

## Original Article

## Female mouthbrooders in control of pre- and postmating sexual selection

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The fertilization mode determines which sex has greater control over the offspring's sires. With internal fertilization, females can strongly influence the chances of different males' ejaculates to fertilize their eggs by the postmating sexual selection process referred to as cryptic female choice. In contrast, when fertilization is external and multiple males compete in this process, the outcome of pre- and postmating sexual selection is largely determined by the competitive quality of males and their sperm. Intermediate modes of fertilization as found in mouthbrooding fishes might allow for a greater maternal influence on her offspring's sire. Here, we show that in the maternal mouthbrooder *Ophthalmotilapia ventralis*, females collect sperm from different males in their mouth, and males can successfully fertilize eggs even if the female did not lay eggs with them. In the field, 25 of 30 clutches had multiple sires, and the fertilization success was significantly biased toward particular males in most clutches. A mate choice experiment revealed that females prefer to spawn with males possessing strongly elongated pelvic fins, a conspicuous secondary sexual character of males in this cichlid. Additionally, the body length of males partly explained their success in sperm competition within the females' mouth, a factor without apparent influence on female choice of partners with which to lay eggs. Hence, successful sires are determined by a 2-step process that is largely under female control; females select which males to spawn with and from which males they collect additional ejaculates for the subsequent sperm competition in their mouth.

**Key words:** cichlidae, lekking, multiple mating, polyandry, sperm competition, sperm shopping. [*Behav Ecol* 22:1033–1041 (2011)]

## INTRODUCTION

Males can generally increase their genetic fitness by mating with multiple females, whereas the fitness benefits gained by multiply mating females are often less obvious (reviewed in Arnqvist and Nilsson 2000; Jennions and Petrie 2000; Simmons 2005) even though polyandry is common (Andersson 1994; Birkhead and Møller 1998; Zeh JA and Zeh DW 2003). Multiple mating by females may strongly influence the direction and intensity of sexual selection (Sugg and Chesser 1994; Eberhard 1996, 1998; Birkhead and Møller 1998; Urbach et al. 2005; Ward 2007; Barbosa et al. 2010). However, the effect of polyandry on selection depends also on which sex is in control of mating (Stockley 1997; Birkhead and Pizzari 2002; Clutton-Brock 2007; Klug et al. 2010).

Polyandry is very common in fishes and may result merely from the attempt of several males to fertilize eggs or from the active choice of females to mate with multiple partners (Taborsky 1994, 1998, 2008). Most fish species show external fertilization, and females have limited control over mating. Polyandry typically occurs during group spawning or by

participation of reproductive parasites during pair spawning (Taborsky 2008). However, in lekking maternal mouthbrooders females appear to be largely in control of the decision with whom to mate. It is the female that moves from one territorial male to the next before deciding which male to mate with (McKaye 1991; Kellogg et al. 1995; Kuwamura 1997; Barlow 2000; Immler and Taborsky 2009). Participation of reproductive parasites in spawning is apparently rare in mouthbrooding cichlids (Albrecht 1968; McKaye 1983; Kuwamura 1987; Haesler et al. 2009), and yet multiple paternity has been reported from a number of species, both in the wild and in aquarium experiments (Kellogg et al. 1995; Parker and Kornfield 1996; Knight et al. 1998; Maan et al. 2004). This suggests that polyandry in these species results mainly from the active choice of females to mate sequentially with multiple males.

Experiments with a mouthbrooding cichlid have shown that most eggs are fertilized in the female's mouth (Mrowka 1987; see also Wickler 1962). Kellogg et al. (1995) hypothesized that sperm competition might occur in the mouths of female mouthbrooding cichlids when they visit different males successively during spawning of a clutch. This has been supported by a comparative study finding that polygamous mouthbrooders have longer sperm than monogamous mouthbrooders (Balshine et al. 2001), which is in agreement with predictions from sperm competition theory (Ball and Parker

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1996). In mouthbrooding Tilapias, sperm are packed into a mucus making ejaculates sticky, so that they can be collected by the female as a package and carried in her mouth until she visits another male (Wickler 1965; Grier and Fishelson 1995). Histochemical examinations have revealed such sperm packaging also in *Ophthalmotilapia ventralis* (Haesler 2007; Immler and Taborsky 2009).

A recent behavioral study tested 4 hypotheses to explain why females mate multiply in the mouthbrooding cichlid *O. ventralis* from Lake Tanganyika (Immler and Taborsky 2009). Females of this species always visit multiple males during a spawning, and this happens in 3 phases 1) before laying eggs, 2) during egg laying, and 3) after the last egg had been laid. The data suggested active inducement of sperm competition by females, which suggests that the sexually selected sperm hypothesis (Keller and Reeve 1995) might partly explain the peculiar spawning behaviour of this cichlid. In particular, this was suggested by the long phase after termination of egg laying during which females continue to visit multiple males to collect sperm (Immler and Taborsky 2009).

Here, we aim to test whether sperm competition occurs in the mouths of female mouthbrooding cichlids by combining behavioral and molecular data from the field and laboratory experiments. We investigated the importance of pre- and post-mating sexual selection mechanisms first by sampling broods from 30 females at a lek and testing for multiple paternity. From spawning observations and with the help of a laboratory experiment, we tested the criteria by which females select multiple mates for spawning and the predictors of success in sperm competition within the female's mouth. We predicted that females influence fertilization success of potential sires at both pre- and postmating levels independently by 1) deciding with whom to lay eggs and 2) visiting successive males to collect sperm at time intervals allowing sperm of different males to compete for the fertilization of eggs in their mouths.

## MATERIALS AND METHODS

### Determination of multiple paternity in the field

Field observations and the collection of clutches in Lake Tanganyika were performed using SCUBA at Kasakalawe point, Mpulungu, Zambia (lat 8°46.849' S, long 31°04.882' E), in March 2003 and October/November 2005. We studied a lek consisting of more than 200 male *O. ventralis* at a depth of 2–4 m. The territories are roughly 1–2 m<sup>2</sup> in size and contiguous. Males make bowers in the form of small circular sand-patches of about 10–12 cm in diameter on top of rocks of approximately 20 × 30 cm in size, which are visited by females for spawning (Immler and Taborsky 2009).

Two types of clutches were collected in the field for paternity analysis: 1) The first sample consisted of a total of 30 clutches of mouthbrooding females that had not been observed during spawning ( $N = 10$  in 2003 and  $N = 20$  in 2005). Twenty-five of these clutches were complete, whereas in the remaining 5 clutches, 1–3 offspring were either lost or DNA extraction was not successful. The mean number of offspring per brood from which DNA was successfully extracted, amplified, and assigned to parents was 12.67 (see RESULTS). 2) The second sample consisted of clutches from 3 females that had been directly observed at spawning (in 2005). The total number of eggs contained in these clutches was 44, of which 25 were successfully genotyped. While observing a spawning sequence, we noted all males the female visited and the number of eggs laid with each male. Males in this species can be individually recognized by their characteristic, irregular black spots on the body. After the female had finished spawning, we caught her with the help of a fine-meshed fence net and hand nets and

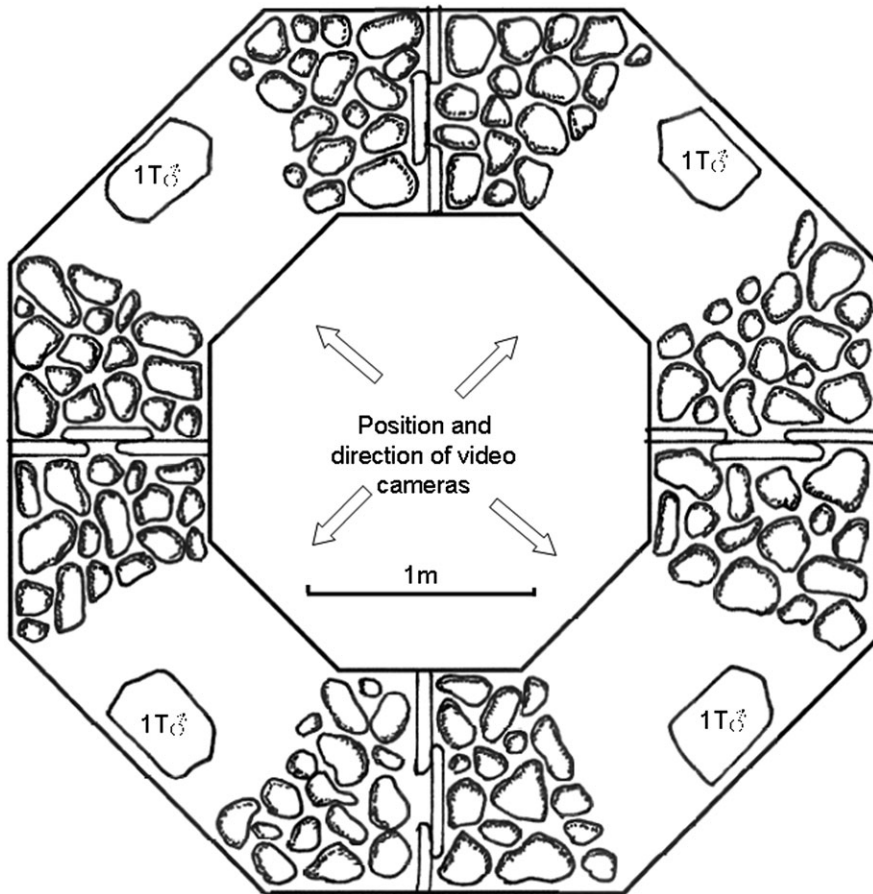
collected the brood, measured female size (standard length, SL) and weight (gram), and took a small fin clip for genetic analysis. Shortly afterward we caught the males the female had spawned with, measured their size and weight, and took a fin clip for paternity analyses. Finally, we caught additional randomly chosen males and females to take fin clips for genotyping and to calculate allele frequencies in the population.

In total, we collected fin clips from 38 males (22 in 2003 and 16 in 2005) and 56 females (13 in 2003 and 43 in 2005), and a total of 390 eggs and fry (118 in 2003 and 272 in 2005) for parentage analysis (see section on paternity analysis). Fin clips and eggs were preserved in 99% ethanol. The eggs from females that had been observed during spawning were kept in an egg tumbler for 1–2 days, so the embryos were developed sufficiently for successful DNA extraction and paternity analysis (Supplementary Figure S1).

### Laboratory experiment

We used a large octagonal ring tank with a volume of 7200 l to test for female choice and sperm competition experimentally. We provided 4 evenly outspaced rocks each suitable to hold a bower, which were similar in size to the rocks used by *O. ventralis* in the field (Haesler 2007; Immler and Taborsky 2009). We put 12 males and 12 females into the tank, that is, there were 3 times more males than potential spawning sites provided in the tank, which caused dynamic territory ownership in the course of the experiment. Territory ownership was checked at least once daily. Additional, upright standing stone slabs extending halfway up the water column induced the males to defend territory boundaries along these structures so that territory sizes were similar. The sandy areas between bowers and vertical rocks were covered with small stones to prevent males from making bowers in the sand (Figure 1). The areas containing bowers were continuously surveyed by video recording throughout the daytime period (0800–2100 h). The eggs are large and therefore were easy to detect on the recordings during spawning. To test for potential deviations of the fertilization pattern from the observed spawning pattern, paternity was determined using microsatellite markers. When a female had spawned, we removed her from the tank after 1–4 days incubation and replaced her by another female to keep the density constant. The eggs were incubated in an egg tumbler for another 1–2 days (Supplementary Figure S1). A total of 20 females were used in this experiment. For practical reasons, the experiment was split up into 2 separate periods (28 March 2006–14 June 2006 and 11 September 2006–7 November 2006) lasting 186 days in total. All fish were taken out between the 2 periods but the setup of the tank was not changed. In the second period of the experiment, the same females and males were used, except for 3 males that had to be replaced because of illness (males 31, 32, and 45 were used only in the second experimental period, whereas the other 9 males used in the second period had also been used in the first period, which adds up to a total of 15 males used in this experiment).

From the video recordings, we scored the total number of eggs spawned, the number of eggs spawned with each male per spawning event, durations, and time intervals between egg deposition and apparent sperm uptake (i.e., the moment a female takes the tassels of the male pelvic fins into her mouth, called mouthing; see below). In a typical courtship sequence, a territorial male leads the female to his bower (scored as "court"), which she may subsequently enter ("follow"). The male ejaculates onto his bower and leaves the tassels of his elongated pelvic fins resting where he apparently ejaculated ("presenting"). The female may then take these tassels, often referred to as egg dummies, into her mouth ("mouthing"), thereby presumably collecting sperm. Thereafter, the male



**Figure 1**

Experimental setup in the octagonal ring tank seen from above. Four territorial males are indicated with “1 T♂” on the rocks on which bowers were located. Additionally, there were 8 floater males and 12 females in the tank. Thin slates reaching to half of the tank height served as territory boundaries. Small rocks were placed on the bottom to prevent males from making bowers somewhere on the sand. All 4 rocks with bowers were continuously monitored with video cameras (as indicated by arrows).

leaves the bower and the female may lay an egg which she immediately takes up. Note that the male ejaculates before the female lays an egg. This sequence may be repeated multiple times both, with the same or a different male (for a detailed description, see Immler and Taborsky 2009).

We measured SL, pelvic fin length (PL), and body weight (wt), and we calculated relative pelvic fin length (PL/SL). The length of each pelvic fin was measured twice to reduce measurement error (the 2 measurements were highly correlated:  $R^2 = 0.956$ ,  $P < 0.0001$ ); the means of the 2 measurements were used to estimate the length difference between both pelvic fins for symmetry estimates. Body condition (BC) was calculated as  $BC = wt/SL^3 \times 100$  (Bolger and Connolly 1989). These measures of male morphology were tested for potential correlation with reproductive success, measured both as number of eggs received and number of eggs sired.

#### Paternity analysis

Eleven (for all field samples from 2003) to 13 (for all field samples from 2005 and for all samples from the laboratory experiment) polymorphic microsatellite loci were used to determine the parentage of the collected clutches (loci NP007, NP773, ULI2: Schlieven et al. 2001; UNH106: Lee and Kocher 1996; Pzeb3, Pzeb4: van Oppen et al. 1997; TmoM7, TmoM11, TmoM13, TmoM25, TmoM27: Zardoya et al. 1996; UME003: Parker and Kornfield 1996; NP101 (LOC101): Brandtman et al. 1999). All loci had between 4 and 31 alleles in 35 unrelated individuals caught in 2003 (females and males combined; Supplementary Table S1) and between 3 and 35 alleles in 57 unrelated individuals caught in 2005 (Supplementary Tables S2 and S3), and they segregated independently.

Genomic DNA was extracted from ethanol-preserved fin clip samples from the females and males or from whole offspring (2003 samples) using the Wizard Genomic DNA Isolation Kit (Catalys Promega AG, Switzerland). Genomic DNA was dissolved in 50  $\mu$ l of DNA Rehydration Solution (Promega) and stored at  $-20^\circ\text{C}$  until further analysis. For DNA extraction from all 2005 samples ( $\sim$ 2-day-old eggs, whole larvae, and fin clip samples) and the samples from the aquarium experiment, we used Magnetic Beads (MagneSil Blue, Promega; White et al. 1998). Tissue lysis was carried out in a Lysis-Buffer containing Nuclei Lysis Solution (Promega), 0.5 M ethylenediaminetetraacetic acid and Proteinase K according to the Wizard Genomic DNA Isolation Protocol (Promega). DNA was captured in solution by adding Paramagnetic Particles (MagneSil Blue, Promega; White et al. 1998) to the lysate, and it was washed 2–3 times with 80% ethanol with the aid of a magnetic separator (Magna-Bot96 Magnetic Separation Device, Promega) to eliminate residual contaminants. Finally, genomic DNA was eluted directly from the paramagnetic particles with 50–100  $\mu$ l of nuclease-free Water.

For polymerase chain reaction (PCR) amplification, up to 7 microsatellite primer pairs were multiplexed in one PCR reaction using the QIAGEN Multiplex PCR Kit (Qiagen AG). PCRs were carried out in a 10  $\mu$ l volume containing: 10 ng of genomic DNA, 1 $\times$  QIAGEN Multiplex PCR Master Mix, and 0.2  $\mu$ M of locus-specific fluorescent-labeled forward primers and non-labeled reverse primers. In order to improve allelicalling efficiency, the sequence 3'-GTTTCTT was added to the 5' end (Brownstein et al. 1996) of 10 of the reverse primers in the 2005 series (NP101, NP773, TmoM7, TmoM11, TmoM13, TmoM25, TmoM27, UME003, ULI2, and Pzeb3). This reverse primer tailing results in nearly 100% adenylation of the 3' end

of the forward strands, thereby facilitating accurate genotyping as a result of consistent allele calls (Brownstein et al. 1996).

Amplification was achieved in a 96-well GeneAmp PCR System 9700 (Applied Biosystems, Switzerland) by using the following sequence of cycling parameters: 15 min at 95 °C; 33–35 cycles at 94 °C for 30 s, 57 °C for 90 s, and 72 °C for 60 s; followed by a final step of 72 °C for 10 min. Fluorescent PCR fragments were visualized by capillary electrophoresis on an ABI PRISM 3100 Genetic Analyzer and analyzed by the GenScan Analysis software v2.1 (2300 samples) and the GeneMapper Analysis Software v3.7 (2005 and 2006 samples; Applied Biosystems). Allele frequencies, observed and expected heterozygosities, and exclusion probabilities were determined using the CERVUS 2.0 software package (Marshall et al. 1998; see Supplementary Tables S2 and S3).

As the offspring were collected together with a fin clip from the mouthbrooding mother, the maternal genotype was known. Genetic analysis confirmed that all offspring had zero mismatching alleles with their mothers. The number of fathers siring a brood was estimated in 2 ways: 1) by counting the paternal alleles in a clutch; the number of paternal alleles from the locus with the greatest number of different paternal alleles per clutch was divided by 2 and rounded up if it was not an integer (e.g., 5 paternal alleles would mean that “at least 2.5 different males” were involved and hence a minimum of 3 fathers was assumed, as each male can transmit only 2 alleles) and 2) by using the program GERUD 2.0 (see Jones 2005; <http://www.bio.tamu.edu/USERS/ajones/JonesLab.htm>). For analysis with GERUD, we used only the 4–6 most variable loci (NP007, NP773, TmoM11, and UME003 for the 2003 samples, exclusionary power = 0.9998; NP007, NP773, TmoM11, UME003, TmoM7, and TmoM13 for the 2005 samples, exclusionary power = 0.9999). This program takes population allele frequencies into account and generates the most likely genotypes for fathers of the known offspring, providing estimates of how many offspring were sired by each father.

### Statistical analysis

The data of offspring number and number of fathers were tested for normality (Kolmogorov–Smirnov test: fathers<sub>2003</sub>:  $N_{2003} = 10$ ,  $P = 0.651$ , clutch size<sub>2003</sub>:  $N_{2003} = 10$ ,  $P = 0.762$ ; fathers<sub>2005</sub>:  $N_{2005} = 20$ ,  $P = 0.084$ , clutch size<sub>2005</sub>:  $N_{2005} = 20$ ,  $P = 0.107$ ) and for homogeneity of variances (Levene’s test: fathers:  $P = 0.447$ , clutch size:  $P = 0.076$ ). Because none of these results showed significant deviations, we used an unpaired *t*-test to check for differences between the samples of the 2 years. Using a poisson generalized linear model (log-link), we tested whether the number of fathers increased with clutch size (based on the number of fathers as estimated by GERUD 2.0). We calculated the expected distribution of offspring over the sires using the hypergeometric distribution for each clutch separately, assuming that females target 4 different sires (the maximum detected in the field and the maximum available in the aquarium experiment). With a Monte Carlo Fisher’s exact test, we tested whether the distribution of the clutch was random with regard to the males a female mated with.

For the analysis of reproductive success and male traits in the laboratory experiment, we used weighted logistic generalized estimating equations (GEEs, logit-link) in a forward stepwise fashion, retaining only the significant variables in the model. Forward stepwise inclusion of significant main effects was chosen, as our data set was too small to test for interaction effects between the main effects. Male and female identities were included as subject effects to account for repeated measures. This method assures that the same pairs (mothers × sires) do not influence the magnitude, standard error, and significance of the main effects.

We used SPSS 17 for Windows for all statistical analyses. All *P* values presented are 2-tailed.

## RESULTS

### Paternity in the field

The 2 methods to determine the number of different sires per clutch (counting paternal alleles vs. GERUD 2.0) generated very similar results (Figure 2 top: hatched bars vs. black bars), so only the GERUD assignments were used for the remainder of the analyses. The GERUD 2.0 analysis revealed that in our sample, only 2 males sired offspring in 2 clutches each: one male sired 6 offspring in clutch 7 and 5 offspring in clutch 19; the other male sired 5 offspring in clutch 11 and 8 offspring in clutch 19. All other fathers sired offspring within only one of the collected clutches, with most males siring only a few eggs (see Supplementary Figures S2 and S3). The 2 samples from 2003 to 2005 did not differ in clutch size or in the number of fathers per clutch (unpaired *t*-test for clutch size:  $n_{2003} = 10$ ,  $n_{2005} = 20$ ,  $\text{mean}_{2003} = 12.5$ ,  $\text{mean}_{2005} = 13.1$ ,  $t = -1.483$ ,  $P = 0.149$ ; for number of fathers:  $\text{mean}_{2003} = 2.75$ ,  $\text{mean}_{2005} = 2.45$ ,  $t = 0.394$ ,  $P = 0.697$ ). Samples from the 2 years were therefore pooled. The means for clutch size and number of fathers per clutch for all 30 clutches collected in the field were  $\text{mean}_{\text{eggs}} = 12.67$  (range 9–17) and  $\text{mean}_{\text{fathers}} = 2.50$  (range 1–4).

Twenty-five of the 30 analyzed clutches (83.33%) had 2 or more fathers (Figure 2 top). The proportion of offspring sired per male declined with the total number of sires in the clutch (Figure 2 middle). As predicted, the total number of sires per clutch tended to increase with the clutch size but leveled off at a maximum of 4 different sires for the largest clutches (Figure 2 bottom,  $P = 0.058$ ), that is, more sires seem to be involved when the clutch size increases. To test whether detection probability might have played a role, we assumed that all females targeted 4 different sires. Under this assumption, there is evidence that females with smaller clutches skewed the proportion of sired offspring toward a particular male: offspring from clutches with 4 sires were all distributed randomly over these 4 males (white circles in Figure 2 bottom: nonsignificant Monte Carlo Fisher’s tests per clutch), whereas offspring from clutches with fewer sires showed increased reproductive skew (black circles in Figure 2 bottom: significant Monte Carlo Fisher’s tests per clutch). This suggests that detection probability is at least not the only cause of the decrease of reproductive skew with increasing clutch size.

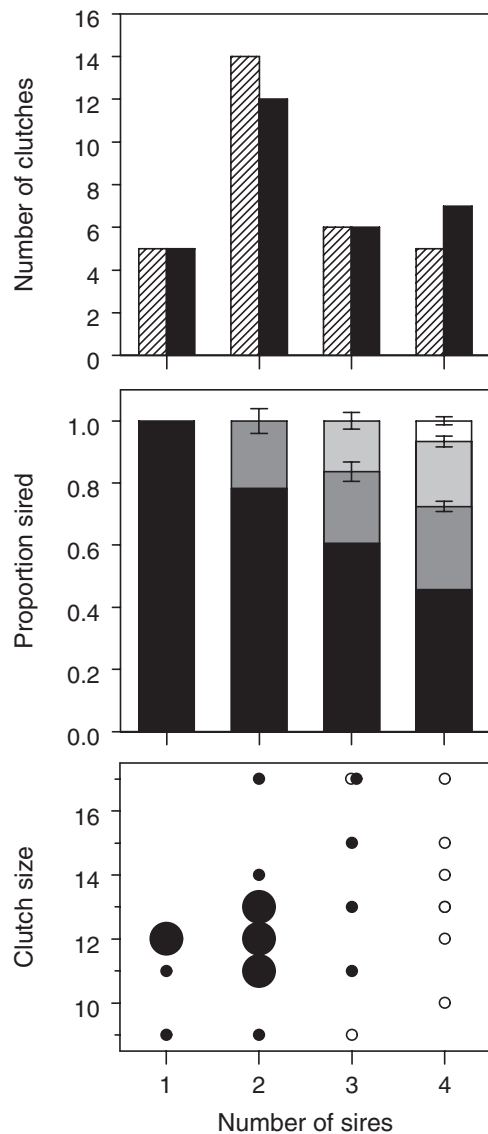
### Spawning observations in the field

We caught 3 females observed at spawning directly after they had finished visiting male bowers, and one of them was observed for the spawning of her entire clutch. This female visited a total of 6 different males and spawned all 10 of her eggs with only one male (O165). She visited a neighboring male (“unmarked neighbor of O165”) many times to collect sperm, but without depositing eggs, switching back and forth between these 2 males. We were able to analyze 6 eggs of this clutch (the DNA of the other 4 eggs was degraded), 5 of which were sired by O165 and 1 by his unmarked neighbor (see Supplementary Table S1). The other field observations of spawning females are described in the Supplementary Material.

### Laboratory experiment

#### *Spawning pattern versus fertilization pattern*

In the first experimental period, we observed the spawning of 13 clutches and analyzed parentage of the offspring from 9



**Figure 2**  
Field data on paternity ( $N = 30$  clutches from 2003 to 2005 combined). Top: Number of clutches with 1, 2, 3, and 4 sires per clutch, respectively. Estimates from counts of paternal alleles (hatched bars) and from the GERUD 2.0 analysis (black bars; see text for details). Middle: Proportion of the clutch sired by up to 4 different males (means  $\pm$  standard error of the mean, based on GERUD) per total number of sires of the clutch (sorted according to share, white: lowest number of sired offspring, to black: highest number of sired offspring; for sample sizes, see (c)). Bottom: The number of different sires tended to increase with the clutch size (poisson generalized linear model:  $\chi^2 = 3.6$ , degrees of freedom = 1,  $P = 0.058$ ). Assuming females targeted 4 different sires in their clutches, black circles (large circles denote 3 overlapping data points) indicate significant reproductive skew (at  $\alpha = 0.05$ ) toward particular males, whereas white circles indicate random reproductive partitioning over 4 different males (Monte Carlo Fisher's exact tests).

of them (numbers 1–9; Supplementary Table S1). In 2 of these clutches, the spawning pattern did not match the fertilization pattern, that is, the number of eggs sired by a male did not match the number of eggs spawned with the corresponding male. In the second experimental period, we observed spawning of 14 clutches and collected 8 of them (numbers 10–17; Supplementary Table S1). In clutch 1, 16 eggs were spawned with male 23, but male 23 ate 2 of these

eggs before the female picked them up. The female also spawned 2 eggs with male 21, that is, a total of 16 eggs were collected. However, all 15 eggs we could genotype were fertilized by male 23, that is, he fertilized at least one egg that was not laid on his bower. There were 2 clutches in which a male fertilized one egg even though none was spawned on his bower (clutch 9, male 26; and clutch 15, male 30). Clutch 2 was split equally between the 2 males 20 and 21, and both males fertilized the number of eggs corresponding to the number laid on their bowers. Clutch 7 was also sired roughly proportionally to where it was laid, but 9 of the 15 eggs of this clutch were eaten by the female before collection, which makes it difficult to interpret this result. Nine clutches (3–6, 8, 11, 12, 16, and 17) were spawned with and sired by only one male. Most eggs of 3 clutches (12, 14, and 17) died in the egg tumbler and therefore could not be successfully genotyped (summarized in Supplementary Table S1).

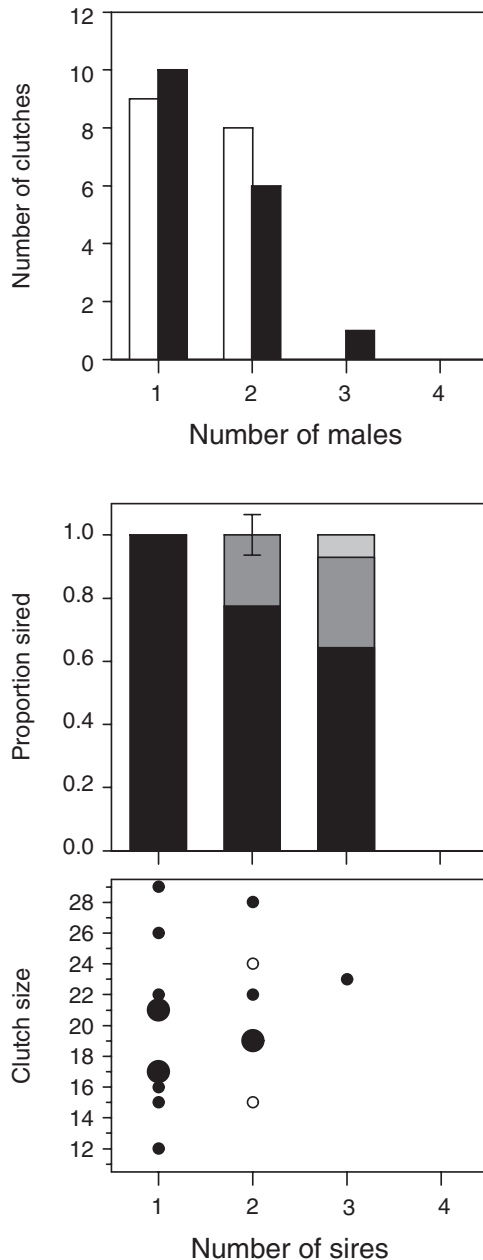
In total, these analyses revealed 3 spawnings in which sperm competition was confirmed by the data, 2 spawnings with no opportunity for sperm competition to occur (i.e., the spawning female did not visit any other males), 5 spawnings in which sperm competition may have occurred but was not detected from the fertilization pattern, and 7 spawnings where sperm competition may have occurred as well but which suffered from substantial egg loss before analysis, hampering interpretation of the results. None of the nonterritorial males secured any spawnings or fertilizations, but territory ownership sometimes changed during the experiment, thus there were more males than available territories that secured spawnings over the entire experimental period.

In contrast to the field situation, where females had a much larger number of males to choose from, the number of males females chose for egg deposition in the laboratory (Figure 3 top, white bars) was only 1 or 2, and the number of sires per clutch varied between 1 and 3 (Figure 3 top: black bars; cf. Figure 2 top). Nevertheless, the proportion of offspring sired by the different males was very similar to the field situation (cf. Figure 3 middle vs. Figure 2 middle). Contrary to the field situation, clutch size did not influence the number of sires detected (Figure 3 bottom,  $P = 0.38$ ), but again like in the field, the proportion of offspring sired was usually not randomly distributed over the available 4 territorial males (Figure 3 bottom, black circles: 15 clutches with significant deviations and white circles: 2 clutches with nonsignificant deviations from random paternity based on Monte Carlo Fisher's tests). This suggests that females preferred to spawn with particular males within the octagonal ring tank, which is analyzed in more detail below.

#### *Causes of variation in male reproductive success*

The males used in the aquarium experiment differed considerably in their body measurements (mean  $\pm$  standard deviation, range): body length ( $87.4 \pm 4.18$  mm, 79–95 mm), body mass ( $17.65 \pm 2.31$  g, 12.4–22.8 g), body condition ( $0.0026 \pm 0.000023$ , 0.0023–0.0033), pelvic fin length (mean of right and left fin  $53.9 \pm 10.15$  mm, 27.25–66.25 mm), ratio of pelvic fin length and body length ( $0.62 \pm 0.11$ , 0.26–0.74), and pelvic fin symmetry (absolute difference in millimeters between right and left fin:  $1.77 \pm 1.67$ , 0–6.5). These measurements did not correlate with each other (except for SL with weight [ $r = 0.815$ ,  $P < 0.001$ ] and pelvic fin length with the ratio of pelvic fin length and body length [ $r = 0.965$ ,  $P < 0.001$ ]).

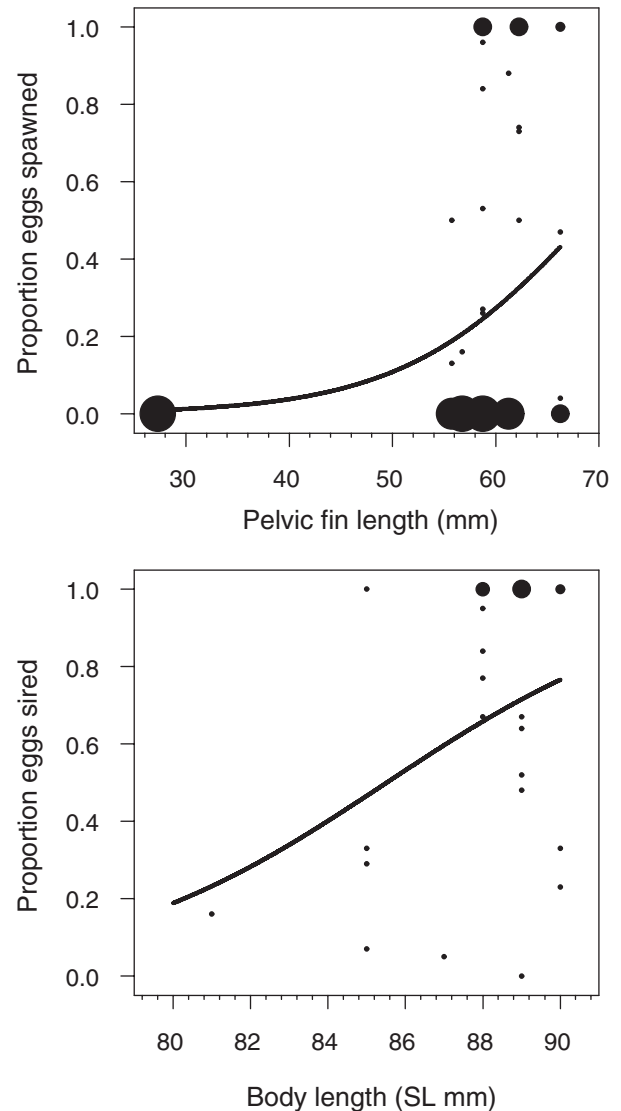
Females spawned a significantly larger proportion of their clutch with males having longer pelvic fins (Figure 4 top, Table 1). For the analysis of siring success, only successful sires were included. A male's siring success depended significantly on his body length (as SL and weight were highly correlated,



**Figure 3**

Laboratory data on spawning and paternity ( $N = 17$  clutches). Top: Number of clutches spawned (white) with up to 4 males (maximum number of territorial male sires available in the octagonal ring tank) and actual number of sires per clutch (black). Middle: Proportion of the clutch sired by up to 3 different males (means  $\pm$  standard error of the mean) per total number of sires of the clutch (sorted according to share, light gray: lowest number of sired offspring, to black: highest number of sired offspring; for sample sizes, see the bottom panel). Bottom: The number of different sires did not increase with the clutch size (poisson generalized linear model:  $\chi^2 = 0.7$ , degrees of freedom = 1,  $P = 0.38$ ). Assuming females targeted 4 different sires for their clutch, black circles (large circles denote 2 overlapping data points) indicate significant reproductive skew (at  $\alpha = 0.05$ ) toward particular males, whereas white circles indicate random reproductive partitioning (Monte Carlo Fisher's exact tests).

only SL was used in the statistical model; Figure 4 bottom, Table 1), although pelvic fin length tended to be important as well when added to the model ( $P = 0.057$ ). Note that siring



**Figure 4**

Top: The males' spawning success (eggs received/clutch size;  $N = 68$ , i.e., 4 potential territorial male sires  $\times$  17 clutches) depending on the males' pelvic fin length (mean of left and right fin). Bottom: The males' siring success (eggs sired/clutch size,  $N = 26$ , i.e., 17 clutches  $\times$  1–3 involved males, see Supplementary Table S1) depending on the males' body length. Data from 17 clutches in the aquarium experiment. Overlapping data points are indicated with increasing symbol sizes, the weighted logistic regression lines result from the two generalized estimating equation models described in Table 1.

success closely matched the number of eggs deposited at a male's bower.

## DISCUSSION

Our results show for the first time conclusively that sperm competition can occur in the mouth of maternal mouth-brooders that take up male and female gametes for fertilization and subsequent brood care. This is especially remarkable in a system where females collect sperm of different males successively, sometimes with long time intervals in between. Female *O. ventralis* collect sperm from a greater number of males than they spawn with (cf. Immler and Taborsky 2009), thereby enhancing the potential level of sperm competition in their mouth. Furthermore, our data

Table 1

Effects of male body measures on male spawning success (number of spawned eggs/clutch size,  $N = 68$  cases including 10 females and 11 males) and male siring success (number of sired eggs/clutch size,  $N = 26$  cases including 10 females and 7 males)

| Male parameter                               | Degrees of freedom | $\chi^2$ | $P$          | Coefficient $\pm$ standard error |
|--|--------------------|----------|--------------|----------------------------------|
| Spawning success                             |                    |          |              |                                  |
| Intercept                                    | 1                  | 5.79     | <b>0.016</b> | $-7.740 \pm 3.216$               |
| Pelvic fin length <sup>a</sup> (mm)          | 1                  | 4.42     | <b>0.035</b> | $0.113 \pm 0.054$                |
| Body length (SL mm)                          | 1                  |          | 0.67         |                                  |
| Body condition                               | 1                  |          | 0.53         |                                  |
| Pelvic fin length symmetry <sup>b</sup> (mm) | 1                  |          | 0.19         |                                  |
| Siring success <sup>c</sup>                  |                    |          |              |                                  |
| Intercept                                    | 1                  | 3.76     | 0.053        | $-22.61 \pm 3.216$               |
| Body length (SL mm)                          | 1                  | 3.94     | <b>0.047</b> | $0.264 \pm 0.133$                |
| Body condition                               | 1                  |          | 0.44         |                                  |
| Pelvic fin length <sup>a</sup> (mm)          | 1                  |          | <u>0.057</u> |                                  |
| Pelvic fin length symmetry <sup>b</sup> (mm) | 1                  |          | <u>0.30</u>  |                                  |

Data from 17 clutches in the laboratory experiment. Depicted are the results of the 2 final weighted logistic generalized estimating equations (logit-link), with male identifier  $\times$  female identifier as subject effects to account for repeated measures and the scaling parameter adjusted using the deviance method. Nonsignificant terms are also depicted with their  $P$  value when entered to the final models.  $P$  values  $< 0.05$  are printed in bold and  $P$  values between 0.05 and 0.1 are underlined.

<sup>a</sup> Mean length of left and right pelvic fins (mm).

<sup>b</sup> Absolute value of the difference in length between the left and right pelvic fin (mm).

<sup>c</sup> Excludes males not receiving any eggs except 2 males who sired one offspring each, even though no eggs were deposited in their bowers.

show that males being visited only for sperm collection can successfully fertilize eggs laid on a competitor's bower (Supplementary Table S1). This is consistent with the "sperm shopping hypothesis" in *O. ventralis* females that was proposed to explain the spawning pattern of these lekking cichlids (Immler and Taborsky 2009). Maternal mouthbrooding seems to put females in control of pre- and postmating sexual selection to a much higher degree than is otherwise the case when fertilization is external. The latter usually limits the influence a female has on how many and which males participate in the fertilization of her eggs, apart from the one male she has selected for spawning (Taborsky 1998, 2008; Taborsky and Brockmann 2010). Mouthbrooding can be viewed as an intermediate form of brood care, in between external care of oviparous species and internal care of viviparous organisms. Our study suggests that this intermediate position of mouthbrooders also holds for the relatively high degree of control of the female as she can decide with whom to mate and whether to spawn with multiple males, and in addition, she can choose the intensity of sperm competition in her mouth by varying the time interval between successive sperm collections from different males.

Our data revealed a very high degree of multiple paternity of clutches, with  $>80\%$  of the broods sampled in the field sired by 2 or more males. Similarly high levels of polyandry were documented in other mouthbrooding cichlids (Kellogg et al. 1995:  $N = 17$  clutches of 7 species, mean = 70.5%; Parker and Kornfield 1996:  $N = 7$  clutches of *Pseudotropheus zebra*, mean = 85.7%; Maan et al. 2004:  $N = 28$  clutches of *Pundamilia nyererei*, mean = 68.33%). The causes of multiple paternity in these other cichlids are unknown, however, because observations of spawnings were previously not reported. Maan et al. (2004) stated that no sneakers were seen during the 5 spawnings they observed. Even though parasitic male tactics occur in some mouthbrooding cichlids (reviewed in Taborsky 1994, 2008), sneaking is apparently rare, and our study implies that multiple paternity in these fishes might mainly result from a female strategy of collecting sperm from multiple males successively (Schaedelin and Taborsky 2010), thereby inducing sperm competition in their mouth.

The degree of polyandry detected by our genetic analyses is consistent with previous behavioral observations of spawnings in *O. ventralis* regarding the number of males at which females laid eggs ( $\bar{X} = 2.5$ ; Immler and Taborsky 2009). However, many more males are usually visited during spawning of a clutch ( $\bar{X} = 8$ ; Immler and Taborsky 2009), and females collect sperm from all these males, which makes postmating sexual selection possible by competition of gametes (sperm) in the female's mouth. Our data confirm that not only sperm of males at which females deposit eggs but also ejaculates of males not receiving eggs can fertilize eggs. If females are selected to induce sperm competition by collecting sperm from different males, they should minimize the time intervals between subsequent male visits, that is, the interval from egg laying to sperm uptake from the next (or previous) male should be short. Our data did not suffice to check for significant differences between these time intervals between clutches with and without evidence for sperm competition, but the results show that even with relatively long intervals between sperm uptake and egg laying, fertilization of eggs was successful. In one case, sperm of a male collected 8 min before an egg was laid in another male's bower successfully fertilized this egg. This reveals an exceptionally long time window for the fertilization of eggs in a species with external fertilization. An accompanying study revealed that sperm of *O. ventralis* are long lived (Haesler 2007), which was observed also in other cichlids (Chao et al. 1987; Fitzpatrick et al. 2006) and is apparently caused by protective mucus (Grier and Fishelson 1995; Immler and Taborsky 2009), just like in many blennies and gobies (reviewed in Taborsky 2008; Taborsky and Neat 2010).

The number of fathers per clutch tended to increase with clutch size in our field sample, which was observed also in a Lake Malawi cichlid (Parker and Kornfield 1996). Nevertheless, it is often unclear whether these relationships are a sampling artifact of the increased likelihood of finding a larger number of sires in larger clutches by default (null hypothesis based on the hypergeometric distribution), that is, whether it is due to a sampling bias. Therefore, we tested the expected hypergeometric distribution against the observed distribution of offspring over the sires. This analysis clearly revealed that

most clutches showed a significant reproductive skew toward one male, except in the clutches with 4 fathers collected in the field, where reproductive success was equally distributed among sires.

Our detailed account of complete spawnings in the laboratory experiment revealed that both pre- and postmating sexual selection are involved. Females selected males with long pelvic fins for egg deposition (and no eggs at all were laid with males having very short pelvic fins; see Karino (1997) for a similar observation in a related crater building cichlid), whereas body size was no predictor of female choice at this level. In contrast, postmating sexual selection by induced sperm competition inside the female's mouth relates strongly to male body size, with larger males siring more offspring. This might hint on greater ejaculate sizes of large males, but this remains to be tested.

Our results suggest that fertilization success of males in mouthbrooding cichlids is determined to a large degree by the female mating behavior, including the choice of males for egg deposition and the sequence and timing of successive sperm collection visits. In contrast to other fishes, males of maternal mouthbrooders cannot fertilize eggs completely against female interests, like sneakers of species with conventional external fertilization may do (Taborsky 1994) or like male livebearers with internal fertilization do that can coerce females to mate (Bisazza 1993). As fertilization occurs within the buccal cavity of females, the latter are in perfect control of the choice of males to spawn eggs with or from which to collect sperm for induction of sperm competition. Both options provide good opportunities for pre- and postmating sexual selection. Thus, in this mating system, the female has full control over from which male to collect sperm. However, it should be noted that there are additional male effects on fertilization success that are not under female control, such as various sperm traits or the properties of the mucus enclosing the sperm.

Sperm shopping to induce sperm competition is also shown by females of the semelparous Australian marsupial *Antechinus stuartii*, where by frequent mating females may increase offspring survival 3-fold (Fisher et al. 2006), and in some snakes and lizards, where multiply mated females also benefit from an increased viability of their clutches (Madsen et al. 1992; Olsson et al. 1996). Future research should focus on potential female fitness benefits of multiple mating and sperm shopping in mouthbrooding fishes.

## SUPPLEMENTARY MATERIAL

Supplementary material can be found at <http://www.beheco.oxfordjournals.org/>.

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