

# Possible effects of sperm limitation on the reproductive success of the Amazon molly, *Poecilia formosa* (Poeciliidae)

Mögliche Effekte der Limitierung von Spermien auf den Fortpflanzungserfolg des Amazonenkärpflings, *Poecilia formosa* (Poeciliidae)

Cornelia Hinz<sup>1,2</sup> & Ingo Schlupp<sup>1</sup>

<sup>1</sup>Department of Zoology, University of Oklahoma, 730 Van Vleet Oval, Norman, OK 73019, USA; schlupp@ou.edu (corresponding author)

<sup>2</sup>Systematic and Evolutionary Biology, Department of Biology and Environmental Sciences, University of Oldenburg, Carl von Ossietzky-Str. 9-11, 26129 Oldenburg, Germany; cornelia.hinz@uni-oldenburg.de

**Summary:** Using artificial insemination we studied the role of sperm number on relative reproductive success in the gynogenetic Amazon molly (*Poecilia formosa*). This species is unisexual, but depends on mating with males for sperm of closely related species (*Poecilia mexicana* and *Poecilia latipinna*) to trigger embryogenesis. The male genome is not incorporated into the female genome; inheritance is strictly maternal. The correlation of sperm number and relative reproductive success is logarithmic and a minimum of about 50,000 sperm was determined. Below this number, the number of offspring drops off. A comparison with another study, which showed that males of the Atlantic molly (*Poecilia mexicana*) provide fewer sperm for Amazon mollies as compared to conspecifics, shows nonetheless that Amazon mollies typically receive far more sperm from males of *Poecilia mexicana* and *Poecilia latipinna* than the minimum determined here.

**Key words:** sperm competition, fertilization success, fecundity, unisexuality

**Zusammenfassung:** Mit Hilfe von künstlicher Befruchtung wurde der Einfluss der Spermienanzahl auf den relativen Fortpflanzungserfolg des gynogenetischen Amazonenkärpflings *Poecilia formosa* untersucht. Diese Art ist unisexuell, jedoch abhängig von Paarungen mit den Männchen nahe verwandter Arten (*Poecilia mexicana* und *Poecilia latipinna*), da die Entwicklung der Eizellen durch Spermien ausgelöst wird, ohne dass dabei das Genom der Spermien in die Eizellen aufgenommen wird. Die Korrelation von Spermienanzahl und dem daraus resultierenden relativen Fortpflanzungserfolg folgt einer logarithmischen Funktion; es konnte ein ungefährer Grenzwert von etwa 50.000 Spermien pro Besamung ermittelt werden, unter welchem der Fortpflanzungserfolg stark reduziert ist. Vergleiche mit den Daten aus einer anderen Studie, die gezeigt hat, dass von Männchen des Atlantikkärpflings (*Poecilia mexicana*) an Amazonenkärpflinge zwar weniger Spermien abgegeben werden als an arteigene Weibchen, deuten dennoch darauf hin, dass Amazonenkärpflinge meist genügend Spermien erhalten, da die gefundenen Spermienzahlen, die sie von *Poecilia mexicana* und auch *Poecilia latipinna* erhalten, deutlich über dem hier ermittelten Grenzwert liegen.

**Schlüsselwörter:** Spermienkonkurrenz, Befruchtungserfolg, Fruchtbarkeit, Unisexualität

## 1. Introduction

The investigation of mating systems involving closely related sexually and asexually reproducing species can be an important step to shed light on the question how sexuality can persist against asexuality despite its costs (VRIJENHOEK

1994, SCHLUPP 2005, 2010). The main cost in this sense is that sexual organisms have to produce males, the so called “two-fold-cost of males” (MAYNARD SMITH 1978). As a consequence in an asexual organism the reproductive rate can be twice that of a sexual organism (SCHLUPP & RIESCH 2011), giving the asexual species a major advantage, which – in theory – could result in the

replacement of the sexual form with the asexual form. One model system in which this problem has been studied for a long time involves the all-female Amazon molly *Poecilia formosa*, which arose from a single hybridization event between the sailfin molly *Poecilia latipinna*, and the Atlantic molly *Poecilia mexicana* (HUBBS & HUBBS 1932, STÖCK et al. 2010). *Poecilia formosa* reproduces asexually via gynogenesis, a special form of parthenogenesis which is characterized by the necessity of sexual hosts as sperm donors (SCHLUPP 2005, 2010). Sperm is typically provided by males of the two parental species (SCHLUPP & RIESCH 2011).

Since in Amazon mollies sperm only triggers embryogenesis, but inheritance is strictly maternal (SCHLUPP 2005, SCHLUPP & RIESCH 2011), this should lead to a conflict between *P. formosa* and males of the sexual host species. On the one hand males can benefit by heterospecific mating because conspecific females copy mate choice decisions of *P. formosa* (SCHLUPP et al. 1994, HEUBEL et al. 2009), on the other hand males do not gain direct reproductive benefits and could reduce the number of sperm provided to Amazon molly females without losing the indirect benefit via mate copying. Furthermore, as the gynogens do not pay the cost of producing males (MAYNARD SMITH 1978), this could lead to an extremely unstable situation (KOKKO et al. 2008, HEUBEL et al. 2009): The asexual Amazon mollies could out-compete their sexual host (because they only have female offspring) and drive the sexual species to extinction. This, however, would subsequently lead to the demise of the Amazon mollies as well, because they rely on their sexual host species for sperm. This raises the question how such a mating system can be stable over time. In essence, by some mechanism reproductive success of the Amazon mollies has to be curbed. Several potential mechanisms have been suggested to be important (reviewed in SCHLUPP & RIESCH 2011). Among these mechanisms, male mate choice and sperm transfer have long been identified as important factors for the stability of the mating system (SCHLUPP 2005, 2010). Simply said males can control the Amazon molly population by selectively mating with their conspecific females.

Since males can actually discriminate between *P. formosa* and conspecific females (SCHLUPP et al. 1998, GABOR & RYAN 2001, DRIES 2003, SCHLUPP & PLATH 2005), males have been suggested to play an important role in population regulation both on the population and on the behavioural level (SCHLUPP 2005). Recently, several studies investigated the role of sperm limitation in the mating system of *P. formosa* and its sexual hosts. *Poecilia latipinna* males prime fewer sperm in the presence of Amazon mollies as compared to conspecific females (ASPBURY & GABOR 2004). Furthermore, SCHLUPP & PLATH (2005) demonstrated that male *P. mexicana* prefer to mate with conspecific females and transfer less sperm to *Poecilia formosa* (numbers flushed from *Poecilia mexicana* females ranged from ca. 0 to  $16 \times 10^6$  sperm and 0 to  $7 \times 10^6$  for *Poecilia formosa*; note that these numbers are based on flushing female genital areas, not from stripping males). The results from that study were also in agreement with field observations using *P. latipinna*, which showed that sexual females had sperm in their reproductive tract more frequently and in higher amounts than unisexual females (RIESCH et al. 2008). Effects of the lower sperm transfer on the reproductive output of the gynogens have not been examined further. However, this is important because in this system differences in sperm quality play a minor role, whereas the number of sperm cells is very likely still important.

To provide a basis for predictions of the fertilization success in *Poecilia formosa*, relative to a certain number of sperm cells observed in the field or in behavioural studies, we examined the relationship between the number of sperm cells transferred by artificial insemination and the number of sired offspring. An approximate threshold of *Poecilia latipinna* sperm cells below which the fertilization success considerably decreases was determined via a sperm dilution series.

## 2. Methods

The fishes for our study were originally collected at Lincoln Park (Brownsville, Texas; N 25°53.98, W 97°28.77) during the years 2006 and 2007. The actual test fishes came from stock popula-

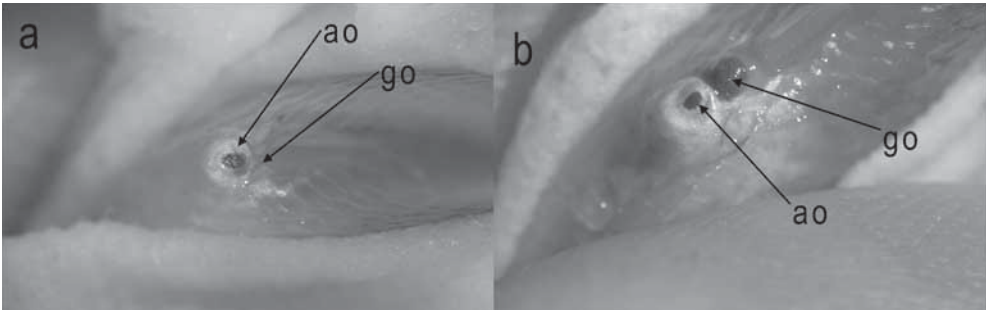
tions that were raised in large (app. 1000 l) cattle tanks in a greenhouse on the Aquatic Research Facility of the University of Oklahoma. Before the sperm transfer experiment fishes were housed in 160 l aquaria in the laboratory under a 12/12 h light-dark cycle. Temperature was kept between 24 and 28 °C via air conditioning and all fishes were fed twice a day ad libitum with

frozen blood worms and white mosquito larvae. We used 28 males of *Poecilia latipinna* which –as a precautionary measure– were separated for a minimum of one week from females to enhance sperm production and storages. We further used 28 *Poecilia formosa* virgins, which had had visual contact with males during the three months prior to the experiment.

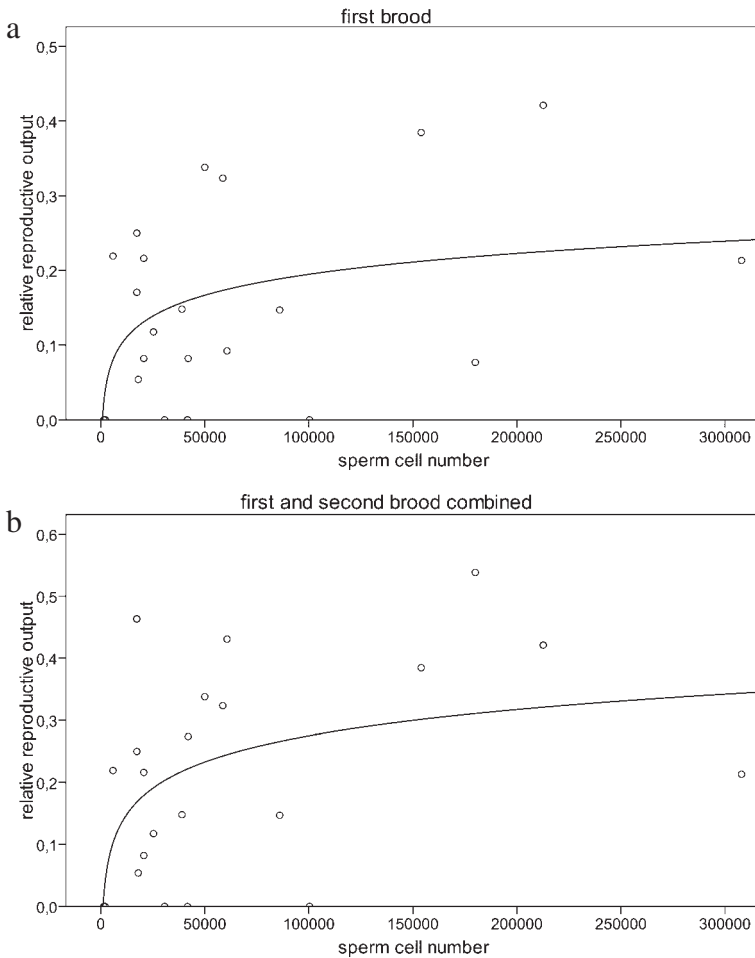
**Tab. 1:** Females (fn) 1-28, their maximum standard length (in mm), amount of saline (in µl) with the approximate number of sperm (scn) they received during artificial insemination, number of offspring in two successive broods (no1, no 2), total number of young (tno), embryos found during dissection (ed), reproductive output of brood 1 (rro1), reproductive output for brood 1 plus brood 2 (rrot), and constitution of the gonad. For further explanations see text.

**Tab. 1:** Weibchen (fn) 1-28, ihre maximale Standardlänge (in mm), Menge an Kochsalzlösung (in µl) mit ungefähre Anzahl von Spermien (scn), die bei der künstlichen Besamung übertragen wurden, Anzahl der Nachkommen in zwei aufeinanderfolgenden Bruten (no 1, no 2), Gesamtzahl der Jungen (tno), Embryonen bei der Sektion gefunden (ed), Fortpflanzungsleistung bei Brut 1 (rro1), Fortpflanzungsleistung bei Brut 1 und 2 (rrot) und Zustand der Gonade. Weitere Erklärungen s. Text.

fn	msl [mm]	vNaCl [µl]	scn	no1	no2	tno	ed	rro1	rrot	od
1	37.5	10	308000	8	0	8	no	0.21	0.21	
2	38	20	212667	16	0	16	yes	0.42	0.42	
3	39	30	154000	15	0	15	yes	0.38	0.38	
4	36.5	40	5867	8	0	8	no	0.22	0.22	
5	41	50	17307	7	12	19	no	0.17	0.46	
6	39	60	41653	0	0	0	yes	0.00	0.00	
7	32.5	70	132000	0	0	0	no	0.00	0.00	no gonad/no embryos
8	35.5	80	50000	12	0	12	yes	0.34	0.34	
9	41	90	21333	0	0	0	no	0.00	0.00	no embryos
10	35.5	100	2160	0	0	0	yes	0.00	0.00	
11	35.5	10	100320	0	0	0	yes	0.00	0.00	
12	34	20	58667	11	0	11	no	0.32	0.32	
13	32.5	30	1467	0	0	0	yes	0.00	0.00	
14	40.5	40	39013	6	0	6	no	0.15	0.15	
15	35.5	50	28000	0	0	0	no	0.00	0.00	no embryos
16	18	60	12000	0	0	0	n.a.	0.00	0.00	dead before test end
17	37	70	18000	2	0	2	no	0.05	0.05	
18	36	80	17333	9	0	9	no	0.25	0.25	
19	36.5	90	20667	3	0	3	no	0.08	0.08	
20	38.5	100	32000	0	0	0	no	0.00	0.00	no embryos
21	37	10	20667	8	0	8	yes	0.22	0.22	
22	39	20	180000	3	18	21	no	0.08	0.54	
23	32.5	30	60667	3	11	14	no	0.09	0.43	
24	17	40	50667	5	0	5	n.a.	0.29	0.29	dead before test end
25	35.5	50	30667	0	0	0	yes	0.00	0.00	
26	36.5	60	42000	3	7	10	no	0.08	0.27	
27	34	70	86000	5	0	5	no	0.15	0.15	
28	34	80	25334	4	0	4	no	0.12	0.12	



**Fig. 1 a and b:** Female genital area. ao anal opening, go genital opening. Note the grey area in b.  
**Abb. 1 a und b:** Genitalregion eines Weibchens. Ao Anus, go für Genitalöffnung. Man beachte das grau gefärbte Areal in b.



**Fig. 2 a and b:** **a** Number of offspring (only first broods); **b** first and second broods, both relative to number of sperm inseminated.  
**Abb. 2 a und b:** Anzahl der Nachkommen; **a** nur der erste Wurf, **b** erster und zweiter Wurf, jeweils pro Anzahl Spermien nach künstlicher Besamung.

Females were inseminated artificially with different amounts of sperm using sperm of one male per female (for a review of methods and sperm competition see EVANS & PILASTRO 2011). Males were anesthetized with MS 222 and stripped to achieve sperm release (LOCATELLO et al., 2006). Sperm was diluted in saline (0.9 % NaCl). Different sperm dilutions were achieved randomly by the naturally occurring differences in ejaculate size combined with varying volumes of saline for dilution (volumes were varied in 10  $\mu$ l steps from a minimum of 10  $\mu$ l to a maximum of 100  $\mu$ l; see table 1). In poeciliids (CONSTANTZ 1989, GREVEN, 2011) sperm is transferred in discrete bundles, called spermatozuogmata. After 30 min at room temperature these spermatozuogmata were mostly dissolved and the motile sperm were available for artificial insemination. Before insemination the dilution was gently mixed. 1  $\mu$ l of sperm suspension was used to count sperm cells. For insemination another 1  $\mu$ l was gently injected into the genital opening of an MS 222 anesthetized female using a fine plastic syringe (EVANS et al. 2003). Only females that showed an easily visible dark grey spot around their genital opening (figs. 1 a, b) were chosen for insemination, because their genital opening was wider and therefore the procedure was much easier to perform and injuries of the genital tract could be minimized. This dark grey spot had not been described before, but may be an indication of receptivity.

Following the artificial insemination, females were housed individually under the same conditions described above, but in individual 5 l aquaria. Tanks were checked for fry twice a day. When a female gave birth the number of offspring was recorded. Females were kept alive for 80 days to allow them to complete multiple (at least two) complete reproductive cycles (PARZEFALL 1973, HUBBS & DRIES 2002). After 80 days, females were sacrificed with an overdose of MS 222 and dissected to control for embryos and the general constitution of the gonads (tab. 1). Two separate analyses were conducted for the reproductive output of the first brood only and first and second brood combined for those females that had multiple broods.

As response variable we used a proportion for fertilization success, the relative reproductive output. It was calculated as the number of offspring divided by the average individual female standard length measured at the beginning and the end of the test period. This correction for size was chosen because TOBLER et al. (2005) demonstrated a linear relationship of female standard length and reproductive output in *P. latipinna*. We did not use that particular dataset to calibrate our data because we were concerned about year and population effects.

The statistical analysis of the data was conducted in SPSS version 15 for Windows. Data showed a normal distribution. To describe the correlation between injected sperm cell number and resulting reproductive output, a curve fit estimation maximizing  $r^2$  was carried out.

### 3. Results

Our results indicated that the best fitting model found by curve estimation was a logarithmic function for both analyses (figs. 2 a, b), but only the result for the relative reproductive output of first and second brood combined was significant (first brood only:  $n = 22$ ,  $r^2 = 0.176$ ,  $P > 0.05$ ; first and second brood combined:  $n = 22$ ,  $r^2 = 0.236$ ,  $P < 0.05$ ; fig. 2 b). For both analyses the relative reproductive output rose steeply up to a sperm count of roughly 50,000 (figs. 2 a, b) and then tapered off. Therefore, a reduced fertilization success in *Poecilia formosa* can be predicted, when the number of transferred sperm falls below 50,000.

Note that the data shows considerable variability and that the  $r^2$  values indicate much unexplained variability in the data set.

### 4. Discussion

Our dissection data (tab. 1) showed that our chosen time span of 80 days was too short to observe all young born resulting from the artificial insemination, because we detected developing embryos in nearly one third of the test females, including females that did not give birth to any offspring during the observation time.

In females that had two broods or had one brood and embryos, the number of offspring in the first brood was below the expected numbers, when compared to a study by SCHLUPP (unpublished data), where the size corrected offspring number per brood was 11 or more. Although it is tempting to speculate about functional explanations, the most likely explanation would be that the artificial fertilization procedure *per se* was at least partly responsible for the observations, although we tried to implement techniques successfully used in guppies and mosquitofish (EVANS & PILASTRO 2011). We speculate that stress or small injuries caused by the artificial insemination procedure might influence the initial fertilization success negatively or delay the time point of egg fertilization in general.

Despite these methodical issues, our data demonstrates the correlation of the relative reproductive success resulting from a given sperm cell number. Furthermore, we could determine an approximate threshold of about 50,000 sperm cells below which the relative reproductive success decreases considerably. One spermiozeugma, which represents the smallest possible unit of sperm transfer, of closely related poeciliid fishes contains around 4,000-5,500 sperm cells (e.g. KALLMAN 1975 for *Xiphophorus*, LOCATELLO et al. 2008 for *Gambusia*). Obviously, these numbers are species specific and variation is to be expected. Since the published numbers are clearly below the threshold found in this study, the reproductive success could theoretically be limited by reduced sperm transfer (if only a few spermiozeugmata were transferred), but studies of SCHLUPP & PLATH (2005) and RIESCH et al. (2008) demonstrated that median sperm cell numbers actually found in the reproductive tract of *P. formosa* were above the threshold of 50,000 sperm cells (see above). In light of the study by SCHLUPP & PLATH (2005) the lower sperm transfer found there might represent a reduction of costs for males by saving sperm, but clearly this does not limit the fecundity of the Amazon mollies.

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