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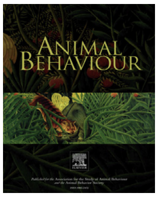

# Audience effects and aggressive priming in agonistic behaviour of male zebrafish, *Danio rerio*

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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).