



Integrative study of multimodal communication in two models of vertebrates : Maylandia zebra and Crocodylus niloticus

Laura Chabrolles

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Integrative study of multimodal communication
in two models of vertebrates: *Maylandia zebra*
and *Crocodylus niloticus*.

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General Introduction

A. Animal communication and multimodality.

Animal communication is an elaborate system encompassing complex signalling displays to interact with conspecifics and heterospecifics across a variety of social contexts (Bradbury & Vehrencamp, 2011). It implies a signal carrying information and transmitted through an environment, from a sender to a receiver. According to Bradbury & Vehrencamp (2011), signals are “stimuli produced by a sender and monitored by a receiver, to the average net benefit of both parties”. It should provide information to another animal about correlated conditions. If those information give mutual benefits for both the sender and the receiver, mutations in either party that improve the process will be favoured over evolutionary time.

When it includes signals from different sensory modalities, simultaneously or in sequence, it is referred to as multimodal communication (Candolin, 2003; Hebets & Papaj, 2005; Partan & Marler, 2005; Smith & Evans, 2013). The complexity of animal communication has been known for some time (Darwin & Prodger, 1998) and is now a topic of research interest with more studies on multimodal communication across species (Duistermars & Frye, 2008; Estramil et al., 2014; Fröhlich & van Schaik, 2018; Gordon & Uetz, 2011; Halfwerk et al., 2014; Wierucka et al., 2018). This growing interest received a new impulse from a study proposing a framework for categorizing multimodal signals based upon receiver behavioural responses to either separate components within a sensory modality or in multimodal composite signals (Partan & Marler, 1999). Even though there is a growing interest for multimodal communication, most studies have investigated signals in isolation or with two modalities combined (Hebets & Papaj, 2005; Higham & Hebets, 2013).

B. Costs and benefits of multimodal communication.

The limitations of the capacities of the different sensory modalities could support the use of the multimodal communication (Bradbury & Vehrencamp, 2011; Higham & Hebets, 2013). Visual and acoustic cues are perceived almost instantaneously but could be easily disturbed by the environment. A visual signal may be blocked by physical barriers. However, although acoustic signals may attenuate with distance they suffer little disruption from physical barriers but will be disrupted by other noise. Olfactory signals travel slowly but remain for longer periods. Multimodal signalling could then compensate the drawbacks of each modality to increase efficacy during communication. But multimodality could also imply costs for the sender and receiver. Complex signalling increase energy expenditure, conspicuousness to predators or eavesdropping (Bro-Jørgensen, 2010; Hebets & Papaj, 2005). Yet, the use of multimodal communication in animals is relatively common (Higham & Hebets, 2013; Rowe & Halpin, 2013; Uy & Safran, 2013).

C. Classification of multimodal signals.

So far, multimodal signals have been categorized as redundant and non-redundant according to the receiver's response and the information they convey (Johnstone, 1996; Partan & Marler, 1999) ([Fig 1](#)).

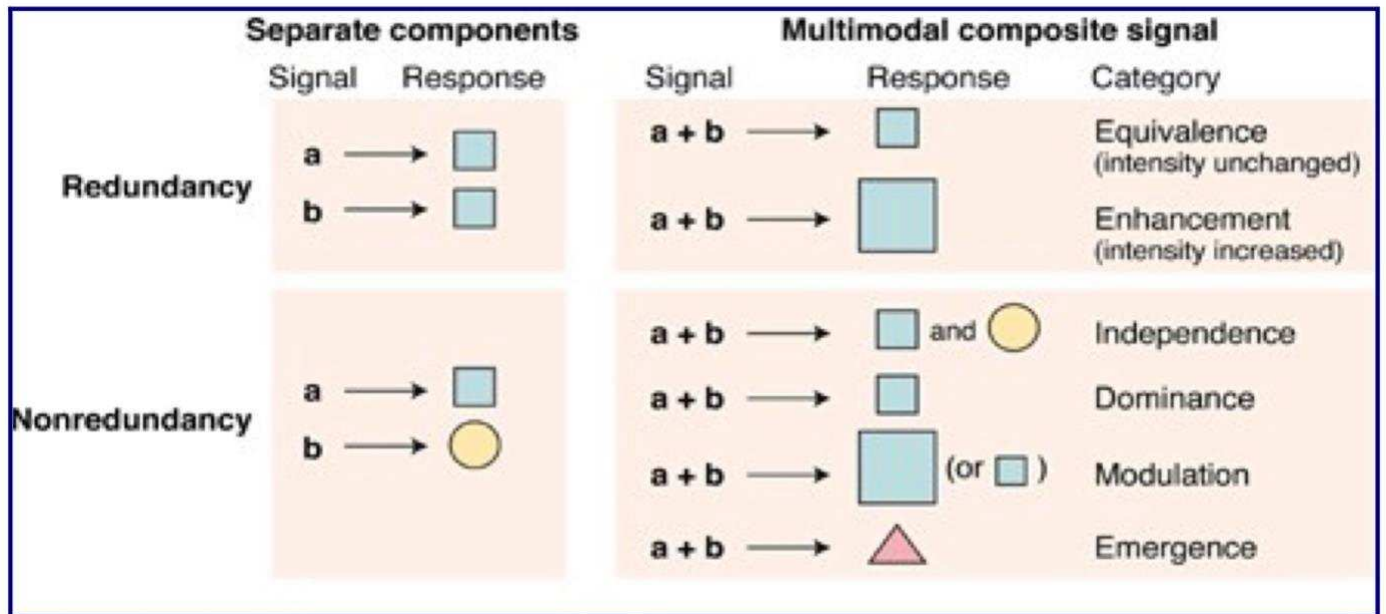


Figure 1. Classification of multimodal signals according Partan & Marler, (1999). Redundant signals are depicted above, nonredundant signals below. The left part represents the responses to two separate components (a and b) represented by geometric shapes (the same shape indicates the same qualitative response; different shapes indicate different responses). The right part represents responses to the combined multimodal signal.

Signals providing the same information can increase the accuracy of the receiver response and are called redundant. They also illustrate the ‘backup signal’ hypothesis as termed by Johnstone (1996). If the receiver response intensity is the same as in an unimodal cue exposure, those redundant signals are qualified as ‘equivalent’, but if the intensity is increased they are ‘enhancement’ signals (Partan & Marler, 1999). Non-redundant signals or multiple messages are signals that provide different information (i.e. different types of information or different aspects of the signaller quality) (Hebets & Papaj, 2005; Johnstone, 1996; Partan & Marler, 1999). Then if the response observed to combined signals correspond to the same separate responses to each signal presented alone, those signals are independent. Dominance is when the response elicited by one signal dominates the other, modulation is when a signal

modulates the other and finally emergence is when a new response emerges, different from when signals are presented separately (Partan & Marler, 1999). But the redundant/non-redundant framework may not always fit the variation observed (Rowe, 1999). Other classifications have been proposed focusing more on inter-signal interactions and signal efficacy (Hebets & Papaj, 2005). And other factors must be taken into account such as the influence of the environment, the physical properties of sensory cues, the interactions among traits, the perceptive/production abilities of the individuals and their recent experience (Hebets et al., 2016; Higham & Hebets, 2013). Exploring this complex system will help to understand the selection pressures acting on multimodality (Candolin, 2003; Hebets et al., 2016; Hebets & Papaj, 2005; Rowe & Halpin, 2013).

D. Territorial defence in a cichlid, *Maylandia zebra*, constitutes a good model for studying multimodal communication.

Conflict resolution constitutes a good context for exploring multimodality and inter-signals interaction. Each individual involved in this social interaction must assess its own fighting ability, motivation and experience compared to its opponent's (Bradbury & Vehrencamp, 2011). Consequently, both individuals are senders and receivers and must exchange information via signals and cues to accurately process the information and make adaptive behavioural responses (Bradbury & Vehrencamp, 2011; Van Staaden et al., 2011). Selection pressure on communication during conflict resolution ought to be strong, as both competitors would benefit if the contest is settled with displays. An escalated fight could lead to serious injuries which could represent a significant cost for both competitors (Bradbury & Vehrencamp, 2011; Van Staaden et al., 2011).

Escobar-Camacho & Carleton (2015) in their review, showed that cichlids rely on different sensory modalities. But we do not know how these modalities interplay during communication. As the different sensory cues do not have the same active space or persistence in time, there could be a hierarchy among the sensory modalities, in the time or the order they are used. For example, when choosing a mate, a female can first evaluate from a distance conspecific males from heterospecific males with visual cues (Fig 2). If the interaction continues at a closer range, more information could be obtained from acoustic and chemical cues (Escobar-Camacho & Carleton, 2015) (Fig 2).

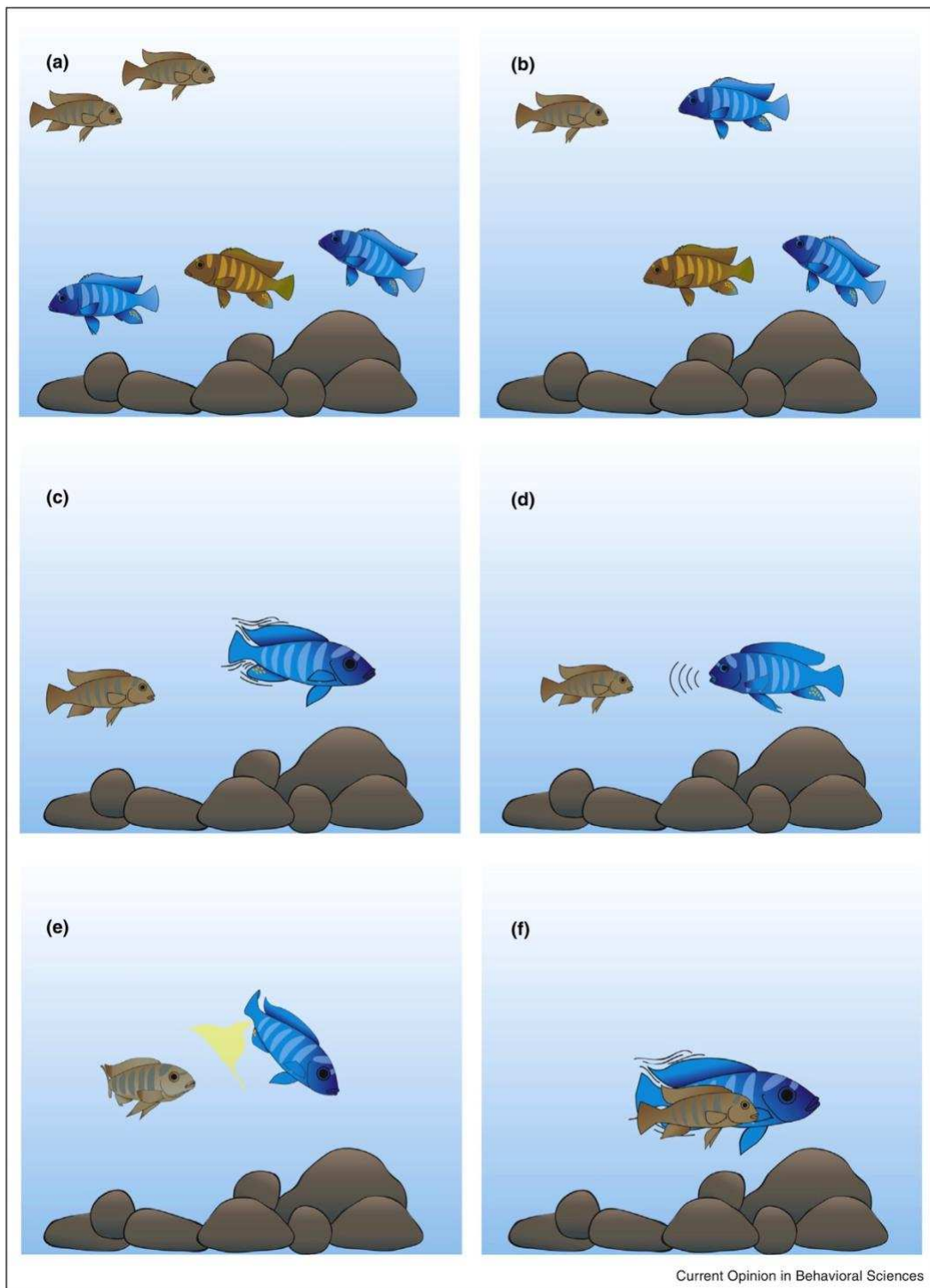


Figure 2. Behaviours associated with multimodal courtship in cichlids according to Escobar-Camacho & Carleton (2015). They show an ethogram for the Malawi species *Maylandia zebra*. **(a)** Females swim freely above the lek and view males on their territories. Females may first use visual cues to detect conspecifics or dominant individuals looking for their pigmentation colour patterns. **(b)** Males approach the females to attract them to their territory. Males may also use visual cues to select which females to approach. **(c)** Males

extending their fins and display in front of the females, quivering their fins and body. **(d)** Males producing vocalizations toward the female which may be coincident with their quivering. **(e)** Males exhibiting several urine pulses in the presence of females to stimulate eggs laying and to indicate his social status. **(f)** Males quiver close to the female potentially stimulating superficial neuromasts of the lateral line.

Maylandia zebra males are consequently ideal for trying to disentangle this complex multimodal communication, because they are highly territorial and they defend their territories by means of conspicuous displays involving multiples senses that are similar to those used during courtship (Escobar-Camacho & Carleton, 2015; Simões et al., 2008) (Fig 2).

Maylandia zebra is a lek-breeding cichlid living in the sediment-free rocky coast of Lake Malawi. They belong to the most diversified cichlids group, called *mbunas*. These territories are not only used for mating but also for feeding, and males defend them from other males for prolonged periods (Holzberg, 1978). Their body is blue with some black strips. Females are less coloured. During agonistic encounters or courtship, the body becomes brighter bringing the black strips out reference.

The agonistic displays of *M. zebra* consist of a series of behaviours not necessarily performed in the same order. Generally, a dominant male will defend his territory by chasing a rival, and by erecting his fins and trembling (Simões et al., 2008). Those behaviours are respectively named lateral display and quiver. They can produce sounds while quivering but also, although rarely, when they are immobile (Simões et al., 2008). It has been shown in this species that sounds provide reliable estimation of the size of the male sender (Bertucci et al., 2012; Simões et al., 2008). If the use of non-contact fight behaviours during mutual assessment is not sufficient, the fights can increase to the use of contact fight behaviours, like darts, tail beating, mouth wrestling and bites. These close-range interactions during territorial encounters,

namely the initial display phase, include communication where males exchange signals in all sensory modalities.

E. Impact of internal physiological processes on multimodal communication in young Nile crocodiles, *Crocodylus niloticus*.



Figure 3. Nile crocodiles, *Crocodylus niloticus*, basking on an artificial riverbank at La Ferme aux Crocodiles, Pierrelatte, France. [Credits : Florence Levrero]



Figure 4. Group of young Nile crocodiles, *Crocodylus niloticus*, in the nursery at La Ferme aux Crocodiles, Pierrelatte, France. [Credits : Florence Levrero]

As it was emphasised by Hebets & Papaj (2005), not enough studies consider how the receivers detect and process signals. And yet the receiver's internal state can greatly influence the signal efficacy. Gong & Gibson (1996), for example, showed that females guppy, *Poecilia reticulata*, exposed to a novel predatory cichlid, entirely change their preferences in mating choice. While half of the females became sexually unreceptive after exposure, the remainder that initially showed strong preference for brighter males, preferred subsequently duller males. In the wolf spider, *Schizocosa uetzi*, Hebets (2003) showed that subadult females exposed to experimentally manipulated mature males (either black or brown forelegs) chose preferentially the male to whom they were previously exposed after maturation. The receiver variability could be linked to different levels of sensory detection but also to different internal processes. If

receivers vary in their capacity to process information in various modalities, then multimodal signalling would permit signallers to reach a broader audience.

Here we emphasise the importance that could have physiological processes, which could turn into internal conflicts about the appropriate behavioural responses that must be displayed. To address this issue, we were then interested in a physiological process that could be easily controlled. Crocodiles are good models to address how the physiological state influences animal responses during multimodal communication as they provide a system where physiological process could be easily controlled (Grigg, 2015). Crocodilians have a very slow metabolism, which allowed us to measure the impact of internal physiological processes on multimodal communication by accurately monitoring the animals' prandial state. Changes in behaviours according to the prandial state have been indeed documented in crocodilians, with sated individuals selecting places with higher temperatures and fasted individuals selecting places with lower temperatures (Grigg, 2015). Nile crocodiles are one of the more social crocodilians species (Vergne et al., 2007) (Fig 3). After hatching, young Nile crocodiles are relatively autonomous and then highly susceptible to predation (Pooley, 1977). While they must find their own food, they also benefit from the mother protection that guards the pod (Pooley, 1977) (Fig 4). They are dependent then from multiple sensory information: information coming from siblings to stay nearby the pod and avoid predation, information coming from preys, all of this according to their physiological state as crocodilians are ectotherms. Vocal communication indeed is really important in this species. Young crocodiles start to vocalize inside the egg and they continue to do so even after hatching (Vergne et al., 2007). Mother protection would be induced by young emitting distress calls and the cohesion among juveniles is maintained by contact calls emitted between young (Vergne, Pritz, & Mathevon, 2009). It is then expected that the influence of the individuals physiological state and the selective pressure on the perceptive abilities of young should be strong in this species. We chose to expose young Niles

crocodiles (fasted or sated) to food odour and/or social and non-social sounds, to see how those two modalities interacted and if this interaction changed when the prandial state was different.

F. Research objectives

This thesis is composed of three chapters. I detail below the questions addressed in each of them.

Chapter 1: Appraisal of unimodal cues during agonistic interactions in *Maylandia zebra*

A first step towards the understanding of how different modalities interact during communication, is to investigate the behavioural response in a 'cue-isolation' experiment (Partan & Marler, 1999; Smith & Evans, 2013). Several communication channels are known to be widely used by fish. Many authors have proposed that visual (Chen & Fernald, 2011; Dijkstra et al., 2010; Mellor et al., 2012; Rosenthal & Ryan, 2000) acoustic (Amorim et al., 2008; Bertucci et al., 2010; Longrie et al., 2013; Maruska, Ung, & Fernald, 2012) and chemical cues (Keller-Costa, Canário, & Hubbard, 2015; Martinovic-Weigelt et al., 2012; Plenderleith et al., 2005) are highly relevant for intraspecific communication. Additional studies revealed the importance of the lateral line system during social encounters (Butler & Maruska, 2015, 2016). However, much remains unknown regarding the role of each sensory modality in eliciting behavioural decisions, particularly their relative hierarchy during multi-modal sensory integration. For example, acoustic and chemical cues are rarely analysed alone and typically need a visual trigger to elicit a behavioural response (Bayani, Taborsky, & Frommen, 2017; Estramil et al., 2014). The aim of this work was to test with a 'cue-isolation' experiment the

prioritization of sensory modalities by investigating how fish respond to isolated chemical, visual and acoustic signals in an agonistic context. It further aimed to develop a bioassay to test the role of each sensory channel on behavioural decisions by assessing the response to different stimulus types, e.g., video playback vs. live male stimulus.

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Chapter 2: Agonistic interactions in *Maylandia zebra* males go multimodal

Animals communicate through multiple senses which allow them to detect and integrate information during social interactions. To better understand animal communication we must consider the role and interaction of the different sensory modalities involved. It is well known that communication usually relies on a multiple sensory system (Darwin & Prodger, 1998), but recently there is a growing interest in understanding how different sensory channels are integrated to produce adaptive behaviour (Higham & Hebets, 2013; Partan & Marler, 2005). Several empirical species-specific studies on multimodal communication used different methodologies and approaches that can be employed in multimodal research and illustrate a variety of behavioural responses (Munoz & Blumstein, 2012). However, the majority of conceptual and empirical studies to date have focused on uni- or bimodal signals (Bertucci et al., 2010; Maruska & Fernald, 2012). Thus, much remains unknown on how composite signals (considering all used modalities) contribute to animal decision making. It is then of prior importance, to fully understand the evolution of the multisensory communication taking into account the full communication repertoire. Following the previous study, we here aimed to investigate how combined chemical, visual and acoustic signals interact to produce an adaptive agonistic behaviour by males of *M. zebra*.

Chapter 3: Cross-sensory modulation in a future top predator, the young Nile crocodile

As animal behaviour is commonly driven by information from different sensory channels, internal conflicts about the appropriate behavioural response regularly arise from concurrent biological stimuli. Inputs from a given sensory modality can thus modulate the perception and behavioural reaction to another stimulus (Munoz & Blumstein, 2012; Otto, Dassy, & Mamassian, 2013; Partan & Marler, 1999). For instance, olfaction has been shown to be modulated by vision (Demattè, Sanabria, & Spence, 2009; Gottfried & Dolan, 2003) and sounds (Wesson & Wilson, 2010). Although probably widespread, this cross-sensory modulation of perception has mainly been studied through sensory analysis, psychological and neurobiological approaches, but paying no or weak attention to its behavioural and adaptive correlates (Belkin et al., 1997; Cohen, Rothschild, & Mizrahi, 2011; Duistermars & Frye, 2008; Lewkowicz & Ghazanfar, 2006; Wallace & Stein, 2007). As other animals, crocodilians routinely experience simultaneous stimuli from distinct sensory channels—chemical, acoustical, visual. From an early age, they have to deal with decisional conflicts emerging from concurrent stimuli, with crucial advantages in integrating various sensory cues to interact with congeners and improve prey localization and capture (Grigg, 2015). Using an experimental paradigm with young Nile crocodiles *Crocodylus niloticus* allowing testing of their behavioural responsiveness to food odour and/or social and non-social sounds, we highlight a cross-modulation effect between olfactory and acoustic inputs. Strikingly, this cross-modulation varies according to an important regulating factor of motivation, the prandial state of the animals.

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-Chapter 1-

Appraisal of unimodal cues during agonistic interactions in *Maylandia zebra*

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Keywords: Playback experiments, Visual, Acoustic, Agonistic interactions, Unimodal signals, Olfaction

Appraisal of unimodal cues during agonistic interactions in *Maylandia zebra*

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ABSTRACT

Communication is essential during social interactions including animal conflicts and it is often a complex process involving multiple sensory channels or modalities. To better understand how different modalities interact during communication, it is fundamental to study the behavioural responses to both the composite multimodal signal and each unimodal component with adequate experimental protocols. Here we test how an African cichlid, which communicates with multiple senses, responds to different sensory stimuli in a social relevant scenario. We tested *Maylandia zebra* males with isolated chemical (urine or holding water coming both from dominant males), visual (real opponent or video playback) and acoustic (agonistic sounds) cues during agonistic interactions. We showed that (1) these fish relied mostly on the visual modality, showing increased aggressiveness in response to the sight of a real contestant but no responses to urine or agonistic sounds presented separately, (2) video playback in our study did not appear appropriate to test the visual modality and needs more technical prospecting, (3) holding water provoked territorial behaviours and seems to be promising for the investigation into the role of the chemical channel in this species. Our findings suggest that unimodal signals are non-redundant but how different sensory modalities interplay during communication remains largely unknown in fish.

Subjects Animal Behavior, Zoology

Keywords Playback experiments, Visual, Acoustic, Agonistic interactions, Unimodal signals, Olfaction

INTRODUCTION

Research on animal conflict is critical to our understanding of social interactions. A common cause of conflict is the dispute for territories which can be key in acquiring food or mates (*Hurd & Enquist, 2001*). Agonistic interactions during territorial contests can provoke serious injuries, but contestants can also avoid costly fights by signalling their status and performing threat displays to elicit the opponent's withdrawal (*Hurd & Enquist, 2001; Van Staaden, Searcy & Hanlon, 2011*). Communication is thus fundamental in resolution of animal conflicts.

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Animals communicate through multiple senses which allow them to detect and integrate information during social interactions. To better understand animal communication, we must consider all the different sensory modalities involved. It is well known that communication is a multiple sensory system (Darwin, 1998), but recently there is a growing interest in understanding how different sensory channels are integrated to produce adaptive behaviour (Partan & Marler, 2005; Higham & Hebets, 2013). A first step towards the understanding of how different modalities interact during communication, is to investigate the behavioural response in a ‘cue-isolation’ experiment (Partan & Marler, 1999; Smith & Evans, 2013). Several communication channels are known to be widely used by fish. Many authors have proposed that visual (Rosenthal & Ryan, 2000; Dijkstra et al., 2010; Chen & Fernald, 2011; Mellor et al., 2012), acoustic (Amorim et al., 2008; Bertucci et al., 2010; Maruska, Ung & Fernald, 2012; Longrie et al., 2013) and chemical cues (Plenderleith et al., 2005; Martinovic-Weigelt et al., 2012; Keller-Costa, Canário & Hubbard, 2015) are highly relevant for intraspecific communication. Additional studies revealed the importance of the lateral line system during social encounters (Butler & Maruska, 2015). However, much remains unknown regarding the role of each sensory modality in eliciting behavioural decisions, particularly their relative hierarchy during multi-modal sensory integration. For example, acoustic and chemical cues are rarely analysed alone and typically need a visual trigger to elicit a behavioural response (Estramil et al., 2014; Bayani, Taborsky & Frommen, 2017). The aim of this work was to test with a ‘cue-isolation’ experiment the prioritization of sensory modalities by investigating how fish respond to isolated chemical, visual and acoustic signals in an agonistic context. It further aimed to develop a bioassay to test the role of each sensory channel on behavioural decisions by assessing the response to different stimulus types, e.g., video playback vs. live male stimulus.

African cichlids use multimodal signals to communicate during social interactions and became important models to evaluate the relative importance of different sensory channels (Escobar-Camacho & Carleton, 2015). *Maylandia zebra* is a lek-breeding cichlid living in the sediment-free rocky coast of Lake Malawi. Males of *M. zebra* defend territories to which they try to attract females by means of conspicuous displays that involve multiples senses (Plenderleith et al., 2005; Miguel Simões et al., 2008). These territories are not only used for mating but also for feeding, and males defend them from other males for prolonged periods (Holzberg, 1978). In this study, we exposed dominant males to the following stimuli: chemical (urine or holding water from dominant males; teleost Ringer solution was used as a control), visual (a real opponent in a jar or video playback), and acoustic (the playback of an agonistic sound). Based on previous studies (Bertucci et al., 2010; Maruska & Fernald, 2012), we predicted that vision would trigger direct agonistic behaviours and acoustic or chemical stimuli alone would rather induce indirect territorial behaviours such as increased activity and nest building.

MATERIALS & METHODS

Subjects and housing conditions

Seventy *Maylandia zebra* were purchased from Oxyfish (Verlinghem, France) and stored in holding tanks (120 cm long, 60 cm wide and 50 m height) at ENES laboratory (University of

Saint-Etienne, France). Each tank contained 10 males that were fed daily with commercial cichlid food (JBL NovoRift sticks and Tetra flakes). All tanks were equipped with an external filter (Rena Filstar xP3; Rena France, Annecy, France), aeration and PVC tubes as shelters. The water temperature was maintained at 25 ± 1 °C with a pH of 8.0 and a 12:12 h light:dark cycle was settled. Each fish was identified by a transponder (PSK Transponder; Dorset Identification B.V., Dorset, Netherlands).

General experimental design

The experimental aquarium (60 cm × 30 cm × 30 cm) was placed on a vibration-insulated shelf (with a layer of wood-fibre) in a sound-proofed chamber (PRIMO Silence-Box, Tip Top Wood, Saint-Etienne, France) to limit background noise. The back of the aquaria was covered on the inside with bubble wrap to break sound reflections on the tank's walls.

Each experimental tank had a filter containing active carbon, aeration, an internal heater, sand substrate, and a terracotta pot usable as a shelter by the fish, all located on the right side of the tank. Because the tested fish stayed in the experimental set up for five days, each aquarium was accompanied by a social group placed in an adjacent tank, to avoid the sense of isolation. These social groups were composed of one male and one female physically separated to avoid any injuries. An underwater loudspeaker developed by [Fonseca & Alves \(2012\)](#) was placed in the aquarium of the tested fish and used for playing back sound stimuli. A hydrophone (Aquarian Audio Products H2a-XLR; AFAB Enterprises, Anacortes, WA, USA, sensitivity: -180 dB re 1 V μ Pa-1, flat frequency response ± 4 dB 20 Hz–4.5 kHz) located in the middle of the tank, monitored sound playback and registered possible sounds produced by the tested fish.

The hydrophone was connected to a preamplifier (Yamaha MLA8; Yamaha Music France, Marne-la-Vallée, France) linked to the video capture card (Osprey-450e) of a PC that synchronized audio and video signals. Behaviour was recorded using a camera (BUL520; brand, Active Media Concept, Vallauris, France) positioned in front of the set-up.

To avoid any effect of novelty, a hermetic jar (20 cm × 11.7 cm × 25 cm) that hosted the intruder fish when the visual live stimulus was presented (see below) was left in the experimental tank during the five days of testing. A plastic tube (TYGON® R-3603; ID = 2.00 mm; Saint-Gobain Performance Plastics Verneret, La Mothe-Aux-Aulnaies, France) hooked up to the hydrophone was used during chemical presentation. When no chemical stimulus was sent, a piece of the same plastic tube was left during the week of experimentation. The presentation of chemical stimuli was either controlled through a peristaltic pump (IPC high precision tubing pump with planetary drive; ISMATEC, Switzerland) or an aquarium pump (Tecatantis EasyFlux300 and EasyFlux600; Aquatantis, Guimarães, Portugal) adjusted with a flow rate of about 1 L/10 min ([Kobayashi, 2002](#)). Both pumps were located outside the sound-proofed chamber (see [Fig. 1](#) for a complete view of the set-up).

Social hierarchies' assessment in community tanks

Only males with a dominance index above 0.7 (range from 0-subordinates, to 1-dominants) were selected for experiments or chemical sampling. To establish social hierarchies between

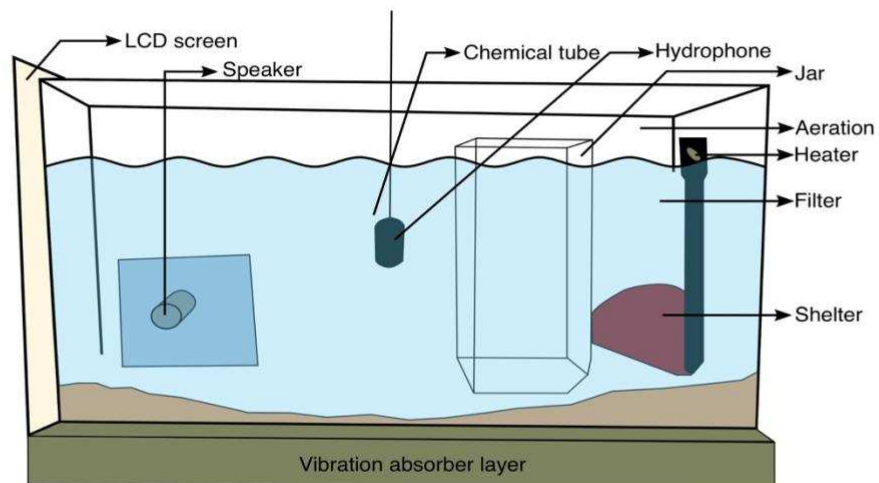


Figure 1 General experimental design. Dominant males were placed in the experimental aquarium forty-eight hours before the beginning of the experiment. First, the visual contact with the social group was interrupted through the LCD screen. The screen was switched on and was behind a removable opaque partition (no image was presented). Trials started with the control period (without stimulus) during five minutes, followed by the stimuli presentation during ten minutes: (1) Teleost Ringer solution, (2) urine from dominant males, (3) real opponent in the jar, (4) agonistic video playback, (5) agonistic sound playback or (6) holding water from dominant males. In experiment 1, fish received treatment (1)–(5) (one treatment per day) on consecutive days in a random order. In experiment 2, fish were exposed to treatment (6) only.

males in stock tanks, we observed the group during ten consecutive minutes before each sampling or experiment. Following [Barata et al. \(2007\)](#), a dominance index (DI) was used to quantify males' social rank. DI was calculated using the ratio between the number of dominant behaviours (aggressive displays or nest-digging) and the number of dominant behaviours plus the number of submissive behaviours (escape from an aggressive opponent).

Protocol for stimuli presentation

Twenty-two dominant males (standard lengths (SL) ranging from 6.5 to 10.9 cm; mean = 9.3 cm) were used to test behavioural responses to each sensory channel. They were placed in the experimental aquarium 48 h before the beginning of stimuli presentation. This study consisted in six different treatments: Teleost Ringer solution (1), urine from dominant males (2), real opponent in the jar (3), agonistic video playback (4), agonistic sound playback (5) and holding water from dominant males (6). In experiment 1, fourteen fish received treatment 1–5 (one treatment per day) on consecutive days in a random order. In experiment 2, nine fish were exposed to treatment 6 only. Experiment 2 was designed to develop another chemical stimulus (holding water) to achieve the best bioassay (holding water or urine) to test this channel. Holding water was tested in a separate experiment from urine to avoid any confounding effects from any potential chemical cues that lingered in the test tank. Different males were used in the two experiments except for one male that was used in both. After experiments, all subject males returned to their original tank and

the whole set up was rinsed with water mixed with white vinegar to eliminate any potential chemical cues in the aquarium, before new males were placed in the experimental tanks.

Each experiment lasted 15 min. Five minutes before the beginning of the test, filter, aeration, and internal heater were switched off. The visual contact with the social group was interrupted through the Samsung's 172x LCD 17" monitor used during video playback. The screen was switched on but covered by a removable opaque partition. At the same time, the loudspeaker was switched on and the real plastic tube for chemical presentation replaced the lure when necessary. The experiment started with a five minute control period while no stimulus was presented. This was followed by a ten minute period of stimulus presentation, in which either sound, chemical, or visual stimulation occurred. Details of the treatments were as follows:

Treatment 1. Teleost Ringer solution: 175 μ L of Ringer solution (Fresh water Teleost Ringer composition was obtained from the Biological Bulletin Compendia website) was presented four times during the stimulus presentation, when the subject fish approached or touched the pump's tube.

Treatment 2. Urine from dominant males: After assessing the fish's social rank, urine was collected by gently squeezing the abdomen from the anterior area to the genital papilla, following the method described in [Barata et al. \(2007\)](#). Once sampled, the fish was immediately placed back in its community tank. The urine from each male was stored at -80°C until use for the experiments. The collected amounts of urine from dominant males ($\text{DI} > 0.7$) ranged from 5 μ L to 340 μ L with a mean of 97 μ L. The chemical composition of the urine was not analysed.

During the stimulus presentation, 175 μ L of diluted urine (1:2 dilution with teleost Ringer solution) was delivered four times when the fish approached or touched the pump's tube. Samples came from the same dominant male, but collected at least on two different dates, and were randomly assigned from six different dominant males ($\text{DI} > 0.7$). To avoid any familiar effect, the urine came from a male unknown to the subject male, i.e., from a different community tank.

Treatment 3. Real opponent in the jar: a male, intermediate or dominant ($\text{DI} > 0.3$), matched in size with the subject, was introduced in the jar located inside the subject's tank, for ten minutes of presentation without previous acclimatization.

Treatment 4. Agonistic video playback: Video playback consisted of three sequences from the same stimulus male: two different biting sequences and one swimming sequence. The two biting sequences were each composed of two bites and lasted five seconds. All sequences (biting and swimming) were recorded with a Logitech c 910 webcam (Logitech HD Pro c910, 1080p) and played back interactively (see below). They were filmed frontally through a one-way mirror associated to a LED light (LED light; wave 5W, TETRA, France) for the biting sequences and a 11 W 230 V light bulb for the swimming sequence. Both biting and swimming sequences were randomly selected from four males with SL ranging from 7.1 to 10.7 cm (mean = 9.4 cm). To avoid any familiar effect, fish used to create the video playback came from different tanks than the subjects. The stimulus male and subject male were matched in size (i.e., SL ratio $\leq 7\%$).

At the beginning of the video playback session, the opaque partition was removed and the swimming sequence was immediately played back. As soon as the subject turned to the screen, one of the two biting sequences was played back. If the individual stayed in that position, the second biting sequence was played back and so on until the two different biting sequences were played back five times each. As soon as the tested fish stopped looking at the screen, the swimming sequence was put on the screen.

Treatment 5. Agonistic sound playback: Agonistic sounds were played back (115 dB re. 1 μ Pa at 3.8 cm, within the range of the natural male sounds) with custom-made underwater loudspeakers (frequency response ± 1.5 dB in the range 20 Hz–2 kHz) and a driver developed by [Fonseca & Alves \(2012\)](#). Sound stimuli were fed to the driver by a D/A converter (Edirol UA-25; Roland, Shizuoka-ken, Japan) controlled by Adobe Audition 3.0 (Adobe Systems Inc., Mountain View, CA, USA) on a laptop.

Two agonistic sounds of the same male were used as stimuli per experiment. These sounds were randomly assigned from six different males with SL ranging 6.0 cm–10.0 cm (mean = 9.4 cm) from the ENES sound archive (see [Bertucci et al., 2012](#)) and from new recordings made at the beginning of the study. All agonistic sounds presented 7–9 pulses (mean = 8.0 pulses) and a pulse rate of 18 ± 7 pulses per second. The pulses mean dominant frequency of the sounds was $289 \text{ Hz} \pm 133 \text{ Hz}$ (see [Bertucci et al. \(2012\)](#), for the description of sounds analysis). Pulses dominant frequencies were obtained with the 'dfreq' function from the 'seewave' R package ([Sueur, Aubin & Simonis, 2008](#)). As in treatment 4, playback was made interactively, simulating close interactions with a potential opponent. The stimulus was played back only when the tested individual approached the loudspeaker area, i.e., within 1–5 cm in front of the loudspeaker. Each sound was played back five times in a random order, which represented ten agonistic sounds in a test.

Treatment 6. Holding water from dominant males: After quantifying the social rank, dominant males ($Di > 0.7$) were selected and placed individually in a jar containing two litres of aerated water at 25 ± 1 °C during three hours. One litre of this solution was used during chemical presentation in the same day, to avoid any chemical degradation. We can assume the presence of urine in the holding water. Indeed, for the purposes of another ongoing study on chemical communication in the same fish, we injected three males with patent blue V (BDH Prolabo Chemicals, Fontenay-sous-Bois, France). Those males with an unknown status were small, median and large fish. Thirty minutes after the injection, we observed that fish were urinating blue and three hours after, we noted a complete blue coloration of the 2 L-aquarium's water. This demonstrates that the fish urinated during those three hours.

For this treatment, one litre of holding water was delivered continuously during the ten minutes of stimulus presentation. Holding water was obtained from eight different dominant males ($Di > 0.7$) and the water from one stimulus male was randomly assigned to subject males. As for urine, the holding water used came from a male unknown to the subject male.

Behavioural analysis

Videos were analysed using JWatcher software (v. 1.0). The aggressive behaviours (i.e., lateral displays, quivers, bites, darts and sounds produced) were counted and then

summed to produce an aggressive score (*Miguel Simões et al., 2008*). We quantified the number of up-and-down swimming (when a territorial fish was swimming up and down against the wall of the aquarium). We also quantified the tank's exploration score, for which the aquarium was artificially divided in three equal zones and the number of shifts between the three zones was counted. Finally, the nest maintenance behaviour (when a territorial fish was moving sand within its territory) and the number of the shelter's visit were also counted.

Statistics

All statistical tests were performed using R software (*R Core Team, 2016*). For each experiment, we analysed the influence of the treatments on five selected behaviours (agonistic, up-and-down swimming, tank's exploration, maintenance and the shelter's visit) separately.

Generalized linear mixed models ('glmer' functions from the 'lme4' R package, *Bates et al., 2014*) were used to assess the effect of the different treatments on the five selected behaviours. For models with Poisson family, overdispersion was tested, and if the model presented overdispersion we used a negative binomial model ('glmmADMB' R package, *Skaug et al., 2013*).

The following model was applied in experiment 1 for the five selected behaviours: **Model 1** <- Behaviour score ~ treatment * period + offset (log (Recording duration)) + (1| day) + (1| subject).

And the following model was applied in experiment 2 for the five selected behaviours: **Model 2** <- Behaviour score ~ period + offset (log (Recording duration)) + (1| subject).

Model 1 tested the two interacting factors (treatment and period). The variable treatment had five levels. For both models, the variable period had two levels: the control and the stimulus presentation. Two random factors were added to the models: the day of the experimentation (1| day) for **Model 1** and the subject identity (1| subject) for **Model 1** and **2**. An 'offset' took into account the different recording durations between the periods of control and stimulus presentation. *P-values* were assessed using the 'Anova' function ('car' R package, *Fox & Weisberg, 2011*). In **Model 1**, if the effect of the variable treatment alone or the effect of the interaction between factors was significant, post hoc tests using Tukey's adjustment were performed using 'lsmeans' function ('lsmeans' R package, *Lenth, 2016*).

Ethical note

All procedures described in this manuscript were conducted in accordance with appropriate French national guidelines, permits and regulations regarding animal care and experimental use (Approval no C42-218-0901, ENES lab agreement, Direction Départementale de la Protection des Populations, Préfecture du Rhône).

RESULTS

Experiment 1 — effect of chemical, visual and acoustic stimuli

The interaction between the treatment and the trial period had an impact on agonistic behaviour (*Fig. 2A*). The view of a real opponent increased the number of agonistic

behaviours observed during the stimulus presentation compared to the control period where no visual stimulus was present (Real opponent: $z = 4.252$, $P < 0.001$). Moreover, the number of agonistic behaviours during the presentation of a real opponent was significantly higher than the number of agonistic behaviours observed during the presentation of all the other treatments (Teleost Ringer solution: $z = 3.378$, $P = 0.005$; Urine from dominant males: $z = 3.300$, $P = 0.005$; Agonistic video: $z = 4.628$, $P < 0.001$; and Agonistic sound: $z = 3.334$, $P = 0.005$).

The interaction between the treatment and the trial period also had an impact on the up and down swimming (Fig. 2B). The view of a real opponent significantly decreased the number of up and down swimming during the stimulus presentation in comparison with the presentation of three other treatments: teleost Ringer solution ($z = -2.750$, $P = 0.051$), dominant males' urine ($z = -3.074$, $P = 0.051$) and agonistic sound ($z = -2.745$, $P = 0.051$).

When a real opponent was present, individuals had the tendency to increase tank's exploration during the stimulus presentation in comparison with the control period where no opponent could be seen (Fig. 2C, $z = 3.058$, $P = 0.056$).

Finally, we did not find any evidence that the number of shelter's visits (Fig. 2D, $\chi^2 = 2.055$, $P = 0.726$) and maintenance behaviour (Fig. 2E, $\chi^2 = 8.292$, $P = 0.081$) were influenced by treatment or trial period.

Experiment 2 — effect of holding water

Holding water had no significant effect on agonistic behaviour (Fig. 3A, $\chi^2 = 0.542$, $P = 0.462$). However, it significantly increased the number of up and down swimming (Fig. 3B, $\chi^2 = 9.161$, $P = 0.002$), tended to increase the tank's exploration (Fig. 3C, $\chi^2 = 2.922$, $P = 0.087$), but decreased significantly the number of visits to the shelter (Fig. 3D, $\chi^2 = 8.605$, $P = 0.003$). We found no evidence that maintenance behaviour (Fig. 3E, $\chi^2 = 0.657$, $P = 0.418$) was influenced by holding water.

DISCUSSION

Our study aimed to test behavioural responses during agonistic interactions to unimodal chemical, visual or acoustic signals in a social fish and to optimise a bioassay to study multimodal communication. Here we showed that only the visual channel elicited changes in the behaviour of dominant *M. zebra* males, including aggression and general activity (explorative behaviour and swimming up and down).

Unimodal signals

We found that the view of a real opponent provoked a highly aggressive reaction from the subject male. It also tended to increase the tank's exploration, which could be related to the subject attempting to reach the opponent confined in a jar. Subject males interacted with the intruder from three sides of the jar, ending up making more shifts between the aquarium zones. In addition, probably because they interacted for long periods with the intruder, the number of up and down swimming decreased. This aggressive response is in accordance with previous studies where visual cues alone were enough to elicit aggressive

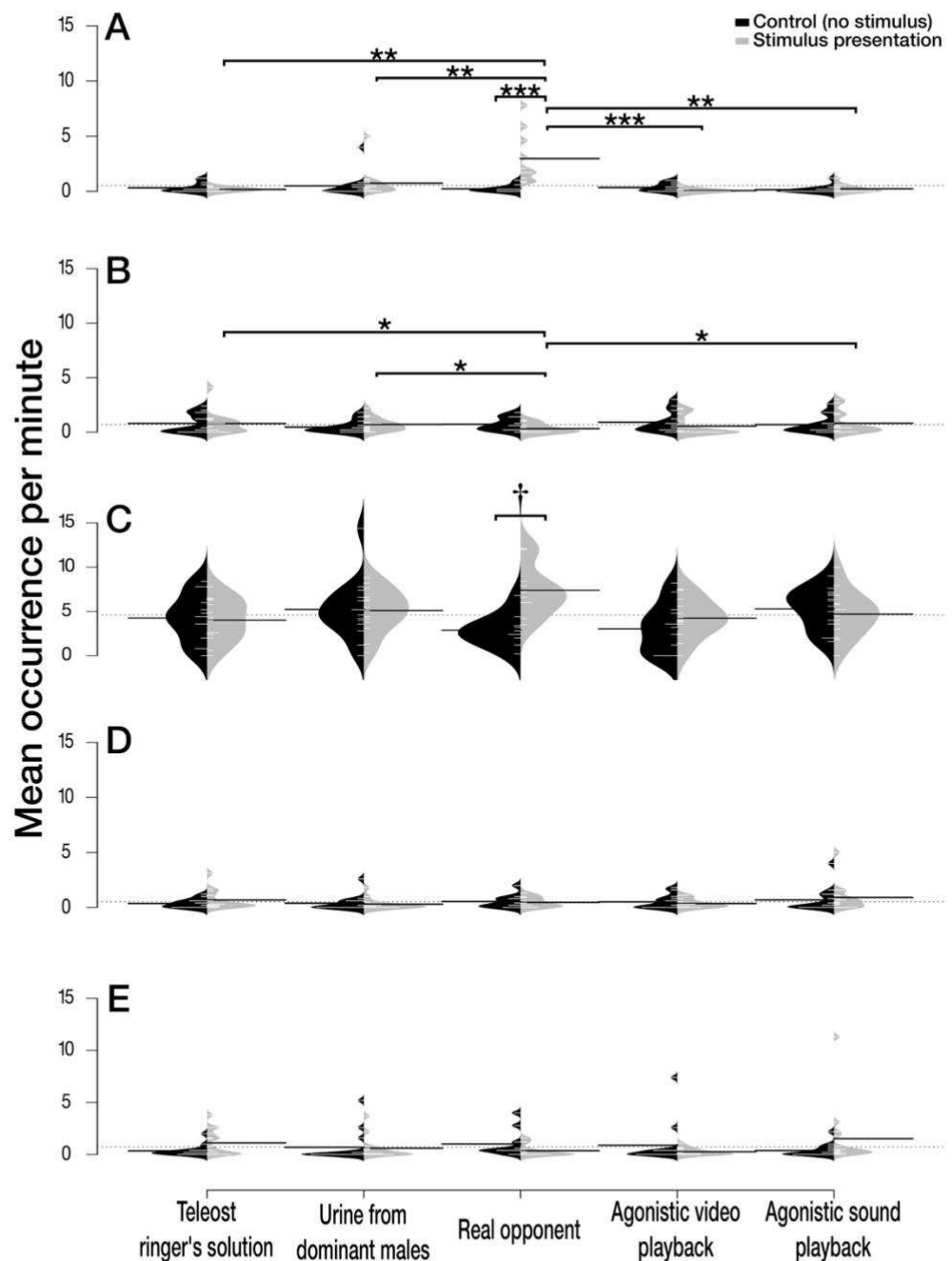


Figure 2 Effect of the treatments on five selected behaviours. Effect of the treatments (Teleost Ringer solution, urine from dominant males, real opponent, agonistic video playback or agonistic sound playback) on the number of (A) agonistic behaviours, (B) up and down swimming, (C) tank's exploration, (D) shelter's visit, and (E) maintenance. Each treatment included two periods, the control period without stimulus (in black) and the stimuli presentation (in light grey). Beanplots ('beanplot' R package (Kampstra, 2008)) combine individual observations (white lines), dataset distribution, the overall dataset average (dashed horizontal line) and the average for each subset (heavy horizontal black line). $^{\dagger}P \leq 0.1$; $*P \leq 0.05$; $**P \leq 0.01$, $***P \leq 0.001$.

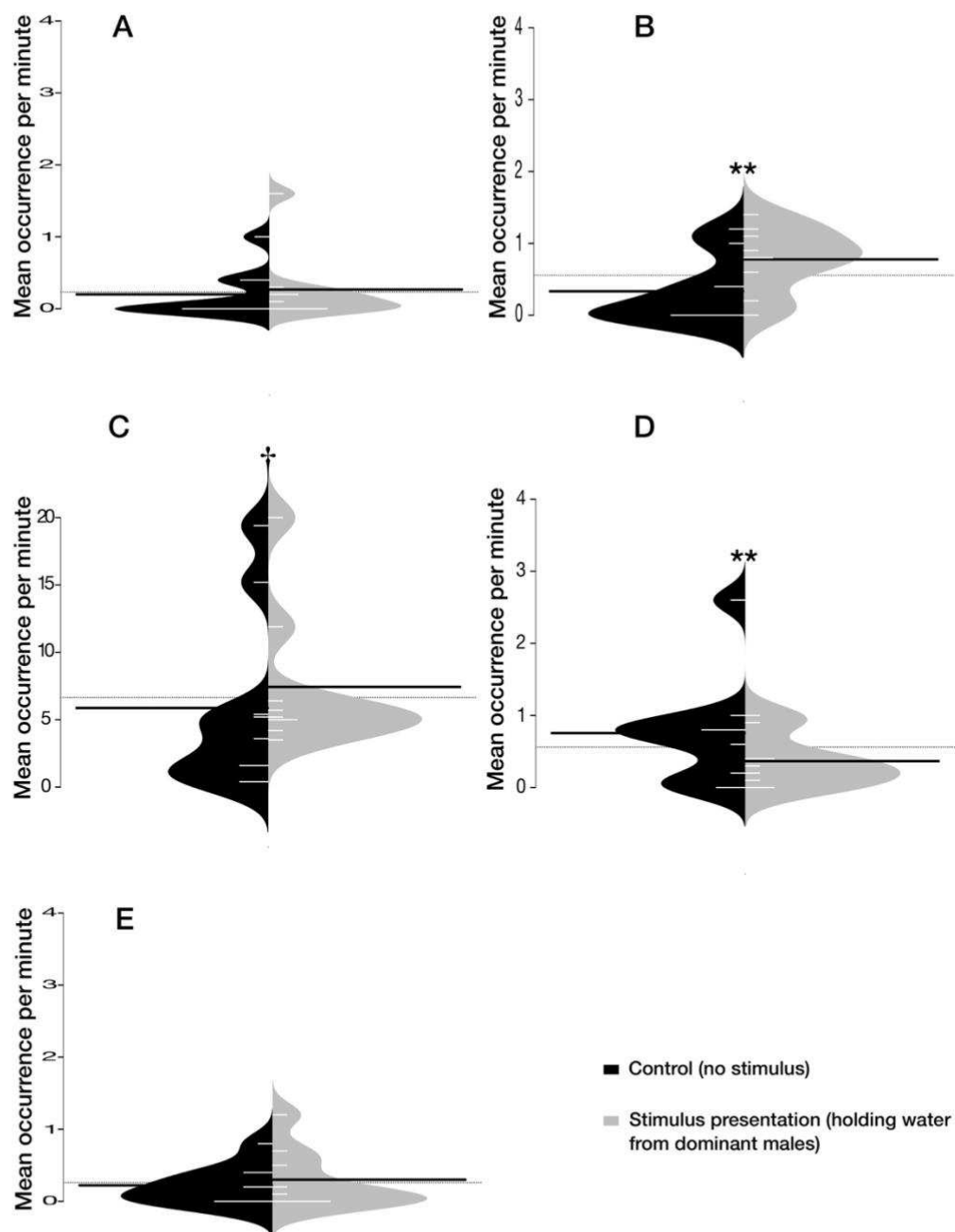


Figure 3 Effect of the holding water on five selected behaviours. Effect of the holding water from dominant males on the number of (A) agonistic behaviours, (B) up and down swimming, (C) tank's exploration, (D) shelter's visit, and (E) maintenance, during the control period without stimulus (in black) and the stimulus presentation (in light grey). Beanplots ('beanplot' R package (Kampstra, 2008)) combine individual observations (white lines), dataset distribution, the overall dataset average (dashed horizontal line) and the average for each subset (heavy horizontal black line). † $P \leq 0.1$; ** $P \leq 0.01$.

behaviour from the subject male. For example, in [Bertucci et al. \(2010\)](#), while an acoustic playback alone did not change the subject's behaviour in comparison to the control period in male *M. zebra*, the only view of a live contestant provoked a high aggressive answer from the tested male.

In the present study, all behavioural responses to unimodal chemical and acoustic treatments did not differ significantly from the control. Indeed, we did not find evidence that the tested fish reacted either to urine from dominant males, or to the playback of agonistic sounds presented alone. Concerning the acoustic treatment, this result is in accordance with previous studies which also found no reaction to sound playback alone as in fish agonistic sounds are typically produced within short distance from the receiver and associated with conspicuous visual displays ([Ladich & Myrberg, 2006](#)). Our first assumption was that acoustic stimulus would induce indirect territorial behaviours such as increased activity and nest building. [Myrberg & Riggio \(1985\)](#) showed that sounds coming from nearest neighbour provoked less diving displays of courtship than sounds coming from other males within the colony. But in *M. zebra*, sounds are associated with behaviours such as quivers during close agonistic interactions ([Miguel Simões et al., 2008](#)). It seems that sounds alone made by a contestant do not suffice to incite territorial males to be involved in aggression or territorial activities. [Butler & Maruska \(2015\)](#) showed in the cichlid *Astatotilapia burtoni* that the lateral line plays an essential role for mutual assessment of opponents during agonistic encounters. This system facilitates the use of non-contact assessment. Additional vibrational information could be essential and the role of the lateral line system during agonistic interactions in *M. zebra* clearly needs further investigation. Regarding the chemical component, [Chien \(1973\)](#) has reported the effect of chemical cues in the South American angelfish (*Pterophyllum scalare*) in a reproductive context. Chemical cues emitted by males increased spawning rates in the females. By contrast, in our study, urine from dominant males did not elicit any behavioural response from the tested males. To our best knowledge this is the first time that isolated chemical stimuli are tested in an agonistic context in fish. Other studies in fish underline that, during agonistic encounters, urine (like sound) is a signal used in close range aggressive interactions and needs concurrent visual stimuli to elicit immediate behavioural responses ([Barata et al., 2007](#); [Keller-Costa et al., 2016](#)).

Under a theoretical framework, these results allow us to hypothesise a categorisation of unimodal signals in *M. zebra* into the proposed categories made by [Partan & Marler \(1999\)](#). Because chemical and acoustic stimuli did not elicit any response in *M. zebra* males but visual stimuli did, unimodal signals should be categorized as non-redundant, since redundant signals have equivalent effects on a receiver. When presented as a composite signal, non-redundant signals can lead to different effects such as an independence effect. When combined, non-redundant signals provoke behavioural reactions which are the same as those observed when signals are presented alone. On the other hand, if one signal overshadows or changes the effect of the other, it has a dominance or a modulation effect. If otherwise the combination of those signals elicits an entirely new response there is an emergence effect. How different sensory modalities interplay during fish communication, in particular during agonistic interactions, remains to be unravelled. In *M. zebra* acoustic

agonistic signals seem to modulate the response to the visual channel ([Bertucci et al., 2010](#)). In contrast, the acoustic sensory modality seems to dominate over the visual one in a small marine goby ([Amorim et al., 2013](#)). In *Neolamprologus pulcher* chemical signals appear to modulate the response to visual signals during an agonistic encounter ([Bayani, Taborsky & Frommen, 2017](#)). Future work needs to address how the different sensory channels interact to produce an adaptive response.

Which bioassay?

In addition to investigate the behavioural response to unimodal stimuli, we aimed to develop a bioassay to assess the role of each sensory channel in eliciting behavioural decisions. Different types of stimuli can be chosen to test the role of one signal modality with each having different pros and cons. For example, in the present study a live opponent was the treatment that elicited the strongest response from subject males compared to a video playback. However, because within one-modality a signal is usually multicomponent ([Candolin, 2003](#)), it is important to be able to control the other parameters, apart from the ones that are being tested. Although we controlled for size effects (live intruders were size-matched to the tested fish) we could not control other aspects such as changes in body colour, behaviour, etc. Because of these drawbacks, other studies have used different visual stimuli such as mirror tests (e.g., [Raffinger & Ladich, 2009](#)) or visual computer-manipulated stimuli ([Watanabe, Shinozuka & Kikusui, 2016](#)). Although more and more research uses computer animation as a tool to control visual cues, including in cichlids ([Balzarini et al., 2016](#)), our results underlined an absence of response towards video playback. This was recently the case in another study using cichlids ([Wackermannova et al., 2017](#)). The use of RGB video screen, the screen frame rate (developed for human sight), the lack of UV components and three-dimensional depth are constraints that may affect visual perception and could explain this absence of response. In any case, natural motion patterns, velocity and interaction should not have been affected here as video playbacks consisted of recordings from real individuals either biting the tank's glass with a one-way mirror, or performing routine swimming.

Finally, we tested another chemical cue, holding water from dominant males, which is classically used in cichlids ([De Caprona, 1974](#); [Thünken et al., 2009](#)). Here, holding water increased the up and down swimming, tended to increase the tank's exploration and decreased the number of shelter's visits. Unlike urine, holding water seems to make the fish aware of the potential presence of a contestant even though no visual signal was present. Holding water represents a more diffuse cue than urine, as urine is generally used during close range aggressive interactions ([Barata et al., 2007](#); [Escobar-Camacho & Carleton, 2015](#)). It also contains in addition to urine, other components coming from the gills, the mucus, and the faeces. These differences could explain that urine without visual signal had no effect on *M. zebra* males' behaviours while holding water without visual signal had an impact. Consequently, the best bioassay in testing behavioural answers in *M. zebra* to chemical signals seems to be the use of holding water. It is a lot easier and less intrusive to collect than pure urine, where collected amounts varied from nothing to very small quantities (<340 µL). Moreover, the handling provokes a stressful response from the handled fish,

typically accompanied by increased circulating cortisol (*Ramsay et al., 2009*). Because we left the fish in a two litres tank during three hours to obtain holding water, the manipulation is less stressful and we can manage larger liquid quantities. Nevertheless, if the question is to seek the existence of active molecules in urine during agonistic encounters, then sampling pure urine should be more promising.

In conclusion, *M. zebra* territorial males seem to rely mostly on the visual sensory modality, as the view of a real opponent provoked intense aggressive reaction but the acoustic signal and chemical cue alone did not elicit obvious changes in their immediate behaviours. The different sensory channels seem to be non-redundant (*sensu Partan & Marler, 1999*) in the study species and likely in most other fishes (*Ladich, 2004*), but how different modalities are integrated to produce adaptive behaviour in fish remains to be answered. Our results also emphasize the need to optimise experimental protocols to test the significance of multimodal communication during specific behavioural contexts, to better understand the evolution of signalling across vertebrates (*Partan & Marler, 1999; Candolin, 2003; Narins et al., 2005*).

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Laura Chabrolles conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Imen Ben Ammar, Joël Attia, M. Clara P. Amorim and Marilyn Beauchaud conceived and designed the experiments, reviewed drafts of the paper.

- Marie S.A. Fernandez analyzed the data, reviewed drafts of the paper.
- Nicolas Boyer conceived and designed the experiments.
- Paulo J. Fonseca conceived and designed the experiments, contributed reagents/materials/analysis tools.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

All procedures described in this manuscript were conducted in accordance with appropriate French national guidelines, permits and regulations regarding animal care and experimental use (Approval no C42-218-0901, ENES lab agreement, Direction Départementale de la Protection des Populations, Préfecture du Rhône).

Data Availability

The following information was supplied regarding data availability:

Raw data is available in the [Supplemental Information](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.3643#supplemental-information>.

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-Chapter 2-

Agonistic interactions in *Maylandia zebra* males go multimodal

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Abstract

The use of multimodal communication in animals is relatively common, however most of the studies have investigated cues in isolation or in bimodal systems. Cichlids of the African lakes constitute an ideal model for studying the multimodal communication as they rely on the different sensory modalities (hearing, lateral line, olfaction, taste and vision) during their social interactions. In *Maylandia zebra*, males engaged in territorial defence use all sensory modalities that are perceived simultaneously by both competitors. We gathered information on responses to each component of this multimodal signal as well as the response to combinations of signal components. We found that stimuli containing visual information provoked strong aggressive response from the tested males. Although agonistic sound playback alone did not trigger changes in behaviour, males exposed to only chemical cues (male holding water) increased their activity in the tank. In association, sound and chemical components modulated visual signals. When the visual stimulus was associated to sound aggression increased whereas the opposite was observed when chemical was associated to visual signals. The chemical signals associated to sound also triggered aggressiveness, although no aggression was observed when they were presented alone. Finally, when the three sensory modalities were available, the intensity of the observed behavioural responses was the highest. All those results clearly show an interaction between the different modalities, allowing to modulate and refine the information transmitted during agonistic interactions in communication network. Thus, it seems that multisensory perception could benefit animal communication by reducing the uncertainty about the environment.

Introduction

Animal communication is an elaborate system encompassing complex signalling displays to interact with conspecifics and heterospecifics across a variety of social contexts (Bradbury & Vehrencamp, 2011). When it includes signals from different sensory modalities, simultaneously or in sequence, it is referred to as multimodal communication (Candolin, 2003; Hebets & Papaj, 2005; Partan & Marler, 2005; Smith & Evans, 2013). The complexity of animal communication is known for some time (Darwin & Prodger, 1998) and multimodal communication across species is now a topic of special interest (Duistermars & Frye, 2008; Estramil et al., 2014; Fröhlich & van Schaik, 2018; Gordon & Uetz, 2011; Halfwerk et al., 2014; Wierucka et al., 2018). This growing interest received a new impulse from a study proposing a framework for categorizing multimodal signals based upon receiver behavioural responses to either separate components within a sensory modality or to multimodal composite signals (Partan & Marler, 1999).

So far, multimodal signals have been categorized as redundant and non-redundant according to the information they convey (Johnstone, 1996; Partan & Marler, 1999). Signals providing the same information can increase the accuracy of the receiver response and are called redundant. They also illustrate the ‘backup signal’ hypothesis as termed by Johnstone (1996), widely used to secure the transmission of information in environments that are restrictive for propagation. If the receiver response intensity is the same as in an unimodal cue exposure, those redundant signals are qualified as ‘equivalent’, but if the intensity is increased they are ‘enhancement’ signals (Partan & Marler, 1999). Non-redundant signals or multiple messages are signals that provide different information (i.e. different types of information or different aspects of the signaller quality) (Hebets & Papaj, 2005; Johnstone, 1996; Partan & Marler, 1999). If the response to combined signals is similar to responses to each signal presented alone, those signals are independent. But we can also observe the establishment of a hierarchy between

signals. Dominance is when the response elicited by one signal dominates the other, modulation is when a signal modulates the other and finally emergence is when a new response emerges, different from when signals are presented separately (Partan & Marler, 1999). But the redundant/non-redundant framework may not always fit the variation observed (Rowe, 1999). Other classifications have been proposed focusing more on inter-signal interactions and signal efficacy (Hebets & Papaj, 2005). And other factors must be taken into account such as the influence of the environment, the physical properties of sensory cues, the interactions among traits and the perceptive/production abilities of the individuals (Hebets et al., 2016; Higham & Hebets, 2013). Exploring this complex system will help to understand the selection pressures acting on multimodality (Candolin, 2003; Hebets et al., 2016; Hebets & Papaj, 2005; Rowe & Halpin, 2013).

The limitations between the capacities of the different sensory modalities could support the use of the multimodal communication (Bradbury & Vehrencamp, 2011; Higham & Hebets, 2013). Visual and acoustic cues are perceived instantaneously but could be easily disturbed by the environment. A visual signal may be completely blocked by physical barriers. However, an acoustic signal would suffer less disruption from physical barriers but could be blocked by other noise. Olfactory signals travel slowly but remain for longer periods. Multimodal signalling could then compensate the drawbacks of each modality to increase efficacy during communication. But multimodality could also imply costs for the sender and receiver. Complex signalling increase energy expenditure, conspicuousness to predators or eavesdropping (Bro-Jørgensen, 2010; Hebets & Papaj, 2005). Yet, the use of multimodal communication in animals is relatively common (Higham & Hebets, 2013; Rowe & Halpin, 2013; Uy & Safran, 2013). Although not well known, this multimodality must therefore play an important role in communication networks.

Conflict resolution constitute a good model for exploring multimodality and inter-signals interaction. Each individual involved in this social interaction must assess its own fighting ability, motivation and experience compared to its opponent (Bradbury & Vehrencamp, 2011). Consequently, both individuals are senders and receivers and must exchange information via signals and cues to accurately process the information and make adaptive behavioural responses (Bradbury & Vehrencamp, 2011; Van Staaden et al., 2011). Selection pressure on communication during territorial conflict resolution ought to be strong, as both competitors would benefit if the contest is settled with displays. An escalated fight could lead to serious injuries which could represent a significant cost for both competitors (Bradbury & Vehrencamp, 2011; Van Staaden et al., 2011).

Escobar-Camacho & Carleton (2015) in their review, showed that cichlids rely on different sensory modalities. But we do not know how these modalities interplay during communication. As the different sensory cues do not have the same active space or persistence in time, there could be a hierarchy among the sensory modalities, in the time or the order they are used. For example, when choosing a mate, a female can first evaluate from a distance conspecific males from heterospecific males with visual cues. If the interaction continues at a closer range, more information could be obtained from acoustic and chemical cues (Escobar-Camacho & Carleton, 2015). The visual component has been thoroughly studied in cichlids as a mechanism responsible for their explosive radiations (Deutsch, 1997; Seehausen & Schluter, 2004). In *Maylandia zebra*, the view of a live contestant elicited a strong aggressive answer from the subject male (Bertucci et al., 2010). In the same species, sounds have been shown to provide reliable estimation of the size of the male sender (Bertucci et al., 2012; Simões et al., 2008). Chemical cues in cichlids can provide multiple information such as information for species recognition (Plenderleith et al., 2005) or for the identification of the social status in males or the reproductive status of the females (Almeida, 2005; Hubbard et al., 2017).

Maylandia zebra represents a good model for trying to disentangle this complex multimodal communication. Males from this species court females with multimodal signals, allowing species recognition and social status identification (Amorim et al., 2008; Escobar-Camacho & Carleton, 2015). Males make conspicuous visual displays, emit sounds and urine pulses when females approach their territories and vibrations that could stimulate the lateral line of the females when males are doing quivers for example (Simões et al., 2008; Escobar-Camacho & Carleton, 2015). Males are also highly territorial and therefore encounter multiple conflict situation whenever two individuals simultaneously attempt to gain access to the same territory (Holzberg, 1978; Mellor et al., 2012). These close-range interactions lead to situations where signals sent by males using all sensory modalities can be perceived simultaneously by both competitors. However, it is yet to be understood how different sensory modalities interplay during conflict resolution in this species and other fishes (Hebets & Papaj, 2005; Higham & Hebets, 2013).

In this study, we examined the role of acoustic, olfactory and visual signals in opponent assessment in *Maylandia zebra*. Dominant males were exposed to bimodal and trimodal signals to test the relative importance of each sensory modality in conflict resolution during territorial encounter. Specifically, we measured the male response in terms of aggressive behaviours, space occupancy and territorial behaviours. This study is part of a classic approach of multimodal communication researches with a previous ‘cue-isolation’ experiment where dominant males were exposed to isolated chemical, visual and acoustic signals in an agonistic context (Chabrolles et al., 2017). Very few studies combined three modalities during their experiments. With this research we provide new inputs for a better understanding of multimodal communication networks.

Materials & Methods

Subjects and Housing Conditions

Maylandia zebra were purchased from Oxyfish (Verlinghem, France) and stored in holding tanks (120 cm long, 60 cm wide and 50 cm height) at ENES laboratory (University of Saint-Etienne, France). Each tank contained 10 males that were fed daily with commercial cichlid food (JBL NovoRift sticks and Tetra flakes). All tanks were equipped with an external filter (Rena Filstar xP3, Rena France, Annecy, France), aeration and PVC tubes as shelters. The water temperature was maintained at $25 \pm 1^\circ\text{C}$ with a pH of 8.0 and a 12:12h light: dark cycle was settled. Each fish was identified by a transponder (PSK Transponder, Dorset Identification B.V., Netherlands).

General experimental design

This work follows the protocol described in Chabrolles et al. (2017). The general experimental design was the same between the two studies, but some details differed.

The experimental aquarium (60 cm x 30 cm x 30 cm) was placed on a vibration-insulated shelf (with a layer of wood-fibre) in a sound-proofed chamber (PRIMO Silence-Box, Tip Top Wood©) to limit background noise. The back of the aquaria was covered on the inside with bubble wrap to break sound reflections on the tank's walls.

Each experimental tank had a filter containing active carbon, aeration, an internal heater, sand substrate, and a terracotta pot usable as a shelter by the fish, all located on the left side of the tank. Because the tested fish stayed in the experimental set up for five days, each aquarium was accompanied by a social group placed in an adjacent tank, to avoid the sense of isolation.

These social groups were composed of one male and one female physically separated to avoid any injuries. An underwater loudspeaker developed by Fonseca & Alves (2012) was placed in the aquarium of the tested fish and used for playing back sound stimuli. A hydrophone (Aquarian Audio Products H2a-XLR, AFAB Enterprises, Anacortes, WA, USA, sensitivity: -180 dB re 1 V μ Pa-1, flat frequency response \pm 4 dB 20 Hz – 4.5 kHz) located in the middle of the tank, monitored sound playback and registered possible sounds produced by the tested fish and the opponent.

The hydrophone was connected to a preamplifier (Yamaha MLA8, Yamaha Music France, Marne-la-Vallée, France) linked to the video capture card (Osprey-450e) of a PC that synchronized audio and video signals. Behaviour was recorded using a camera (BUL520, brand, Active Media Concept, Vallauris, France) positioned in front of the set-up.

When the visual live stimulus was presented (see below), a hermetic jar (20 cm x 11.7 cm x 25 cm) that hosted the intruder fish was left in the experimental tank during the five days of testing to avoid any effect of novelty. A silicone tube (ZOLUX, reference 323 206, ID = 4.00 mm, France) attached to a partition and used during chemical presentation was also left in the experimental tank during the week of experimentation. The presentation of chemical stimuli was controlled through an aquarium pump (Tecatantis EasyFlux300 and EasyFlux600, Aquatantis, Guimarães, Portugal) adjusted with a flow rate of about 1 L/10min (Kobayashi, 2002). The pump was located outside the sound-proofed chamber (see [Fig. 1](#) for a complete view of the set-up).

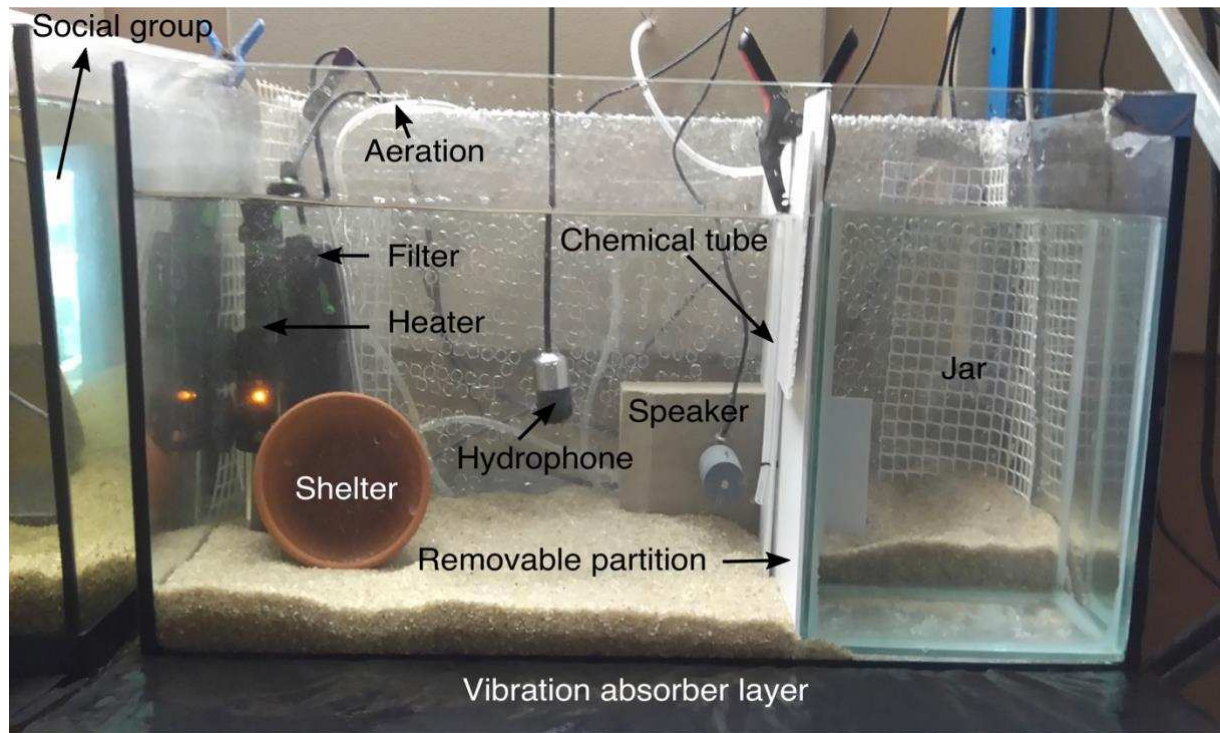


Figure 1. Behavioural experiment set-up. Dominant males were placed in the experimental aquarium forty-eight hours before the beginning of the experiment. A vibration absorber layer was placed under the aquarium to limit background noise. Each experimental tank had a filter, aeration, an internal heater, sand substrate, and a terracotta pot usable as a shelter by the fish, all located on the left side of the tank. In order to avoid sense of isolation for males, each aquarium had a social group placed in an adjacent tank. An hydrophone was placed in the middle to record possible sounds produced by the tested fish and the opponent. The underwater loudspeaker was used for sound playbacks. A silicone tube attached to the partition and linked to an external pump was used for chemical presentation. The hermetic jar hosted intruder fish during visual presentation which was allowed by a removable partition.

Social hierarchies' assessment in community tanks

To establish social hierarchies between males in stock tanks, we observed the group during ten consecutive minutes before each sampling or experiment. Following (Barata et al.,

2007), a DI was used to quantify males' social rank. DI was calculated using the ratio between the number of dominant behaviours (aggressive displays or nest-digging) and the number of dominant behaviours plus the number of submissive behaviours (escape from an aggressive opponent).

Only males with a dominance index (DI) above 0.7 (range from 0 – subordinates, to 1 – dominants) were selected for experiments or chemical sampling.

Protocol for stimuli presentation

Twenty dominant males (standard lengths (SL) ranging from 8 to 11.7 cm; mean = 9.7 cm) were used to test behavioural responses to each condition. They were placed in the experimental aquarium 48 hours before the beginning of stimuli presentation. This study consisted in five different treatments: (1) real opponent in the jar (Visual only), (2) real opponent in the jar + agonistic sound playback (Visual + Sound), (3) real opponent in the jar + holding water from dominant males (Visual + Chemical), (4) real opponent in the jar + agonistic sound playback + holding water from dominant males (Visual + Sound + Chemical), (5) agonistic sound playback + holding water from dominant males (Sound + Chemical). Fish received treatment 1 to 5 (one treatment per day) on consecutive days in a random order. After experiments, all subject males returned to their original tank and the whole set up was rinsed with water and white vinegar to eliminate any potential chemical cues in the aquarium, before new males were placed in the experimental tanks.

Each experiment lasted 25 min. Ten minutes before the beginning of the test, filter, aeration, and internal heater were switched off. The visual contact with the social group was interrupted through a partition and the loudspeaker was switched on. The experiment started with a five minutes control period where no stimulus was presented. This was followed by a

ten minutes period of stimulus presentation, in which potential combined sound, chemical, and visual stimulation occurred. The stimuli presentation was then followed by a ten minutes of post stimulation period where the visual contact with the real opponent was interrupted through the removable partition. Details of the treatments were as follows:

Treatment 1. Real opponent in the jar (V): a male matched in size with the subject, was introduced thirty minutes before the beginning of the test in the jar located inside the subject's tank to acclimatize. Before the capture, the opponent was observed in the group to ensure that he did not become subordinate.

Treatment 2. Real opponent in the jar + agonistic sound playback (VS): Agonistic sounds were played back (115 dB re. 1 μ Pa at 3.8 cm, within the range of the natural male sounds) with custom-made underwater loudspeakers (frequency response \pm 1.5 dB in the range 20 Hz – 2 kHz) and a driver developed by Fonseca & Alves (2012). Sound stimuli were fed to the driver by a D/A converter (Edirol UA-25, Roland, Japan) controlled by Adobe Audition 3.0 (Adobe Systems Inc., Mountain View, CA, USA) on a laptop.

Two agonistic sounds of the same male were used as stimuli per experiment (Chabrolles et al., 2017). These sounds were randomly assigned from seven different males with SL ranging 8.4 cm to 10.8 cm (mean = 9.4 cm) from the ENES sound archive. All agonistic sounds presented 7-9 pulses (mean = 8.0 pulses) and a pulse rate of 18 ± 7 pulses per second. The pulses mean dominant frequency of the sounds was $289 \text{ Hz} \pm 133 \text{ Hz}$ (see Bertucci et al. (2012), for the description of the sounds analysis). Pulses dominant frequencies were obtained with the 'dfreq' function from the 'seewave' R package (Sueur, Aubin, & Simonis, 2008).

During the ten minutes treatment phase, the playback was made interactively, sounds were sent each time the opponent displayed a behaviour which is usually associated with a

sounds production during real interactions. The stimulus was played back only when the tested individual approached the loudspeaker area, i.e. within 1 to 5 cm in front of the loudspeaker. Each sound was played back five times in a random order, which represented ten agonistic sounds in a test. All individuals approached the loudspeaker ten times or more. The removable partition was then put back during the post period of ten minutes. The visual contact was interrupted and no more sounds were played back.

Treatment 3. Real opponent in the jar + holding water from dominant males (VC): After quantifying the social rank, dominant males ($DI > 0.7$) were selected and placed individually in a jar containing two litres of aerated water at $25 \pm 1^\circ\text{C}$ during three hours. One litre of this solution was used during chemical presentation in the same day, to avoid any chemical degradation. Preliminary experiments on chemical communication in the same fish species have shown that if we injected three males with patent blue V (Prolabo, France), they urinated during the next three hours thereby confirming the presence of urine in the holding water.

During the ten minutes treatment phase, the pump was plugged in and one litre of holding water was delivered continuously. Holding water was obtained from fifteen different dominant males ($DI > 0.7$) and the water from one stimulus male was randomly assigned to tested males. The used holding water came from a male unknown to the subject male. The partition was then put back during the post period of ten minutes interrupting the visual contact. The pump was also turned off stopping the holding water delivery. But as the aquarium filter was also turned off during the experiment, the chemical information sent during the stimulation period could persist during the post stimulation period, unlike the acoustic or the visual stimuli.

Treatment 4. Real opponent in the jar + holding water from dominant males + agonistic sound playback (VCS): After the control period (five minutes), visual interaction occurred by

removing the partition, the holding water pump was plugged in and the playback was sent interactively during the ten minutes of stimulus presentation. The partition was then put back during the post period of ten minutes. The visual contact was interrupted and no more sounds were played back. But the chemical information could persist.

Treatment 5. Holding water from dominant males + agonistic sound playback (CS): After the control period (five minutes), in order to have the same conditions for each treatment, the partition was removed even if no opponent was present. The holding water pump was plugged in and the playback was sent during the ten minutes of stimulus presentation. The sounds were played back only when the tested individual approached the loudspeaker area, i.e. within 1 to 5 cm in front of the loudspeaker, simulating close interaction. The partition was then put back during the post period of ten minutes and no more sounds were played back. But the chemical information could persist.

Behavioural analysis

Videos were analysed using Jwatcher software (v. 1.0) which allows registering systematically the frequency of occurrence and the duration of each variable. Both measures were considered in the statistical analysis.

For both the subject and the opponent, the aggressive behaviours (i.e. lateral displays, quivers, bites, attempted chase, darts and sounds produced) were counted and then summed to produce an aggressive score (Chabrolles et al., 2017). For the subject only, we quantified the number of up-and-down swimming (when a territorial fish was swimming up and down against the wall of the aquarium). We also quantified the tank's exploration score, for which the aquarium was artificially divided in two equal zones and the number of shifts between the two

zones was counted. Finally, the nest maintenance behaviour (when a territorial fish was moving sand within its territory) and the number of the shelter visits were also counted.

Statistics

All statistical tests were performed using R software (R Core Team, 2016).

I. Effect of treatment and trial period on the behavioural variables scores (PC1 and PC2)

In order to reduce the number of variables for statistical analysis, the nine selected behavioural measures (aggression (duration and occurrence), up-and-down swimming (duration and occurrence), tank's occupation, maintenance (duration and occurrence) and shelter visits (duration and occurrence)) were grouped using Principal Component Analysis (PCA; McGregor, 1992). The first three principal components (PCs), with eigenvalue above 1 were selected (Table 1). These explained 82% of the total variance. This first analysis tested the effect of treatment and the test periods (control, stimulation and post-stimulation) on overall behaviour.

	PC1	PC2	PC3
Variance explained a (% cumulative)	41.10	65.12	82.33
Eigenvalue a	3.70	2.16	1.55
Aggressiveness (duration) (Box-Coxb)	-0.77	0.17	-0.41
Aggressiveness (occurrence) (Box-Coxb)	-0.80	0.25	-0.38
Maintenance (duration) (Box-Coxb)	0.71	0.02	-0.65
Maintenance (occurrence) (Box-Coxb)	0.68	0.03	-0.69

Shelter (duration) (Box-Coxb)	0.82	0.08	0.30
Shelter (occurrence) (Box-Coxb)	0.83	0.04	0.23
Up-and-down swimming (duration) (Box-Coxb)	-0.17	-0.95	0.03
Up-and-down swimming (occurrence) (Box-Coxb)	-0.19	-0.95	0.03
Tank's exploration (Box-Coxb)	0.28	-0.50	-0.43

Table 1. Principal component analysis of the behavioural variables.

a Decomposition of total inertia for each principal component ('inertia.dudi' function from 'ade4' R package).

b Column coordinates and transformations used before the PCA.

Aggressiveness was inversely correlated with the maintenance and the shelter visits on PC1. Positive values of PC1 values represent increased aggressiveness and negative values mean increased maintenance and shelter visits (see Fig 2). The Up-and-down swimming and the tank's exploration contributed mainly to PC2. Positive values represent an increase in Up-and-down swimming and tank's exploration whereas negative values represent a decrease of both parameters (see Fig 3). Although, maintenance also contributed to PC3, we did not represent it to simplify the analysis and because it explained a small amount of variance of this third component (17%).

Because the data distributions were not Gaussian, we performed a Box-Cox transformation which computes one parameter transformation using the following formula: $\text{parameter}(\lambda) = (\text{parameter}^\lambda - 1) / \lambda$, if $\lambda \neq 0$ and $\text{parameter}(\lambda) = \ln(\text{parameter})$ if $\lambda = 0$. The 'boxcox' function from the 'Mass' R package (Venables & Ripley, 2002) automatically finds the appropriate λ value to reach a distribution as close as possible to the Gaussian distribution.

To assess the effect of the different treatments, linear models ('lmer' functions from the 'lme4' R package (Bates et al., 2014)) were performed on values of each principal component that has been kept. Residuals equivariance and distribution were checked using the 'plotresid' function from the 'RVAideMemoire' R package (Hervé, 2017).

To test the two interacting factors, treatment and period the following model was applied:

Model 1 <- lmer (PC values ~ treatment * period + offset (log (Recording duration)) + (1| day) + (1| subject) + (1| opponent sounds production) + (1| opponent aggressive score))

Model 1. The variable period had three levels: the control, the stimulus presentation and the post stimulation period. The variable treatment had five levels. Four random factors were added to the model: the day of the experimentation (1| day), the subject identity (1| subject), if the opponent produced sounds during the stimuli presentation (binary answer: yes or no) (1| opponent sounds production) and the aggressive score of the opponent calculated during the interaction for the stimuli presentation (1| opponent aggressive score). An 'offset' took into account the different recording duration of the control compared to the stimuli presentation and the post stimulation period. *P-values* were assessed using the 'Anova' function ('car' R package (Fox & Weisberg, 2011)). If one factor had a significant effect, post hoc tests using Tukey's adjustment were performed using 'lsmeans' function ('lsmeans' R package (Lenth, 2016)). When all the pairwise comparisons were not necessary, contrast matrices were build and used with the 'contrast' function ('lsmeans' R package (Lenth, 2016); 'RVAideMemoire' R package Hervé (2017)).

II. Contribution of the behavioural variables for each treatment

This second analysis aimed to see what really changed in each treatment, namely if the contribution of the behavioural variables was different across trial period in the different

treatments. This was achieved with five principal component analyses, one per treatment, using transformed data (Box-Cox transformation). For each treatment, the nine selected behavioural measures were grouped in a principal component analysis and only the first three principal components were selected (Table 2).

Treatment	Principal Components	Eigenvalue	Variance explained (% cumulative)
CS	PC1	3.28	36.49
CS	PC2	1.91	57.76
CS	PC3	1.79	77.61
V	PC1	4.05	44.97
V	PC2	2.16	68.95
V	PC3	1.63	87.01
VS	PC1	4.05	44.96
VS	PC2	2.09	68.23
VS	PC3	1.48	84.64
VC	PC1	3.94	43.77
VC	PC2	2.20	68.26
VC	PC3	1.13	80.87
VCS	PC1	3.79	42.16
VCS	PC2	2.09	65.39
VCS	PC3	1.72	84.46

Table 2. Decomposition of total inertia for each principal component ('inertia.dudi' function from 'ade4' R package) from principal component analyses realised on each treatments (V: visual, VS: visual + sound, VC: visual + chemical, VCS: visual + sound + chemical and CS: chemical + sound) for the nine selected behavioural measures (aggression (duration and occurrence), up-and-down swimming (duration and occurrence), tank's

occupation, maintenance (duration and occurrence) and occupation of the shelter (duration and occurrence)).

III. Change in behaviour between the control (Pre) and the stimuli presentation (Stim)

To quantify the magnitude of the behavioural response during treatment stimulation, an index was built to measure if each behavioural variables increased or decreased between the control and the stimuli presentation for each treatment. To have comparable measures between treatments, the mean value of each behavioural variable was centred and scaled for the control and the stimuli presentation. This value was then multiplied by its absolute value of their contributions on PC1, PC2 and PC3 and then summed (for detail of calculation, see example below). The mean values of each behavioural variable for the control and the stimuli presentation was calculated from the 'tab' object with the 'dudi.pca' function from 'ade4' R package (Dray & Dufour, 2007). The 'tab' object returned the data frame containing the behavioural values but centred and scaled. The behavioural variables contribution to the principal components were obtained from the 'co' object with the 'dudi.pca' function from 'ade4' R package (Dray & Dufour, 2007).

Index (for behaviour A) = [Mean value of the behaviour A (centred and scaled) * |Contribution of the behaviour A on PC1|] + [Mean value of the behaviour A (centred and scaled) * |Contribution of the behaviour A on PC2|] + [Mean value of the behaviour A (centred and scaled) * |Contribution of the behaviour A on PC3|]

For example, to obtain the index for the aggressiveness (duration) during the control, the contributions on PC1, PC2 and PC2 were 0.52, 0.60 and -0.41 respectively (see [Table 3](#)). The mean value of aggressiveness (duration) during the control, obtained from the data frame centred and scaled, was -0.42 (see [Table 4](#)). The index was then :

$$[-0.42 * |0.52|] + [-0.42 * |0.60|] + [-0.42 * |-0.41|] = - 0.64$$

The same calculation was done for the stimulation period and then the difference between the index for the control and the index for the stimulation period was plotted.

	PC1	PC2	PC3
Variance explained a (% cumulative)	36.49	57.76	77.61
Eigenvalue a	3.28	1.91	1.79
Aggressiveness (duration) (Box-Coxb)	0.52	0.60	-0.41
Aggressiveness (occurrence) (Box-Coxb)	0.51	0.65	-0.39
Maintenance (duration) (Box-Coxb)	-0.50	-0.39	-0.74
Maintenance (occurrence) (Box-Coxb)	-0.55	-0.36	-0.71
Shelter (duration) (Box-Coxb)	-0.69	0.15	0.46
Shelter (occurrence) (Box-Coxb)	-0.70	0.05	0.39
Up-and-down swimming (duration) (Box-Coxb)	0.74	-0.50	0.16
Up-and-down swimming (occurrence) (Box-Coxb)	0.79	-0.49	0.17
Tank's exploration (Box-Coxb)	0.24	-0.57	-0.04

Table 3. Principal component analysis of the behavioural variables for the chemical + sound (CS) treatment.

a Decomposition of total inertia for each principal component ('inertia.dudi' function from 'ade4' R package).

b Column coordinates and transformations used before the PCA.

	Control period	Stimulation period
Aggressiveness (duration) (Box-Cox)	-0.42	0.56
Aggressiveness (occurrence) (Box-Cox)	-0.44	0.67
Maintenance (duration) (Box-Cox)	-0.01	-0.12
Maintenance (occurrence) (Box-Cox)	-0.06	-0.13
Shelter (duration) (Box-Cox)	0.13	-0.21
Shelter (occurrence) (Box-Cox)	0.17	-0.31
Up-and-down swimming (duration) (Box-Cox)	-0.26	-0.09
Up-and-down swimming (occurrence) (Box-Cox)	-0.30	-0.09
Tank's exploration (Box-Cox)	-0.63	-0.12

Table 4. Mean values for each behavioural variables for the control and the stimulation period.

It was calculated from the 'tab' object with the 'dudi.pca' function from 'ade4' R package (Dray & Dufour, 2007), containing the behavioural values but centred and scaled.

This index allow us to represent the behavioural changes observed during stimulation in comparison to the control, while taking into account the different contributions that the variables had according to the treatments. The difference between the index calculated for the stimuli presentation and the control was used to represent if the behavioural variables increased or decreased during the stimuli presentation for each treatment.

Ethical Note

All procedures described in this manuscript were conducted in accordance with appropriate French national guidelines, permits and regulations regarding animal care and experimental use (Approval n°C42-218-0901, ENES lab agreement, Direction Départementale de la Protection des Populations, Préfecture du Rhône).

Results

I. Effect of treatment (V: visual, VS: visual + sound, VC: visual + chemical, VCS: visual + sound + chemical and CS: chemical + sound) and trial period (control, stimulation and post-stimulation) on the behavioural variables scores (PC1 and PC2)

The treatment, the trial periods and the interaction between the two factors had an impact on PC1 (Table 5). For all treatments, resident males were more aggressive, and consequently did less maintenance and visited less their shelter, during the stimuli presentation (Stim) compared to the Pre- and Post- stimulation, since PC1 was significantly higher in Stim (Table 6; Fig 2). During the stimuli presentation period (Stim), the addition of visual cues to the combined chemical and sound stimuli (blue) increased the aggressiveness of the tested fish compared to the combined chemical and sound (red) stimuli alone (Table 6; Fig 2). During the post period (Post), the presence of the remaining chemical during the chemical/sound treatment (in red) and the chemical/sound/visual treatment (in blue) decreased the maintenance and the visit to the shelter of the tested fish in comparison to the control period (Pre) (Table 6; Fig 2). Likewise the tested fish performed less maintenance and the visits to the shelter during the post

period (Post) in the presence of the remaining chemical of the chemical/visual treatment (green) when comparing to the post period (Post) of the visual treatment (grey) (Table 6; Fig 2).

Significant differences were observed for the control period (Pre) amongst treatments due to individual variation in maintenance and shelter visits before stimulation, (Table 6; Fig 2).

	Chisq	Df	P
Treatment	33.542	4	<0.001 ***
Period	330.562	2	<.0001 ***
Treatment: Period	57.712	8	<.0001 ***

Table 5. Effect of the five treatments (Visual, V + Sound, V + Chemical, V + S + C and C + S) according to the period (Control, stimuli presentation and post-stimulation period) on PC1 values (aggressive behaviours, maintenance and the shelter visits). Linear mixed-effect models ('lmer' function from the 'lme4' R package (Bates et al., 2014)) tested the effect of the treatments, the periods and the interaction between treatments and periods. An 'offset' took into account the different recording duration of the control in comparison to the stimuli presentation and post-stimulation. Day of experimentation, subject identity, opponent sound production and opponent aggressive score were included in the model as random factor. *P-values* were assessed using the 'Anova' function ('car' R package (Fox & Weisberg, 2011)).

Contrast	Estimate	SE	df	t.ratio	p.value
CS Stim vs CS Post	-0.965	0.294	251.324	-3.277	0.003 **
CS Post vs CS Pre	-0.696	0.294	251.324	-2.363	0.037 *
CS Stim vs CS Pre	-1.661	0.294	251.324	-5.639	<0.001 ***
V Stim vs V Post	-3.463	0.35	66.872	-9.899	<0.001 ***
V Post vs V Pre	0.029	0.294	251.324	0.098	0.922

V Stim vs V Pre	-3.434	0.35	66.872	-9.816	<0.001 ***
VC Stim vs VC Post	-3.067	0.353	59.601	-8.686	<0.001 ***
VC Post vs VC Pre	-0.238	0.294	251.324	-0.81	0.506
VC Stim vs VC Pre	-3.306	0.353	59.601	-9.361	<0.001 ***
VCS Stim vs VCS Post	-3.166	0.366	41.078	-8.651	<0.001 ***
VCS Post vs VCS Pre	-0.972	0.294	251.324	-3.301	0.003 **
VCS Stim vs VCS Pre	-4.138	0.366	41.078	-11.308	<0.001 ***
VS Stim vs VS Post	-3.543	0.366	45.72	-9.672	<0.001 ***
VS Post vs VS Pre	-0.461	0.294	251.324	-1.566	0.189
VS Stim vs VS Pre	-4.004	0.366	45.72	-10.931	<0.001 ***
CS Stim vs VCS Stim	1.962	0.366	41.078	5.36	<0.001 ***
V Stim vs VC Stim	0.353	0.3	254.305	1.177	0.337
V Stim vs VS Stim	0.232	0.308	208.71	0.753	0.511
VCS Stim vs VC Stim	0.393	0.302	233.329	1.305	0.282
VCS Stim vs VS Stim	0.272	0.311	181.23	0.875	0.496
CS Post vs VCS Post	-0.239	0.294	251.324	-0.813	0.506
V Post vs VC post	0.749	0.294	251.324	2.542	0.026 *
V Post vs VS Post	0.152	0.294	251.324	0.517	0.662
VC Post vs VCS Post	-0.492	0.294	251.324	-1.671	0.168
VCS Post vs VS Post	-0.104	0.294	251.324	-0.355	0.744
CS Pre vs V Pre	0.229	0.294	251.324	0.776	0.511
CS Pre vs VC Pre	0.71	0.294	251.324	2.411	0.034 *
CS Pre vs VCS Pre	-0.516	0.294	251.324	-1.751	0.149
CS Pre vs VS Pre	-0.109	0.294	251.324	-0.371	0.744
V Pre vs VC Pre	0.481	0.294	251.324	1.635	0.172

V Pre vs VCS Pre	-0.744	0.294	251.324	-2.528	0.026 *
V Pre vs VS Pre	-0.338	0.294	251.324	-1.147	0.34
VC Pre vs VCS Pre	-1.226	0.294	251.324	-4.162	<0.001 ***
VC Pre vs VS Pre	-0.819	0.294	251.324	-2.782	0.015 *
VCS Pre vs VS Pre	0.407	0.294	251.324	1.381	0.257

Table 6. Pairwise comparisons of the five treatments (V: visual, VS: visual + sound, VC: visual + chemical, VCS: visual + sound + chemical and CS: chemical + sound) according to the period (Control (Pre), stimuli presentation (Stim) and post-stimulation period (Post)) on PC1 values (aggressive behaviours, maintenance and the shelter visits). Least-squares means ('lsmeans' function from 'lsmeans' R package (Lenth, 2016) and 'contrast' function from 'RVAideMemoire' R package (Hervé, 2017)) allowed post-hoc comparisons when significant main or interaction effects were found. For the pairwise comparisons *P-values* were adjusted using Tukey's method. Estimates were extracted from the summary of the least-squares means function.

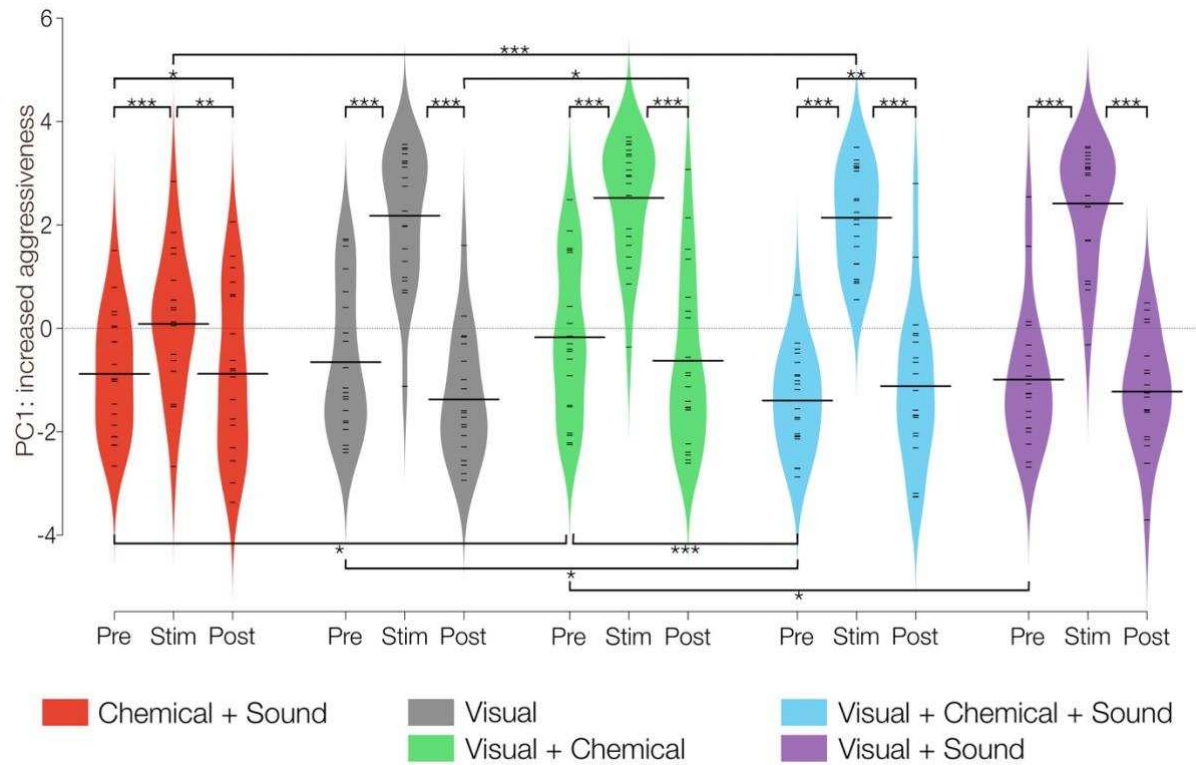


Figure 2. Effect of the five treatments (Chemical + Sound (red), Visual (grey), V + C (green), V + S + C (blue) and V + S (violet),) according to the period (Control (Pre), stimuli presentation (Stim) and post-stimulation period (Post)) on PC1 values (aggressive behaviours, maintenance and the shelter visits). PC1 explained 41% of variance. The interaction between treatment and trial period was significant. Aggressiveness was inversely associated with maintenance and shelter visits. Beanplots ('beanplot' R package (Kampstra, 2008)) combine individual observations (black lines – when represented inside the beans), dataset distribution, the overall dataset average (dashed horizontal line) and the average for each subset (heavy horizontal black line). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

Only trial period had an impact on PC2 values (up-and-down swimming and tank's exploration) (Table 7). Up-down-swimming and tank's exploration kept increasing between the control, the stimuli presentation and the post-stimulation period. PC2 was significantly higher during the stimuli presentation in comparison to the control. It was also significantly higher

during the post-stimulation period in comparison to both control and stimuli presentation (Table 8; Fig 3).

	Chisq	Df	P
Treatment	6.648	4	0.1557
Period	172.840	2	<.0001 ***
Treatment: Period	7.662	8	0.4672

Table 7. Effect of the five treatments (Visual, V + Sound, V + Chemical, V + S + C and C + S) according to the period (Control, stimuli presentation and post-stimulation period) on PC2 values (up-and-down swimming and tank's exploration). Linear mixed-effect models ('lmer' function from the 'lme4' R package (Bates et al., 2014)) tested the effect of the treatments, the periods and the interaction between treatments and periods. An 'offset' took into account the different recording duration of the control in comparison to the stimuli presentation and post-stimulation. Day of experimentation, subject identity, opponent sounds production and opponent aggressive score were included in the model as random factor. *P-values* were assessed using the 'Anova' function ('car' R package (Fox & Weisberg, 2011)).

Contrast	Estimate	SE	df	t.ratio	p.value
Stim vs Post	1.278	0.324	79.3	3.948	<0.001 ***
Stim vs Pre	-0.786	0.324	79.3	-2.427	0.046 *
Post vs Pre	-2.063	0.157	225.5	-13.112	<0.001 ***

Table 8. Pairwise comparisons of the three periods (Control (Pre), stimuli presentation (Stim) and post-stimulation period (Post)) on PC2 values (up-and-down swimming and tank's exploration). Least-squares means ('lsmeans' function from 'lsmeans' R package (Lenth, 2016)) allowed post-hoc comparisons when significant main or interaction effects were found. For the

pairwise comparisons *P-values* were adjusted using Tukey's method. Estimates were extracted from the summary of the least-squares means function.

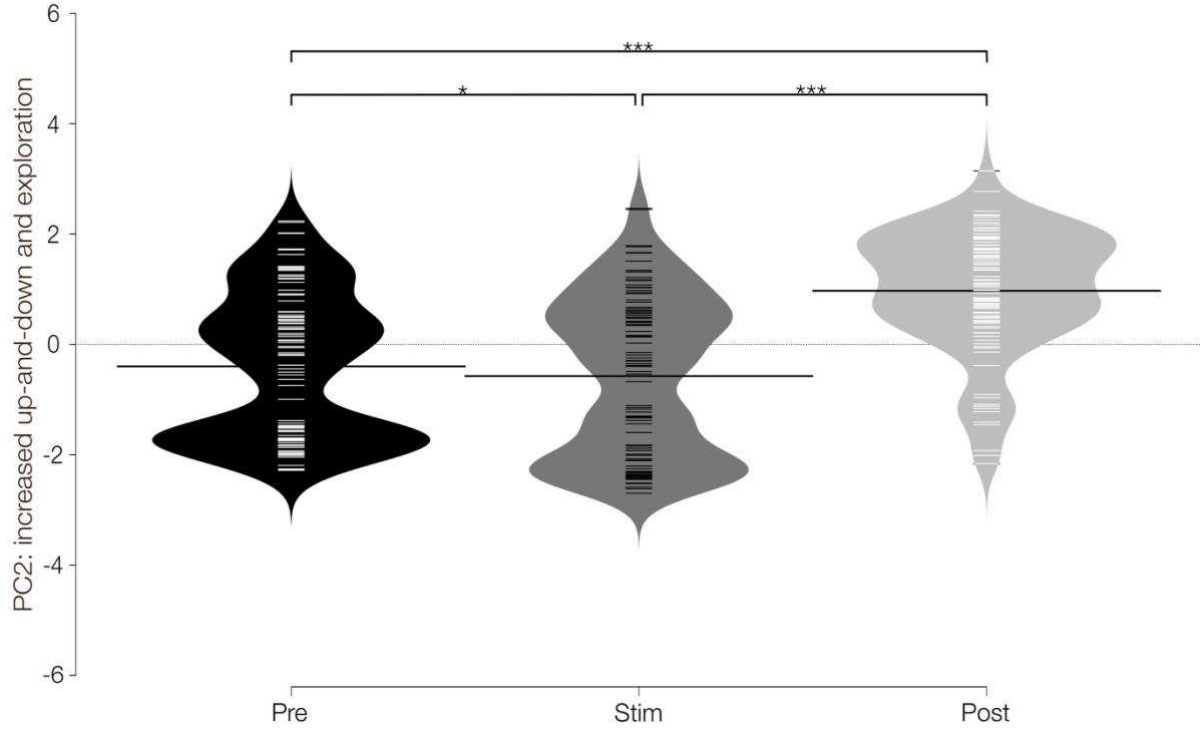


Figure 3. Effect of the period (Control (Pre), stimuli presentation (Stim) and post-stimulation period (Post)) on PC2 values (up-and-down swimming and tank's exploration). The different treatments did not have an effect on PC2 values. PC2 explained 24% of variance. Beanplots ('beanplot' R package (Kampstra, 2008)) combine individual observations (black or white lines – when represented inside the beans), dataset distribution, the overall dataset average (dashed horizontal line) and the average for each subset (heavy horizontal black line). * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

II. Contribution of the behavioural variables for each treatment

In the first analysis we showed an effect of treatment and trial period on male behaviour. This second analysis aimed to examine if the contribution of the behaviour variables was

different across trial period according to the treatments. This was achieved with five Principal Component Analyses, one per treatments (Fig 4).

For all five treatments, the period of stimulation (Stim) was characterised by an increase of aggressiveness and a decrease of shelter visits and maintenance in comparison to the control period (Pre) and the post-stimulation period (Post). Similarly, the same applied to the up-and-down swimming which increased during the post-stimulation period (Post) for the different treatments (Fig 4). Although the contributions of the behavioural variables were similar, individuals responded less in the chemical + sound treatment, with overlapping ellipses, compared to the other treatments.

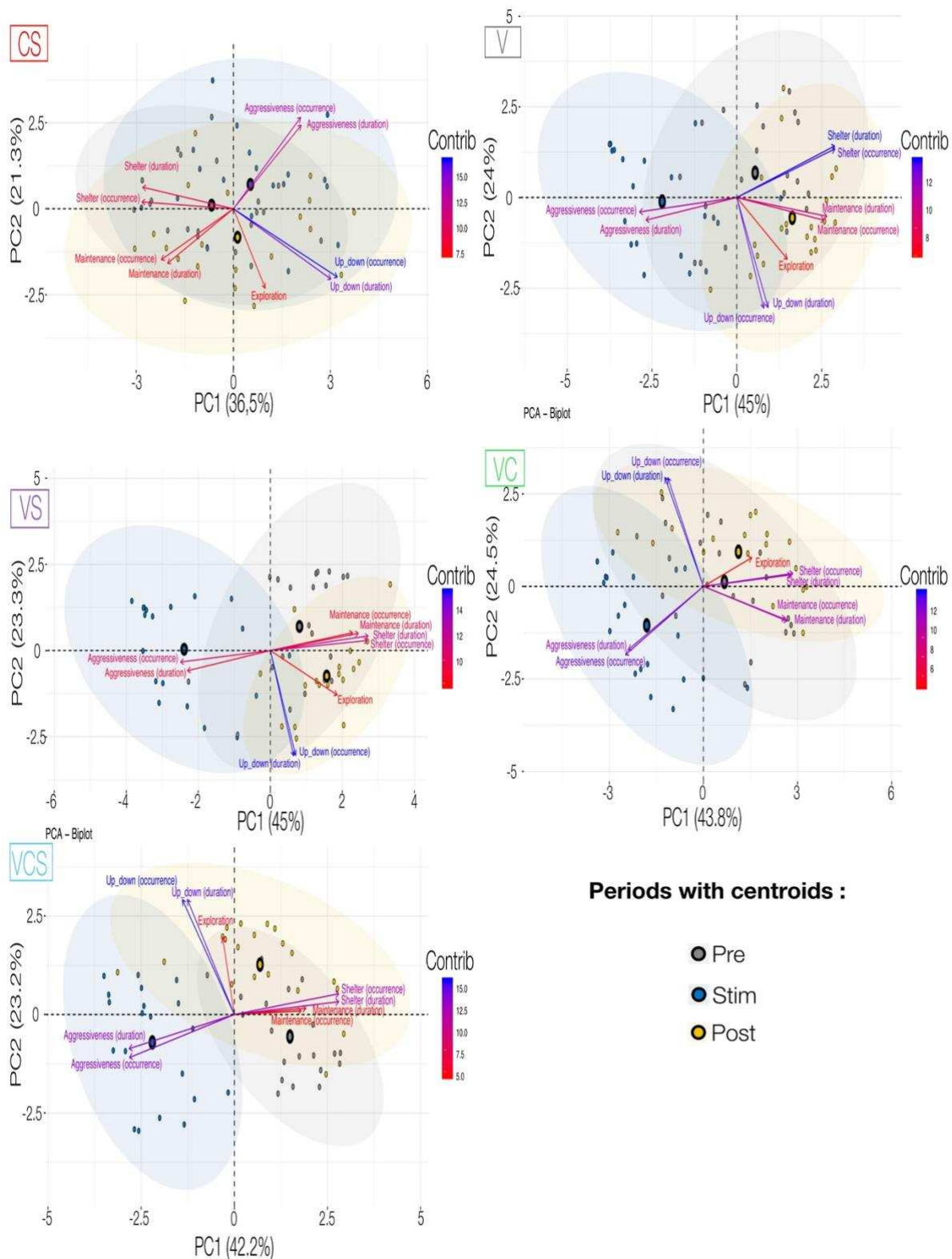


Figure 4. Principal component analysis biplot showing for each treatment (Chemical + Sound (red), Visual (grey), V + C (green), V + S + C (blue) and V + S (violet)) the contribution of each behavioural variables (aggressive behaviours (duration and occurrence), maintenance

(duration and occurrence), the shelter visits (duration and occurrence), the up-and-down swimming (duration and occurrence), and the tank's exploration score) according to the period (Control (Pre), stimuli presentation (Stim) and post-stimulation period (Post)). The 'fviz_pca_biplot' function ('factoextra' R package (Kassambara, 2017) combine the graph of individuals with concentration ellipses and centroids (larger symbols) and the graph of variables with their contributions.

III. Change in behaviour between the control (Pre) and the stimuli presentation (Stim)

To assess how behavioural variables changed when stimuli were presented, the difference between Stim and control periods of the behavioural Index (the mean value of each behavioural variable weighed by its loading in the 3 first PCA axis; see Methods) was calculated.

During stimuli presentation, the aggressiveness (duration and occurrence) increased for all treatments, especially when the visual component was present (Fig 5).

The maintenance (duration and occurrence) decreased during the stimuli presentation for all treatments, as well as the shelter visits (duration and occurrence) (Fig 5).

Up-and-down swimming (duration and occurrence) increased during the stimuli presentation for the CS and VCS treatments (Fig 5). For the V, VS, and VC treatments, the up-and-down swimming, decreased slightly or did not change (Fig 5).

Finally, the tank's exploration decreased for the VS and VC treatments while it increased for the other treatments (Fig 5).

With the exception of three behavioural variables, the other behaviours decreased or increased during the stimuli presentation more importantly for the VCS treatment, while there was no strong behavioural variability between the other treatments (Fig 5).

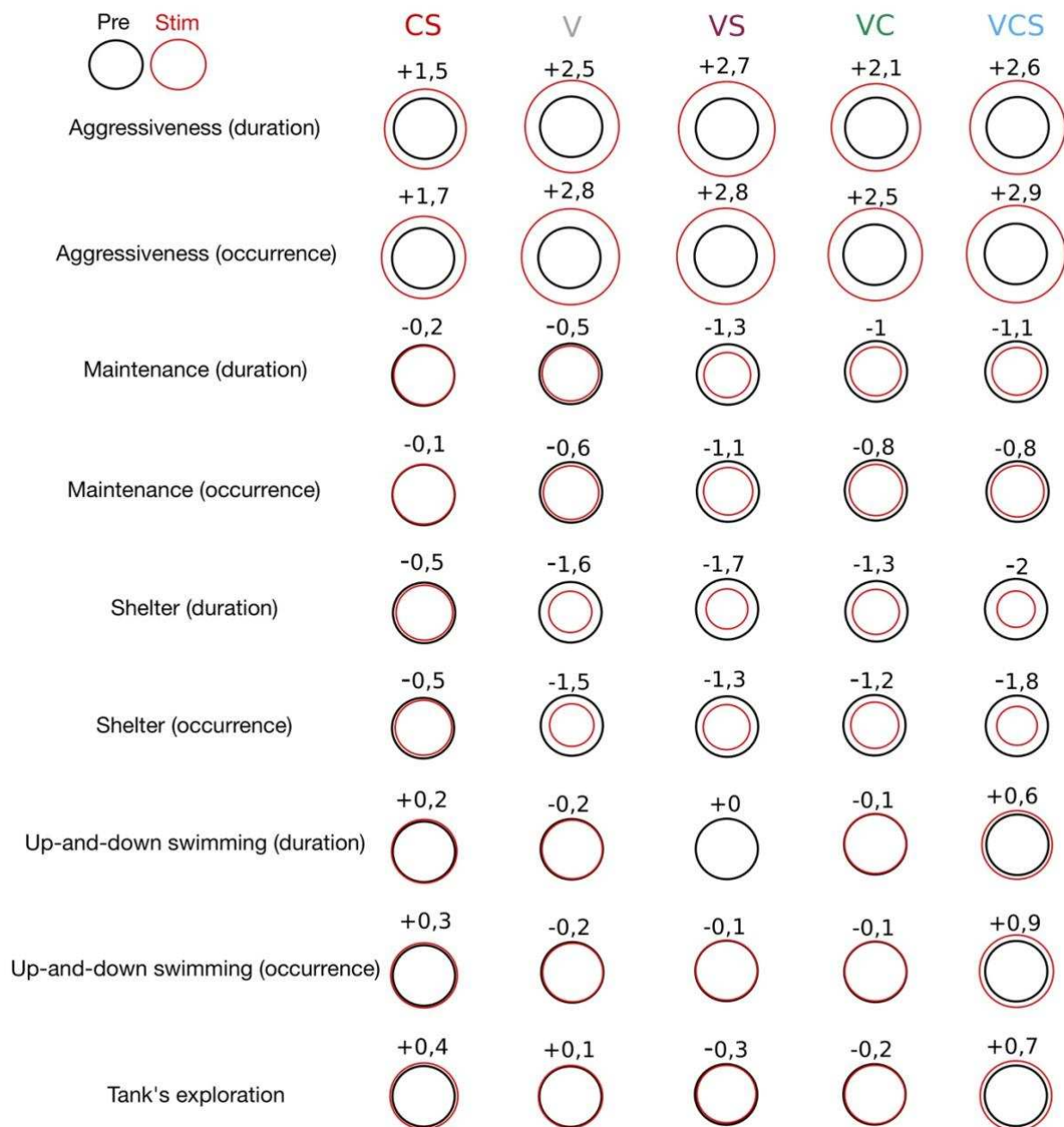


Figure 5. Index of the relative importance of the behavioural variables (aggressive behaviours, maintenance, the shelter visits, the up-and-down swimming, and the tank's exploration score) for the control (Pre in black) and the stimuli presentation (Stim in red) according to the treatments (Chemical + Sound (red), Visual (grey), V + C (green), V + S + C (blue) and V + S (violet)). The difference between the index calculated for the stimuli presentation and for the control was used to represent if the behaviour increased (positive values) or decreased (negative values) during the stimuli presentation (red circles) for each treatment.

Discussion

The use of multiple sensory cues in animal communication is relatively common but although more and more studies explored multimodality, most of the studies available up to now have investigated isolated or bimodal signals (Higham & Hebets, 2013; Uy & Safran, 2013). As a result, interactions between the sensory modalities and their actions on the receiver behaviour remain largely unravelled. Here, we investigated the interaction in a trimodal system during territorial encounter between males of a cichlid *Maylandia Zebra*. The results of this paper have to be interpreted with the results of a previous paper (Chabrolles et al., 2017) where dominant males were exposed to isolated chemical, visual and acoustic signals also in an agonistic context.

In this study we found that when the visual component (alone and combined with the two other modalities) was available for the receiver, his response was essentially agonistic with a high level of aggressiveness towards the opponent. This was associated to a decrease of other territorial behaviours such as maintenance and shelter visits. They also increased the up and down swimming and tank's exploration, especially during the post-stimulation period. Even when the different treatments (V, VS, VC, CS and VCS) were analysed separately (Fig 4), changes in behaviour were similar across trial periods. This could suggest that the visual component could be the most important sensory modality in this species. Indeed, Escobar-Camacho & Carleton (2015) consistently suggested that visual system could be the most important sensory system for cichlids in general. Regarding the chemical component, males exposed to male holding water changed their behaviours, by increasing both the up and down swimming and the tank's exploration, and decreasing the shelter visits (Chabrolles et al., 2017). Similar behavioural responses were observed in this study when the chemical component was

associated with the sound and when both sound and chemical were associated with the visual component. We also found that visual signals associated to chemical signals decreased aggression compared to the visual treatment alone. This suggests that chemical component could provide information about status and motivation in this species in accordance with previous studies on another cichlid, the Mozambique tilapia, which showed that males of the latter species could advertise their social rank and motivation to fight with urine pulses and odorants molecules contained in urine during territorial encounter (Barata et al., 2007; Keller-Costa et al., 2016). In addition, the association of the sound with the chemical component triggered aggression even though no visual component was present. They elicited the same behavioural responses as the visual component alone did, except for the number of up and down swimming which increased for the chemical + sound treatment and decreased for the visual treatment. To the best of our knowledge this is the first study looking into the receiver's response to chemical stimuli associated to agonistic sounds but without any visual component. It highlights the importance to look for inter-signals interaction as it was emphasised by Hebets & Papaj (2005).

As proposed by Escobar-Camacho & Carleton (2015) in their review, the chemical component could be the next most important modality in cichlids after the visual and could be of relative importance during close-range interactions. In comparison, the sound treatment in Chabrolles et al. (2017) did not elicit changes in behaviours by itself. Like in other studies, the view of a live contestant triggers a highly aggressive response from the tested male, while an acoustic playback alone did not change the behaviour of the targeted individual (Bertucci et al., 2010; Ladich & Myrberg, 2006). Agonistic sounds are typically used within short distance and associated with conspicuous visual displays (Ladich & Myrberg, 2006a). This could be linked to the order signals perception, which can be influenced by the modalities active space. In fact, the assessment of multimodal signals could be sequential in this species. Uy & Safran (2013)

have shown that sequential assessment is widespread across diverse taxa and modalities, with long-range signals attracting conspecifics for further assessment of close-range signals. In *Maylandia zebra* visual signals are used at both short and long ranges. They can be perceived first, however chemical signals could also be carried by water and remain for longer period. In the case where visual signals are disrupted by a physical barrier, it seems plausible that chemical signals are then perceived before the visual signals, while agonistic sounds are used only for close range interactions. If the different modalities in this species are assessed sequentially, visual signals could be perceived and processed before other signals and could elicit a strong response. This is of great importance in conflict resolution when both competitors would benefit if the contest is settled with displays and not an escalated fight (Bradbury & Vehrencamp, 2011; Van Staaden et al., 2011). Future work needs to address if the different sensory modalities in this species are indeed processed sequentially.

Using the conceptual framework proposed by Partan & Marler (1999) and the results from Chabrolles et al. (2017) we can suggest that the visual, the chemical and the sound component seem to be non-redundant signals, as those signals in isolation elicited different responses (aggression, arousal and nothing respectively). With this paper we showed in addition that the chemical and the acoustic signals modulated the visual component. For example, when visual signals were combined with agonistic sounds (VS) and with both sound and chemical signals (VCS), it increased a little bit aggressiveness. But this is in contrast with a previous study of Bertucci et al. (2010) where territorial males decreased their aggressiveness when visual signals were associated to agonistic playbacks. This fact highlight that response could be shaped by prior individuals experiences (Hebets & Vink, 2007). In our experiment, tested males were accompanied by a social group placed in an adjacent tank, as they were maintained in the experimental set up for five days. In contrast, in Bertucci et al. (2010), tested males were visually and acoustically isolated for 24h before the beginning of the experiment. They also

used repeated measures with three treatments (visual alone, acoustic alone and visual and acoustic stimuli) in one experiment (one day) while we used repeated measures with five treatments over five days (one treatment per day). These issues emphasise the importance to consider social experience when analysing multimodal communication.

When considering the behavioural responses to the different treatments in this paper, we saw that those responses were expressed in the same way (with increased aggressiveness, decreased maintenance and shelter visits and increased up-and-down swimming and tank's exploration). While the response is less intense for the chemical + sound treatment (CS) and more intense for the visual + chemical + sound treatment (VCS) there is no strong behavioural variability between the other treatments (Fig 5). If you consider the signal content, it might not be completely correct to categorise them as non-redundant. Sound in this species provide information about the size (Bertucci et al., 2012). Another study showed that *Maylandia zebra* males are able to perceive differences in frequency during playback and use that information for size assessment (Amorim et al., unpublished). Simões et al. (2008) also suggested that in this species, sounds produced by males during agonistic encounters could convey information on the motivation of the producer similar to the chemical signals (Keller-Costa et al., 2015). The visual component also provides direct information about the individual's size and coloration which is also related to the social status (Holzberg, 1978; Korzan & Fernald, 2007; Simões et al., 2008). In summary, these three signal modalities seem to provide information that overlap and could be used as a 'backup'. Whether redundant or not they could reduce the uncertainty about the environment as proposed by Munoz & Blumstein (2012). In this study, multimodality seem to reinforce the receiver responses with a gradient of intensity and with the number of signals received. This could be directly related to the distance between the two protagonists, which could vary across the situations. It is then possible to secure information according to the propagation constraints (modalities active space in a given environment).

With these results, we stress the complexity of characterising multimodal communication. Regarding how you frame the sensory modalities, you can classify them as redundant or non-redundant if you look into the receiver response. But if you look into the information that signals could carry, redundant and non-redundant classification is no longer properly adapted. A new, more complex classification correlating the receiver response and signal content needs to be developed to improve our understanding of multimodal communication. In addition, if you take into account the ecological context and the prior experience of the receiver, the framework may change suggesting that a more dynamic framework should be considered. More studies, empirical, theoretical and integrative, on multimodal signalling systems should help to clarify why animal communication became so complex.

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-Chapter 3-

Cross-sensory modulation in a future top predator, the young Nile crocodile

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Cross-sensory modulation
in a future top predator, the
young Nile crocodile

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Animals routinely receive information through different sensory channels, and inputs from a modality may modulate the perception and behavioural reaction to others. In spite of their potential adaptive value, the behavioural correlates of this cross-sensory modulation have been poorly investigated. Due to their predator life, crocodilians deal with decisional conflicts emerging from concurrent stimuli. By testing young *Crocodylus niloticus* with sounds in the absence or presence of chemical stimuli, we show that (i) the prandial (feeding) state modulates the responsiveness of the animal to a congruent, i.e. food-related olfactory stimulus, (ii) the prandial state alters the responsiveness to an incongruent (independent of food) sound, (iii) fasted, but not sated, crocodiles display selective attention to socially relevant sounds over noise in presence of food odour. Cross-sensory modulation thus appears functional in young Nile crocodiles. It may contribute to decision making in the wild, when juveniles use it to interact acoustically when foraging.

1. Introduction

As animal behaviour is commonly driven by information coming from different sensory channels, internal conflicts about the appropriate behavioural response regularly arise from concurrent biological stimuli. Inputs from a given sensory modality can thus modulate the perception and behavioural reaction to another stimulus [1–3]. For instance, olfaction has been shown to be modulated by vision [4,5] and sounds [6]. Although probably widespread, this cross-sensory modulation of perception has mainly been studied through sensory analysis, psychological and neurobiological approaches, but paying no or weak attention to its behavioural and adaptive correlates [7–11]. As crocodilians routinely experience simultaneous stimuli

from distinct sensory channels—chemical, acoustical, visual—from an early age, they have to deal with decisional conflicts emerging from concurrent stimuli, with crucial advantages in integrating various sensory cues to interact with congeners and improve prey localization and capture [12]. Using an experimental paradigm with young Nile crocodiles *Crocodylus niloticus* allowing testing of their behavioural responsiveness to food odour and/or social and non-social sounds, here we highlight a cross-modulation effect between olfactory and acoustic inputs. Strikingly, this cross-modulation varies according to an important regulating factor of motivation, the prandial state of the animals.

2. Material and methods

2.1. Animals and housing conditions

The study was conducted on 30 Nile crocodile juveniles (7–10 months old, undetermined sex, size: 33–41 cm), provided by the zoo 'La Ferme aux Crocodiles' (Pierrelatte, France). The experiments started after three weeks of acclimatization in the laboratory. The crocodiles were separated in two groups and kept in rest tanks (180 × 120 × 50 cm) under a 12:12 light:dark cycle. Since these animals are mostly nocturnal, their circadian cycle was reversed gradually during the first 3 days post-arrival to allow performing experiments during daytime. A low intensity red light allowed the monitoring of animals during the nocturnal phase. Air and water temperatures were maintained at 26 and 28°C respectively. Each animal was identified by a white mark drawn on its skin. Animals were fed twice a week, at the end of the diurnal phase, with chicken pieces (2–3 g/individual/feeding).

2.2. Acoustic stimuli

Contact calls of four different young Nile crocodiles (4–6 days old) were used for the playback experiments. These calls had been previously recorded in the laboratory from unfamiliar individuals, which were released into a tank where they could meet other siblings for the first time. In this situation, young crocodiles use contact calls to communicate [13,14].

An acoustic stimulus consisted of a series of repeated contact calls from the same individual (three renditions of the same call emitted in 2 s, which corresponds to a natural emission rhythm). During a test, a series was repeated nine times. These renditions were separated by silence periods of variable durations (1 s, 1 min or 5 min) to mimic natural sequences of acoustic interactions (detail of these durations: 1 s between the first and second call series, 1 min between the second and the third, 1 s between the third and the fourth, 5 min between the fourth and the fifth, and then 1 s between the remaining four series). To limit pseudo-replication, each tested individual was challenged with contact calls coming randomly from one of the four recorded crocodiles.

A white noise was used as control (bandwidth = 100–5000 Hz). The control sequence was organized identically to the call playback, except that the series of three successive calls was replaced by a continuous 2 s white noise to avoid the rhythmic effect produced by the alternation of calls and short silences.

2.3. Double-choice set-up

The behavioural tests were run in a rectangular arena (50 × 30 × 30 cm) with a wire-mesh platform (41 × 28 × 2 cm; mesh size: 1 mm²) divided in half by a median line. Below the platform, two Petri dishes (diameter: 4.7 cm) were placed at equal distance (2.5 cm) from each of the extremities, which delimited two zones containing the Petri dishes (figure 1; see [15,16] for an initial use of such apparatus in young rabbits). The Petri dishes contained either water or meat. This experimental set-up was located in a sound-proofed chamber (PRIMO Silence-Box, Tip Top Wood©). The temperature and luminance of the chamber were the same as for the housing room. Two loudspeakers (Kinyo, SW 201, Taiwan, frequency response : 80–16 000 Hz) were settled in each of the extremities of the arena. During experiments with sounds, only one of the two loudspeakers was used for playback. The active loudspeaker was changed alternatively between tests. PRAAT software [17] was used to control the playback of the acoustic stimuli.

Behavioural answers were recorded using a camera (BUL520, Active Media Concept, Vallauris, France) suspended above the arena and connected to a computer located outside the sound-proofed chamber. Just before releasing a crocodile into the arena, the experimenter started the video-monitoring. Each crocodile was introduced into the arena with its sagittal axis aligned on the midline of the platform and was left free over the wire platform. At the end of the experiment, the animal was reintroduced into



Figure 1. Experimental arena divided into two zones (1 and 2) and four orientation areas (north, south, east and west). An active loudspeaker was set up on one side, while there was either water or meat on the other side. To avoid spatial bias, both sides were permuted between tested individuals. Cardinal points were used to identify the animal's head orientation.

its rest tank, and the arena rotated at 180° to limit bias due to potential chemical cues in the environment. Every three individuals, the arena and the wire mesh platform were rinsed with 100° ethyl alcohol and pure water and dried; meat and water were replaced by new samples. To control for side biases, we balanced the stimuli position between the left and the right sides. Each individual was tested with only one treatment per day, with 2–6 days between two consecutive tests. An individual was submitted to a minimum of three and a maximum of seven different assays.

2.4. Assessment of the individuals' feeding state

We conducted preliminary observations to determine how long animals remain sated after a feeding episode. We manually fed individuals ($n = 13$) until they refused the food during 10 consecutive minutes. Two other feeding sessions were proposed respectively 1 and 2 h after the first one. During the first feeding session, individuals ate between 5 and 14 g of chicken (median weight: 9.13 g). Only two individuals ate again during the second session, while only one accepted to be fed during the third session. For the experimental tests, we therefore considered that an individual remained 'sated' during the 2 h following a feeding session. 'Fasted' individuals were not fed during 2 consecutive days prior to an experimental test.

2.5. Experiments

In a first experiment, we tested whether juvenile crocodiles display differential locomotor activity in response to food odour depending on their prandial state. One of the Petri dishes of the double-choice arena contained water and the other one contained a piece of meat. No sound was emitted by the loudspeakers. We tested 13 'sated' crocodiles and 10 'fasted' crocodiles.

Then, in the second and third experiments, we did two different playback assays:

- Playback 1 ('no food' condition) challenged both fasted ($n = 26$) and sated ($n = 24$) individuals with juveniles' contact calls or white noise *in the absence of any odour source*. One of the loudspeakers emitted the sounds while water was present on the other side of the arena.
- Playback 2 ('presence of food' condition) challenged both fasted ($n = 24$) and sated ($n = 24$) individuals with juveniles' contact calls or white noise *in the presence of an odour source*. Here we thus assessed if the responsiveness of crocodiles to sound can be modulated by food odour, and whether this putative effect depends on both the nature of the sound (contact call versus noise) and the prandial state (sated versus fasted) of the animal. One of the loudspeakers emitted the played back sounds while a piece of food was present on the other side of the arena (figure 1).

2.6. Analysis of behavioural responses

We characterized the behavioural responses of crocodiles to the experimental stimuli by measuring two variables:

- (1) the time (in seconds) spent in the two zones containing either meat or water (figure 1); an animal was considered to be in one zone when its nostrils and eyes were in that zone;
- (2) the head orientation time (in seconds), displayed alone or in addition to other body movements. Each movement of the head or head + body was quantified according to the cardinal points (figure 1). For example, an individual was considered facing east if its head orientation was north-east, east or south-east. This parameter allowed us to determine the time spent oriented towards the acoustic stimulus (sound or noise) emitted by the loudspeaker (while the active loudspeaker was on the right (east) or on the left (west)).

When no sound was broadcasted, we assessed only the presence time in the two pre-defined zones of the arena. Behavioural responses were analysed using ETHOLOG v. 2.2 software [18].

2.7. Statistical analysis

Due to the limited size of the groups, non-parametric tests were used. When no sound was broadcasted, the mean presence time spent on the zones above food versus water was compared with non-parametric permutation *t* tests for paired data ('perm.t.test' function from 'RVAideMemoire' R package [19]). In Playback 1 'no food' condition, and Playback 2 'presence of food' condition, we compared the head orientation times and the presence times spent in a specific area of the arena in different manners depending on the variance's homogeneity (the variance's homogeneity was tested with a non-parametric permutation Fisher test, 'perm.var.test' function from 'RVAideMemoire' R package): when the variance was homogeneous, non-parametric permutation *t* tests were used for comparisons between individuals submitted to a given choice, while Mann–Whitney tests were used when the variance was not homogeneous. To compare the behaviour of same individuals in different conditions of choice tests, non-parametric permutation *t* tests for paired data were used. For all comparisons, statistical differences were considered significant when $p \leq 0.05$ (without Bonferroni correction). Statistical tests were conducted using R v. 2.14.0 software [20].

3. Results and discussion

When challenged in the double-choice test with meat odour versus water (first experiment, without sound), fasted crocodiles were more attracted by the odour of meat ($n = 10$, $t = -2.39$, $p = 0.01$) while sated individuals did not display any preference between stimuli ($n = 13$, $t = -0.62$, $p = 0.56$; figure 2). In this experimental condition where no sound stimulus was emitted, the prandial state thus unsurprisingly modulates the responsiveness of young crocodiles to a congruent, i.e. food intake-related, olfactory stimulus.

However, the first playback assay (Playback 1), in absence of any odour (food) source but with a sound stimulus, further showed that the feeding state of juvenile crocodiles also alters their responsiveness to an incongruent—i.e. independent of food—stimulus. Although all tested crocodiles oriented their head towards both sound stimuli during the playback compared to the silent pre-playback period (fasted animals challenged with calls, $n = 14$, or noise, $n = 12$: $t = 3.86$ or 2.84 , $p < 0.01$; sated individuals challenged with calls, $n = 13$, or noise, $n = 11$: $t = 5.57$ or 3.52 , $p < 0.01$; Playback 1, figure 3*a*), only sated individuals explored equivalently both sides of the arena—including the sound side—during playback (time spent by sated individuals on opposite versus loudspeaker side, for calls or noise respectively: $t = -1.45$ or -0.90 , $p = 0.18$ or 0.43 ; Playback 1, figure 3*b*). Conversely, fasted individuals stayed preferentially on the water side ($t = 2.80$ or 2.25 , $p < 0.01$ or 0.02 ; Playback 1, figure 3*b*). Thus, in the absence of food odour, both fasted and sated crocodiles display attention (head orientation) to social and non-social sounds, but these stimuli fail in eliciting a locomotor response in fasted individuals.

Finally, the second playback assay (Playback 2, 'presence of food' condition) directly investigated our main question, i.e. how the olfactory and auditory channels interact during behavioural decisions. When meat odour was opposed to sound, fasted and sated animals mainly explored the food side compared to the loudspeaker side of the arena, whatever the sound (time spent by fasted individuals on food odour versus loudspeaker side, for calls or noise respectively: $n = 14$ or 10 , $t = 3.14$ or 1.89 , $p < 0.01$ or $p = 0.05$; for sated individuals: $n = 13$ or 11 , $t = 2.08$ or 1.67 , $p = 0.03$ or 0.07 ; Playback 2, figure 3*b*). However, although fasted crocodiles still oriented their head towards the loudspeaker in response to calls ($n = 14$, $t = 1.56$, $p = 0.05$; Playback 2, figure 3*a*), the noise stimulus was no longer efficient to elicit such head orientation ($n = 10$, $t = 0.05$, $p = 0.56$; Playback 2, figure 3*a*). Conversely, sated individuals

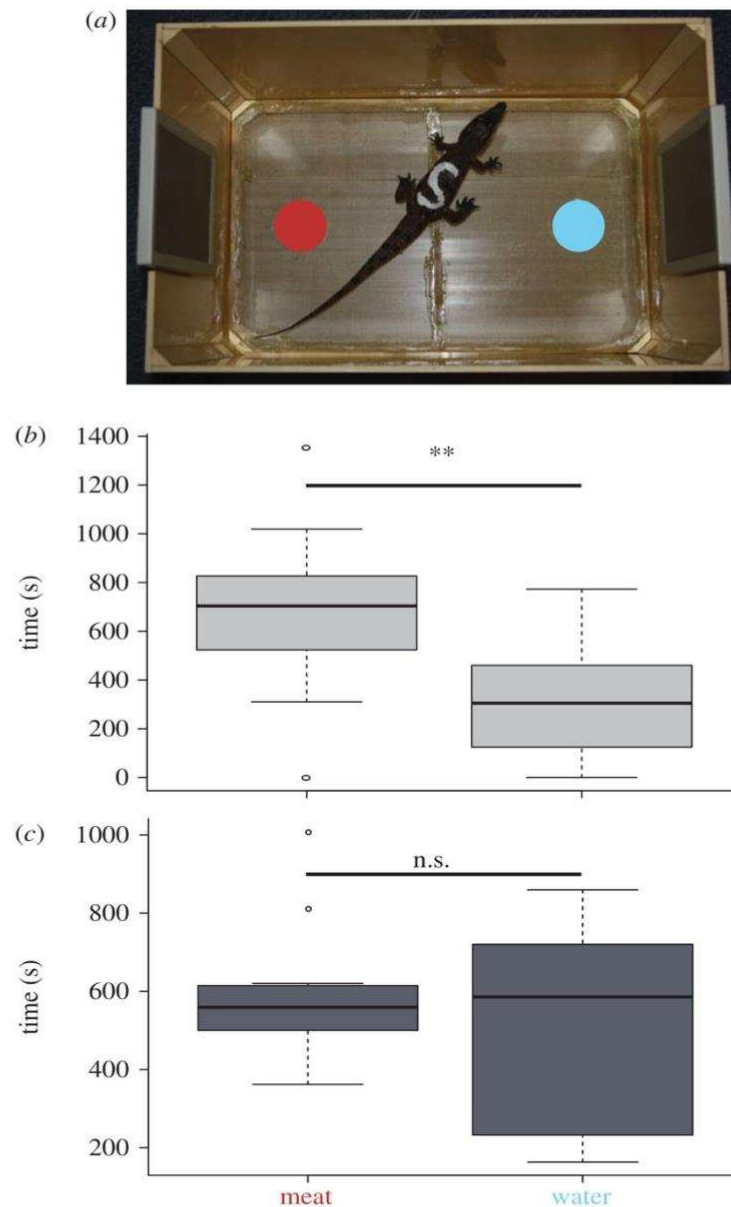


Figure 2. Young Nile crocodiles' preference to food odour versus water according to their prandial state. (a) Double-choice testing device (red dot: area with meat; blue dot: area with water). The boxplots show the presence time over each of the areas for (b) fasted ($n = 10$) and (c) sated ($n = 13$) crocodiles. n.s., non-significant difference; $**p \leq 0.01$.

oriented their head significantly towards the loudspeaker in response to both playback signals (calls: $n = 13$, $t = 5.95$, $p < 0.01$; noise: $n = 11$, $t = 2.94$, $p = 0.01$; Playback 2, figure 3a). Thus, young crocodiles prioritize food over sound stimuli whatever their prandial state and the nature of sound, but when fasted they display selective attention to socially relevant acoustic signals over noise in presence of food odour.

In summary, the prandial state influences behavioural responsiveness to food odours but also to sounds in juvenile crocodilians. In addition, and strikingly, the odour of food modulates their response to social calls, i.e. to signals from the acoustic modality. This cross-sensory modulation varies according to the prandial state of the animal, therefore to physiological processes related to motivation. Thus, young Nile crocodiles, which are known to gather together in pods and to strongly depend on their mother for protection [12], may already benefit from prioritizing distinct environmental sensory cues including intraspecific sounds, to maintain cohesion between siblings, interaction with the mother, and to be alerted for the presence of food. It is also noticeable that young crocodilians have been observed producing contact calls when being fed or in the presence of food [21]. This may explain why, in

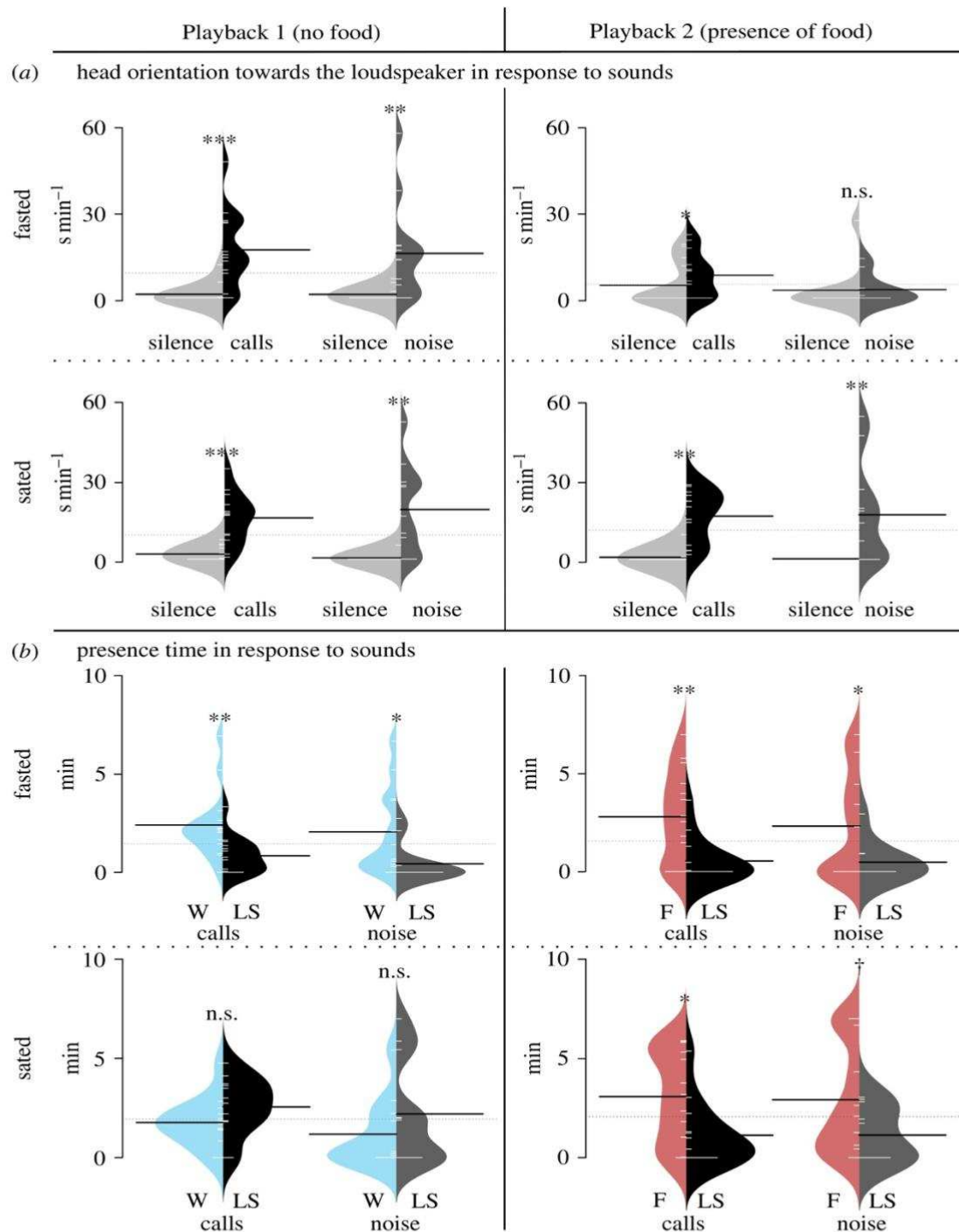


Figure 3. Behavioural response of young Nile crocodiles to sound stimuli according to their prandial state (fasted or sated) and the absence or presence of food. (a) The time spent oriented towards the loudspeaker (head orientation) was assessed during both the pre-playback period (silence) and the playback period (calls or noise). (b) The time spent close to the loudspeaker (LS) and the time spent at the opposite side (containing either water (W) or food (F)) were measured during the playback of sound signals (calls or noise). Beanplots ('beanplot' R package) combine individual observations (white horizontal lines), dataset distribution, the overall dataset average (dashed horizontal line) and the average for each subset (black horizontal lines). n.s., non-significant, $^+p < 0.1$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

our study, fasted individuals—i.e. young animals likely to search for food—responded selectively to this variety of calls compared to noise. Furthermore, at the adult age, they could use this ability in a multimodal sensory context including also interspecific sounds emitted by prey. Further research is required to determine how such cross-sensory modulation evolves during development and what are the physiological processes involved in its expression.

Ethics. All procedures described in the manuscript were conducted in accordance with appropriate French national guidelines, permits and regulations regarding animal care and experimental use (Approval n°C42-218-0901, 'Direction Départementale de la Protection des Populations' committee, Préfecture du Rhône).

Data accessibility. Raw data are included in the electronic supplementary material.

Authors' contributions. M.B., G.C. and N.M. initiated the study and contributed to the design of the experiments. L.C. collected the data and performed statistical analysis. N.B. was in charge of the animals. M.B., L.C., G.C. and N.M. contributed to the manuscript. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

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General Discussion

A. Research context and original contributions of this study.

The use of multiple sensory signals in animal communication is relatively common (Higham & Hebets, 2013). While the function and role of each signal separately has often been investigated, interactions between the sensory modalities and their actions on the receiver behaviour remain to be unravelled (Smith & Evans, 2013). Moreover, knowledge is limited about the perceptive abilities, physiological state and psychological framework of the receiver, which is independent of signal content, but should also influence signal evolution (Hebets & Papaj, 2005; Smith & Evans, 2013).

This thesis aimed to disentangle the complex system of multimodal communication. The originality of my studies lies in the fact that I conciliated both the interactions that could exist between sensory modalities with the potential influence of the receiver physiological state. I tried to provide a research as complete as possible about multimodal communication.

We used two species of vertebrates as models: a cichlid fish *Maylandia zebra*, and a crocodile *Crocodylus niloticus*. The first step was to characterize the role of different sensory modalities during territorial defence in *Maylandia zebra* males, and to understand the synergy between those modalities. The second step was to evaluate the impact of internal physiological processes on multimodal signals processing. We measured the behavioural responses of young Nile crocodiles who were exposed to olfactory and/or acoustic signals. We controlled their physiological state by rigorously modulating the prandial state of the animals.

In order to help with the reading of this discussion, [Table 1](#) summarises the results of both the first and the second chapters.

Behavioural response	Chemical (holding water)	Sound	Visual	C + S	V + S	V + C	V + C + S
Aggressiveness (duration)			↑	↑	↑	↑	↑
Aggressiveness (occurrence)	-	-	↑	↑	↑	↑	↑
Maintenance (duration)			↓	↓	↓	↓	↓
Maintenance (occurrence)	-	-	↓	↓	↓	↓	↓
Shelter (duration)			↓	↓	↓	↓	↓
Shelter (occurrence)	↓	-	↓	↓	↓	↓	↓
Up and down swimming (duration)			↓	↑	-	↓	↑
Up and down swimming (occurrence)	↑	-	↓	↑	↓	↓	↑
Tank's exploration	↑	-	↑	↑	↓	↓	↑

Table 1. Summary of the results from Chabrolles et al. (2017) where dominant males of the cichlid fish *Maylandia zebra* were exposed to isolated chemical, visual and acoustic signals in an agonistic context (chapter 1) and results from the second chapter where dominant males were exposed to combined chemical, visual and acoustic signals in an agonistic context. Arrows upward correspond to an increase of the observed behavioural response, while arrows downward correspond to a decrease of the measured behaviour. The arrows size indicates the intensity of the increase or decrease in behaviours. (-) means no behavioural change.

B. Role of each sensory modality in *Maylandia zebra* males in an agonistic context.

Question: What is the role of each sensory modality in this species? What are the behavioural responses of males *Maylandia zebra* exposed, during agonistic interactions, to isolated chemical, visual or acoustic signals?

In *M. zebra* males, the visual channel elicited strong response (Table 1). The view of a real opponent triggered a highly aggressive reaction and increased the subject activity (Table 1). In Bertucci et al. (2010) the view of a live contestant also provoked a high aggressive answer from the subject male. The visual component has been thoroughly studied in African great lake cichlid fishes as it was proposed that the diversity of colours in cichlids could be part of the mechanisms responsible for the explosive radiations observed in cichlids (Deutsch, 1997; Seehausen & Schluter, 2004). Consistently with this hypothesis, it has been shown that females select conspecific mates using visual cues (Kidd, Danley, & Kocher, 2006; Selz et al., 2014), though chemical and acoustic signals also seem to play an important role (Amorim et al., 2008; Plenderleith et al., 2005). More recently, male-male competition has also been proposed to be an important agent of diversification among cichlids since owning a territory is a prerequisite to mating (Dijkstra & Groothuis, 2011; Seehausen & Schluter, 2004). Males use colour patterns during their territorial defence from conspecifics and heterospecifics.

Acoustic signals, when presented in isolation, did not change the behaviour of males *M. zebra* in our study (Table 1). This result reinforces previous studies with similar results when studying agonistic context in fishes (Bertucci et al., 2010; Ladich & Myrberg, 2006). Nevertheless, agonistic sounds may impart information about size as the dominant frequency is negatively related to size (Bertucci et al., 2012). Simões et al. (2008) also suggested that in this species, sounds produced by males during agonistic encounters could convey the motivation of the sender. Fish agonistic sounds are typically produced within short distance from the receiver and associated with conspicuous visual displays (Ladich & Myrberg, 2006b). The absence of behavioural changes during playback could be explained by the fact that it may not be adaptive to respond to sounds unless visual cues confirm the presence of a nearby opponent, as sounds may travel long distances underwater (Bradbury & Vehrencamp, 2011).

As proposed by Escobar-Camacho & Carleton (2015) in their review, the chemical component could be the next most important modality in cichlids after the visual and could be of relative importance during close-range interactions. In the *Mozambique tilapia*, males could advertise their social rank and motivation to fight with urine pulses and odorants molecules contained in urine during territorial encounter (Barata et al., 2007; Keller-Costa et al., 2016). In *Neolamprologus pulcher*, fish that had only had access to visual information increased their urination frequency and showed more aggressive displays than fish that had both visual and chemical information available (Bayani et al., 2017). In males *Maylandia zebra*, the chemical channel presented alone did not elicit aggressiveness, but increased males' arousal (Table 1). These results suggest that males *M. zebra* could communicate social information and their aggressive propensity via the chemical component. The role of the chemical communication, during agonistic interactions in *M. zebra*, remains to be clarified.

In contrast to the wealth of research in cichlids available on visual, auditory and chemical communication, little is known about the role of the lateral line system, especially during territorial and aggressive encounters. Although this component is missing in my study it is likely that it also plays a significant role in this species agonistic interactions. Consistent with this suggestion, Butler & Maruska (2015) showed that in the cichlid *Astatotilapia burtoni* the lateral line plays an essential role for the mutual assessment of opponents during agonistic encounters, facilitating non-contact mutual assessment. Vibrational information could be essential and the role of the lateral line system during agonistic interactions in *M. zebra* clearly needs further investigation. For this purpose, the ablation of the lateral line would be possible in *M.zebra* according to the method used in Butler & Maruska (2015).

C. Inter-signals interaction, individual perceptive abilities and impact of the internal physiological state: multimodality as a complex system.

Question: How does the interaction between sensory modalities and the individual prior experience influence the perception of multimodal signals?

For the purposes of another ongoing study on chemical communication in *Maylandia zebra*, we injected males with patent blue V (Prolabo, France) and measured aggressive behaviours and urination patterns during agonistic contests (Ben Ammar et al., unpublished). The aim of this study was to understand the role of chemical communication during multimodal territorial defence in this species. During agonistic encounters, males of different social rank (dominant or subordinate) were exposed to an opponent with visual contact only, then visual and chemical contact was allowed, but the chemical provided came from holding water produced by unknown males with also different social rank (dominant or subordinate). We found that visual information triggered a strong aggressive response from subjects and the association of the chemical stimulus with the visual lowered this aggressiveness (Fig 1). The rank of the subject and the rank of the individual used for the holding water did not have a significant effect on the subject aggressive score. Data from dominant and subordinate males were then pooled for the Figure 1.

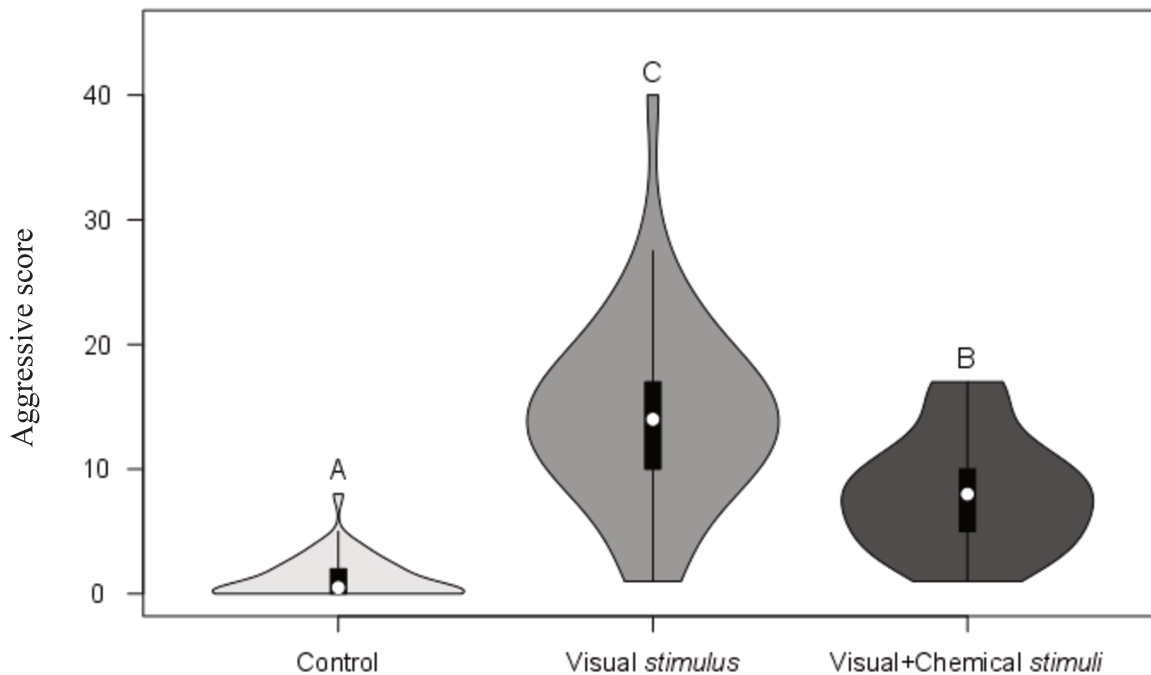


Figure 1. Aggressive score depending on the test phases: control (light grey), visual (grey) and visual + chemical (dark grey) phases. The Violin plots (combination of a box plot and a kernel density plot) span the minimum to maximum values. The boxes inside span the first to third quartiles and the white point inside the boxes show the median. Different letters indicate significant differences ($p < 0.001$).

Similar results were found in the second chapter where the association of visual and chemical signals also decreased the aggressiveness (duration and occurrence) in comparison to the visual treatment alone (Table 1). Those results reinforce the idea that chemical component could provide information about status and motivation in this species and highlights the significance of multimodal signalling during territorial defence as the chemical component clearly modulates the visual component in this species. Additional research about urine chemical components could improve the understanding of the role of chemical communication during agonistic interactions in males *Maylandia zebra*.

This is also the case for the acoustic component. Although males exposed to only acoustic or only chemical signals did not display aggressiveness, the association of the sound with the

chemical component triggered aggression even without the visual component (Table 1). When visual signals were combined with agonistic sounds (VS) or with both sound and chemical signals (VCS), they increased aggressiveness (Table 1) pointing to an interaction between the sensory modalities during multimodal communication in *Maylandia zebra*. The chemical and sound components modulate the visual signals. Another study using mismatched acoustic and visual signals carried out with this species, supports these results. They showed that when the opponent matched in size the subject fish, the sound was prevalent to visual information when it indicated a larger size (lower frequency) than the subject fish (the sound lowered aggression), but when the sound indicated a smaller size than the subject fish, visual information was prevalent (the sound did not lower aggression) (Amorim et al., unpublished). Taken together, these studies highlight the importance to look for inter-signals interaction when studying multimodality, as emphasised by Hebets & Papaj (2005).

The interaction and modulation among sensory modalities could be directly linked to the order of signal perception (i.e. the assessment of multimodal signals could be sequential in this species), which could be influenced by the modalities' active space. In fact, Uy & Safran (2013) have shown that sequential assessment is widespread across diverse taxa and modalities, with long-range signals attracting conspecifics for further assessment using close-range signals. An ongoing study using a cross-modal paradigm, has exposed *M. zebra* males to two experimental conditions: either a congruent context where the speaker that provide the playback is located near the jar containing the opponent, or an incongruent context where the speaker is distant from the visual stimulus. Results from this study should bring new inputs that should shed some light on this question. Synchronisation between different sensory modalities could be of great importance in the relevance and the efficacy of the multimodal communication as emphasised by Narins et al. (2005).

Apart from measuring behavioural responses, neural mechanisms of multimodal

communication should also be addressed. Future work on brain areas activation could be considered in this species by measuring the level of neural activity and expression of immediate early genes (IEGs). Numerous studies have already focused their research on the transcription factors *egr-1* and *cfos* (Burmeister, Jarvis, & Fernald, 2005; Desjardins, Klausner, & Fernald, 2010; Fernald & Maruska, 2012; Maruska et al., 2013; Okuyama et al., 2011). In *Astatotilapia burtoni*, social opportunity induced expression of the immediate-early gene *egr-1* in the anterior preoptic area (Burmeister et al., 2005; Fernald & Maruska, 2012). This change in expression could subsequently induce physiological changes that would increase males' fertility. It has also been shown that social information activates specific brain areas that differ significantly depending on what the female sees (Desjardins et al., 2010). It should thus be most relevant to test *M. zebra* behavioural responses in both males and females and IEG expression with an audience effect.

Here we emphasised the complexity of multimodal communication and how behavioural responses may differ according to combinations of stimuli, congruency of signal information or social context (e.g. audience effect). A remaining issue is the effect of internal physiological state which could cause internal conflicts about the appropriate behavioural responses that must be displayed. To address this issue, we were interested in a physiological process that could be easily controlled. Crocodiles were a very good model to test the effect of physiological state as they have a very slow metabolism, allowing to measure the impact of internal physiological processes on multimodal communication by accurately monitoring the animals prandial state.

Question: How the internal physiological state, more specifically the prandial state, can influence this inter-signals interaction during multimodal communication?

In young Nile crocodiles, the prandial state unsurprisingly modulates the responsiveness

of the individuals to a congruent (food intake-related) olfactory stimulus. However, in absence of any odour the prandial state also alters the responsiveness to an incongruent (independent of food) stimulus, a social sound (juveniles' contact calls) and a non-social sound (white noise). Only sated individuals explored the whole experimental arena during playback. Thus, in absence of food odour, social and non-social sounds elicited a locomotor response in sated individuals. Finally, when food odour was available, fasted crocodiles still oriented their head towards the social sound but the noise stimulus was no longer efficient to elicit the same behavioural response. Sated individuals still oriented their head towards the loudspeaker for both playback signals. Thus, young crocodiles prioritize food over sound stimuli whatever their prandial state and the nature of sound, but, when fasted, they display selective attention to socially relevant acoustic signals over noise in presence of food odour.

As young Nile crocodiles must find their own food, the influence of the prandial state ought to be strong in this species. Nevertheless, they must stay close to their siblings in order to be still protected by their mother who guards the pod. In this study, we could clearly see an interaction between acoustic and olfactory modalities, modulated by the internal physiological state of the individuals. Young animals that must be relatively autonomous at the beginning of their life should benefit of this cross-sensory modulation according to the prandial state by prioritizing distinct environmental sensory cues.

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Laura CHABROLLES

Title : Integrative study of multimodal communication in two models of vertebrates: *Maylandia zebra* and *Crocodylus niloticus*.

Abstract : The use of multimodal communication in animals is relatively common, however most of the studies have investigated cues in isolation or in bimodal systems and did not consider how the receivers detect and process signals. This thesis aims to disentangle the complex system of multimodal communication by studying the receiver behavioural responses to unimodal or multimodal stimuli and addressing the potential influence of the receiver physiological state on these responses. I used two vertebrates models that use multimodal signals: a cichlid fish *Maylandia zebra*, and a crocodile *Crocodylus niloticus*. The results show an interaction between the different modalities, allowing to modulate and refine the information transmitted during agonistic interactions in *Maylandia zebra*. In young Nile crocodiles, the prandial state (fasted or sated) is an important regulating factor of motivation which can influence the cross-modulation between olfactory and acoustic inputs. This thesis brings new inputs for a better understanding of multimodal communication in vertebrates. It calls for further studies, empirical, theoretical and integrative, on multimodal signalling systems that should help to clarify why animal communication became so complex.

Titre : Étude intégrative de la communication multimodale chez deux modèles de vertébrés : *Maylandia zebra* et *Crocodylus niloticus*.

Résumé : Bien que l'utilisation de la communication multimodale chez les animaux soit relativement courante, la plupart des études jusqu'à maintenant ont porté sur des systèmes uni- ou bimodaux et n'ont pas examiné comment l'individu qui reçoit les signaux les détecte et les traite. Cette thèse a pour but d'étudier le système complexe que représente la communication multimodale en conciliant à la fois les interactions qui pourraient exister entre les modalités sensorielles et l'influence potentielle que pourrait avoir l'état physiologique de l'individu recevant les signaux. J'ai utilisé deux espèces de vertébrés comme modèles utilisant des signaux multimodaux : un poisson cichlidé *Maylandia zebra*, et un crocodile *Crocodylus niloticus*. Les résultats montrent une interaction entre les différentes modalités, permettant de moduler et d'affiner l'information transmise lors des interactions agonistiques chez *Maylandia zebra*. Chez les jeunes crocodiles du Nil, l'état prandial (à jeun ou à satiété) est un facteur régulateur important de la motivation qui peut influencer la modulation croisée entre les signaux olfactifs et acoustiques reçus. Cette thèse apporte de nouveaux éléments pour une meilleure compréhension de la communication multimodale chez les vertébrés. Elle appelle à la réalisation d'autres études, empiriques, théoriques et intégratives, sur les systèmes de signalisation multimodale qui devraient aider à comprendre pourquoi la communication animale est devenue si complexe.

Discipline : Ethologie, Biologie

Mots-clés : communication multimodale, cichlidés, *Maylandia zebra*, interactions agonistiques, signaux visuels, signaux acoustiques, signaux olfactifs, modulation cross-sensorielle, crocodile du Nil, *Crocodylus niloticus*, comportement, état prandial, playback.

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