

# Sequential polyandry affords post-mating sexual selection in the mouths of cichlid females

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**Abstract** Females mating with multiple males may obtain direct benefits such as nuptial gifts or paternal care or indirect (i.e. genetic) benefits resulting in higher-quality offspring. While direct benefits are easily identified, it is difficult to determine indirect benefits, and it is hence largely unclear how they are obtained. This is particularly true in species with external fertilisation, where females seem to have little control over fertilisation. In cichlids, most maternal mouthbrooders show sequential multiple mating, where females visit several males for egg deposition. Genetic data revealed that multiple paternity of eggs and young in the mouth of females is common, but behavioural data of female spawning decisions are missing. Here, we test four hypotheses to explain female multiple mating in the maternally mouthbrooding cichlid, *Ophthalmotilapia ventralis*: (1) fertilisation insurance, (2) genetic bet-hedging, (3) female choice and (4) ‘sperm shopping’ (i.e. induction of sperm competition resulting in sexually selected sperm). Detailed observations of spawning behaviour in the field combined with histological analyses of the male reproductive organs suggest that fertilisation insurance, genetic bet-hedging and pre-mating female choice are

unlikely to explain the sequential female multiple mating in *O. ventralis*. Instead, cryptic female choice by sperm shopping, i.e. post-mating sexual selection, is most compatible with our data and might be the major ultimate cause of multiple mating in females of this species and of mouthbrooding cichlids with maternal care in general. Our study provides new insight into ultimate causes of sequential polyandry in species with external fertilisation, as hitherto post-mating sexual selection by cryptic female choice has been assumed to be incompatible with external fertilisation mechanisms except by components of the ovarian fluid.

**Keywords** Polyandry · External fertilisation · Sperm competition · Cryptic female choice · *Ophthalmotilapia ventralis* · Lake Tanganyika · Testis histology

## Introduction

Multiple mating of females is widespread in animals (Gowaty 1994; Zeh and Zeh 2003; e.g. mammals: Gomendio and Roldan 1991; Stockley 2003; birds: Griffith et al. 2002; fish: Avise et al. 2002; Nordeide 2007; insects: Arnqvist and Nilsson 2000; Wiklund et al. 2001; Brown and Schmid-Hempel 2003; Torres-Vila et al. 2005), yet its functional significance is still poorly understood and highly debated (Hosken and Blanckenhorn 1999; Simmons 2001, 2003, 2005; Colegrave et al. 2002; Zeh and Zeh 2003). Females are thought to gain either (1) direct benefits such as nutrients (e.g. from nuptial gifts), fertility insurance or paternal investment (Birkhead et al. 1987; Simmons 1992; Sheldon 1994; Andersson 2005) or (2) indirect, that is, genetic benefits based on male quality (Kempnaers et al. 1992; Keller and Reeve 1995; Zeh and Zeh 1996; Yasui

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1998; Jennions and Petrie 2000; Tregenza and Wedell 2000; Colegrave et al. 2002; Ivy and Sakaluk 2005; Rudolfson et al. 2005). Genetic benefits raise the fitness of a female's offspring, which is either achieved by chance when females use multiple partners without choosing among them (i.e. female bet-hedging to increase the genetic variance of their offspring; Yasui 2001) or by female preference for high-quality males. Traditionally, females have been thought to choose males by evaluating male traits and resources such as secondary sexual traits, body size, territory quality and social rank (Andersson 1994; Maynard-Smith and Harper 2003). However, there is also growing evidence for post-mating sexual selection where females appear to assess male quality by sperm quality (Eberhard 1996; Wirtz 1997; Stockley 1999; Birkhead and Pizzari 2002; Pizzari and Birkhead 2002). 'Sperm selection' is considered to be a possible way of cryptic female choice (Birkhead et al. 1993; Wirtz 1997), and there is empirical evidence that better sperm competitors sire higher-quality offspring (Konior et al. 2001; Hosken et al. 2003; Neff 2004; Fisher et al. 2006; Simmons and Kotiaho 2007). However, cryptic female choice by sperm selection so far has been studied mainly in species with internal fertilisation, as in external fertilisers female control over fertilisation is limited (Taborsky 1998; but see Urbach et al. 2005).

In fish with external fertilisation, polyandry is widespread and may be divided into 'passive' and 'active' polyandry. Passive polyandry occurs in species with group spawning or parasitic spawning, where females seem to have very limited influence on who is fathering their offspring (Taborsky 1994). In these mating systems, sperm competition may strongly influence the mating success of each participating male (Parker 1990a,b; Taborsky 1998, 2008; Vladoic and Järvi 2001; Schulte-Hostedde and Burness 2005; Linhart et al. 2005). In contrast, in 'actively' polyandrous systems such as in many lekking species, females visit several males sequentially and hence have increased control over paternity. Sequential polyandry appears to be widespread in maternally mouthbrooding African cichlids (McKaye 1991; Höglund and Alatalo 1995; Kellogg et al. 1995; Kuwamura 1997; Schaedelin and Taborsky 2006), but its functional significance is poorly understood, mainly because detailed observations of spawning behaviour in nature are missing. Fertility insurance has been proposed to be a possible direct benefit of sequential multiple mating in external fertilisers (Barlow 2000). Furthermore, sequential multiple mating may provide indirect (i.e. genetic) benefits for females by assessing male quality either before or at mating (i.e. female choice) or thereafter (i.e. sexually selected sperm).

In species with *sequential* polyandry, post-mating sexual selection has received only limited attention, as the combination of sequential mating and external fertilisation

seems to exclude the possibility of sperm competition and hence the hypothesis of sexually selected sperm (Keller and Reeve 1995; Hosken et al. 2003). Reasons for this assumption are (1) the separation in time between matings with different males and (2) limited sperm longevity in externally fertilising species (especially in fresh water; Billard 1978; Morisawa 1994; Takai and Morisawa 1995), which in combination both seem to exclude the encounter between active sperm of different males. However, preliminary behavioural observations of mouthbrooding cichlids suggest that sperm of different males might meet inside the female's mouth and compete amongst each other: In most mouthbrooding cichlids, eggs are picked up by the female immediately after deposition (Wickler 1962a, b), and fertilisation takes place in the female mouth (Mrowka 1987; Rossiter and Yamagashi 1997; Barlow 2000). Various mechanisms have evolved in different species to facilitate the sperm uptake into the female's mouth (Wickler 1962a, b; Mrowka 1987). In some species, females nibble directly at the male genital papilla, in others more complex mechanisms such as the use of 'egg dummies' located near the male genital papilla induce the female to snatch them, which results in sperm uptake (Wickler 1962a, b; Hert 1989). The limited sperm longevity in aquatic environments due to osmolality stress has been overcome by many fish species by enclosing sperm in a mucous substance to protect them, and such sperm are able to fertilise eggs for hours (Trippel and Morgan 1994; Marconato et al. 1996; Scaggiante et al. 1999; Rasotto and Mazzoldi 2002; Elofsson et al. 2003). A similar substance has been found in the gonads of the mouthbrooding cichlid *Oreochromis aureus* (Grier and Fishelson 1995).

Here, we study female multiple sequential mating in an externally fertilising teleost to understand why females mate with multiple males. We recorded the spawning behaviour of the maternal mouthbrooder *Ophthalmotilapia ventralis* in the field and analysed the data with respect to four non-exclusive, alternative hypotheses to explain this mating pattern: (1) fertilisation insurance, (2) bet-hedging, (3) female choice by pre-mating sexual selection and (4) cryptic female choice by post-mating sexual selection ('sperm shopping'). In addition, we measured the gonadosomatic index and used histological analyses of testes to check for indications of sperm competition, mechanisms of sperm release and ejaculate characteristics.

## Material and methods

### Study species and spawning behaviour

*O. ventralis* (Ectodini; Cichlidae) is an endemic cichlid of Lake Tanganyika in which lekking males build bowers of

fine sand and algae debris on boulders, which are visited by females for spawning. Territorial males are larger in body size (mean standard length, SL: head to beginning of tail fin,  $8.15 \text{ cm} \pm 0.25 \text{ SD}$ ) than females (SL,  $7.33 \text{ cm} \pm 0.47 \text{ SD}$ ) and have elongated pelvic fins (mean length,  $5.6 \text{ cm} \pm 0.4 \text{ SD}$ ) with bright yellow tips (Pellegrin 1904; Liem 1981). Territorial males show a flashing whitish nuptial colouration, which makes them visible over large distances. The cryptically coloured females live in loose shoals, which they leave for spawning.

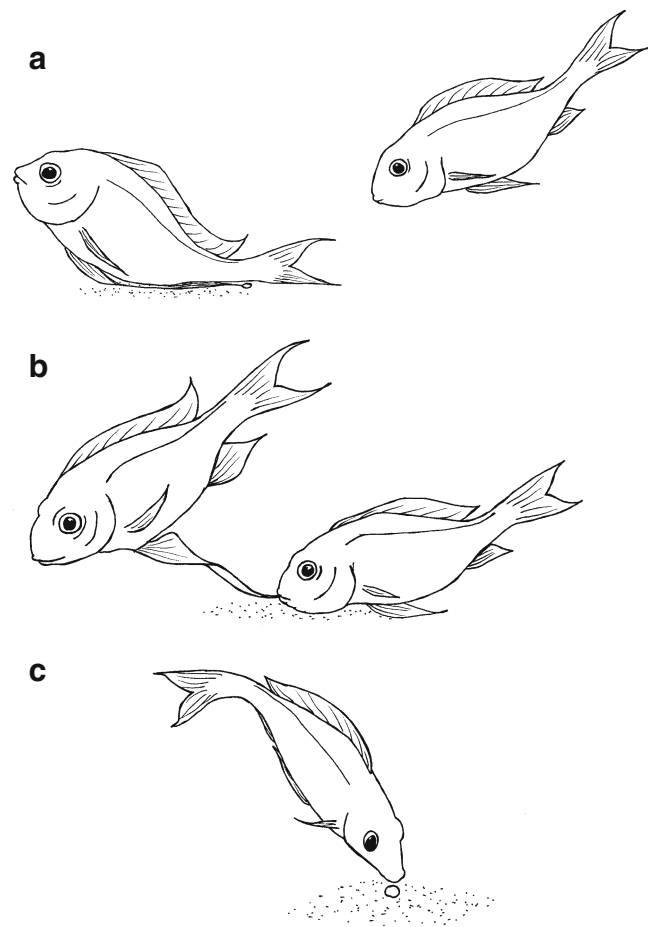
The spawning behaviour of *O. ventralis* is very characteristic and involves three main steps: (1) Females ready to spawn have an extended abdomen and when they approach a lek they are courted by males that try to lead them to their bower by lateral display and exaggerated lead swimming. (2) The female may follow the male onto his bower (hereafter referred to as a 'visit'), and 'spawning bouts' will ensue: The male places his genital papilla onto the bower and quivers (probably the moment of sperm release; Fig. 1a). Then, he raises some centimetres from the bower and stretches his elongated pelvic fins maximally from the body, so that their brightly coloured tips are placed approximately where he had just released sperm. This stimulates the female to approach and snatch at the gaudy fin tips (likely to be the moment of uptake of the deposited sperm; Fig. 1b). Thereafter, the male leaves the bower, whereas the female stays and starts circling on the bower, where she may lay up to three eggs in successive turns, which she takes up quickly (Fig. 1c). (3) After the spawning bout, the female leaves the bower, and the male resumes lateral displays and lead swimming to bring the female back to the bower for another spawning bout. Females often switch between males for subsequent spawning bouts and usually visit several males for the deposition of a whole clutch (hereafter referred to as 'spawning event'). Females visit different males during all stages of a spawning event (1) before starting to lay eggs, (2) during egg laying and (3) after the last egg has been laid.

#### Hypotheses accounting for sequential polyandry

To explain multiple mating of *O. ventralis* females, we tested predictions derived from four mutually non-exclusive, alternative hypotheses.

#### Fertilisation insurance hypothesis

The number of sperm released by one male is not sufficient to ensure the fertilisation of an entire clutch of eggs (Birkhead et al. 1987), and hence, females are forced to visit several males to get all eggs fertilised. One possible



**Fig. 1** Spawning bout: **a** The male presses his genital papilla against the substrate and quivers (probably moment of sperm release); **b** the male raises some centimetres from the bower and stretches his elongated pelvic fins maximally from the body to display their brightly coloured tips, which are snatched by the female (likely to be the moment of uptake of the deposited sperm); **c** the male leaves the bower, and the female stays and starts circling on the bower and may lay up to three eggs, which she picks up (drawings by S. Immler)

reason for sperm limitation might be sperm depletion of males due to repeated mating (Nakatsuru and Kramer 1982; Warner 1997; Wedell et al. 2002). This hypothesis predicts (1) a positive correlation between the total number of eggs laid and the number of separate spawning bouts induced by a female during a spawning event at a particular bower, as more eggs need more sperm to be fertilised and (2) the probability of sperm depletion of male *O. ventralis* due to frequent ejaculations is high.

#### Genetic bet-hedging hypothesis

Females mate with many different males to increase the genetic diversity of their offspring (Yasui 2001; Fox and Rauter 2003). It is difficult to predict specific mating patterns according to this hypothesis. However, females laying larger clutches may be expected to visit more

different males than females with smaller clutches to make use of their potential to increase genetic diversity of their offspring. We would therefore expect that the number of different males selected for spawning should rise with an increasing number of eggs laid per clutch in order to increase the genetic diversity of offspring.

#### *Female choice hypothesis*

Females choose males by assessing their genetic quality in relation to correlating traits such as body size, body colouration or territory size (Andersson 1994). In this case, females may be expected (a) to continue their search for higher-quality males after they started to spawn, (b) to inspect several males without actual spawning and (c) to return to specific males. In addition, we would expect females to stop or reduce seeking for more males over time since the probability to find a better male decreases with time (Dombrovsky and Perrin 1994; Collins et al. 2006). Predictions from this hypothesis are (1) a positive correlation between both, male quality traits such as body size, and spawning site quality (e.g. bower size, area and height of defended boulder) with mating success; (2) that females pay short visits without spawning bout to males which they visit again later for egg laying to assess male quality; (3) a declining number of different visited males during the course of egg laying as the chance of encountering higher-quality males decreases over time; (4) females visit different males before and during egg laying, but not after egg laying has stopped.

#### *Sexually selected sperm hypothesis (sperm shopping)*

There may be positive genetic associations between a male's sperm competitiveness and the general viability of his offspring ('good sperm' models; Yasui 1997; Fisher et al. 2006) or multiply mating females produce sons that will be successful in sperm competition and daughters that will induce sperm competition through multiple mating ('sexy sperm' models; Harvey and May 1989; Keller and Reeve 1995). If such a sexually selected sperm process occurs, females would incite sperm competition by switching between males to collect their sperm without much delay. This hypothesis predicts (1) a short time interval between subsequent spawning bouts with different males; (2) initiation of ejaculation and sperm collection from different males after short delays only, e.g. without spawning additional eggs with the previous male in between, to maximise the sperm competition potential; (3) performance of spawning bouts also without egg laying and female visits of males even after egg laying has ceased as during this phase females may still carry unfertilised eggs in their mouth if eggs are not immediately fertilised at deposition (Mrowka 1987); (4) shorter time intervals between egg

deposition and the subsequent spawning bout if the latter is performed with a different male than with the same male to increase the chance of sperm competition; (5) a mechanism prolonging sperm longevity in water.

#### Sexual conflict

We tested for a possible conflict of interest between the sexes caused by pre-oviposition ejaculation as the male has limited information about the female's egg allocation. Sperm are costly (Nakatsuru and Kramer 1982; Wedell et al. 2002), and therefore, males are expected to reduce costs by optimising ejaculate expenditure. They should be able to decide about sperm and ejaculate allocation dependent on whether a visiting female is able to lay eggs or carries fertilisable eggs in her mouth. We therefore compare (1) the number of spawning bouts performed by males with full information about a female's spawning condition, i.e. after the female has laid an egg with this male, with (2) the number of spawning bouts performed when no eggs can be fertilised (i.e. no eggs are laid or carried); and we estimate (3) the proportion of males performing spawning bouts without any chance to fertilise eggs.

#### Behavioural observations

We marked 52 male territories with numbered stones during March and April 2001 at Kasakalawe point near Mpulungu, Zambia, at the southern end of Lake Tanganyika (8°46.849' S, 31°04.882' E). We measured the distances between the centres of bowers of neighbouring males with a tape measure and determined maximum length, maximum width and maximum height of 44 bowers with bowers. In addition, we measured the diameter of 39 bowers with a ruler, which is straightforward because the bowers are usually perfectly round. Territory owners were observed for spawning activity for 5 min/day at different times during daylight hours, and the numbers of visiting and egg laying females were recorded. Independently, 67 females ready to spawn (round belly and empty buccal cavity) were detected and followed for continuous focal animal watches. Time was recorded continuously from the moment of the first visit of a male until the female left the last male she visited. The observation of a female was ended after a female had not visited any male for 15 min. We divided a spawning event into three phases: (1) The pre-egg laying phase lasted from a female's first visit of a male's bower until the visit during which the first egg was laid; (2) the egg laying phase extended from the first to the last visit during which an egg was laid; and (3) the phase after egg laying had stopped lasted from the last visit in which an egg was laid until the female left the last male she visited. Of each bower visit we recorded (a) arrival and departure time of the female on the bower, (b) time of each spawning bout and (c)



time of each egg deposition; we counted (d) the number of visits, (e) the number of short visits (visits without spawning bout), (f) the number of spawning bouts performed, (g) the number of different males visited and (h) the number of eggs laid with each male.

We tested the data for deviation from normal distribution using Shapiro–Wilk's tests. To test for correlations, we used Spearman rank correlation analyses, and to test for correlations across females, we performed a derived variable analysis using Spearman's rho in a one-sample *t* test against zero. To compare data between different phases, we used paired *t* tests for normally distributed data and Wilcoxon matched-pairs signed-ranks tests for other data. Statistical analyses were performed using SPSS version 12.0.

### Testis size, histology and histochemistry

Fifteen male *O. ventralis* were collected in the field at the end of the observation period, i.e. after they had been observed on their bowers for at least 3 weeks. Individual males were identified by their specific dark markings on the flanks, which is very easy and reliable in this species. They were killed with a lethal dose of anaesthetic (MS222, Sandoz) and measured for standard length (SL), maximum body height and maximum belly width, and they were weighed for body weight (WT).

The entire reproductive tract was removed, weighed to the nearest mg and fixed for a minimum of 6 days in Dietrich's solution (900 ml distilled water, 450 ml 95% ethanol, 150 ml 40% formaldehyde, 30 ml acetic acid). Then, it was dehydrated in ethanol, embedded in paraffin and sectioned serially at 6–7  $\mu\text{m}$ . Sections from each specimen were stained with haematoxylin and eosin. For polysaccharide detection, sections were stained using the reaction of periodic acid-Schiff (PAS; Pearse 1985). Alcian Blue staining at pH 1.0 and 2.5 was performed to detect sulphated and non-sulphated mucins (Pearse 1985).

## Results

### Male and bower measures

Mean SL of collected territorial males was 8.05 cm  $\pm$  0.24 SD, which was strongly correlated with body weight (13.3 g  $\pm$  1.68;  $N=15$ ;  $r=0.85$ ,  $P<0.001$ ) and mean maximum body height (2.87 cm  $\pm$  0.12;  $r=0.63$ ,  $P=0.009$ ), whereas there was no significant correlation between SL and mean maximum belly width (1.14 cm  $\pm$  0.09,  $r=0.25$ ,  $P=0.36$ ). Bower rocks were on average 27.3 cm long ( $\pm 7.5$ ;  $N=44$ ), 20.8 cm wide ( $\pm 3.6$ ; surface area 582.5 cm<sup>2</sup>  $\pm$  245.4) and 13.2 cm ( $\pm 3.5$ ) high, and the mean distance

between neighbouring bowers was 2.73 m ( $\pm 1.6$ , min=0.5 m, max=10 m;  $N=66$  nearest neighbour measures). Bowers had a mean diameter of 11.56 cm  $\pm$  1.64 SD ( $N=39$ ).

### Spawning observations

During 92.75 observation hours of 52 different territorial males, we counted a total of 67 spawnings (defined as observation of a female visiting a bower, with or without egg deposition), during which we observed the laying of a total of 57 eggs. We observed nine complete spawning events (i.e. laying of all eggs of a clutch), in six of which all individual males participating could be identified. In the remaining three spawning events, females left the marked area during spawning, and male identification was impossible as the visited males had not been registered beforehand. A complete spawning event lasted on average 35.5 min ( $\pm 9.2$  SD; range 22.5–50.3). Females switched between eight different males on average (range 4 to 15) in 19.6 visits ( $\pm 11.3$ ) and laid eggs with 2.5 different males (range one to six). On average, a female laid 13.2 eggs per clutch ( $\pm 3.7$ ).

### Fertilisation insurance hypothesis

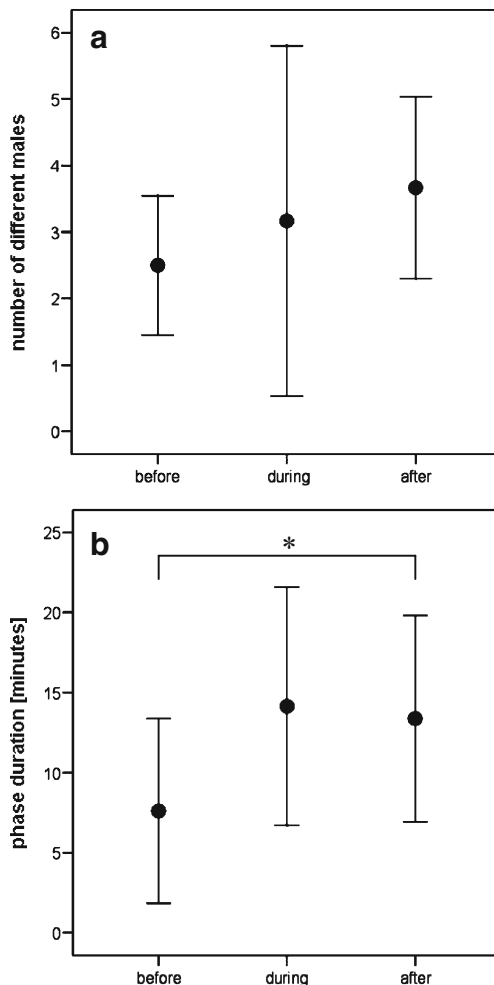
(1) There was no correlation between the total number of spawning bouts and the total number of eggs laid during one visit at a particular bower (Spearman,  $r_s=-0.14$ ,  $P=0.72$ ,  $N=9$ ). (2) The mean number of spawning bouts performed by an individual male during one spawning event of one female was 6.5  $\pm$  5.3 SD; ( $N=6$ ). We also estimated the maximum number of spawning bouts performed by one male during a spawning event. We did this in four steps: (a) In each completely observed spawning event, we identified the male which performed most spawning bouts with that particular female. (b) From these six identified males (in six complete spawning events, all males could be identified, see above), we calculated the number of spawning bouts performed, which was 16.5  $\pm$  8.0 SD ( $N=6$ ). (c) Furthermore, from our general observations of the lek, we estimated that each male was involved in two spawning events per day. (d) If the most successful males performed 16.5 spawning bouts per spawning event and two spawning events per day, this results in a maximum of 37 spawning bouts per male and day.

### Genetic bet-hedging hypothesis

There was no significant correlation between the number of eggs laid and the number of visited males (males visited before egg laying had started were excluded;  $r_s=-0.66$ ,  $P=0.15$ ,  $N=6$ ; note that the relationship is negative, which is opposite to the prediction. The lack of significance may be due to small sample size).

### Female choice hypothesis

(1) No significant correlation existed between any of the male and territory traits measured and the number of female visits per male (SL:  $r_S = -0.11$ ,  $P = 0.69$ ,  $N = 15$ ; pelvic fin length:  $r_S = 0.14$ ,  $P = 0.61$ ,  $N = 15$ ; rock surface area:  $r_S = -0.13$ ,  $P = 0.4$ ,  $N = 44$ ; bower height:  $r_S = -0.24$ ,  $P = 0.12$ ,  $N = 44$ ; bower diameter:  $r_S = 0.022$ ,  $P = 0.9$ ,  $N = 39$ ); (2) 19.3% of all males were visited by a short visit only, and 22.2% of all males were visited by a short visit before the respective females returned to them later to induce spawning bouts. (3) The number of different males visited did not differ between the different phases (before egg laying, 2.5 males  $\pm$  1.1 SD; during egg laying, 3.2 males  $\pm$  2.6 SD; after egg laying, 3.7 males  $\pm$  1.4 SD,  $N = 6$  complete spawning events; Wilcoxon signed-ranks test: before vs during,  $Z = 0.27$ ,  $P =$



**Fig. 2** **a** The number of different males visited during the three phases (before, during and after egg laying) of a spawning event (mean  $\pm$  SD,  $N = 6$ ). **b** The durations of the phase before egg laying had started, the laying phase and the phase after egg laying had stopped (mean  $\pm$  SD,  $N = 8$ ). Significant differences between phases are indicated by an asterisk ( $p < 0.01$ )

0.79; before vs after,  $Z = 1.16$ ,  $P = 0.25$ ; during vs after,  $Z = 0.64$ ,  $P = 0.52$ ; Fig. 2a). Although the difference between before and after egg laying is not significant probably due to low sample size, the effect size for the difference is about 1 SD, which is remarkable. (4) The phase after egg laying had stopped (983.13 s  $\pm$  388.65 SD,  $N = 9$ ) was significantly longer than the pre-laying phase (524.0 s  $\pm$  368.68;  $N = 9$ ; paired  $t$  test,  $t_8 = -3.82$ ,  $P = 0.007$ ; Fig. 2b).

### Sexually selected sperm hypothesis (sperm shopping)

(1) The mean time between the last spawning bout in a visit and the first spawning bout with a new male was 126.07 s ( $\pm$  87.2 SD;  $N = 12$ ). (2) In 65.9% of visits with egg deposition ( $N = 41$  visits), the female induced one to three spawning bouts of the bower owner after the last egg of her visit was laid and before she switched to a new male. (3) All observed females continued inducing spawning bouts with different males after the last egg had been laid. In addition, the phase after egg laying was significantly longer than the phase before egg laying (see results reported above, “Female choice hypothesis”, (4) of the “Results” section; Fig. 2b). (4) Time intervals between the last egg laid in a visit and the subsequent spawning bout induced with the *same* male in a next visit after having left the nest (40.1 s  $\pm$  40.5 SD,  $N = 7$ ) did not differ significantly from the time intervals between egg laying and the subsequent spawning bout induced with a *different* male (76.6 s  $\pm$  48.5 SD,  $N = 6$ ;  $t$  test,  $t_{11} = 1.46$ ,  $P = 0.18$ ). (5) Males produce a mucous substance, which is likely to protect sperm against osmolality stress (see results of “Testis size, histology and histochemistry”, below).

### Sexual conflict

(1) In 16% of all spawning bouts, males could be certain about the presence of eggs since they witnessed egg laying in their own bower before they performed a spawning bout with the same female. To be conservative in these estimates, we assumed that males cannot identify individual females, and therefore, returning females were considered to be ‘new’ to the male. (2) In 55.2% of all male visits, the female carried eggs that had been laid earlier, in her buccal cavity; 23.7% of all spawning bouts were performed without any eggs laid or present in the female's buccal cavity; (3) 10.4% of all males performed spawning bouts with a female without any fertilisable eggs present and without being visited by the same female again later. (4) The time measured between sperm release and egg deposition within a spawning bout was 24.7 s ( $\pm$  23.0 SD;  $N = 12$  time intervals between sperm release and egg deposition). The time between the last egg laid in one visit and the first spawning bout with a different male was 177.9 s ( $\pm$  124.4 SD,  $N = 12$ ).

### Testis size, histology and histochemistry

Testes had a mean length of 2.36 cm $\pm$ 0.28 SD and a mean weight of 0.103 g $\pm$ 0.027 SD. We calculated a mean GSI (=gonad weight/body weight $\times$ 100) of 0.779 $\pm$ 0.186 SD. Testes are elongated, paired and rounded tubes suspended from the dorsal wall of the coelomic cavity by a short mesorchium. Gonoducts are present as two main testicular ducts (Grier 1981) running along the testis length, fusing in a common sperm duct shortly before reaching the urogenital opening.

Testes are organised in lobules, which are separated from each other by a thin layer of fibrous connective tissue. Lobules open into a main testicular duct, located ventro-medially, on the testis side facing the dorsal mesentery. The lobule walls are lined with a germinal epithelium. Leydig cells build interstitial compact groups between the lobules throughout the whole length of the testes. The germinal epithelium shows spermatogonia distributed along its length and, consequently, can be defined as an unrestricted spermatogonial type, following Grier (1981). Spermatogenesis was fully active as all stages from spermatogonia to spermatids were observed, and all males showed mature sperm in their gonads. The main testicular ducts running along each testis are multi-chambered, and the chamber walls comprise a layer of connective tissue, a single-layered columnar epithelium, and they are rich in smooth muscle cells. The inner epithelium of the chamber walls and the testicular duct consists of columnar cells, of which the apical part reacted positively to PAS and to Alcian Blue at pH 1 and 2.5. The sperm duct, arising from the fusion of the two main testicular ducts, has a similar structure. A homogeneous material staining for sialomucins was present in the chamber lumina in the testes mixed with sperm (M.P. Häslér, S. Immler & M. Taborsky, unpubl. data).

### Discussion

Sequential multiple mating in the maternally mouthbrooding cichlid *O. ventralis* appears to be driven primarily by post-mating sexual selection. Our data suggest that direct benefits in the form of fertilisation insurance are unlikely to explain female spawning behaviour in this species. Furthermore, we found no evidence that females adopt a bet-hedging strategy to improve the fitness of their offspring or that they choose mating partners according to the quality of males or the spawning sites they provide. Below, we discuss our results in relation to each of the four hypotheses we considered.

#### Fertilisation insurance hypothesis

Fertilisation insurance is an unlikely explanation for polyandry in *O. ventralis* because: (1) Sperm depletion is

unlikely to occur in male *O. ventralis* as the spawning frequency and the number of spawning bouts are relatively low (a maximum estimate of 37 ejaculations per male and day) in comparison to other cichlids (e.g. *Lamprologus callipterus*; Sato et al. 2004). All dissected males showed a high sperm content in their gonads with chambers filled with sperm. We found muscles around the testicular duct that could allow males to adjust ejaculate size. This mechanism is known from the blue-headed wrasse (*Thalassoma bifasciatum*), where dominant males may adjust ejaculate size to mating frequency and the intensity of sperm competition (Shapiro et al. 1994; Warner et al. 1995; Rasotto and Shapiro 1998). (2) Female *O. ventralis* produce small clutches compared to other fish species (Stockley et al. 1996), which should require a comparatively small number of sperm for fertilisation. We found no correlation between the number of eggs per clutch and the respective number of spawning bouts, i.e. females did not request more sperm when laying more eggs to guarantee fertilisation. (3) The site of fertilisation (i.e. the female buccal cavity) makes large numbers of sperm redundant, which contrasts with species where fertilisation occurs in the water column or on the substrate. In fact, a comparative study of Lake Tanganyika cichlids showed that substrate brooders have relatively larger testes (and probably much more sperm) than mouthbrooders (Balshine et al. 2001). Furthermore, in a mouthbrooding tilapia, for instance, densely packed sperm drops are directly taken up by spawning females, which creates high sperm concentrations in their buccal cavity (Wickler 1962a). Although we did not determine sperm packaging in *O. ventralis*, the mucous we found in the testicular duct might serve a similar function (Grier and Fishelson 1995).

#### Genetic bet-hedging hypothesis

Bet-hedging may be advantageous especially in small populations and unstable environments, where females may raise offspring fitness by mating with different males to increase the genetic diversity of their offspring (Yasui 2001). The ecology of *O. ventralis* makes bet-hedging an unlikely explanation for sequential polyandry because (a) the sublittoral zone of Lake Tanganyika is a remarkably stable environment (Coulter 1991) where large genetic variation among a female's offspring is unlikely to yield great fitness advantages, and (b) *O. ventralis* is a common species in the lake, and hence, population size is large (Konings 1988). Furthermore, we expected that if bet-hedging is important, females laying larger clutches should visit more different males to make use of their potential to increase genetic diversity among offspring. However, there was no positive but a negative correlation between the number of eggs laid and the number of visited males, albeit statistically non-significant. Together, these observations

suggest that females do not adopt a bet-hedging strategy by spawning with many males randomly.

#### Female choice hypothesis

We found no evidence that pre-mating female choice is the major reason for female multiple mating in *O. ventralis*. Females visit different males before egg laying but return only rarely for egg deposition, and they spend almost twice as much time visiting different males after they have stopped to lay eggs than before laying has started. Furthermore, we found no correlation between the quality of males and their spawning sites (bowers and bower locations) with female preference, even though the traits measured included the clearest secondary sexual characters of this species. In the closely related *Cyatopharynx furcifer*, crater size and male building activity are apparently used by females as indicators of male quality (Schaedelin and Taborsky 2006). Other factors such as the bower position on the lek might be important for mate choice (Alatalo et al. 1991; Rintamäki et al. 1995). However, in *O. ventralis*, positions of bowers within the lek seem to be fixed, and males take over territories from previous owners. The 15 males we removed from their territory for the histological analyses were often immediately replaced by new males, and by the following day, all bowers were occupied by new owners. A possible criterion for female choice that we have not tested is that males might release pheromones during ejaculation, which might allow females to assess male quality (see also Plenderleith et al. 2005). The presence of Leydig cells in the testes could indicate the production of pheromones (M.P. Häslner, S. Immler & M. Taborsky, unpubl. results), which may play an important role in male reproduction as shown in the black goby, *Gobius niger* (Colombo et al. 1980; Locatello et al. 2002; Immler et al. 2004).

#### Sexually selected sperm hypothesis

Our results support the hypothesis that post-mating sexual selection may be primarily responsible for multiple mating of female *O. ventralis*. The long phase after egg laying has stopped during which females continue visiting males and collecting sperm is the strongest support of the sexually selected sperm hypothesis. In addition, the frequent switch between males and the short intervals between subsequent visits with apparent uptake of sperm from different males suggest a high potential for sperm competition. The induced spawning bouts after laying eggs at a bower and immediately before switching to a new male increase the chance that active sperm of different males meet and compete. Besides serving the function of dense sperm packaging (see above), the mucous found in the testicular

duct of *O. ventralis* is also suited to increase sperm longevity and to prolong sperm competitiveness. In two marine goby species, sperm packed in mucous similar to *O. ventralis* survive and fertilise eggs even for hours (Scaggiante et al. 1999). In *O. ventralis*, stripped sperm were found to be viable on a microscope slide in excess of 15 min (Haesler 2007). Sperm of different males are therefore likely to compete for fertilisation in the female buccal cavity, despite the fact that the interval between two subsequent ejaculations of different males may last up to 8 min. Furthermore, females often switch to and fro between two males, laying only a small number of eggs at a time for each male. For all these reasons, the sexually selected sperm hypothesis seems to be the most likely explanation for female multiple mating in *O. ventralis*. This was also corroborated by a paternity study showing that 83% of the clutches studied were sired by two or more males (Haesler 2007). Notably, males indeed sired offspring when a female did not lay eggs at their bowers but only collected their sperm; the longest interval between egg laying and sperm uptake resulting in successful fertilisation was 8 min (Haesler 2007).

There is empirical evidence from other species that sperm competition may influence the quality of resulting offspring. In bluegill sunfish, *Lepomis macrochirus*, parasitic males produce better sperm (Burness et al. 2004) and sire fitter offspring (Neff 2004). In the yellow dungfly, *Scatophaga stercoraria*, males that outcompete rivals in sperm competition sire higher-quality offspring (Hosken et al. 2003). Similarly, in the Trinidadian guppy, *Poecilia reticulata*, multiply mated females sired higher-quality offspring, suggesting that fitter males outcompete lower quality males in sperm competition (Evans and Magurran 2000). In addition, quantitative genetic data obtained from the dung beetle *Onthophagus taurus* support the role of a sexually selected sperm process in the evolutionary divergence of sperm (Simmons and Kotiaho 2007).

#### Alternative explanations

Which alternative mechanisms might be responsible for the reproductive pattern we found in this study? In particular, the post-spawning sperm collection of females at different males deserves to be scrutinised, as it is this trait that most clearly points towards the sexually selected sperm hypothesis. It is possible that females cannot determine in time that they have no further eggs ready to be laid. Such constraint might exist if their spawning behaviour is triggered by hormones, without any feedback from the content of the ovary. However, in the cichlid *Astatotilapia burtoni*, it has been shown that the gravidity state of females has a major impact on the hormones and hence the reproductive behaviour of females (Clement et al. 2005). In



addition, the bower visits involve costs of time, energy and risk to the female and her offspring, which is important especially during brood care and should hence be selected against if they lack a function. Another possibility is that females are collecting sperm of different males for nutrition, which might be important particularly at the beginning of a long period of fasting caused by their mouthbrooding. However, this seems unlikely because sperm is poor in nutrition, and benefits for the female through sperm consumption seem too small to justify the additional visits. One could argue that females go for the accessory mucous produced by the testes, but this only shifts the question by one level: Why should males be selected to produce such substances if it is not for the protection of sperm, but only for the nutrition of females they have not even spawned with? We think, therefore, that the post-mating sexual selection hypothesis is the most parsimonious and hence most likely explanation for multiple sequential mating and sperm collection in this species, particularly during the post-laying period.

#### Sexual conflict

Pre-oviposition sperm release has been suggested to increase fertilisation success in three-spined sticklebacks (Le Comber et al. 2004). In *O. ventralis*, it may lead to a conflict of interest between the two sexes. For most spawning bouts, males lack information about whether a female carries fertilisable eggs or will lay eggs on their bower. Therefore, to maximise the chances to sire offspring, they must release sperm in every spawning bout before the female is laying. This is wasteful because half of all female visits and a quarter of all spawning bouts are performed without presence of fertilisable eggs. Sperm production is costly, and therefore, males are selected to optimise the number of sperm released (Nakatsuru and Kramer 1982; Wedell et al. 2002). The muscles surrounding the testicular duct are one way to do so. Males might use possible indicators such as a full, round belly of females (i.e. with eggs to be laid), a full buccal cavity (i.e. a female has laid many eggs already) or chemical cues to decide how much sperm to release. However, females seem to be largely in control over fertilisation in this species and probably in many other mouthbrooders with similar spawning habits (see Kuwamura 1997; Barlow 2000).

#### Conclusions

When interested in evolutionary mechanisms underlying multiple mating of females in the absence of paternal care, sequential polyandry in mouthbrooders is a suitable study system to evaluate alternative hypotheses. Nevertheless, to

our knowledge, this is the first detailed quantitative study of mating behaviour of a lekking mouthbrooder in the field. Our data suggest that the fertilisation insurance and genetic bet-hedging hypotheses are unlikely explanations for female polyandry in this species. Regarding female choice for male quality, the mere existence of secondary sexual characters in males, such as elongated pelvic fins with bright yellow tips (Konings 1988, Salzburger et al. 2007), might indicate that female choice at the pre-mating stage does exist, which has been confirmed by an experimental laboratory study (Haesler 2007). The female choice hypothesis and the sexually selected sperm hypothesis assume female choice and sexual selection to take effect at different stages of the spawning event, which selects for very different traits. Our results suggest that inducement of sperm competition in the buccal cavity of females is an important mechanism responsible for multiple mating in female *O. ventralis*, which is particularly demonstrated by the long post-laying phase during which females collect sperm from different males without laying eggs on their bowers.

Among mouthbrooders, multiple mating is exclusively found in species with maternal care, where males do not invest in the offspring. In contrast, biparental and paternal mouthbrooders are monogamous at least for the fertilisation of one clutch (Kuwamura 1997; Goodwin et al. 1998; Neat and Balshine-Earn 1999; Okuda 1999). Due to the fact that females are largely in control of fertilisations in maternal mouthbrooders, multiple mating may have evolved to increase offspring quality. From genetic data, this seems to be widespread (Kellogg et al. 1995; Parker and Kornfield 1996; Knight et al. 1998; Maan et al. 2004).

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