Male body size and breeding tubercles are both linked to intrasexual dominance and reproductive success in the minnow

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Antagonistic encounters between males are often costly (Smith 1974) and dominance hierarchies may be established to reduce the intensity of such interactions (Collias 1943). Dominant males usually have larger and higher-quality territories (Foote 1990; Candolin & Voigt 2001; Andersson et al. 2002), better access to females (Fleming & Gross 1994; Quinn & Foote 1994; Creighton 2001; Wong & Candolin 2005) and they often have higher fertilization success (Wiley 1973; Dewsbury 1982; Andersson 1994; Esteve 2005).

Across various taxa, dominance is usually well indicated by body size and weight (Andersson 1994; Qvarnstrom & Forsgren 1998), but often also by secondary sexual characters that are not directly used as weapons (Clutton-Brock et al. 1980; Berglund et al. 1996; Kortet & Taskinen 2004; Kortet et al. 2004). Sometimes, male dominance seems to be linked more closely to body size and secondary sexual characters (Zucker & Murray 1996; Hudman & Gotelli 2007), and sometimes secondary sexual characters may be the better indicators of male dominance (Kitchen et al. 2003; Kortet et al. 2004; Setchell et al. 2006; Stuart-Fox et al. 2006), especially so if secondary sexual characters indicate good health and vigour. Indeed, high resistance or tolerance to pathogens has been found in dominant males (Rantala & Kortet 2004; Ahtiainen et al. 2006) and in males with elaborate secondary sexual characters (Milinski & Bakker 1990; Wedekind 1992; Taskinen & Kortet 2002; Kortet & Taskinen 2004; Ezenwa & Jullo 2008). Secondary sexual characters can therefore be important not only in female choice but also in male–male competition (Andersson 1994).

We studied male reproductive success in regard to dominance and secondary sexual characters in the European minnow, Phoxinus phoxinus, a cyprinid whose mating system has so far only been qualitatively described as ‘communal spawning’ (Breder & Rosen 1966; Bless 1992). Mature males seem to establish a dominance hierarchy and to defend territories before females begin to spawn (Bless 1992). Males often display secondary sexual characters during the reproductive period. These characters can include conspicuous skin colours (e.g. melanin-based patterns and/or red colours: the latter are usually most pronounced around the mouth and the pectoral and pelvic fins) and breeding tubercles that are mostly located on the head. Breeding tubercles are small, colourless and horny epidermal structures that are common in many fish.
species. Their functional significance is not fully understood yet (see discussion in Wiley & Collette 1970 and Wedekind et al. 2008). In the case of the minnow, breeding tubercles may simply facilitate the maintenance of body contact between the mating partners, be used as weapons during male fights, or act as signals that provide information about male genetic quality and parasitic load through visual or sensory hydrodynamic signals (Wiley & Collette 1970; Müller & Ward 1995).

We analysed the spawning behaviour of minnows in a controlled seminatural set-up. We then tested whether male body size and/or breeding tubercles are linked to dominance and to male reproductive success as confirmed by microsatellite typings of parents and offspring.

METHODS

Recording of Spawning Behaviour

Minnows were caught by electrofishing from a natural population in the catchment of the river Venoge, Vaud canton, Switzerland, in April 2007, some weeks before the spawning season. Males were anaesthetized (with Aqui-S, Aqui-S New Zealand Ltd, Lower Hutt, New Zealand; 0.04 ml/litre) and individually marked with different black and white combinations of plastic beads (diameter 2 mm) on a nylon filament that was fixed to the dorsal fin by penetrating its basal part from one side with a small needle and attaching the perforated bead on the other side. After marking, they were transferred to a basin filled with oxygenated water to recover. After 30 min, all fish had fully recovered and the anaesthesia had no apparent negative effects. The fish were then introduced intro two aquarium (50 x 50 cm and 100 cm high, eight males with two females per aquarium) in a climate chamber. Individuals were fed twice a day with dry food (Tetra Min, BioActive formula), and with live zooplankton on the weekends. Individuals were prevented from eating eggs. In each tank, an area without gravel was left for potential use as a refuge for individuals to avoid predation. The gravel and the perforated metallic boxes were fitted into four Plexiglas boxes that had been put into each aquarium before the fish were introduced. The gravel and the perforated metallic boxes allowed the spawned eggs to fall through the gravel down to a 2 cm gap between the Plexiglas and the metallic box. This way, the fish were prevented from eating eggs. In each tank, an area without gravel was left for potential use as a refuge for individuals to avoid predation. The fish were then introduced into two aquarium (50 x 50 cm and 100 cm high, eight males with two females per aquarium) in a climate chamber. Individuals were fed twice a day with dry food (Tetra Min, BioActive formula), and with live zooplankton on the weekends. Four perforated metallic boxes were filled with gravel, 2–3 cm diameter, a substrate known to be preferred by minnows for spawning (Bless 1992). These filled metallic boxes fitted into four Plexiglas boxes that had been put into each aquarium before the fish were introduced. The gravel and the perforated metallic boxes allowed the spawned eggs to fall through the gravel down to a 2 cm gap between the Plexiglas and the metallic box. This way, the fish were prevented from eating eggs. In each tank, an area without gravel was left for potential use as a refuge for individuals to avoid predation.

To describe the males’ dominance hierarchy and their spawning behaviour, we monitored the aquaria with eight surveillance cameras (CCD cam 1/3” SONY Super HAD, lens angle 78°, minimum illumination 0.05 Lux, Profile, two cameras per side and per aquarium), which were linked to a MultiCam GV-1000 System (Ecoline; see Jacob et al. 2007 for further description). We recorded all behaviour between 10 May and 1 June 2007. A seasonal change was simulated by increasing the water temperature from 7 °C to 14 °C (1 °C every 2 days) and by changing the light cycle from 8 to 13 h of light per day. Observations during the first few days indicated that fish activity depended on the light regime, with low activity in darkness and increased activity when the light was switched on in the morning. The cameras were therefore programmed to film the aquaria from 0800 hours to 2100 hours. Boxes were checked every morning for the presence of eggs. Eggs were collected and individually distributed to 24-well multiwell plates (BD Falcon, San Jose, CA, U.S.A.; nontreated polystyrene, flat bottom). Each well had been filled before with 2 ml of water (14 °C) that was chemically standardized according to the OECD guidelines (OECD 1992). The isolated embryos were then incubated at 10.7 °C until hatching (no water exchange occurred in between).

On 14 June 2007, all males were anaesthetized, as described above, for biometry. We took digital photos of their foreheads so we could count the breeding tubercules later on. The diameters of individual breeding tubercules were also measured with the open-access software IMAGEJ (http://rsb.info.nih.gov/ij/). For this measurement we first sampled the four largest tubercules of the anterior part of the forehead, which are situated more or less in a rectangular pattern between the nostrils, two on the left and two on the right side of the mesial sagittal line (Figure 1 in Frost 1943). We also measured four randomly picked forehead tubercles that were situated posterior to the eyes (the tubercules were numbered on both sides of the mesial sagittal line and then selected for measurements using a random number generator).

We described the dominance hierarchy based on the antagonistic behaviour during three different kinds of observation periods. The first covered 2 h shortly before female spawning activity started (pfore), the second covered 1 h from the moment female spawning activity had started (p during), and the third (pend) covered 30 min starting 1 h after the end of the second period. An antagonistic act was defined as an interaction between two males that ended by one male swimming away and being followed or chased by the other male. The total number of recorded antagonistic interactions was aquarium 1: N1 = 445 (60 in pfore, 254 in p during, 131 in pend); in aquarium 2: N2 = 978 (428 in pfore, 382 in p during, 168 in pend). We assigned a winner and a loser for each of these interactions and calculated dominance hierarchies for each aquarium using the David’s score method. This method takes the relative strength of the opponents into account (Gammell et al. 2003; De Vries et al. 2006) and results in continuous scores (instead of ranks). We calculated an overall dominance hierarchy per aquarium. We also determined dominance hierarchies for each of the three observation periods per aquarium.

To identify male territories, we used the same video sequences as for the calculations of the dominance scores. The gravel area in each aquarium was divided into 16 sections of the same size. The position of each male was recorded every 5 min but only if no female showed any spawning activity on the spawning area. Otherwise, we skipped to the next observation point 5 min later. The size of a male territory was estimated using a score $s_{ij}$ for male i in section j, calculated as $s_{ij} = 1/n_j$ where $n_j$ is the number of males in section j at the time of observation, that is, a male’s score was weighted for the presence of other males in a given section. This procedure was followed for all observations separately to produce a sum of scores for each male in each section. We then computed a relative score for each male per section by dividing a male’s score by the sum of all scores for the given section. We summed these relative scores for each male over all sections to obtain the overall territoriality per male. This way, we obtained an index that was weighted by the presence of other males in each section of the potential spawning area.

Genetic Analyses

We used microsatellite markers to genotype all adults and a random sample of offspring (that had been killed with a lethal dose of Aqui-S, 0.1 ml/litre for 30 min, at the hatchling stage). To estimate male fertilization success per clutch (c) we genotyped the following hatchling numbers: aquarium 1: $N_1 = 40; N_2 = 63; N_3 = 80; N_4 = 28$; aquarium 2: $N_5 = 32; N_6 = 33; N_7 = 30; N_8 = 6$; that is, a total of 211 individuals were analysed for aquarium 1 and 101 individuals for aquarium 2.

Genomic DNA was extracted from tissue samples using the Qiagen DNAeasy Kit (Qiagen Inc., Valencia, CA, U.S.A.), following the manufacturer’s protocol. We used five microsatellite loci (Ca1, Ca12, Ca3, Ca5, Ppro126) previously developed in other cyprinids.
PCR amplification was carried out separately for Ca5 in 10 µl final volume containing 100–250 ng DNA, 1.5 mM MgCl2, 0.5 µM of each primer, 0.2 mM of dNTPs and 0.5 U of Qiagen Taq polymerase. All other loci were multiplexed in 8 µl final volume containing 2.5 µl of Qiagen Multiplex PCR Kit, 0.28 µM of Ca1 primer, 0.12 µM of Ca12 primer and 0.06 µM of Ca3 and Ppro126 primers. The PCR profile for Ca5 was: (1) 94 °C for 3 min, (2) 94 °C for 30 s, (3) 51 °C annealing for 30 s, (4) 72 °C for 30 s, (5) return to step 2 for 30 cycles, (6) 72 °C for 10 min. The PCR profile for the multiplexed loci was: (1) 95 °C for 15 min, (2) 94 °C for 30 s, (3) 58 °C annealing for 90 s, (4) 72 °C for 60 s, (5) return to step 2 for 35 cycles, (6) 60 °C for 30 min. The forward primers were labelled with a fluorescent dye (HEX, FAM or NED) on the 5’ end. PCR products were run on an ABI 3100 Automated Sequencer (Applied Biosystems Inc., Foster City, CA, U.S.A.) and analysed with the GeneMapper software (Applied Biosystems).

The assignment of the hatchlings to their parents was done by simple exclusion since all potential parents were known. The number of hatchlings sired by a male seemed to reveal directly the number of eggs that the male fertilized, because total hatching rate was high (96.1% in aquarium 1 and 95.7% in aquarium 2).

**Statistical Analyses**

We used a randomization test on the Kendall coefficient of concordance (W) to test whether the dominance hierarchies of the three periods were in agreement with each other. We therefore randomized the order of the dominance scores for each of the three periods and calculated W to get a null expectancy (i.e. a distribution of expected W based on 10 000 runs each) with which the observed W could be compared.

We fitted linear mixed-effect models with overall dominance score per male as the response variable and one or two fixed effects as an explanatory variable (i.e. male size, number of tubercles per male and male weight). To control for potential differences between the two aquaria, we added a random aquarium effect to each model. The aquarium effect was tested by randomizing the aquarium origin between the males and recording the quality of fit (log likelihood) of the model. The procedure was repeated 10 000 times to obtain an empirical distribution of log likelihoods for the aquarium affiliation per male. We then tested a potential effect of aquarium on this empirical distribution. The aquarium effect never explained a significant part of the variance (all respective P values > 0.2). For further analyses we thus excluded this effect and pooled the data of both aquaria. We then calculated Pearson correlations (r) or Spearman correlations (rs) when graphical inspection of the data suggested a significant deviation from normality. We also fitted a linear multiple regression model with dominance as a function of male size and number of breeding tubercles (the same was done for male weight instead of male size).

During a study on sperm motility that directly followed the present one, we found that all males except one from aquarium 1 had well-developed gonads. This one nonmature individual was excluded from all present analyses. The individual reproductive success of the other males was estimated for every clutch by multiplying the proportion of eggs the male had fertilized by the total number of eggs in the given clutch. These numbers were then summed to compute the total individual reproductive success, which was log transformed and included in linear mixed-effect models as the response variable. A random aquarium effect was introduced in each model and fixed effects included the dominance score, the number of tubercles, male size and territory size. The significance of the fixed effects was tested with likelihood ratio tests in separate models (see Jacob et al. 2007 for further explanation), but the effects of the number of tubercles and male size were also tested together in a single model. The aquarium effect on male reproductive success was significant in two of five models (based on randomization tests as described above), so we kept this random effect in all five models. Analyses were done with the R software (R Development Core Team 2007). We used the lme4 package for the mixed-effect models analyses (Bates et al. 2008).

**Figure 1.** Male positions on the spawning ground in relation to their breeding tubercles, their body size and the females’ spawning behaviour. Each aquarium (a, b, c, d) was divided into 16 sections. The bar plots represent the territoriality of each male in the aquarium. In (a) (aquarium 1) and (b) (aquarium 2), the males are sorted based on their body size with the largest male on the left and the smallest on the right. In (c) (aquarium 1) and (d) (aquarium 2), the males are sorted by decreasing tubercle number from left to right. The same bar colours are used for the same males in (a) and (c), or (b) and (d), respectively. Sections in which a female laid eggs have a grey background.
Ethical Note

The electrofishing was performed by professional fishery managers who routinely use this technique to monitor wild fish populations. We observed no adverse effects on the fish or the other wildlife in the river. The filament marking method was chosen because it does not seem to affect the fish adversely (Jacob et al. 2007). The filament is fixed with a single puncture in the dorsal fin. We believe this marking procedure results in less disturbance to the fish compared with other marking methods that are usually used for individual visual recognition (e.g. branding or tag fixation with a wire perforating the body cavity). We observed no difference in behaviour between marked and unmarked fish and the degree of stress was classified as 0 ('minimal degree of severity') by the veterinary office of the Vaud canton (the authority that gave permission for our experiments). During dominance fights, bites were never observed and actual physical contact was rare, that is, in the majority of cases the antagonistic interactions were chases. During the spawning events males were close to each other so that physical contact usually occurred (see videos 1 and 2 in the Supplementary Material). Throughout the study the fish were able to retreat to a refuge to avoid antagonistic encounters. The study conforms to Swiss laws and was done with permission of the Centre de conservation de la faune et de la nature and of the Service Vétérinaire of the Vaud canton. The fish were used in a follow-up study on sperm physiology and were killed at the end of these studies, as a condition of the permits. To kill the fish, they were exposed for at least 30 min to Aqui-S (0.1 ml/litre) following the manufacturer’s instructions. To confirm death, we transferred the fish to oxygenated water; none recovered from the anaesthetic.

RESULTS

The males displayed antagonistic behaviours soon after release into the aquaria and long before the first spawning event (see video 1.)

![Figure 2](image-url)  
**Figure 2.** Male dominance scores based on antagonistic encounters over three different kinds of observation periods for (a) aquarium 1 and (b) aquarium 2. Dashed lines connect male dominance scores measured before female spawning activity, dotted lines during spawning activity and straight lines towards the end of spawning activity. The filled symbols show the overall dominance scores that include all antagonistic interactions. Males are ordered by decreasing overall dominance from left to right.

![Figure 3](image-url)  
**Figure 3.** Relationship between male dominance status and (a) male body size or (b) number of breeding tubercles. Individuals from aquaria 1 and 2 are represented by open and solid symbols, respectively. Regression lines are given to illustrate the trends. See text for statistics.
in the Supplementary Material). Males seemed to defend territories by trying to maintain their position and chasing away other males (Fig. 1).

The dominance hierarchies from the different observation periods (before, during and at the end of spawning) appeared to be consistent over time when we combined the two tests for both aquaria (Fisher’s combination test: $\chi^2 = 18.2$, $P < 0.01$; concordance: in aquarium 1: $P = 0.11$; in aquarium 2: $P < 0.001$; Fig. 2).

The scores for male territoriality were positively linked to the overall dominance scores ($r_{13} = 0.65$, $P < 0.01$). Male dominance was also positively linked to male size ($r_{13} = 0.69$, $P < 0.01$; Fig. 3a) and weight ($r_{13} = 0.74$, $P < 0.01$). During the observation period, all mature males developed breeding tubercles on their forehead. The number of breeding tubercles was not significantly correlated with male size ($r_{13} = 0.39$, $P = 0.15$) or weight ($r_{13} = 0.43$, $P = 0.11$). Male dominance was, however, strongly and positively related to the number of breeding tubercles ($r_{13} = 0.71$, $P < 0.01$; Fig. 3b). Neither the mean diameter of the low forehead tubercles ($r_{13} = 0.25$, $P = 0.37$) nor the mean diameter of the high forehead tubercles ($r_{13} = 0.21$, $P = 0.48$) was significantly correlated with an individual’s number of tubercles. Our measures of tubercle size were also not significantly correlated with male dominance (anterior forehead: $r_{13} = 0.39$, $P = 0.16$; posterior forehead: $r_{13} = 0.42$, $P = 0.13$; one male was excluded as he lacked posterior forehead tubercles).

About 70% of the variance in dominance could be explained in a linear model that included body size and number of tubercles ($N = 15$; body size: $r_{12} = 2.87$, $P = 0.014$; tubercle number: $r_{12} = 3.02$, $P < 0.01$). The interaction term was not significant ($r_{13} = 0.63$, $P = 0.54$) and was thus removed from the models. We found analogous results when male size was replaced by male weight (data not shown).

At the beginning of the experiment, females tended to swim by themselves and to approach males only rarely. Later in the spawning season, females frequently swam close to the gravel, sometimes touching it. They were then usually closely followed by most of the males. Females that spawned batches of eggs into the gravel were always closely accompanied by all or almost all the males of an aquarium (see video 2 in the Supplementary Material).

We found a total of 707 eggs in aquarium 1 and 805 in aquarium 2. Of the 15 males, 12 could be confirmed by later genetic analyses to have sired offspring (all seven males in aquarium 1 and five of eight males in aquarium 2). The number of sires per batch of eggs ranged from one to six ($X \pm SD = 4.5 \pm 2.4$) in aquarium 1 and from three to four ($3.25 \pm 0.5$) in aquarium 2. Male reproductive success ranged from 11 to 199 embryos ($X \pm SD = 101.1 \pm 62.6$) in aquarium 1 and from 0 to 488 ($100.6 \pm 176.3$) in aquarium 2. Because male reproductive success differed between aquaria (10,000 permutations of model 2 in Table 1; $P = 0.04$), a random aquarium effect was included in the respective models (Table 1). Male dominance was positively linked to reproductive success (Fig. 4, Table 1). Tubercle number, body size and overall territoriality were also positively related to male reproductive success (Table 1). When male tubercle number and body size were entered together in a single model (Akaike’s information criterion, AIC = 63.076), the effect of male tubercle number was significant (likelihood ratio test; LRT: $X^2 = 4.82$, $P < 0.03$), but not male body size (LRT: $X^2 = 1.13$, $P = 0.29$). The model with the best AIC was the one including dominance (Table 1).

**DISCUSSION**

The minnows in our study showed a lek-like breeding system with males defending territories on the spawning ground. The males quickly established dominance hierarchies, long before the beginning of female spawning activity. These dominance hierarchies seemed to be important for the breeding system of minnows: dominance was positively linked to territory size and to reproductive success. However, our behavioural observations and our genetic analyses of the offspring revealed that multimale fertilizations were common. This suggests that the spawning territories could not usually be defended to allow for pairwise spawning, confirming previous observations on European minnows in the wild (Breden & Rosen 1966).

As expected from findings in other fish, male body size was a reliable indicator of dominance rank, but what about the breeding tubercles? Male size and tubercle number were not significantly correlated in our sample (in contrast to other samples, see Müller & Ward 1995). However, dominance status and the induction of breeding tubercles are based on similar physiological pathways, that is, both dominance (Cardwell et al. 1996; Fitzpatrick et al. 2008) and breeding tubercles (Kortet et al. 2003) can be positively linked to androgen concentration (mainly to 11-ketotestosterone but also to testosterone). Indeed, both male size and tubercle number were reliable indicators of dominance rank. This latter result contrasts with findings on roach, *Rutilus rutilus* where tubercle size, but not number, was linked to dominance (Kortet et al. 2004), and with findings on fathead

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**Table 1**

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Effects were tested by comparing each model against the reference model 1. Such likelihood ratio tests are based on a $\chi^2$ distribution with $df = 1$. Akaike's information criterion (AIC) describes the quality of fit of each model. The table also gives the Pearson correlation coefficients ($r$) between male traits and male reproductive success.

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**Figure 4.** Relationship between male dominance and reproductive success (see text for statistics; line fitted according to model 2 in Table 1). Males from aquaria 1 and 2 are represented by open and solid circles, respectively.
minnow, *Pimephales promelas*, where tubercle number was also not linked to dominance (Hudman & Gotelli 2007). In our study, male size and tubercle number seemed to capture different aspects of dominance, as both were significant when included simultaneously in a single model. We found no significant correlation between tubercle size and tubercle number or male dominance.

Our results confirm the assumption made by other authors that the European minnow is a group-spawning species (Bless 1992; Stockley et al. 1997). Such a mating system is expected to make female choice difficult, but females may still be able to increase the relative fertilization success of some males. During the spawning period the females performed upward and downward movements often at the same spot and at a high frequency. This was often but not always followed by the spawning of a batch of eggs, that is, females may only release eggs, or more eggs than usual, when an attractive male is close and thus more likely to fertilize a larger share of the eggs. The number of breeding tubercles could act as a stimulus that would trigger the egg release by the female. In line with this hypothesis, breeding tubercles were significantly linked to reproductive success and, when entered in a model together with male size, they still significantly predicted reproductive success, whereas male size did not. It is still unclear whether this is any female choice in minnows, but, if so, females may gain two-fold by choosing dominant males with elaborate secondary ornamentation. On the one hand, they may get high-quality males with possibly better surviving offspring (Wedekind et al. 2001, 2008). On the other hand, if tubercle number and preference for it are heritable, their sons may have a higher fertilization success (Fisher 1930). Alternatively, female choice could be based on the quality of male territories. In some fish, the better territories are occupied by more dominant males (Foote 1990; Dijkstra et al. 2005). In the prespawning period, most male–male interactions ended after some male display and without any direct physical contact. Contact between males usually occurred only shortly before and during spawning when several males tried to get close to a female. We therefore believe that breeding tubercles are signals that indicate a male’s determination to fight for its territory and/or signals that are used in female choice.

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Supplementary Material


References


