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Population and individual consequences of breeding resource availability in the European bitterling (*Rhodeus amarus*)

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Abstract Resource availability may affect both individual fitness and population demography and the effects can interact. We used two experiments to test how breeding resource abundance and its spatial distribution, combined with female abundance, affected male reproductive behavior, population spawning rate, and embryo development and recruitment in the European bitterling (*Rhodeus amarus*), a small cyprinid fish that lays its eggs in living unionid mussels. In the first experiment, we found that at the population level the abundance of breeding resources (freshwater mussels) was more important for bitterling recruitment than resource spatial distribution (clumped or regular). In contrast at the individual level, (variability in reproductive success) the spatial distribution of resources was more important, but only when resource abundance was not limiting. Territorial males obtained almost exclusive access to fertilizations when resources were abundant and distributed regularly, but were unable to defend large clusters of resources (when rival abundance was always high) and abandoned territoriality. Surprisingly, territorial males remained aggressive and successfully defended their territories when resources were grouped into a single cluster, but at a low abundance. In the second experiment,

more rapid embryo development and larger juvenile body size at the end of the growing season was detected at high resource abundance and low female abundance, indicating that early hatched juveniles survived better and hence investment in offspring production early in the season yields a higher fitness pay-offs. The abundance of females in spawning condition was the best overall predictor of the intensity of male reproductive behavior in both experiments.

Keywords Acheilognathinae · Demography · Recruitment · Sexual selection · Variation in reproductive success

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Introduction

The abundance and distribution of resources critical to reproduction are fundamental determinants of mating systems (Emlen and Oring 1977). When resources are rare they are likely to be controlled by a small number of individuals, resulting in a bias in reproductive success among individuals with and without access to such resources. Typically, resources are guarded by males, who establish territories around resources, and thereby enjoy superior reproductive success by attracting females to mate in their territories. A bias in reproductive success (reproductive skew) is generally predicted to increase with the scarcity of resources (Emlen and Oring 1977; Shuster and Wade 2003). However, recent theoretical and empirical research suggests that extreme resource scarcity leads to resource monopolization being uneconomical since the cost of aggressive defense against constant intrusions from other competitors may exceed the benefits of holding that resource or make such a resource indefensible. This situation leads to a dome-shaped rather than linear relationship between resource scarcity and fitness variation

(Grant et al. 2000; Dubois and Giraldeau 2005; Noël et al. 2005). Resource scarcity may be manifested by its low abundance or high clustering. Both low abundance and high clustering of a resource may independently result in small number of territories available for reproduction and hence may have a similar effect on the success of individual males.

At the population level, however, the effects of resource abundance and resource clustering may be different. Egg production and recruitment may be a function of resource abundance regardless of its spatial distribution (Newton 1994). Hence, egg production and recruitment are often not linked to the number of territories, but to the overall amount of the resource (Sutherland 1996). On the other hand, behavioral interactions arising from resource clustering are known to have significant consequences at the population level. For example, in the strawberry poison frog, *Dendrobates pumilio*, nursery sites may be limiting to female reproduction. Females concentrate around nursery sites, and males focus their activity in areas where female density is highest. Males compete for sites with the highest female density, which thereby determines male reproductive success (Pröhl and Berke 2001). Similarly, aggressive male-male territorial disputes decreased population rate of egg production in an experimental populations of the European bitterling (*Rhodeus amarus*; Reichard et al. 2004a) and zebrafish (*Danio rerio*; Spence and Smith 2005). In a cavity-nesting duck (*Bucephala albeola*) territorial behavior and not nest site availability was shown to limit breeding density (Gauthier and Smith 1987).

Resource abundance, female abundance, and its relative ratio may also have consequences arising from egg and embryo crowding during development (Beekey and Karlson 2004; Kitamura 2005). The competition for limiting supplies of space, oxygen, or other key resources for embryo development is often strong (Kamler 1992; Ahnesjö 1996). The lack of such resources can inflict direct mortality or limit developmental rate, resulting in slower rates of development and smaller juvenile size (Kamler 1992; Payne et al. 2002; Beekey and Karlson 2004; Barrett et al. 2009).

Here, we investigated how the abundance and spatial arrangement of resources critical to reproduction, in association with female abundance, affected the spawning rate, duration of development, and recruitment in the European bitterling (*R. amarus*), a small cyprinid fish. We also investigated the underlying behavioral mechanisms for these effects.

European bitterling have a resource-based mating system; females lay their eggs in the gills of living freshwater mussels. During the reproductive season (April to June, with a peak during a 3-week interval in late April-May), male bitterling compete aggressively for mussels and successful males establish territories in the immediate proximity of mussels (single or a cluster of several), court females and lead them to

their territories to spawn. Females with ovulated eggs develop a long flaccid ovipositor that they use to place their eggs inside the mussel gill cavity. Females undergo repeated cycles of spawning activity (five to ten during the reproductive season), and during each spawning cycle they lay ten to 50 eggs in three to 15 clutches. A seasonal female fecundity varies from 80 to 250 eggs per year (Smith et al. 2004; Reichard et al. 2009). Mussels differ in their quality for developing bitterling embryos. Mussels of certain species and those that contain smaller number of bitterling embryos are superior hosts (Smith et al. 2000b). Most embryo mortality stems from asphyxiation and ejections by host mussels (Kitamura 2005). Male and female bitterling are able to detect differences in mussel quality and alter their spawning decisions accordingly (Smith et al. 2001). Many males (typically, though not exclusively, smaller) do not establish their own territories and engage in sneaking (i.e., non-territorial) behavior by releasing sperm into a mussel guarded by another male. Male mating behavior is opportunistic; all males may become territorial or sneak, and frequently switch between these tactics (Smith et al. 2004). Territoriality confers higher fitness at low and intermediate levels of competition, but may break down at a high male density when the fitness of territoriality is no higher than that of a non-territorial tactic (Reichard et al. 2004a). Bitterling do not form pairs and provide no parental care. Embryo development is completed inside the mussel gills and lasts 4–6 weeks (depending on ambient temperature and oxygen availability; Smith et al. 2004). Juveniles emerge at a body size (standard length, SL) of approximately 10 mm (Aldridge 1999) and are immediately capable of active swimming and feeding (Smith et al. 2004).

Previous research on the individual and population consequences of reproductive behavior in bitterling has concentrated on the effects of male density and sex ratio (Mills and Reynolds 2003; Reichard et al. 2004a, b), temporal pattern in female reproductive activity (Reichard et al. 2008) and the spatial distribution of resources (Przybylski et al. 2007; Reichard et al. 2009). The present study builds on these previous findings. We examined several hypotheses arising from these earlier studies and extended the scope of possible population consequences of behavioral interactions from data on egg production (Reichard et al. 2004a) and juvenile emergence from mussels (Reichard et al. 2009), to recruitment measured as juvenile abundance at the end of the growing season. Specifically, we tested the following predictions:

1. Spawning rate is constrained by a limitation in resource abundance with a regular resource distribution, but not a clumped distribution. This pattern arises through a failure of males to defend territories when the availability of territories is severely limited (Reichard et al. 2004a) and

assumes a dome-shaped rather than linear relationship between territory availability and reproductive skew. Accordingly, we predicted a decrease in aggression with a clumped resource distribution (when territoriality collapses), with a stronger effect when resources are abundant since there is a greater opportunity for males to defend a smaller cluster of mussels.

2. Territorial aggression is more pronounced at low resource abundance and low female density, and territorial behavior confers higher reproductive success with a regular resource distribution (due to a failure to control resources when they are distributed in a single clump) and low female abundance (when there is better opportunity to control females).

3. The higher female-to-resource ratio (and hence more bitterling embryos laid per mussel) increases the duration of embryo development through a density-dependent effect on developmental rate (Kamler 1992; Payne et al. 2002). The slower development is manifested in a later emergence of bitterling juveniles with high female and low resource abundance compared to low female and high resource abundance, with consequences for subsequent juvenile growth and recruitment.

Material and methods

Experiment 1

The experiment was conducted in a large outdoor concrete pool at the Institute of Vertebrate Biology (IVB), Brno, Czech Republic from 26 April to 19 May 2007. The pool measured 12.4×6.0 m and was filled with water to a depth of 60 cm. Fish used in this experiment were from a stock collected in the River Kyjovka (Danube Basin, Czech Republic). All experimental fish were overwintered in facilities at the IVB in 1,000-L holding tanks with a sand substrate, artificial plants, aeration, and an external filter to maintain water quality. The tanks were exposed to natural light and temperature fluctuations, except that the temperature was never allowed to fall below 4°C. Throughout overwintering, fish were fed with the mixture of frozen chironomid larvae, *Cyclops* spp. and commercial flake food. Mussels (*Unio tumidus*) used in this experiment were collected in Spring 2007, before the beginning of the bitterling spawning season, from a small oxbow lake adjacent to the River Kyjovka and kept in a garden pond until their use in the experiment. Mean (± SE) mussel size was 71.7±0.7 mm (range 50–93 mm, n=192).

A total of 30 male and 60 female bitterling were stocked in the pool in mid-April and allowed to settle. After

10 days, 12 observation arenas were designated at regular distances along three walls of the pool. Arenas were visually separated by clumps of artificial plants. The fourth wall was left without mussels to avoid fish disturbance while entering the pool for observations. Each day of the experiment, mussels were positioned according to one of four treatments. Either four or 12 mussels (low and high resource abundance, respectively) were positioned in sand-filled plastic pots at different arenas, or clumped in a single arena (regular and clumped resource distribution, respectively). Observations were completed over a total of 24 days. Water temperature varied naturally between 15 and 21°C during the morning observation period and 17-26°C during the afternoon observation period. Fish were fed daily with frozen chironomid larvae.

On each day of the experiment, before behavioral observations began, the number of arenas occupied by territorial males was counted. One occupied territory was then randomly chosen for observation. A diver recorded fish behavior in the chosen territory for 20 min twice each day (morning at 08:00, afternoon at 15:00) using an underwater video camera. After the afternoon observation, the diver censused females in spawning condition (clearly identifiable by an extended ovipositor) in the pool. The territory holder from the observed arena was captured and replaced by another size-matched male. The captured male was measured (standard length), placed in a stock tank and not used again in the experiment. Mussels were collected, measured, and dissected to count the number of bitterling eggs in their gills for the population level analysis. The next set of mussels was placed in the pool according to a randomly predetermined order.

Experiment 2

Experiment 2 was conducted in 30 large fiberglass tanks (130×130 cm) at the IVB from 26 April to 6 November 2007 and from 10 April to 31 October 2008. All experimental fish originated from the River Kyjovka. Tanks were situated in the garden of the IVB and filled to a depth of 60 cm with tap water. To facilitate capture of juvenile fish, a fine mesh (mesh size 1 mm) was used to line the inside of each tank. Tanks were furnished with a thin layer of sand and six artificial plants were placed at fixed positions to create refuges for fish.

Four experimental treatments were used. Resource abundance was either low (two mussels) or high (ten mussels), and female abundance was also either low (two females) or high (ten females). Female-to-resource ratio was 0.2 (2:10), 1 (2:2, 10:10), or 5 (10:2). All tanks contained five males. Therefore, by manipulating female density, we also manipulated population sex ratio and adult density (seven or 12 fish). Mussels (*U. tumidus*) in sand-filled plastic pots were placed in the tanks in a regular

pattern. Mussel collection and maintenance prior to this experiment was identical to experiment 1.

Each tank was stocked with two females (large and small) and two males (large and small) from our stock of wild fish housed at the IVB. In addition, we used fish collected from the River Kyjovka 14 days prior to experiment. Hence, all tanks additionally contained three medium-sized males and tanks with a high female abundance treatment additionally contained eight medium-sized females. This design generated a natural size distribution of males and females in replicates. The 95% confidence intervals for fish SL was 32.0–34.7 mm and 33.2–36.4 mm for small males in 2007 and 2008, respectively, 38.9–41.0 mm and 39.3–41.6 mm for medium-sized males, 52.3–56.5 and 45.0–47.6 mm for large males, 33.8–36.3 mm and 32.4–35.2 mm for small females, 39.3–41.2 mm and 37.5–39.2 mm for medium-sized females, and 45.7–54.8 mm and 40.3–44.3 mm for large females. Fish were fed every day with frozen chironomid larvae. After approximately 3 weeks fish, were additionally fed on algae and invertebrates that established in the tanks. Water temperature and photoperiod varied naturally.

Observation of fish behavior in tanks was conducted from 27 April to 3 May 2007. An observer recorded specific behaviors (territorial male aggression, territorial and sneaker male sperm release, territorial male courtship, female skimming, and spawning) for 15 min. between 13:00 and 16:00 using a palm computer with the FIT-system behavior-recording software (Held and Manser 2005). A total of 20 randomly selected tanks were observed.

Once juveniles appeared, tanks were censused every fortnight (first census on 19 June 2007 and 9 June 2008). Juveniles were categorized into four classes—small (10–15 mm), medium (15–20 mm), large (20–25 mm), and very large (<25 mm). To keep interference with the tanks to a minimum, fish were collected with a plastic jar with the net lining the tank raised and separated into one of the four size categories. After the census, juvenile fish were released back into the tank. A total of 11 censuses were completed over the season. All juveniles were measured to the nearest 1 mm during the last census in 2008.

Experiment 2 was conducted over 2 years, with 15 replicates completed each year. From the original target of 20 replicates (five per treatment), fish in five replicates suffered from infection by white spot disease (*Ichthyophthirius multifillis*), and were excluded from the analysis. Hence, an additional 15 replicates were completed in 2008, yielding seven or eight replicates per treatment.

Data analyses

In experiment 1, the relationship between mussel size and the number of eggs inside their gills was examined using a Pearson correlation. Wilcoxon paired tests were used to compare behavioral data from the morning and afternoon observations. Given that there was no difference in any of the behaviors recorded between the two periods (Table 1), data collected within the same day were pooled for all subsequent analyses.

General linear models with full factorial design (factors were resource abundance and resource distribution) were used to analyze the data. Data were compared using analysis of covariance (ANCOVA), with the number of females in spawning condition (estimated as the number of females with an extended ovipositor during behavioral observation) as covariate. Mussel size was an additional covariate in analyses involving the number of eggs. For male behavior, six behaviors were recorded (territorial male aggression towards non-territorial males, non-territorial male aggression towards a territorial or non-territorial male, territorial male sperm release, non-territorial male sperm release, territorial male leading of a female towards a mussel, and non-territorial male leading of a female towards a mussel; for a full description of each behavior, see Reichard et al. (2004a) and Smith et al. (2004). Additionally, overall male aggression in the population was calculated as the sum of territorial and non-territorial aggression. To compare the rates of territorial and non-territorial sperm releases, an ejaculation index (E_i, the proportion of sperm releases by a territory holder from the total number of sperm releases counted at the focal territory) was used (Reichard et al. 2004b). In the case of a clustered mussel distribution, the whole cluster was considered as one territory for analysis of male aggression, because territorial males always guarded the entire cluster of mussels. For analysis of sperm release rate and leading behavior, only one mussel from the cluster was randomly chosen for analysis. No change in direction of the effect and statistical significance of the results was found when sperm releases over all mussels in the cluster were analyzed and

Table 1 Results of comparison of behavioral rates during morning (8:00) and afternoon (15:00) observations using a Wilcoxon paired test

Behavior	N	T	z	P
Total aggression	24	77.0	0.72	0.469
Territorial aggression	24	75.0	0.80	0.421
Non-territorial aggression	24	48.5	0.25	0.802
Territorial sperm release	24	69.5	1.03	0.305
Non-territorial sperm release	24	88.0	0.28	0.778
Ejaculation index	13	14.0	1.01	0.314
Territorial leading	24	46.0	0.41	0.683
Non-territorial leading	24	21.0	1.07	0.286

divided by the number of mussels; i.e., the mean sperm release rate per one mussel. Log_{10} and square root transformations were employed to normalize data distributions when they deviated from normality. In cases where a behavior was rare, data followed a Poisson distribution and GLZ with a Poisson distribution were used.

In experiment 2, resource abundance and female abundance were manipulated in a full factorial design. A total of 30 replicates were completed over 2 years; seven replicates were completed in low mussel-low female abundance and high mussel-high female abundance (both with three replicates in 2007, four in 2008) and eight replicates were completed for low mussel-high female abundance (five in 2007, three in 2008) and high mussel-low female abundance (four in 2007, four in 2008). Given the effects of inter-annual variability in fish, and environmental conditions on fish recruitment, data on juvenile abundance were standardized within years for the analysis by subtracting the mean from each value and dividing it by the standard deviation. Juvenile abundance was tested using repeated measures ANOVA, with 11 consecutive censuses as repeated measures. Behavioral data were tested by ANCOVA with the number females in spawning condition (estimated as the number of females with extended ovipositor during behavioral observations) as covariate, and resource and female abundance as factors.

In addition to conventional statistical significance, we report effect size. The effect size measures the magnitude of a treatment effect independent of sample size (Nakagawa and Cuthill 2007). We used eta-squared (η^2), which represents the proportion of the total variance that is attributed to the treatment effect and is calculated as a ratio of the effect variance to the total variance. Small, medium and large effects for η^2 are defined as 0.01, 0.058, and 0.20 (Cohen 1988). All statistical analyses were conducted in Statistica 8. We used eta-squared values that were calculated manually from mean square values and not partial eta-squared as presented in Statistica output.

Results

Experiment 1

The total number of eggs laid per day ranged from 98 to 310 and was higher with the high mussel abundance ($F_{1,18}=9.9$, $P=0.006$, $\eta^2=0.32$), but there was no difference between a clustered and regular mussel distribution ($F_{1,18}=1.1$, $P=0.315$, $\eta^2=0.03$) and no significant interaction between spatial distribution and density ($F_{1,18}=0.6$, $P=0.439$, $\eta^2=0.02$; Fig. 1a). The number of females with an extended ovipositor ($F_{1,18}=1.7$, $P=0.211$, $\eta^2=0.05$) and mussel size ($F_{1,18}=0.01$, $P=0.937$, $\eta^2<0.01$) were not

significant covariates. The number of eggs laid in an individual mussel ranged from 0 to 84 and was positively correlated with mussel size (Pearson correlation: $r=0.236$, $n=192$, $P=0.010$). More eggs per mussel were laid at low mussel abundance ($F_{1,186}=50.2$, $P<0.001$, $\eta^2=0.20$) and when mussels were clustered ($F_{1,186}=5.8$, $P=0.017$, $\eta^2=0.02$), but there was no significant interaction between the two factors ($F_{1,186}=0.001$, $P=0.983$, $\eta^2<0.01$; Fig. 1b). Mussel size was a significant covariate ($F_{1,186}=15.1$, $P=0.001$, $\eta^2=0.06$), while the number of females with extended ovipositors was not ($F_{1,186}=0.2$, $P=0.636$, $\eta^2<0.01$). The rate of territorial aggression did not vary across treatments (log_{10} transformed data, resource abundance: $F_{1,19}=1.1$, $P=0.301$, $\eta^2=0.04$; resource distribution: $F_{1,19}=3.5$, $P=0.076$, $\eta^2=0.11$; interaction: $F_{1,19}=3.7$, $P=0.069$; $\eta^2=0.12$; Fig. 2a), but did depend on the number of females in spawning condition (covariate: $F_{1,19}=5.0$, $P=0.038$, $\eta^2=0.15$). The aggression of non-territorial males was higher at high resource abundance (log_{10} transformed data, $F_{1,19}=10.2$, $P=0.005$, $\eta^2=0.21$) and with a clustered distribution ($F_{1,19}=5.9$, $P=0.025$, $\eta^2=0.12$). A significant interaction showed that the aggression of non-territorial males was higher when 12 mussels were clustered (interaction: $F_{1,19}=8.7$, $P=0.008$, $\eta^2=0.18$; Fig. 2b). The number of females in spawning condition was a significant covariate ($F_{1,19}=5.6$, $P=0.028$, $\eta^2=0.11$). Overall male-male aggression did not vary across treatments (log_{10} transformed data; resource abundance: $F_{1,19}=0.5$, $P=0.497$, $\eta^2=0.02$; resource distribution: $F_{1,19}<0.1$, $P=0.850$, $\eta^2<0.01$; interaction: $F_{1,19}<0.1$, $P=0.867$, $\eta^2<0.01$; Fig. 2c), but was dependent on the number of females in spawning condition (covariate: $F_{1,19}=4.9$, $P=0.039$, $\eta^2=0.20$).

The rate of sperm release of territorial males was higher with a regular mussel distribution (Poisson distribution, $\chi^2=173.0$, df=1, $P<0.001$), but there was no effect of mussel abundance ($\chi^2=1.2$, df=1, $P=0.281$) and no interaction ($\chi^2=2.9$, df=1, $P=0.091$; Fig. 2d). The number of females in spawning condition was a significant covariate ($\chi^2=5.1$, df=1, $P=0.024$). Mean non-territorial male sperm release over a single mussel was not directly affected by mussel abundance (Poisson distribution, $\chi^2=1.5$, df=1, $P=0.220$) or mussel spatial distribution ($\chi^2=0.2$, df=1, $P=0.694$), but a significant interaction ($\chi^2=15.7$, df=1, $P<0.001$) showed that sperm releases by non-territorial males increased with resource abundance with a clustered mussel distribution, but decreased with a regular distribution (Fig. 2e). Non-territorial male sperm release increased with the number of females in spawning condition (covariate: $\chi^2=17.1$, df=1, $P<0.001$). The E_i was not directly affected by resource abundance (square root-transformed data: $F_{1,19}=0.1$, $P=0.783$; $\eta^2<0.01$) or mussel distribution ($F_{1,19}=4.3$, $P=0.052$; $\eta^2=0.012$), but there was a significant interaction $F_{1,19}=10.0$, $P=0.005$; $\eta^2=0.28$), which revealed differences with a high resource

Fig. 1 Total number of eggs (**a**) and number of eggs per one mussel (**b**) laid by an experimental bitterling population during a 24-h period
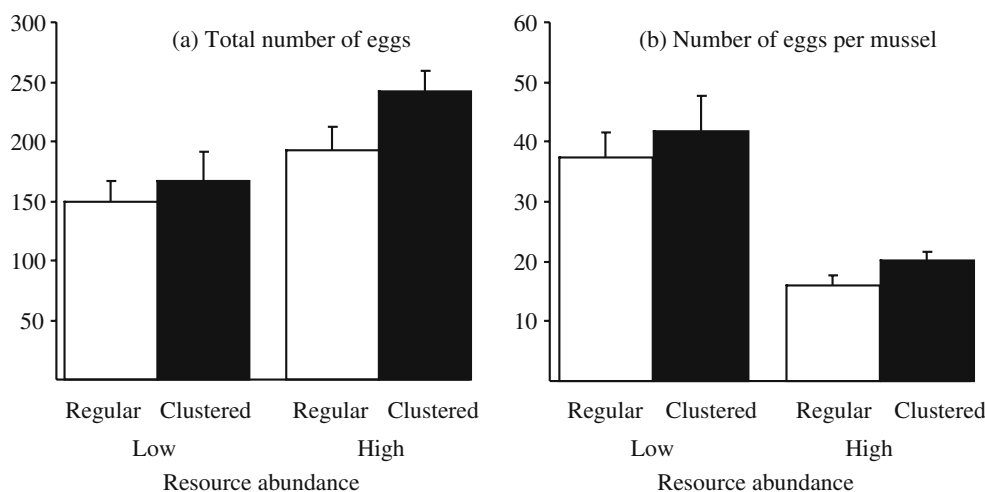
abundance, where territorial males gained the highest E_i with a regular resource distribution and lowest with a clumped distribution (Fig. 3f). The effect of the number of females in spawning condition was not significant (covariate: $F_{1,19}=2.4$, $P=0.136$; $\eta^2=0.07$).

Territorial male leading rate was higher with a regular mussel distribution (Poisson distribution, $\chi^2=68.7$, df=1, $P<0.001$) and high mussel abundance ($\chi^2=5.4$, df=1, $P=0.021$), though there was no significant interaction ($\chi^2=0.2$, df=1, $P=0.669$). More leading behavior was recorded with more females in spawning condition present (covariate: ($\chi^2=18.8$, df=1, $P<0.001$). Non-territorial leading was rare except with a high resource abundance and a clustered mussel distribution (Poisson distribution, resource abundance: $\chi^2=2.2$, df=1, $P=0.134$, resource abundance: $\chi^2=2.2$, df=1, $P=0.134$, resource distribution: $\chi^2=0.1$, df=1, $P=0.725$, interaction: $\chi^2=4.3$, df=1, $P=0.038$, females in spawning condition: $\chi^2=1.8$, df=1, $P=0.186$).

Experiment 2

The recruitment of juvenile fish was not affected by mussel density (RM ANOVA, $F_{1,26}=1.1$, $P=0.297$, $\eta^2=0.04$), female density ($F_{1,26}=0.1$, $P=0.760$, $\eta^2<0.01$), nor their interaction ($F_{1,26}=1.1$, $P=0.307$, $\eta^2=0.04$). There was a significant overall seasonal effect ($F_{10,260}=13.4$, $P<0.001$, $\eta^2=0.31$) and a significant interaction between census dates (repeated measure) and mussel density ($F_{10,260}=2.6$, $P=0.005$, $\eta^2=0.06$). Juvenile abundance was higher with a high mussel density treatment during the first and second censuses (univariate results: $F_{1,26}=5.3$, $P=0.030$, $\eta^2=0.16$ for the first census and $F_{1,26}=4.4$, $P=0.046$, $\eta^2=0.14$ for the second census, respectively) and equivalent from the third census onward (all other univariate results non-significant; Fig. 3). At the high resource abundance,

juvenile abundance peaked earlier than at the low resource abundance (Fig. 3).

Mean body size of juveniles at the end of the experiment was higher at a high resource density ($F_{1,11}=6.1$, $P=0.032$, $\eta^2=0.27$). The effect of female density was not statistically significant ($F_{1,11}=4.7$, $P=0.053$), though this variable explained over 20% of variation ($\eta^2=0.21$) and hence had a strong effect on juvenile size. The interaction was not significant ($F_{1,11}=0.5$, $P=0.479$, $\eta^2=0.02$; Fig. 4).

Territorial aggression was not directly affected by female or mussel density in experimental populations (mussel density: $F_{1,15}=0.2$, $P=0.697$, $\eta^2<0.01$; female density: $F_{1,12}<0.01$, $P=0.963$, $\eta^2<0.01$; interaction: $F_{1,12}=2.0$, $P=0.182$, $\eta^2=0.06$), but was strongly dependent on the number of females in spawning condition (covariate: $F_{1,15}=18.4$, $P=0.001$, $\eta^2=0.52$). The same effect was found for territorial sperm releases (covariate: $F_{1,15}=29.8$, $P<0.001$, $\eta^2=0.63$; mussel density: $F_{1,15}<0.01$, $P=0.971$, $\eta^2<0.01$; female density: $F_{1,15}=0.2$, $P=0.669$, $\eta^2<0.01$; interaction: $F_{1,15}=2.5$, $P=0.136$, $\eta^2=0.05$). As expected, there were more females in spawning condition in the high female density treatment (Poisson distribution, $\chi^2=5.0$, df=1, $P=0.025$), while mussel density had no effect ($\chi^2=0.1$, df=1, $P=0.808$) and there was no interaction between the variables ($\chi^2=1.7$, df=1, $P=0.194$), therefore more territorial aggression and territorial sperm releases were detected in the high female density treatment when tested without the covariate (factorial ANOVAs, results not shown, $\eta^2=0.24$ and $\eta^2=0.22$ for the effect of female density on aggression and sperm releases, respectively). No effect of experimental manipulation of female or resource abundance on non-territorial sperm releases was recorded (covariate: $F_{1,15}=0.7$, $P=0.429$, $\eta^2=0.04$; mussel density: $F_{1,15}=1.6$, $P=0.226$, $\eta^2=0.09$; female density: $F_{1,15}<0.1$, $P=0.913$, $\eta^2<0.01$; interaction: $F_{1,15}=0.8$, $P=0.388$, $\eta^2=0.05$).

Fig. 2 Reproductive behavior of bitterling males. The rate of territorial aggression (**a**), non-territorial aggression (**b**), total aggression (sum of territorial and non-territorial aggression; **c**), territorial sperm release (**d**), non-territorial sperm release (**e**), and ejaculation index (proportion of territorial sperm releases as a proportion of all sperm releases; **f**) recorded by diver during 40-min observation periods
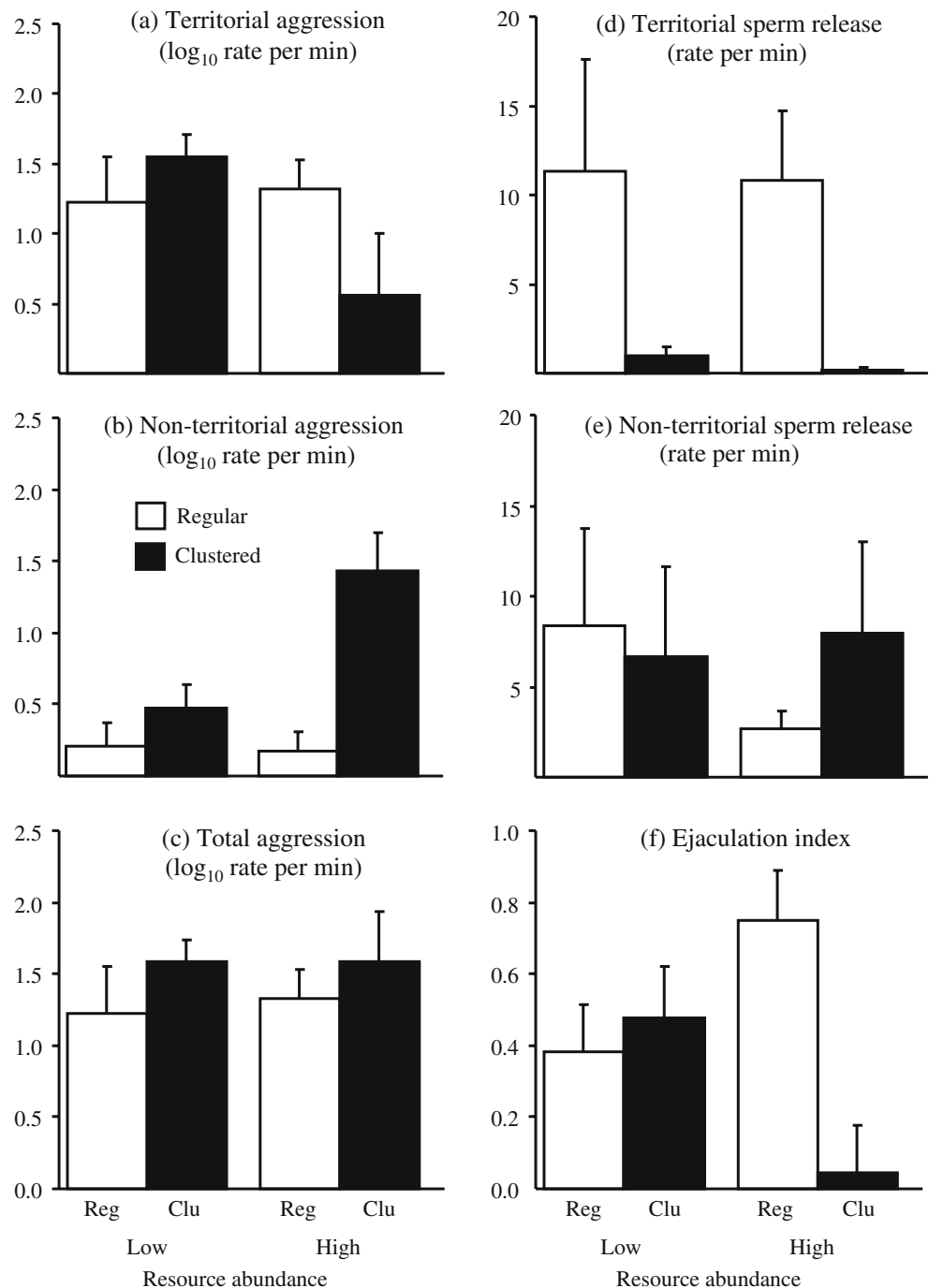
Discussion

The aims of the present study were to test how breeding resource availability (manipulated as abundance and distribution of live mussels used for oviposition) and female abundance affected male reproductive behavior, population spawning rate, embryo development, and recruitment in the European bitterling. Two separate experiments were conducted. Experiment 1 tested the effect of resource abundance and its spatial clustering on the number of eggs oviposited by the bitterling population, and on the behavior and success of individual males in a semi-natural population. In experiment 2, we studied a seasonal pattern of emergence of juvenile bitterling, their survival over the growing season and the behavioral interaction among males, and between males and females at contrasting female and resource abundances.

In experiment 1, we anticipated a significant reduction in spawning rate due to experimental limitation of breeding resources. As predicted, we found a strong effect of mussel
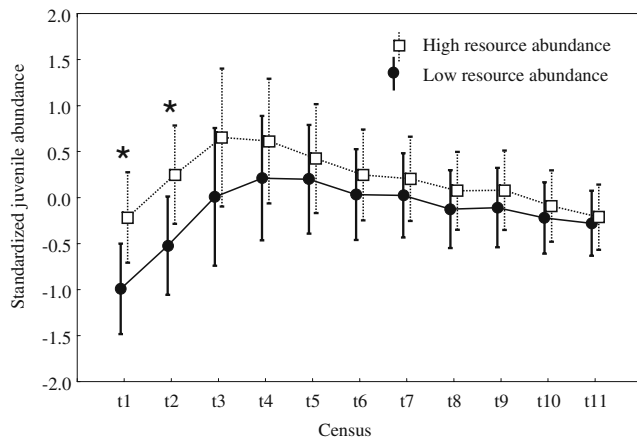
Fig. 3 Seasonal pattern of juvenile abundance in experimental tanks with high and low resource abundance. Values are standardized within each study year to account for environmental inter-annual variability. *Asterisks* denote statistically significant differences between resource abundance treatments

abundance on the number of bitterling eggs laid in a large outdoor pool (Fig. 1a). We further predicted the effect to be mediated by intense aggression among territorial males, and hence more pronounced at a regular resource distribution when territorial disputes were expected to be more intense. With a clumped resource distribution we predicted a breakdown of territoriality with a consequent stabilizing effect on spawning rate. However, no significant effect of resource distribution on spawning rate was detected (Fig. 1a), although territorial aggression strongly decreased at a high resource abundance and clumped resource distribution (Fig. 2a).

The total number of eggs at a high resource abundance treatment was numerically greater with a clustered resource distribution in experiment 1. The lack of a statistically
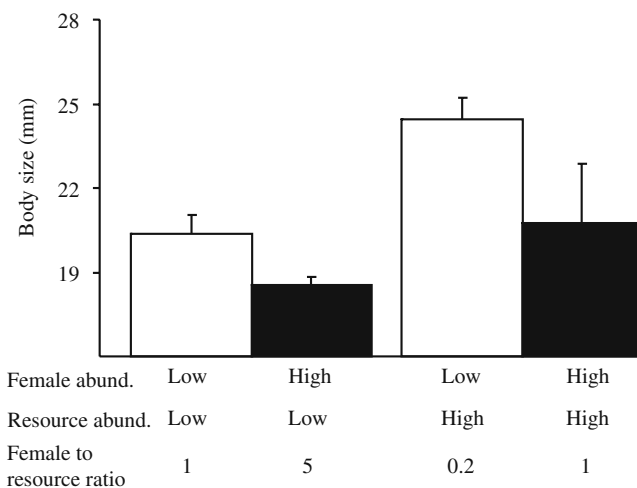

Fig. 4 Mean body size (measured as standard length to the nearest millimeter) of juvenile bitterling across four experimental treatments at the last census (November) in experimental tanks in 2008

significant effect may have been affected by our relatively low sample size (six replicates in each of the four treatments) caused by the short interval of peak bitterling reproductive activity. A total of 13 replicates (for a two-tailed test) would be required to detect a statistically significant difference between regular and clustered distributions with a high resource treatment at alpha 0.05, with a power of 0.80 for recorded variance, while 136 replicates was required to detect a statistically significant difference at a given level with a low resource distribution. It suggests that the effect was not negligible at the high resource abundance, but trivial at the low resource abundance.

The population level effects were in general agreement with our hypothesis of the lower effect of territorial aggression at low resource abundance. In fact, no decrease in territorial aggression was observed in experiment 1 with a clumped distribution at low resource abundance, in marked contrast to high resource abundance (Fig. 2a). Territorial males were able to successfully defend territories formed by a cluster of four mussels, as indicated by estimates of ejaculation index (Fig. 2f), in spite of the fact that they were the only mussels available to the entire population. However, a cluster of 12 mussels was too large to be defended successfully (Fig. 2f) and group spawning, with the participation of several males was assumed. In aquarium experiments (Przybylski et al. 2007), bitterling males that defended patches of four mussels enjoyed higher reproductive success than males defending solitary mussels because females were attracted to groups of mussels more than to single mussels (Przybylski et al. 2007). In contrast, a cluster containing 12 mussels occupied a three times larger area (1,200 cm^2 vs. 400 cm^2) and its defense was probably not economical (Dubois and Giraldeau 2005), due to the substantial cost of breeding resource defense for small fishes (Lindström 2001).

Aggressive territorial disputes between males offers the most plausible explanation for constraints on the population spawning rate (Reichard et al. 2004a). Nevertheless, other possible explanations are that: (1) the number of eggs in individual mussels reached a threshold value between high and low resource quality that made females less likely to lay their eggs, or (2) egg ejections by mussels increased dramatically in mussels with >40 eggs. Female bitterling are choosy with respect to resource quality (Smith et al. 2000a) and prefer mussels with a lower number of eggs and embryos (Smith et al. 2002), which are less capable of egg ejection (Mills and Reynolds 2002a). Embryo mortality in mussels is density dependent (Smith et al. 2000b), the density dependent effect arising largely through egg ejections (Mills and Reynolds 2002a; Kitamura 2005; Reichard et al. 2007). In the field, the number of eggs and embryos in a single mussel may exceed 250 (Smith et al. 2004); more than 100 eggs and embryos are commonly

found in mussels under natural conditions (Smith et al. 2004; Przybylski et al. 2007) and during long-term experiments (Reichard et al. 2004a, 2007, 2009). Nevertheless, some circumstantial evidence suggests that a density of approximately 40 eggs might be a critical density that influences female spawning decisions or radically increases mussel ejection rates (Reichard et al. 2005). For example, Reichard et al. (2004a) observed a mean number of 41 eggs per mussel in their experiment on the effect of mussel density on the spawning rate of an experimental bitterling population. While their second experiment confirmed a clear effect of male aggressive behavior on spawning rate (given that the behavioral effect was observed with the number of eggs in a mussel being controlled when only empty mussels were used), their results of the first experiment may have been magnified due to a mussel fullness effect on female spawning decisions and/or mussel ejection rate, in addition to the reported male aggression effect. Similarly, Reichard et al. (2007) reported egg abundance close to 40 eggs per single native mussel after a 24-h period of exposure to bitterling spawning. Here, we observed a mean of 42 eggs per mussel at low resource density with a clumped distribution, while 50-60 eggs per mussel was expected according to the prediction of an equal spawning rate with a clustered resource distribution. A dedicated experimental study is needed to demonstrate the importance of female choosiness and egg ejections on the number of eggs in mussels.

At the level of individual consequences, we confirmed the prediction that territorial males obtained the highest reproductive success at high resource density with a regular resource distribution (experiment 1). As indicated by the ejaculation index, territorial males achieved 75% of sperm releases over mussel inhalant siphons. The ability of males to outcompete their rivals in sperm competition, measured by the ejaculation index, is the most powerful predictor of paternity success (Reichard et al. 2004b). Territorial males achieved 43% of sperm releases at low resource abundance, with no difference between resource distributions. Territoriality broke down at the high resource abundance with a clustered distribution (Fig. 2f). This outcome of the experiment 1 has clear consequences for variability in male mating success, resulting in high mating skew (few males gain most paternity) when resources are defendable.

Importantly, we found a decrease in variability in reproductive success among males with a clustered resource distribution when clusters contained high numbers of mussels. This runs counter to the expectation that clustering of resources leads to their monopolization by dominant males (Emlen and Oring 1977; Shuster and Wade 2003), and highlights the importance of economic defensibility (Grant et al. 2000; Dubois and Giraldeau 2005; Noël et al. 2005) and alternative mating behavior when considering the

significance of sexual selection on population parameters, including the effects on effective population size (Jones et al. 2001; Reichard et al. 2007) and directional selection on particular phenotypic traits (Reichard et al. 2009).

Behavioral data further showed that the mating system of R. amarus is flexible and matches the environmental and demographic setting. In both experiments, males were able to track changes in mate availability and modified their behavior according to the number of females ready to mate. Aggressive behavior, sperm release and courtship behavior of territorial and non-territorial males in experiment 1 were positively related to the number of females in spawning condition, used in analyses as a covariate. In experiment 2, we predicted territorial behavior to be more pronounced and conferring higher reproductive success at low female abundance when males were hypothesized to be more able to control females. However, we did not detect any direct effect of female abundance on male behavior at the population level, because males adjusted their behavior to the number of females in spawning condition rather than to the number of females in the population. Both experiments clearly demonstrated that male behavior follows subtle changes in the operational sex ratio (OSR, the proportion of male and females ready to mate) or direct changes in the abundance of females in spawning condition. The effects of the OSR and density of females in spawning condition were invariably correlated in our study, since alternative, non-territorial mating tactics are commonly adopted by all bitterling males (Smith et al. 2004) and all males are, hence, capable of reproduction throughout the breeding period (Reichard et al. 2008). Consequently, the effect of density of females in spawning condition was strong even when OSR was always male-biased.

Embryo development inside mussels was extended at the high female-to-resource ratio in experiment 2 and more juvenile bitterling departed from their host mussels early in the reproductive season in treatments with a high resource abundance (and hence, lower embryo density in mussels; Fig. 3). Fish embryo development is strongly affected by oxygen availability (Kamler 1992; Wootton 1998) and bitterling embryos consume a considerable proportion of the oxygen circulating through a host mussel's gills (Smith et al. 2001; Kitamura 2005). Bitterling embryos also reduce the capacity of the mussel to filter water (Mills and Reynolds 2002b), further limiting their host's access to oxygen, as well as that of other bitterling embryos. We assumed that the relatively high embryo density inside mussels in the low resource abundance treatment delayed embryo development (Payne et al. 2002), and resulted in the late departure of juveniles from mussels (Beekey and Karlson 2004). A predicted effect of female to mussel ratio on recruitment, in addition to the effect of mussel abundance, was not detected at the

level of juvenile abundance (Fig. 3), but appeared at the level of juvenile body size at the end of the growing season (Fig. 4).

The earlier juvenile emergence in the high resource treatment was prominent. The bitterling that departed the mussels early in the season probably survived until the last census in November rather than being replaced by a younger cohort of juveniles, as indicated by differences in juvenile size at the end of the growing season in experiment 2. Juvenile bitterling from the high mussel-low female abundance had achieved almost a 30% larger mean body size than fish from low mussel-high female abundance. This finding has three clear consequences. First, the fitness value of offspring sired early in the season may be higher due to higher survival expectancy (Cargnelli and Gross 1996; Forchhammer et al. 2001), with an outcome for males to invest more in competition for fertilizations at the start of the reproductive season (Reichard et al. 2008). Second, overwinter survival of temperate cyprinids increases with body size (Griffiths and Kirkwood 1995; Kirjasniemi and Valtonen 1997) and earlier departure may result in a higher adult population size. Third, poorer conditions in early life may have long-term consequences for an individual's competitive ability and fitness (Birkhead et al. 1999; Hunt et al. 2005; Barrett et al. 2009).

In conclusion, we showed that, at the population level, the number of resources necessary for reproduction was more important for recruitment than resource distribution. On the other hand, for individual consequences (variability in reproductive success), resource distribution was more important, but only when resource abundance was not limiting. Territorial males were unable to defend large clusters of resources and abandoned territoriality when rival abundance was high, though they obtained almost exclusive access to fertilizations when resources were abundant and distributed regularly. Surprisingly, territorial males remained aggressive and successfully defended their territories when resources were clumped, but at a low overall abundance. The abundance of females in spawning condition rather than population density of females were the best predictor of male reproductive behavior. A high resource-to-female abundance ratio resulted in more rapid emergence of juvenile bitterling from mussels, with an effect of larger body size in juveniles at the end of the growing season. No effect of resource abundance was found, likely due to a limited carrying capacity of our experimental tanks. Our results showed that the mating system of the European bitterling is flexible, with changes from strong territoriality to group spawning occurring over the course of a single day. An effect of decreased spawning rate due to resource limitation appears to be diminished during the reproductive season by juvenile mortality, but can still be detected in the final body size of recruiting juveniles.

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