

## Male mating behavior and sexual harassment in population hybrids of cave- and surface-dwelling Atlantic mollies (*Poecilia mexicana*, Poeciliidae)

Paarungsverhalten der Männchen und sexuelle Belästigung bei Populationshybriden von höhlenbewohnenden und oberirdischen Atlantikkärpflingen (*Poecilia mexicana*, Poeciliidae)

MARTIN PLATH<sup>1</sup>, MICHAEL TOBLER<sup>2</sup>, OLE KLAWONN<sup>3</sup> & JAKOB PARZEFALL<sup>3</sup>

<sup>1</sup>J.W. Goethe Universität Frankfurt, Abteilung Ökologie & Evolution, Siesmayerstraße 70a, D-60323 Frankfurt am Main, mplath@bio.uni-frankfurt.de, corresponding author

<sup>2</sup>Texas A&M University, Department of Biology and Department of Wildlife and Fisheries Sciences, 2258 TAMU, College Station, TX 77843-2258, USA

<sup>3</sup>Universität Hamburg, Abteilung für Verhaltensbiologie, Zoologisches Institut, Martin-Luther-King-Platz 3, 20146 Hamburg

**Summary:** Atlantic mollies (*Poecilia mexicana*) in the southern Mexican Cueva del Azufre system inhabit a variety of different habitat types, some of which are characterized by perpetual darkness (caves) and/or the presence of toxic hydrogen sulfide. Previous studies demonstrated heritable differences among populations from adjacent but ecologically divergent habitat types in morphology, behavior and life history. Here we examined male mating behavior in fish from the most extreme habitat, the sulfidic Cueva del Azufre ("cave mollies"), and a nearby non-sulfidic river (i.e., the least extreme habitat type) as well as hybrids (F<sub>1</sub> and F<sub>2</sub>) between both populations. Compared to surface-dwelling males, mollies showed strongly reduced sexual activity, which may be an adaptation to life in a stressful, energy-poor environment. However, sexual activity of first-generation hybrids even exceeded the level of the surface fish, suggesting that a heterosis effect leads to strongly increased sexual activity. To avoid unwanted copulations, surface females are more vigilant in the presence of a male than in the presence of a female, which detracts from the time spent feeding. Reduced sexual activity in cave mollies comes along with reduced vigilance in females, so cave molly females do not feed less when accompanied by a male. Despite increased sexual activity in F<sub>1</sub> hybrid males, F<sub>1</sub> females did not feed less in the presence of a male (i.e., resumed feeding even though males frequently attempted to mate) and also no effect was seen in the F<sub>2</sub> hybrids. This could indicate a breakdown of co-evolved gene complexes of (i) male persistence traits (i.e., population-specific levels of male sexual activity) and (ii) female resistance traits (vigilance and flight behavior).

**Key words :** cavefish, coercive mating, heterosis effect, hydrogen sulfide, sexual conflict

**Zusammenfassung:** Im Cueva del Azufre-System in Süd Mexiko bewohnen Atlantikkärpflinge (*Poecilia mexicana*) eine Vielzahl unterschiedlicher Habitattypen, darunter dauerhaft dunkle Höhlen („Höhlenmollies“) oder Gewässer, die giftigen Schwefelwasserstoff enthalten. Frühere Untersuchungen haben erbliche Unterschiede zwischen Populationen aus nahegelegenen, jedoch ökologisch stark unterschiedlichen Habitaten hinsichtlich äußerlicher Körpermerkmale, des Verhaltens und der Lebenszyklusstrategien aufgezeigt. Hier untersuchten wir das Paarungsverhalten der Männchen bei Fischen aus dem extremsten Habitattyp (der schwefelhaltigen Cueva del Azufre) und einem nahegelegenen schwefelfreien Fluss (d.h. dem am wenigsten extremen Habitattyp) sowie bei F<sub>1</sub>- und F<sub>2</sub>-Hybriden zwischen beiden. Verglichen mit oberirdischen Männchen zeigten Männchen des Höhlenmollies eine stark reduzierte sexuelle Aktivität, was als Anpassung an das Leben in einer stressvollen, energielimitierten Umwelt angesehen werden kann. F<sub>1</sub>-Hybriden zeigten mehr sexuelles Verhalten als die Flusspopulation, was auf einen Heterosiseffekt hindeutet. Um ungewollten Kopulationen zu entgehen, müssen oberirdische Weibchen ihre Wachsamkeit erhöhen, wodurch sie in Gegenwart eines Männchens weniger fressen können als in

Gegenwart eines anderen Weibchens. Beim Höhlenmolly geht erniedrigte männliche sexuelle Aktivität mit einem Verlust dieses Effektes einher. Trotz der erhöhten sexuellen Aktivität der F<sub>1</sub>-Männchen zeigte sich bei deren Weibchen keine Reduktion der Fresszeit, ebenso nicht bei den F<sub>2</sub>-Hybriden (d.h. die Weibchen fraßen trotz der häufigen Kopulationsversuche der Männchen weiter). Dies deutet auf ein Aufbrechen koevolvierter Genkomplexe hin, die für (i) das Paarungsverhalten der Männchen und (ii) Verhaltensweisen der Weibchen, um sich der sexuellen Nachstellung zu widersetzen (Wachsamkeit und Fluchtverhalten), verantwortlich sind.

**Schlüsselwörter:** Höhlenfisch, erzwungene Paarungen, Heterosiseffekt, Schwefelwasserstoff, sexueller Konflikt

## 1. Introduction

In the southern Mexican Cueva del Azufre system, the widespread Mexican livebearing toothcarp *Poecilia mexicana* (Poeciliidae) has colonized at least two caves (GORDON & ROSEN 1962, PARZEFALL 2001, TOBLER et al. 2008a, b). *Poecilia mexicana* in this system are especially interesting to study, because several proximate habitats are characterized by strongly divergent abiotic environmental conditions, i.e. the presence or absence of naturally occurring hydrogen sulfide (H<sub>2</sub>S) and/or light (TOBLER et al. 2006, 2008a). Both, the presence of H<sub>2</sub>S and the absence of light, are potential sources of divergent natural selection, and any combination of the two selective agents is found in the Cueva del Azufre system (TOBLER et al. 2008b). H<sub>2</sub>S is correlated with extreme hypoxia in aquatic environments and is a potent respiratory toxicant lethal for most metazoans even in micromolar amounts (BAGARINAO 1992, GRIESHABER & VÖLKEL 1998). In the Cueva del Azufre itself, H<sub>2</sub>S is present in acutely toxic concentrations of up to 300 µM (TOBLER et al. 2006). Similarly, the absence of light in caves inhibits the use of visual senses, and cave-dwellers are under selection to cope with darkness, especially if they evolved from a diurnal surface-dwelling ancestor (CULVER et al. 1995, HOWARTH 1993, LANGECKER 2000).

Previous studies revealed different evolutionary changes in *P. mexicana* in response to the divergent environmental conditions. Reduced pigmentation and reduced eye size was found in subterranean populations and may be linked to the absence of stabilizing selection in darkness (PETERS et al. 1973, TOBLER et al. 2008b). Populations from all sulfidic habitats

in the Cueva del Azufre system have larger heads and a larger gill surface area (TOBLER et al. 2008b), which facilitates more efficient oxygen uptake under hypoxic conditions via aquatic surface respiration (PLATH et al. 2007c). This morphological differentiation is paralleled by pronounced genetic differentiation among populations from different environments despite small geographic distances and the lack of physical barriers that would prevent migration (PLATH et al. 2007a).

Populations of *P. mexicana* from dissimilar habitats also diverged in a number of behavioral traits. Energy limitation, either due to resource scarcity and/or costly adaptations that allow for coping with the toxic environment, is thought to have selected for the regression of costly behaviors (PLATH & TOBLER 2009, TOBLER & PLATH 2009). Cueva del Azufre cave fish are less aggressive (PARZEFALL 1974, 1979) and exhibit a heritable reduction in shoaling (PLATH & SCHLUPP 2008) and male sexual behavior (PLATH 2008, PLATH et al. 2003, 2007b). In the present study we examined male sexual activity (numbers of sexual behaviors per unit of time) in population hybrids of *P. mexicana*. Investigations of hybrid other behaviors in this system revealed that hybrids exhibit intermediate aggression and shoaling tendencies compared to parental populations (PARZEFALL 2001). We therefore also expected male sexual activity to be intermediate in population hybrids.

A central aspect of our present study was male sexual harassment and the costs male sexual activity inflicts for females. Males often respond to female resistance—i.e., their unwillingness to mate—by using coercion to inseminate them (CLUTTON-BROCK & PARKER 1995, ARNQVIST & ROWE 2005). Females usually

try to avoid coercive copulations (i.e., raise their vigilance levels), and male sexual behavior that alters females' time allocations can be defined as sexual harassment (MAGURRAN 2001, MAGURRAN & SEGHERS 1994). Sexual harassment can affect females' fitness, e.g., through a reduction in foraging time, as females constantly need to monitor surrounding males (SCHLUPP et al. 2001, PILASTRO et al. 2003, DADDA & BISAZZA 2006). In *P. mexicana*, low male sexual activity in populations from cave and sulfidic habitats comes along with a lack of female feeding time reduction (PLATH et al. 2003, 2007b, PLATH 2008). In this study, we re-analyzed data for the two parental populations (Río Oxolotan and cave mollies from the sulfidic Cueva del Azufre), and compared population-specific rates of male sexual activity as well as costs of male sexual harassment among the parental populations, F<sub>1</sub>, and F<sub>2</sub> crosses.

## 2. Methods

### 2.1. Study organisms and fish maintenance

We compiled and re-analyzed all available data on male sexual activity for the populations from the sulfide-free Río Oxolotan [ $n = 20$  (PLATH et al. 2003),  $n = 6$  (PLATH 2008)] and from cave chamber XIII of the Cueva del Azufre [ $n = 25$  (PLATH et al. 2003),  $n = 20$  (PLATH et al. 2004),  $n = 26$  (PLATH et al. 2007b)]. These populations were collected in different years and stocks were reared in different laboratories (see Plath 2008 for details). Theoretically, prolonged periods of captivity could have affected sexual activity, e.g., if inbreeding plays a role (e.g., *Drosophila melanogaster*: MILLER et al. 1993, *Poecilia reticulata*: MYLENE et al. 2006). Also, differences among labs might have affected the results. However, it was shown beforehand that male sexual activity of the two studied populations in the compiled dataset is not affected by any of these factors (PLATH et al. 2007b, PLATH 2008).

Novel data were collected from F<sub>1</sub> hybrids, which were bred in the laboratory in Hamburg. Virgin females of the surface population (Río

Oxolotan) were mated to cave molly males ( $n = 5$  crosses). Offspring from the tanks containing F<sub>1</sub> hybrids (i.e., F<sub>2</sub> hybrids) were transferred into new aquaria. In the course of this study  $n = 36$  trials were completed ( $n = 18$  each). All fish used in this study were sexually mature and had interacted with the opposite sex; thus, all females were most likely pregnant.

All stocks were maintained under common garden conditions, i.e., under daylight conditions, in the absence of H<sub>2</sub>S, and under *ad libitum* feeding, so any differences observed represent evolved (heritable) differences, and are not just due to different rearing conditions. Fish kept in the laboratory of the Zoological Institute and Zoological Museum of the University of Hamburg were maintained in 50-200 l tanks at 25-30 °C under a 12:12 hour light: dark cycle. We fed the fish twice a day with commercially available flake food, *Artemia* naupliae, water fleas, and *Tubifex* sp. Some fish came from stocks in a greenhouse of the Aquatic Research Facility of the University of Oklahoma (Norman, OK) and were maintained in 1,000-liter tanks under ambient light conditions. The tanks contained naturally growing algae as well as a variety of invertebrates like chironomid larvae, copepods, and amphipods, on which the fish could feed. In addition, the fish were fed flake food every two days.

All fish were acclimated to the experimental room for 24 hrs and were fed Tetra Tibi-Tabs food tablets, making sure that the fish would be habituated to, and feed on food tablets during the tests. Then, males were isolated from females in small, visually separated aquaria for another 24 hrs. Meanwhile the focal females were isolated in small groups (4-5 individuals) in 50-liter aquaria and were not fed for 24 hrs before the tests, making sure that focal females would be motivated to feed throughout the test. Males and partner females were fed to saturation in the morning just prior to the tests.

### 2.2. Experimental design

The experimental design of the behavior tests followed PLATH et al. (2003). Each test

consisted of two parts during which a focal female could feed while interacting either with another female or with a male partner fish. The effect of male harassment was estimated from the difference in feeding times with the female partner (“baseline”) and the male. Moreover, numbers of male sexual behaviors were scored.

A food tablet as feeding source was attached to a small Petri dish and placed in the center of a test aquarium (49 x 24 x 24 cm), which was filled to three quarters with aged tap water of 27-30°C. The sides and back were covered with black plastic foil. The observer was sitting behind a black curtain in front of the test tank and could observe the fish through a small hole in the curtain. Illumination was provided by a 40 W fluorescent lamp from above. A focal female was habituated in a clear plexiglass cylinder (8.2 cm diameter) and a male or a female partner fish was introduced into the tank. Both fish were given five minutes for acclimation. The focal female was then released, and the behavior observation started. We measured the time the focal female spent feeding on the presented

stationary food source, from the surface of the water, the aquarium walls and bottom, and on floating matter during a five-minute observation period. Whenever a male partner was present, we also recorded the number of sexual behaviors, namely pre-mating behavior involving body contact (i.e., nipping at the female gonopore) and gonopodial thrusts (i.e., copulations and copulation attempts). Nipping at the female genital pore typically precedes copulation attempts in mollies (PARZEFALL 1973).

After the first part of a trial, the focal female was introduced into the cylinder again, the first partner was removed, and a partner fish of the opposite sex was introduced. The order of presentation (male or female partner first) was balanced. After a trial, all fish involved were measured for standard length to the closest millimeter (tab. 1)

### 2.3. Statistical analysis

All statistical analyses were carried out using SPSS 12.0. Male sexual activity (sum of male sexual behaviors) was compared across popula-

**Tab. 1:** Descriptive statistics for the measurements of body size (standard length, SL), the focal females’ feeding times in the presence of a female and a male partner, and male sexual behaviors [nipping and copulation attempts, and estimated marginal means for the combined male sexual activity as calculated from a GLM (see main text)]. Data are given as mean  $\pm$  S.E.

**Tab. 1:** Deskriptive Statistik zu den Körperlängen (Standardlänge, SL), Fresszeiten der Fokusweibchen in Gegenwart eines weiblichen bzw. männlichen Partnerfisches und zum sexuellen Verhalten der Männchen [Nippen und Kopulationsversuche, sowie *estimated marginal means* für die kombinierte sexuelle Aktivität der Männchen, berechnet mit Hilfe eines generellen linearen Modells (s. Text)]. Daten sind als Mittelwerte  $\pm$  S.A. wiedergegeben.

Population	n	Male SL [mm]	Focal female SL [mm]	Partner female SL [mm]	Time spent feeding with female [s]	Time spent feeding with male [s]	Male nipping	Male copulation attempts	Male sexual activity, estimated marginal means*
Surface	26	30.80 $\pm$ 1.01	35.46 $\pm$ 1.34	35.75 $\pm$ 1.30	40.77 $\pm$ 8.60	31.31 $\pm$ 8.41	26.08 $\pm$ 5.65	5.54 $\pm$ 2.11	2.07 $\pm$ 0.15
F <sub>1</sub>	18	28.44 $\pm$ 1.21	38.89 $\pm$ 1.05	36.11 $\pm$ 1.08	45.94 $\pm$ 9.78	49.61 $\pm$ 14.45	141.56 $\pm$ 21.85	40.44 $\pm$ 8.27	3.05 $\pm$ 0.20
F <sub>2</sub>	18	32.06 $\pm$ 1.17	32.33 $\pm$ 0.89	32.33 $\pm$ 0.89	39.17 $\pm$ 17.00	19.00 $\pm$ 11.29	24.72 $\pm$ 6.31	15.22 $\pm$ 4.44	2.32 $\pm$ 0.22
Cave	71	29.89 $\pm$ 0.57	34.13 $\pm$ 0.58	32.91 $\pm$ 0.58	65.54 $\pm$ 7.37	64.54 $\pm$ 760	13.83 $\pm$ 2.48	3.35 $\pm$ 0.77	1.62 $\pm$ 0.09

\*Estimated marginal means are calculated for male SL = 30.16 mm, focal female SL = 34.52 mm.

tions using a general linear model (GLM), in which ‘population’ was treated as a between-subjects factor (for simplicity, the two groups of hybrids are henceforth also referred to as ‘populations’). To test for a potential effect of male body size on male mating activity (i.e., small males showing higher sexual activity than larger males: SCHLUPP et al. 2001, PILASTRO et al. 2003, PLATH et al. 2003) and female body size (i.e., male mating preferences for large females: PLATH et al. 2006, PLATH 2008), male standard length and the focal females’ standard lengths were included as covariates.

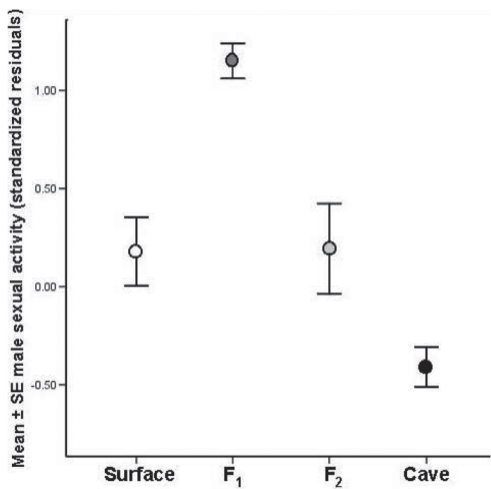
Furthermore, we tested for a reduction of females’ feeding times in the presence of a male (i.e., an effect of male sexual harassment). Female feeding times were log-transformed, and were compared between the two experimental situations (with male or female partner) using paired *t*-tests. In another approach, we compared female feeding time reduction (determined as the relative time the focal female spent feeding in the presence of a male) across populations. The relative time spent feeding with a given male was calculated as [time spent feeding with that male / (time spent feeding with the respective partner female + time spent feeding with the male)]. Hence, values < 0.50 would indicate that the focal females fed less with the male partner, 0.50 would indicate no change at all and values > 0.50 would indicate that the females fed more with the male partner. All relative data were arcsine (square-root)-transformed for the analyses. Again, we employed GLM, in which ‘population’ was a between-subjects factor and male sexual activity (individual values for each male) was included as a covariate. Because the overall feeding motivation of females might also have an effect, the total feeding time of the focal females (absolute feeding time with female + with male partner) was included as another covariate. Where interactions with one of the covariates were significant, standardized residuals from GLM were used to calculate *post hoc* Spearman rank-correlations, which allowed qualitatively comparing correlation coefficients. All reported *P*-values are two-tailed.

### 3. Results

#### 3.1. Male sexual activity

##### 3.1.1. Population comparison

In the GLM, a significant effect of the factor ‘population’ was detected (tab. 2a). *Post hoc* pairwise comparisons [Fisher’s least significant difference (LSD)] revealed that the  $F_1$  hybrids exhibited more sexual behaviors than any other population examined ( $P < 0.0001$  in all cases; tab. 1; fig. 1). The cave population also showed significantly fewer sexual behaviors than the surface population ( $P = 0.0014$ ) and the  $F_2$  males ( $P = 0.047$ ). There was no significant difference between the surface population and the  $F_2$  hybrids ( $P = 0.47$ ).



**Fig. 1:** Mean (± S.E.) male sexual activity (standardized residuals for the sum of all sexual behaviors) in surface dwelling *P. mexicana* (left),  $F_1$  hybrids (surface x cave),  $F_2$  hybrids, and cave mollies (chamber XIII of the Cueva del Azufre).

**Abb. 1:** Die mittlere (± Standardfehler) sexuelle Aktivität der Männchen (standardisierte Residuen für die Summe aller sexuellen Verhaltensweisen) bei oberirdischen *P. mexicana* (links),  $F_1$ -Hybriden (oberirdisch x Höhle),  $F_2$ -Hybriden sowie bei Höhlenmollies (Kammer XIII der Cueva del Azufre).

**Tab. 2:** Results from GLMs on male sexual activity (sum of all male sexual behaviors, log-transformed, **a**) and female feeding time reduction [using the proportion of time spent feeding with a male (arcsine-square root-transformed) as dependent variable, **b**]. All other interaction terms were not significant ( $F = 1.91$ , mean square = 0.978,  $P = 0.13$ ) and hence removed from the final models.

**Tab. 2:** Ergebnisse zweier GLM zur sexuellen Aktivität der Männchen (Summe aller sexuellen Verhaltensweisen der Männchen, log-transformiert, **a**) und zur Fresszeitreduktion der Weibchen [wobei die relative Zeit, die zusammen mit einem Männchen gefressen wurde (arcsin-Wurzel-transformiert), als abhängige Variable verwendet wurde, **b**]. Alle weitere Interaktionsterme waren nicht signifikant ( $F = 1.91$ , mittlere Quadratsumme = 0.978,  $P = 0.13$ ) und wurden daher aus den finalen Modellen entfernt.

	<i>df</i>	Mean square	<i>F</i>	<i>P</i>
<b>(a) Male sexual activity</b>				
Population	3	3.403	6.64	<b>0.0003</b>
Focal female SL	1	0.011	0.02	0.89
Male SL	1	0.164	0.32	0.57
Population x focal female SL	3	2.220	4.34	<b>0.006</b>
Error	124	0.512		
<b>(b) Female feeding time reduction</b>				
Population	3	0.466	3.07	<b>0.031</b>
Total feeding time	1	3.245	21.34	<b>&lt;0.0001</b>
Male sexual activity	1	0.005	0.03	0.86
Population x total feeding time	3	0.369	2.43	0.069
Error	124	0.152		

### 3.1.2. Male mating preference for large female body size

In the GLM, the interaction term ‘female standard length x population’ was also significant (tab. 2 a), suggesting differences across populations in male preferences for large female body size. *Post hoc* correlations between the focal females’ body size and male sexual activity (standardized residuals) revealed the predicted positive correlation in cave molly males (Spearman rank-correlation:  $r_s = +0.43$ ,  $P < 0.0001$ ,  $n = 71$ ), but a negative relationship in surface-dwelling males ( $r_s = -0.41$ ,  $P = 0.039$ ,  $n = 26$ ), and no significant correlation at all in both groups of hybrids ( $F_1$ :  $r_s = +0.26$ ,  $P = 0.31$ ,  $n = 18$ ;  $F_2$ :  $r_s = +0.39$ ,  $P = 0.11$ ,  $n = 18$ ; fig. 2).

### 3.2. Effect of male harassment on females’ feeding times

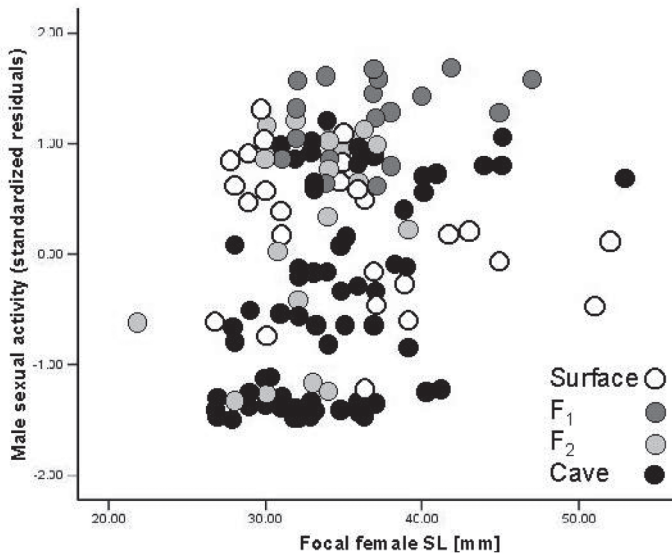
#### 3.2.1. Pair-wise comparison of feeding times

Only in the surface population did females spend less time feeding in the presence of a

male compared with the part of the tests involving a partner female (paired *t*-test:  $t_{25} = 2.54$ ,  $P = 0.018$ ), but this was not the case in the cave form ( $t_{70} = 0.39$ ,  $P = 0.70$ ) and the  $F_1$  hybrids ( $t_{17} = 1.16$ ,  $P = 0.26$ ). The mean time spent feeding with a male partner was distinctly lower than the time spent feeding with a female partner in the case of the  $F_2$  hybrids (tab. 1), but again, the difference was not statistically significant ( $t_{17} = 1.59$ ,  $P = 0.13$ ).

#### 3.2.2. Population differences in females’ overall feeding times

We first compared the absolute times females spent feeding (with a male + with a female partner combined) across populations. Mean ( $\pm$  S.E.) feeding times were  $72.08 \pm 15.47$  s in surface-dwelling females and  $130.08 \pm 13.20$  s in cave molly females. The  $F_1$  hybrids showed intermediate feeding behavior ( $95.56 \pm 20.46$  s), but the  $F_2$  hybrids showed low feeding ( $58.17 \pm 23.38$  s). This difference among populations was significant (one way ANOVA:  $F_{3,129} = 3.72$ ,  $P = 0.013$ ). A *post hoc* test revealed that cave molly females differed significantly from the



**Fig. 2:** Scatter plot depicting the relationships between male sexual activity (standardized residuals for the sum of all male sexual behaviors) and the focal females' body size (standard length, SL). Note the positive correlation in cave molly males and the negative correlation in surface dwelling males.

**Abb. 2:** Streudiagramm, welches die Beziehung zwischen der sexuellen Aktivität der Männchen (standardisierte Residuen für die Summe aller sexuellen Verhaltensweisen der Männchen) und der Körpergröße der Weibchen (Standardlänge, SL) darstellt. Man beachte die positive Korrelation im Falle der Höhlenmollies und die negative Korrelation bei oberirdischen Männchen.

surface form (LSD test:  $P = 0.014$ ) and from the  $F_2$  hybrids ( $P = 0.008$ ), but not from the  $F_1$  hybrids ( $P = 0.20$ ). All other pair-wise comparisons yielded non-significant ( $P = 0.27$ ). Due to the observed differences among populations in the overall motivation of the focal females to feed, the total feeding time was included as a covariate in the between-population comparison of female feeding time reduction (see below).

### 3.2.3. Population comparison of female feeding time reduction

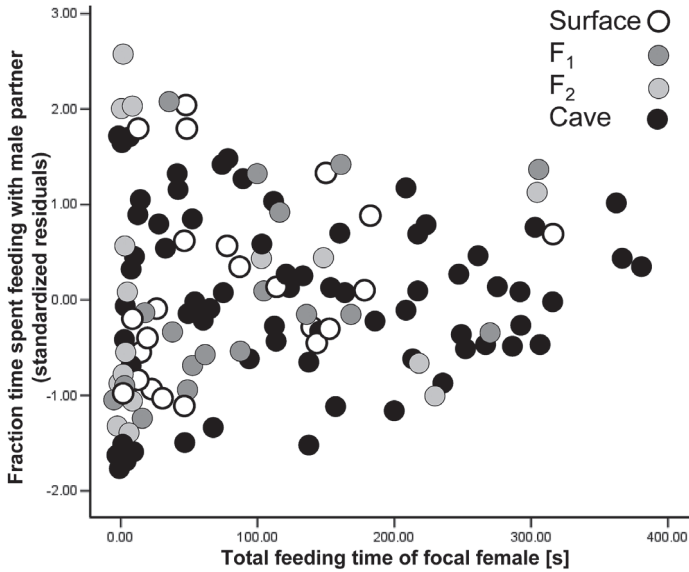
In the GLM, there was a significant effect of the focal females' overall feeding motivation on the decline of female feeding rates (tab. 2 b). A *post hoc* Spearman rank-correlation revealed a significant positive correlation between the females' total feeding time and the fraction time spent feeding with the male (standardized residuals,  $r_s = +0.299$ ,  $P < 0.0001$ ,  $n = 133$ ), suggesting that those females that were more

motivated to feed avoided male harassment less (fig. 3). Moreover, a significant overall difference among populations was detected (tab. 2b; fig. 4). *Post hoc* Fisher's LSD tests revealed that the cave population differed significantly from the surface form ( $P = 0.028$ ) and the  $F_2$  hybrids ( $P = 0.035$ ), whereas all other pair-wise comparisons were not significant ( $P > 0.18$ ).

## 4. Discussion

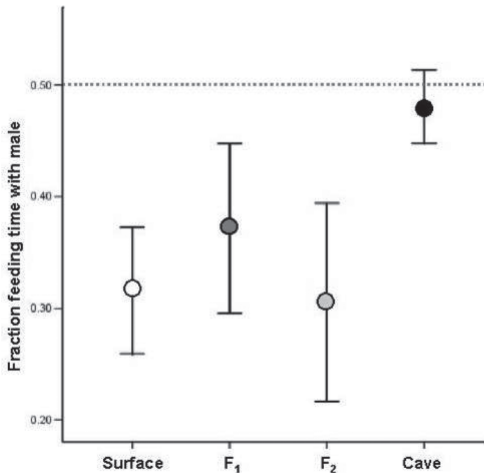
### 4.1. Reduced male sexual activity in cave mollies

*P. mexicana* males from the Río Oxolotan exhibited high sexual activity, and male sexual activity was distinctly lower in the cave population from the sulfidic Cueva del Azufre. It seems likely that energy-limitation is the driving force selecting for reduced male sexual activity (PLATH & TOBLER 2009, TOBLER & PLATH 2009), since *P. mexicana* from habitats with presence of  $H_2S$  show a low body



**Fig. 3:** Scatter plot depicting the correlation between the focal females' motivation to feed (determined as the combined feeding time) and the reduction of females' feeding times by male harassment (determined as the fraction time spent feeding in the presence of a male). Overall, there was a slight positive correlation (see main text).

**Abb. 3:** Streudiagramm, das die Beziehung zwischen der Motivation der Weibchen zu fressen (als kombinierte Fresszeit bestimmt) sowie der Reduktion der Fresszeit der Weibchen durch die sexuelle Belästigung der Männchen (bestimmt als relativer Anteil der Fresszeit in Gegenwart eines Männchens) darstellt. Eine schwach positive Korrelation wurde gefunden (s. Text).



**Fig. 4:** The relative time (mean  $\pm$  S.E.) focal females spent feeding with the male partner. Values  $< 0.50$  (dotted line) indicate that the females spent, on average, less time feeding with the male partner compared with the part of the tests with the female partner.

**Abb. 4:** Die relative Zeit (Mittelwerte  $\pm$  Standardfehler), welche die Fokusweibchen in Gegenwart eines männlichen Partners mit dem Fressen verbrachten. Werte  $< 0,50$  (gepunktete Linie) zeigen an, dass die Weibchen im Durchschnitt weniger Zeit mit dem männlichen Partner fraßen als während des Versuchsteils, bei dem ein weiblicher Partner zugegen war.

condition factor under natural conditions (TOBLER 2008). A recent study has demonstrated that cave molly males have even higher oxygen demands than females, i.e., males spent more time at the surface showing aquatic surface

respiration in sulfidic water (PLATH et al. 2007c, TOBLER et al. 2008c). Altogether, males should reduce metabolic expenses wherever possible, which—in the long run—may have selected for a genetic reduction of energy-demanding male

mating behavior (PLATH 2008, this study). Another study has shown that also male sperm production is reduced in *P. mexicana* males from sulfidic sites, and cave molly males have lighter testes (FRANSEN et al. 2008). It seems, therefore, that physiochemical stressors induce fundamental shifts in several behavioral and life-history traits, with relatively less energy allocation in reproduction, but relatively more investment in somatic maintenance (see also RIESCH et al. 2009).

## 4.2. Heterosis effect

An unexpected result was that  $F_1$  hybrids showed a higher sexual activity than males of the surface population. This is at odds with the predicted patterns of either intermediate or dominant inheritance under the assumption of an additive genetic mechanism. Intermediate expression of behavioral traits was documented for aggressive behavior: surface males were found to be highly aggressive, cave molly males showed reduced aggressive behavior, and the mean number of several aggressive behaviors ranged between those values in population hybrids (PARZEFALL 1974, 1979). Cave mollies have also reduced shoaling, probably because benefits of shoaling are lacking in the cave, since predatory birds and fish predators are absent, and increased food competition selects against shoaling (PLATH & SCHLUPP 2008). Again, hybrids from crosses of surface and cave mollies showed intermediate shoaling (PARZEFALL 2001).

A likely explanation for the observed increase of male sexual activity in the population hybrids is hybrid vigor (the heterosis effect). In other systems, so-called transgressive segregation in morphological characters is described for species hybrids, i.e. the expression of traits in hybrids is outside of the range of variability of the parental species (SEEHAUSEN 2004). Cave mollies are genetically distinct from surrounding populations residing in ecologically different habitat types, i.e., they have private alleles at several nuclear loci (PLATH et al. 2007a, TOBLER

et al. 2008b). Moreover, the genetic variability is reduced in cave mollies, which can be attributed to a smaller population size compared with surface populations (PLATH et al. 2007a). Under such circumstances, the combination of different alleles in  $F_1$  hybrids leads to high levels of heterozygosity.

## 4.3. Male harassment, female vigilance, and reduced female feeding time

It is important to note that we detected no overall effect of male sexual activity on female feeding rates ( $P = 0.86$ ; tab. 2b), but a significant difference among populations in the effect of the presence of a male on females' feeding rates was detected. This hints towards differences among populations not only in male mating activity (see above), but also the females' readiness and/or ability to avoid unwanted sexual attention.

Even though  $F_1$  hybrid males showed the highest sexual activity, females did not show a stronger feeding time reduction than surface females. Also when only the  $F_2$  animals were analyzed, no correlation between male sexual activity and female feeding time reduction was found (results not shown). These findings are congruent with the idea that male persistence traits and female resistance traits (i.e., behavioral mechanisms females employ to avoid male harassment) co-evolve (antagonistic sexual co-evolution: ARNQVIST & ROWE 2005), and seem to be inherited independently. In the cave molly, male sexual activity is low, and females seem to have lost vigilance behavior. In the  $F_1$  hybrids, female avoidance behavior seems to be lower than in females from the surface population (maybe due to intermediate inheritance), while male sexual behavior is increased likely due to a heterosis effect (see above). Disruption of the balance between male persistence traits and female resistance in the  $F_2$  hybrids may thus be the cause for the lack of a correlation between male sexual activity and an effect of male harassment on the females' feeding behavior, but this aspect certainly warrants further investigation.

#### 4.4. Male preference for large female body size

In female fish fecundity almost invariably covaries with body size (HELPMAN et al. 1997, WOOTON 1990). In the present study, an effect of female body size on male sexual activity (i.e., a positive correlation) was detected in the case of the cave molly, while in the surface form even a negative correlation was found. However, surface and cave molly males have been shown to have an intrinsic preference for large females in simultaneous association preference tests, where two different-sized females were presented to the choosing male (PLATH et al. 2006, 2008). Hence, the results from the present study suggest that large Río Oxolotan females were more apt to escape from male harassment than smaller females, and the escape abilities may be linked to the physical strength of a female. Consequently, male sexual behavior in our set-up reflects not only male mate choice for large female body size, but also female resistance to male harassment needs to be considered (see 4.3). Again, this result is congruent with the above-mentioned interpretation of antagonistic co-evolution between the sexes taking place in the Río Oxolotan population, but not in the cave molly (ARNQVIST & ROWE 2005, PLATH et al. 2007b).

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in this paper comply with the current laws on animal experimentation of Germany and the European Union, and the USA.

#### Literature

- ARNQVIST, G., & L. ROWE. 2005. Sexual conflict. Princeton University Press, Princeton.
- BAGARINAO, T. 1992. Sulfide as an environmental factor and toxicant: tolerance and adaptations in aquatic organisms. *Aquatic Toxicology* 24, 21-62.
- CLUTTON-BROCK, T., & G. PARKER. 1995. Sexual coercion in animal societies. *Animal Behaviour* 49, 1345-1365.
- CULVER, D. C., T. C. KANE, & D. W. FONG. 1995. Adaptation and natural selection in caves. Harvard University Press, Cambridge.
- DADDA, M., & A. BISAZZA. 2006. Lateralized female topminnows can forage and attend to a harassing male simultaneously. *Behavioral Ecology* 17, 358-363.
- FRANSSSEN, C., M. TOBLER, R. W. RIESCH, F. J. GARCIA DE LEON, R. TIEDEMANN, I. SCHLUPP, & M. PLATH. 2008. Sperm production in an extremophile fish, the cave molly (*Poecilia mexicana*, Poeciliidae, Teleostei). *Aquatic Ecology* 42, 685-692.
- GORDON, M. S., & D. E. ROSEN. 1962. A cavernicolous form of the Poeciliid fish *Poecilia sphenops* from Tabasco, México. *Copeia* 1962, 360-368.
- GRIESHABER, M. K., & S. VÖLKEL. 1998. Animal adaptations for tolerance and exploitation of poisonous sulfide. *Annual Reviews in Physiology* 60, 33-53.
- HELPMAN, G. S., B. B. COLLETTE, & D. E. FACEY. 1997. The diversity of fishes. Blackwell Science, Malden, Massachusetts.
- HOWARTH, F. G. 1993. High-stress subterranean habitats and evolutionary change in cave-inhabiting arthropods. *American Naturalist* 142, S65-S77.
- LANGHECKER, T. G. 2000. The effect of continuous darkness on cave ecology and cavernicolous evolution. In: WILKENS, H., D. C. CULVER, & W. F. HUMPHREYS, eds. of the world 30: Subterranean Ecosystems. Amsterdam: Elsevier Science.
- MAGURRAN, A. E. 2001. Sexual conflict and evolution in Trinidadian guppies. *Genetica* 112, 463-474.
- MAGURRAN, A. E., & B. H. SEGHERS. 1994. A cost of sexual harassment in the guppy, *Poecilia reticulata*. *Proceeding of the Royal Society London Series B: Biological Sciences* 258, 89-92.
- MILLER, P., J. GLASNER, & P. W. HEDRICK. 1993. Inbreeding depression and male-mating behavior in *Drosophila melanogaster*. *Genetica* 88, 29-36.

- MYLENE, M., J. L. KELLEY, R. BROOKS, & J. P. EVANS. 2006. The effects of inbreeding on male courtship behaviour and coloration in guppies. *Ethology* 112, 807-814.
- PARZEFALL, J. 1973. Attraction and sexual cycle of poeciliids. Pp. 177-183 in: SCHROEDER, J., ed. *Genetics and mutagenesis of fish*. Springer Verlag, Berlin.
- PARZEFALL, J. 1974. Rückbildung aggressiver Verhaltensweisen bei einer Höhlenform von *Poecilia sphenops* (Pisces, Poeciliidae). *Zeitschrift für Tierpsychologie* 35, 66-84.
- PARZEFALL, J. 1979. Genetics and biological significance of the aggressive behavior of *Poecilia sphenops* (Pisces, Poeciliidae): Studies on hybrids of epigeous and hypogeous populations. *Zeitschrift für Tierpsychologie* 50, 399-422.
- PARZEFALL, J. 2001. A review of morphological and behavioural changes in the cave molly, *Poecilia mexicana*, from Tabasco, Mexico. *Environmental Biology of Fishes* 62, 263-275.
- PETERS, N., G. PETERS, J. PARZEFALL, & H. WILKENS. 1973. Über degenerative und konstruktive Merkmale bei einer phylogenetisch jungen Höhlenform von *Poecilia sphenops* (Pisces, Poeciliidae). *Intentionale Revue der Gesamten Hydrobiologie* 58, 417-436.
- PILASTRO, A., S. BENETTON, & A. BISAZZA. 2003. Female aggregation and male competition reduce costs of sexual harassment in the mosquitofish *Gambusia holbrooki*. *Animal Behaviour* 65, 1161-1167.
- PLATH, M. 2008. Male mating behavior and costs of sexual harassment for females in cavernicolous and extremophile populations of Atlantic mollies (*Poecilia mexicana*). *Behaviour* 145, 73-98.
- PLATH, M., & M. TOBLER. 2009. The evolutionary ecology of the cave molly (*Poecilia mexicana*, Poeciliidae) from the Cueva del Azufre system. In: TRAJANO, E., E. M. BICHUETTE, & B. G. KAPOOR (eds.) *The Biology of Subterranean Fishes*, Science Publishers, Enfield, NH (in press).
- PLATH, M., & I. SCHLUPP. 2008. Parallel evolution leads to reduced shoaling behavior in two cave dwelling populations of Atlantic mollies (*Poecilia mexicana*, Poeciliidae, Teleostei). *Environmental Biology of Fishes* 82, 289-297.
- PLATH, M., J. PARZEFALL, & I. SCHLUPP. 2003. The role of sexual harassment in cave- and surface-dwelling populations of the Atlantic molly, *Poecilia mexicana* (Poeciliidae, Teleostei). *Behavioral Ecology and Sociobiology* 54, 303-309.
- PLATH, M., A. BRÜMMER, & I. SCHLUPP. 2004. Sexual harassment in a livebearing fish (*Poecilia mexicana*): influence of population-specific male mating behaviour. *Acta Ethologica* 7, 65-72.
- PLATH, M., U. SEGCEL, H. BURMEISTER, K. U. HEUBEL, & I. SCHLUPP. 2006. Choosy males from the underground: male mating preferences in surface- and cave-dwelling Atlantic mollies (*Poecilia mexicana*). *Naturwissenschaften* 93, 103-109.
- PLATH, M., S. J. HAUSWALDT, K. MOLL, M. TOBLER, F. J. GARCIA DE LEON, I. SCHLUPP, & R. TIEDEMANN. 2007a. Local adaptation and pronounced genetic differentiation in an extremophile fish, *Poecilia mexicana*, inhabiting a Mexican cave with toxic hydrogen sulfide. *Molecular Ecology* 16, 967-976.
- PLATH, M., A. MAKOWICZ, I. SCHLUPP, & M. TOBLER. 2007b. Sexual harassment in live-bearing fishes (Poeciliidae): comparing courting and noncourting species. *Behavioral Ecology* 18, 680-688.
- PLATH, M., M. TOBLER, F. J. GARCIA DE LEON, O. GIÈRE, & I. SCHLUPP. 2007c. Survival in an extreme habitat: the role of behaviour and energy limitation. *Naturwissenschaften* 94, 991-996.
- PLATH, M., S. RICHTER, R. TIEDEMANN, & I. SCHLUPP. 2008. Male fish deceive competitors about mating preferences. *Current Biology* 18, 1138-1141.
- RIESCH, R. W., M. TOBLER, M. PLATH, & I. SCHLUPP. 2009. Offspring number in a livebearing fish (*Poecilia mexicana*, Poeciliidae): reduced fecundity and reduced plasticity in a population of cave mollies. *Environmental Biology of Fishes* 84, 89-94.
- SCHLUPP, I., R. MCKNAB, & M. J. RYAN. 2001. Sexual harassment as a cost for molly females: Bigger males cost less. *Behaviour* 138, 277-286.
- SEEHAUSEN, O. 2004. Hybridization and adaptive radiation. *Trends in Ecology and Evolution* 19, 198-207.
- TOBLER, M. 2008. Divergence in trophic ecology characterizes colonization of extreme habitats. *Biological Journal of the Linnean Society* 95, 517-528.
- TOBLER, M., & M. PLATH. 2009. Living in extreme habitats. In: EVANS, J., A. PILASTRO, & I. SCHLUPP (eds.) *Ecology and Evolution of Livebearing Fishes (Poeciliidae)*. Chicago University Press, Chicago (in press).
- TOBLER, M., I. SCHLUPP, K. U. HEUBEL, R. W. RIESCH, F. J. GARCIA DE LEON, O. GIÈRE, & M. PLATH. 2006. Life on the edge: Hydrogen sulfide and the fish communities of a Mexican cave and surrounding waters. *Extremophiles* 10, 577-585.
- TOBLER, M., I. SCHLUPP, R. W. RIESCH, F. J. GARCIA DE LEON, & M. PLATH. 2008a. A new and morphologically distinct cavernicolous population

- of *Poecilia mexicana* (Poeciliidae, Teleostei). Environmental Biology of Fishes 82, 101-108.
- TOBLER M, T. J. DEWITT, I. SCHLUPP, F. J. GARCÍA DE LEÓN, R. HERRMANN, P. G. D. FEULNER, R. TIEDEMANN, & M. PLATH. 2008b. Toxic hydrogen sulfide and dark caves: phenotypic and genetic divergence across two abiotic environmental gradients in *Poecilia mexicana*. Evolution 62, 2643-2659.
- TOBLER M, C. FRANSSSEN, & M. PLATH. 2008c. Male-biased predation of a cave fish by a giant water bug. Naturwissenschaften 95, 775-779.
- WOOTON, R. 1990. Ecology of teleost fishes. Chapman & Hall, London.

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