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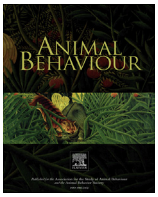

Audience effects and aggressive priming in agonistic behaviour of male zebrafish, *Danio rerio*

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Animals communicate through the exchange of signals. However, third-party individuals can detect and intercept signals not directly sent to them, a phenomenon known as eavesdropping, and the presence of bystanders can influence the signalling behaviour of interacting conspecifics, a phenomenon named the audience effect. So far, research done on audience effects and eavesdropping has been mainly focused on their function, rather than on their proximate mechanisms. For this reason, we were interested in testing the occurrence of audience effects on male zebrafish, a genetically tractable model organism that is emerging as a major candidate for the study of the neural basis of social behaviour. Here, pairs of males were exposed to a mixed-sex shoal, which was used as an audience, at two different times: (1) during a contest between them, to test for an audience effect and (2) before the contest, to test whether this prior exposure influences subsequent agonistic behaviour (i.e. aggressive priming). We analysed the pairs' aggressive signalling during the contest by measuring variables that characterize both the individuals' behaviour and the interaction, and found that pre-exposure to an audience induced a shorter latency to display, an increase in the time dominants spent chasing subordinates and a shorter time to resolve the agonistic interaction. Also, exposure to the audience during the interaction led to a higher number of interactions in which displays occurred, a higher number of resolved interactions with displays and a decrease in the escalation of aggression for resolved interactions. These results add zebrafish to the literature on the audience effect and, most importantly, open the way for the study of the neural mechanisms involved in the processing of social information in a model organism.

The social environment is composed of conspecifics that communicate with each other through the exchange of signals. However, communication is not restricted to dyadic interactions, as exchanged information is also available to other individuals within the range of signal transmission. Therefore, communication occurs in the form of a network comprising signallers, receivers and bystanders (McGregor & Peake, 2000). These third-party individuals can detect and intercept signals, which can be relevant to them, in a phenomenon known as eavesdropping (McGregor, 1993). Thus, bystanders can effectively gather information about observed conspecifics without the costs associated with trial-and-error tactics (Danchin, Giraldeau, Valone, & Wagner, 2004). On the other hand, the presence of bystanders, capable of detecting and intercepting signals, can potentially influence the signalling behaviour of interacting conspecifics. Thus, animals might alter their signalling behaviour in the presence of potential eavesdroppers, by manipulating either the conspicuousness or the intensity of their signals, a phenomenon named the audience effect (Marler, Dufty, & Pickert, 1986). Therefore, bystanders within communication networks are simultaneously influencing the interactions of others as well as being influenced by the signals on which they eavesdrop (Earley & Dugatkin, 2002). Several studies have reported the occurrence of audience effects in both mating and agonistic contexts. Indeed, the presence of bystanders can influence the mating decisions of observed animals (Blum, Plath, Tiedemann, & Schlupp, 2008; Plath & Schlupp, 2008) and the aggressive behaviour of both males (Doutrelant, Mcgregor, & Oliveira, 2001; Dzieweczynski, Earley, Gree, & Rowland, 2005; Dzieweczynski, Gill, & Perazio, 2012; Fitzsimmons & Bertram, 2013; Matos, Peake, & McGregor, 2003) and females (Dzieweczynski, Greaney, & Mannion, 2014). Interestingly, the effect of the presence of bystanders on signallers' behaviour can vary depending on the

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composition of the audience (Doutrelant et al., 2001; Fitzsimmons & Bertram, 2013). For example, the sex composition of an audience affects aggressive signalling towards a competitor in Siamese fighting fish, *Betta splendens*, in which case males decrease their aggressive signalling and increase the number of behaviours commonly used in aggression and courtship, when a female, rather than a male audience, is present (Doutrelant et al., 2001). Finally, the presence of an audience may affect not only current but also future signalling behaviour. This effect, where the pre-exposure to an audience induces a change in the signalling behaviour of the focal individual, has been called priming (Matos et al., 2003). It is important to note here that priming can refer to either an increase (positive priming) or a reduction (negative priming) in the behavioural response.

So far most of the literature on audience effects and eavesdropping has focused on their function and little attempt has been made to understand the underlying mechanisms. The few studies that have addressed this topic have focused on hormonal responses to social information both in bystanders and in signalling individuals exposed to an audience (Dzieweczynski & Buckman, 2013; Dzieweczynski, Eklund, & Rowland, 2006; Oliveira, Lopes, Carneiro, & Canário, 2001). However, the neural correlates of social information use in the scope of communication networks have remained elusive. Interestingly, in the closely related research area of social learning, the neural mechanisms of observational learning in humans have recently been uncovered (Burke, Tobler, Baddeley, & Schultz, 2010). Thus, a first step for the study of the proximate mechanisms underlying eavesdropping and audience effects is to demonstrate their occurrence in a genetically tractable model organism, which allows the dissection of the underlying neural circuits and of the neuromolecular processes involved in the use of social information in signalling networks. Of the model organisms most commonly used and for which the genetic tools are available for the visualization and manipulation (i.e. gain/loss of function) of the nervous system in relation to behaviour (e.g. *Caenorhabditis elegans*, fruit flies, zebrafish, mice), the zebrafish is emerging as a major candidate for the study of the neural basis of social behaviour and cognition (Miller & Gerlai, 2008; Oliveira, 2013; Saverino & Gerlai, 2008). The zebrafish is a highly social species that lives in shoals with structured dominance hierarchies and transient territoriality (Grant & Kramer, 1992; Paull et al., 2010; Spence, Gerlach, Lawrence, & Smith, 2008) which suggests they use social information available in the environment. Indeed, zebrafish have been shown to have their attention tuned towards social interactions (Abril-de-Abreu, Cruz, & Oliveira, in press) and to be able to learn both about others (e.g. social recognition, (Barba-Escobedo & Gould, 2012) and from others (aka social learning, e.g. Hall & Suboski, 1995; Lindeyer & Reader, 2010)). In parallel, a large number of genetic tools and resources are currently available for this species that allow genetic manipulation and visualization of specific neural circuits or candidate genes in relation to behaviour (e.g. Asakawa et al., 2008; Baier & Scott, 2009; Muto, Ohkura, Abe, Nakai, & Kawakami, 2013), and their small brains also allow the use of 3D whole brain imaging through confocal or light-sheet microscopy (e.g. Ahrens, Orger, Robson, Li, & Keller, 2013). Moreover, detailed brain atlases are also available (Ullmann, Cowin, Kurniawan, & Collin, 2010; Wullimann, Rupp, & Reichert, 1996) and homologies, based on topological and functional data, between zebrafish and mammalian brain areas have been established (Ganz et al., 2012, 2014; Wullimann & Mueller, 2004). Together, these facts make the zebrafish a highly attractive model for studying the neural basis of normal and pathological social behaviour (Oliveira, 2013; Stewart, Braubach, Spitsbergen, Gerlai, & Kalueff, 2014) due to both their lower complexity than other vertebrate organism models and high homology to humans (Stewart et al., 2014). Also, social behavioural paradigms developed in zebrafish might be used both in social neuroscience studies and in toxicological studies investigating potential drugs for treatment of social disorders (Stewart et al., 2014).

In this study we tested the effects of both pre-exposure and the presence of an audience in the agonistic behaviour of zebrafish. For this purpose, pairs of male zebrafish were exposed to a mixed-sex shoal either (1) during a contest between the interacting males, to assess the occurrence of the audience effect, or (2) before the contest, to test whether this pre-exposure influences subsequent agonistic behaviour during the interaction (i.e. aggressive priming). We predicted that both the pre-exposure and the presence of conspecifics would influence the aggressive behaviour of the interacting males. However, no directionality of response (i.e. increase or decrease in aggressive behaviour) is predicted in either case, since studies in other social species show that although these are widespread phenomena, the directionality of the response depends on the audience composition (Doutrelant et al., 2001; Matos & McGregor, 2002).

METHODS

Animal Housing

There are significant behavioural differences between zebrafish laboratory strains, and therefore it is important to select the most appropriate line for the behavioural task to be studied. In this study we used the AB strain, given that, although more anxious (Sackerman et al., 2010; Vignet et al., 2013), individuals from this strain are capable of social discrimination in a conspecific visual discrimination test, an ability that is absent in other laboratory strains (Barba-Escobedo & Gould, 2012; Sackerman et al., 2010). Moreover, males of the AB strain have also been shown to change their social profile based on recent social experience (Oliveira, Silva, & Simões, 2011). Together, these two studies suggest the potential for individuals of the AB strain to use social information, which makes them a good model to test the occurrence of audience effects. The AB strain stock at the Instituto Gulbenkian de Ciência (IGC), Portugal, was initially established with fish imported from the Zebrafish International Resource Center, University of Oregon Eugene, OR, U.S.A. All individuals used in this study were bred and reared at the IGC Fish Facility, and were 7–9 months old. They were kept in tanks (50 × 25 cm and 30 cm high), in mixed-sex groups of 30 males and five females, and their environment was enriched with small rocks and artificial plants. The fish were kept in a 12:12 h light:dark photoperiod at a temperature of 25 °C. They were fed in the morning with crushed TetraMin tropical fish food flakes and with live food (*Artemia salina*) twice in the afternoon.

Experimental Treatments

To test the audience effect, half of the sample was exposed to a conspecific audience during the aggressive signalling event while the other half was not. We also tested for aggressive priming in zebrafish by pre-exposing half of our sample to an audience while the other half was not pre-exposed. We used a 2 × 2 experimental design with two independent factors (audience effect, priming effect) with two levels each (audience present, audience absent). Therefore four experimental treatments were considered: (AA) audience present both before and during the interaction; (AN) audience present before but not during the interaction; (NA) audience not present before but present during the interaction; (NN) audience absent both before and during the interaction. We used a total of 96 pairs of males (24 pairs per treatment). To avoid any potential effect of past experience, each pair was used only once and within each pair males were matched for body size (see below), and were unfamiliar to each other (i.e. they came from different home tanks).

Audience Composition

We used a mixed-sex audience since the zebrafish is a highly social species in which shoals include both males and females (Spence et al., 2008). We decided to use shoals of four individuals (two males and two females) as an audience because zebrafish males do not have a preference for a specific shoal size (Ruhl & McRobert, 2005), and this shoal size was adequate for the size of the tanks (15 × 15 cm and 9 cm high) holding the audience (Fig. 1). Audiences were left to habituate to the experimental set-up for 3 weeks prior to the experiment, so that dominance relationships between individuals would stabilize before the experiment started, thereby diminishing the probability of aggressive contests occurring within the audience at the time of testing. The same two audiences were used throughout the experiment, so that they represented stable shoals and also to standardize as much as possible the audience stimuli across focal individuals.

Experimental Set-up

The experimental set-up was composed of four 15 × 15 cm arenas and two 30 × 15 cm arenas (Fig. 1). The longer ones were the audience tanks (A and B) in which the audience individuals were placed. The shoals that served as audiences remained in only half (15 × 15 cm) of these 30 × 15 cm arenas since, on the other half, water was oxygenated and filtered. A light bulb was placed on top of the audience tanks. On the side of each audience arena, we had two interaction arenas (1, 2, 3 and 4), in which we placed our focal individuals (one pair per interaction arena), physically (but not chemically) separated by opaque partitions. Between the interaction and the audience arenas, one-way mirrors were placed (Fig. 1, grey lines), in such a way that focal individuals could see the audience but the audience could not see them, thereby avoiding interactions between the audience and the focal pair. One removable opaque partition was placed between each arena and the audience tank, so that we could manipulate the subjects' visual access to the audience (Fig. 1, dotted lines).

To avoid any effects of the presence of the experimenter, we had white barriers around the interaction arenas (30 cm height; Fig. 1), with an opening for us to record the subjects' behaviours.

Experimental Protocol

The protocol involved two distinct periods, for all treatments: (1) the acclimatization period, which lasted 24 h, (2) and the interaction period, with a duration of 30 min. In the AA and AN treatments, subjects acclimatized to the experimental set-up while exposed to the audience (priming), that is, no opaque partitions were placed between them and the audience tank during this period (Fig. 1). The NA and NN pairs acclimatized without visual contact with the audience (no priming). After this period, the aggressive interactions took place. In all treatments, the opaque partitions between subjects within the same arena were lifted and focal males were allowed to interact. During the interaction visual contact with the audience was allowed in the AA and NA treatments (i.e. no opaque partition between the subjects and the audience tanks), whereas in the AN and NN treatments subjects interacted with each other without the presence of an audience (i.e. with the opaque partitions between them and the audience tanks). The placing or removal of these partitions, right before the contest started, was also mimicked for treatments in which this did not occur (i.e. the AA and NN treatments). In each session four pairs of males were tested (not simultaneously). The four *experimental* treatments (one for each arena) were used in a balanced order, such that all treatments were applied in all arenas, throughout the 24 sessions of this experiment.

At the end of each session, all subjects were anaesthetized with a Tricane (MS222) diluted solution (1:25 ml), weighed (g) and their standard length measured (cm). There were no differences in body weight and length between opponents among the four experimental treatments (ANOVA: body weight: $F_{1,85}$: 0.268, $P = 0.606$; standard length: $F_{1,85}$: 0.648, $P = 0.423$).

Video Recording and Behavioural Analysis

To measure agonistic behaviours of the interacting pairs, a side view of the aggressive interactions between subjects was recorded using a video camera (SONY Handycam DCR–SR58E camera, 25 frames/s). The available ethogram of the agonistic behaviour of zebrafish was used to recognize relevant behaviour patterns (Oliveira et al., 2011). For each pair of males the following variables were measured: (1) latency to approach (s); (2) latency until the first aggressive event (s); (3) time in display/total time of the interaction (s); (4) frequency of displays (number of occurrences); (5) latency until the first display (s); (6) time in chasing/total time of the interaction (s); (7) frequency of chases (number of occurrences); (8) latency to chase (s); (9) interaction resolution point (i.e. point in time at which one fish became dominant and the other subordinate); (10) preresolution bite rate (number of occurrences/s) between the first aggressive event (display or bite) and the

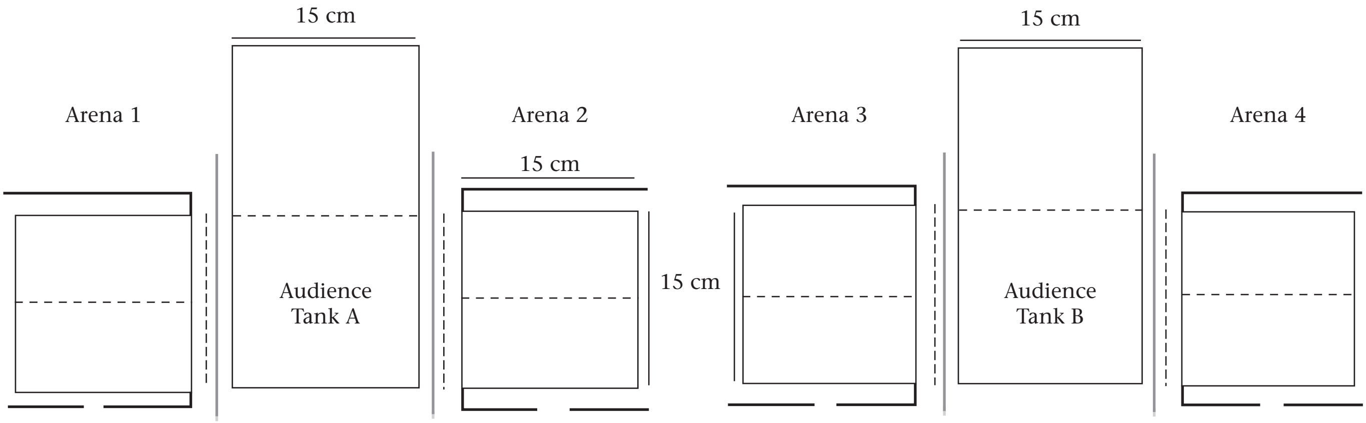

Figure 1. Experimental set-up. The light grey lines represent one-way mirrors and the dotted lines represent removable opaque partitions. Barriers surrounding the arenas protected the fish from the experimenter's presence. Audience tanks A and B accommodated the audience, while arenas 1 to 4 were used to promote the interactions between focal subjects (four pairs per session).

resolution point; (11) postresolution bite rate (number of occurrences/s) between the resolution point and the end of the interaction; (12) total number of bites; (13) latency to first bite (s); (14) number of resolved interactions (i.e. where a resolution point was present) per group. The interaction resolution point was used to estimate two different measures: (1) the latency (i.e. the time it took the opponents) to resolve the interaction (continuous measure) and (2) the number of interactions that were resolved (categorical measure). When behaviours were not observed their latencies assumed the total time of the interaction. Similarly, when the interactions were not resolved (i.e. a dominance relationship was not established) the interaction resolution point assumed the value of the total time of the interaction. All behavioural variables were measured using a multi-event recorder software (OBSERVER XT version 7, Noldus Inc., Wageningen, the Netherlands).

The occurrence of the different behavioural patterns in zebrafish agonistic interactions follows a temporal pattern (Oliveira et al., 2011). Displays are characteristic of the preresolution phase of the interaction, that is, when no dominance relationship between individuals has yet been established. Chasing is typical of a postresolution phase, when a dominant individual actively chases a subordinate one. Biting can occur throughout the agonistic interaction, although it is more frequent in the postresolution phase. However, in some interactions dominance was established without the occurrence of displays (see Results).

For each interaction, an escalation of aggression index was also computed as the frequency of chasing plus total bite rate over the frequency of display and frequency of chasing plus total bite rate. This escalation index is, therefore, a measure of the relative amount of overt aggression for each interaction. We also scored how many interactions included mutual display behaviour and how many had a clear resolution point (i.e. with clear dominance established between males) per group.

Statistical Analysis

Of the 96 pairs tested, seven were excluded from the analysis because males did not interact (two from the AA group; four from the AN group and one from the NN group). A two-way factorial ANOVA was used to assess the effects of priming and audience on the measured variables and escalation index. Data normality was tested using the Shapiro–Wilk's test and homoscedasticity of variances was tested using the Levene's test. A square root transformation was performed for the latency to display, time in chasing/total time, latency to chase, latency to bite and the frequencies of display and chase. For the escalation index variable of resolved interactions, we performed an arcsine square root transformation. The proportion of interactions with displays and the proportion of resolved interactions were analysed using Pearson chi-square tests, in order to determine the influence of priming and exposure to an audience during the contest on these variables. When necessary, multiple comparisons between groups were performed using Bonferroni corrections ($\alpha = 0.05$). To compare the proportion of resolved interactions with displays (in relation to the total number of resolved interactions), we used a two-tailed Fisher's exact test. Also in this case, multiple comparisons followed by Bonferroni corrections ($\alpha = 0.05$) were made. All statistical tests were performed with the software package STATISTICA v.12 (Statsoft Inc., Tulsa, OK, U.S.A.).

Ethical Note

No fish was injured as a result of the aggressive interactions. Audience tanks' (A and B) ammonia and nitrate concentrations were monitored throughout the experiment. All fish were returned to their home tanks at the end of the experiment. Sample sizes took in consideration the expected effect size of the experimental manipulations. This experiment was conducted under the permit for animal experimentation no. 008955 issued by the relevant Portuguese Authority (Direção Geral de Alimentação e Veterinária).

RESULTS

Effect of Priming and Presence of an Audience

There were no main effects of the presence of an audience on any of the behavioural measures (Table 1). There were main effects of priming both in the latency to display and in the time spent chasing/total time, and a marginally nonsignificant effect in the frequency of chases (Table 1). There were no interactions between audience and priming effects for any of the behavioural variables (Table 1). Also, there were no main effects either of the presence of the audience or of priming, or of their interaction, on the escalation index (Table 1).

The structure of the agonistic interactions observed in this study varied qualitatively in terms of both expression of aggressive displays by the opponents and their outcome. Based on these two parameters (i.e. occurrence or not of aggressive displays and establishment or not of a dominance relationship), three types of agonistic interactions were considered: (1) resolved interactions with aggressive displays ($N = 38$); (2) resolved interactions without aggressive displays ($N = 26$); (3) unresolved interactions, which could or could not have aggressive displays ($N = 25$; Appendix Table A1, Fig. A1). The effects of the experimental treatments on these two parameters are analysed below.

Occurrence of Aggressive Displays

The presence of an audience (i.e. AA+NA versus AN+NN) during an aggressive interaction was associated with a significantly higher proportion of interactions with displays (Pearson chi-square test: $\chi^2_1 = 4.088$, $P = 0.043$). However, no influence of priming (i.e. AA+AN versus NA+NN) was found ($\chi^2_1 = 3.487$, $P = 0.062$; Fig. 2a). Furthermore, when the proportion of interactions with displays was compared across all treatments (i.e. AA versus AN versus NA versus NN), it was found that their proportion in the AA group differed significantly from all other groups (Pearson chi-square test: AA versus AN: $\chi^2_1 = 14.438$, $P = 0.0001$; AA versus NA: $\chi^2_1 = 13.252$, $P = 0.0003$; AA versus NN: $\chi^2_1 = 10.761$, $P = 0.001$), while no differences were found between the AN, NA and NN groups (Fig. 2a). Both priming and exposure to an audience during the contests had a marginally nonsignificant influence on the escalation index of interactions with display (factorial ANOVA: priming: $F_{1,55} = 3.923$, $P = 0.053$; exposure to audience: $F_{1,55} = 2.890$, $P = 0.095$; priming*exposure to audience: $F_{1,55} = 0.289$, $P = 0.593$).

Interaction Resolution

Neither the presence of an audience nor priming influenced the occurrence of the interaction resolution point (Pearson chi-square test: priming: $\chi^2_1 = 1.747$, $P = 0.186$; exposure to an audience: $\chi^2_1 = 0.822$, $P = 0.365$; Fig. 2b). However, the presence of an audience influenced the occurrence of resolved contests in which display had occurred (two-tailed Fisher's exact test: $P = 0.002$; Fig. 2b). Moreover, there was a main effect of priming, but not of the presence of the audience during the interaction, on the time to the interaction resolution point (Table 1). When multiple comparisons were performed, we found that pairs in the AA group had more postdisplay resolutions than AN (two-tailed Fisher's exact test: $P < 0.0001$), NA (two-tailed Fisher's exact test: $P = 0.003$) and NN

Table 1
Summary of the factorial ANOVA results for all measured behavioural and interaction variables

Variables	Mean±SE				Priming		Audience		Priming×audience	
	AA	AN	NA	NN	F_{85}	*P*	F_{85}	*P*	F_{85}	*P*
Behavioural variables										
Latency to first aggression (s)	73.8±18.5	158.7±47.7	103.3±23.7	111.0±30.7	0.09	0.77	2.23	0.14	1.55	0.22
Time in display/total time (s)	0.14±0.04	0.12±0.05	0.14±0.03	0.19±0.05	0.48	0.49	0.07	0.79	0.81	0.37
Frequency of display (*N*)	41.0±6.9	30.7±11.6	42.5±9.2	46.4±11.1	0.01	0.91	1.14	0.29	1.91	0.17
Latency to display (s)	578.0±112.9	848.7±163.3	1049.0±146.1	1154.1±121.0	8.29	0.005*	2.37	0.13	0.08	0.78
Time in chasing/total time (s)	0.19±0.03	0.16±0.03	0.10±0.02	0.11±0.03	8.07	0.006*	0.87	0.35	0.18	0.67
Frequency of chasing (*N*)	42.6±6.3	43.3±7.7	30.7±6.8	30.5±7.5	3.68	0.058	0.13	0.71	0.09	0.76
Latency to chase (s)	629.3±78.5	874.6±119.1	907.9±101.1	910.7±132.5	2.05	0.16	1.27	0.26	1.22	0.27
Preresolution bite rate (*N*/s)	0.12±0.02	0.09±0.02	0.14±0.05	0.16±0.03	1.67	0.20	0.01	0.91	0.62	0.43
Postresolution bite rate (*N*/s)	0.29±0.02	0.27±0.06	0.25±0.04	0.26±0.08	0.15	0.70	0.01	0.90	0.08	0.78
Total number of bites (*N*)	401.7±34.4	342.7±40.0	345.6±33.8	394.1±51.5	0.003	0.96	0.02	0.90	1.74	0.19
Latency until first bite (s)	73.1±18.5	158.7±47.7	112.2±23.4	111.0±30.7	1.07	0.30	0.01	0.91	1.90	0.17
Interaction variables										
Resolution point (s)	1078.5±106.6	966.6±130.1	1201.8±109.7	1314.2±123.1	4.01	0.048*	0.000	1.0	0.91	0.34
Escalation index	0.91±0.01	0.92±0.02	0.90±0.02	0.88±0.03	0.02	0.90	0.33	0.57	1.08	0.30

Asterisks indicate significant *P* values.

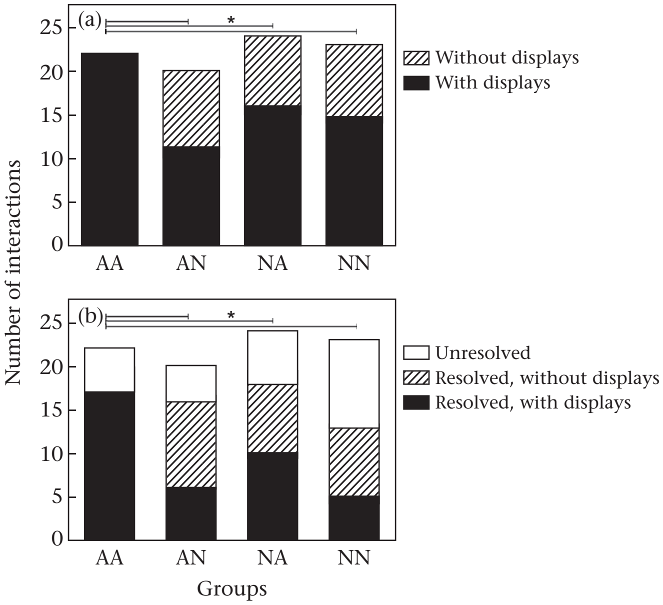

Figure 2. Proportion of (a) agonistic interactions with mutual display and (b) resolved interactions with and without displays, for the four experimental treatments: (AA) audience present both before and during the interaction; (AN) audience present before but not during the interaction; (NA) audience not present before but present during the interaction; (NN) audience absent both before and during the interaction. Significant differences between groups are marked with an asterisk.

groups (two-tailed Fisher's exact test: $P = 0.0002$). All other comparisons were not significant (two-tailed Fisher's exact test: AN versus NA: $P = 0.327$; AN versus NN: $P = 1.000$; NA versus NN: $P = 0.473$). Finally, resolved interactions between males that occurred in the presence of an audience had a lower escalation index value (0.921 ± 0.014, $N = 35$) than contests without an audience (0.962 ± 0.011, $N = 29$; $F_{1,60} = 5.008$, $P = 0.028$).

DISCUSSION

The results presented here demonstrate that the presence of an audience affects agonistic behaviour in male zebrafish. Moreover, the timing of exposure to the audience relative to the interaction (i.e. before versus during the interaction) had different effects on the behaviour of the interacting individuals. Pre-exposure to an audience induced a shorter latency to display, an increase in the time dominants spent chasing subordinates and a shorter time to interaction resolution (i.e. for the establishment of a dominance relationship). Together, these results indicate that pre-exposure to an audience induces aggressive priming in zebrafish. Exposure to the audience during the interaction led to a higher number of interactions in which mutual displays occurred, a higher number of resolved interactions with display behaviour and a decrease in the escalation index for resolved interactions. Together, these results indicate a decrease in overt aggression when interacting in front of an audience.

The positive priming effect on aggressive behaviour described here has already been reported for other species and has been interpreted as a change in motivational state (e.g. Hogan & Bols, 1980; Matos et al., 2003; Potegal & Popken, 1985). This interpretation is compatible with our results in zebrafish that show a shorter latency to engage in the contest, as indicated by the shorter latency to display, and an increased persistence to fight, as indicated by the increase in the time dominant males spent chasing the subordinates. An increase in aggressive motivation in response to the presence of a mixed-sex conspecific audience before the interaction occurred can be explained by the fact that, in zebrafish, dominant males have a higher reproductive success than subordinates (Paull et al., 2010). Therefore, once a breeding opportunity in the presence of other male competitors has been identified it is adaptive to increase aggressive motivation in order to gain access to females. Moreover, by increasing their aggressive behaviour, males also reinforce their dominance status towards bystanders and consequently guarantee their access to females, while keeping away potential rivals, such as those in the audiences.

The decrease in overt aggression when interacting in front of an audience observed here contrasts with the game theory analysis of eavesdropping, which predicts an increase in aggressiveness given the extra benefit of a positive image (reputation) to be gained by winning the agonistic interaction in the presence of an audience (Johnstone, 2001). This model has the assumption that a mechanism similar to image scoring, which has been proposed to explain indirect reciprocity in cooperative interactions (Nowak & Sigmund, 1998; for an example of image scoring in fish see Bshary & Grutter, 2006), is also in place in agonistic interactions. Accordingly, the winner of an eavesdropped interaction would be more likely to win the next interaction with the eavesdropper, because the latter would have a 'positive image' of the observed winner and would be less likely to challenge it. However, if there is an asymmetry in the payoff matrix of the agonistic interaction such that the costs of losing are higher than the benefits of winning, then the costs of a 'negative image' should make the contenders behave in a less aggressive way. There is some evidence for such asymmetry in the literature of winner and loser effects that shows that the effects of losing last longer and are typically more acute than those of winning, even in the absence of an audience (Rutte, Taborsky, & Brinkhof, 2006). These differential gains for winners and losses for losers are expected to be amplified when interacting in front of an audience, since not only the opponent but also the bystanders will gain information on the competitive ability of the interacting individuals (Peake & Mcgregor, 2004). The increasing number of studies that have reported that bystanders can eavesdrop on others' agonistic interactions and use the gathered information in subsequent interactions with the observed individuals (Amy & Leboucher, 2009; Earley & Dugatkin, 2002; McGregor, Peake, & Lampe, 2001; Oliveira, McGregor, & Latruffe, 1998; Peake, Terry, McGregor, & Dabelsteen, 2001) reinforce the idea that losing in front of conspecifics might come at a higher price. This asymmetry between winner and loser effects is also present in zebrafish (Oliveira et al., 2011) and hence may help to explain the observed decrease in overt aggression when interacting in the presence of the audience.

The occurrence of aggressive priming and audience effects in a model organism, such as zebrafish, for which optogenetic and transgenic techniques are available for the visualization and manipulation of neural circuits in relation to behaviour, opens the way for future studies on the neuroethology of communication networks. Indeed, brain imaging of zebrafish using genetically encoded calcium indicators, such as GCaMP, allow single-cell resolution when mapping behaviour into brain activity (Baier & Scott, 2009; Portugues, Severi, Wyart, & Ahrens, 2013). Moreover, the limitation of having to use head-fixed preparations for image acquisition with these fluorescent reporters, which limits the behavioural tasks that can be investigated, has been recently overcome by the development of a virtual reality system. In this system the fish is stationary but its fictitious movement in the virtual environment is directed in real time by the putative motor output recorded from its motor neuron axons in the tail (Ahrens et al., 2013). On the other hand, the use of opsin photoswitchable probes, such as channel rhodopsin and halorhodopsin, which activate neurons in a reversible way in response to light pulses, has allowed the study of gain and loss of function at the cell type level (e.g. Arrenberg, Del Bene, & Baier, 2009; Douglass, Kraves, Deisseroth, Schier, & Engert, 2008). Finally, the Gal-UAS binary transgenic system has been used to genetically target specific cell populations for the expression of the abovementioned reporters and manipulators of neural activity (Zhu et al., 2009), which allows the mapping of specific neural circuits to specific behaviours (e.g. Agetsuma et al., 2010; Muto et al., 2013; Okamoto, Agetsuma, & Aizawa, 2012). Therefore, the available zebrafish optogenetic toolbox offers an unprecedented opportunity to study the neural circuits underlying social communication and social abilities in a vertebrate model.

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Appendix

Table A1

Characterization of different types of interaction

Measured variables	Interaction type		
	Resolved with displays	Resolved without displays	Unresolved
Time in display/total time (s)	0.17±0.03	–	0.27±0.05
Time to fight resolution (s)	1056.6±60.7	711.5±94.3	–
Time in chasing/total time (s)	0.19±0.02	0.15±0.02	–
Preresolution bite rate (*N*/s)	0.12±0.01	0.07±0.02	0.22±0.05
Postresolution bite rate (*N*/s)	0.45±0.05	0.26±0.03	–

Resolved interactions with display had a clear resolution point, preceded by display behaviour. Resolved interactions without display also had a clear resolution point, but with no displaying behaviour before it. Unresolved interactions did not have a resolution point and, therefore, no postresolution phase behaviours such as chasing or postresolution bites. Displaying behaviours in the preresolution phase might or might not have occurred.

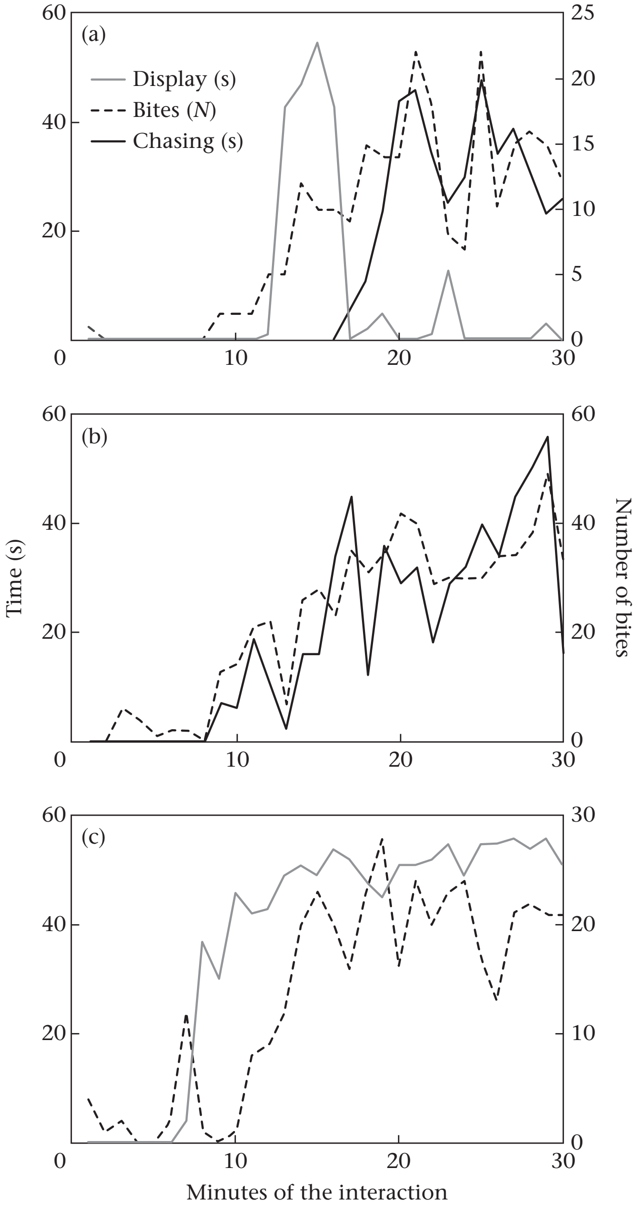

Figure A1. Representative examples of different types of agonistic interactions in zebrafish: (a) interaction resolved with mutual displays (i.e. with the occurrence of a displaying phase before resolution was established); (b) interaction resolved without mutual displays (i.e. displaying did not occur in the preresolution phase); (c) unresolved interactions, in this case with prolonged mutual displaying and no clear resolution point.