



RESEARCH ARTICLE

REVISED Casanovas are liars: behavioral syndromes, sperm competition risk, and the evolution of deceptive male mating behavior in live-bearing fishes [v3; ref status: indexed, <http://f1000r.es/1zi>]

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Abstract

Male reproductive biology can be characterized through competition over mates as well as mate choice. Multiple mating and male mate choice copying, especially in internally fertilizing species, set the stage for increased sperm competition, i.e., sperm of two or more males can compete for fertilization of the female's ova. In the internally fertilizing fish *Poecilia mexicana*, males respond to the presence of rivals with reduced expression of mating preferences (audience effect), thereby lowering the risk of by-standing rivals copying their mate choice. Also, males interact initially more with a non-preferred female when observed by a rival, which has been interpreted in previous studies as a strategy to mislead rivals, again reducing sperm competition risk (SCR). Nevertheless, species might differ consistently in their expression of aggressive and reproductive behaviors, possibly due to varying levels of SCR. In the current study, we present a unique data set comprising ten poeciliid species (in two cases including multiple populations) and ask whether species can be characterized through consistent differences in the expression of aggression, sexual activity and changes in mate choice under increased SCR. We found consistent species-specific differences in aggressive behavior, sexual activity as well as in the level of misleading behavior, while decreased preference expression under increased SCR was a general feature of all but one species examined. Furthermore, mean sexual activity correlated positively with the occurrence of potentially misleading behavior. An alternative explanation for audience effects would be that males attempt to avoid aggressive encounters, which would predict stronger audience effects in more aggressive species. We demonstrate a positive correlation between mean aggressiveness and sexual activity (suggesting a hormonal link as a mechanistic explanation), but did not detect a correlation between aggressiveness and audience effects. Suites of correlated behavioral tendencies are termed behavioral syndromes, and our present study provides correlational evidence for the evolutionary significance of SCR in shaping a behavioral syndrome at the species level across poeciliid taxa.

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- 1 **Katja Heubel**, University of Tuebingen Germany
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We discuss in more detail whether sperm was actually transferred during the mating trials and why we find it reasonable to assume that average 'sexual activity' is a good proxy for sperm competition risk at the species level (even though we did not assess sperm competition directly). We further included a brief discussion as to the question of whether or not we can rule out avoidance of aggressive interactions as another factor explaining the evolution of deceptive mating behavior, as sexual activity correlates not only with deceptive mating behavior but also aggressiveness. We also propose future experimental approaches that may provide additional insights into those questions.

See referee reports

Introduction

Female mate choice and male competition are widely acknowledged as the principal forces of sexual selection^{1,2}, while male mate choice has received comparatively little attention (but see³⁻⁵). Over the past decades, however, it has become apparent that males also express mating preferences^{3,6-12}, especially if females show pronounced differences in mate quality (e.g., through size–fecundity relationships¹³). Nonetheless, male reproductive biology is clearly influenced by competition over mates^{1,14-16}, and, at least in species in which females tend to mate with multiple males, this competition extends well into the period after a successful copulation, as sperm of several males can compete for fertilization of the female's ova¹⁷⁻¹⁹. However, the level of male competition, male mate choice and behavioral responses to perceived sperm competition risk (SCR), may vary between taxa²⁰⁻²². An interesting group to study interspecific variation in male aggressive and reproductive behavior is the family Poeciliidae (livebearing fishes), which comprises at least 260 species²³. Several members of this family are model organisms for a range of topics in behavior, ecology and evolution²⁴. Nonetheless, comparative approaches in this group mostly considered morphological or physiological traits^{25,26}, while comparisons of behavioral traits are usually limited to population-level differences (guppy, *Poecilia reticulata*²⁷), or to a few species commonly used in scientific laboratories^{28,29} for exceptions see Dugatkin *et al.*³⁰, and Westneat *et al.*³¹. Our present study compared ten different species (13 populations) of poeciliid fishes and thus, provides comprehensive insights into potential interspecific variation in male aggressive and reproductive behavior within the family Poeciliidae. Beside aggressiveness and sexual activity, we particularly focused on the presumed role SCR plays for males of this family²².

Theory predicts that males should adjust their mating behavior strategically to imminent SCR^{19,32}, and several studies on species exhibiting frequent multiple mating confirm that perceived SCR affects male mate choice behavior^{10,11,18,33-35}. In the Atlantic molly, *Poecilia mexicana*, for instance, males temporarily decrease their sexual activity and cease showing mating preferences when another male is eavesdropping^{9,18,21,36,37}. It has been hypothesized that those audience-induced changes in male mating behavior prevent rivals from copying mate choice decisions^{19,32}. Moreover, males initially interact more with a previously non-preferred female in the presence of a rival, which has again been interpreted in the context of mate choice copying — and ultimately, SCR — as males could thus

lead the copying male away from the preferred mate (“deceptive mating behavior”^{21,36,38}).

Theoretical considerations identify avoidance of aggressive interactions as another potential mechanism explaining audience-induced changes in male mating behavior³². Specifically, if different males share intrinsic mating preferences (e.g., for large female body size^{8,21}), males could interact more equally with different females to reduce the risk of injuries resulting from aggressive interactions over commonly preferred female phenotypes³². If avoiding aggression plays a role, then the magnitude of audience-induced changes in male mating behavior (at the species level) should correlate positively with mean aggressiveness. To test this hypothesis, we examined the intensity of aggressive interactions in size-matched dyadic (paired) male combats for the set of poeciliid species included herein and in an independent approach quantified audience-induced changes of male mate choice in response to an audience (see above) for the same taxa.

Consistency in the expression of a certain behavioral type across different environmental contexts at the inter-*individual* level has received considerable scientific interest³⁹⁻⁴², and suites of correlated behavioral types have been termed behavioral syndromes^{39,43}. Réale *et al.*⁴⁴ proposed five different axes of animal personality: shyness–boldness, exploration–avoidance, general activity, aggressiveness, and sociability. Conrad *et al.*⁴³ highlighted several correlations of those behavioral axes in teleost fishes, but audience-induced changes in male mating behavior have not yet been investigated in the context of behavioral syndromes. Recent studies exemplified the importance of population differences in behavioral syndromes^{45,46}, and the concept of behavioral syndromes was expanded to the comparison of groups of animals or populations. Chapman *et al.*⁴⁷, for example, demonstrated correlations between mean colony (and caste) behavioral types in *Myrmica* ants. Here, we apply this concept to the comparison of different poeciliid taxa, thus evaluating species-specific behavioral types.

In summary, we assembled a unique data-set comprising ten different poeciliid species (in some cases, several sub-species or ecotypes, or multiple populations) and sought for variation at the taxon level (“species-specific behavioral types”) in (1) audience-induced changes in male mate choice, (2) deceptive male mating behavior, (3) sexual activity (previously published, re-analyzed own data, see Table 1), and (4) aggressiveness (newly generated data as well as previously published own data, Table 1). We tested for correlations of these behavioral tendencies, *i.e.*, we asked whether there are behavioral syndromes at the taxon level.

Methods

Study organisms and their maintenance

The experiments reported here comply with the current laws of Germany (approved by Regierungspräsidium Darmstadt V-54-19c-20/15-F104/Anz.18) and the USA (approved by the Institutional Animal Care and Use Committee of the University of Oklahoma; AUS-IACUC approved protocols: R06-026 and R09-023).

Test subjects were lab-reared descendants of wild-caught fish. We included Atlantic mollies from the coastal lagoons around the

Table 1. The mean (\pm SE) standard length (SL [mm]) of the test fish used in the experiments examining (a) male aggressiveness and (b) male sexual behavior and mate choice. In (a) SL differences between the two opponents are given along with the results from paired *t*-tests comparing winner and loser SL after dominance was established. In (b) N_{audience} indicates the number of trials with an audience presented during the second part. * indicates species imported by "Aquarium Dietzenbach GmbH".

(a) Aggressive behavior	N_{dyads}	Dyad SL	SL difference	<i>t</i>	<i>df</i>	<i>P</i>	Source
<i>G. sexradiata</i>	8	18.6 \pm 0.7	1.3 \pm 0.3	1.09	5	0.33	this study
<i>H. milleri</i>	14	22.5 \pm 0.5	1.2 \pm 0.2	1.41	3	0.25	this study
<i>P. reticulata</i> (feral)	8	16.2 \pm 0.8	1.1 \pm 0.2	0.58	3	0.60	this study
<i>P. reticulata</i> *	11	22.5 \pm 0.7	1.2 \pm 0.3	0.00	6	1.00	this study
<i>P. picta</i> *	9	23.3 \pm 0.6	2.3 \pm 0.4	0.36	6	0.73	this study
<i>L. tridens</i> *	18	24.8 \pm 0.8	1.5 \pm 0.2	0.37	7	0.72	this study
<i>L. sulphurophila</i>	12	32.7 \pm 1.1	1.9 \pm 0.4	2.11	11	0.58	this study
<i>P. latipinna</i>	9	43.6 \pm 2.8	2.2 \pm 0.4	0.01	5	1.00	this study
<i>P. latipunctata</i>	9	25.3 \pm 1.3	1.4 \pm 0.3	1.57	3	0.22	this study
<i>P. orri</i>	9	33.1 \pm 0.7	2.0 \pm 0.4	1.20	8	0.27	this study
<i>P. m. limantouri</i>	12	37.1 \pm 1.8	2.7 \pm 0.4	1.01	10	0.30	[51]
<i>P. m. mexicana</i> (sulfide)	9	28.7 \pm 0.9	2.0 \pm 0.4	2.05	8	0.12	[51]
<i>P. m. mexicana</i>	18	35.7 \pm 1.3	1.4 \pm 0.3	1.06	15	0.27	[51]
(b) Male mating behavior	N_{trials}	Focal male SL	Large female SL	Small female SL	N_{audience}	Audience male SL	Source
<i>G. sexradiata</i>	20	21.4 \pm 0.6	37.9 \pm 0.7	30.3 \pm 0.6	10	21.7 \pm 0.6	[42]
<i>H. milleri</i>	25	22.4 \pm 0.5	33.3 \pm 0.5	25.0 \pm 0.7	14	21.4 \pm 0.5	[58]
<i>P. reticulata</i> (feral)	32	14.9 \pm 0.2	19.3 \pm 0.4	14.8 \pm 0.2	16	14.9 \pm 0.2	[55]
<i>P. reticulata</i> *	47	21.8 \pm 0.4	33.4 \pm 1.1	24.2 \pm 1.0	25	21.2 \pm 0.5	[42]
<i>P. picta</i> *	43	23.0 \pm 0.2	34.0 \pm 0.8	26.7 \pm 0.6	26	22.6 \pm 0.4	[42]
<i>L. tridens</i> *	46	23.6 \pm 0.3	30.2 \pm 0.9	25.6 \pm 0.2	23	22.6 \pm 0.4	[42]
<i>L. sulphurophila</i>	28	31.0 \pm 0.7	38.6 \pm 1.0	31.6 \pm 0.8	14	32.2 \pm 0.8	[42]
<i>P. latipinna</i>	31	36.4 \pm 1.0	45.4 \pm 0.5	33.8 \pm 0.8	18	35.2 \pm 1.0	[42]
<i>P. latipunctata</i>	21	25.9 \pm 0.8	35.0 \pm 0.5	27.6 \pm 0.5	11	25.5 \pm 0.8	[42]
<i>P. orri</i>	18	32.2 \pm 0.8	37.8 \pm 0.8	32.1 \pm 0.7	9	32.4 \pm 1.0	[42]
<i>P. m. limantouri</i>	36	34.0 \pm 0.9	49.9 \pm 0.4	33.8 \pm 0.6	18	35.8 \pm 1.0	[41]
<i>P. m. mexicana</i> (sulfide)	22	29.0 \pm 0.6	47.6 \pm 1.3	35.3 \pm 0.6	11	30.2 \pm 0.7	[42]
<i>P. m. mexicana</i>	39	32.5 \pm 1.0	47.4 \pm 0.8	37.4 \pm 0.8	19	35.2 \pm 1.3	[42]

Central Mexican city of Tampico (belonging to the subspecies *P. mexicana limantouri*); another population was collected in the Río Oxolotan in Tabasco, South México (*P. mexicana mexicana*). Recent phylogenetic analyses argue in favor of full species status of the two subspecies⁴⁸. We further included a locally adapted and genetically differentiated (*i.e.*, independently evolving) ecotype from the *P. mexicana mexicana* clade: the hydrogen sulfide-adapted form inhabiting El Azufre, a tributary to the Río Oxolotan^{49,50}. As another representative of short-fin mollies^{23,51} we included mangrove mollies (*P. orri*) from Roatán Island, Honduras. Two species of long-fin mollies were tested: sailfin mollies (*P. latipinna*) stemmed from the Comal River in Central Texas, USA, while Tamésí mollies (*P. latipunctata*) were collected near Ciudad Mante in Tamaulipas, México. We further included guppies (*P. reticulata*) from Venezuela and a feral population from the San Antonio River, Texas, USA⁵²,

as well as Venezuelan swamp guppies (*P. picta*). As representatives of the genus *Limia*, we included *L. tridens* and sulfur limia (*L. sulphurophila*), both originating from the Dominican Republic. *Gambusia sexradiata* from the Río Teapa, and Grijalva mosquitofish (*Heterophallus milleri*) from the Río Oxolotán (both Tabasco, México) were included as representatives of mosquito fishes.

Test fish came from large, randomly outbred single-species stocks maintained at the Department of Ecology and Evolution of the University of Frankfurt (*P. m. mexicana*, *P. m. limantouri*, *P. reticulata* from Venezuela, *P. picta*, *L. tridens*), or at the Department of Zoology at the University of Oklahoma in Norman (*P. m. mexicana* from El Azufre, *P. latipinna*, *P. latipunctata*, *P. orri*, feral *P. reticulata*, *L. sulphurophila*, *G. sexradiata*, *H. milleri*; Table 1). Fish were reared as single-species, mixed-sex stocks in 200-l (Frankfurt) or

1,000-l (Norman) tanks at 25–27°C under an 12:12 hours light:dark cycle (Frankfurt) or under ambient light conditions in a greenhouse (Norman). At the University of Frankfurt, fish were fed twice daily *ad libitum* with commercial flake food. Stock tanks in Norman contained naturally growing algae as well as a variety of naturally occurring invertebrates such as chironomid larvae, copepods and amphipods, on which the fish could feed. In addition, fish were supplied with flake food every two days. However, at least 1 week prior to the behavioral experiments, fish were fed *ad libitum* at least once daily with flake food.

Experimental design

Aggressive behavior

We determined male aggressive behaviors during dyadic encounters by analyzing contests staged between pairs of males in a small test tank measuring 30 × 20 × 20 cm⁵³. To avoid confounding effects of previously established dominance and/or familiarity^{54,55}, males were taken from different stock tanks. Males in a dyad differed by less than 15% in standard length (SL), which has previously been established as the threshold below which fights typically escalate⁵³; nevertheless, size difference was included as a covariate in the statistical analyses (see below). We separated males by an opaque filter sponge while three sides of the test tank were taped with gray paper to minimize disturbances from the outside. The bottom of the tank was filled with black gravel, and water was aerated and maintained at 27–29°C. Males could habituate to the test tank overnight, and observations took place the next day between 09:00 and 13:00. To initiate a trial, the sponge divider was gently lifted, and we noted behavioral interactions for a maximum of 10 minutes, starting with the first interaction. We focused on three frequent aggressive behaviors^{56,57}: (1) S-position: this threat display usually initiates a fight. Males swim in a parallel or anti-parallel position and bend their bodies in an S-shaped manner with all unpaired fins erect; (2) tail-beats: S-positions are often followed or superimposed by tail-beats, which are fast movements of head and tail in opposing directions that either touch the opponent's body or send shock waves to the opponent; and (3) bites – we defined all incidences of ramming and bite-like attacks as bites, because both these behaviors occur extremely quickly and thus are indistinguishable to the human eye. For some species examined in this study no formal description of aggressive behavior was available from the literature, and so we confirmed in pre-trials that the aforementioned behaviors are part of their behavioral repertoire.

We also recorded fight duration until dominance was established. Contest outcome could be inferred from behavioral differences between the contestants. Folded fins, head-down posture and a position at the periphery of the tank typically characterize contest losers, while winners constantly chase and further attack the loser with fins fully erect, occasionally performing S-positions or bites⁵³. We met all requirements for animal well-being in behavioral experiments; apart from the occasional loss of single scales, no severe injuries were observed, as we separated males immediately once dominance was established. If no dominance was established within 10 minutes of the first interaction, we terminated the fight; those trials were discarded from the analysis of fighting durations ($N = 52$ cases discarded), while fight durations were scored as "0" when no aggressive behavior occurred at all (those trials were

terminated after a total of 15 minutes of observation). SL of both contestants was taken after a contest by laying the fish flat on plastic foil-covered millimeter paper (Table 1). Afterwards we transferred males back to their respective stock tanks. In total, we successfully completed $N = 146$ trials (Table 1).

Male mate choice

We reanalyzed previously published data on audience-induced changes in male mate choice (Table 1). Focal males were isolated in 25- to 38-l tanks for two to four days prior to the tests to ensure that they were motivated to mate¹². We tested each focal male only once; however, owing to the limited number of males available from our stocks, some males were also used as audience males after they had served as a focal male, but never on the same day and not in the same dyadic constellation. As familiarity among males affects the strength of audience effects in *P. mexicana*⁹, focal and audience males were taken from different stock tanks.

Each focal male was tested for its mating preference in a binary choice situation and was then retested with the same stimulus females either without audience (control treatment) or with an audience male present (50% of trials each). We were thus able to examine changes in focal males' behavior from the first to the second part of the tests and could discern between effects induced by the audience and changes that would occur over the course of the experiment even without audience. In theory, we could have used an alternative design of presenting an audience in all trials while starting the tests with or without audience in alternating order; however, in such a design, prior exposure to the audience male (when presented during the first part) could still affect the focal males' behavior during the second part of the tests⁵⁸.

The test tank (50 × 30 × 30 cm, length × width × height) was filled to 20 cm height with aged tap water. Water temperature was maintained at 27–28°C using an aquarium heater. In addition, the water was aerated between trials, but both the heater and the airstone were removed for all trials. Black plastic covered all sides except the front. Prior to the tests, we choose two different-sized stimulus females (for SL see Table 1) from a stock tank and introduced them into the test tank. Poeciliid males prefer to mate with larger, more fecund females (*e.g.*,^{8,59–61}, but see Baerends *et al.*⁶²). Afterwards, we introduced a focal male into a transparent Plexiglas cylinder (10 cm diameter) located in the center of the tank and left the fish undisturbed for 5 minutes. After the habituation period, we gently lifted the cylinder. During a 10-min observation period, we scored male sexual behaviors directed toward either of the two females and noted with which female the focal male interacted with first. We decided *a priori* to terminate trials if the male did not show any sexual behavior during the first part of the test; $N = 3$ trials with *P. orri*, $N = 5$ (*P. latipinna*), $N = 2$ (*P. latipunctata*), $N = 4$ (*P. reticulata*, Venezuela), $N = 1$ (*P. picta*), $N = 1$ (*P. reticulata*, San Antonio), and $N = 6$ (*H. milleri*) were discarded from the statistical analyses based on this criterion.

Genital nipping is a typical pre-copulatory behavior in poeciliids, whereby the male approaches the female from behind and touches her genital region with his snout^{30,56}. During thrusting, males swing their gonopodium forward while attempting to introduce it into the

female's gonopore. However, in most poeciliids it is not possible to discriminate with certainty between a successful mating (defined as a mating with sperm being transferred) and the pure mating attempt. Courtship behavior is absent in *P. mexicana*³⁰, *P. orri*, the examined *Limia* species (authors, personal observation) and *Gambusia* spp. (⁶³ for *G. holbrooki*). *Poecilia reticulata* males court in front of females in an S-shaped body posture (sigmoid displays^{64,65}), while the primary courtship display of *P. picta* males consists of circling around the female (the so-called 'orbit'^{56,65}), but males also court with their fins raised in front of the female (⁶⁵; D.B., personal observation). *Heterophallus milleri* males circle around the female and swing their gonopodium forward when in the female's visual field⁶¹. Large *P. latipinna* and *P. latipunctata* males occasionally court in front of females with raised dorsal fins^{56,66}. As not all species examined herein show courtship displays and courtship was by far the least frequent behavioral category, we excluded numbers of courtship displays from our main analyses.

Upon completion of the first preference test, we immediately repeated measurement of male mating preferences, but in one half of the trials, an audience male was presented, while the other half of the trials was repeated without audience (control). To initiate this second part of a trial, we reintroduced the focal male into the acclimatization cylinder. An audience male was placed in another transparent cylinder in the central back of the tank, while for the control only an empty cylinder was presented. The audience male was confined in his cylinder throughout the test. After another 5 minutes of habituation (during which all four fish could interact visually), measurement of male preferences was repeated, as described above. Interactions between males were not quantified, but aggressive displays were not observed. In total, we successfully completed $N = 408$ trials (Table 1). Once a trial was completed, all fish were measured for SL to the closest millimeter (Table 1).

Statistical analyses

First, we asked whether species show consistent variation in the behavioral traits examined in this study (on the individual level often referred to as "character" or "behavioral type", e.g.,⁴²). In analogy to individual-level analyses of behavioral consistency (where each individual is tested repeatedly), our species-level analysis defined each tested individual as a repeated measure of the subject 'species'. We used univariate mixed models (MM) in which we treated the mean of each behavioral trait as a fixed effect and included random intercepts for each species. This approach was recently recommended to decompose phenotypic variance into a within-subjects variance component (i.e., the variance around the species-specific intercept) and a between-subjects variance component (i.e., the variance between species-specific intercepts)⁶⁷. Consistent differences among species – species-specific 'behavioral types' – for a given behavioral trait can be inferred when the between-subjects variance component significantly differs from zero. Based on the variance decomposition through MMs, we furthermore calculated a metric for the repeatability of each behavioral trait, i.e., the proportion of the total variance accounted for by differences among species (*sensu*⁶⁸):

$$R = \frac{\text{Variance (between species)}}{\text{Variance (between species) + Variance (within species)}}$$

The three members of the *Poecilia mexicana* species-complex used in our study clearly represent three phylogenetically independent groups (two sub-species and one derived ecotype⁴⁸) and, thus, were treated statistically as independent species. However, this was not the case for the two populations of the guppy (*P. reticulata*) and so we re-ran all analyses without data from the feral guppy population (San Antonio), but this did not alter the direction of the results (not shown).

We then proceeded to ask whether the different behavioral traits are correlated among species (i.e., if behavioral syndromes can be inferred;³⁹). To this end, we calculated pair-wise non-parametric Spearman's rank correlations with species means for all behavioral traits. We are aware of other methods to test for a syndrome structure, namely, multivariate MMs⁶⁷, but based on our limited sample size of $N = 13$ independent subjects (species/populations) we decided to use non-parametric tests instead (which is also an accepted technique, see⁶⁹).

We depict mean values (\pm standard error) of the investigated behaviors for all species examined.

Aggressive behavior

In order to compare variation in aggressive behavior across species, we employed Principal Component Analysis (PCA) to reduce the number of dependent variables (numbers of S-positions, tail-beats and bites per male dyad) and extracted one independent component (PC1; eigenvalue = 2.47) that explained 82.3% of the variance. The three aggressive behaviors had axis loadings of 0.85 (S-positions), 0.93 (tail-beats) and 0.94 (bites). PC1 was checked for normal distribution using a Kolmogorov-Smirnov test and used as dependent variable in a linear mixed model (LMM, 'mixed' procedure in SPSS 21) with species-specific random intercepts (see above). To test whether the variance between intercepts differed significantly from zero (thus indicating consistent differences between species in aggressive behavior) we compared a model with random intercepts to a reduced model without random intercepts via likelihood ratio tests. Male body size may influence aggressiveness⁷⁰, and this could affect apparent between-species effects (with larger species being more aggressive than smaller ones) as well as within-species effects (larger males within a given species can be more aggressive than smaller ones). However, the within-species effect of body size can also vary between species (when larger males are more aggressive than smaller ones in one species but not in another). To separate within- from between-species effects, we followed the "within-subject centering" approach proposed by van de Pol and Wright⁷¹ and included species means for the mean SL of a dyad (termed 'between-species dyad SL') as well as each dyad's deviation from the respective species mean (termed 'within-species dyad SL') as fixed covariates in our model. To test whether the within-species effect of mean dyad SL differed between species, we included random slopes of 'within-species dyad SL' for each species in our model and tested for slope heterogeneity through likelihood ratio tests (model with random slopes vs. model without random slopes, see⁶⁷). Furthermore, the opponents' body size difference influences fight intensity⁵³, which again can be a species-specific trait. As our experimental setup largely prevented between-species variation in 'opponent body size difference' as we had chosen pairs of males that differed by less

than 15% in SL, we were interested in whether fights with smaller SL differences between both opponents were more intense than fights with larger differences and thus included ‘opponent body size difference’ (arcsine (square root)-transformed $SL_{\text{small}}/SL_{\text{large}}$) as a fixed covariate. To test whether there were between-species differences in the effect of ‘opponent body size difference’ we again included species-specific random slopes and tested for slope heterogeneity using likelihood ratio tests. Non-significant fixed effects and random slopes were excluded from the final model. We, thus, excluded the covariates ‘between-species dyad SL’ (estimated slope: 0.013 ± 0.021 ; $F_{1,111.24}=0.37$; $P=0.54$), ‘within-species dyad SL’ (estimated slope: -0.039 ± 0.190 ; $F_{1,119.9}=4.00$; $P=0.12$) and random slopes for ‘within-species dyad SL’ (estimated variance: 0.003 ± 0.003 ; $P=0.12$) from the final model.

Repeatability was calculated based on the final model; as we retained the covariate ‘opponent body size difference’ as a fixed effect with random slopes for each species in the model, our measure of repeatability represented conditional repeatability⁶⁷ where opponent body size difference equals zero.

Fight durations (log-transformed prior to the analysis to approach normal error distribution) were analyzed in a similar LMM. All three covariates and random slopes were removed from the final model since none was significant (‘between-species dyad SL’, estimated slope: -0.017 ± 0.021 ; $F_{1,111.1}=0.66$, $P=0.434$; ‘within-species dyad SL’, estimated slope: -0.027 ± 0.031 ; $F_{1,2.1}=0.76$, $P=0.47$; random slopes for ‘within-species dyad SL’, estimated variance: 0.003 ± 0.007 ; $P=0.58$; ‘opponent body size difference’, estimated slope: -1.435 ± 0.897 ; $F_{1,61.3}=2.55$, $P=0.11$; random slopes for ‘opponent body size difference’, estimated variance: 0.408 ± 2.241 ; $P=0.84$). Repeatability was calculated as described before.

Male sexual behavior

As a measure of sexual activity we used numbers of sexual behaviors directed to both stimulus females in the first part of a mate choice trial (without audience male). As described for the analysis of aggressive behavior, we used PCA to condense sexual behavior (genital nipping and thrusting) to one principle component (PC1, eigenvalue = 1.79) that explained 89.7% of the total variance. Both variables had equal axis loadings of 0.95. We used PC1 (checked for normal distribution by means of a Kolmogorov-Smirnov test) as dependent variable in a LMM (see above). Small males show more sexual behaviors than larger ones in at least some of the species examined here as part of a ‘sneak-like’ alternative mating strategy⁷², so we included species-wise means for focal males’ SL (‘between-species focal SL’) as well as each focal male’s SL deviation from the species mean (‘within-species focal SL’) as fixed covariates. As described for aggressive behavior, we included species-specific random slopes for the within-species covariate to test for between-species differences in the relation between sexual activity and focal males’ body size. Also, poeciliid males typically prefer to mate with large females, and so we included the SL difference of each stimulus female dyad [arcsine (square root)-transformed $SL_{\text{small}}/SL_{\text{large}}$] as another fixed effect covariate and accounted for potential between-species differences by including random slopes. However, all three covariates and the random slopes had no significant effect (‘between-species focal SL’, estimated slope: 0.049 ± 0.035 ; $F_{1,110.9}=1.94$,

$P=0.19$; ‘within-species focal SL’, estimated slope: 0.017 ± 0.010 ; $F_{1,393.9}=2.94$, $P=0.087$; random slopes for ‘within-species focal SL’, estimated variance: 0.002 ± 0.010 ; $P=0.67$; ‘stimulus SL difference’, estimated slope: 0.083 ± 0.380 ; $F_{1,398.1}=0.05$, $P=0.83$; random slopes for ‘stimulus SL difference’, estimated variance: 0.002 ± 0.007 ; $P=0.72$), and were removed from the final model. Repeatability was calculated based on the final model as described for aggressiveness.

Audience-induced changes in preference expression

To compare the magnitude of audience-induced changes in individual male mate choice behavior across species, we calculated a preference score³⁶ as:

(fraction of sexual behaviors with the initially preferred female during the second part of a trial) – (fraction of sexual behaviors with the same female during the first part),

such that negative values would indicate that individual preferences decreased. We analyzed scores as dependent variable in a LMM with species-specific random intercepts and ‘treatment’ as another random factor. ‘Treatment’ was also used as a fixed factor such that we could evaluate first whether there was an overall treatment effect on the dependent variable and secondly decompose the variance into treatment-specific between- and within-species components. Again, focal male body size as well as stimulus size difference could have influenced preference expression and so we initially included ‘between-species focal SL’, ‘within-species focal SL’ and ‘stimulus SL difference’ as fixed covariates (and random slopes for the latter two) but removed them from the final model as none had a significant effect (‘between-species focal SL’, estimated slope: 0.004 ± 0.010 , $F_{1,110.9}=1.62$, $P=0.22$; ‘within-species focal SL’, estimated slope: -0.001 ± 0.004 , $F_{1,347.3}=0.16$, $P=0.68$; random slopes for ‘within-species focal SL’, estimated variance: -0.007 ± 0.012 , $P=0.27$; ‘stimulus SL difference’, estimated slope: 0.136 ± 0.130 , $F_{1,308.1}=1.09$, $P=0.30$; random slopes for ‘stimulus SL difference’, estimated variance: -0.003 ± 0.004 , $P=0.41$). Repeatability was calculated for both treatments separately, as described above.

Deceptive mating behavior

The first sexual approach of focal males is assumed to be another indicator of male preference³⁶. We sought to corroborate this assertion and thus, tested whether males on average interacted more with the females they approached first in the first part of our tests. In all species most males first approached the female they also interacted most often with during the entire first preference test (in 76–100% of trials those females approached first also received the majority of sexual behaviors; *chi*²-tests significant for all species, results not shown). In the context of deceptive mating behavior, the first sexual approach of focal males is of interest as interacting first with the previously non-preferred female has been interpreted as an attempt to mislead the rival³⁶. Thus, we analyzed the fraction of males that first interacted with the opposite (“1”) or same female during the second part (“0”) using a Generalized Linear Mixed Model (GLMM) with a binary error distribution and a logit-link function. As described for the LMMs analyzing audience-induced changes in mating preferences, ‘species ID’ was used as a grouping variable in combination with ‘treatment’, while ‘treatment’

also served as a fixed factor. We initially included ‘between-species focal SL’, ‘within-species focal SL’ and ‘stimulus SL difference’ as fixed covariates but removed them from the final model as they had no significant effects (‘between-species focal SL, estimated slope: -0.005 ± 0.022 , $F_{1,398}=0.052$, $P=0.82$; within-species focal SL’, estimated slope: 0.078 ± 0.560 , $F_{1,398}=0.044$, $P=0.89$; ‘stimulus SL difference’, estimated slope: 0.888 ± 1.157 ; $F_{1,399}=1.53$, $P=0.22$). It was not possible to fit random slopes in the GLMM model, but as neither covariate had a significant effect, differences between species likely can be neglected. Repeatability was calculated for each treatment separately based on the variances obtained from the final model, and thus represents link-scale repeatability⁶⁹.

Correlations of behavioral types at the species level

The central question of our present paper was whether there are correlations between the aforementioned behaviors at the species level. Owing to the limited sample size ($N = 13$ groups), we used non-parametric, pair-wise Spearman’s rank order tests to correlate species means for (1) aggressiveness (log(sum of aggressive interactions per fight)), (2) fight duration (log(time)), (3) sexual activity (sum of nipping and thrusting behavior during the first part of the tests), (4) consistency in preference expression without an audience (preference score), (5) the strength of changes in preference expression when an audience male was presented (preference score), (6) consistency in first approached females without an audience male presented (fraction of males that changed their first interaction without audience present), (7) deceptive male mating behavior (fraction of males that changed their first interaction in the audience treatment). We are aware of a possible error inflation due to multiple comparisons, but did not use alpha-corrections (such as Bonferroni) since the investigated behaviors were not independent. To further show the intercorrelative character of the investigated behaviors, we condensed them through PCA and extracted two principle components with Eigenvalues above 1 (Eigenvalues:

PC1=2.49; PC2=1.92) that explained 35.5% and 27.6% of the total variation, respectively. The principle components were varimax-rotated for better interpretation.

Results

Male aggressive behavior

There was significant between-species variation in aggressiveness (Table 2a) indicating that some species are consistently more aggressive than others (Figure 1a). On average, the amount of aggressive behaviors decreased with increasing size-difference between the opponents even though this effect was not significant when random slopes for each species were included (fixed covariate ‘opponent body size difference’: estimated slope: -1.492 ± 1.249 , $F_{1,12,9}=1.60$, $P=0.23$). Nevertheless, species-specific random slopes differed significantly between species (variance estimate: 13.020 ± 6.923 ; $P<0.001$) and were negatively correlated with the species-specific random intercepts ($r_{\text{intercept-slope}}=-0.95$, $P<0.001$) indicating that highly aggressive species reduced aggressive behavior more when opponent SL difference increased than less aggressive species. The repeatability value — by inclusion of random slopes for opponents’ body size difference representing the conditional between-species variance at an extrapolated opponent body size difference of zero — was relatively high at 0.71 (Table 2a).

When analyzing fight durations, we again found significant variation between species (Table 2a, Figure 1b), while repeatability was much lower than for numbers of aggressive behavior (Table 2a).

Male sexual behavior

There was pronounced variation among species in male sexual activity (Table 2c) with some species (especially Atlantic mollies) being far more active than others (Figure 2a). Repeatability for sexual activity was comparably high as for aggressive behavior (Table 2b).

Table 2. Results from mixed models (LMM, linear mixed model; GLMM, generalized linear mixed model) analyzing. (a) aggression in dyadic male fights, (b) male sexual behavior in dichotomous choice tests, (c) changes in male preference expression with or without an audience male presented, and (d) deceptive male mating behavior with or without an audience male present. The between- (Var_{between}) and within-species variances (Var_{within}), and the associated repeatability values (R) are shown. Significant between-species variances (P -values obtained from likelihood ratio tests) are given in bold typeface.

Test	Dependent	Var_{between}	P	Var_{within}	R
<i>(a) Aggressive behaviors (LMM)</i>					
Number of aggressive behaviors	PC1	1.38	< 0.001	0.57	0.71
Fight duration	ln (duration)	0.19	0.008	0.74	0.20
<i>(b) Sexual activity (LMM)</i>					
Number of sexual behaviors 1 st part	PC1	0.64	< 0.001	0.51	0.55
<i>(c) Change in mating preference (LMM)</i>					
Without audience	Preference score	0.003	0.24	0.055	0.048
With audience	Preference score	0.006	0.19	0.085	0.065
<i>(d) Deception (GLMM)</i>					
Without audience	Number of males that changed 1 st interaction	0.21	0.10	0.960	0.18
With audience	Number of males that changed 1st interaction	0.58	0.002	0.95	0.38

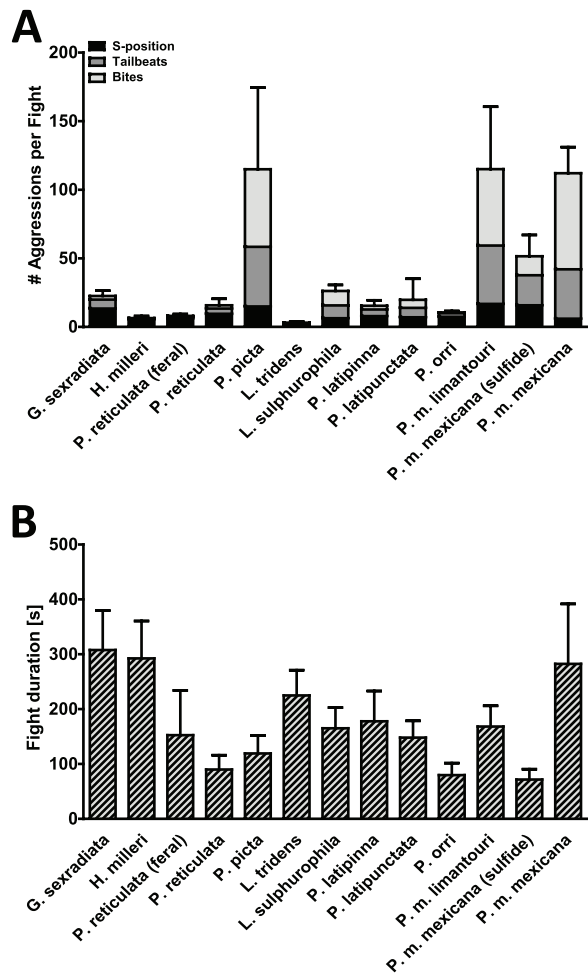


Figure 1. Means (\pm SE) of (a) numbers of aggressive interactions per male fight and (b) fight duration in the different poeciliid species examined.

Audience-induced changes in preference expression

When comparing the change in individual males' mating preferences from the first to second part of the tests (preference score), we detected no significant between-species variation — both with and without an audience male presented (Table 2c). The fixed factor 'treatment' had a significant effect ($F_{1,21.5}=6.87, P=0.016$) indicating that preference scores, overall, differed in response to whether or not an audience male was presented. All species except *H. milleri* showed similar responses: males were consistent in their mate choice behavior when no audience male was presented and decreasing preferences when observed by an audience (Figure 2b).

Deceptive mating behavior

Our GLMM did not detect significant between-species variation in fractions of males that changed the initially preferred female from the first to the second part of the mate choice tests when no audience was presented (Table 2d). In other words: species were similarly consistent in their preferences in the control treatment. In the treatment where an audience male was presented during the second part of the test, we found significant between-species variance, along with a comparably high repeatability value (Table 2d). The fixed factor 'treatment' was significant in our final model, indicating that males were generally more likely to interact with the opposite species in the treatment involving an audience male (Figure 2c).

Correlations of behavioral types at the species level

In line with our prediction derived from the interpretation that SCR explains the occurrence of audience-induced behavioral changes, we found a strong, positive correlation between sexual activity and the amount of deceptive behavior at the species level (Figure 3a). The alternative prediction, that avoidance of aggressive behavior drives audience effects (leading to positive correlations between the degree of preference change and aggressiveness as well as between deceptive behavior and aggressiveness), received no support (not statistically significant; Table 3). However, there was also a significant positive correlation between the amount of aggressive behavior and sexual activity (Figure 3b).

Table 3. Pair-wise Spearman's rank order correlations between species-wise mean values for all seven behaviors investigated. An asterisk indicates significant correlations.

Behavior	Aggressiveness	Fight duration	Change in preference (without audience)	Change in preference (with audience)	Sexual activity	Change in first interaction (without audience)	Change in first interaction (with audience)
Aggressiveness	-	$r_s=-0.21$; $P=0.48$	$r_s=0.07$; $P=0.82$	$r_s=-0.31$; $P=0.31$	$r_s=0.61$; $P=0.027^*$	$r_s=-0.05$; $P=0.88$	$r_s=0.36$; $P=0.23$
Fight duration		-	$r_s=-0.22$; $P=0.47$	$r_s=0.15$; $P=0.62$	$r_s=-0.33$; $P=0.27$	$r_s=0.41$; $P=0.16$	$r_s=-0.28$; $P=0.36$
Change in preference (without audience)			-	$r_s=0.19$; $P=0.54$	$r_s=0.13$; $P=0.68$	$r_s=-0.35$; $P=0.24$	$r_s=-0.34$; $P=0.26$
Change in preference (with audience)				-	$r_s=-0.08$; $P=0.79$	$r_s=-0.18$; $P=0.57$	$r_s=-0.41$; $P=0.16$
Sexual activity					-	$r_s=-0.14$; $P=0.65$	$r_s=0.67$; $P=0.013^*$
Change in first interaction (without audience)						-	$r_s=-0.06$; $P=0.84$
Change in first interaction (with audience)							-

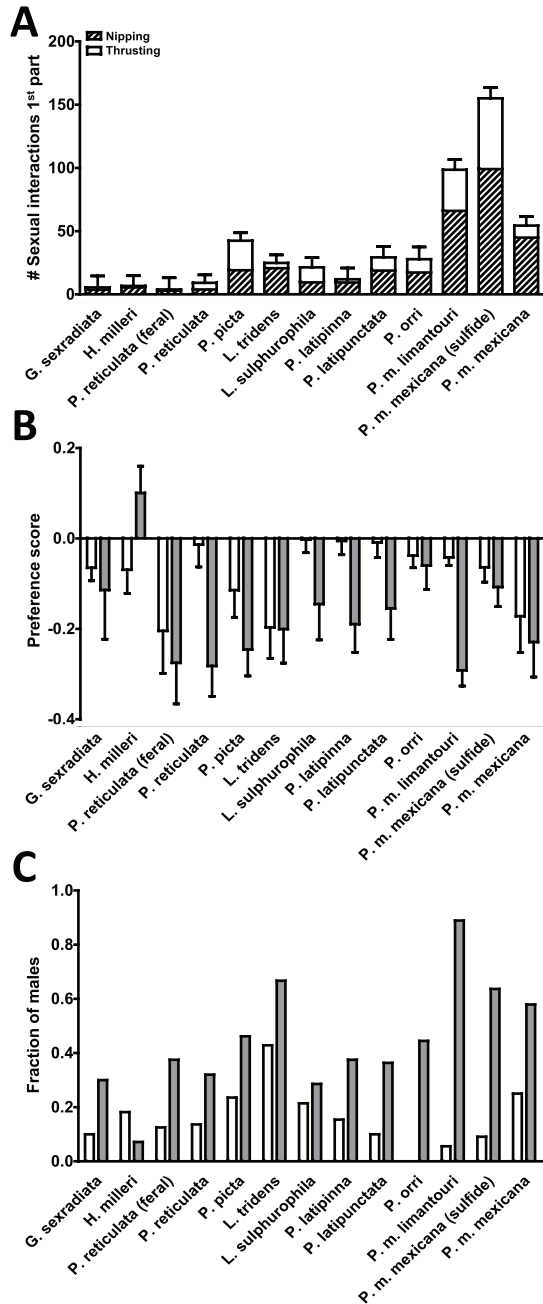


Figure 2. (a) Mean (\pm SE) numbers of male sexual behaviors during the 10 min observation period. (b) Changes in individual focal males' mate choice behavior in the presence of an audience male. Depicted are mean (\pm SE) preference scores (see main text), whereby negative values indicate that male preferences decreased in strength. (c) Proportion of males that first interacted with the opposite female when released from the acclimation cylinder in the second part of the tests. Open bars in (b) and (c) represent the control treatment (no audience) while gray bars represent the audience treatment.

PCA with all seven behaviors retrieved two principle components accounting for 63.1% of the total variance. While PC1 received strongest loadings from deceptive male mating behavior (fraction of males that changed their first interaction in the audience treatment; axis loading: 0.90), sexual activity (0.78), aggressiveness (0.63) and preference changes due to an audience (-0.67; all other axis loadings between -0.40 and -0.01), PC2 received strongest loadings from both control treatments (change in preference without audience: -0.84; fraction of males that changed the initially approached female without audience: 0.85; all other axis loadings between -0.28 and 0.59) and thus reflects general consistency in mate choice behavior (Figure 3c).

Data on dyadic male contests

1 Data File

<http://dx.doi.org/10.6084/m9.figshare.427708>

Discussion

Our current study identified aggressiveness, male sexual activity, and deceptive mating behavior in presence of an audience as consistent, species-specific behavioral traits, while decreased preference expression due to an audience ('audience effects' *sensu*³⁷) was found to be a universal feature in all but one of the investigated species. Also, species did not differ in their consistency during mate choice in the control treatment without audience — whether evaluated as the change in preference expression or numbers of males that changed the female with which they interacted first. Subsequent correlation analyses uncovered two effects: (a) males of species with high sexual activity are more likely to show deceptive mating behavior, *i.e.*, they initially approached more often the non-preferred female when an audience male was presented; while species-level mean aggressiveness did not predict the occurrence of audience effects. (b) Mean aggressiveness, by contrast, correlated positively with mean sexual activity. Hence, we detected two correlations of behavioral types at the species level.

One of the behavioral syndromes at the species level we uncovered in our present study — the correlation between aggressiveness and sexual activity — can be partly explained mechanistically through species differences in plasma concentrations of sexual corticosteroids (testosterone and its derivatives^{73,74}). Individual androgen concentrations predict aggressiveness in male swordtails, *Xiphophorus hellerii*⁷⁵; furthermore, plasma testosterone levels correlate positively with sexual behavior in male mosquito fish (*G. holbrooki*)⁷⁶, so physiological pleiotropy could also explain species differences in aggression and sexual activity as detected here.

The main focus of our present study was on audience-induced changes in male mating behavior, and we asked if those behaviors can be linked to mean sexual activity and SCR. The rationale behind our prediction was that males of taxa with high overall sexual activity

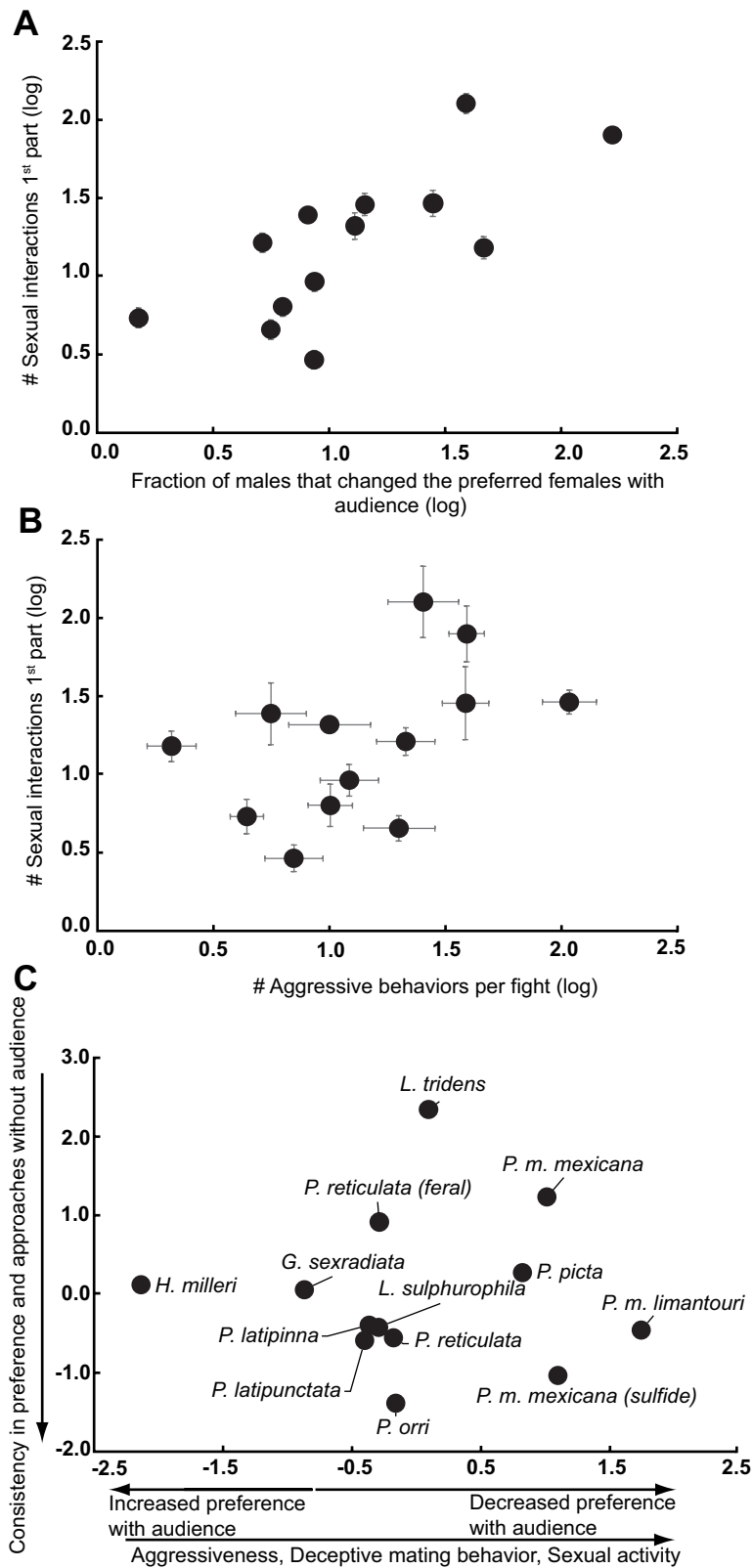


Figure 3. Correlations between species-level means (\pm SE) of male aggressiveness (log(number of aggressions per fight)) and (a) sexual activity (log(number of sexual interactions during the 1st part)) and (b) deceptive mating behavior (fraction of males that changed the first approached female from 1st part to 2nd test part with an audience present). (c) First two principle components from PCA with species-level means of all seven behaviors determined in this study. Axis loadings >0.63 are given along the respective axes.

face a higher risk of by-standers making use of socially acquired information when eavesdropping on sexual interactions. It seems reasonable to assume the propensity for male mate choice copying to be a common feature of poeciliid mating systems^{10,77}, but the likelihood of mate copying in natural systems should correlate positively with mean sexual activity. We found sexual activity (but not aggressiveness — despite some degree of inter-correlation between aggressiveness and sexual activity, see above) to correlate positively with the level of presumed deceptive mating behavior. This finding lends support to our hypothesis that SCR is a driving force behind the evolution of this behavior and is in line with our interpretation that focal males thus attempt to lead the rival away from their preferred mate, exploiting male mate choice copying to reduce SCR^{19,21,32}.

A general objection to our interpretation of deceptive mating behavior could be that leading the audience away from a preferred mating partner to deceive the rival may increase the risk of losing the preferred female, as poeciliid females tend to flee from male sexual harassment^{30,78,79}. We argue that this male behavior still offers advantages even if the preferred female flees: on the one hand, a pattern of last male sperm precedence was uncovered in guppies^{22,80}, which renders mate choice copying a profitable option for the eavesdropping (copying) male¹⁰. However, the longer the time between copulations by the first and second male in the mating trials conducted by Evans and Magurran⁸⁰, the higher the proportion of offspring fathered by the first male was. This implies that leading the by-standing rival away from (or at least delaying its approaches toward) a recently inseminated female would indeed be beneficial for the deceiving male even though it risks losing contact with the initially preferred (but already inseminated) female. Our interpretation assumes that males initially transferred sperm to the preferred female, which could not be determined unambiguously by simply counting copulation attempts. We thus recommend future experiments that will extract and quantify the amount of transferred sperm from females after the first preference test (see Evans *et al.*⁸¹ for a protocol).

Since our analyses were based on species/population differences in aggressiveness, sexual activity and audience-induced changes in male mate choice behavior, we strongly recommend future experiments concentrating on within-population variation (*e.g.*, individual “behavioral types”,^{42,44}) that define a male’s response to a by-standing rival. For example, males are sensitive to the perceived sexual activity of a rival when exhibiting audience effects⁹, and future studies could elaborate on the question of whether also perceived aggressiveness — a correlate of sexual activity — might influence the occurrence of audience effects. Such an experiment could also shed new light on the observed cross-correlation between sexual activity and aggressiveness as well as between sexual activity and deceptive behavior. However, such an approach requires multiple

testing of the same individuals, which imposes logistic constraints on comparative analyses like our present study. Furthermore, future studies ought to elaborate on potential factors affecting the observed consistent behavioral differences among species. In this context, both phylogenetic considerations (for example through phylogenetically adjusted generalized linear models on a larger set of poeciliid species) and a comparison of shared and unique ecological features of different poeciliids are promising fields of investigation.

In summary, using a comparative approach, we were first able to quantitatively characterize behavioral types at the species level for several poeciliid species and further found correlational support for the hypothesis that SCR arising from male mate choice copying drives the evolution of audience-induced changes in male mate choice behavior. We argue that taxa with elevated sexual activity face a higher risk of males making use of socially acquired information (*i.e.*, copying mate choice decisions), and so focal males in those species are more likely to respond to the presence of an audience with altered mate choice behavior.

Author contributions

DB, IS, BS and MP designed the study. DB, AMM and HG conducted the experiments. DB and MP analyzed the data. DB prepared the first draft of the manuscript. All authors were involved in the revision of the draft manuscript and in incorporating the valuable comments provided by the three reviewers. All authors have agreed to the final content of this article.

Competing interests

No competing interests were disclosed.

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Current Referee Status:

Referee Responses for Version 3



Lisa Locatello

Evolution and Ecology of Fish Reproduction, Department of Biology, University of Padova, Padova, Italy

Approved: 14 November 2013

Referee Report: 14 November 2013

In this updated version of the manuscript the Authors dealt with most of the raised issues.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Responses for Version 2



Lisa Locatello

Evolution and Ecology of Fish Reproduction, Department of Biology, University of Padova, Padova, Italy

Approved with reservations: 06 September 2013

Referee Report: 06 September 2013

The Authors fulfilled properly all the statistical problems of the previous version and the manuscript is definitively improved.

However, even if the new analyses confirm the previous results, I still feel that the Authors' conclusions are too far stretched and I would suggest a more cautious approach to the discussion. This is due to three main reasons:

1. The rationale behind predictions is that a higher sexual activity is linked with a higher SCR, but this is not directly demonstrated by this or by previous studies on the selected species. Therefore, I would be more cautious in definitively concluding that the positive correlation between sexual activity and deceptive mating behaviour support the hypothesis that SCR is the driving force behind the evolution of deception.
2. The firm conclusion that deceptive behaviour is independent from aggressiveness (derived from the absence of correlation between the two) has a weak point if considering that sexual activity correlates with deceptive behaviour but it also correlates with aggressiveness. As a consequence the Authors' should be less categorical in attributing the whole weight to sexual activity while totally excluding an implication of aggressiveness.

3. Main conclusions are drawn in the perspective of males that, after having inseminated a female, take advantage of a deceptive behaviour by standing rivals away from recently inseminated female even considering the risk of losing contact. But in fact, during the experiment it was impossible to discriminate a successful mating from a simple mating attempt. As a consequence, insemination (mating) should not be taken for granted. I would be more cautious in interpreting the adaptive behaviour of these experimental males as they have inseminated the female when this did not happen with certainty. Therefore, the Authors' might want to consider the following assumptions with more prudence:
- i) That patterns of sperm precedence are the cause that renders the risk of losing a high quality and inseminated female beneficial. This may be true only if insemination definitely occurs.
 - ii) That male mate choice copying renders recently mated females more attractive to rivals, as they can't be sure that experimental females have mated.

I have also a last minor comment on the phylogenetic comparative analyses that I suggested as a future perspective. By excluding the population of guppies that were most closely related to the Venezuelan guppies the Authors are not definitively controlling for independence of the results from phylogenetic relationship across species.

What I meant is that only a phylogenetic comparative study, implying statistical analyses that account for phylogeny (see for example phylogenetic generalized linear models) on a large species set, would allow the pattern observed on this study to extend to a broad-scale; excluding the possibility that this pattern is explained by phylogenetic factors other than sexual selection. This is why I suggested a phylogenetic comparative study as a promising future approach, taking advantage of data on a higher number of species and of a resolved phylogenetic tree.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

1 Comment

Author Response

David Bierbach, Goethe-University of Frankfurt, Germany

Posted: 23 Sep 2013

Dear reviewer,

Please find our responses to your points below. We have also indicated the parts of the manuscript which were changed accordingly. We hope our revision has dealt appropriately with your points of critique.

1. You are right, we did not quantify the degree of sperm competition in the species investigated here; nevertheless, we have reason to believe that "sexual activity" is a correlate of sperm competition risk. For example, in [Bierbach *et al.* \(2011\)](#) we were able to

demonstrate that *P. mexicana* males do not respond with “audience effects” to males that focal males perceive as sexually inactive (equally low SCR), while strong reactions were found in response to males they previously perceived as sexually active (high SCR). On the population (or species) level, the likelihood of females receiving sperm from more than one male ought to be a function of male sexual activity, especially because poeciliid populations in nature tend to be female-biased.

2. We have addressed this point in the discussion: “*Such an experiment could also shed new light on the observed cross-correlation between sexual activity and aggressiveness as well as between sexual activity and deceptive behavior.*”
3. Sure, the adaptive significance of deceptive behavior is linked to sperm being transferred before the rival enters the mating arena. Still, previous studies have demonstrated rapid sperm transfer in poeciliid fishes when males and females are kept together in similar experimental tanks (for example: [Riesch et al., 2008](#) and [Nöbel & Witte, 2013](#)). Investigations on wild Trinidadian guppies suggest high rates of transferred sperm even through forced copulations ([Evans et al., 2003](#)). However, we have added the following sentence to the Discussion: “*Our interpretation assumes that males initially transferred sperm to the preferred female, which could not be determined unambiguously by simply counting copulation attempts. We thus recommend future experiments that will extract and quantify the amount of transferred sperm from females after the first preference test.*”

In response to the minor comment; we added the following half-sentence to the discussion, “*for example through phylogenetically adjusted generalized linear models on a larger set of poeciliid species*”. However, we have never stated that excluding the feral guppy population will provide phylogenetically independent data.

Competing Interests: no competing interests



Katja Heubel

Department of Biology, Institute for Evolution and Ecology, Animal Evolutionary Ecology, University of Tuebingen, Tuebingen, Germany

Approved: 30 August 2013

Referee Report: 30 August 2013

In the current version of the article, the authors dealt with most of the issues and concerns addressed by the referees.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.



Clelia Gasparini

Centre for Evolutionary Biology, The University of Western Australia, Crawley, WA, Australia

Approved: 23 August 2013

Referee Report: 23 August 2013

The manuscript was already interesting and well written in its first version and the revised version now includes/clarifies the unclear points. I am satisfied with the new version and authors' replies to my specific comments, and I feel overall that the revisions have resulted in a very interesting paper.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Responses for Version 1



Clelia Gasparini

Centre for Evolutionary Biology, The University of Western Australia, Crawley, WA, Australia

Approved with reservations: 16 July 2013

Referee Report: 16 July 2013

The authors present a study in which audience effects on male mating behaviour was analysed in several species of poeciliids (a family of livebearing freshwater fish) and related to mean sexual activity (used as a proxy for sperm competition risk) and aggressiveness. This is an attempt to study if sperm competition risk (SCR) can explain the occurrence of audience effects on male choice in this family. The rationale behind this is that males should adjust their mating behaviour by modulating, or even reversing, their initial mate choice in the presence of a rival. The change in male mate choice in the presence of another male has been mainly interpreted as a deceptive signal to lead competitors away from the preferred females, therefore lowering sperm competition risk. Given the complexity of factors (abiotic or biotic) that can contribute simultaneously to shape male mating decisions, explanations other than SCR (though not necessarily mutually exclusive) are also possible, although SCR is certainly likely to be important. Indeed, sperm competition is pervasive in poeciliids, and it is therefore likely that sperm competition is a major force in shaping the evolution of male mating strategies in this family. The hypothesis tested in this paper is that a higher sperm competition risk (SCR) should positively correlate with stronger audience effects across different species. Aggressiveness was also considered, as males could adjust their mate choice to avoid aggressive rivals.

This is a well written paper, addressing an interesting topic in evolutionary biology. Unfortunately, as the study is only correlative and phylogeny was not accounted for, results can only suggest a general trend, but this can certainly set the stage for future work in this area. No data was collected or analysed to directly quantify SCR in the different species, but total sexual activity (measured in the initial test) was used as a proxy.

Aggressiveness tests:

The authors performed aggressiveness tests, controlling for a number of factors that can possibly confound interpretation of results, for example, choosing males from different tanks to prevent previously established dominance. However, would aggressiveness scores differ when males are tested in the presence of a female during these encounters? Indeed, two males may have a lot more reasons to exhibit aggressive behaviour when a potential partner is present.

Male mate choice tests:

In these tests the focal male and two females were free to interact. Methods are described in detail, but I wonder if this is the exact protocol used in all experiments. I am guessing that the method used is probably similar across experiments, but it seems unlikely to me that it is exactly as described here for all of them. Authors also exclude courtship from the sexual activity variable because this behaviour is not present in all species. However, courtship is an important component of sexual behaviour in some of the species considered and including this aspect of male behaviour may therefore change results.

Main conclusions:

The main finding that lead the authors to support the hypothesis “SCR is a driving force behind the evolution of this behaviour” is the positive correlation (depicted in fig 3e) between the intensity of sexual behaviour (proxy for SCR) recorded in the first test and the level (occurrence) of deceptive behaviour (the fraction of males that reverse their first choice, based on the first interaction with female, page 7). I would like to know how well the first sexual interaction reflects a male’s sexual choice in these species; is there any direct evidence? In guppies, for example, researchers have tested whether the time spent in front of a female during a binary dichotomous test is a good predictor of actual mating preference (Jeswiet & Godin 2011). Are there any studies that show that first sexual interaction is a reliable sign of male sexual interest in most of the species considered here?

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

1 Comment

Author Response

David Bierbach, Goethe-University of Frankfurt, Germany

Posted: 12 Aug 2013

Thank you very much for the positive view of our paper! The reviewer is right, we did not account for phylogeny in our current paper and the main focus of our study was to provide a general comparison of Poeciliid male reproductive behavior given the strong SCR assumed in this family.

Aggressiveness tests: Your assumptions might be right; in Siamese fighting fish effects of

by-standing females on male aggressive behavior has been found (see work by McGregor). However, a recent study showed that Atlantic molly females did not prefer males after they had won a fight (Bierbach *et al.* 2013) which could lead to reduced aggressive behavior between males when being observed by a female in Poeciliids.

Male mate choice tests: The described experimental setup was exactly the same in all studies from which we extracted the mate choice data. The reviewer is right, courtship is an important aspect of some of the investigated species' sexual behavior. Nevertheless, in order to draw general conclusions across a wide range of Poeciliid species that differ in several behavioral and ecological aspects, we focused on sexual behaviors that are directly linked to copulations (thus sperm transfer). Surely, courtship is an aspect that should be investigated in future studies.

Main conclusions: Thank you for this comment! To show that first sexual interactions and mating preferences are congruent, we added another paragraph to the methods section where we explain that in 76%-100% of the trials the first approached females were also subject to the majority of males' sexual behaviors makes the first sexual approach a good proxy for male preference in all species examined.

Competing Interests: no competing interests



Lisa Locatello

Evolution and Ecology of Fish Reproduction, Department of Biology, University of Padova, Padova, Italy

Approved with reservations: 17 June 2013

Referee Report: 17 June 2013

Bierbach and co-authors investigated the topic of the evolution of the audience effect in live bearing fishes, by applying a comparative method. They specifically focused on the hypothesis that sperm competition risk, arising from male mate choice copying, and avoidance of aggressive interactions play a key role in driving the evolution of audience-induced changes in male mate choice behavior. The authors found support to their hypothesis of an influence of SCR on the evolution of deceptive behavior as their findings at species level showed a positive correlation between mean sexual activity and the occurrence of deceptive behavior. Moreover, they found a positive correlation between mean aggressiveness and sexual activity but they did not detect a relationship between aggressiveness and audience effects.

The manuscript is certainly well written and attractive, but I have some major concerns on the data analyses that prevent me to endorse its acceptance at the present stage.

I see three main problems with the statistics that could have led to potentially wrong results and, thus, to completely misleading conclusions.

- First of all the Authors cannot run an ANCOVA in which there is a significant interaction between factor and covariate Tab. 2 (a). Indeed, when the assumption of common slopes is violated (as in their case), all other significant terms are meaningless. They might want to consider alternative statistical procedures, e.g. Johnson—Neyman method.
- Second, the Authors cannot retain into the model a non significant interaction term, as this may affect estimations for the factors Tab. 2 (d). They need to remove the species x treatment interaction (as they did for other non significant terms, see top left of the same page 7).

- The third problem I see regards all the GLMs in which species are compared. Authors entered the 'species' level as fixed factor when species are clearly a random factor. Entering species as fixed factors has the effect of badly inflating the denominator degrees of freedom, making authors' conclusions far too permissive. They should, instead, use mixed LMs, in which species are the random factor. They should also take care that the degrees of freedom are approximately equal to the number of species (not the number of trials). To do so, they can enter as random factor the interaction between treatment and species.

Data need to be re-analyzed relying on the proper statistical procedures to confirm results and conclusions.

A more theoretical objection to the authors' interpretation of results (supposing that results will be confirmed by the new analyses) could emerge from the idea that male success in mating with the preferred female may reduce the probability of immediate female's re-mating, and thus reduce the risk of sperm competition on the short term. As a consequence, it may be not beneficial to significantly increase the risk of losing a high quality and inseminated female for a cost that will not be paid with certainty. The authors might want to consider also this for discussion.

Lastly, I think that the scenario generated from comparative studies at species level may be explained by phylogenetic factors other than sexual selection. Only the inclusion of phylogeny, that allow to account for the shared history among species, into data analyses can lead to unequivocal adaptive explanations for the observed patterns. I see the difficulty in doing this with few species, as it is the case of the present study, but I would suggest the Authors to consider also this future perspective. Moreover, a phylogenetic comparative study would be aided by the recent development of a well-resolved phylogenetic tree for the genus *Poecilia* (Meredith 2011).

Minor comments:

Page 3: the authors should specify that also part of data on male aggressiveness (3 species from Table 1) come from previous studies, as they do for data on deceptive male mating behavior.

Page 5: since data on mate choice come from other studies is it so necessary to report a detailed description of methods for this section? Maybe the authors could refer to the already published methods and only give a brief additional description.

Page 6: how do the authors explain the complete absence of aggressive displays between the focal male and the audience male during the mate choice experiments? This sounds curious if considering that in all the examined species aggressive behaviors and dominance establishment are always observed during dyadic encounters.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

1 Comment

Author Response

David Bierbach, Goethe-University of Frankfurt, Germany

Posted: 12 Aug 2013

Thank you very much for your overall positive view! In the revised version of our manuscript we rigorously tried to answer all your questions and clear up all points of critique raised. We analyzed our data with the recommended mixed model approach and added a PCA that depicts the species' behavioral characteristics.

Statistics: We re-analyzed all data using mixed models with "species ID" included as a subject grouping factor and random intercepts for each species. We then established whether there was significant between-species variation through likelihood ratio tests (model with random intercepts for species vs. reduced model). In the new analysis, random slopes for 'opponent size difference' were included for each species in our model so that the final analysis appropriately accounts for species-specific reactions towards this covariate. In the new analysis only significant interaction terms and covariates were retained. For the analysis of the changes in mating preferences (linear mixed model) as well as changed first interactions (generalized linear mixed model) we included 'treatment' as a random factor as suggested.

"male success in mating with the preferred female may reduce the probability of immediate female's re-mating": This idea opposes our assumption of general "male mate choice copying" which renders recently mated females more attractive to rivals. If we understand correctly, you suggest some kind of "mate guarding" that would delay re-mating. This is however not a feature of any Poeciliidae mating system known so far. In this context, we would like to refer to our paragraph in the discussion dealing with patterns of sperm precedence in Poeciliids. Up to now, last male sperm precedence is at least verified in one of the species investigated here (for guppies) but it was not the focus of investigations into the other species. Thus, as audience-induced changes in preferences are found in all but one species (namely *H. milleri*), we assume the occurrence of last male sperm precedence is one cause that renders the "risk of losing a high quality and inseminated female" beneficial.

Phylogenetics: Phylogenetic analysis may be useful, and we re-ran our analysis while excluding the population of feral guppies that were most closely related to the Venezuelan guppies. However, the results remained unchanged. Furthermore, the new PCA that includes all behaviors investigated in the current study does not show any phylogenetic grouping.

Minor comments: The reviewer is right, the protocol for the mate choice tests as well as the aggression tests are already published but we would like to keep it in the current manuscript for reasons of clarity (also taking advantage of the less restrictive word limits of an online-only journal). In our mate choice tests, focal and audience males were separated as the audience males were fixed in a Plexiglas cylinder. Thus, direct aggression was not observable. Furthermore, a recent study showed that Atlantic molly females did not prefer males after they had won a fight which could have resulted in focal males showing low aggressiveness in front of the two female stimulus fish (Bierbach *et al.* 2013)

Competing Interests: no competing interests



Katja Heubel

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Approved with reservations: 17 June 2013

Referee Report: 17 June 2013

I very much appreciate the effort of putting results together to compile a dataset that makes comparison across taxa possible. The authors could take more advantage of that. See more detailed comments below. Unfortunately, the manuscript does not have line numbers.

Title: Your title does not really suit your story. In particular, I am not in favour of your Casanova analogy. It is not clear to me how and in which part of your paper you may have tested the males properties of being “Casanovas” – I would have expected you to test a males multiple mating success – which you did not. Furthermore, the “liar” is not clear to me either. You did not test how male’s fitness correlated traits are dishonest or something like this. Anyways, analogies from the liberal arts always come with the problem of being a bit inaccurate since the definition of such characters are always somewhat blurred (is a “Casanova” a male with many matings? Many mates? High reproductive success? Is also male-male competitive ability part of the syndrome? What about the male aggression level?). I suggest refraining from using such non-scientific terms in your title and rather find a suitable title. Reading the abstract and introduction would suggest a title on sperm competition risk and audience effect.

Abstract: your start is a bit odd. You start with what seems to me to be the most exceptional example for the cause of sperm competition. Mate copying and even more specific male mate copying is not required for sperm completion. A simple scenario of multiple mating in a promiscuous mating system is a much more straightforward explanation.

Article content: the methods section is well explained. Looking at Table 1, I started wondering how many cases were excluded because a clear winner-loser situation could not be established. Comparing N dyads and the d_f 's, it seems that some species (*H. milleri*, *P. reticulata feral*, *L. tridens*, *P. latipunctata*) have more difficulties in establishing hierarchies under the given size differences. In the mentioned species, no more than 50% of trials ended with a winner-loser situation (*H. milleri* 4 out of 14 dyads). Thus, the n of trials with established hierarchy is 103, not 146.

Your experimental procedure on male mating behaviour comes with the side-effect that some of the males (and females) got to mate successfully in the first round, but others maybe not. Does this potentially affect their behaviour in front of an audience?

I was wondering whether there would be an alternative and more efficient approach to test species level regressions/correlations? Maybe you can compare regression slopes. Figure 2c may suggest that real deception only occurs in *L. tridens*, *P. mex. lim*, *P. mex sulf*, and *P. mex mex* which have an average likelihood to swap preferences and approach the other female of more than 50%.

Introduction: the introduction is a bit weak in underlining the novelty and achievements of the current study compared to the already existing body of articles on SCR and audience effects in poeciliids. A reader skimming through the paper may wonder why another study on SCR and audience in poecillids would be an interesting read.

I very much appreciate the effort of putting results together to compile a dataset that makes comparison across taxa possible. Take more advantage of that.

In particular, I was wondering how encounter rates and population density may affect SCR and related coping styles. A life-history approach by looking at shifts in the reproductive periods may be interesting. Depending on whether first or last males sperm precedence is applicable, one may predict an advancement of the mating activity on the population level. Nevertheless, I am aware that this is beyond

the scope of this paper.

Discussion: I am not convinced that your first sentence is supported by your data: where do you show that variation in audience effects is less pronounced among taxa? Why is personality and behavioural syndromes not touched upon in your introduction? Not sure it really belongs to your story. There is no real data on SCR in your paper. Your introduction deals with SCR in great detail, but it is not really in your data. Is there any solid data that supports your proposed link between sexual activity and SCR? Data: having 13 taxa at hand, it would be interesting to see which and how some species cluster together. Could you include multiple contrasts or a factor analysis to illustrate similarity vs dissimilarity among species?

As it is, Table 2 with “species” being significant, only reports that at least one species is different from the others. Would be useful to add more information. What you really want to show is how the species are clustering and how this relates to their mating system and sperm competition risk.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

1 Comment

Author Response

David Bierbach, Goethe-University of Frankfurt, Germany

Posted: 12 Aug 2013

Thank you for your positive view of our comparative approach! We tried to outline this cross-taxa character in more detail in the new version of the manuscript. I am afraid the lack of line numbers is a feature of the journal's publishing and editing concept, sorry.

You are right to be wary of analogies from the liberal arts. However, the Oxford dictionary defines a “Casanova” as a man notorious for seducing women. As the most sexually active species in our study readily switch their preferred females, we believe that referring to those males as “Casanovas” is not far from reality. The second part of our title adequately describes our current study from a scientific point of view, also incorporating sperm competition as one of your suggested key words.

Abstract: Thank you for this point! We changed the beginning of the abstract, several parts of the introduction and discussion, as well as the statistical analysis to underpin the comparative approach more precisely.

Article content: The reviewer is right about the number of dyads in which a clear dominance hierarchy was established. Nevertheless, even when no dominance was established we analyzed the number of aggressive behaviors that occurred and counted those trials as successful. We now precisely state how many trials dominance was established in.

The reviewer is right, successful and unsuccessful matings could have influenced the behavior in front of an audience. However, in most Poeciliids it is not possible to discriminate with certainty between a successful mating (defined as a mating with sperm being transferred) and the pure mating attempt. We followed our analysis with a protocol from [Hammond-Tooke *et al.* \(2012\)](#) where Spearman's rank order tests were used to test for behavioral syndromes. We also depict results from PCA to cluster species based on their behaviors (see below). The new analysis found significant between-species variation in the level of deceptive behavior while the general tendency to swap more often between females when confronted with an audience male was found in all but one species. Our current analysis is focused on general behaviors across Poeciliids, more species-based analysis can be found in [Bierbach *et al.* \(2013\)](#) (in press).

Discussion: Our new analysis shows that between-species variation is much smaller in the audience behavior. We have switched the paragraph dealing with personality and behavioral syndromes from the discussion to the introduction so that the reader will be introduced to these considerations at the beginning. Several studies used the presence of a by-standing rival during mate choice to simulate SCR (see introduction) and sexual activity as a proxy for sperm competition in Poeciliids with high rates of multiple matings (which in turn leads to broods normally sired by multiple males). However, quantifying direct sperm competition (e.g., through offspring genotyping) was beyond the scope of our study.

Data: To provide an overview about similarity vs. dissimilarity among species we now provide results from PCA.

Competing Interests: no competing interests