Personality traits, reproductive behaviour and alternative mating tactics in male European bitterling, *Rhodeus amarus*

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Abstract

Individual differences in behavioural traits may play a role in reproductive behaviour and it is likely that different personality types have different reproductive success across specific social environments. This suggests a role for sexual selection for personality types, including a link between behavioural traits and alternative reproductive tactics. While research on morphological differences between guarders (bourgeois males defending resources) and sneakers (males parasitizing the territories of bourgeois males) is well characterized, the role of personality in the adoption of alternative mating tactics has hitherto been largely ignored. We investigated individual behavioural differences in a sneaker–guarder mating system using size/age-matched males of a small freshwater fish, the European bitterling (*Rhodeus amarus*). We predicted distinct behavioural responses by guarder and sneaker males that were consistent in different contexts, indicating the existence of behavioural syndromes associated with male mating tactic. No behavioural syndromes were detected in male *R. amarus*, despite ability of individual male bitterling to establish dominance, boldness and investment in sperm competition being relatively repeatable across three consecutive trials. Male aggression, though not repeatable, was negatively correlated with the number of ejaculations, indicating a trade-off between aggression and sperm loading. No association between the tendency of males to guard a territory and behavioural traits was found, despite a significant association between the tendency to guard and morphological and physiological traits, with higher relative testis size and more breeding tubercles in guarder males. Our data suggest male bitterling mating tactics are largely unconstrained by innate factors and likely to be the product of prevailing environmental and social conditions.

1. Introduction

Research on animal behaviour increasingly recognizes that behavioural traits can be consistent through time and contexts, resulting in the emergence of distinct behavioural types (Stamps & Groothuis, 2009). Such types have been termed 'personalities' (defined as a set of personality traits consistent through time). Additionally, 'behavioural syndromes' are recognized as an inter-correlated set of individual personality traits (Sih & Bell, 2008). For example, 'personality' can be recognized when an individual is boldest among a group of conspecifics as a juvenile and expresses a relatively high level of boldness later in life, when compared to similarly-aged conspecifics. A 'behavioural syndrome' is observed when a positive or negative correlation is found between traits, such as aggression directed at conspecifics and boldness towards predators (Huntingford, 1976). Such correlated suites of behaviours may be favourable in certain environments or situations (Reaney & Backwell, 2007), while maladaptive in others (Johnson & Sih, 2005). Consequently the existence of distinct behavioural types has important implications for the way in which selection acts on behavioural traits, and thereby on the maintenance of behavioural variation among and within populations.

To investigate personality, a group of individuals can be observed through a suite of contexts and their behavioural traits compared. A typical approach is to expose animals to new or threatening stimuli (Weinstein et al., 2008) and score their behaviour with respect to a shy–bold axis, with the relative position of an individual along this continuum representing its willingness to take risks (Wilson et al., 1994). Bold individuals are more prone to engage in risky behaviour, such as predator inspection, more readily switch to unfamiliar diets, interact readily with novel objects or individuals, and are generally more explorative (Budaev, 1997; Godin & Clark, 1997; Wilson, 1998). In contrast, shy individuals engage in behaviours yielding lower profits but also less risk (Ward et al., 2004). The position of individuals on the shy-bold axis can predict their behaviour in the presence of predators, under competition, and their ability to monopolize resources (Weinstein et al., 2008).

Behavioural traits can play a role in reproductive behaviour, either directly or by their covariance with other traits via pleiotropic effects. For example, Colléter & Brown (2011) have shown that the most dominant males of the crimson-spotted rainbowfish (*Melanotaenia duboulayi*) were also more aggressive, bold and more active than subordinates. Males at the top of rainbowfish social hierarchies have better access to females and are able to

monopolize them, which significantly enhances their reproductive success (Young et al., 2010). Personality also plays a role in individual reproductive success in the Trinidadian guppy (*Poecilia reticulata*). Godin & Dugatkin (1996) demonstrated that females preferred mating with bolder males who were more likely to inspect a predator in a female's presence, and apparently were better informed about imminent attack from predators. Under certain demographic and environmental conditions, females may, thus, increase their fitness by mating with bolder males in the case there is heritable component to boldness (Brown et al., 2007).

While it appears that different personality types may have different re-productive success across specific social environments, the impact of sexual selection on personality traits has only rarely been studied (Schuett et al., 2010), including the link between personality traits and alternative repro-ductive tactics (ARTs) (Brockmann et al., 2008; Bergmüller & Taborsky, 2010). ARTs represent mating polymorphisms, often maintained through negative frequency-dependent selection (Shuster, 2010). ARTs are a general phenomenon among competing males, particularly in taxa with external fer-tilization reflecting a functional distinction between 'producers' (bourgeois males) and 'scroungers' (parasitic males) (Taborsky et al., 2008). One of the most common forms of ART is the sneaker–guarder system, in which guarders monopolize females or critical breeding resources, while sneakers attempt to mate deceptively and engage in sperm competition (Parker et al., 1997; Taborsky, 1998). The relative success of an ART depends on indi-vidual competitive capability, environmental and population parameters, and the level at which reproductive competition occurs (Reichard et al., 2004b; Taborsky, 2008; Konečná et al., 2010; Shuster, 2010). While research on morphological differences between guarders and sneakers has received some attention, the role of behavioural syndromes and personality in the adoption of alternative tactics has so far been largely ignored (Brockmann et al., 2008; Bergmüller & Taborsky, 2010).

Here, we investigated individual behavioural differences in bourgeois-parasitic tactics using males of a small freshwater fish, the European bit-terling (*Rhodeus amarus*). Because ARTs are associated with contrasting roles, we predicted that guarder (bourgeois) and sneaker (parasitic) males would differ in personality traits across contexts. We anticipated that the expression of discrete behavioural syndromes would predispose individual males to largely act in one mating role, with guarder (bourgeois) males being

bolder, more aggressive and dominant. We further tested the association between particular behavioural traits and morphological (body size, abundance of breeding tubercles, fluctuating asymmetry), physiological (testis size, red colour in eye iris, growth rate) and genetic (heterozygosity at microsatellite loci) characters. We tested a group of bitterling males in a series of aquarium and semi-field experiments. We controlled for the effect of body size, since this trait is known to affect dominance and territorial status in *R. amarus*.

2. Methods

2.1. Study species

The European bitterling is a cyprinid fish (subfamily Acheilognathinae) that uses freshwater mussels for oviposition (Smith et al., 2004). Bitterling have a promiscuous resource-based mating system (Reichard et al., 2008), with adult body size ranging from 35 to 65 mm (Smith et al., 2004). Females deposit their eggs in the gills of mussels using an unusually long ovipositor, while males release sperm over the inhalant siphon of mussels, so that water filtered by a mussel carries the sperm to the eggs. During the reproductive season males defend small territories around living freshwater mussels. Guarder males actively court females and attempt to lead them to mussels in their territory. Males can also act as sneakers, interrupting spawning pairs by darting next to a spawning pair from a shelter and fertilizing eggs by releasing sperm into the mussel defended by another male. Larger males are more likely to become guarders than smaller males (Reichard et al., 2008), but there are no apparent morphological differences between guarders and sneakers. Male tactics are sensitive to prevailing environmental and social conditions (Przybylski et al., 2007) and it appears that all males are capable of both tactics, though disproportionally perform one of the roles (Smith et al., 2004). For further details on bitterling mating systems see Smith et al. (2004).

2.2. Subjects

Experimental fish were collected in the River Kyjovka in the southeast of the Czech Republic using a portable electrofishing backpack, modified to capture small fish efficiently (Lena, www.r-bednar.cz), and transported in aerated river water to the Institute of Vertebrate Biology (IVB) in Brno. Prior to experiments, a stock of wild fish was held in a large pond in the garden of

the IVB under natural conditions. In March (prior to the onset of the bitter-ling spawning season), a sample of males was measured for Standard Length (SL, body size excluding caudal fin) and 24 selected on the basis of their sim-ilar body size (mean $\pm$ SE $= 53.8 \pm 0.29$ mm, range 51.5–57.0 mm). All the males were uniquely marked with coloured visible implant elastomer tags (VIE, Northwest Marine Technology Company). After marking, fish were housed in a large aquarium (126 $\times$ 41 $\times$ 40 cm) (henceforth referred to as the social tank) with a sand substrate and artificial plants as refuges. Fish experienced natural daylight (approx. 13 h of light) and water temperature fluctuation (mean 18°C, range 16–22°C) and were fed once each day with frozen chironomid larvae. The start of experiments coincided with the on-set of the bitterling reproductive season (early April). Experimental mussels were collected from an oxbow lake adjacent to the River Kyjovka prior to the start of bitterling spawning season.

2.3. *Experimental procedures*

Individual boldness was measured as the time taken for a male to emerge from a shelter after introduction to a novel aquarium. The test aquarium was 75 $\times$ 40 $\times$ 38 cm and contained a 2 cm layer of river sand. Three sides of the aquarium were covered with opaque barriers to minimize external dis-turbance. Two large artificial plants were provided as shelters and situated in both of the rear corners. Before the start of the experiment all the experi-mental males were carefully removed from the social tank to six plastic tubs to standardize the effects of capture in fish that were tested. Trials were con-ducted on 7, 8 and 10 April 2009. After a short period of habituation in the tubs (15 min), an experimental fish was gently captured and released into the right front corner of the experimental tank. All fish immediately hid in the fronds of the artificial plants. The time taken to emerge from the shelter and swim in open water (i.e., to start tank exploration) was measured as index of boldness. Some individuals did not leave the shelter within 1200 s and were assigned a score of 20 min (maximum shyness). After emerging from shelter, or after 1200 s, whichever was sooner, the test male was captured and gen-tly returned to the social tank. Note that our measure of boldness could also reflect a measure of stress resistance, because it measures the recovery time after being caught, handled and moved. The test consisted of three replicates for each male. Each male was tested once each day of the experiment. The order of testing males followed a predetermined random sequence.

Male dominance was measured as the ability of a male to establish a territory under competition with other males (conducted 14, 15 and 17 April 2009). All males likely interfered with each other prior to the test in the social tank, though their potential interactions before the test were standardized by using a single social tank for all males. Pre-experimental and tank conditions were identical to the test for boldness, except that a freshwater mussel, placed in a sand-filled plastic box (10 × 6 × 6 cm), was located in the centre of the tank. Three males were captured from a plastic box and simultaneously released into the experimental tank. The establishment of a dominance hierarchy was checked each 15 min. Dominant males were identified through mussel defence and aggressive displays directed towards other males (Reichard et al., 2005). Physical contact between males was occasionally recorded, but no injuries were observed. Territorial disputes are a common feature of male reproductive behaviour and are typically settled prior to physical contact (Smith, 2011). Subordinate males were provided with artificial vegetation and were able to readily escape and hide from dominant individuals. Once one male had unambiguously established dominance (chased the rivals from the territory and patrolled over the mussel), he was removed. This process was repeated with the two remaining males to enable the second and third ranked males to be identified. The trials took between 30 and 75 min before dominance was established. This period corresponds with the rapid establishment of territoriality observed in the field (Smith et al., 2003). Dominance was scored on a 3-point ordinal scale from 2 (the most dominant) to 0 (no dominance). Winner/loser effects were not considered. Each male was tested three times and male combinations were random except to ensure that no individual males were ever tested together more than once. Each male was tested once each day of the experiment.

Male investment in aggressive behaviour and sperm competition was tested as a response by each male to a rival (conducted 21–28 April 2009). Pre-experimental and aquarium conditions were identical to the previous experiment, but with the addition of rival male in a glass jar (10 × 10 × 28 cm) positioned in the centre of the aquarium, 15 cm from the mussel, with a mesh top to permit ventilation. Small bitterling males (SL ± SE = 35 ± 0.7 mm) were used as rivals. Test males could not attack the bottled rival males directly, but the presence of a rival elicited the focal male to respond to the risk of sperm competition (Smith et al., 2003, 2009). The response of focal males was recorded 30 s after the first response to the rival male

and lasted 10 min. Head butting and parallel swimming (Smith et al., 2004) were recorded as aggressive bouts and their frequency was used in analyses. No other aggressive behaviour was observed. At the same time, investment in sperm competition was quantified as the number of ejaculations over the inhalant siphon of the mussel, which is a good approximation of sperm investment by male bitterling (Smith et al., 2009). Male bitterling frequently ejaculate over mussels in the absence of females. This behaviour probably relates to the importance of sperm precedence in fertilization success, with preoviposition ejaculation rate (often in the absence of females) being the only significant predictor of paternity success (Reichard et al., 2004b). Rival males and mussels were replaced in each trial (to minimize their stress when constrained) and each male was tested on three separate occasions.

In a final, large-scale trial, the ability of each male to defend a territory under semi-natural conditions, their adoption of a specific mating tactic (guarder, sneaker), and a measure of their reproductive success were measured in an outdoor concrete pool measuring 12.6×6 m filled to a water depth of 0.6 m (approximately 45 000 l). Large (1 m long) artificial plants were placed around the perimeter of the pool as shelters. On 30 April, 23 experimental males and 135 females were released into the pool and allowed to settle for one week. Fish fed on naturally occurring food items (detritus, algae, aquatic insect larvae) supplemented with a daily ration of frozen chironomids. Note that only 2–20% of female bitterling are in reproductive condition on any given day (Konečná & Reichard, 2011) yielding a local operational sex ratio (Seger & Stubblefield, 2002) typically encountered under natural conditions, despite the population sex ratio being significantly female biased. Both reproductively active and non-reproducing females swim in large shoals during the reproductive season, forage and visit male territories (Smith et al., 2004), and this behaviour was also observed in the experimental pool (Reichard et al., 2004a; Konečná et al., 2010). After one week, live mussels were placed in small sand-filled plastic boxes (18×14 cm) to represent varying number of territories. A total of 18 plastic boxes were placed along three walls of the pool (one side was left empty to allow a diver to enter and leave the pool without disturbing fish) 1.7 m apart. A mussel was placed in a box to enable an active territory to be formed. The number of potential territories was varied between 1 and 18, with 1, 2, 3, 4, 6, 8, 10, 14 or 18 territories active on a given day. The number of active territories was randomized among days. The position of active territories within the pool was

selected to maximize distances between adjacent territories. On each day of the experiment, a snorkeler observed every mussel for 15 min and recorded male behaviour. For each marked male, the number of defended territories, number of sneaking attempts, leading of females to a mussel, and number of spawnings were recorded. After completion of behavioural observations, all mussels were removed and replaced with new set of mussels that were positioned in accordance with the next experimental treatment. Behavioural observations took place between 9:00 and 17:00 Central European Summer Time; i.e., >3.5 h after sunrise and <3 h before sunset. A single treatment was completed each day.

At the end of the experiment, the males were recaptured using baited net traps. Nine males were not recovered (and not observed during snorkelling after the end of the experiment) and those males appeared to have died over the course of the experiment. Intrinsic male mortality is naturally high during the reproductive season of the bitterling (Smith et al., 2000), with only 4–5% of males surviving until the end of reproductive season under natural conditions in the study population (Konečná & Reichard, 2011). Only recovered males were used in data analysis. Captured males were photographed (Olympus Mju 1030 SW) in a glass cell under identical light conditions from a distance of 25 mm to estimate the extent of red area in their iris, serving as carotenoid-based nuptial signal (Reichard et al., 2009). The male was killed with an overdose of anaesthetic (clove oil), a finclip taken for DNA extraction and fixed in ethanol, and the body preserved in an 8% formaldehyde solution.

After preservation, each male was measured for SL to the nearest mm and weighted to the nearest 0.001 g (wet weight). A count was made of the number of ventral fin rays, scales in the longest row along the body, and breeding tubercles on each side of the body and head. Males were dissected and their testes were weighted to the nearest 0.001 g. Gonad mass measured at the end of reproductive season denotes residual gonad mass and represents a valid relative index of investment in gonad tissue in the foregoing breeding season (Reichard et al., 2009). This measure 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 et al., 2009).

Fish were individually marked using elastomer tags, which are widely used in experimental studies and have proven harmless (Halls & Azim, 1998;

Weston & Johnson, 2008), which was also the case in the present study. Mortality of a single male during the first phase of experimental work (aquarium), and a further nine males during the second phase was recorded. These mortalities were not associated with experimental treatments. Our target sample size was estimated based on previous comparable experiments (Smith et al., 2003; Reichard et al., 2004b, 2005, 2006; Smith & Reichard, 2005) to achieve a trade off between maximizing experimental power while minimizing the number of experimental fish used.

2.4. Data analysis

The repeatability of behavioural traits was measured as intraclass correlation *sensu* Lessells & Boag (1987) (ICC_1). However, our experimental design (constrained by the duration of the bitterling reproductive season) necessitated identical trials to be repeated within a relatively short period, with a risk that subjects habituated to experimental conditions (Chervet et al., 2011), which was indeed the case for some measures of personality traits (see Results). To account for this, we calculated alternative intraclass correlations according to Shrout & Fleiss (1979) and implemented in package 'psych' for R 2.9.1 (R Development Core Team, 2009). Those intraclass correlations remove mean differences in response variables between trials (McGraw & Wong, 1996) and we present the outcomes of analyses where subjects were modelled as either random (ICC_2) or fixed (ICC_3) effects. We acknowledge, however, that our experimental design did not control for potential carry-over effects (Dochtermann, 2010).

A fluctuating asymmetry index (F_A) was calculated according to formula FA11 of Palmer (1994), whereby $F_A = \sum |R - L|$, where R is the value for a trait on right side of the fish and L the value for the same trait on left side. Meristic data for ventral fins, breeding tubercles and the number of scales along the body axis were summed. The extent of eye redness was estimated as the proportion of the total iris that was red using ImageJ 1.43n (Abramoff et al., 2004; Reichard et al., 2009). The gonadosomatic index (GSI) was calculated as $GSI = (W_G / W_E) \times 100$, where W_G is gonad weight and W_E is the weight of the eviscerated fish. The use of W_E (weight of internal organs not included) minimized the effect of gut fullness. Growth rate was calculated as a difference in the natural log of body size measured on 1 April and 15 June, which coincided with the reproductive period and can be considered as growth rate over the breeding season (Reichard et al., 2009).

Individual heterozygosity was estimated on the basis of 10 microsatellite loci (Rser01, Rser03, Rser05, Rser06, Rser08, Rser09, Rser10, Rser11, Rser12 and Rser13) (Dawson et al., 2003; Reichard et al., 2008) using Genepop 4.0.10 (Rousset, 2008). DNA extraction and microsatellite analysis followed established protocols given in Bryja et al. (2010).

Prior to statistical analysis, all data were checked for assumptions of parametric tests (normality, homoscedasticity of variances) and transformed if necessary. Proportional data were square-root arcsine transformed. Final sample size was 23 males in aquarium experiments and a subset of 14 males in the outdoor experiments due to mortalities (see below). Given that some mortalities are common among males during the reproductive season, we anticipated that final sample size (at the end of reproductive season) to be lower than the number of males used at the beginning of experiments (at the start of reproductive season). Consequently, with a target final sample size of 18 males, we initially used 24 males. Therefore, our final sample size in aquarium experiments ($N = 23$) was slightly higher than planned, while the sample size for outdoor experiments was slightly lower ($N = 14$).

Associations between personality traits and morphological traits were tested using summed values across all three trials. To test whether fish habituated to experimental conditions and changed their response among consecutive trials, we fitted linear mixed models (nlme package in R 2.9.1, LMM) with particular personality trait as response variable and fish identity as random factor. The relationships between male territoriality in the outdoor pool, personality traits, and morphological characters were tested using a Spearman correlation. A measure of territoriality (tendency of the males to guard a territory) was expressed as the number of cases when a particular male was observed to guard a territory.

3. Results

Individual differences in the capacity to establish dominance, boldness, and investment in sperm competition were repeatable, though male aggression was not (Table 1); the significance of repeatability increased when habituation was accounted for (ICC_2 and ICC_3 measures in Table 1). There was a clear increase in boldness score across trials, indicating that fish decreased their time to emerge from a refuge with increasing experience with experimental conditions (LMM, trial effect $F_{2,44} = 8.75$, $p < 0.001$). Investment

Table 1.

Results of repeatability analysis ($N = 23$ males, aquarium).

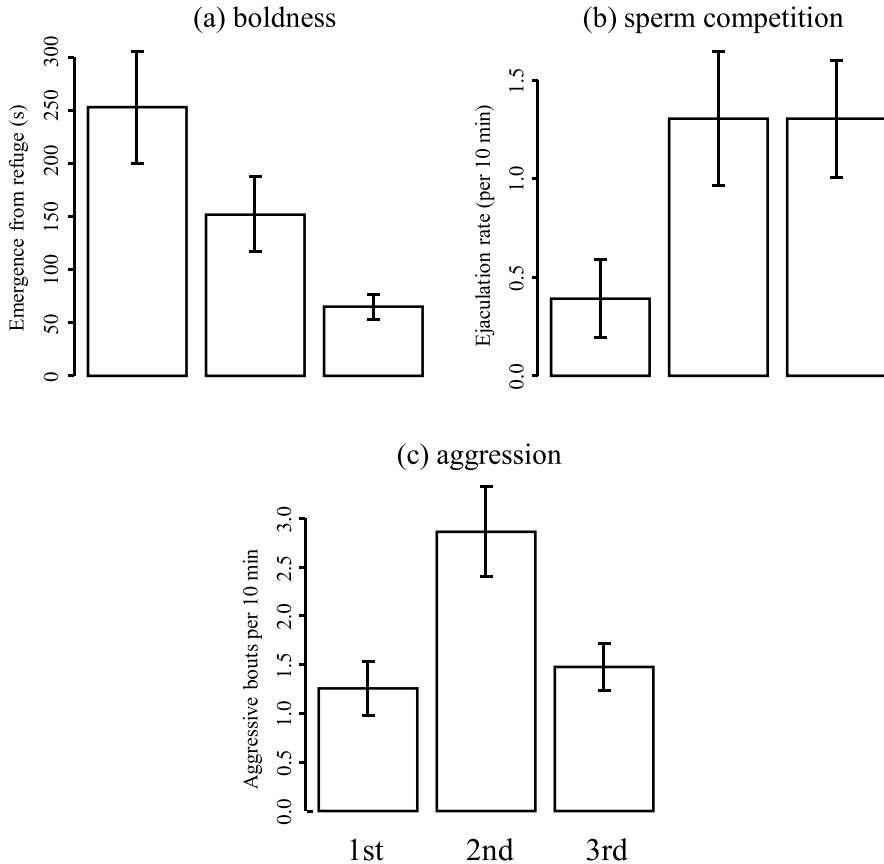| | ICC_1 | | | ICC_2 | ICC_3 | | |
	r	$F_{22,46}$	p	r	r	$F_{22,44}$	p
Dominance	0.385[*]	2.88	0.001	0.379[*]	0.369[*]	2.76	0.002
Boldness	0.160	1.27	0.098	0.210[*]	0.267[*]	2.09	0.017
Sperm competition	0.178	1.65	0.076	0.207[*]	0.230[*]	1.90	0.035
Aggression	−0.195	0.51	0.955	−0.119	−0.147	0.62	0.889

Intraclass correlations were calculated sensu Lessells & Boag (1987) (ICC_1) and with mean differences between trials taken into account, with subjects modelled as random (ICC_2) or fixed (ICC_3) effects.

[*] Statistically significant result.

in sperm competition (LMM, trial effect $F_{2,44} = 4.45$, $p = 0.017$) and aggression rate varied among trials (LMM, trial effect $F_{2,44} = 6.62$, $p = 0.003$) (Figure 1). These personality traits were significantly repeatable when differences in mean values among trials were accounted for (Table 1), indicating that despite overall numerical changes in the response variable, rank order of boldness and sperm competition score for individual fish was consistent across trials. Dominance was measured on a rank scale, resulting in no differences in mean values across trials and significant repeatability using all three intraclass correlation coefficients (Table 1).
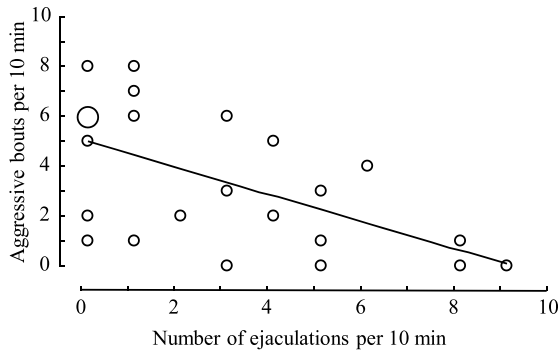
There was a significant negative correlation between male aggression and investment in sperm competition (Pearson correlation, $r = −0.544$, $N = 23$, $p = 0.004$; Figure 2). The statistical significance was retained after Bonferroni correction (critical $p = 0.008$). Other associations between personality traits were not significant (all $p > 0.229$), giving little support for the existence of behavioural syndromes, at least under our experimental conditions.

Male body size was experimentally constrained in the study, and as anticipated did not correlate with any behavioural traits (boldness: $r = 0.237$, $p = 0.276$; investment in sperm competition: $r = −0.111$, $p = 0.615$; aggression: $r = −0.150$, $p = 0.494$; dominance: $r = 0.367$, $p = 0.085$). Within individual dominance trials, male body size still had some importance. It was not associated with the rank order among males during the first (Kendall coefficient of concordance $W = 0.204$, $p = 0.195$) and second ($W = 0.094$, $p = 0.472$) trial, though it did affect the outcome of the third trial, with the relatively largest males being most dominant ($W = 0.717$, $p = 0.003$).

Figure 1. Mean values of personality traits across individual trials in aquarium experiments (*N* = 23 males). Whiskers represent one standard error.

There was no association between the tendency to act as a guarder in the outdoor experiment and personality traits (dominance: $r_S = 0.401$, $p = 0.156$, investment in sperm competition: $r_S = 0.460$, $p = 0.098$, boldness: $r_S = 0.325$, $p = 0.257$, aggression: $r_S = -0.077$, $p = 0.793$). For morphological and physiological traits, a greater tendency to act as a guarder was positively associated with larger GSI and more breeding tubercles (Table 2). The number of spawning acts a male participated in (either as guarder or sneaker) was positively related to the ability of a male to establish a territory (measured as the overall number of territories a male defended) (Spearman correlation, $r_S = 0.740$, $N = 14$, $p = 0.002$). The ability of males to estab-

Figure 2. Relationship between aggression and number of ejaculations during simultaneous assessment in male *R. amarus* in aquarium experiments ($N = 23$ males). The larger point identifies an overlap between two individuals.

lish dominance in aquarium tests correlated positively with the number of breeding tubercles, but no other relationship between morphological (and physiological) traits and personality traits proved significant, including the effect of body size (Table 2).

Table 2.

Association between territoriality (tendency to act as a guarder in an outdoor study) and dominance (in an aquarium study) and morphological and physiological traits in male European bitterling ($N = 14$ males).

	Territoriality		Dominance	
	r_S	p	r_S	p
GSI	0.697*	0.006*	0.399	0.158
Breeding tubercles	0.610*	0.021*	0.633*	0.015*
Growth rate	0.407	0.149	0.117	0.692
Eye redness	0.191	0.514	0.045	0.879
Body size	0.093	0.753	0.230	0.430
Body mass	0.038	0.898	0.173	0.555
Heterozygosity	−0.137	0.641	0.197	0.499
Fluctuating asymmetry	−0.153	0.601	−0.294	0.308

Territoriality was expressed as the number of cases when a particular male was observed to defend a territory. Results of a Spearman correlation and its statistical significance are reported.

* Statistically significant result.

4. Discussion

We investigated individual behavioural differences in a sneaker–guarder system using males of the European bitterling. We predicted distinct behavioural responses by guarder and sneaker males that were consistent in different contexts, indicating the existence of behavioural syndromes. We demonstrated the ability of individual male bitterling to establish dominance was highly repeatable, while boldness and investment in sperm competition were repeatable only at relative scales. Individuals retained their relative ranks, but numerical measures of their personality traits varied. Male aggression was not repeatable. Overall, the existence of a behavioural syndrome was not supported, because personality traits were not correlated. The exception was the association between male aggression and sperm competition which were negatively correlated, indicating that individual males varied in their response to the presence of a rival along the bourgeois-parasitic continuum. Despite significant association between the tendency to act as a guarder and morphological and physiological traits (with greater relative testis size and more breeding tubercles in guarders), no difference between males along the guarder–sneaker continuum was found in personality traits. High repeatability in dominance was detected even though male size was constrained within a narrow (5 mm) range. This suggests that an individual's ability to establish and successfully defend a territory is consistent even among similarly-sized rivals. However, even within this size-constrained cohort, slight body size differences explained variance in dominance in one experimental trial. Male body size is the major predictor of dominance hierarchy in most animals, including fishes (Andersson, 1994).

Male boldness gradually increased across trials, with the time to emerge from a shelter declining across successive trials (Figure 1). This indicates that the measure of boldness might also reflect stress resistance or 'training effect'. Chervet et al. (2011) report a 'training effect' in a Tanganyikan cichlid (*Neolamprologus pulcher*). If two behavioural tests were conducted only a short time apart in their study, individuals were usually bolder and more explorative in the second test. Wilson & Godin (2009) also found that individual bluegill sunfish varied in their response to predators across trials due to individuals habituating to the novel stimuli at different rates. This implies that different capacities of individual males to learn and habituate to experimental conditions can limit repeatability of behavioural traits. In our study, however, repeatability of male boldness was high after accounting for

habituation in the calculation of intraclass correlation coefficient, consistent with a situation of relatively minor differences to habituate to experimental conditions among individual fish.

Behavioural syndromes in males were not demonstrated by the study. This result may be an artifact of our experimental design (using size-matched 1-year-old males tested during the reproductive season or the choice of behavioural measures and their order). Alternatively it may be a feature of our study population or, more generally, of the European bitterling. Study subjects may have come from an environment where strong correlations between behaviours across contexts were not adaptive. For example, Bell (2005) reported a behavioural syndrome in a population of three-spined sticklebacks (*Gasterosteus aculeatus*) that experienced strong predator pressure. In an adjacent population subjected to limited predation pressure no behavioural syndrome was detected. However, the results may have been confounded by testing different populations at different phases of reproduction. Ontogenetic patterns may also affect expression of behavioural syndromes. Bell & Stamps (2004) found a weaker correlation across behaviours in subadult sticklebacks compared with adults, probably linked to hormonal changes associated with sexual maturation.

Male aggression and investment into sperm competition were measured in the same experiment and a negative correlation between aggression and number of ejaculations supports the observation that male bitterling traded-off between the two behaviours. These two activities are time and energy expensive and males may be constrained in their ability to express both simultaneously (Candolin & Reynolds, 2002). A trade-off between aggression and sperm competition has been observed in other fish species, including the Mediterranean wrasse (*Symphodus ocellatus*) (Alonzo & Warner, 2000), three-spined stickleback (Le Comber et al., 2003) and zebrafish (*Danio rerio*) (Spence & Smith, 2005), and may be a general feature of animal mating systems.

Dominant male bitterling were shown to have more breeding tubercles; epidermal keratin-based structures found on the head of reproductively active males. The development of breeding tubercles in fish is controlled by androgens (Wiley & Collette, 1970), themselves influenced by social status (Oliveira et al., 1996). Dominant individuals with elevated levels of these hormones express more breeding tubercles (Oliveira et al., 2002) and they may be perceived as an indicator of androgen levels. Tubercles have also

been hypothesized as functioning as signals of male quality affecting female mate choice (Huuskonen et al., 2009), though whether this occurs through direct or indirect selection is unclear. A large number of breeding tubercles in dominant males has also been demonstrated in the roach (*Rutilus rutilus*) (Kortet et al., 2004), a species of fish with a lek mating system (Wedekind, 1996). Breeding tubercles may provide protection from mechanical friction during spawning (Ahnelt & Keckeis, 1994). In the European bitterling, tubercles are concentrated on the tip of the snout and may function as a weapon in male–male combat. During territorial fights males frequently engage in bouts of what has been termed headbutting (Smith et al., 2004), but which actually involves males striking each other with their snouts. Hence, the number of breeding tubercles may potentially be under intrasexual selection and further research is needed to establish their function in the bitterling mating system.

Male tendency to act as a guarder positively correlated with testis mass. This finding appears to contradict a key prediction of sperm competition theory (Parker, 1998). An assumption of classical sneak-guard models is that sneakers face sperm competition in all their reproductive attempts, while guarder males are confronted with sperm competition only in a subset (Parker, 1998). However, in *R. amarus* this assumption may be violated, with the overall level of sperm competition risk consistently high for all males in a population, irrespective of mating tactic (Candolin & Reynolds, 2001; Reichard et al., 2004a,b; Smith et al., 2004, 2009). Male *R. amarus* display specialized reproductive adaptations for high levels of sperm competition and sperm economy in comparison with other bitterling species (Pateman-Jones et al., 2011), and with no distinction in sperm quality or testis structure in relation to mating tactic (Pateman-Jones, 2008). Male bitterling release sperm both before and after a female deposits her eggs, and each spawning is accompanied by a mean $\pm$ SE of 4.4 $\pm$ 0.47 ejaculations/mating, with an excess of 250 ejaculations per guarder male on each day of the reproductive season in a wild population (Smith et al., 2009). Our observations that guarder males had greater testis mass than sneakers may better reflect the pattern in resource-based mating systems than is generally recognized (see also Zbinden et al., 2001; Fitzpatrick et al., 2006; Reichard et al., 2007), with only males in sufficiently good condition being able to afford the high cost of sperm production and territorial defence simultaneously. Indeed, the ability of a male to guard a territory was positively related to the overall number of spawning acts a male participated in as guarder or sneaker and, therefore,

with reproductive success under our experimental conditions. However, the relative success of guarders and sneakers varies extensively across demographic parameters (Reichard et al., 2004a,b).

We predicted that specific morphological, physiological and genetic traits would be associated with personality traits and mating tactics. Contrary to predictions (Tiira et al., 2003, 2006; Vilhunen et al., 2008), we failed to detect a relationship between genetic heterozygosity, the level of fluctuating asymmetry (an index of developmental instability), carotenoid-based red coloration and behaviour, especially the ability to establish dominance. While this may be partially ascribed to our relatively low sample size, no apparent trend was detected and any potential relationship must have had only a relatively small effect.

Males that employ alternative mating tactics are sometimes morphologically dissimilar and their behaviour may be, at least temporarily, dictated by morphological and physiological conditions related to ontogenetic stage or phenotype. However, the majority of known cases of alternative mating tactics reflect conditional responses to transitory social conditions (Oliveira et al., 2008), including in *R. amarus* (Smith et al., 2004). Social conflict can select for the stable coexistence of different behavioural types and Bergmüller & Taborsky (2010) hypothesized that consistent individual differences in personality traits may lead to a diversification into discrete social roles, including guarder and sneaker males as alternative avenues to maximize fitness. Our data do not support Bergmüller & Taborsky's (2010) hypothesis; there was no difference in any personality trait between guarder and sneaker males, nor was there consistency in behaviour across contexts. The assumption that bolder individuals are more likely to become guarders was also not confirmed. However, we acknowledge a relatively small sample size in our analysis, and the fact that our experimental design excluded the role of predators, despite an attempt to mimic natural conditions in a large semi-natural setting. Predators can have a significant impact on individual behaviour (Bell, 2005), and guarder males may be more susceptible to predation when courting or displaying to females (Magurran, 2005; Konečná & Reichard, 2011).

In conclusion, we did not detect evidence for behavioral syndromes corresponding with male mating tactics in male *R. amarus*. We further failed to find strong associations between individual behavioural, morphological, physiological and genetic traits, despite repeatability of some personality

traits. These data suggest male bitterling mating tactics are largely unconstrained by innate or ontogenetic factors, implying male bitterling mating tactics to be the product of the prevailing environmental and social conditions rather than being evolutionarily constrained (Duckworth, 2006), making the bitterling an especially attractive model for research on conditional male reproductive behaviour.

Acknowledgements

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