Female vibration discourages male courtship behaviour in the Amarillo fish (Girardinichthys multiradiatus)

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\textbf{ABSTRACT}

Amarillo fish females (Girardinichthys multiradiatus) vibrate when conspecific males approach them; the reason behind this behaviour is unclear. Hypotheses are that females vibrate either to avoid aggression from males or to court them. We prevented females from vibrating by temporarily blocking their lateral line organs and eyes, on the assumption that they rely on these senses to detect approaching males. Females with the lateral line organs obstructed vibrated less frequently than females with the lateral line intact, indicating that the mechanosensory lateral line system is necessary for perceiving approaching males. Males displayed more courtship behaviour to sighted females with the lateral line organs obstructed than to sighted females with the lateral line intact. A general tendency indicated that the less the females vibrated the more the males courted them. These findings indicate that female vibration discourages male courtship behaviour.

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1. Introduction

The importance of the lateral line system in the behaviour of fish has frequently been demonstrated (Bleckmann, 1993). But, except for the relatively complex inter-sexual vibrational communication of the himé salmon (Satou et al., 1994), most studies have dealt with relatively simple topographic interactions with the environment, including prey, or conspecifics. Examples include orientation to water currents in several fish species (Montgomery et al., 1997), schooling (e.g. in saithe Pollachius virens; Pitcher et al., 1976) and the predatory behaviour of the bluegill (Lepomis macrochirus; Enger et al., 1989). Interactions involving more than just adjustments of position or orientation, and controlled by more than one sensory modality (e.g. mechanoreception and vision) have seldom been examined (Rüschenbaum and Schlupp, 2013). This is in part due to the difficulty in separating the effects of mechanical sensory cues from those produced by other sensory modalities. Here we used temporary blockade of the lateral-line organs and eyes to discriminate between two competing hypotheses about the significance of vigorous vibration shown by females of the Amarillo fish (Girardinichthys multiradiatus). This fish is one of several species of Goodeinae, a subfamily of freshwater, viviparous fish endemic to central México (Doadrio and Domínguez, 2004; Webb, 2004; Miller, 2005). As with other goodein species, the Amarillo fish shows conspicuous sexual dimorphism (Macías Garcia and Valero, 2010). Males have larger and more colourful dorsal and anal fins than females, which they display during courtship (Macías Garcia, 1994) and male–male encounters (Macías Garcia, 1990). Females are dull except for a pigmented black spot around the vent, possibly derived from a pregnancy signal, which is also used by submissive males to mimic females (Macías Garcia and Valero, 2001).

Amarillo females of most goodein species show vibration, that is, they wag the head while leaning forwards when conspecific males approach them (Moyaho, 2002). Previous observations in the Amarillo fish suggested that females vibrate to avoid sexual harassment by males (Macías Garcia et al., 1994). Thus, vibration could serve as an appeasement signal, albeit an expensive one (Valero et al., 2005). This putative appeasement signal does not work in female–female interactions, indicating that it is a signal directed particularly to males. If so, this suggests the alternative, non-exclusive hypothesis that female vibration is a courtship display, as it was considered to be the case in related Goodea atripinnis (Nelson, 1975).

Disentangling the possible functions of female vibration requires evaluating the behaviour of males exposed to females whose tendency to vibrate has been manipulated. Consequently we first established the nature of the sensory cues that evoke female vibration, and secondly we used this knowledge to manipulate the female tendency to vibrate in the presence of males. We
then evaluated the male responses to females and related them to the amount of vibration performed.

Although interacting cyprinodontiform fish use a variety of sensory modalities, we focused on hydrodynamic cues because they are effective over short ranges (Webb et al., 2008; Bradbury and Vehrencamp, 2011). As some lateral line receptors provide information on water currents (Dijkgraaf, 1962; Münz, 1979), which may be produced by approaching conspecifics or predators (e.g., aquatic snakes), we used cobalt-dependent pharmacological blockade of the lateral line mechno-sensitivity (Karlsen and Sand, 1987; Satou et al., 1994; Janssen, 2000). In this way we could determine whether mechanical sensory cues were responsible for female vibration. In addition, we temporarily concealed the female's eyes because there is evidence that the sight of males also evokes female vibration (Valero et al., 2005).

2. Materials and methods

2.1. Study population

Twenty-four male and female Amarillo fish (G. multiradiatus) from a dam in Lázaro, State of México, were used in this study. The fish were collected using a 4.5-m long seine and brought to the laboratory, where they were kept in mixed-sex groups in 40L glass aquaria and daily fed twice commercial food flakes. The aquaria were equipped with sponge filters, aeration and aquatic weed for shelter. The aquaria with the fish were put in a regulated-temperature room (18–22 °C) with a constant 12/12 h light/dark photoperiod (lights were turned on at 0700 h). Twenty-five days before the experiment began the fish were separated by sex to standardize their sexual response. All the fish were maintained in accordance with institutional guidelines for the use of animals in research.

2.2. Experimental tank

We used 4 glass tanks (51 cm × 26 cm × 30 cm deep) for observing and recording the agonistic and courtship behaviour of male–female dyads. Each tank had a gravel bottom, a diffuser connected to a pump for aeration, and aquatic weed. We covered the back and sides of each tank with opaque paper. The aquatic weed and paper were used for minimizing disturbance of the fish and for presenting a uniform background during the observation of behaviour. Four 20-W fluorescent lamps, each placed on the top of each tank, provided light for the observation. The four tanks were put back-to-back and side-to-side on a table in a quiet room. A blind with a hole through which we observed the fish, provided us an appropriate condition for the observation.

2.3. Experimental design

We used four treatments to distinguish between the effect of mechanical and visual cues on female vibration; females had their lateral line system intact or blocked and their eyes covered or uncovered. The treatments were arranged in an incomplete six-block balanced design (Cox, 1958) with two replicates per block. Body size was used to group male–female dyads into blocks, as it could be an indirect indicator of fecundity, mating success and survival (Werner and Gilliam, 1984; Gross and Sargent, 1985; Andersson, 1994; Macias Garcia et al., 1998). Body mass and standard length, which is the distance from the snout tip to the end of the caudal peduncle, were used as measures of body size. To estimate body mass, which was used to group the fish in blocks, each fish was put in a plastic bowl filled with water and weighted to the nearest g. To estimate standard length, which was used as a covariate (see below), each fish was put in a small glass tank (25 cm × 26 cm × 20 cm height) filled with water. Then, the fish was gently pushed with a net against the tank's front wall; from there it was measured using a plastic rule. Body mass of male and female fish was separately sorted into descending order. The four heaviest males and females formed the first block; the same procedure was repeated until the six blocks were completed. Each home aquarium was divided into 3 compartments (17 cm × 26 cm × 30 cm each) with acrylic dividers, and the fish, one-sex per aquarium, were individually placed within the compartments.

The order in which the blocks were tested was randomly selected, with one block run per day. The day before each block was run males and females were weighed again and four male–female dyads were formed according to body mass. Each dyad was randomly assigned to a treatment and to an observation tank, and the order of observation was randomly selected. On the eve of each trial the dyads were put in their corresponding observation tanks to familiarize them with the experimental condition. A volunteer, who was as blind as it was possible to the treatments applied to the fish, observed and recorded from 1230 to 1400 h the following behaviours for 20 min (or less if the blindfolds covering the eyes (see below) became detached from these): (1) Approaches, the number of times a male oriented himself towards and faced a female. Concurrently, he moved towards her within approximately one body length. This is the average distance to which lateral line neuromasts detect water flows produced by fish (Dijkgraaf, 1962; Schellart and Wubbels, 1998; Wullimann, 1998). (2) Vibra- tion, the number of times a female folded the dorsal and anal fins and lowered her head while flicking it from side to side in a rapid manner (Nelson, 1975; Macias Garcia, 1990). Tilting of the body varies in intensity from 0 to about 45°. (3) Flight, the number of times a female fled from a male approaching her. (4) Courtship, the number of times a male either waived the median fins or displayed the figure-of-eight dance, which consists of the male swimming forth and back in front of the female (Macias Garcia, 1990; Gonzalez-Zuath et al., 2011). (5) Chase, the number of times a male orientated himself towards a female and pursued her.

2.4. Mechanoreception (MI) and vision (VI) impairment

Mechanoreception impairment consisted of exposing females to concentrations of either 0 mmol (MI−) or 1 mmol (MI+) L−1 of cobalt chloride hexahydrate (CoCl2) (FlukaTM, Germany), which is within the range of concentrations reported to inhibit the functioning of the lateral-line organs (Karlsen and Sand, 1987; Satou et al., 1994). Ninety minutes before each trial began, each female was taken out of the observation tank and placed in a glass jar containing 1 L of water with the corresponding concentration of CoCl2. The jar had a diffuser connected to a pump to aerate the water. We diminished the concentration of calcium (CaCl2) in the water of the observation tanks to allow the CoCl2 to exert its effect as a calcium channel antagonist (Stoven and Sognen, 1960). To diminish the Ca2+ concentration we added 0.5 mmol L−1 of ethylene glycol-bis tetraacetic acid (EGTA) (Sigma–AldrichTM, St. Louis, MO, U.S.A.), which is a chelate with high affinity for Ca2+. The addition of EGTA to the water of the observation tanks diminished the pH, and hence we added 2 mol L−1 of sodium hydroxide (Sigma–AldrichTM, St. Louis, MO, U.S.A.) to restore the pH to the values recorded in the water of the home aquaria (between 7.5 and 7.9).

After the treatment with CoCl2, each fish was anesthetized by placing it in a plate containing 100 ml of water with EGTA and 12 drops of a solution containing benzocaine (Sigma–AldrichTM, St. Louis, MO, U.S.A.) diluted in acetone (1 g of benzocaine per 100 ml of acetone). Then we put the fish on a sponge soaked with water containing EGTA, and two small patches of black plastic (5 mm × 3 mm) were stuck on each eye (VI−) using adhesive tissue (3M™ Animal Care Products, St. Paul, MN, U.S.A.). The patches of the sighted fish
mechanoreception (VI+) had a hole (about 1.5 mm diameter) cut out of for the pupil. The patches were carefully fixed so that no adhesive reached either the operculum or the eyes.

2.5. Data analyses

The number of male approaches was analyzed using linear mixed models (LMMs) (Bolker et al., 2008), in which block was defined as a variable of random effects because we were not interested in estimating differences between blocks. Frequency of approaches (square root transformation of data) was the response variable. MI, VI, female standard length (FSL) and male standard length (mSL) were the explanatory variables. Since frequency of male approaches was a function of FSL (see below), as previously reported (Bisazza, 1997), agonistic and courtship behaviours were analyzed as proportion of male approaches. In this way we also corrected any bias arisen from those cases in which the blindfolds became separated from the female's eyes. Generalized Linear Mixed Models (GLMMs) were applied to data of female vibration, courtship behaviour, flight and chase using a binomial structure of errors and a log link function (Crawley, 2002). Again, block was defined as a variable of random effects and each category of behaviour as the response variable. MI and VI and the interaction term between them were explanatory variables. In all these statistical analyses deletion tests were used for model simplification so as to obtain a minimal adequate model (Crawley, 2002). Likelihood ratio tests (\(\chi^2\)) were used for model selection, and Wald Z and t for hypothesis testing. In addition, we used a loess smoothing method (Martínez and Martínez, 2002) to model the relationship between the proportion of female vibration and courtship behaviour. This model, which was run using MATLAB (v. 7.9.0.529), estimates two parameters: \(\alpha\), which determines the degree of smoothing with high values yielding smoother curves, and \(\lambda\) which determines the degree of the local regression. Cases in which occurred neither courtship behaviour nor female vibration were discarded to make the model robust. All other statistical analyses were done using R (RDCT, 2011) and the lme4 package (Bates et al., 2011). An alpha-value lower than 0.05 was considered statistically significant.

3. Results

Overall, the behaviours recorded as responses to male approaches varied among treatments and in some cases they were absent (Table 1). Since males approached females to a similar extent regardless of the treatment (Table 1), the variation in response to male approaches was a result of the effect of the sensory impairment imposed on the females.

Only FSL contributed significantly to total variation in frequency of approaches (\(t_{12} = 2.51, p = 0.022, n = 12\), Table 2 and Fig. 1). Approaches increased with FSL although the proportion of the variance explained by the statistical model was small (regression analysis, \(R^2 = 0.22, F_{1,22} = 6.32, p = 0.019\)). This result is consistent with previous studies with other species [see e.g., (Turner, 1993)], and indicates that female body size might reflect fecundity, assuming that both traits correlate positively (Andersson, 1994; Herdman et al., 2004; Macías García et al., 1998).

As regards female vibration, the minimal adequate model only included MI (Table 2). The mean proportion of vibration in the females exposed to MI+ (1.3 ± 1.3%, ±SEM) was significantly lower (\(Z = -2.09, p = 0.036, n = 12\) than that of females exposed to MI− (12 ± 3.56%; Fig. 2).

The minimal adequate model for courtship behaviour included all the terms (Table 2). However, only MI contributed significantly to the variation in courtship behaviour; males courted VI+MI− females more frequently (27.02 ± 7.3%, \(n = 6\)) than VI+MI− females (4.87 ± 3.36%, \(n = 6\); \(Z = 2.43, p = 0.015, \text{Fig. 3}\)).

Neither MI nor VI affected the mean proportion of female flights (Table 2), possibly as result of the variation in response within females.

### Table 1

<table>
<thead>
<tr>
<th>MI</th>
<th>MI+</th>
</tr>
</thead>
<tbody>
<tr>
<td>VI+</td>
<td>6.83 ± 1.49&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>VI−</td>
<td>7 ± 3.04</td>
</tr>
<tr>
<td>VI</td>
<td>14.81 ± 10.99</td>
</tr>
<tr>
<td>VI−</td>
<td>3.17 ± 3.17</td>
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<tr>
<td>VI</td>
<td>2.78 ± 2.78</td>
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<tr>
<td>VI−</td>
<td>7.14 ± 5.46</td>
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<tr>
<td>VI</td>
<td>6.34 ± 3.85</td>
</tr>
<tr>
<td>VI−</td>
<td>–</td>
</tr>
<tr>
<td>VI</td>
<td>2.77 ± 2.77</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mehanoreception (MI) and vibration (VI) impairment. MI−, 0 mmol L<sup>−1</sup> of cobalt; MI+, 1 mmol L<sup>−1</sup> of cobalt. VI−, females with blindfolds; VI+ sighted females.

### Table 2

<table>
<thead>
<tr>
<th>Step</th>
<th>Simplification</th>
<th>df</th>
<th>LogLik</th>
<th>Deviance change ((\chi^2))</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>Maximal model</td>
<td>1</td>
<td>MI + VI + FSL + mSL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7</td>
<td>−27.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>MI + VI + FSL</td>
<td>6</td>
<td>−27.79</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>MI + FSL</td>
<td>5</td>
<td>−27.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>FSL</td>
<td>4</td>
<td>−28.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>Intercept</td>
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<td>−31.36</td>
</tr>
<tr>
<td>(b)</td>
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<td>MI + VI</td>
<td>4</td>
<td>−13.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>MI</td>
<td>3</td>
<td>−13.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>Intercept</td>
<td>2</td>
<td>−17</td>
</tr>
<tr>
<td>(c)</td>
<td>Maximal model</td>
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<td>MI + VI</td>
<td>4</td>
<td>−16.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
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<td>−9.76</td>
</tr>
<tr>
<td>(d)</td>
<td>Maximal model</td>
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<td>MI + VI</td>
<td>4</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>VI</td>
<td>3</td>
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<tr>
<td></td>
<td></td>
<td>3</td>
<td>Intercept</td>
<td>2</td>
<td>−12.03</td>
</tr>
<tr>
<td>(e)</td>
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<td>MI + VI</td>
<td>4</td>
<td>−6.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>MI</td>
<td>3</td>
<td>−6.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>Intercept</td>
<td>2</td>
<td>−6.64</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mehanoreception (MI) and vibration (VI) impairment, female (FSL) and male (mSL) standard length. The plus mark denotes inclusion of an explanatory variable in the model. \(p\)-Values refer to the change in deviance when the respective terms are removed from the model.
treatments (Table 1). The minimal adequate model was a null model \((-3.232 \pm 0.416, \text{coefficient } \pm \text{SE})\), and none of the deletion tests indicated a significant loss of the explanatory power of the reduced models (Table 2, \(p > 0.05\)).

The mean proportion of male chases directed towards females did not vary regardless of the treatments applied to females (Tables 1 and 2). Indeed the minimal adequate model was a null model \((-4.356 \pm 0.711\)). Finally, the loess-smoothing model revealed a negative association between the proportions of vibration and courtship (Fig. 4). When the females did not vibrate at all, males also did not show courtship behaviour, very likely because the females ran away (Table 1).

4. Discussion

We assessed whether female vibration serves as a courtship display or as an appeasement response to sexual harassment engendered by males. We used a stimulus control approach, temporarily blocking the perception of the cues (mechanical or visual) that we had reason to believe were involved in female detection of approaching males. We did not directly demonstrate the physiological effect of Co\(^{2+}\) on the functioning of the lateral line organs of
the fish used in this study. Nonetheless the behavioural difference between treated and untreated females, as well as the known effect of CO2 (Karlsen and Sand, 1987, Satou et al., 1994), convinced us that the observed effects were the result of the action of CO2. As to the patches of plastic used to cover the females’ eyes, pilot trials indicated that the females with blindfolds swam around the aquarium close to the walls. They moved as if they wanted to remove the patches from their eyes. These observations thus suggest the females with blindfolds were unable to see. After a few minutes the females seemingly became accustomed to the blindfolds.

Our results indicate that hydrodynamic cues play a determining role in eliciting female vibration. However, both acoustic and hydrodynamic components could have been important for females to be able to identify approaching males. There are examples where the two sensory modalities are involved in the spatial localization of stimulus sources (Webb et al., 2008) for a review concerning multimodal sensory integration). In fact, sighted females also vibrated when mechanoreception was impaired suggesting that visual cues are also capable of eliciting vibration albeit to a lesser degree. This is consistent with the report that G. multiradiatus females vibrated within a closed respirometer on sight of a male (Valero et al., 2005), although such outcome could also be the result of acoustic cues.

In contrast to the decrease of female vibration, courtship displays of males increased with the blockade of the females’ lateral line system. This opposite effect suggests that female vibration discourages males from performing courtship. Male courtship therefore does seem to represent some form of harassment to females as was hypothesized earlier (Macías García et al., 1994). Yet, G. multiradiatus females sequentially exposed to two males during their receptive period tend to bear more offspring of the male who makes the most courtship displays (Macías García et al., 1998). In addition, vibration frequently occurs in gravid females, probably as an anticipatory strategy to evaluate potential sexual partners (Macías García, 1994). Therefore, female vibration, which is a costly display (Valero et al., 2005), seems also to benefit females byPromoting their mating with high-condition males (see e.g., Barbosa and Magurran, 2006). It is uncertain what information precisely may females gather from male courtship. Courtship may allow males to exhibit their morphology and colour, both targets of female choice in this species (Macías García and Valero, 2010). At the very least courtship may reveal the male endurance if it involves performing prolonged energetic displays (Eberhard, 2002). This would go in line with recent evidence showing that G. multiradiatus males exposed to pesticides early in life perform less dynamic courtship than control males, who are mated preferentially by females (Arellano-Aguilar and Macías García, 2008). If, as it seems to occur, females vibrate in response to an approaching male regardless of their reproductive state, there should be some feature in such response that makes males either to diminish or to increase courtship. Variation in some component of female vibration (e.g., amplitude, frequency, angle of inclination) may be an indicator of acceptance or refusal of courtship. Male courtship displays are ubiquitously diverse amongst the Goodeidae (Moyano, 2002), and also differ substantially among populations of G. multiradiatus (Gonzalez-Zuárrth and Macías García, 2006). There is now evidence that the latter is, at least in part, due to local differences in the female responses to the behaviours that constitute the male courtship display, with the frequency and intensity of vibration playing a preponderant role (Gonzalez-Zuárrth et al., 2011).

Therefore, can we rule out a role of female vibration as a courtship display in favour of abating aggression? Females perform vibration in two contexts, when approached by males and when approached by dominant females. As female–female aggression is unaffected by whether the subordinate vibrates, it has been argued that this is an appeasement signal directed specifically towards males (Valero et al., 2005). Our present findings do not modify this conclusion, but instead suggest that female vibration inhibits male aggression by discouraging males from escalating courtship. Both female flight and male chase, which are indicators of male aggression, were not observed when females vibrated more.

In conclusion the findings of this study indicate that female vibration of G. multiradiatus is an appeasement signal and suggest that it may also have a function in eliciting male courtship. Further studies will reveal which components of female vibration either attract males and stimulate them to display courtship or deter them from escalating courtship.

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