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BREEDING RESOURCE DISTRIBUTION AFFECTS SELECTION GRADIENTS ON MALE PHENOTYPIC TRAITS: EXPERIMENTAL STUDY ON LIFETIME REPRODUCTIVE SUCCESS IN THE BITTERLING FISH (*RHODEUS AMARUS*)

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The spatial distribution of breeding resources can have pronounced demographic and evolutionary consequences. We used 20 experimental groups of the bitterling (*Rhodeus amarus*), an annual fish with a promiscuous, resource-based mating system, and extended breeding season to investigate how the spatial distribution (clumped or regular) of bitterling oviposition sites (live freshwater mussels) affected offspring production, variation in reproductive success, and directional selection on phenotypic traits over their entire reproductive lifetime. We did not detect any effect of resource distribution on offspring production or variation in reproductive success among individual fish, although variation between replicates was higher with a clumped distribution. This finding is discussed with regard to the incidence of alternative mating behaviors (sneaking) within the limitations imposed by our experimental design. Breeding resource distribution had a significant effect on selection on male phenotypic traits. Stronger directional selection on traits associated with intrasexual competition for fertilizations, gonad mass (an indicator of sperm competition), and the extent of red, carotenoid-based pigment in the iris (an index of dominance status), was detected with a clumped resource distribution. With a regular resource distribution, a stronger positive selection on male body size was detected. We discuss the implications of our results for natural populations.

KEY WORDS: **Directional selection gradient, individual and population consequences of behavior, opportunity for selection, parentage analysis, reproductive effort, sexual selection.**

The spatial distribution of breeding resources is crucial to understanding the demographic and evolutionary consequences of sexual selection (Kokko and Rankin 2006). The availability of breeding resources affects the number of individuals that breed in a given reproductive season, variance in reproductive success among individuals of the same sex, and success of alternative mating behaviors, hence affecting the intensity and direction of selection (Village 1983; Kwiatkowski and Sullivan 2002; Lehtonen and Lindström 2004; Larison 2007; Twiss et al. 2007). In their broadest sense, breeding resources include sites of oviposition or nesting, through territories, leks, and other mating arenas, to defensible groups of individuals of the opposite sex (Emlen and Oring 1977), with the spatial arrangement of these resources directly influencing the demographic and evolutionary effects of sexual selection.

At population level the interplay between sexual selection and the distribution of breeding resources influences demographic parameters such as recruitment, juvenile distribution and competition (Einum et al. 2008), effective population size (Twiss et al. 2007), and population (Levin et al. 2000) and metapopulation dynamics (Menendez and Thomas 2000). The influence of breeding resource distribution can be detected across mating systems. For example, in the socially monogamous European kestrel, *Falco tinnunculus*, high spatial clustering of nest sites limited their use through the territorial aggression of breeding pairs, excluding some individuals from breeding (Village 1983), although the number of males able to mate was limited by the availability of shallow pools in polygynous grey seals, *Halichoerus grypus* (Twiss et al. 2007).

The evolutionary consequences of changes in the spatial distribution of breeding resources typically arise through effects on the strength of sexual selection on traits, a consequence of variation in reproductive success among members of the same sex (Andersson 1994). Resource distribution can directly affect the proportion of breeding individuals in a population (Lehtonen and Lindström 2004), their relative reproductive success (Reichard et al. 2004a), and the success of alternative mating behaviors (Bergman et al. 2007; Larison 2007). The impact of resource availability is especially pronounced in species in which reproduction is limited to nesting sites or breeding territories defended by individual males (Emlen and Oring 1977; Wade 1995). In these circumstances the direction and intensity of selection can vary among populations that differ in critical features such as male and nesting-site density (Borg et al. 2002; Kwiatkowski and Sullivan 2002) or territory quality (Formica et al. 2004).

Sexual selection theory predicts greater variance in reproductive success and, therefore, a higher opportunity for sexual selection for populations with a clumped distribution of breeding resources because a relatively small proportion of males are able to control them, thereby constraining the reproductive success of other males through intrasexual competition (Wade 1995). Under these circumstances females may be obliged to mate with dominant males (Qvarnström and Forsgren 1998), and sexual selection is predicted to favor traits important to intrasexual selection. In contrast, where resources are abundant and regularly distributed, males may be unconstrained by resource defense and females may choose mates without constraints imposed by male dominance (Qvarnström and Forsgren 1998). A consequence might be selection for different phenotypic traits under contrasting regimes of sexual selection (Candolin 2004; Seamons et al. 2007).

Alternative male mating behavior may further complicate the outcome of selection. When intrasexual selection is intense, some males circumvent energetically expensive investment in courtship and territorial possession and use alternative tactics to achieve fertilizations (Gross 1996). Such males typically acquire fertilizations through sperm competition, with a specialized suite of behavioral, physiological, and morphological adaptations (Taborsky 1998). Sperm competition risk and intensity tend to be significantly higher, although not constrained to habitats in which resources are limiting or clumped (Birkhead and Møller 1998; Singer et al. 2006), but the final effect of sperm competition intensity on opportunity and strength of sexual selection may be complex and difficult to predict (Jones et al. 2001a; Mills and Reynolds 2003).

Variation in environmental and population parameters can interact and it is sometimes difficult to separate the roles of natural and sexual selection under natural conditions (Endler 1986). Here, we overcome the problems associated with the complexities of natural habitats by using replicated experimental groups as surrogates of populations to investigate the effect of the spatial distribution of resources on recruitment and lifetime reproductive success of male and female bitterling fish (*Rhodeus amarus*). The bitterling has a promiscuous, resource-based mating system with territorial males defending living freshwater mussels that are their only substrate for oviposition (Smith et al. 2004). Previous studies that involved single oviposition events for each individual revealed that the opportunity for sexual selection varies in relation to the operational sex ratio (OSR) and resource availability (Reichard et al. 2004a) and demonstrated that different male traits are selected under intra- and intersexual selection (Reichard et al. 2005). Sneaking is a common male tactic both in nature (Smith et al. 2002) and under experimental conditions (Smith et al. 2003), with a potential to diminish the variance in reproductive success among males (Reichard et al. 2004b). Bitterling population size and resource availability can vary 90 and 500-fold, respectively, (Smith et al. 2000) making it likely that differential selection regimes exist between even adjacent populations.

Although short-term studies have proven valuable in identifying sources of variation in reproductive success they may provide little information on their significance in natural populations (Westneat and Mays 2005). Lifetime data are needed for more complete insights into the processes that are important in nature. Lifetime reproductive success, the total number of offspring produced in a lifetime, is considered the best available measure of fitness that can be collected relatively economically (Grafen 1982), although measures that account for the number of grandchildren generated are superior (Schmoll et al. 2005). Lifetime reproductive success consists of four main components; survival to reproductive age, reproductive life span, offspring production (that combines effects of fecundity or mating success) and offspring survival (Clutton-Brock 1988). In the present study, we analyzed experimental data that encompass the entire reproductive life span of parental fish in terms of offspring production corrected for offspring survival during the most critical part of their life history to investigate population and individual consequences of the spatial distribution of breeding resources. Bitterling are ideal for studies aimed at tackling the effect of the spatial distribution of resources on reproductive parameters because: (1) they oviposit in a discrete spawning substrate that can easily be manipulated; (2) they have a resource-based, promiscuous mating system that is prone to sneaking; (3) they readily adapt to experimental conditions and perform their full behavioral repertoire in captivity; (4) their offspring can be collected after the period of greatest mortality when they depart their mussel hosts (comparable to the number of fledgling chicks in bird study systems). Given that bitterling in our study area are annual (Smith et al. 2000; M. Konečná and M. Reichard, unpubl. data), we estimated lifetime reproductive success by parental assignment of offspring departing from mussel hosts over an entire reproductive season.

We varied resource distribution in 20 experimental groups of adult fish (10 clumped resource, 10 regular) while keeping density constant. At the population level, we predicted that offspring production would be lower in groups with a clumped resource distribution as a consequence of territorial aggression reducing female oviposition rate (Reichard et al. 2004b). We further predicted that variation in reproductive success and therefore opportunity for selection on males and opportunity for sexual selection would be higher with a clumped resource distribution. At the individual level, we predicted that the spatial distribution of mussels would lead to different selection regimes between treatments, with stronger selection on traits important in intrasexual competition for matings with a clumped distribution. In particular, we predicted that selection for eye redness (an index of male dominance), male size (the best predictor of dominance), and gonad weight (an important trait in sperm competition) would be higher with a clumped mussel distribution. For females, we predicted no differences in the intensity of selection between treatments.

Materials and Methods

STUDY SYSTEM

Bitterling fish can lay their eggs only in the gill cavities of freshwater mussels. In the European bitterling the reproductive season lasts from mid April to July, with a peak in spawning during an approximately three-week period in late April–May. Male bitterling establish territories around living freshwater mussels and attract females to deposit their eggs into mussels in their territory. Females with ovulated eggs develop a long ovipositor and use it to place their eggs into mussel gills. Mussels vary in their suitability to host bitterling eggs and embryos and both male and female bitterling are able to discern differences in mussel quality, probably using olfactory cues. Males compete aggressively for mussels and large males typically establish superior territories. A typical male territory has a diameter of approximately 100 cm, but males patrol a much larger area in search of females, frequently invading the territories of neighbors (Reichard et al. 2004b). A territory may contain a single mussel or a cluster of several. Smaller males often do not establish their own territories, especially when mussels are scarce relative to male abundance. Males often engage in sneaking behavior by releasing sperm into a mussel guarded by another male, either before or after oviposition. Male mating behavior is opportunistic and both large and small males may be territorial and sneak, and there is no genetic or morphological difference between territorials and sneakers (Smith et al. 2004). Territoriality generally confers higher fitness, but the success of a territorial male is compromised in competition with two or more rivals (Reichard et al. 2004a).

Females produce 80–250 eggs over the reproductive season and eggs are spawned in several (approx. 5–10) bouts. Each spawning bout lasts one or two days and within each bout the eggs are laid in 5–12 separate clutches consisting of one to six (typically two to four) eggs. Mating is promiscuous and each spawning act may involve different partners. Bitterling do not provide parental care; the eggs are deposited deep inside the mussel gills and adults do not have access to them. Embryonic development lasts four to six weeks (depending on ambient temperature). During embryonic development, two main sources of embryo mortality are ejections by host mussels (Mills and Reynolds 2002; Reichard et al. 2007a) and asphyxiation related to bitterling–mussel and sibling competition for oxygen (Smith et al. 2001). At emergence from mussels, bitterling are approximately 10 mm long and actively swimming and feeding (Aldridge 1999; Smith et al. 2004).

EXPERIMENTAL GROUPS

Data on reproductive success were collected from 20 experimental groups. Each experimental group consisted of three males and three females housed in large fiberglass tubs (135 × 135 cm, 90 cm deep) in the garden of the Institute of Vertebrate Biology (IVB) in Brno, Czech Republic. Each tub was furnished with a layer of sand, six artificial plants at fixed positions, and three sand-filled plastic pots containing a single *Unio pictorum* mussel. The position of the pots was assigned to two treatments according to a predetermined random pattern; either regular (the distance between pots was 120 cm, allowing formation of three territories) or clumped (the three pots positioned adjacent to each other in one corner, allowing establishment of a single territory).

Mussels were collected from an oxbow lake adjacent to the River Kyjovka before the onset of bitterling reproduction and placed in the experimental tubs four days prior to the introduction of fish. Experimental fish were collected from the River Kyjovka on 1 March 2005, before the start of the bitterling reproductive season. After a four-day period of acclimatization, fish were assigned to groups. Every fish was measured, fin-clipped (a small part of lower or upper lobe of the caudal fin was removed and fixed in 96% ethanol), and photographed. Fish represented a random sample from the wild population, their body size ranged from 29.6 to 53.2 mm, and were likely one year of age. To standardize size structure among replicates and mimic natural conditions in which fish of a broad size range interact, individuals of each sex were assigned to three broad size categories (large, medium, and small). Three male and three female bitterling (one of each size class) were released into each tub on 5 April 2005 and allowed to spawn throughout the reproductive season until late July when spawning had ceased. Selecting a size range of fish reduced variation among replicated groups in starting conditions while retaining a natural degree of size variation within groups.

Fish were fed daily with frozen chironomid larvae and frozen copepods (a standard amount to each group) and were also able to forage on algae and small invertebrates that established in tubs within two weeks. Every tub was monitored daily (0900–1300) and any juvenile bitterling that had emerged from mussels were captured using a fine-mesh dip net. Bitterling emerge from mussels at night and shoal near the water surface (Reichard 2002); therefore their collection did not disrupt adult fish. We cannot conclusively reject the possibility that some juveniles disappeared from tubs before their collection, although there is no evidence that adult bitterling cannibalize juveniles (Smith et al. 2004). All juvenile fish were fixed in 96% ethanol. Fish emergence lasted from 27 May to 26 August, indicating that spawning started in approximately mid April. In this study, we did not systematically collect data on individual male spawning behavior and territoriality, and we were also unable to compare offspring production from different territories or track territory ownership over the season.

We estimated the fecundity of each female using a size-fecundity relationship for our study population that took the form $F = aL^b$, where F was fecundity, L was female Initial Size in millimeters, and the fitted parameter a was 0.07 and parameter b was 2.165 (full model: $r^2 = 0.81$, $F_{1,75} = 318.33$, $P < 0.001$) (Smith et al. 2000). Embryo mortality in mussels was calculated as the proportion between the number of juveniles that were collected and assigned using parentage analysis (see below) to a particular female and that female's estimated fecundity. Thus the period of embryo mortality spanned oviposition to emergence from the mussel host, a phase that usually lasts from four to six weeks. Estimated mortalities may have been caused by ejections of eggs by mussels immediately after oviposition (Reichard et al. 2007b) or later (Mills and Reynolds 2002), or from suffocation inside the mussel gill chamber (Smith et al. 2001), but also potentially by the failure of females to oviposit due to overt male–male competition, with consequent egg resorbtion through the process of ovarian atresia (Tyler and Sumpter 1996; Wootton 1998; Reichard et al. 2004b).

PHENOTYPIC TRAITS

For all adult fish, with the exception of a single female that died during the experiment (on 18 June), a set of phenotypic traits was measured. Between 29 July and 3 August (after the end of breeding season), all adult fish were captured and returned to the laboratory for parasitological dissection. All males were photographed under standardized conditions less than 60 sec after capture, with standard flash light and aperture setting, a plastic strip of red, blue, and green color, and size scale to serve as a reference during image analysis. Fish were humanely killed by cutting the spine at the base of the skull, dissected and parasites were removed from host tissue (fins, body surface, gills, brain, eyes, internal organs, muscle), and fixed according to an established protocol (Ergens and Lom 1970). All parasites were subsequently identified under a light microscope equipped with phase-contrast, differential interference contrast, and Digital Image Analysis.

The following phenotypic traits were considered. **Initial Size** is Standard Length (body size from tip of the mouth to the base of the caudal fin) measured to the nearest 0.1 mm from photographs taken at the start of the experiment. **Final Size** was measured at the end of the experiment immediately before parasitological dissections using digital calipers to the nearest 0.01 mm. **Growth** was calculated as the difference between $\log_e$-transformed Initial and Final Size (Wootton 1998; Kotiaho 1999). **Body Height** was represented by the residuals from a regression of body depth (the perpendicular height from the anterior base of the dorsal fin; i.e., the deepest point on the body, measured before parasitological dissection) regressed against Final Size. This trait approximates body condition (Wootton 1998), but may also be under directional sexual selection. In a closely related species, the rose bitterling (*Rhodeus ocellatus*), the male predorsal region is excessively deep and accentuated by green iridescent scales, and relative body depth correlates with male mating success in this species (Casalini 2007). **Condition Factor** is a standard ichthyological index calculated from the function: $K = W_E \times 100/SL^3$ where W_E is eviscerated body weight in milligrams (all organs from inside the body cavity excluded before weighing, measured to the nearest milligrams on digital scales) and SL is Final Size in millimeters (Wootton 1998). This trait is the best predictor of individual energetic state and overall somatic condition and is strongly positively correlated to nonpolar lipid density (Neff and Cargnelli 2004). **Gonad Weight** represents the total mass of gonads (to the nearest 1 mg) at the time of dissection; i.e. at the end of the reproductive season. Because bitterling gonads go through resorbtion in autumn and early winter (Nishi and Takano 1979; Solomon et al. 1984), gonad mass measured after the cessation of reproduction denotes residual gonad mass and represents a relative index of investment in gonad tissue in the foregoing breeding season. The estimate of Gonad Weight after the cessation of reproduction overcomes the problem of temporary sperm depletion, which commonly affects ejaculate size and sperm density (traits that can be measured during the reproductive season) in bitterling (Smith and Reichard 2005; Pateman-Jones 2007). Measurement of ejaculate and sperm traits would further disrupt the experimental goal of leaving fish without interference over the entire breeding season.

Parasite Load is the sum of all individual metazoan parasites found on a given fish, irrespective of parasite taxa. Preliminary analysis revealed that this measure of Parasite Load best explained differences among individual fish. In total, 13 parasite species were found (three Monogenea, one Nemotoda, eight larval stages of Trematoda), with a mean prevalence of 19% (range: 0.8–81% fish infected by a particular species). The number of individual parasites of all common species (six parasite species with $N >$ 18 individuals distributed on a total of 119 hosts) positively correlated (Pearson correlations, $P < 0.05$) with Parasite Load; i.e. there was no particular parasite species that dominated. The distribution of parasite taxa was further investigated with a correlation analysis between the abundances of individual parasite species and reproductive success (data not shown). One female carried an exceptionally high number of metacercariae (larval stages of Trematoda) of two species. This datum exerted high leverage in subsequent correlation analyses and positively skewed the dataset. Consequently, the value was arbitrarily reduced from 119 to 31 (calculated as the second highest value for Parasite Load in females (20) plus 200% of the mean. **Parasite Species Richness** is the number of metazoan parasite species found on a given individual.

In males, analysis of red coloration was performed. We estimated the extent and intensity of red color in the iris (Reichard et al. 2005) using ImageJ software (Mennill et al. 2003). The coloration of males was quantified following the protocols of Barber et al. (2000), Candolin and Reynolds (2001), Smith et al. (2002), and Reichard et al. (2005). **Red Area** was estimated as the ratio between the size of the Red Area and pupil. Red color area (in the upper part of the iris), pupil, and total eye areas were identified in ImageJ using freehand and elliptical selection tools. The transition from carotenoid-based Red Area and surrounding silver pupil area is abrupt making the outlined methods satisfactory (see also Head et al. 2008). Red Area was estimated three times for each male from a single photograph before the reproductive season and from two photographs (i.e., six times) at the end of the reproductive season. Repeatability (Lessels and Boag 1987) was high ($r =$ 0.989 before and $r = 0.969$ after the reproductive season). Other possible measures of Red Area (proportion of red color in total iris area and proportion of red in total eye area) gave concordant results (all measures were strongly correlated). **Red Intensity** was estimated as red index (Frischknecht 1993). Five pixels from the red patch were randomly selected and their red index (proportion of the brightness of the red component to the sum of red, blue, and green component values) was calculated. Repeatability of Red Intensity estimates was lower than for Red Area ($r = 0.747$ before and $r = 0.472$ after the reproductive season), which likely arises from variation in Red Intensity across red patches in an individual male's iris. Three measures of Red Area (**Initial Red Area** measured before reproductive season, **Final Red Area** measured after reproductive season, and **Red Area Difference** calculated as difference between Initial and Final Red Areas) were obtained, but for Red Intensity only **Initial Red Intensity** and **Final Red Intensity** were calculated. The failure to estimate Red Intensity Difference was due to a slight change in lighting conditions between the two measurements due to logistical constraints, which meant the two estimates were not comparable. At the start of the experiment, there were no differences in male coloration among spatial treatments (ANCOVA with Initial Size as covariate, Initial Red Area: $F_{1,57} = 0.28$, $P = 0.597$, Initial Red Intensity: $F_{1,57} = 0.30$, $P = 0.588$) and no difference in Initial Size of males (ANOVA: $F_{1,58} = 0.62$, $P = 0.434$) or females ($F_{1,58} =$ 0.40, $P = 0.528$).

PARENTAGE ANALYSIS

The DNA from 120 parental fish and 4058 offspring was isolated from fin tissue. For each parental individual, two samples (taken at the start and at the end of the experiment) were isolated, except for a single female that died before the end of experiment for which only a single sample was obtained. The parental samples were initially genotyped for 12 variable microsatellite loci *Rser01–06*, *Rser08–Rser12* (Dawson et al. 2003), and *Rser13* (Reichard et al. 2008). Based on their informative value and compatibility, five loci were combined in a single multiplex PCR reaction (*Rser03*, *04*, *08*, *11*, *13*), with a mean of 30 (range: 4–64) alleles per locus. Mean observed heterozygosity in parental fish was 0.74 (range: 0.31–0.93). The mean combined nonexclusion probability estimated in Cervus 3.0 (Kalinowski et al. 2007) was 0.088 for the first parent and 0.019 for the second parent. All offspring were genotyped at five loci. A detailed description of DNA isolation and genotyping is presented in Reichard et al. (2008).

All parental fish were analyzed twice on at least five loci (the set used in the multiplex reaction). In addition, 147 juveniles on five loci and 94 juveniles on four loci were read twice (total of 1111 loci). For all repeated analyses a new PCR was performed. A total of 83 loci were read for the second time because the peak position could not be read reliably and the remaining 1028 loci were read either when a nonparental allele was detected, for logistical reasons (sample position checks), or to estimate error rate. In seven cases, juveniles possessed genotypes incompatible with candidate parents. In five cases, a mismatch was observed at single loci and incompatible alleles were considered as new mutations. In the remaining two cases, we observed mismatch between putative parents and offspring at multiple loci and juveniles were assumed to emerge from eggs laid in mussels prior to field collection of mussels (they appeared among the first releases from mussels) and were disregarded. Paternity was estimated using Cervus 3.0 and checked manually by exclusion. The observed heterozygosity enabled parental assignment by the exclusion of incompatible paternal and maternal genotypes in most cases.

DATA ANALYSIS

We considered two measures of reproductive success. **Total Reproductive Success** (Total RS) is the number of juveniles assigned to a particular parent; that is it corresponds to the total number of offspring that survived the egg and free embryo stages inside the mussel gill cavity and successfully emerged from their host mussel. **Relative Reproductive Success** (Relative RS) is a value standardized among groups and calculated as the proportion of offspring assigned to a particular parent in a particular group. The major difference is that the Relative RS of each individual is dependent only on competition with other members of the same sex and choice and compatibility with the opposite sex and controls for differences in fecundities and mortality rates among groups (i.e., it assumes that intergroup differences in mortality rates were caused by abiotic factors). The measure of Total RS assumes that parental quality and reproductive investment (e.g., egg survival due to maternal effects, sperm limitation) rather than abiotic factors caused intergroup differences in mortality rate.

The parentage dataset was used to calculate variation in the reproductive success of males and females as the opportunity for selection on each sex (I_{M}, I_{F}; variance in reproductive success of the given sex divided by the square of mean reproductive success of that sex) (Shuster and Wade 2003) for each replicate separately. The opportunity for sexual selection (I_{mates}) was calculated for each replicate as the sum of the differences between the opportunity for selection on each sex (I_{M}–I_{F}) (Shuster and Wade 2003).

For comparison of population characteristics between mussel spatial treatments, *t*-tests and Bartlett's test were used. A mixed-model ANCOVA with spatial treatment as a fixed factor, group identity as a random factor, and female fecundity as a covariate was used to compare embryo mortality between spatial treatments. Given the limited number of experimental groups (20), we additionally estimated the proportion of variation explained by treatment effect using Cohen's *r* and used power analysis based on the variation in our dataset to calculate the number of experimental groups that would be necessary to demonstrate a statistically significant effect at $\alpha = 0.05$ with a statistical power of at least 80%. Mixed-model ANCOVAs (group identity as random factor and Initial Size as covariate) with Tukey post hoc tests were used to compare phenotypic traits between spatial treatments. Satterthwaite's method was applied to calculate degrees of freedom for error terms in mixed-model analyses. Means are reported ± 1SE. Log_{10}, square root, and power transformations were applied to achieve normal data distributions and homogeneity of variance where appropriate.

Partial correlations among phenotypic traits were calculated. Traits that were strongly intercorrelated were removed from the matrix before further analysis to minimize multicollinearity. This procedure prompted exclusion of Red Area Difference (strongly correlated with Final Red Area), Parasite Species Richness (with Parasite Load), Final Size (with Growth), and Condition Factor (with Body Height) from regression analysis. Linear selection gradients (Lande and Arnold 1983) were calculated for the reduced trait matrix of both Total RS and Relative RS. Linear selection gradients are standardized coefficients (ß) from a multiple regression analysis and describe directional selection acting on a given trait. Our sample size did not permit calculation of nonlinear selection (using quadratic regression coefficients to describe stabilizing and disruptive selection) or correlational selection (interactions between traits) (Lande and Arnold 1983) due to a high number of potential interactions between parameters. We did not use Principal component analysis (PCA) to reduce the trait matrix. Although PCA is an efficient technique for dealing with intercorrelation of explanatory variables, demonstrating selection gradients on PC axes has many shortcomings (summarized in Mitchell-Olds and Shaw 1987); interpretation of component loadings in our dataset was problematic.

We used the approach of Chenoweth and Blows (2003) and Head et al. (2008) to test for significant overall patterns of selection and to compare the strength and direction of selection on phenotypic traits between mussel spatial treatments. The method compares the fit of three models; Model 1 contains only the treatment, Model 2 contains the treatment and all linear terms as covariates, and Model 3 contains the treatment, all covariates, and all treatment by covariate interactions. The relative fit of the three models was compared using a likelihood approach. Comparison of the relative fit of Model 2 against Model 1 determined whether linear selection occurred whereas comparison of Model 3 against Model 2 tested whether selection gradients differed between spatial treatments. Statistical analyses were performed using the R 2.4.0 statistical package (R Development Core Team 2006) and Statistica 6.0.

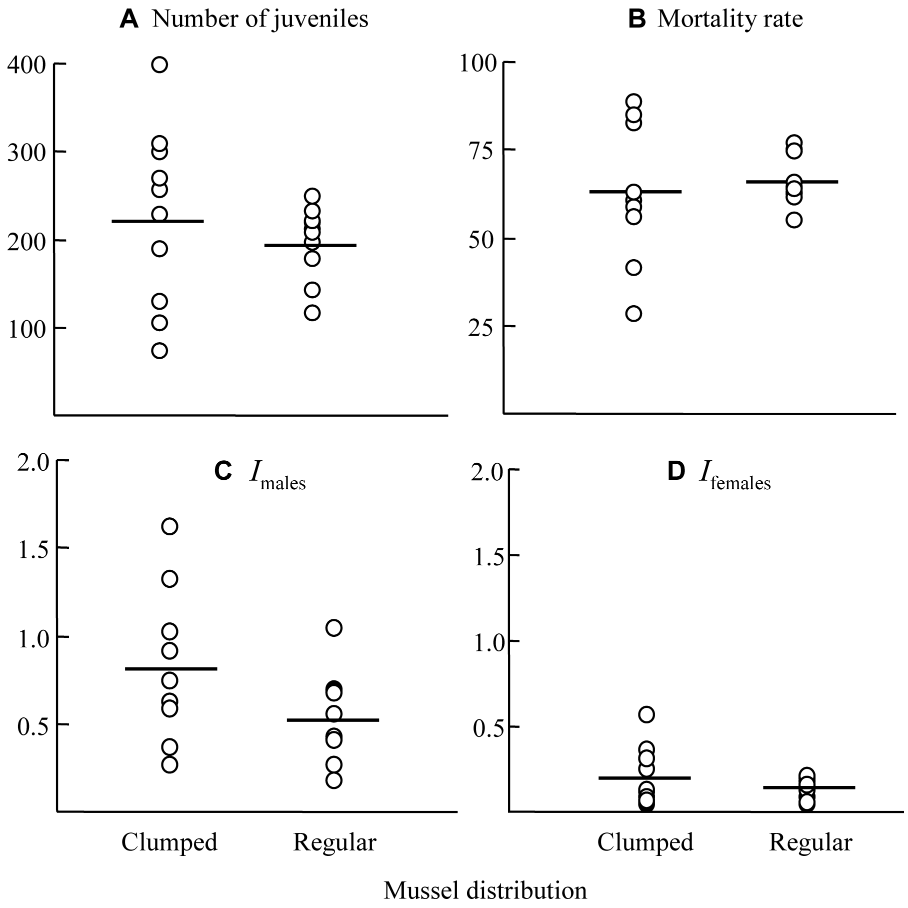

Figure 1. The effect of spatial resource distribution on (A) number of offspring released from mussels per group, (B) group-level mortality rate (combined fecundity of all females divided by the number of offspring released, in percentage), (C) variation in reproductive success among males and (D) females. Each dot represents one experimental group, horizontal lines are treatment means.

Results

POPULATION CONSEQUENCES OF RESOURCE DISTRIBUTION

We detected no difference in the number of juveniles released from mussels between mussel spatial treatments (t-test, $t_{18} = 1.12$, $P = 0.28$; Fig. 1A), with an overall mean of 203 ± 17.1 (range: 69–385) juveniles per experimental group. The treatment effect explained only 6% of variability (Cohen's $r = 0.24$) and power analysis showed that 43 groups of each treatment would be necessary to demonstrate the significant effect at $\alpha = 0.05$ with a statistical power of at least 80%. However, there was significantly higher variance in the number of juveniles released with a clumped compared to a regular mussel distribution (Bartlett's test, $\chi^2 = 6.61$, df = 1, $P = 0.010$). The estimated embryo mortality rate in mussels, calculated for individual females, did not differ between spatial distribution treatments (Mixed model ANCOVA, $F_{1,18} = 0.13$, $P = 0.719$) and female fecundity was not a significant covariate of mortality ($F_{1,56} = 3.01$, $P = 0.088$). However, group identity (a random factor) significantly affected offspring mortality ($F_{18,39} = 2.32$, $P = 0.014$). Population level embryo mortality ranged from 29.6 to 87.4% (mean 63.3 ± 2.0) and was significantly more variable with a clumped mussel distribution (Bartlett's test, $\chi^2 = 7.21$, df = 1, $P = 0.007$; Fig. 1B).

For our sample size of 10 experimental groups per treatment, the opportunity for selection on males was not statistically different between mussel spatial treatments ($t_{18} = 1.76$, $P = 0.096$; Fig. 1C), but the treatment explained 14% of variability (Cohen's $r = 0.38$). Twenty-five groups per treatment would be necessary to demonstrate a statistically significant effect at $\alpha = 0.05$ and power $\geq 80\%$. There was no difference in variance between treatments (Bartlett's test, $\chi^2 = 2.29$, df = 1, $P = 0.131$). The opportunity for selection on females did not differ between treatments ($t_{18} = 0.98$, $P = 0.34$; Fig. 1D) and explained only 5% of variability (Cohen's $r = 0.23$). A total 79 groups per treatment would be necessary to demonstrate a statistically significant effect at $\alpha = 0.05$ and power $\geq 80\%$. However, there was higher variance in the opportunity for selection on females exposed to a clumped compared to a regular mussel distribution (Bartlett's test, $\chi^2 = 7.86$, df = 1, $P = 0.005$). There was no relationship between the opportunity for selection on males or females or relationship between variance in male body size and offspring production with either mussel distribution (Pearson's correlation, all $P > 0.39$). Across treatments, the opportunity for selection was significantly higher on males than on females (paired $t_{19} = 5.72$, $P < 0.001$) with a mean $I_M = 0.64$ (±0.08) and mean $I_F = 0.15$ (±0.03). The opportunity for sexual selection was not different between spatial treatments ($t_{18} = 1.21$, $P = 0.24$), with a mean $I_{\text{mates}} = 0.49$ (±0.09). The treatment effect explained only 7.5% of variance in this variable (Cohen's $r = 0.27$) and 52 experimental groups per treatment would be necessary to demonstrate a significant effect at $\alpha = 0.05$ and power ≥ 80%.

INDIVIDUAL CONSEQUENCES OF RESOURCE DISTRIBUTION

All fish, except for one female, produced some offspring. Individual males sired 4–332 juveniles (95% confidence intervals 52–86), whereas females produced 18–151 juveniles (95% confidence intervals 61–80; female with no offspring excluded). The frequency distribution of total and relative RS of male and female bitterling is shown in Figure 2.

At the end of the experiment males from groups with a clumped mussel distribution had a higher Gonad Weight and Red Area ($P = 0.032$ and $P = 0.037$, respectively; Table 1). In females, there was no difference between treatments in any trait measured (Table 1). Growth was negatively related to Initial Size in males and females ($P < 0.001$; Table 1) and Gonad Weight, Final Red Area and Final Red Intensity were positively related to male Initial Size ($P < 0.05$; Table 1). Final and Initial Size were positively related in females, but not in males (Table 1).

SELECTION GRADIENTS

There was significant linear selection on male phenotypic traits; model 2 (treatment with phenotypic traits as covariates) explained significantly more variance than model 1 (treatment only) (deviance = 2532.3, df = 9, $P < 0.001$). The spatial distribution of mussels affected the strength and significance of selection gradients in males; model 3 (treatment, phenotypic traits as covariates and treatment by covariate interactions) explained significantly more variance than model 2 (deviance = 252.1, df = 9, $P = 0.01$). Male linear selection gradients for Total RS and Relative RS for each mussel distribution treatment are shown in Table 2. Generally, there was strong positive selection on male Initial Size in both treatments, but positive selection on Gonad Weight only with a clumped mussel distribution. Positive linear selection on Final Red Area was not significantly stronger with a clumped mussel distribution, although it did approach statistical significance ($P = 0.051$ and $P = 0.056$ for relative and total RS, respectively; Table 2).

There was significant linear selection on female phenotypic traits; model 2 (treatment and covariates) explained more variance than model 1 (treatment only) (deviance = 154.0, df = 5, $P < 0.001$). However, linear selection gradients were not different between spatial distribution treatments in females, because model 3 (treatment, covariates and treatment by covariates interactions) did not explain significantly more variance than model 2 (deviance = 78.5, df = 5, $P = 0.16$). Both Total RS and Relative

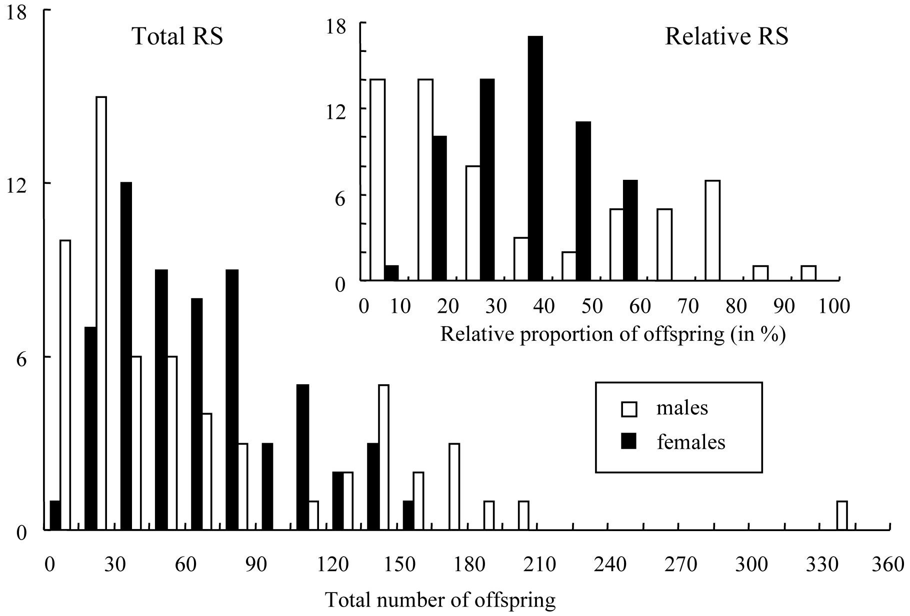

Figure 2. Total (total number of offspring produced) and relative (proportion, in percentage) reproductive success of individual male (open bars) and female (filled bars) bitterling over one entire breeding season.

Table 1. Results of mixed model ANCOVA on the effect of the spatial distribution of breeding resources (fixed factor) on (A) male and (B) female phenotypic traits measured at the end of the experiment, with initial body size as covariate. Group identity was used as a random factor. Difference (Diff) is based on Tukey post hoc comparisons that identified the direction and significance of difference between resource treatments.

	(A) Males				(B) Females			
	F	df	*P*	Diff	*F*	df	*P*	Diff
Final size								
Spatial	0.7	1,18	0.304		0.6	1,18	0.441	
Initial Size	2.6	1,57	0.111		31.1	1,50	<0.001	
Group	2.4	18,39	0.012		3.9	18,38	<0.001	
Growth								
Spatial	0.6	1,18	0.439		0.6	1,18	0.439	
Initial Size	362.4	1,57	<0.001		132.2	1,53	<0.001	
Group	2.1	18,39	0.029		3.2	18,38	0.001	
Body height								
Spatial	0.8	1,18	0.382		1.1	1,18	0.310	
Initial Size	1.5	1,57	0.225		1.0	1,53	0.330	
Group	2.5	18,39	0.008		1.1	18,38	0.410	
Gonad weight								
Spatial	5.4	1,18	0.032	Clu>Reg	3.7	1,18	0.070	
Initial Size	21.0	1,56	<0.001		1.6	1,56	0.210	
Group	2.5	18,39	0.073		1.8	18,38	0.062	
Final red area								
Spatial	5.1	1,18	0.037	Clu>Reg	*NA*			
Initial Size	11.1	1,53	0.001					
Group	1.2	18,39	0.073					
Final red intensity								
Spatial	0.0	1,18	0.987		*NA*			
Initial Size	12.6	1,48	0.001					
Group	0.6	18,39	0.857					
Total parasite load								
Spatial	0.3	1,18	0.591		3.2	1,18	0.093	
Initial Size	1.1	1,55	0.299		3.6	1,51	0.063	
Group	1.6	18,39	0.115		0.9	18,38	0.605	

RS of females were negatively related to Growth and positively related to Gonad Weight (Table 3).

Discussion

We experimentally investigated how the spatial distribution of oviposition sites affected offspring production, variation in reproductive success among individuals, and directional selection on phenotypic traits in the bitterling fish over their entire reproductive lifetime. We found that mean group offspring production and variation in reproductive success among individual males and females was not affected by resource distribution, although intergroup variation was higher with a clumped distribution. Although the number of experimental groups used in the analysis was limited (20), our results proved robust in the light of power analyses. To achieve a statistical power of at least 80%, more than 50 experimental groups per treatment would be required to demonstrate a statistically significant effect for all measures, except for the effect on the opportunity for selection on males, where a sample size of 25 experimental groups per treatment would be sufficient to detect a significant effect in the direction we predicted. Therefore, there was a weak tendency for a higher opportunity for selection on males with a clumped mussel distribution. This result was confirmed in the analysis of selection gradients, where more traits were under selection with a clumped distribution. No difference in the direction or strength of selection was detected on females.

We hypothesized that a clumped resource distribution may decrease overall offspring production at the population level. This effect was predicted as a consequence of male–male competition over possession of breeding sites. Intense aggression between males can directly interfere with courtship (Spence and Smith

Table 2. Linear selection gradients (standardized regression coefficients) on male phenotypic traits with respect to the Relative Reproductive Success (related to production of offspring within a particular group) and Total Reproductive Success (absolute number of offspring produced by individual males) with regular and clumped resource distributions. Regression analysis is based on 30 males in each treatment and used a normalized distribution, with group identity entered as random factor.

Phenotypic trait	Regular distribution				Clumped distribution			
	Relative RS		Total RS		Relative RS		Total RS	
	β±SE	*P*	β±SE	*P*	β±SE	*P*	β±SE	*P*
Initial size	0.99±0.29	0.005*	0.56±0.23	0.037*	0.57±0.26	0.049*	0.75±0.23	0.009*
Growth	0.40±0.29	0.194	−0.10±0.24	0.699	−0.04±0.23	0.875	0.05±0.21	0.815
Body height	0.12±0.12	0.335	0.07±0.10	0.520	0.13±0.09	0.168	−0.04±0.10	0.728
Gonad weight	0.40±0.22	0.104	0.16±0.19	0.417	0.25±0.10	0.032*	0.23±0.10	0.043*
Initial red area	0.12±0.11	0.287	0.08±0.09	0.353	0.06±0.13	0.641	−0.02±0.11	0.851
Final red area	0.02±0.13	0.893	−0.05±0.12	0.656	0.30±0.14	0.051^	0.27±0.13	0.056^
Initial red intensity	−0.22±0.13	0.126	−0.14±0.11	0.225	−0.01±0.12	0.955	0.11±0.12	0.369
Final red intensity	−0.10±0.09	0.277	−0.02±0.07	0.838	−0.21±0.15	0.196	−0.10±0.13	0.443
Total parasite load	0.04±0.12	0.738	−0.06±0.10	0.580	−0.03±0.09	0.742	−0.01±0.09	0.932

*$P < 0.05$; ^$P < 0.10$.

2005; Watters 2005), often resulting in females leaving breeding sites (Smith et al. 2006). Alternatively or additionally, territorial males may refuse to spawn when the risk of sperm competition is high (Alonzo and Warner 1999; Le Comber et al. 2003), which may ultimately lead to decreased egg production at the population level (Alonzo and Warner 2000; Smith et al. 2006). Our prediction was based on a previous observation that time to spawning was higher and daily egg production significantly decreased as a consequence of oviposition site distribution (Reichard et al. 2004b). Two reasons may explain why decreased offspring production was not observed in clumped resource distributions over a longer temporal scale in the present study. First, given that bitterling embryo mortality inside mussels is density dependent (Smith et al. 2000), a decrease in spawning rate may result in an improved survival of already deposited eggs residing in mussels, with an attenuating effect on subsequent recruitment. Second, territorial aggression and courtship interference would not necessarily be elevated with a clumped mussel distribution over a longer time interval if dominance rank was settled within the first few days of the experiment.

Table 3. Linear selection gradients (standardized regression coefficients) on female phenotypic traits with respect to Relative Reproductive Success (related to offspring production within a group) and Total Reproductive Success (absolute number of offspring produced by individual females). Regression analysis was based on 59 females and used a normalized distribution, with group identity entered as a random factor.

Phenotypic trait	Relative RS		Total RS	
	β±SE	*P*	β±SE	*P*
Initial size	−0.03±0.20	0.888	−0.29±0.17	0.096
Growth	−0.45±0.20	0.029*	−0.74±0.18	<0.001*
Body height	−0.07±0.12	0.578	−0.03±0.10	0.766
Gonad weight	0.27±0.12	0.027*	0.36±0.10	0.002*
Total parasite load	0.14±0.12	0.249	0.17±0.10	0.104

*$P < 0.05$; ^$P < 0.10$.

Although mean offspring production did not differ between treatments, variation in offspring production was considerably higher with a clumped distribution (Fig. 1). If the relationship between territorial aggression and offspring production is valid, the clumped resource distribution treatment may have contained groups with variable levels of territorial aggression. Territoriality may have been stable and the aggression level low in some groups with a clumped mussel distribution resulting in high offspring production, but ongoing male–male disputes over unresolved dominance may have decreased spawning rate in others. Under this scenario, the most successful males in groups with well-established dominance were predicted to have higher reproductive success than in groups where dominance was continually contested. Because behavior was not recorded during the present study for logistical reasons, it was impossible to directly determine whether territorial dominance had a positive effect on offspring production. However, there was no relationship between variation in reproductive success or body size among males and offspring production, discounting the negative effect of male–male interference competition on offspring production. Improved survival of spawned eggs and the failure of male aggression to have an impact on female spawning over a longer time interval remain equally plausible explanations for the observed pattern and more research is needed to address their respective significance.

Individual reproductive success varied considerably among males and females, but all fish, except for one female, produced some offspring. The proportion of successfully breeding individuals, especially males, may be dramatically low in some polygynous mating systems. Only 28% of adult males achieved any fertilization and over 90% of juveniles were sired by dominant, harem-holding males in elephant seals, *Mirounga leonina* (Fabiani et al. 2004). In contrast, differences in lifetime reproductive success among individuals of the monogamous California mouse, *Peromyscus californicus*, were minimal in males and females (Ribble 1992). In the present study the Relative Reproductive Success of the most successful males varied from 47% to 92% (Fig. 2). Assuming that an equal share of paternity among the three males in an experimental group results in a 33% of the Relative Reproductive Success, the observed variation is relatively high. It is likely that in nature, where a greater number of fish interact, interindividual variation in reproductive success may be even higher.

Sperm competition probably affected variation in reproductive success among males. Unlike in socially monogamous mating systems in which sperm competition increases sexual selection intensity through extra-pair matings (Webster et al. 2007), its consequences for the opportunity for selection on males in species with polygynous or promiscuous mating systems are less predictable (Jones et al. 2001a). This is because sperm competition is often associated with alternative male mating behavior (Taborsky 1998) and, especially when female preference for specific males is strong, sperm competition may actually decrease the intensity of sexual selection (Reichard et al. 2004a). In bitterling, dominant males constrain opportunities for female choice through resource monopolization and females, despite being choosy, have limited control over the paternity of their offspring (Reichard et al. 2005). Female bitterling engage in behavior that increases the intensity of sperm competition (Smith et al. 2007) and it is hypothesized that this behavior may be a form of cryptic female choice under dominant male harassment (Smith and Reichard 2005). Therefore, the lack of a strong effect of breeding resource aggregation on the variation in reproductive success among males as predicted by Shuster and Wade (2003) might be a consequence of the expression of alternative mating behaviors, which are well described in bitterling (Smith et al. 2004).

As anticipated, variation in reproductive success among females was lower than that of males and no female contributed more than 59% of offspring to recruitment within an experimental group, equivalent to 151 juveniles released from mussels. One female did not produce any offspring and that female was the smallest fish in our experiment, with an Initial Size of 23.3 mm. It is possible that the female was not sexually mature and did not ovulate during the experiment. Apart from sex-role reversed species, females are typically not considered to be under strong sexual selection (Andersson 1994), except from mate selection in monogamous taxa (Jones et al. 2001b) and under strong female biases in the OSR (Forsgren et al. 2004), which was not applicable to our study system.

We found that variation in reproductive success among individual males and females was not significantly different with respect to the resource distribution treatment. Variation in reproductive success is an essential prerequisite for selection to occur but selection may favor different phenotypic traits under different conditions. Indeed, our data revealed that resource distribution treatments differed in the strength of selection on different phenotypic traits in males. Stronger directional selection on male Gonad Weight and Red Area was detected with a clumped mussel distribution (Table 2) and this also resulted in higher absolute measures of those traits for individual males (Table 1).

Greater investment into Gonad Weight is associated with increased sperm competition risk (Byrne et al. 2002) and intensity (Hosken and Ward 2001) and our results are consistent with this prediction. Our measure of investment in gonad tissue was estimated after the end of the reproductive season and, therefore, represents the residual mass of gonad tissue. If gonad mass was measured during the breeding season, the recorded value could be affected by a reduction of gonad mass through acute sperm depletion (Linklater et al. 2007), that is common in some fish (Warner et al. 1995), including bitterling (Smith and Reichard 2005; Pateman-Jones 2007). The positive association between individual male reproductive success and Red Area in the iris and absolutely higher values of Red Area at clumped mussel distributions are consistent with our predictions. The Red Area is an index of dominance in the bitterling (Reichard et al. 2005) and selection on redness is strongest when dominance is continuously challenged (Candolin 1999; Casalini et al. 2008).

These results show that when breeding resource distribution was limited to a single territory, a single male dominated the resource, although his position was challenged by other males, either directly (as indicated by selection on Red Area) or indirectly through sperm competition (selection on Gonad Weight). With a regular resource distribution there was the potential for each male to establish his own territory (Reichard et al. 2004b; Reichard et al. 2008), although some males may have failed to establish territories in some experimental replicates. Some bitterling males may never become territorial, even if the number of vacant territories greatly exceeds the number of males (Smith et al. 2004). Further, territory ownership may be temporary with dominant males sometimes taking control of adjacent territories, because territorial quality varies during the breeding season because it decreases with the number of eggs already present in mussel gills (Smith et al. 2001).

Positive linear selection on male Initial Size was consistent across resource treatments and confirms the predominant role of male size on sexual selection in bitterling (Candolin and Reynolds 2001; Smith et al. 2002; Reichard et al. 2005) and in other mating systems (Andersson 1994; Seamons et al. 2007). Linear selection on female traits was not affected by resource distribution and strong positive selection on Gonad Weight, with a trade-off between Growth and reproductive success, was observed. Investment in gonad tissue is positively related to fecundity in fish (Wootton 1998) and strong negative selection on Growth agrees with a prediction for annual species with an extended breeding season (Reznick 1983), as is the study population. Given that bitterling populations in Southern Europe and Asia Minor likely reproduce for up to five breeding seasons (Tarkan et al. 2005), it would be valuable to compare the strength of the fecundity–growth trade-off among populations with contrasting prospects of future reproduction (Reznick 1983; Candolin 1998).

One component of lifetime reproductive success is survival over a reproductive season and our experimental design probably underestimated its importance; natural mortality of parental fish from predators was excluded for ethical reasons, and because recreating a sufficiently natural predatory regime was impractical. A higher mortality risk from predation is expected during courtship displays (Andersson 1994) and there is empirical evidence of this prediction in some taxa (Houde and Endler 1990; Quinn et al. 2001). In the bitterling, all males including small nonterritorial sneakers court females (Smith et al. 2002) and courting greatly increases their visibility and probably the risk of detection by aquatic and avian predators. At present we have no data on variation in mortality risk among males or between males and females. Levels of courtship activity by territorial males are significantly higher than nonterritorials (Reichard et al. 2004b) and predation risk could offset strong selection on male dominance traits (Reichard et al. 2005).

In conclusion, we found a significant effect of the spatial distribution of breeding resources on selection for male phenotypic traits. Greater investment in gonad tissue and the extent of red, carotenoid-based pigment in the iris in males was matched with higher reproductive success in those experimental groups with a clumped mussel distribution that allowed formation of a single territory. Males maintaining strong dominance over rivals and success in sperm competition enjoyed greater reproductive success. In groups with a regular mussel distribution, with the potential for each male to establish his own territory, variability in reproductive success among males was, surprisingly, not significantly lower than that in a clumped mussel distribution, but selection on male initial body size was stronger and there was no selection on gonad mass and red coloration. No effects of resource distribution on demographic parameters were detected.

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