

**Male vocalizations**

A quantitative analysis of the mating calls that we used in our playback experiments confirmed the previously described differences between the two species (Fig. 4a-c). Mating calls of the four *R. lessonae* were significantly longer than those of the four *R. esculenta* and had a higher repetition rate of pulse groups, both per call and per second (Mann-Whitney U test: $U=0$, $N_1=N_2=4$, $P<0.014$ for all three variables). Lengths of pauses between consecutive solo calls did not differ ($P = 0.248$), as we controlled pause length for technical reasons (see Methods).

**Female choice**

**General behaviour of females and overall interest in calls**

When the cage in the centre of the test arena was lifted, females swam either directly to the basin rim within one of the loudspeaker sectors (grey in Fig. 3) or only a short distance and then stopped for some seconds to a few minutes, usually close to a stone. They then continued their approach towards the loudspeaker, either directly or along the rim of the basin. Some females spent the whole 10-min period in a loudspeaker sector and others spent some time in neutral areas (white in Fig. 3), but once a female had reached a loudspeaker sector she usually remained there. Only six LL (three each in experiments 1 and
2) and four RL females (one in experiment 1 and three in experiment 2) changed from one loudspeaker sector to the other.

In 1998, *R. lessonae* females showed little interest in male mating calls. They made significantly more invalid runs than *R. esculenta* in experiment 1 (62.5 versus 30.8%; chi-square test: $\chi^2 = 6.797, P=0.009$) and among those that did cooperate the proportion of time spent in the loudspeaker sectors during experiment 2 was not significantly higher than expected by chance (Wilcoxon signed-ranks test, two-tailed: $Z=1.087, P=0.277$). Therefore, both experiments were repeated with *R. lessonae* females in 1999, and that year they showed a proportion of invalid runs in experiment 1 as low as *R. esculenta* had the previous year (33.3 versus 30.8%; chi-square test: $\chi^2 = 0.038, P=0.846$) and spent significantly more time in the loudspeaker sectors than expected by chance (Wilcoxon signed-ranks test: $Z=2.599, P=0.01$). We limit the following presentation of our results to those two experimental series in which females did express a clear interest in male vocalization, namely the *R. esculenta* data from 1998 and the *R. lessonae* data from 1999. Each of the two data sets comprises 16 females that fulfilled the choice criteria in both experiments.

In terms of their overall interest in male vocalizations (Fig. 5a), the proportions of time females spent in the two loudspeaker sectors were significantly higher in experiment 1 than in experiment 2 (repeated measures ANOVA: $F_{1,30}=8.44, P=0.007$), with no difference between the two species ($F_{1,30}=1.38, P=0.249$) and no significant species*experiment interaction ($F_{1,30}=0.16, P=0.694$). Pairwise comparisons revealed that both female types spent significantly more time in the two loudspeaker sectors than expected by chance, both in experiment 1, testing solo calls (Wilcoxon signed-ranks test: $Z=-2.500, P=0.012$ for *R. esculenta* and $Z=-2.579, P=0.01$ for *R. lessonae* females). The same result was obtained with first choice as the measure for preference: females of both species directed more first choices to LL than to RL solo calls (binomial test: $P<0.021$ for both comparisons). In experiment 2, we

We used two measures of female preference: first choice and proportion of time in one of the two loudspeaker sectors (Fig. 5b, c). In terms of time, we again found a significant experiment effect (repeated measures ANOVA: $F_{1,30}=6.29, P=0.018$), no species effect ($F_{1,30}=0.72, P=0.404$) and no species*experiment interaction ($F_{1,30}=0.91, P=0.349$). In experiment 1, females of both species spent a significantly higher proportion of time in the LL sector than in the RL sector (Wilcoxon signed-ranks test: $Z=-2.500, P=0.012$ for *R. esculenta* and $Z=-2.579, P=0.01$ for *R. lessonae* females). The same result was obtained with first choice as the measure for preference: females of both species directed more first choices to LL than to RL solo calls (binomial test: $P<0.021$ for both comparisons). In experiment 2, we
found no significant preference for a chorus type in either species for either first choice or
time (all $P > 0.105$).

The higher attractiveness (Fig. 5a) and apparently better discrimination (Fig. 5b, c)
between solo calls than between choruses is further supported by the following data: the
proportion of females spending the whole 10-min period in one of the two loudspeaker
sectors was higher in experiment 1 than in experiment 2 (40.6 versus 15.6%; Chi-square
test: $\chi^2 = 4.947, P = 0.026$); conversely, the proportion leaving a once chosen sector tended to
be lower in 1 than in 2 (15.6 versus 28.1%), but the difference is not significant ($\chi^2 = 1.463,$
$P = 0.226$).

Factors potentially influencing the preference

Female identity

There was no consistency of female performance in the two experimental series. For
both species, first choice in the chorus experiment was unrelated to first choice of the same
female in the previous solo call experiment (Fisher’s exact probability test: both $P > 0.250$).
There was also no significant correlation between the proportions of time a female spent in
both loudspeaker sectors and in the LL sector, respectively, in experiments 1 and 2
(Spearman rank correlation: $r_s < 0.256, N = 32, P > 0.16$ for both experiments). Thus, a
female’s interest in and preference for male solo calls was no predictor of her response to
choruses. Hence, data from the two series can be considered independent, which justifies
the separate analyses for solo calls and choruses.

Male vocalization, water temperature and body size of females

Water temperature and female body size as well as the quality of the individual LL and
RL playback tapes in a choice experiment can all affect the preference of females (see
Methods). We therefore analysed whether the above results can be partially attributed to
additional effects from any of these variables. This, however, was not the case. When the
proportion of time spent with LL calls (dependent variable) was related to the independent
variables species, LL tape, RL-tape, LL*RL-tape, water temperature and female snout-vent
length by means of a stepwise ANCOVA, none of the variables remained in the final model
for either experiment 1 or 2. Stepwise logistic regressions, testing for the effects of the same
independent variables on first choice, confirmed the results: choice between solo calls and
choruses, respectively, was not related to any of the variables in either $R. esculenta$ or $R.$
lessonae females.
DISCUSSION

In all four data sets (two each from experiments 1 and 2), females spent significantly more time close to the loudspeakers than expected by chance (Fig. 5a). This indicates that females were attracted by both solo calls and choruses of males. It is usually assumed that such approach and proximity to male vocalizations reflects an interest in sexual partners because it results in amplexus in most anuran species (Dyson et al. 1992; Ryan & Rand 1993a; Schwartz 1993; Gerhardt 1994a; Murphy & Gerhardt 1996). The differences between the two experiments discussed below suggest, however, that the effectiveness of attraction differs between nearby solo calls and distant choruses.

Experiment 1: Response to solo calls

Females of both species showed a clear preference for mating calls of _R. lessonae_ males. This was true whether we used first choice (Fig. 5b) or time in the loudspeaker sector (Fig. 5c) as a measure for preference. The preference of female _R. lessonae_ for conspecific males is not surprising; but what makes _R. esculenta_ females prefer _R. lessonae_ males over their own? One possible explanation is that the hybrid inherits the preference with the L genome from the parental species. Another possibility is that the preference reflects a pre-existing sensory bias in females that originally evolved for reasons other than sexual selection (Ryan & Rand 1993b; Ryan 1997). In a review of over 150 studies of mate choice, Ryan & Keddy-Hector (1992) found that female preferences are regularly biased towards traits of greater quantity, which elicit greater sensory stimulation. In terms of acoustics, the preferred traits include louder calls, higher repetition rates and larger repertoires. Since calls of LL males are longer and contain more pulse groups (Fig. 4) than those of RL males, they may have transmitted more energy and, hence, better stimulated the female for purely mechanistic reasons. However, to test these ideas, we need further experiments that also consider the fact that under natural conditions _R. esculenta_ males often call louder than _R. lessonae_, which might improve the stimulation properties of hybrid vocalization.

The fact that choice experiments with _R. lessonae_ females failed in 1998 (see Results) is probably best explained by insufficient reproductive motivation in that year. It is true that in 1998 the body condition index, used as a measure for presence of ripe eggs and readiness to mate, was the same for _R. lessonae_ and _R. esculenta_ (1.04 and 1.06, respectively); but _R. lessonae_ seems to be more susceptible to experimental stress. Previous experiments required many more _R. lessonae_ than _R. esculenta_ to demonstrate female choice (Engeler 1994) and some tests even had to be restricted to hybrid females because _R. lessonae_ tended to panic in the experimental situation (Abt 1992; Bergen et al. 1997). This stress
response seems to decrease only in highly gravid females which must get rid of their eggs fairly soon and will even release some of them without being amplexed (Reyer et al. 1999). In line with this argument is the fact that *R. lessonae* females from 1999, which showed more interest in vocalizations in general (Fig. 5a) and significantly preferred those of *R. lessonae* (Fig. 5b,c), had a significantly higher condition index than their conspecifics from 1998 (1.25 versus 1.04). Thus, the indiscriminate behaviour in 1998 may have reflected a fear-motivated general approach to a familiar acoustic environment. Sexual interest in and clear preference for a specific species seems to require a higher reproductive urge in *R. lessonae* than in *R. esculenta*, at least under experimental conditions.

**Experiment 2: Response to choruses**

The response to choruses was weaker than that to solo mating calls in all three choice measures: overall, females of both species spent less time in both loudspeaker sectors (Fig. 5a) and did not discriminate between choruses with a 3:1 excess of *R. lessonae* and *R. esculenta*, respectively (Fig. 5b,c). The lower interest in and poorer discrimination between male vocalizations has probably both methodological and biological reasons.

In terms of the method, the lower SPL in experiment 2 (51 dB) than experiment 1 (74 dB) might have been too low. This, however, is unlikely. Auditory nerves of *Rana* (and also *Hyla*) females respond to SPLs above 20-25 dB (Capranica & Moffat 1983; Schmitz et al. 1992; Christensen-Dalsgaard et al. 1998); and in *Hyla gratiosa* a behavioural phonotactic response towards a single chorus has been observed at SPL values as low as 38-40 dB and preference for a mixed chorus with conspecific males at a level of 55 dB (Gerhardt & Klump 1988b). In our study, too, females spent more time close to the loudspeaker sectors than expected by chance. Hence, they were attracted, but did not discriminate.

Another potential methodological explanation could arise from the fact that choice between choruses was always tested after choice between single calls. With increasing time in captivity, females may have lost their sexual motivation and/or may have learned in experiment 1 that their approach to the vocalization does not guide them to a real male; as a result, they may have reduced their response in experiment 2. So far, however, there is no evidence to support this idea. In experiments with *R. lessonae* and *R. esculenta*, Engeler (1994) observed no loss of motivation between first and second choice experiments. Gerhardt (1981) showed for *Hyla cinerea* that a female’s preference was not affected by her behaviour in a previous experiment, which agrees with our finding that individual females did not respond consistently in experiments 1 and 2. Murphy & Gerhardt (1996) even showed that experience with a choice situation reduces the time a female takes to choose in
subsequent experiments. It remains unclear whether these different results are due to
differences between species and/or experimental situations.

In terms of biology, the poorer response to male choruses (Fig. 5a) and the lack of a
clear preference (Fig. 5b,c) might be a consequence of an unrealistic experimental situation,
if females do not normally hear choruses of very different male composition. This, however,
is not true. Breeding ponds, separated by less than 100m, can have very different LL/RL
ratios (Holenweg 1999; A.-K. Holenweg & H.-U. Reyer, unpublished data), and within a pond
there can be spatially segregated concentrations of LL and RL males, respectively (Heym

A more likely explanation is that the poor response to choruses reflects a greater
difficulty in discriminating between vocalizations in experiment 2 than in experiment 1.

Choruses present a complex acoustic environment with interference between calls of
different males. As a result, females can probably assess only a few of the closest males
(Gerhardt & Klump 1988a; Wollerman 1999). In such an environment, anuran female choice
is usually less clear than in the classic choice experiment where two loudspeakers alternately
sound mating calls from opposite ends of a soundproof room (Gerhardt 1982; Schwartz
1987; Fritsch et al. 1988; Telford et al. 1989). Increasing the distance between loudspeakers
and decreasing the acoustic overlap will decrease interference and improve discrimination

Similarly, increased ‘quality’ differences between the two choruses (i.e. more extreme
LL/RL ratios) might have yielded better discrimination. The LL/RL ratios of 3:1 and 1:3 used
in our experiments are fairly moderate compared with several natural populations where one
species can outnumber the other by a factor of 10 or more (Berger 1977; Blankenhorn 1977;
Holenweg 1999). Under such conditions, detection of the ‘right’ chorus may be easier and
the benefits of avoiding strongly RL-biased choruses can be assumed to be higher than in
our experiment. Moreover, the ability to discriminate between choruses may be related to the
LL/RL ratios in the population the females come from, provided populations have been
isolated long enough for selection regimes to result in different responses. In our source
population at Hellberg, *R. lessonae* males outnumber *R. esculenta* males by about 5:1 (H.-U.
Reyer, unpublished data). Hence, selection against RL-male-biased choruses is probably
weak, because such choruses almost never occur. It would be interesting to test the
discriminative abilities of females from populations with a high excess of *R. esculenta* males.

Evidence for discrimination related to species ratios comes from Gerhardt’s (1994a) study.
When exposed to calls from different males, female *Hyla chrysocelis* living in sympatric
populations with *Hyla versicolor* discriminated better between than within species, whereas
females from allopatric populations paid more attention to differences within than between
species.
Conclusions

Within the range of LL/RL ratios used in our study (3:1 versus 1:3), there is no evidence that females use the composition of distant choruses for choosing ponds, or arenas within a pond, that hold an excess of the preferred _R. lessonae_ males (experiment 2). Gerhardt & Klump (1988b) have suggested that chorus sound may have little effect in species where the composition of male choruses is stable in time and space and, hence, females have other means to locate breeding sites with reproductively active conspecific males. LL/RL-ratios are indeed pond specific and do not change much from year to year (Berger 1977; Blankenhorn 1977; Holenweg 1999; A.-K. Holenweg & H.-U. Reyer, unpublished data), but we do not yet know whether this stability also holds for the male arrangement within ponds. Once females have arrived in a chorus, they can discriminate between the closest LL and RL males not only on the basis of visual, olfactory and tactile cues (Abt & Reyer 1993; Engeler 1994) but also on the basis of mating calls (experiment 1). Whether this discrimination ability increases their chances of achieving amplexus with an LL male will depend not only on the relative numbers of LL and RL males but also on how freely a female can move within a chorus without being sexually coerced by males. Since anurans respond to rapid movement (Grüsser & Butenandt 1968), females can, to some extent, improve this freedom by swimming slowly (Notter 1974; Engeler 1994). The freedom will decrease, however, with increasing density, spatial clumping, reduced distances between males and male-biased operational sex ratios. These factors will not only increase male-male competition, but also affect the relative powers of the sound sources to which females are exposed (cf. Forrest & Raspet 1994). How precisely these interactions influence female choice is being investigated both in the field and under experimental conditions. The results will be important for understanding why the relative mating success of LL and RL males differs between ponds, between years and during the season (G. Abt & H.-U. Reyer, unpublished data).

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References


Figure legends

Fig. 1: Call sequences in (a) experiment 1 with a choice between solo calls and (b) experiment 2 with a choice between choruses. Each solo call consisted of two different mating calls from the same male (call 1, call 2) followed by a pause (—). A chorus consisted of three mating calls from different males of one species (A1, A2, A3) and one call from a male of the other species (B1). The call of the rarer species (either *R. lessonae* or *R. esculenta*) could occur in any of the four possible locations in the sequence. For further details see Methods.

Fig. 2: Composition of (a) choruses and (b) design of playback experiments. (a) C-LL 1 to C-LL 4 = *R. lessonae* choruses in which three *R. lessonae* calls (○) were combined with one *R. esculenta* call (●); C-RL1 to C-RL 4 = *R. esculenta* choruses with the reverse ratio. (b) the 16 combinations in which tapes with vocalisations of *R. lessonae* and *R. esculenta*, respectively, were presented. S-LL/C-LL1-4 and S-RL/C-RL 1-4: solo call/chorus tapes with *R. lessonae* and *R. esculenta* vocalisations, respectively. Symbols indicate whether playback began with *R. lessonae* (○) or *R. esculenta* (●) and whether the respective vocalisation was sounding from the left (l) or right (r) loudspeaker. Numbers (1-4) refer to different vocalisations.

Fig. 3: Sketch of the experimental arena with the grid used for recording the locations of frogs. Also shown are the positions of stones (dark grey) and of the two loudspeakers (Sp), placed between the edge of the basin (double line) and a black curtain (thick line). Females were considered to choose only when they stayed in the light grey sectors of circles 2-4. For further explanation see text.

Fig. 4: Call characteristics for *R. lessonae* (□) and *R. esculenta* (●). (a) Length of call, (b) number of pulse groups per call and (c) per second for the two calls from each of the four males (1-4) of each of the two species. In *R. esculenta*, within-male similarity of the two calls was so close that the dots often lie on top of each other. Also shown are the species-specific means ± SE calculated from the eight calls per species.
Fig. 5: Measures of interest in and preference for male vocalizations in females of *R. esculenta* (■) and *R. lessonae* (□). (a) Percentage of time spent in any of the two loudspeaker sectors, (b) frequency of first choices for the *R. lessonae* (LL) sector, and (c) proportion of time spent in the *R. lessonae* sector. Results are shown for both experiment 1 presenting solo calls and experiment 2 presenting choruses. ★ = observed value significantly different (*P*<0.05) from that expected under the assumption of a random distribution (———). For further explanations and details see text.
Roesli & Reyer, Figure 1

(a) Solo call

Call 1     Call 2

(b) Chorus

Calls

B1  A1  A2  A3

Calls

A1  A2  A3  B1
### a) Chorus

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### b) Combination

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Roesli & Reyer, Figure 2
Roesli & Reyer, Figure 4

(a) Length of call (s) vs. Males

(b) Pulse groups/call vs. Males

(c) Pulse groups/s vs. Males