

## Summary and Conclusions

### Summary of results

Behavioral observations of the spawning behaviour of the Lake Tanganyika cichlid *Ophthalmotilapia ventralis* (Ectodini) by Immler & Taborsky (unpublished data) suggested a high incidence of multiple paternity and the possibility of sperm competition induced by the large number of males visited by the females (Immler & Taborsky, unpublished data). Indeed, most broods of *O. ventralis* showed multiple paternity, 25 of 30 broods (or 83.33%) had two or more fathers (mean = 2.5, range = 1-4; chapter 5). Similarly high levels of polyandry, have been found in other mouthbrooding cichlids (Kellogg et al. 1995: N = 17 clutches of 9 species, 70.5%; Parker & Kornfield 1996: N = 7 clutches of *Pseudotropheus zebra*, 85.7%; Maan et al. 2004: N = 28 clutches of *Pundamilia nyererei*, 68.33%). In 80% of all multiply fathered broods, paternity appeared biased towards one father within one brood, resulting in a pattern of few males fertilizing many eggs and many males fertilizing few eggs, i.e. a strong reproductive skew. This can occur through the spawning behaviour itself or through a selective process, such as sperm competition.

One way of how spawning behaviour could be influenced and shaped into such a pattern is via disturbances by other individuals, i.e. egg predators or sneakers. To investigate this idea I did some modeling work together with a postdoctoral researcher in our group, Ian Hamilton (Chapters 2 & 3). Both models were inspired by the lekking systems of cichlids from Lakes Malawi and Tanganyika (McKaye 1983, McKaye 1984, Karino 1996). In particular, the paper by Kellogg et al. (1995), which had already suggested a role for egg predators in the evolution of multiple mating in mouthbrooding cichlids from Lake Malawi, motivated us to do this work. We wanted to theoretically explore the possible effects of disturbances by egg predators, or sneakers (or other harassing males, etc., collectively referred to as enemies) on sequential female mating decisions. In Chapter 2 (Hamilton et al. 2006), we explored this question on a population level with dynamic game modeling. We show that the presence of costly enemies can lead to the persistence of poor males on a lek if there is some spatial structure to the lek. Good males attract more females and therefore also more enemies. This makes good males more costly to females. Hence some females will choose to mate with poor males. This provides a very simple solution to the lek paradox (Borgia 1979, Taylor & Williams 1981). Furthermore, in a version where enemies are sneakers, which may not necessarily be costly to females but certainly are to males, sneakers are more likely to persist if the quality difference between good and poor males is large.

The results of that model (Hamilton et al. 2006) can also be interpreted such that females mate with both, good and poor, males in a certain proportion, i.e. are polyandrous. However, it does not specifically predict that, but is consistent with such a prediction.

Therefore, we wanted to explore the same situation on an individual level (Chapter 3). We approached this problem using a stochastic state-variable model (Mangel & Clark 1988, Clark & Mangel 2000). We followed an individual female's mate choice decisions under predation risk. We modeled two kinds of predation risk: (i) predation risk on the eggs being laid and (ii) predation risk on the adult female *on* and *off* the lek (possibly with differing survival probabilities). The first kind of risk (egg predation) is a time-dependent risk, that is, it increases with time spent with one male. Another interpretation of what was modeled as egg predation, is loss of fitness due to mating with subfertile males. Males may economize on their sperm expenditure or suffer sperm depletion such that females do not gain the optimal fertilization benefits (Warner et al. 1995). Our model predicts that polyandry can be an adaptive response by females to such time-dependent costs of mating. However, if risk increases slowly or decreases quickly then females frequently shift to remating, leaving and mating again with the same male or multiple mating with the same male. Furthermore, it also predicts that females will trade-up, i.e. the last-mated male quality is predicted to be higher than that of previous males.

Both models predict that females should trade-off male quality with predation risk, thus females should be sensitive to the presence of enemies (e.g. sneakers or egg predators). Therefore, I wanted to test in the field whether females respond to the presence of either or both of sneakers or egg predators (Chapter 4). First, we demonstrated that there were sneakers, that is parasitic males, in this system through observations of "sneakings" by floater males and by comparing these floaters with territorial males morphologically. We called them parasitic males and not sneakers because they do not perform simultaneous parasitic spawnings, but "sneak in" and court the female on the territorial male's bower while the territorial male is chasing other intruders away. We found that only the larger floater males had mature gonads and that all floaters had much fat in their coelomic cavity, also those with very mature gonads. All territorial males had highly mature gonads but always lacked fat. We therefore suggested that they switch ontogenetically from sneaker to territorial males.

In the experiment, we presented either an empty plastic bag, a plastic bag containing a "sneaker", or a catfish of the species *Synodontis multipunctatus*, the cuckoo catfish, as an egg predator. Males reacted aggressively to both kinds of intruders but far more strongly to the former. Females did not differ significantly in their response to the courtship of males in an overall test of the three treatments. Nevertheless, the responsiveness to males under the "sneaker" treatment greatly was reduced, significantly so when compared only to the empty bag treatment. Observations of females responding negatively to parasitic male courtship additionally underlined that this trend to avoid sneakers was real.

To test the sperm competition idea, I conducted an experiment in the laboratory in a large circular tank (Chapter 5). The "ringtank" is a large octagonal ring-shaped tank holding about 7'200l of water. I offered four territories with rocks for bowers. I put in 12 males and 12 females. The ringtank was continuously monitored by video during daylight hours. I recorded male courtship behaviour and female spawning behaviour, so that I knew with which male a female spawned how many eggs. Afterwards, we analysed paternities with microsatellite markers to test whether spawning patterns match with fertilization patterns. I did two runs of this experiment. We found that sometimes males did not fertilize eggs that were spawned in their bower. This strongly suggests that sperm competition occurred in these cases. It further proved that eggs were fertilized in the female's mouth. This also means that sperm competition occurs in the female's mouth.

There were 3 out of 17 analysed spawnings, in which sperm competition was found. In 5 spawnings the female visited other males, and thus sperm competition may have occurred but the spawning pattern did not differ from the fertilization pattern. In 2 spawnings the females did not visit any other males and hence there was no opportunity of sperm competition. Of the remaining 7 spawnings we lost too many offspring to compare the spawning patterns with the fertilization patterns. As an alternative explanation to sperm competition, males may have run out of sperm and thus another male got the chance to fertilize eggs that were not spawned with him. Sperm depletion or economy is not mutually exclusive to sperm competition as an explanation for the observed patterns of fertilization. Sperm economy may mean that one male's ejaculate reduced and thus smaller than another male's ejaculate and therefore had smaller chances of fertilizing an egg (Parker 1990). However, from the female's perspective, it may be sperm limitation (or fertilization insurance) rather than sperm competition that selected females to mate multiply.

To distinguish between these two hypotheses, I measured time intervals between egg deposition at one male and presumed sperm uptake at another male and compared these intervals between spawnings where sperm competition occurred and spawnings where the fertilization pattern matched the spawning pattern. We expected that these intervals were shorter when fertilization patterns did not match the spawning patterns if sperm competition is an important selective force selecting females to mate multiply. Additionally, we calculated the average time interval between egg deposition and mouthing with the "same" male. We expected that these time differences were longer when fertilization patterns did not match the spawning patterns if sperm competition is selecting females to mate multiply. There was no significant difference, however, the range overlap was only marginal and the sample size was very small ( $n_1 = 3$ ,  $n_2 = 5$ ). It is therefore suggestive that shorter time intervals strongly influences the occurrence and outcome of sperm competition. However, time intervals between visits to different males were still rather long.

Females were rather choosy in the ringtank experiment, although not always consistent in their choice. In the first run, the most preferred male was male 20. In the second run he slowly lost his position to male 26. It is plausible that the most popular males may economize on their sperm expenditure in a way that females may be sperm limited. Warner et al. (1995) reported in their study of fertilization patterns of *Thalassoma bifasciatum* that the most popular males give the lowest fertilization benefits to females. This may indicate that sperm depletion or sperm economy may have played an important role both in fertilization patterns as well as in the evolution of multiple mating. This idea is also supported by what can be seen on some of the histological slides from wild-caught males. One can see that the germinal tissue is exhausted on some slides and so those males may experience sperm depletion.

We further studied the testes, ejaculates, and sperm characteristics of *O. ventralis* males (Chapters 6 & 7). Simone Immler excised gonads of males observed in the field and measured their length and weight. In the laboratory the histological slides were stained with the following staining agents: eosin, haematoxylin, PAS and alcian blue at pH 1 and pH 2.5. This revealed that sperm were packed into sialoglycoprotein-mucus, similar to what was found by Grier (1995) in mouthbrooding Tilapias. This mucus protects sperm from osmotic stress, only slowly dissolves in water and hence increases longevity of the ejaculate. Furthermore, the histology indicates the possibility that males can control ejaculate size. The seminal duct is made up of smooth muscles and structures that may serve like a valve to divide ejaculates into discrete amounts. Sperm economy is very common in fishes (Shapiro et al. 1994, Marconato & Shapiro 1995, Warner et al. 1995, Pilastro et al. 2002) and it is thus not very surprising to find the structures that allow for sperm economy in a fish with a lekking mating system. A lekking mating system provides a territorial male with many opportunities for mating and thus sperm economy may be expected.

The third interesting finding resulting from the histological analysis is that the testes contain many large compact clusters of Leydig cells. These are cells that produce steroid hormones (Miura et al. 1991). They are present in most teleost fishes' testes as interstitial cells but normally only in small numbers (Grier 1981). All examined *O. ventralis* males had large clusters of Leydig cells in their testes. The relative numbers of Leydig cells, however, varied significantly between males. Currently, we can only speculate about their significance. It seems most likely that they serve to produce a pheromone much like in the black goby, *Gobius niger*, (Colombo et al. 1980, Rasotto & Mazzoldi 2002, Mazzoldi & Rasotto 2002, Locatello et al. 2002, Immler et al. 2004), where territorial males produce hormonal pheromones to attract females to their spawning site. Grier (1981) describes such clusters of Leydig cells in three distantly related cichlids, so these clusters may be more common in cichlids in general. Furthermore, a recent publication on mate choice in Malawi cichlids

suggested that olfactory cues might play a more important role than previously thought (Plenderleith et al. 2005).

However, it is unclear what the pheromone may signal. The amount of pheromone released could give some information on the quantity of sperm released. There was no significant correlation of relative investment into Leydig cells with the absolute area or volume of mature sperm. However, there was a nearly significant correlation relative investment into Leydig cells with the absolute area of mature sperm multiplied by the GSI. Thus, we do not have strong support for this hypothesis. Alternatively, it is also plausible that the hormones produced in the Leydig cells influence the quality and thus competitiveness of sperm (Folstad & Skarstein 1997, Hillgarth et al. 1997, Skau & Folstad 2005). Androgens can suppress the immunesystem and thus protect the sperm, that are recognized as non-self, from immune-system activity and thereby positively influence the ejaculate quality (Folstad & Karter 1992, Folstad & Skarstein 1997, Hillgarth et al. 1997, Skau Folstad 2005).

Additionally, I measured sperm length, longevity and swim speed. I found that sperm are roughly 21  $\mu\text{m}$  long (mean  $\pm 1\text{SE}$ : 20.825  $\mu\text{m}$   $\pm 0.351$ ). Sperm length varied significantly between males but was consistent within a male. There were significant differences between males in terms of initial swimming speed and longevity. Interestingly, there was also an interaction effect of longevity and swimming speed, depending on sperm length. This meant that longer sperm swam faster initially but also became slower more quickly, and shorter sperm swim slower at first but then stayed motile for longer. This is, to our knowledge, the first time that this trade-off between swimming speed and longevity depending on sperm length has been shown conclusively within a species. The small and long-living sperm and also the mucus that increases the longevity of the ejaculate are certainly of advantage in such a mating system and with such a tricky fertilization mode. Balshine et al. (2001) found that mouthbrooding cichlids have shorter and thus longer-living sperm than substrate-spawning cichlids underlining that sperm with a long motile period may be advantageous with such mating system. However, these authors also found that polygamous mouthbrooders have longer sperm than monogamous mouthbrooders (Balshine et al. 2001), which is in agreement with expectations based on sperm competition theory (Ball & Parker 1996). This result suggests that longer sperm do have an advantage in competition. The spawning mode, however, favours long-living sperm. Due to the trade-off it is not possible to maximize both at the same time. This and the fact that both fast and long-living sperm are favoured by different selective forces may be a reason why we could not find a correlation between morphological characters and sperm characteristics.

## Conclusions

The discovery of polyandry in socially monogamous birds only in the late 1980ies (Burke & Bruford 1987, Wetton et al. 1987) had great impact on the research to come and on the way researchers today think about sexual selection. It gave rise to a whole new debate over multiple mating, optimal mating rates for males and females and hence, sexual conflict and today's vast literature on multiple mating. Before these two publications, everything in sexual selection conformed to the Darwinian paradigm of choosy, essentially monogamous females and male-male competition for access to females. However, a growing number of examples appear not to fit this paradigm but rather one of female resistance and inter-sexual conflict (Holland & Rice 1998).

Multiple mating is now a frequently observed mating pattern (Birkhead & Møller 1998) and also very common in mouthbrooding cichlids of the East African Great Lakes (Kellogg et al. 1995, Paker & Kornfield 1996, Knight et al. 1998, Maan et al. 2004, Chapter 5 this thesis). Neither the benefits to females from multiple mating, nor the mechanisms causing it have been investigated in mouthbrooding cichlids. A number of hypotheses for multiple mating seem inadequate for these mating systems. Inbreeding avoidance is unlikely because of large population sizes and male biased dispersal (Knight et al. 1999). Further, these fishes live in a stable and predictable environment (Coulter 1991); hence we would not expect strong selection for increased genetic variation within clutches. Most direct benefits can be excluded as well because there is no paternal care for the offspring (Kuwamura 1986). Possible direct benefits of multiple mating in these cichlids could be fertilization assurance or uptake and consumption of nutritious sperm (see e.g. Vahed 1998 and Arnqvist & Nilsson 2000 for reviews on nuptial gifts in insects). Indirect benefits could be accrued via trading-up, bet-hedging (Yasui 2001) or induced sperm competition (Keller & Reeve 1995, Yasui 1997).

It appears that in the case of the lekking mouthbrooding cichlids, females have total control over with whom and how many times they mate. There are no forced matings in mouthbrooding cichlids. Thus, females may appear to have won the inter-sexual conflict over mating rates. Nevertheless, it appears that intra-sexual conflicts (e.g. sperm competition) and conflicts with a third party, i.e. interruptions of matings by sneakers or predators, have an important influence on the evolution of multiple mating in mouthbrooding cichlids (intra-sexual conflict or male-male competition in the case of sneakers) and it may be these influences that result in females possibly mating at a suboptimal rate. In *O. ventralis* it may be mainly interruptions by sneakers (Chapter 3 & 4, pers. obs.) rather than egg predators, which may be more important in Lake Malawi cichlids (McKaye 1983, pers. obs.) as there are many specialized egg predators in Lake Malawi (McKaye 1983, McKaye 1990, Stauffer & McKaye 1986, Kellogg et al. 1995, pers. obs.). Another important driving force in the

evolution of multiple mating in mouthbrooding cichlids may be sperm depletion, i.e. fertilization insurance as a direct benefit to multiply mating females. Both of the two presented mechanism – risk of egg predation and male infertility – can be summarized as time-dependent costs of mating. The longer the mating takes, the more likely it is that either the spawning pair is detected by a predator or that the male's sperm supply becomes depleted.

Another mechanism that has not been very well studied so far, particularly in cichlids, is the influence of pheromones on mate choice decisions. We showed that *O. ventralis* has large clusters of hormone producing cells in their testes and it appears most likely that these fish thereby produce a hormonal pheromone. However, we do not know what it may signal. It could give the female some information on the number of sperm released but we did not find good support for this hypothesis. Another possibility is that the presumed high level of testosterone in the blood suppressed the immune-system and that may result in sperm of greater quality (Hillgarth et al. 1997, Folstad & Skarstein 1997, Skau & Folstad 2005). If this is the case, we would expect to find differences in parasite load both between males and females (greater variation and higher parasite load in males) and among males (Hamilton & Zuk 1982, Folstad & Karter 1992).

Nevertheless, sperm competition certainly occurs, underlined both by comparative work on sperm length in mouthbrooding cichlid species (Balshine et al. 2001) and by my own work (Chapter 5). Sperm competition is primarily a selective force on males to produce more competitive sperm, it is therefore not necessarily so that females benefit from sperm competition, i.e. linkage between "good genes" for "good sperm" and "good genes" for survival is not necessarily given (Curtisinger 1991, Pizzari & Birkhead 2002, Zeh 2004, Birkhead et al. 2005; but see also Hosken et al. 2003, Fisher et al. 2006). It appears from our data that only a relatively small number of eggs were fertilized as a result of sperm competition. However, with our data we cannot tell whether females gain indirect benefits from sperm competition, i.e. whether males that are competitively superior under sperm competition carry better genes. Thus, it is unclear whether sperm competition is intense enough to promote the evolution of multiple mating in this species. Sperm competition may also simply increase the chances that all eggs will be fertilized and hence is actually a direct rather than an indirect benefit (cf. Keller & Reeve 1995, Smith & Reichard 2005). To understand the mechanisms of sperm competition, we have to further investigate fertilization mechanisms. We found that there was a trade-off between sperm swimming speed and longevity. This was predicted by Stockley et al. (1997) based on their comparative work with data from various species, but has not been found before within species (Vladic et al. 2002, Gage et al 2004). This trade-off is important because theory predicts that sperm size is only affected by intensity of sperm competition such a trade-off exists (Ball & Parker 1996).

Sperm size appears to be affected by sperm competition intensity in various species (mammals: Gomendio & Roldan 1991; birds: Briskie et al. 1997, but see also Immler & Birkhead 2006; butterflies: Gage 1994) and the trade-off has also been suggested in mammals (Gomendio & Roldan 1991 and 1993).

This study investigated mechanisms that could lead to the evolution of multiple mating in mouthbrooding cichlids. We showed that there are three ways how females could potentially benefit from multiply mating: (i) by avoiding predation risk or sneakers (Chapters 3 & 4), (ii) through sperm competition, or (iii) by fertilization insurance (the latter two are discussed in Chapters 5-7). It is plausible that all three of these suggested mechanism played a role in the evolution of multiple mating. Predation avoidance has been suggested as an important driving force in the evolution of mouthbrooding (Fryer & Iles 1972). As a next step it is imaginable that fertilization had to become independent of male presence while the egg was spawned so that the male could defend the territory while the female spawned eggs. However, despite this intruders still sometimes manage to enter the territory and the female leaves to mate with another male. Once multiple mating is frequent enough, genetic benefits may become important in the evolution of the frequency and degree of polyandry.

It would be interesting to study how these mechanisms translate into population dynamics and how it affects sexual selection in these fish, particularly because sexual selection is thought to be a powerful force driving speciation in these fish (Dominey 1984, Seehausen 2000, Kocher 2004). We looked at this to some extent in our first models (Hamilton et al. 2006, Chapter 2). We showed that more females may mate with poor males under risk of predation and thus that sexual selection may become relaxed by the presence of what we called enemies (egg-predators, predators, harassing males, sneakers, etc.). If this is the case, this could have great influence on intensity of sexual conflict, sexual selection and thus population divergence and speciation (e.g. Gavrillets 2000, Gage et al. 2002). Further investigations of patterns of polyandry on a between-population, and between-species level are needed to examine the effects of polyandry on sexual selection.