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„Decoding the function of the ring dove multimodal courtship display using audio-visual integrated analysis“

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To all the women trying to make it in a man's world

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Publications associated with this thesis and authors contributions

Chapter 1

Mitoyen, C., Quigley, C., and Fusani, L. (2019). Evolution and function of multimodal courtship displays. *Ethology*, *125*(8), 503-515. https://doi.org/10.1111/eth.12882

CM performed the literature review, drafted the manuscript. CQ critically revised the manuscript. LF critically revised the manuscript.

Chapter 2

Mitoyen, C., Quigley, C., Boehly, T., and Fusani, L. (*In press*). Female behaviour is differentially associated with specific components of multimodal courtship in ring doves. Animal behaviour.

CM designed the study, trained the animals, ran the experiment, performed statistical analysis, and drafted the manuscript. CQ designed the study, critically revised the manuscript. TB pre-processed the acoustic and visual data and coded the videos. LF critically revised the manuscript.

Chapter 3

Mitoyen, C*., Quigley, C*., Canoine, V., Colombo, S., Wölfl, S., and Fusani, L. Audio-visual synchronization of multimodal courtship signals affects female sexual receptivity in the ring dove (*Streptopelia risoria*). *Submitted to Proceedings of the Royal Society of London. Series B, Biological sciences*

*both authors contributed equally to the work

CM designed the study, carried out the experiment, performed the statistical analyses of the data, critically interpreted the results and drafted the manuscript. CQ designed the study, developed and tested the playback system, created the stimuli, critically interpreted the results and revised the manuscript. VC carried out estradiol assays and drafted the manuscript. SW trained the animals, carried out the experiment and coded the videos. SC coded the videos. LF conceived the study, critically interpreted the results and revised the manuscript.

GENERAL INTRODUCTION

The diversity of animal communication signals

Communication is the process through which information is produced and sent by one individual to another, resulting in an alteration of the receiver's behavioural or physiological state (Bradbury & Vehrencamp, 2011). The study of animal communication started thousands of years ago and the first writing traces come from Aristotle (384–322 BC) who quite precisely described acoustic communication in several bird species in his book "History of Animals". Animal communication studies considerably gained in popularity in the last few decades (Pepperberg, 2017), following the first Nobel Prize attributed to ethologists von Frisch, Lorenz, and Tinbergen in 1973 for their work on communication in bees. However, we are still far from fully understanding the diversity, complexity, evolution and function of communication signals observed in nature.

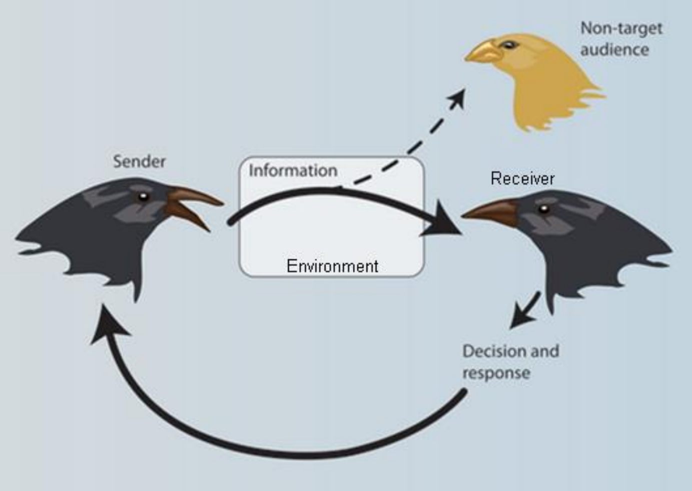

Figure 1. A model of animal communication (from Gillam, 2011).

Several situations require communication between at least two individuals, such as agonistic interactions (Aidan-Martin, 2006; Shelton & Grace, 1996), parent-offspring communication (Rojas Ripari et al., 2018; Trivers, 1974), group behaviour coordination (Fisher & Zinner, 2010) and sexual communication between potential mates (Andersson, 1994). Communication has been documented in virtually every existing taxon and can range from transmission of a single molecule (for instance, pheromones produced by female Lepidopterans to attract males, Myers, 1972), to remarkably complex messages in some specific social context in animals. When a piece of information intentionally changes the state of one or several receivers by inducing a response, this information is commonly referred as being a "signal". From an evolutionary point of view, signals are beneficial for both senders and receivers, differently from "cues" which unintentionally affect non-targeted receivers and are not usually shaped by natural selection to transmit information (Figure 1, Laidre & Johnstone, 2013).

Communication signals can be transmitted through different sensory channels and perceived by different sensory organs (**Table 1**). While as humans we are familiar with some communicative modalities such as visual and acoustic signals, our sensory systems can perceive only a small subset of the whole spectrum animals use to communicate. Among signals that humans cannot perceive, we find for example acoustic signals such as the low frequency (infrasound) singing of some whale species (Nieukirk, 2004; Edds-Walton, 2012) or high frequency (ultrasound) calls used by bats or dolphins (Liu et al., 2010; Puechmaille et al., 2014). This is also the case for some visual signals we cannot see, like ultraviolet colours used by bees to locate flowers (Chittka et al., 1994; Silberglied, 1979), or olfactory signals we cannot smell, like active compounds of pheromones used by Lepidopterans (Myers, 1972). Some sensory modalities are even fully unfamiliar for us as we do not possess the appropriate sensory organs to perceive and evaluate them. This is true for instance for electric signals used by elasmobranchs to hunt, perceived through specific electro-sensory organs, the ampullae of Lorenzini (von der Emde, 1998). Another example comes from snakes using the pit organ to perceive infrared temperature signals emitted by prey (Gracheva et al., 2010). As studies of animal behaviour has typically focused only on those displays we can perceive, such as those of many bird species, it is not surprising that our understanding of communication signals remains very incomplete.

Table 1. Example and functions of sensory modalities used by animals to communicate

Sensory modality	**Example and function**	**References**
Visual	Color patches used by fish to indicate condition	Price et al., 2009
Acoustic	Birds song used to defend territory	De Kort et al., 2009
Chemical	Butterfly pheromones to attract mates	Myers, 1972
Electrical	Use of electromagnetic field to hunt by sharks	von der Emde, 1998
Temperature	Infrared perception of prey by snakes	Gracheva et al., 2010
Tactile	Use of grooming to reinforce social bonds in chimpanzees	Fedurek & Dunbar, 2009

The diversity of sensory channels used to transmit signals, as well as the sensory systems adapted to perceive, integrate and process the information contained in these signals make the diversity and complexity of animal communication both fascinating and highly challenging to investigate. In this respect, technological development greatly serves the study of animal communication, by allowing to record a diversity of signals that was previously impossible to investigate. For instance, high-speed cameras now make it possible to record visual displays invisible to humans' eyes. One recent example comes from the blue-capped cordon bleu (*Uraeginthus cyanocephalus*), and the impressively fast tapping display made by both females and males during courtship which could be observed and quantified only by using video recordings acquired at 300 frames per second (Ota et al., 2015).

On the top of the diversity of sensory modalities used by animals to communicate, an additional level of complexity arises from the structure of the transmitted information. Not only can animals produce and perceive signals belonging to different sensory modalities, but they can also alter their temporal and spatial synchronization. These signals are referred as being multi-component if they are composed of signals occurring in the same sensory modalities, and multimodal or multisensory in the opposite case. The multimodality aspect of animal communication signals has started to be investigated relatively recently and has gained increasing attention over the past decades.

From a theoretical point of view, multimodal signaling raises many conceptual questions (Partan, 2013). One of the most obvious questions relates to the function of such complex communication signals when unimodal ones are simpler and less costly to produce and perceive. Indeed, using concomitant signals in different sensory modalities is potentially more energetically costly in term of physiological and anatomical adaptations required to produce the signal, but also more costly to integrate and assess from the receiver perspective (Partan & Marler, 2005; van Doorn & Weissing, 2006). Additionally to those intrinsic costs, external costs also arise as multimodal signals are often more conspicuous than simpler ones, making them more salient to predators (Rubi et al., 2019) or parasites (Zuk & Kolluru, 1998). Ultimate explanations for the existence of multimodal signals can be essentially assigned to two non-mutually exclusive hypotheses, based on what type of information is transmitted: a content-based hypothesis and an efficacy-based hypothesis. The first hypothesis (multiple message hypothesis, Doucet & Montgomerie, 2003) suggests that those signals could first evolve because each component would carry different pieces of information. This hypothesis has received empirical support both within and between species (Mowles et al., 2017; Takeshita et al., 2018; Candolin, 2003). Alternatively, the efficacy-based hypothesis suggests that some parts of the signal could improve the transmission of the main information by either facilitating its physical transmission in the environment (back-up signal hypothesis, Johnstone, 1997) or by improving receivers' capacity to perceive, assess or remember the signal (receiver psychology hypothesis, Guilford & Dawkins, 1991; Lynch, 2017).

Several studies in the last decade have attempted to set a theoretical background for multimodal signalling (Partan, 2013; Halfwerk et al., 2019), and we now need to formulate and empirically test hypotheses to understand how those multimodal signals are built and why they are so prominent. When it comes to the investigation of complex signals and multimodality in general, sexual signaling and courtship displays are particularly good model behaviours. Indeed, they are impressively diverse, often conspicuous and are virtually always multimodal or at least multicomponent.

How to attract mates? Sexual signaling and courtship displays

When Darwin first proposed his theory about evolution by means of natural selection (Darwin, 1859), the presence among animals of extravagant and costly traits detrimental for survival was a puzzling topic. Indeed, how to explain the prevalence of traits reducing their carrier's survival, by making them slower or very conspicuous to predators, for example? To justify the existence of those traits, Darwin additionally proposed sexual selection theory, explaining the differential in reproductive success between individuals due to the competition over mates (Darwin, 1871).

The reasoning is that features detrimental for survival can evolve if they provide an advantage in competing with the same sex to gain access to mates (intra-sexual selection), or helping to be chosen by the opposite sex (inter-sexual selection) (Andersson, 1994; Kokko et al., 2006). In this competition for mate access, both physical characters and behaviour can be sexually selected and become "secondary sexual characters" in opposition to primary sexual characters, such as external and internal genitalia in both females and males. Traits evolving by intra-sexual selection are generally referred as being "armaments" (e.g. weapons or color badges) and those evolving by intersexual selection as "ornaments" (e.g. conspicuous colors or long tails of some birds' species). Sexual selection sometimes leads to an exaggeration of those traits (Jennions et al., 2001). For instance, in guppies (*Poecilia reticulata*) colorful ornamental spots indicating male overall body condition can be beneficial in term of female choice, but also make males more obvious to predators (Kodric-Brown, 1993; Godin & McDonough, 2003). Another famous example of over-exaggerated sexually selected traits are the horns of several rhinoceros beetles species (McCullough et al., 2015), which presumably evolved by intra-sexual and inter-sexual selection but drastically reduce male lifespan (Emlen, 2001). In extreme cases, species could even go extinct (Bro-Jørgensen, 2014), which was likely the case for the Irish elk (*Megaloceros giganteus*) (Moen et al., 1999).

Among traits that evolved to increase the reproductive success of their bearer, behaviours are quite common. All behaviours aiming at attracting and ultimately reproducing with the opposite sex are referred as courtship behaviour. Similarly to ornaments and armaments, courtship displays can sometimes have a detrimental effect on survival. Indeed, because of their complexity and conspicuousness they can make the displayer more exposed to predators or parasites (Candolin, 1997, Endler, 1987, Koga et al., 1998). The energetic cost of courtship is also non-negligible, and these displays can be extremely metabolically demanding. In the golden-collared manakins (*Manacus vitellinus*) for example, heart rate during courtship is one of the highest recorded in avian or mammal species (Barske et al., 2014). In fact, the costly nature of courtship itself could be under sexual selection by female choice, as it would indicate male condition (Seymour & Sozou, 2009).

One field of research that has gained interest over the past years is the study of how the choosing sex can use more than one character to assess and choose a potential mate (Candolin, 2003). In early studies of mate choice and multiple traits, scientists usually focused on morphological characteristics of individuals. For instance, females can assess male body size (Cooper & Vitt, 1993), or colors (Morehouse & Rutowski, 2010) or length of some body part (Graham et al., 2020). But what happens when males present different types of ornaments? How do females assess different traits? How are multiple components differentially weighted in the context of sexual selection? Recently, these questions have been tackled by studies assessing the relative effect of different traits and females' preferences for different characteristics.

Many of the first studies investigating multiple traits in the sexual selection context assessed how courtship display in itself could interact with morphological characteristics. For example, Zeng et al. (2019) studied how female jumping spiders (*Cosmophasis umbratica*) responded to both the UV reflectance of males, and their vibratory courtship. In another study, Simpson & McGraw (2019) investigated the evolutionary relationship between iridescent plumage, courtship display and solar environment in six different humming bird species. Movement and courtship in general are thought to enhance male morphological features and to expose their ornaments. This happens for instance in guppies (*Poecilia reticulate*), where male movements likely serve to more efficiently display the orange spots that are indicators of good genetic condition and dominance (Kodric-Brown, 1993).

The specific case of multimodal courtship (*ie*. behaviour aimed to attract a mate displayed in more than one sensory modality) is worth considering when looking at the evolution of complex signaling. Indeed, the costly nature of courtship is especially true in the context of multimodal displays (Cady et al., 2011). The reasons for the prevalence of complex signals in courtship are still not fully understood. In order to counterbalance those costs, courtship must have a very important role in increasing the displayer's reproductive success. The role of courtship is to convince a potential mate (most often, a female) to mate with the sender. Females can use information contained in displays to assess direct and indirect benefits a male would be able to provide (Andersson, 1994). Courtship can also in some cases increase female sexual receptivity, or decrease her aggressiveness prior to mating.

Courtship can occur in different sensory modalities, and a given courtship component (i.e. courtship call) can vary both within and between individuals (i.e. call duration or call frequency can be variable). This adds a new level of complexity, as both the presence of a component and its inter and intra-individual variation could be assessed by the potential mate. For instance, the role of the presence of courtship calls can be investigated by cue-isolation experiment, presenting female with a courtship display lacking the acoustic component. Alternatively, one can also investigate how the quantitative variation of those calls (pitch of the calls, or the duration of the syllables for example) impacts the receiver's response. It is only by quantifying and investigating the variation present both within and between courtship components that we can better understand the forms and functions of multimodal courtship displays.

The ring dove as a model species to study multimodal signaling

When interested in multimodal courtship displays, the choice of a model species, and especially the choice of a relevant courtship display is important. The ring dove (*Streptopelia risoria*), also called Barbary dove or ringneck turtle dove, belongs to the Columbidae family and is purely domesticated. Ring doves are thought to have originated from the African collared dove (*Streptopelia roseogrisea,* Figure 2), a wild species native from northern Africa and from which they are morphologically indistinguishable (Baptista et al., 1997; van Grouw 2018, Goodwin, 1967). Its classification remains debatable today, as it can still hybridize with a variety of species from the *Streptopelia* family (Davis, 1970). In Europe, the first traces of domestication go back to the 16th century (van Grouw, 2018), and today, they can be found in various breeding pools across the continent.

Figure 2. *Streptopelia roseogrisera*, the wild species from which the ring dove probably originated. Illustration from John Gerrard Keulemans, in *Onze vogels in huis en tuin,* 1869.

Ring doves are monogamous and monomorphic. Usually males and females engage in courtship interactions for several days before the pair forms, and several reproductive episodes can take place within one year. The courtship form varies over this time, and different phases with different types of behaviour occur (Lovari & Hutchison, 1975). From the male side, sexual interaction usually starts with a chasing and bowing phase, followed by preening and stuttering, and ends with copulation-oriented behaviour such as nest-soliciting display, nest-cooing and wing-fluttering behaviour. In this thesis, I exclusively investigated the bow-call display, typically occurring at the beginning of the pair interaction (Figure 3, Video Supplementary S1, Appendix A). The bow-call is an audio-visual display composed of two concomitant, highly temporally synchronized signals. The visual part consists of the male bending repeatedly toward the ground, facing the female ("the bow"). An acoustic display, the bow-call, always accompanies the bowing. Males can perform this courtship from a few seconds to a few minutes duration without interruption.

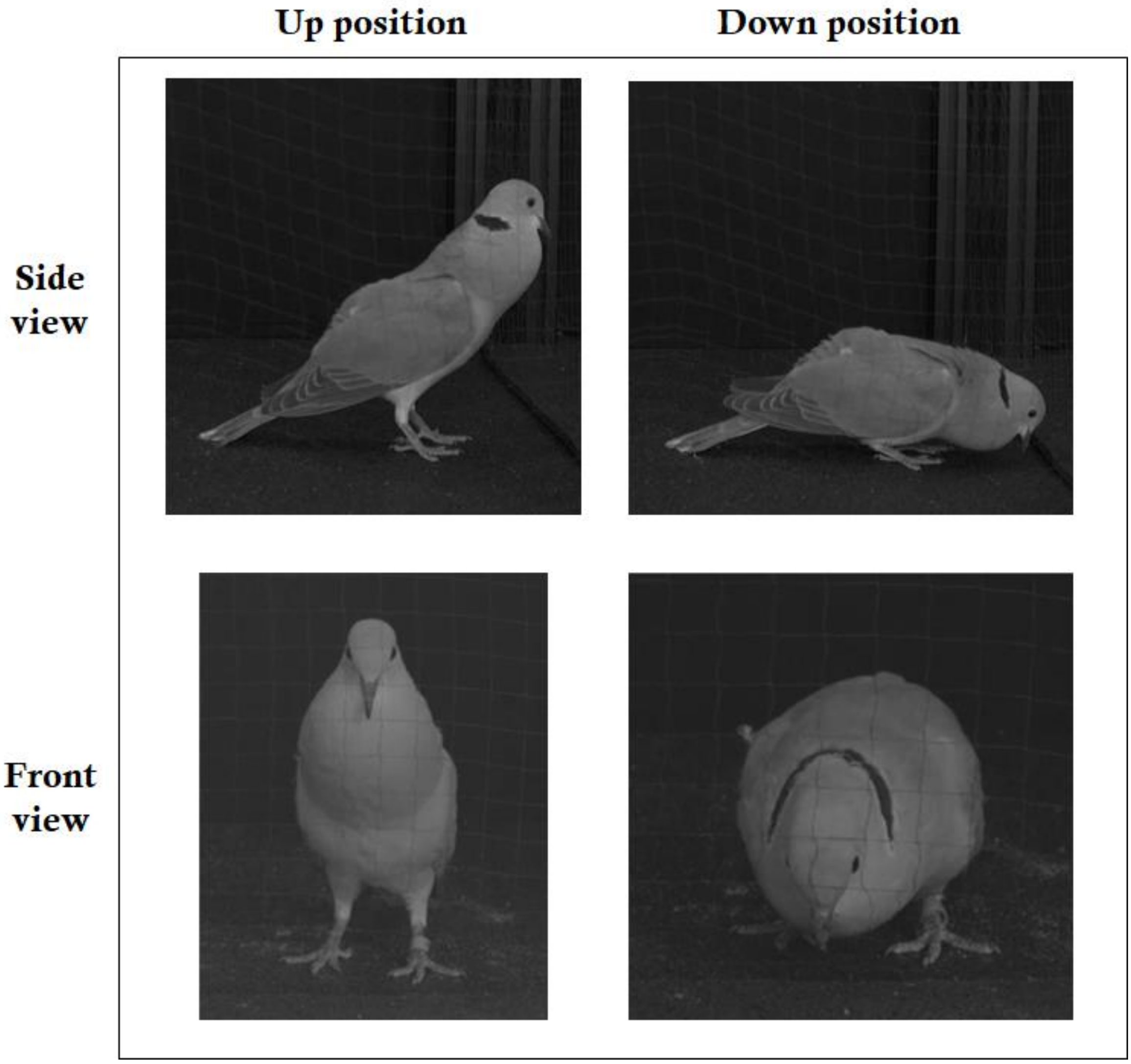

Figure 3. Side and front view (up and down position) of the male bowing display. During the first phases of the courtship interaction, males can perform this bowing display for several minutes without interruption.

Several factors make the ring dove an ideal model species to study and quantify a multimodal courtship in controlled laboratory conditions. Ring doves are tame, can be kept easily in captivity and trained efficiently to behave naturally in an unnatural laboratory setup. This aspect was particularly important as my hypothesis and analyses relied on semi-automatic video tracking, requiring highly controlled laboratory conditions regarding image background and lighting.

My analyses also required a relatively high number of courtship interactions to be recorded and quantified. Another advantage of the ring dove is that is it relatively easy to trigger courtship in males. Indeed, after an adequate period of visual isolation prior to the experiment (Hutchison, 1970), simply putting a male in contact with a female would easily trigger a courtship display.

Although being multimodal and therefore suitable to investigate mechanisms of elaborate and complex signaling in general, the ring dove display remains relatively simple. It can therefore be used as a basic model, in the context of the relatively young field of empirical studies on multisensory courtship. Indeed, it is only after elucidating the mechanisms at play in a bi-sensory stereotyped repetitive courtship that we might be able to focus on more elaborate displays where both male movements and acoustic parameters of calls are more complex. The ring dove display is therefore an interesting starting point to open up the way to study more than two concomitant sensory modalities within one display.

Finally, the last advantage of the ring dove as a model species is linked to its history in being broadly used from 1950 on as a model for behavioural endocrinology studies. Those studies precisely characterized the male ring dove courtship and behavioural transitions over a reproductive episode (Miller & Miller, 1958; Davies, 1974; Lovari & Hutchison, 1975; Hutchison & Lovari, 1976), but also the associated plasma androgen changes (Hutchison, 1970; Fusani & Hutchison, 2003). Previous studies also provided a wide knowledge on how male courtship influences female physiological response, which in turn influences female behaviour and anatomical changes such as their ovarian development (Lehrman, 1964; Cheng, 1973; Cheng, 1986; Cheng et al., 1988, Erickson, 1986). In particular, female estrogens rise in response to male courtship (Korenbrot, 1974) and female's own vocalizations (Cheng, 2003), and how these hormonal changes affects behaviour has been well documented (Lehrman, 1958; Cheng & Silver, 1975). This series of studies yielded a very good understanding on how different male courtship phases, as well as females' own vocal stimulation are related to females' neuro-endocrine system stimulation (Cheng 1979, Cheng, 2003; Cheng 2008).

One pioneering study even investigated the role of multimodality, by comparing physiological responses of females exposed to different modalities of the male courtship (Friedman, 1977). This study revealed that multimodal courtship was more efficient in stimulating females than unimodal signals. However, how specific parameters of the male courtship differently affected various females' responses remains unknown. The two experiments performed during my PhD were aimed at answering those questions.

How to investigate the role of multimodality? Methodology, conceptual approach and thesis plan

Currently, studies of complex signals and multimodality typically lack an integrative approach. Investigating how the presence or absence of a signal influences receiver response can give us hints regarding the intended recipient of the signal (for instance, is it a signal used in the context of inter or intra-sexual selection?). However, this approach is not sufficient to fill the gaps in the evolutionary history of communication signals. The function of multimodal signals can only be assessed by studying how their variability changes a receiver's response. While this has been shown in the context of unimodal signals, where intensity of female response has been linked to the variation of the signal (e.g. female preferences vary depending on the intensity or level of male courtship parameters, Ritchie et al., 2001), it remains to be investigated for multimodal signals (but see Ronald et al., 2017).

I hypothesized that males differ from each other regarding acoustic and visual courtship parameters, and that this variation carries different types of information. Females should be able to perceive small quantitative variation and respond accordingly. Females' wide range of response (and not only female choice *per se*) to variation in a number of sensory modalities has rarely been taken into account and needs to be considered. My aim was to investigate the effects of variation in male courtship parameters on female response, with the hypothesis that different female responses can indicate different functions of male signals. Rather than exclusively studying the role of independent signal components, I further hypothesized that the signal configuration and architecture itself is important and carries additional information that can be assessed by females.

During this PhD, I proposed an integrative approach, with the aim of quantifying both multimodal signal emission and receiver response to unravel the function of both individual signal variation and multimodal signal configuration. To this aim, I investigated several variables of the visual and acoustic components of courtship and assessed their range of variation both within and between individuals. I then investigated a number of female behavioural and physiological responses.

To understand how multimodal complex sexual behaviours evolved, it is important to precisely define courtship displays, to describe their forms and what is currently known of their different functions. In **Chapter 1**, I review what is known about the occurrence and diversity of multimodal courtship displays, the specificity of multimodality in the context of sexual signaling, and what gaps in the literature exist regarding examination of these displays. Finally, I suggest some directions that new studies should take to better understand complex signaling in the context of sexual selection.

The goal of the cross-over experiment presented in **Chapter 2** was to analyze the intra- and inter-individual variation of male multimodal courtship and to assess for the first time how females respond to those variations on a short time scale.

In **Chapter 3**, I focused on the configuration of the courtship signal, and tested if the temporal association between acoustic and visual components of courtship affects female response. This study was based on an innovative approach using audio-visual playback, where I used cutting-edge technology to create naturally looking video stimuli to be presented to female ring doves. I investigated both female behaviour and physiological responses to alterations in courtship synchrony, with the idea that courtship synchronization could carry information affecting immediate female behavioural response, but also act to physiologically stimulate them prior to mating. I first hypothesized that females would show a stronger behavioural response to playback of male courtship than to a control playback. I further hypothesized that multimodal playback would physiologically affect females and that circulating estradiol concentrations would be higher after repeated exposure to playback stimuli than at the beginning of the experiment. Finally, I hypothesized that multimodal synchronization would affect both behavioural and physiological states of female, showing differential behavioural response and differential circulating estradiol concentration depending on the type of synchronization between acoustic and visual courtship stimuli.

In this thesis, I used two different, yet complementary experimental approaches, illustrated in **Chapter 2** and **Chapter 3**. In **Chapter 2**, I used a correlative approach to associate male behavioural variation to female response. This approach has the advantage of allowing to unravel general patterns underlying the link between male behavioural variation and female associated response, without a priori hypotheses. However, the association between behaviours remains correlational and makes it difficult to infer a strict causal link between male courtship and female response. To overcome those potential limitations, **Chapter 3** presents a study based on playback techniques. Presentation of experimentally modified stimuli within a playback setup has the advantage of allowing to test how the variation of one specific parameter affects the receiver's response, as every other display parameter can be kept constant by the experimenter. This makes playback experiments a very powerful tool when it comes to study the effect of a given behavioural variation. However, one has to keep in mind that the artificial nature of the interaction potentially leads to issues linked to the lack of feedback in the communication (Butkowski et al., 2011) especially relevant in the context of sexual communication, as well as perceptual issues regarding colors or polarization of the presented videos (Rosenthal, 1999, Muheim, 2011).

Differently from classical studies on female choice for specific components of male courtship, my work attempted to unravel mechanisms behind the evolution and function of elaborate communication signals. Indeed, the experimental approach I present here, and especially the study of how the variation of different signal components and of their interaction triggers different responses, can be equally applied to non-sexual signals, such as signals used in agonistic interactions, for example. Additionally, I think that this thesis adds knowledge to the broad field of sexual selection, by giving more insight into how individuals use complex signals to communicate to attract and be chosen by potential mates.

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Chapter 1. Evolution and Function of Multimodal Courtship Displays

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Evolution and Function of Multimodal Courtship Displays

Running title: Multimodal courtship review

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Abstract

Courtship displays are behaviours aimed to facilitate attraction and mating with the opposite sex and are very common across the animal kingdom. Most courtship displays are multimodal, meaning that they are composed of concomitant signals occurring in different sensory modalities. Although courtship often strongly influences reproductive success, the question of why and how males use multimodal courtship to increase their fitness has not yet received much attention. Very little is known about the role of different components of male courtship and their relative importance for females. Indeed, most of the work on courtship displays has focused on effects on female choice, often neglecting other possible roles. Additionally, a number of scientists have recently stressed the importance of considering the complexity of a display and the interactions between its different components in order to grasp all the information contained in those multimodal signals. Unfortunately, these methods have not yet been extensively adapted in courtship studies. The aim of the present paper is to review what is currently known about the functional significance of courtship displays, particularly about the role of multimodality in the courtship communication context. Emphasis is placed on those cases where a complete picture of the communication system can only be assessed by taking complexity and interaction between different modalities into account.

Keywords

multisensory signals, sexual behaviour, elaborate displays, female preference, sexual stimulation

Introduction

Courtship is the suite of behaviours displayed by an individual to attract and eventually reproduce with an individual of the opposite sex (Bastock, 1967). Because courtship takes place within the scope of competition among conspecifics of the same sex, it is generally assumed to have evolved through sexual selection mechanisms. Courtship is usually performed by males towards females, but often involves an interaction between the two sexes (Huxley, 1914; Ota et al., 2015; Soma & Iwama, 2017), or a reversal of the usual sex roles. Historically, male courtship behaviour has been studied far more than female behaviour, and as a result most of the examples we provide in this review refer to male courtship. However, our arguments apply equally well to female courtship.

Courtship displays are extremely diverse. They are known to occur in many sensory modalities and can vary substantially even between closely related species (Andersson, 1994; Bastock, 1967). The most studied courtship displays in the animal kingdom are the visually conspicuous dances and acoustic calls of birds. Vibratory and olfactory signals are also very common, especially in arthropods (Hebets & Uetz, 1999; Houck & Reagan, 1990). Courtship can vary in its duration, with some species having only a few seconds courtship interaction before mating (Bastock & Manning, 1955), to several days of mutual interaction before copulation, as in the dwarf seahorse (*Hippocampus zosterae*) (Masonjones & Lewis, 1996) or emperor penguin (*Aptenodytes forsteri*) (Ancel et al., 2013). Secondary sexual characters and courtship are generally more conspicuous and intense in polygamous species, but are also present in socially monogamous species (Kirkpatrick, et al., 1990).

Past work focused mainly on the most conspicuous component of courtship signals (typically visual or auditory). However, with the possible exception of courtship occurring in environments in which some modalities cannot be transmitted, such as in complete darkness, most courtship displays occur in at least two sensory modalities, and include more than one signal aimed at separate sensory systems of the receiver. Those components may occur sequentially at different times during courtship. This is the case when males use a first component to attract females from a distance, and then another when the female is at a closer range, for example in the ring-necked pheasant *Phasianus colchicus* (Mateos & Carranza, 1999). Alternatively, the components can occur simultaneously, leading to very complex signals such as in birds performing conspicuous courtship composed of dances and calls (Andersson, 1994). We refer to these types of courtship as multicomponent if the components occur in the same sensory modalities, and multimodal or multisensory if they occur in two or more sensory modalities (Partan & Marler, 2005; Rowe, 1999).

Complex multicomponent and multimodal courtship displays have now been described for a large number of taxa (Knörnschild et al., 2014; Manica et al., 2017; Mowles et al., 2017; Ota et al., 2015; Preininger et al., 2013), and for both sexes. For example, in the blue-capped cordon-bleu (*Uraeginthus cyanocephalus*), Ota et al., (2015) documented a multimodal courtship display composed of visual, auditory, and tactile components performed by both males and females during courtship interactions.

Courtship plays an essential role in reproduction as it is often required for copulation to occur, with the exception of sneaky and forced copulations**.** Yet the ultimate causes at work are still poorly understood, as the links between the multiple signals of complex, elaborate displays and fitness benefits are far from being clear. Especially in the context of multicomponent courtship displays, it is still unclear what the role of each component is and why very elaborate behaviours have sometimes evolved in cases where simpler displays should suffice. As Candolin (2003) noted, courtship behaviour is usually studied as a simple structure, even though it almost invariably includes several components. Hundreds of studies have focused on single components of male courtship and investigated their relevance for female choice or female stimulation. With surprisingly few exceptions, studies on multimodal courtship rarely examine how females integrate multiple components to reach mating decisions, or whether courtship signals have effects beyond mate choice. The traditional 'trait-based' approach assesses the effects of individual signals sent by males on female preference and choice (Schacht & Grote, 2015). Experimentally, this is achieved via cue-isolation experiments, where single stimuli are presented separately to the choosing sex, typically the female. This approach raises at least three issues. First, experiments of this type rarely accurately reflect the complexity of courtship interactions as they occur in nature. Even in cases in which single modality components may reach the receiver in isolation from the rest of the signal, this does not always occur in a symmetric way in the wild. For example, in audio-visual communication the sender could be visually hidden but easily heard, whereas the opposite scenario is unlikely, unless there is masking by strong background noise. Cue-isolation experiments have rarely taken this into account and usually involve play-back stimuli separated from other stimuli that typically would accompany them. Such experiments therefore often disregard the natural conditions in which the behaviour evolved. Second, by focusing on the role of courtship on female choice and preference, researchers often neglect other potential functions of courtship. For instance, few studies have examined the role of male courtship components on female sexual stimulation, which may have a strong influence on male reproductive success (Beach, 1975; Lehrman & Friedman, 1969). Finally, the trait-based approach ignores information contained in the interaction between different components of courtship signals. An increasing number of theoretical (Partan & Marler, 1999; Stein & Stanford, 2008; Hebets & Papaj, 2005; Candolin 2003) and empirical studies (Taylor & Ryan, 2013; Stange et al., 2016; Ronald et al., 2017) have shown that the response to multiple signals differs from the simple sum of the responses to each component taken separately. This emphasises the potential additional value provided by the interaction between components (Taylor & Ryan, 2013).

Our overall goal is to provide a concise overview of what is known today about multimodal courtship displays, from both the empirical and theoretical point of view. For recent reviews about more general themes linked to the present paper, see Rosenthal (2017) for a thorough review of mate choice, Prum (2012) for a novel perspective on the role of beauty and attractiveness in sexual selection, and Ryan (2018) for a complete overview of the sensory bias theory. Because identifying the different functions of a particular behaviour is fundamental to understanding its evolution, the first aim of this paper is to review what is currently known about the general roles of courtship displays. Then, we will discuss how different signals can be composed to form complex multicomponent or multimodal courtship displays. Finally, with the help of recent theoretical work regarding multimodal communication, we will focus on assessing the function of complex behavioural signalling in the reproductive context to stress the importance of studying such signals as a complex unity. See Table 1 for definitions of key terms employed in this review.

Table 1. Definitions of key terms employed in this review

Term	Definition	Example	References
Multicomponent courtship	Courtship with two or more distinct signals occurring in a single sensory modality	The vocal courtship of the túngara frog composed of whines and chucks	Partan & Marler, 2005; Rowe 1999; Stange et al. 2016
Multimodal / multisensory courtship	Courtship comprising two or more signals occurring in at least two different sensory modalities	The bowing display of a ring dove, including a visual signal (the bow) and the bow-call	Partan & Marler, 2005; Rowe 1999, Fusani et al. 1997
Complex courtship	Courtship composed of multiple signaling elements, in one or more sensory modalities	The acrobatic dance of a manakin, jumping in a courtship arena while displaying brilliant plumage and snapping its wings	Hebets & Papaj, 2004; Fusani et al. 2007; Perrot et al. 2016; Miles & Fuxjager, 2018
Female sexual stimulation	Physiological changes occurring in females following male courtship	Courtship-induced follicular growth in ring doves	Lehrman, 1961

I - Functional significance of courtship

The most studied function of courtship is that of highlighting male quality and thus increasing reproductive success by enhancing the chances of the male to obtain a mate. However, courtship displays have other important functions.

A- Sex and species recognition

The ability of an organism to recognize members of its own species, and in particular of the opposite sex, is fundamental to produce offspring. In various taxa, closely related species might be morphologically very similar. In such cases, courtship behaviour can serve to identify members of a particular species, thereby reducing the risk of inter-specific mating in sympatric species. In the *Drosophila* genus, several features of courtship songs vary between species and are thought to be responsible for maintaining sexual isolation between closely related species (Saarikettu, et al., 2005). Another example comes from a recent study on birds of paradise (*Lophorina* genus), where the authors analysed the audio-visual courtship displays of individuals in New Guinea (Scholes & Laman, 2018). By highlighting differences in ornament exposition in the courtship displays of birds previously thought to belong to only one species, they could support previous molecular and morphological analysis (Irestedt et al., 2017) and confirm the existence of several allopatric species. As a further example, in field crickets of the *Teleogryllus* genus, male calls that serve to attract females are thought to be important for pre-zygotic isolation (Hoy et al., 1977). Finally, in *Heliconus* butterflies, male multimodal courtship based on olfactory and visual signals is thought to be a powerful driver of reproductive isolation (Southcott & Kronforst, 2017). Similarly, in monomorphic species where the two sexes look alike, courtship and response to courtship provide important information about the sex of the potential partner, for example in the ring dove (Lehrman, 1964; Lovari & Hutchison, 1975). Even though reproductive isolation due to mate choice has been long seen as a main driving force of speciation (Kirkpatrick, 1982), in some cases, courtship might not be sufficient to maintain reproductive isolation. In *Drosophila heteroneura* and *Drosophila silvestris*, for example, experimental crosses between the two species showed that courtship behaviour has a minor role in reproductive isolation (Boakes et al., 1997) and that the isolation between the species is due to the failure of heterospecifics to perform courtship behaviour at all, rather than differences in courtship repertoire such as courtship duration (Boakes et al., 2000).

B- Sexual stimulation and synchronization of mating behaviour

In species with a distinct breeding season, the transition to reproductive status is triggered by environmental factors such as photoperiod, temperature, or light intensity (Farner, 1964; Gemeno & Haynes, 2001). In some species, additional stimulation is sometimes necessary for mating to occur, and courtship and mating can induce the female to become physiologically responsive and eventually allow fecundation.

Within this context, a considerable amount of research has been carried out on web-building spiders. In orb-web spiders, one function of the abdominal wagging performed by the courting male on the female is thought to be an increase in pressure of the haemolymph which facilitates sperm transfer after copulation (Huber, 2004; Wignall & Herberstein, 2013). In the wolf spider (*Stegodyphus lineatus)*, courtship pre-mating vibratory behaviour seems to stimulate the receptive female to mate (Maklakov et al., 2003). In salamander of the Plethodontidae family, sexual pheromones delivered by males during courtship shorten the latency of females to mate and increase female sexual receptivity (Houck & Reagan, 1990; Houck et al., 2008). In the ring dove (*Streptopelia risoria*), male courtship is responsible for hormonal and physiological changes that trigger oviduct growth in females, making reproduction possible (Lehrman, 1964). Lovvorn et al. (2012) described the courtship behaviour of spectacled eiders (*Somateria fischeri*) and hypothesized that the more likely role of courtship in this species was to accelerate female hormonal development in order for them to be ready to mate during the short time windows their polar habitat offers.

Courtship can also be useful for spatial synchronisation of mating behaviour in species where individuals are spatially dispersed, for example as a means to attract females to a courting male's territory or to a breeding site. In field crickets, males use a long-range calling song to attract distant females (Alexander, 1961). The use of long-distance infrasound calls has also been proposed to attract females to mating leks in cetacean species where individuals can be several hundreds of kilometres apart (Herman, 2016).

C- Female choice process

According to sexual selection theory, females choose a sexual partner because of the relatively greater benefits potentially acquired through mating with this individual. Those benefits are traditionally classified as direct if females gain access to territory, help in parental care, or other resources; and indirect if they are gained only by the offspring, e.g. good genes and/or the capacity to attract mates (Andersson, 1994). With courtship, males may signal potential benefits to females, and females can assess these signals to make a mating decision. A large number of male morphological traits have been shown to play a role in the female choice process, among them size

(Harari et al., 1999), symmetry (Little et al., 2008) and colour (Kodric-Brown & Nicoletto, 2001). Some studies examined the link between particular features of courtship and female preference and choice. Among the features under female selection, we find rate and intensity of postural (Mowles et al. 2018) or auditory (McComb, 1991) displays, as well as total courtship duration (Seymour & Sozou, 2009) and overall courtship rate (Berson & Simmons, 2018). In the golden-collared manakin (*Manacus vitellinus*), females show a preference for good motor skills by choosing to mate with males who display faster and longer (Barske et al., 2011; Fusani & Schlinger, 2012). All those characters are thought to indicate physical ability or general qualities of the males that can be passed to the offspring.

Not all male characters necessarily reflect some intrinsic quality. In some species, males are known for exploiting female sensory biases to influence their decisions. Sensory bias theory states that the most successful courtship displays are those which best stimulate specific aspects of the female sensory system which evolved through natural selection (Fuller et al., 2005; Rowe, 1999). Although the sensory bias hypothesis has been applied to specific morphological and acoustic traits involved in mate choice (see reviews by Ryan & Cummings, 2013), examples of its extension to more elaborate courtship displays are rather scarce. One example comes from a study on grasshoppers (*Chorthippus biguttulus)* where the authors studied the shape of the female preference function after artificially adding a new element to a naturally simple male courtship and thereby making it more complex (Reichert et al., 2017). They found a complex relationship between female preference and the timing and the type of novel elements added to the original song, and concluded that sensory bias could in some cases promote the evolution of male courtship signals. Another example comes from different species of bowerbirds, where it has been found that male preference for the coloured decorations they use to adorn their bowers matches with female colour preferences for food items during foraging (Madden & Tanner, 2003). A more sophisticated case of exploitation of a courtship receiver's perception has been suggested in the case of the great bowerbird (*Ptilonorhynchus nuchalis*), where males place objects in a particular size order when building the court in front of their display avenue, creating a visual illusion which might make the displaying male look larger than he really is (Kelley & Endler, 2017).

During the early discussions about mate choice within an evolutionary context, Wallace argued that courtship vigour was the primary focus of females, while Darwin thought that the choice for ornamentation prevailed (Hoquet & Levandowsky, 2015; Prum, 2012). Both of them were probably right, as ornaments and vigour are often closely related (Cornuau et al., 2012), as motor displays or specific postures are necessary to expose ornaments and to make them more conspicuous (Hebets & Uetz, 2000; Jones et al., 2014).

D- Moderation of female aggression

Additionally, courtship may act as a moderator of female aggressiveness, and is particularly important in species where female cannibalism is common. In these cases, males should greatly benefit from displaying a behaviour that may prevent them from being killed. In orb-web spiders (*Argiope keyserlingi)* for example, male shuddering behaviour during courtship seems to have an effect on female cannibalism (Wignall & Herberstein, 2013)

II- Why so much complexity in courtship displays?

The presence of complex communication signals raises questions about their advantage over simpler ones. Producing complex signals might be energetically more costly and might increase predation rate (Partan & Marler, 2005) (but see Clark, 2012 for an alternative view on the potential cost of courtship, and Munoz & Blumstein (2012) regarding the cost of multisensory signals in general). Although it is clear in some cases that multi-modal signalling improves mating success (Berson & Simmons, 2018; Girard et al., 2015; Stafstrom & Hebets, 2013) or increases physiological responses in females (Friedman, 1977), the proximate and ultimate mechanisms involved are unclear and elaborate behavioural signalling still lacks a unitary and broadly accepted theoretical framework.

The evolution of multi-component signalling has recently received a great deal of attention, and several hypotheses have been proposed (Partan, 2013). For example, Hebets & Papaj (2005) stressed the fact that selection pressure can also act on the composed signal and not only on its independent components. Following the classification first proposed by Guilford & Dawkins (1991), the authors distinguish between "content-based" and "efficacy-based" hypotheses as possible mechanisms of complex signalling evolution. While the former focuses on the information carried by the signal, typically identity or quality in the context of courtship displays, the latter includes mechanisms improving the production, transmission and reception of a signal, including factors in the signalling environment or the receiver's sensory system. Later, Rowe & Halpin (2013) applied this same classification to the specific case of aposematic signals. Candolin (2003) conceived a similar classification for cues used in mate choice in a large number of taxa, but did not specifically address courtship or courtship components other than those involved in mate choice. Table 2 lists empirical studies reporting evidence for the benefits of multimodal courtship displays

Table 2. Empirical studies reporting a benefit for sender and/or receiver for courtship composed of more than one sensory modality

Function of multicomponent/multimodal signals	**Sensory modalities involved**	**Species**	**References**
Improve signal efficiency			
Vocal sac helps females to better discriminate and detect male signal	Visual and acoustic	Anurans *sp*	Starnberger et al., 2014
Part of the auditory courtship increases discriminability of the entire call	Acoustic	Magicicada *sp*	Cooley and Marshall, 2001
Redundancy i.e. suppression of one modality does not alter copulation success	Visual, acoustic, chemical and tactile	Drosophila (*Drosophila saltans*)	Colyott et al., 2016
Provide multiple information about male qualities			
Vibration vigor and display duration advertise different aspect of male quality and differentially predict mating success	Visual and tactile	Peacock spider (*Maratus volans*)	Girard et al., 2015
Different male display traits predict different cognitive abilities of the males	Visual	Satin bowerbird (*Ptilonorhynchus violaceus*)	Keagy et al., 2012
Trigger different females responses			
Pheromones serves for sex recognition and head bobbing attracts the attention of females and communicates male's location	Chemical and visual	Iguana (*Liolaemus pacho)*	Vicente and Halloy, 2016; Vicente and Halloy, 2017
Frequency and temporal patterns of sounds give information about species identity while call intensity and visual signals influences mate choice	Acoustic and visual	Sand goby (*Pomatoschistus minutus*)	Pedroso et al., 2013
Shuddering behaviour increases female acceptance and reduces aggressiveness while abdominal wagging facilitates sperm transfer	Visual and tactile	Orb-web spider (*Argiope keyserlingi*)	Wignall and Herberstein, 2013; Huber 2004
Reach different receivers			
Females differs in their preference for individual components of courtship according to their own sensory configuration	Visual and acoustic	Brown-headed cowbird (*Molothrus ater*)	Ronald et al., 2018
Acoustic part help males in discriminating between female visual aggressive and courtship display	Visual and acoustic	Red-winged blackbirds (*Agelaius pboeniceus*)	Beletsky, 1983

Signal at different environmental scales			
Feeding courtship attracts female attention and the lateral display triggers copulation solicitation displays by females	Visual	Ring-necked pheasant (*Phasianus colchicus*)	Mateos and Carranza, 1999
Acoustic component determines whether females visit a male and display rate then predicts of the likelihood of mating	Visual and acoustic	Sage grouse (*Centrocercus urophasianus*)	Gibson, 1996
Signal good neuro-muscular coordination			
Courtship elicit female response only if all the components are present	Visual and tactile	Drosophila (*Drosophila virilis*)	LaRue et al., 2015
Males synchronize acoustic with visual components	Visual and acoustic	Montezuma oropendolas (*Psarocolius montezuma*)	Miles and Fuxjager, 2018
Temporal synchrony of signals increases female receptivity	Visual and tactile	Brush-legged wolf spider (*Schizocosa ocreata*)	Kozak and Uetz, 2016
Females reject courtship when the signal lacks synchrony and synchronized signals are more attractive	Visual and acoustic	Túngara frogs (*Physalaemus pustulosus)*	Taylor et al., 2011; Taylor et al., 2017
Interaction between components yields new information			
Two artificial courtship signals individually not attractive combine into an artificially attractive signal	Visual and acoustic	Túngara frogs (*Physalaemus pustulosus)*	Taylor and Ryan, 2013
Female integration of component signals is not additive and the preference varies with signal complexity	Visual and acoustic	Túngara frogs (*Physalaemus pustulosus)*	Stange et al., 2016
Intensity of the visual component of male courtship modulates the attractiveness of male song	Visual and acoustic	Brown-headed cowbird (*Molothrus ater)*	Ronald et al., 2017

A- Improving signal efficiency

Rather than carrying information for the receiver, some parts of a multicomponent signal can instead act to improve signal efficiency, which is defined as the probability that the receiver perceives the signal in the intended way. This can be achieved by improving the way the receiver perceives the signal (receiver psychology hypothesis), or by improving transmission in the environment (back-up signals hypothesis).

a) Receiver psychology in the courtship context

The receiver psychology hypothesis states that some signal components function to facilitate improved perception, discriminability, assessment or memorization of the information contained in the main signal (Guilford & Dawkins, 1991; Rowe, 1999). For instance, a sound can function to draw attention to a visual display, or vice versa. In anurans, for example, vocal sac inflation during courtship calling helps females to detect and discriminate male signals, thereby increasing male attractiveness (Starnberger et al., 2014). Receiver psychology in the courting context is particularly relevant in noisy environments where assessment of mates is difficult. For example, in a study on sexual signalling in several *Magicicada* species, Cooley & Marshall (2001) hypothesized that some parameters of the auditory courtship display increase discriminability of individual calls among a chorus. It has long been established that low intensity signals are detected faster when they occur in more than one modality (e.g. Gielen et al., 1983). This seems to equally apply to multicomponent and multimodal signals. Indeed, in an experiment with swordtails (*Xiphophorus nigrensis*), it was found that females were faster to approach one of two males when the males differed on two rather than only one visual signal (body size and courtship vigor) (Reding & Cummings, 2017).

b) Back-up signals hypothesis

The back-up signal hypothesis specifies that multicomponent signals carry redundant information to limit errors in signalling, allowing the receiver to assess the final message with more accuracy (Johnstone, 1996; Møller & Pomiankowski, 1993). In those cases, we expect different traits to be correlated as the multiple 'back-up' components are redundant if the receiver's response to each is the same. Bro-Jørgensen (2010) hypothesised that even though one signal might be sufficient to communicate a message, temporal and/or spatial environmental variability could lead to the evolution of multi-component sexually selected signals ("fluctuating environment hypothesis", Partan (2017) and Munoz & Blumstein (2012)). Multi-component displays could prevent interference from unpredictable variation and thereby ensure signal transmission under varying environmental conditions. In such cases, it is predicted that back-up signals would evolve. For example, Colyott, et al. (2016) found that removing one courtship component (it being either visual, auditory, chemical or tactile) in *Drosophila saltans* did not alter the females' decision to mate. This indicates at least some degree of redundancy between different components. In the satin bowerbird (*Ptilonorhynchus violaceus*), Keagy, et al. (2012) found that females used multiple traits of the bower constructed by the male, such as the size of the sticks used, the symmetry of the bower, or the coloration of decorations, to better estimate a composite measure of male cognitive abilities. They additionally found that some of those traits were redundant, for example, stick size and bower symmetry seem to convey the same information. In canaries (*Serinus canaria*), female responses to male courtship are multimodal and composed of a visual (copulation solicitation display, CSD) and auditory part (female-specific trill, FST and contact calls, CC) (Amy et al., 2015). Salvin (2018) found that the number of modalities used by both males and females within courtship interactions affected the response of the other sex. For example, males responded to a female's behaviour during courtship only when they could see and hear the female, and not when they could only hear them. However, no enhancement effect between female visual and auditory signals seemed to occur and those two signals seemed to be redundant for males.

B- Multi-component courtship for multiple pieces of information

In contrast with the above hypotheses, the multiple message hypothesis states that each component of the multimodal signal carries different information, and therefore each component taken separately should trigger a different response (Johnstone, 1996; Møller and Pomiankowski, 1993). For example, theoretical work by Wilson et al. (2013) investigated which constraints would favour the evolution of multimodal signals over simpler signals, and found that having multiple receivers, or multiple qualities to display would all favour the emergence of multimodal signalling.

Most of the studies on multicomponent signals in the context of courtship displays focus on behavioural responses indicating female preference (Ronald et al., 2017; Taylor & Ryan, 2013), but less has been done on multicomponent courtship where separate components might have different functions (such as sexual stimulation of the female, cf. part 1 above).

a) Signalling different aspects of male quality

According to sexual selection theory, females choose a male who can increase her reproductive success or the quality of her offspring. In an interesting theoretical paper, van Doorn & Weissing (2004) showed that the evolution of multiple male ornaments was plausible if they displayed different aspects of male quality. Although they focussed on ornaments, the same evolutionary process could be involved in the behavioural components of courtship.

Surprisingly, very few studies have focused on the multiple aspects of male quality that courtship could potentially advertise. In some cases, multimodal signals have been found to display different aspects of a male's quality (multiple message hypothesis). Girard et al. (2015) found that in peacock spiders (*Maratus volans)* different components of courtship, such as vibration vigour and display duration, advertise separate aspects of male quality and differentially predicted mating success. Another example comes from the satin bowerbird, where features of the bower and plumage related to courtship were found to reflect different aspects of male quality (Doucet & Montgomerie, 2003).

b) Triggering different female responses

There is also evidence that different parts of the male display can trigger differential responses in females. In the iguana *Liolaemus pacho* for example, chemical signals are thought to have a role in sex recognition (Vicente & Halloy, 2016), while other behaviours like head-bobbing might have a role in attracting the attention of the receiver and communicating the signaller's location (Vicente & Halloy, 2017). In the sand goby (*Pomatoschistus minutus*), the frequency and temporal pattern of sounds are thought to communicate species identity, whereas call intensity and the visual part of male courtship are more relevant for female choice (Pedroso et al., 2013). In the orb-web spider, as mentioned above, some of the courtship signals have an influence on female acceptance and probably reduce female aggressiveness (Wignall & Herberstein, 2013), while other signals might facilitate sperm transfer (Huber, 2004). In the lizard *Anolis carolinensis*, early investigations by Crews (1975) on the relative importance of each component of the male courtship display showed that physical movements of the male dewlap during courtship triggered hormonal changes and follicular growth in females, while dewlap colour was used by females for mate choice.

c) Reaching different receivers

In some cases multimodal signalling can help the signaller to reach more than one receiver. Female preference has long been considered to be homogeneous within a studied population, as if a consensus had been reached about attractiveness of male attributes. A commonly used approach is therefore to use the mean female response of the population as the response variable. However, it is becoming more and more evident that mate preference varies greatly depending on a female's age, condition, or environment (Burley & Foster, 2006; Jennions & Petrie, 1997). Female preference for male colouration can vary according to level of predation risk (Godin & Briggs, 1996). It is thus likely that female preference for individual courtship components varies within a population. Displaying on different sensory channels, and having more than one information-containing signal, would potentially allow a male to attract different types of females, thereby dealing with variation in female preferences. In the brown-headed cowbird (*Molothrus ater*), females differ in their preferences for individual components of the complex male courtship display as a function of their sensory acuity (Ronald et al., 2018). In this species, the visual and auditory temporal resolution of females has an influence on which type of visual display and songs they prefer during male courtship, for example females with better auditory temporal resolution preferred shorter songs.

In other cases, some components might be relevant not only in the context of courtship, but also function as signals directed to other males or predators. In the flamboyant lizard (*Sarada superba)*, different colours of the same display elicit different responses in rival males and courted females, stressing the role of simultaneous selection pressures from intra- and inter-sexual selection in the evolution of multimodal signals (Zambre & Thaker, 2017). In several bird of paradise species, display complexity is also driven by those two forces (Miles & Fuxjager, 2018). Female choice seems to influence sexual dichromatism, while male-male competition is related to carotenoid-based ornaments. In the peacock (*Pavo cristatus*), different signalling ornaments and behaviour have evolved in response to pressure coming from both intra- and inter-sexual selection (Loyau et al., 2005) and in the ochre-bellied flycatcher (*Mionectes oleaginous)*, male songs in leks are used both for courtship to females and intra-sexual competition (Westcott, 1992). The case of co-option of courtship behaviour from aggressive displays is also quite common in the animal kingdom (Berglund et al., 1996). In the ring dove, for example, the bow-coo display used as an aggressive display towards other males does not differ from the courting display males perform to attract females (Craig, 1909). When the courtship display and the aggressive display are similar, an additional signal component may act as an indicator of intention (Baptista, 1978). For example in female red-winged blackbirds (*Agelaius pboeniceus*), the visual signals used for courtship and aggressive display by territorial females are similar, while two types of female song have been reported. This suggests that the auditory part of the signal allows a male to disambiguate the female's behaviour (Baptista, 1978; Beletsky, 1963).

d) Signalling at different geographical and temporal scales

Sometimes, having several courtship components can reduce the cost of mate choice by reducing the time females spend in close inspection of available males. For example, females can use one signal to choose which males are worth observing and then use another signal component for their subsequent choice among this subset of males. In other words, different cues are used for attraction and for mate assessment. Although the sequential assessment of cues to gain information from a conspecific is well documented in general (Uy & Safran, 2013), less evidence exists in the context of mate choice, and even less in the general context of courtship. In the fiddler crab for example, females first select certain males for their size, and then assess them for their burrow quality in order to decide which male to mate with (Backwell & Passmore, 1996). Regarding the specific case of courtship, in the ring-necked pheasant (*Phasianus colchicus*), different messages sent at separate times by males during courtship elicit different responses in females (Mateos & Carranza, 1999). The first signals are used to attract females' attention, whereas the 'lateral' display triggers a copulation solicitation display from the female. At a larger scale, some elements of courtship can attract the potential mate and guide her towards the sender. In the sage grouse (*Centrocercus urophasianus)*, females use the acoustic component of the display to determine whether to visit a male, but the display rate is a better indicator of the likelihood of mating (Gibson, 1996).

C- Information in complex signal structure and component interactions

Partan & Marler (1999) were the first to propose a classification of multimodal signals in animal communication. They pointed out that adding a second component to a unimodal signal could modulate the first component's effects on the receiver's behaviour (enhancement or suppression, for example), or even create a new "emergent" response in the receiver. Their article was an important contribution to multi-sensory communication theory in the field of animal behaviour, because historically multicomponent signals were studied by analysing each component separately and not by taking the whole signal or the relationship between components into account. More recently, an increasing number of authors have emphasized the need for studying complexity itself, as the different components of a signal are likely to have evolved conjointly (Cooper & Goller, 2004; Groyecka et al., 2017).

Smith & Evans (2013) described an interesting heuristic for the study of multicomponent signals. In particular, they proposed a method to better understand and visualize how concomitant variation within and between two modalities influences the receiver's response. The resulting three-dimensional surface plot (with the magnitude of each component signal represented on two axes and magnitude of female response on the third) was later used by Hebets and colleagues (2016) to study female preference in response to two courtship stimuli, using the multi-sensory wolf spider courtship display as a model. This type of graphical representation helps to visualize the complexity of a receiver response when exposed to different levels of components inside the same complex display. Here, we use a similar graphical representation to describe how two male courtship components can separately or jointly influence two female responses (Figure 1). The two-plane multi-dimensional plots illustrate a theoretical case where two sensory modalities of male courtship (e.g. a visual and an auditory signal) can interact or not to influence two aspects of female response (e.g. female preference and female sexual stimulation).

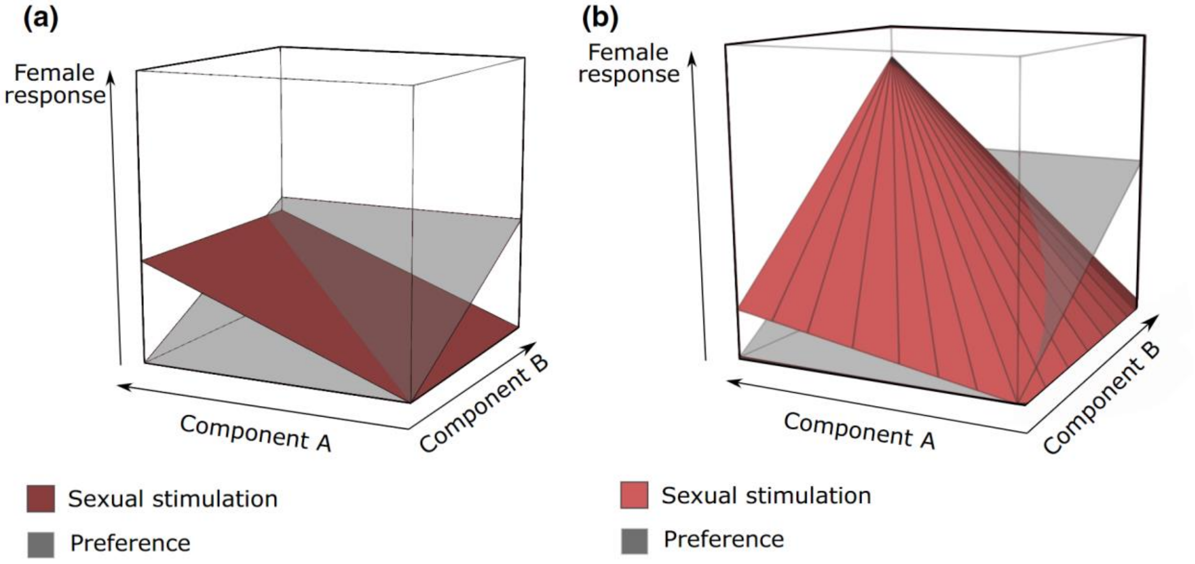

Figure 1. Multi-dimensional plots of female responses to multi-component male courtship. Components A and B have different effects on female preference and female sexual stimulation, resulting in two 3-dimensional planes. a) No interaction between components A and B. As component A increases, female sexual stimulation increases, but not female preference. Conversely, component B has a positive effect on female preference but does not influence female sexual stimulation. b) An interaction exists between courtship components A and B. The effect of component A on female sexual stimulation increases as the magnitude of B increases. Component B alone does not have any effect on female sexual stimulation. [Based on Smiths & Evans (2013) and Hebets et al. (2016)]

One of the first examples of a behavioural response that derives specifically from a multisensory display comes from the study from Rowe & Guilford (1996) who investigated multicomponent anti-predator warning signals found in prey. They found that neither the chemical released by the prey nor the warning colour they display could, when presented alone, trigger aversive behaviour in the domestic chick (*Gallus gallus domesticus*), and that the repulsive effect emerged only when the two signals (visual and chemical) were combined. This stresses the importance of taking each component into account, as well as paying attention to the overall structure of the complex signal and the relation between its different components. In the specific case of courtship, most of the studies reporting an interaction between signals do not directly concern interactions between different behavioural components of courtship, but rather the relative role of courtship behaviour and morphological traits (Reynolds, 1993, Table 2).

a) Multi-signalling as a marker of quality *per se*

Independently of its content, multicomponent signalling can intrinsically be a sign of the quality of the displaying individual. Indeed, complex courtship displays are often more energetically costly than single-component displays and could therefore be under female selection for energetically demanding behaviour (Byers et al., 2010). For example, in *Drosophilia virilis,* courtship elicits a response from a female only if all elements of a sequence of signals are present. This might be a way for females to select those males that are able to send accurately timed and energetically costly signals (LaRue et al., 2015). In the wolf-spider (*Schizocosa ocreata*), females prefer multi-modal courtship signals over unimodal ones (Stoffer & Uetz, 2017). Additionally, in peacock spiders (*Maratus volans*), Girard et al., (2015) found that total courtship effort (a variable capturing the time a male spends courting across different sensory modalities) positively affected female preference. We know that in some species, courtship is costly and condition-dependent. In the fiddler crab (*Austruca lactea*) for example, males produce a multisensory courtship display to attract females from a distance and then to court them when closer. Tekeshita et al., (2018) showed that the male visual waving signal used to attract females was condition dependent, and therefore, could be used by females to assess the individual quality of potential partners.

In addition, the timing of different components of courtship does not occur randomly. Signal synchronisation could itself indicate good neural control. In the Montezuma oropendolas (*Psarocolius montezuma*), males synchronize the loudest note of their song with a specific visual courtship display (the bow and wing-spread), which could indicate the quality of an individual motor's skills (Miles & Fuxjager, 2018). In the brush-legged wolf spider (*Schizocosa ocreata*), accuracy in the temporal synchrony of separate courtship signals increases female receptivity (Kozak & Uetz, 2016). In Túngara frogs (*Physalaemus pustulosus)*, females reject the courtship when the multimodal elements of the mating signal lack synchrony (Taylor et al., 2011). In the same species, synchronised visual and acoustic displays are more attractive to females than asynchronous signals (Taylor et al., 2017). However, an asynchronous multimodal signal is still more attractive than a unimodal signal. This underlines the complex relationship that can exist between all sensory modalities contributing to a signal and the information contained in their interactions and their relative timing.

b) Multi-modal courtship displays are more than the sum of their parts

When we consider the response to a complex signal that involves several sensory modalities, we now realise that this response is not always additive. Experiments investigating multisensory integration in the midbrain have found clear evidence for super-additive multisensory enhancement when comparing single neurons' responses to uni- and multi-modal stimuli (Meredith & Stein, 1983). Multi-sensory responses have also been found in cells located in cortical areas of the rodent brain which were traditionally assumed to be entirely modality-specific (Wallace, Ramachandran, Stein, 2004). This may explain how a signal in one modality can influence the processing and perception of a signal in another modality. It is not clear though whether such cross-modal effects are exploited in courtship displays as only few examples have been documented so far.

A study on mate choice in Túngara frogs showed that two courtship signals which are not attractive individually, i.e. the two parts of the vocal signal, become attractive for the females when combined with a visual signal (the inflation of the vocal sac) (Taylor & Ryan, 2013). The multimodal integration of signals by females is therefore not additive but rather involves a complex emergence. In another recent paper on Túngara frogs, Stange et al., (2016) tried to assess the relative importance of each part of a multicomponent signal by manipulating the complexity of the courtship display. They found that female integration of the multicomponent male signal was not additive, and that the preference varied with display complexity i.e. with the number of components of the signal. This suggests the presence of some higher-order interaction between the visual and acoustic components of the courtship that goes beyond a simple enhancement effect. Finally, in the brown-headed cow-bird (*Molothrus ater*), the intensity of the visual component of male courtship modulates the attractiveness of male songs (Ronald et al., 2017). This study is an interesting example where two components of male courtship interact in a complex way to modify overall attractiveness. Examples of this kind are still scarce, but there is no doubt that the growing interest in multimodal signalling displays will allow more research to reveal similar interactions in the courtship displays of other species.

Conclusion

Over the past years, many authors have proposed new theoretical backgrounds for the study of the role and function of multimodal displays (Bro-Jørgensen, 2010; Candolin, 2003; Partan, 2013; Rowe & Halpin, 2013). Even though courtship displays typically involve signals coming from more than one sensory modality, relatively little theoretical and experimental work exists on multimodal and multicomponent courtship displays, and most of the work so far has focused on auditory or visual courtship. Empirical studies are now needed to specifically test how the variation in different modalities and the interaction between them influence female response and choice in the context of courtship. In addition, we know very little about the neural mechanisms involved in multi-sensory processing in the courtship context. As mentioned above, uni-sensory information from different sensory channels is integrated and transformed into multi-sensory responses in the midbrain (e.g. Meredith & Stein, 1986; Gandhi & Katnani, 2011) and cross-modal stimuli yield faster responses and can be detected with higher accuracy than modality-specific stimulus presentations (Gingras et al., 2009). Whether these neural and behavioural principles of multi-sensory enhancement also hold in the context of multimodal courtship displays remains to be investigated.

Finally, courtship is often an interactive process between two sexes rather than the production of signals by a courting individual and its evaluation by a receiver. In a number of dynamic courtship interactions, the emitter modifies its signals on the basis of the response of the receiver. Therefore, many types of multimodal courtship can be fully understood only by analyzing their variation across time and in response to signals coming from the receiver. This is a further level of complexity that we have only started to explore.

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Chapter 2. Female behaviour is differentially associated with specific components of multimodal courtship in ring doves

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Female behaviour is differentially associated with specific components of multimodal courtship in ring doves

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Highlights

- Courtship is often multimodal and elaborate, yet most studies focus on single traits
- We studied the synchronized vocal-visual courtship of the ring dove
- Spectral auditory features and courtship duration influence female sexual response
- Repeated encounters affect female response and temporal structure of male display

Abstract

Courtship displays are typically conspicuous, elaborate, and composed of concomitant signals occurring in different sensory modalities. Although multimodal signals have received increasing attention over the past years, technical issues, in particular the lack of appropriate recording and analytical methods, have long restricted large-scale systematic study of their function. Here, we investigated in detail in 100 dyads if the multimodal, audiovisual courtship signals of the male ring dove (*Streptopelia risoria*) are associated with different female immediate behavioural responses, and if such responses depend on the courting individual. We used synchronized high-speed video and audio recordings followed by semi-automatic image and audio processing techniques to precisely quantify variation in male courtship and female response. In particular, we investigated the structure of acoustic and visual courtship components, as well as aspects related to multimodal synchronization. We found that the fundamental frequency of male calls, as well as the total courtship duration and the duration of courtship bouts, influenced female tail quivering behaviour, confirming that this behaviour is a sign of sexual interest in doves. On the other hand, some courtship variables frequently investigated in the literature, such as courtship rate, did not affect any aspect of female response. Additionally, we demonstrated an effect of repeated encounters with an individual of the opposite sex both on male courtship variables and on female sexual response to courtship. Females also responded differently to different males, that is, we found variation in female behavioural response to courtship variables related to identity and courtship effort. Further empirical studies are needed to assess how different male courtship elements influence female behaviour and ultimate mating decisions.

Keywords

Birds, elaborate signals, female choice, multisensory signaling, sexual selection.

Introduction

A large number of social signals are elaborate and composed of elements occurring in different sensory modalities (Candolin, 2003; Rowe & Halpin, 2013; Higham & Hebets, 2013). Such signals are referred to as multimodal (or multisensory), and they have received increasing interest over the past years. Sexual signaling and courtship behaviour are particular interesting cases, as courtship displays are among the most elaborate, diverse and conspicuous signals described in the animal kingdom. We can think of the impressive visual-acoustic courtship of some tropical bird species (Frith & Frith, 1988; Fusani et al., 2007), or the visual-vibratory display of wolf spiders (Scheffer et al., 1996). Courtship is typically displayed in at least two sensory modalities and considerable theoretical literature exists with several hypotheses have been proposed to explain the function of these multi-component signals (Guilford & Dawkins, 1991; Hebets & Papaj, 2005; Rowe & Halpin, 2013; Mitoyen et al., 2019). The multiple message hypothesis suggests that every component of an elaborated signal carries different information and therefore is responsible for different responses in receivers (Møller & Pomiankowski, 1993; Johnstone, 1996). Alternatively, different courtship traits are redundant and act as a mutual back-up (Møller & Pomiankowski, 1993; Johnstone, 1996). A more recent hypothesis proposes that different components interact with each other, leading to the emergence of new information (Johnstone, 1996; Møller & Pomiankowski, 1993; Hebets & Papaj, 2005). Yet, relatively few empirical studies have attempted to precisely characterize, quantify, and explain the proximate and ultimate functions of these multiple components (Wiens & Tuschhoff, 2020).

The classical approach in sexual selection studies has been to investigate courtship behaviour as a whole, examining the effects of gross variables (such as courtship duration) or the occurrence of specific behavioural patterns (circling, jumping, occurrence of a type of call etc.) on female response or female choice (Candolin, 2003). Some recent studies examined in a more quantitative way the role of different physical parameters of each component signal in the sexual selection context, e.g. the speed or amplitude of limb movements or specific acoustic parameters of songs. In those studies, associations were tested between female choice or male reproductive success and some specific courtship characteristics like song structure (Christie et al., 2003), song and call frequency (Nemeth & al., 2012; Hasegawa & Arai, 2015), or amplitude of courtship display movements (Murai & Backwell, 2006). In laboratory studies, the role of specific courtship components is usually investigated using cue isolation experiments where females are exposed to only a subset of the usual courtship components, and her response recorded in terms in term of preference (Uetz & Roberts, 2002) or physiological response (Crews, 1975). In both cases, the complexity and the multimodality of the courtship display is generally not addressed *per se*, and there are few or no studies on the effect of every component of the courtship.

Another limitation originates from the way the value of those components is usually assessed. In most studies, the function of signal components is investigated by looking at their effects on the courted sex (typically the female) in terms of preference or choice. Obviously, courtship plays a major role in partner choice and thus has a major effect on individual fitness through reproductive success (Andersson, 1994; Bastock, 1967). However, this approach though useful does not always allow a direct investigation of the mechanistic function of the whole signal. According to the multiple message hypothesis, the diversity of responses triggered by various components can potentially provide valuable information about the function of these complex signals and should not be overlooked. For instance, females could respond behaviorally to some courtship components signaling species or sex identity, and physiologically (Pedroso et al., 2013, Vicente & Hallow, 2016) to other components carrying information about male quality. In fact, the signal could even carry information about undesired features such as high aggressiveness, low parental investment, or kinship, which may adversely influence the choice made by the receiver (Burley, 1986; Borgia & Coleman 2000; von Hippel, 2000). Choice and preference experiments with only two possible outcomes or investigating only one female response (time spent next to a male, or latency to approach for example) therefore cannot grasp the potentially conflicting effects or simply the different responses associated with diverse signal elements. The diversity of sensory modalities involved in the courtship interaction, the relation between those different sensory components but also the different responses from the receiver all contribute to courtship signaling. Trying to investigate as many of those aspects as possible in a more comprehensive way could increase our understanding of the evolutionary mechanisms at play in the sexual selection context (Mitoyen et al., 2019; Halfwerk et al., 2019).

In this study, we aimed to investigate in more detail how inter-individual variability in male courtship components and their interaction affect female response. To do so, we focused on the courtship display of the ring dove (*Streptopelia risoria*). The ring dove is a domesticated, socially monogamous species that forms strong pair bonds over consecutive years (Morris & Erickson, 1971). Its courtship is relatively simple, making it a good model for the study of multimodality in controlled laboratory condition. Males typically court a female for several days before a pair forms, and courtship is repeated before each reproduction event. The first and very typical courtship phase consists of the male performing a bowing display, where he bends repeatedly toward the floor, facing the female. A call, the bow-call, always accompanies the bowing, creating a multimodal "stereotyped" courtship display (Video S1). Even when the pair is formed, courtship continues to occur and successful reproduction depends on a successful reciprocal courtship (Cheng et al., 1981;

Dios, 2015). This indicates that female doves do not use male courtship only to choose their partner; for example, courtship could also have a role in consolidating pair bonds (Morris & Erickson 1971; Dios, 2015). Additionally, in doves the multimodal aspect of courtship is important, as physiological stimulation is higher when females are exposed to audio-visual courtship rather than unimodal auditory courtship lacking the visual component (Friedmann, 1977). Finally, we know that male inter-individual variability exists for several courtship elements (Davis, 1970; Fusani et al., 1997), suggesting that these traits undergo sexual selection by female choice or may carry information about individual qualities and/or physical condition.

We hypothesized that variation in visual and auditory components of the ring dove courtship display is associated with variation in female responses. We focused on one particular male courtship display that is predominant during the first phases of courtship, the bowing display. We investigated a range of courtship variables falling into three main categories: temporal and spectral structure of bow-calls, temporal structure of the bowing movements, and synchronization between vocal and visual signals. We performed a cross-over experiment in which each of 10 females encountered 10 males repeatedly (3 times). High-speed video and audio recordings of those interactions, associated with semi-automatic movement tracking and analysis allowed us not only to describe the structure of the displays and their multimodal synchronization, but also to precisely quantify the variability of all these components. In addition, the experimental design allowed us to assess how female response and male courtship changed over repeated encounters between the same birds and throughout the experiment. It is well known that both female (Barfield, 1971; Cheng 2008) and male (Lovari & Hutchison, 1976) behavioural and physiological responses change after several days of courtship and sexual interaction. Lastly, we asked if some individual males consistently induced stronger responses in most of the females they encountered, in an attempt to search for male behavioural variables that reflect objective courtship values.

Materials and methods

Study species

The ring dove (*Streptopelia risoria*) is very likely the domesticated form of the African collared dove (*Streptopelia roseogrisea*), a native species of northern Africa (Baptista et al., 1997; van Grouw, 2018). The ring dove is usually sexually active between April and October, and several clutches can be laid every year. The birds studied here came from different breeders across Austria and most of them were sexually naïve and had never completed a complete breeding cycle when we acquired them. All birds were between 10 months and 2 years old. The birds were housed in homogeneous sex groups prior to experiments to reduce any effect of familiarity on behaviour (Erickson & Morris, 1973). In order to ensure that the males were sexually motivated and thus ready to perform courtship during testing, we housed them indoors in individual cages (50 x 38 x 60 cm) from one week before and during the whole duration of the experiment (Hutchison, 1970). Males were visually but not auditorily separated from each other. We maintained the housing room under a light regime of 14D:10N. We housed females in groups of six in two outdoor aviaries (2.95 m depth x 2.80 m high x 1.20 m width).

Ethical note

This work was approved by the local ethics committee of the Faculty of Life Sciences, University of Vienna, and by the national committee of the Austrian Federal Ministry of Education, Science and Research (BMWFW permit 66.006/0042-WF/V/3b/2017). This study adheres to the ASAB/ABS Guidelines for the Use of Animals in Research and the ARRIVE Guidelines. All efforts were made to limit stress before and during the experiment, and ring doves are very tame and used to be handled. Prior to the experiment, doves underwent a habituation phase with regular handling and exposure to the experimental setup, which was done daily for several weeks. When not in the setup, birds had constant access to seed mix, grit, and water, *ad libitum*. Males housed in individual cages were never socially isolated from each other, as this species relies on acoustic communication, and the use of pre-experiment visual separation is standard in this species (Hutchison, 1970; Lovari & Hutchison, 1976). We monitored birds during the entire duration of the experiment for any signs of acute stress, which was never observed during testing.

Recording apparatus

In order to record in controlled conditions the male courtship and the associated female response, we built a two compartment (each 50 x 50 x 50cm) recording apparatus in which one male and one female could interact (Figure 1.D). For each compartment, a camera (Basler acA1920-155uc) recorded a side view of the bird. We placed two microphones (Sennheiser ME66 directional head with battery-powered K6 power module) behind black fabric walls on each side of the apparatus to record vocalizations. The microphones were chosen for their broad (40-20000 Hz) and reasonably flat frequency response. Care was taken with hardware and software audio settings and the placement of the microphone in the setup to avoid clipping of audio recordings. We separated the two compartments by a net to prevent birds from entering the compartment of the partner but the birds could see and hear each other without restriction. A movable partition was placed between the two compartments before recording in order to prevent birds from seeing each other prior to testing. For optimal video recording, we placed 10 LED lights above each compartment (powered by a DC power supply to eliminate flicker, set at 0.30 V and 0.26 A), as well as one 30 W LED panel behind each camera to suppress shadows. We fixed acoustic foam on the walls to suppress echoes, and a double layer of thick curtains was additionally used for acoustic dampening and to separate the birds from the experimenter. Video acquisition was done using a Motif Video Recording System (loopbio gmbh, Vienna, Austria). Video recordings were controlled through a web interface, and audio recording was controlled using Audacity (Audacity Team). We recorded video images at 60 frames per second, with an exposure time of 0.015 seconds. A synchronization signal from the Motif camera system hardware synchronizer (one pulse per frame) was recorded as an audio signal synchronously with the microphone recordings (Allen & Health, ZEDi 10), which allowed us to synchronize audio and video recordings with a precision of +/-1 video frame (16.67 ms). The recording room temperature was between 25°C and 28°C during the whole duration of the experiment.

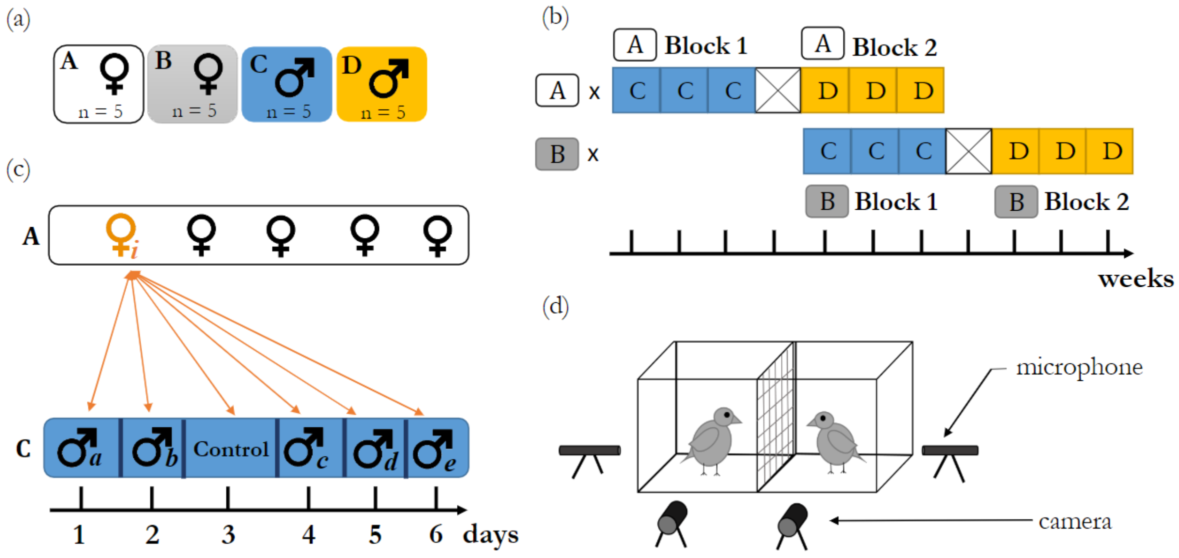

Figure 1. Scheme of the cross-over experimental design. (a) Experimental groups. We divided the birds into four groups of 5 birds. (b) Temporal organization of the testing. Every male-female dyad was tested three times (three repetitions) between July and August 2018. For females, each block corresponded to a 3 week period during which they encountered each individual male 3 times. This led to recordings of 300 test sessions (between 100 unique couples) and 120 control treatments. Crossed boxes indicate a week without testing. (c) Example of the experiment repetition pattern for one female, over a six day period (example for group A and C). Female *i* (from Group A) meets male *a* (from Group C) on day 1, male *b* on day 2 etc. Female *i* is tested in front of an empty box on day 3 (Control). The same pattern applies for every female with a different random order for each female and for each week of the experiment. At the end of the week of 6 days, every female had met every male once, and faced an empty box once. (d) A sketch of the recording apparatus

Experimental design

We used ten sexually mature males and ten sexually mature females to conduct this experiment. We used a cross-over design experiment where every male was presented to every female resulting in the recording of multiple test sessions between 100 unique dyads (Figure 1). For logistical reasons, it was not possible to test all individuals each day, so we split males and females into two groups and the experiment was staggered into two blocks in June and August 2018 (Figure 1c).

After we retrieved one female from the aviary and one male from the individual cages, we put both individuals in the experimental setup with the partition closed to let them habituate. After 10 minutes, we removed the partition between the two compartments and the birds could freely interact for 5 minutes. We started video and audio recording one minute before the removal of the partition and stopped it 5 minutes after the partition was opened. As a control, we recorded every individual alone in the setup once a week, following the same timing of partition removal and recording. Instead of encountering another individual, the test bird faced an empty compartment.

Behavioural analysis

Female behaviour

We coded video recordings of female subjects using Solomon Coder v17.03.22 (Peter, 2017). The coding started when the partition separating the two compartments was completely removed and continued for five minutes until the end of the recording. Two coders coded the videos, and we used Cohen's kappa coefficient to assess inter-coder reliability on a subset of 20 videos coded by both coders. Depending on behaviour investigated, there was at least 95% reliability between coders ($P < 0.001$). Coding resolution was 0.2 seconds. We coded female behaviours that had previously been found to be associated with sexual response or sexual interest in doves and other bird species (Cheng, 1973; Witte, 2006; Amy et al., 2015). These included tail quivering, self-preening, approach to the male, and time spent near the male. We additionally coded behaviours related to female activity in general, such as the number of steps made, number of pecks to the ground or net, and duration of periods during which the female had its eyes closed. Females did not vocalize during the experiment thus we did not perform any acoustic analysis of female behaviour. A summary of the coded female behaviour is provided in Table 1.

Table 1. Female behaviours measured in response to male courtship

Behaviour	Description
Tail quivering	Number of times the female quivered her tail rapidly
Preening	Number of times the female performed self-preening
Approach attempts	Number of approach attempts the female made toward the male. Defined as the female touching the net to try to go through it and then taking a step back
Time near adjacent box	Proportion of time the female spent in the third of the test cage closest to the adjacent box. This is synonymous to "Time near male" in the non-control condition
Steps	Total number of steps made by the female
Pecks	Total number of pecking events (to ground or net)
Time eyes closed	Proportion of time during which the female remained with its eyes closed

Male behaviour

All male acoustic and visual courtship variables that were measured are illustrated in Figure 2 and described in Table 2. For every variable calculated (except courtship total duration, total number of calls, number of bouts, and call rate), we averaged the values over each test session in order to obtain only one average value per male per day.

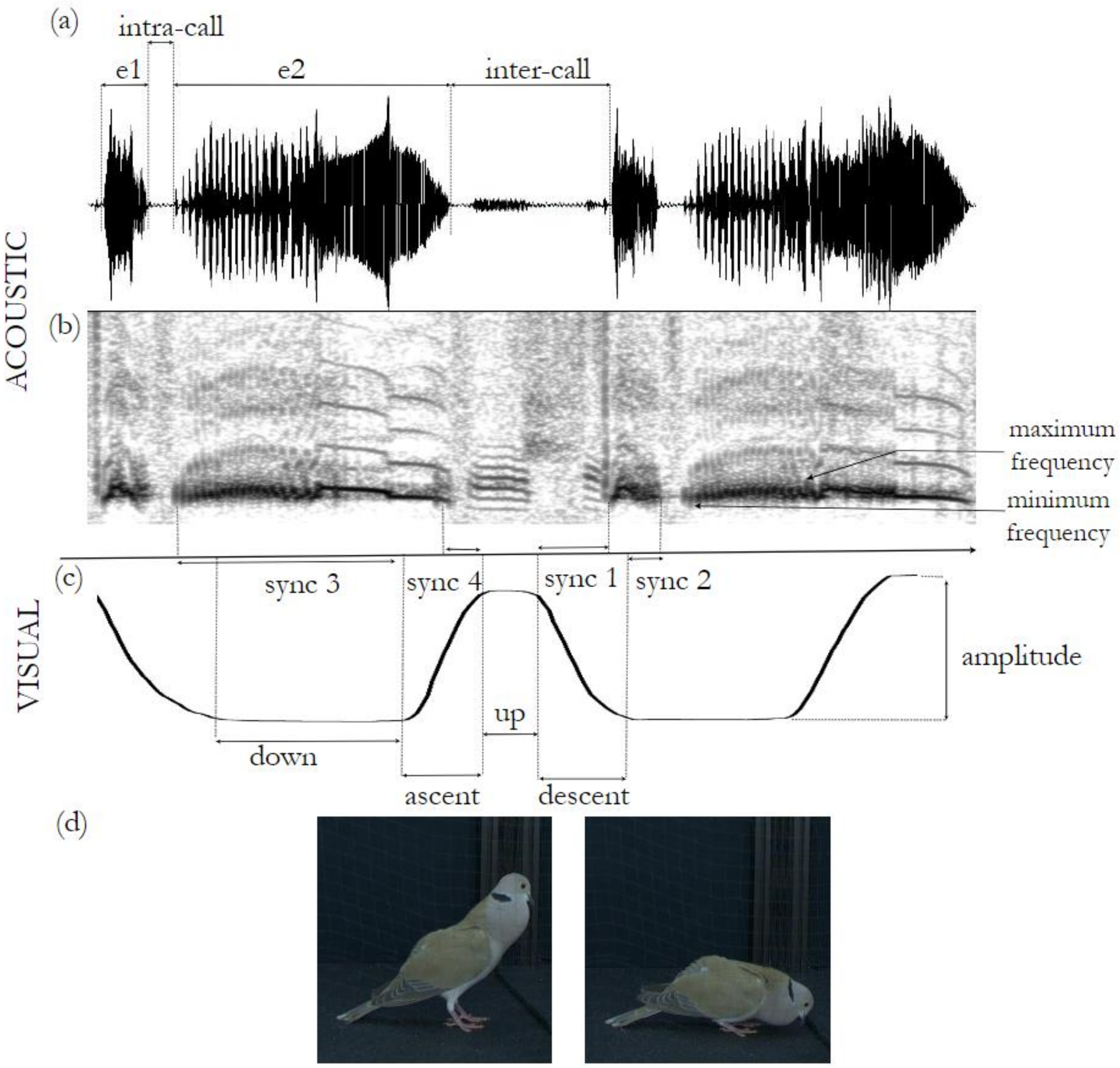

Figure 2. Representation of the two data channels recorded of the ring dove multimodal courtship display and the extracted courtship variables. (a) Oscillogram of the ring dove acoustic display. Acoustic display is composed of repetitive calls composed of two elements (e1 and e2). (b) Spectrogram of the acoustic display where the maximum and minimum frequency of e2 are described. (c) Representation of the visual bowing display. The line is the trajectory of the eye of the dove seen from a side view, during the bowing and extracted from an automatic annotation. The variables describing the synchronization between acoustic and visual display are shown. (d) Picture of a male in the experimental apparatus in the up (left) and down (right) position of the bowing display.

Table 2. Variables measured from courtship of male ring doves.

Courtship parameters	**Description**
Call: temporal structure	
CD total duration	Total courtship display duration during each session (5 minutes encounter) (s)
Calls number	Total number of calls during the session
Bouts number	Number of courtship bouts during the session
Call rate	Average call frequency within bouts (n/s)
e1 duration	Average duration of element 1 of the call (s)
e2 duration	Average duration of element 2 of the call (s)
Intra-call duration	Average duration of the interval between the end of element 1 and the beginning of element 2 (s)
Inter-call duration	Average duration between the end of element 2 of a call and the beginning of element 1 of the following call (s)
Call: Spectral structure	
e1 max f0	Average maximum fundamental frequency of element 1 (Hz)
e1 min f0	Average minimum fundamental frequency of element 1 (Hz)
e1 median f0	Average median fundamental frequency of element 1 (Hz)
e2 max f0	Average maximum fundamental frequency of element 2 (Hz)
e2 min f0	Average minimum fundamental frequency of element 2 (Hz)
e2 median f0	Average median fundamental frequency of element 2 (Hz)
Bow	
Bowing amplitude	Average vertical distance between the eye position before ascent start and the eye position when ascent ends (pixel)
Up duration	Average time the bird spends in the up position of the bow (s)
Descent duration	Average time between up position and down position (s)
Down duration	Average time the bird spends in the down position of the bow (s)
Ascent duration	Average time between down position and up position (s)
Audio-visual synchronization	
Sync 1	Average difference between onset of e1 and onset of descent movement (s)
Sync 2	Average difference between offset of e1 and offset of descent movement (s)
Sync 3	Average difference between onset of e2 and onset of ascent movement (s)
Sync 4	Average difference between offset of e2 and offset of ascent movement (s)

Apart from courtship total duration, number of calls, number of bouts, and call rate, all other variables were averaged for each 5-min test session, yielding one number per male per day.

Acoustic analysis

We recorded sound in wav format with a sampling rate of 44.1 kHz and a bit-depth of 16-bits. Analysis of the auditory part of the courtship was performed using PRAAT v6.0.26 (Boersma & Weenink, 2020). We automatically annotated our recordings (function: annotate) by defining a courtship call as being higher than 100 Hz and longer than 0.05 seconds and by setting a silence threshold relative to higher intensity at -30 dB. We then manually differentiated between the first and the second part of the call (Figure 2). For the spectral analysis, we used a Fourier transform method (Spectrogram settings: frequency range of 0-3000 Hz and window length of 0.05 seconds). For both element 1 and element 2 of every call of every courtship recording, we extracted maximum, minimum and median fundamental frequency (f0). We also calculated temporal variables of courtship elements by extracting the duration of both call elements. We defined a bout as a courtship display sequence that is separated from other bouts by at least 2 seconds. We calculated the duration of every courtship bout and the total courtship duration, the number of calls per bout, the total number of calls, the call rate and the number of courtship bouts per test session.

Visual analysis

Male behaviour was semi-automatically quantified using Loopy (http://loopb.io, loopbio gmbh, Vienna, Austria). Loopy software is a suite of image processing applications including pose detection, which uses machine learning to automatically track user-defined points of interest on the object of interest. It returns frame-by-frame estimates of the pixel coordinates of these points. We either tracked the right or left eye, depending on which box the male was placed (Video S2). We manually annotated these reference points on 864 sample frames from three courtship videos from three different males. The model was trained with those annotations and the obtained model was used to quantify all courtship video recordings. We manually checked the accuracy of predictions on a sample of our recordings to confirm that the points of interest were properly tracked. With the resulting coordinates, we calculated the amplitude of the bows (in pixels). We also extracted temporal variables of the visual courtship, including bowing ascent time, bowing descent time, up time, and down time (see Figure 2 for a description of all calculated postural variables).

Audio-visual synchronization

Video capture timing was controlled using hardware synchronization. In order to allow post-recording synchronization of audio and video recordings, the synchronization signal was recorded as an acoustic signal synchronously with the microphone audio tracks. During recordings, the experimenter manually synchronized the video cameras, which was recorded in the video meta-data as a frame index and frame time. The corresponding audio synchronization track contained a pulse corresponding to each frame of the video. The sync frame pulse was characterized as the first pulse onset after a long period of no pulses. This allowed us to use the same timeline for video and audio. To quantify audio-visual synchronization of male courtship, we calculated the following durations: difference between the time of the video frame containing the start of descent movement and of the audio sample corresponding to the beginning of part 1 of the call, difference between end of descent movement and end of part 1 of the call, difference between start of ascent movement and beginning of part 2 of the call and difference between end of ascent movement and end of part 2 of the call (Figure 2).

Statistical analysis

We performed all statistical analysis using R (version 3.3.2, R Core Team 2019). To assess correlation among same-sex behaviour variables we used a Spearman correlation test with a 0.05 significance level (R package Hmisc v.4.3-1, Harrel et al., 2020). For every female behaviour considered, the duration of the behaviour was highly correlated to the number of occurrences of the same behaviour ($r > 0.98$). We used MANOVA followed by Tukey HSD post-hoc tests to examine whether repetition number, experimental block, and male identity had an effect on male courtship variables (separated between structural, spectral, visual and synchronization variables).

For all the models described hereafter, we ran separate models with each female behaviour of interest as the response variable (number of tail quivering events, number of approaches, number of self-preening events, number of steps, number of pecks, proportion of time female spent near the adjacent box).

We first aimed to investigate if female behavioural response was different depending on social context, i.e. when encountering a male versus facing an empty box. We modelled the impact of social context on female behaviour using generalized linear mixed models (GLMM). We fitted the models with a negative binomial distribution that deals with overdispersion when female behaviour was expressed as counts. We fitted the models with a beta distribution when investigating behaviour expressed as percentage (time spent near male). We fitted the models using the glmmTMB function from the glmmTMB packages (version 1.0.0, Brooks et al., 2017). Full models included male identity, female identity and male-female dyad as random effects. We included the interaction between repetition number and female experimental block (Figure 1) as a control. Social context (empty box versus male) was included as a fixed effect. In order to test the effect of social context, we used a full-null model comparison approach. The null model lacked the social variable, but the random effects and the controls remained. We used the DHARMa package (v. 0.2.7, Hartig 2020) to check our model assumptions. We then aimed to investigate if female behaviour would vary depending on whether they faced a courting or a non-courting male. We used the same approach to model the effect of presence of courtship behaviour per se on female number of behaviour events. As a fixed effect, we replaced social context by courtship status (yes/no). The dataset for the latter models only comprised the test sessions where females encountered a male (N=295).

To further investigate the influence of male courtship variables on female behavioural responses, we ran additional models. As explanatory variables (fixed effects), we used 14 uncorrelated male courtship variables (see Results section). We standardized the data by scaling each continuous variable beforehand by subtracting the variable mean and dividing by its standard deviation. The resulting z-scores were used in the model. We included the interaction between dyad repetition and experimental block, as well as the side of the experimental apparatus (left/right) where the male was placed. We tested the effect of explanatory variables using the drop1 function with a likelihood ratio test (LRT) (stats package v.4.0.1, R Core team 2019). To assess effect size, we used the function r.squaredGLMM from the package MuMIn (v.1.43.15, Barton 2019). We used the emmeans function from the emmeans package (v.1.4.5, Lenth 2020) to perform post-hoc tests.

To investigate whether female behaviour was influenced by repeated encounters with males (one repetition per week in each block), we fitted a model where we included the repetition number, the experimental block as well as their interaction as fixed effects. Female identity, male identity and dyad were included as random effects. Finally, to investigate whether specific males triggered stronger behavioural responses from females we fitted a final model where male identity was entered in the model as a fixed effect, and we used female identity as random effect. The null model only comprised female identity as random effect and repetition number and experimental block as controls. Female behaviour was strongly influenced by the experimental block (see Results section) and due to our experimental design, males of group C were only tested in the female block 1 and males of group D only in the female block 2 (see Methods section). Therefore, for each female behaviour we ran two models: one comprising the males from group C and the other comprising the male from group D. The dataset comprised all the dyad test sessions (N=295) and not only the ones where males were courting.

Results

We recorded 300 male-female test sessions. Four sessions could not be analyzed due to technical issues, and one session had to be stopped because the male managed to intrude into the female compartment. Our final dataset thus comprises 295 sessions with male-female interactions. Males courted females in 253 sessions, whereas in the remaining 42 sessions males did not perform any courtship. We also recorded 60 control sessions in which both females and males faced an empty box. As no male ever courted the empty box, no male data were collected from these sessions.

Females response to social interaction and to courtship

Out of 295 recorded test sessions where males and females interacted, females displayed preening in 159 sessions, tail quivering in 133 sessions and attempted to approach the male in 78 sessions. Every female displayed tail quivering behaviour even though there was a large variation between females regarding the number of events, ranging from only 3 tail quivering events over the whole experiment for one female to 285 events for another. We observed the same pattern for approach behaviour (range across females: 1 to 275) and self-preening (3 to 904 events). Number of steps and pecking events were positively correlated with every coded behaviour, apart from the time with eyes closed, with which they were negatively correlated (steps: r = -0.32, $P < 0.001$, pecks: -0.19, $P < 0.001$). The duration of events (tail quivering, preening and approach) was highly positively correlated to their respective number of occurrence (r > 0.99, $P < 0.001$ for the three behaviours). We therefore decided to use only the number of events in further analyses. Because of the small number of females closing their eyes during testing, and the negative correlation of this variable with steps and pecks number, we did not investigate this behaviour further and only kept steps and pecks as a measure of activity.

Female number of tail quivering events was impacted by social context (whether a male was present in the other box or not; full-null model comparison: likelihood ratio test: $\chi^2_{11} = 33.57$, $P < 0.001$). On average, females quivered their tail 2.33 ± 5.19 times (mean ± SD, $N = 295$, range 0–34) when a male was present and never if they faced an empty box (Table 3, Figure 3). Number of pecking events was also influenced by male presence ($\chi^2_1 = 4.51$, $P = 0.03$). Females pecked significantly more often in front of a male (1.32 ± 4.10 times, $N = 295$, range 0-45) than an empty box (0.62 ± 2.48 times, $N = 60$, range 0-18). Females also showed a difference in the number of steps depending on whether they faced a male or not ($\chi^2_1 = 5.69$, $P = 0.01$), making more steps during encounters with males (84.88 ± 112.07 times, $N = 295$, range 0-529), than in front of an empty box (52.75 ± 84.73 times, $N = 60$, range 0-283, Figure 3). The number of self-preening events was not related to male presence (χ^2_1 = 2.22, *P* = 0.13), nor was the number of approach attempts (χ^2_1 = 0.33, *P* = 0.56), or the proportion of time females spent next to adjacent box (χ^2_1 = 1.29, *P* = 0.25).

When investigating the female response to a courting vs. a non-courting male, we found that the number of tail quivering events was related to the presence of courtship (χ^2_1 = 4.41, *P* = 0.03, Figure 3), with females showing more tail quivering during encounters with a courting male than in front of a non-courting male (Table 3). Females also showed a higher number of steps when males were courting than when males were not courting (χ^2_1 = 3.96, *P* = 0.04, Figure 3). The number of self-preening events was not related to courtship (χ^2_1 = 0.91, *P* = 0.33), and neither was the number of approach attempts (χ^2_1 = 0.22, *P* = 0.63), number of pecking events (χ^2_1 = 1.10, *P* = 0.29), or the proportion of time female spent near the adjacent box (χ^2_1 = 0.35, *P* = 0.55).

Table 3. Summary of female behavior when facing different contexts

Female behaviour	**Contexts**		
	Control (N = 60)	**Non-courting male (N = 42)**	**Courting male (N = 253)**
Proportion of time spent near adjacent box	0.26 ± 0.39	0.24 ± 0.31	0.34 ± 0.38
Preening events	2.38 ± 4.18	3.5 ± 5.79	12.24 ± 26.84
Approach attempts	3.33 ± 10.80	0.62 ± 1.94	2.37 ± 7.24
Tail quivering events	0 ± 0	0.57 ± 1.17	2.62 ± 5.53
Steps	52.75 ± 84.74	52.74 ± 74.14	90.21 ± 116.44
Pecking events	0.62 ± 2.48	2.17 ± 5.36	1.18 ± 3.86

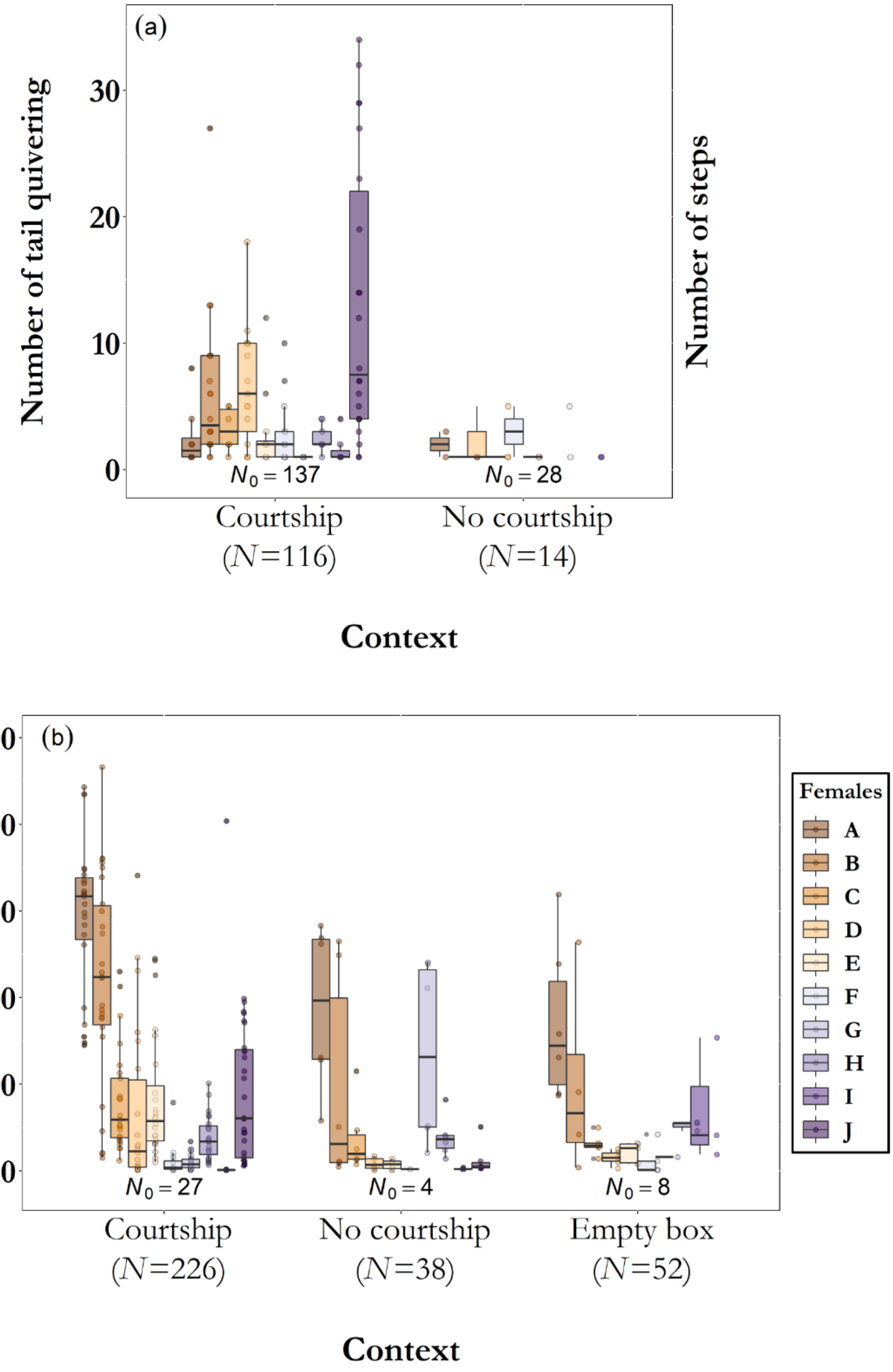

Figure 3. Number of behavioural events recorded from females in different social contexts. For clarity, we did not plot observations where number of tail quivering and number of steps was equal to zero. Number of sessions with zero events for each social context is indicated below the x-axis (N0) as well as the number of observations where females showed the behaviour at least once (N). (a) Number of tail quivering shown in presence or absence of courtship. Females never quivered their tail in front of an empty box. (b) Number of steps made in the three different behavioural contexts. Boxplots' horizontal lines display the median, lower and upper quartiles. Whiskers represent values within 1.5 times the interquartile range. Dots represent each observations.

Intra and inter-male variability in courtship structure

To avoid multicollinearity issues, we only kept a set of 14 relatively uncorrelated variables as explanatory factors for our models which were chosen on the basis that they would grasp a representative spectrum of male audio-visual courtship parameters (call temporal structure, call spectral structure, bow parameters and audio-visual synchronization; Table A1). The intra-coo duration was strongly negatively correlated with the duration of the first element of the coo-call (e1: r = -0.82, $P < 0.001$), meaning that the longer the e1, the shorter the interval between the two elements. The total duration of the coo, however, was highly positively correlated with the e2 duration (r = 0.90, $P < 0.001$). All together this indicates that call duration was not dependent on the interval between the two elements, but rather on the e2 duration. We therefore discarded the intra-coo duration from our models but kept both coo durations and inter-call duration as they better represented temporal acoustic aspects of courtship. Median f0 of both e1 and e2 were highly positively correlated with the maximum (e1: r = 0.84, $P < 0.001$; e2: r = 0.61, $P < 0.001$) and minimum f0 of both elements (e1: r = 0.88, $P < 0.001$; e2: r = 0.94, $P < 0.001$). We therefore only used the median frequencies as explanatory factors as they better represented how high-pitched calls were on average. The amplitude of bowing was moderately negatively correlated with the inter-coo duration (r = -0.52, $P < 0.001$), i.e. the larger the bowing movements, the shorter the interval between two consecutive bows. We kept the bowing amplitude in the models as this was the only estimation of courtship movement that we measured. Total courtship duration and bout duration were highly correlated with the total number of coos (r = 1, $P < 0.001$) and the number of coos within bouts (r = 1, $P < 0.001$), respectively. We chose to use durations as explanatory factors as they better represent the courtship effort a female is exposed to. The total courtship duration was also positively correlated with the number of bouts (r = 0.58, $P < 0.001$). Finally, all audio-visual synchronization timing values were strongly positively correlated (r > 0.74, $P < 0.001$) and we only used the timing difference between the start of descent movement and the start of call part 1 in our models. The strongest correlation between temporal characteristics of bowing was between time in down position and ascent time and was only -0.32 (P < 0.001), therefore we integrated all of these variables in our models.

Males were statistically different from each other for dependent variables describing courtship acoustic temporal structure (Pillai's trace = 3.30, $F_{81,2187} = 15.69$, $P < 0.001$), acoustic spectral structure (Pillai's trace = 3.12, $F_{36,972} = 95.80$, $P < 0.001$), bow variables (Pillai's trace = 2.32, $F_{45,970} = 15.18$, $P < 0.001$) and audio-visual synchronization (Pillai's trace = 0.85 , $F_{36,652} = 4.95$, $P < 0.001$). A set of one-way analysis of variance analyses revealed that males individually differ for each single variable of the courtship (Figure A1, A2, A3, A4, Table A2).

Variation of courtship structure after repeated interactions

We asked if male behaviour was affected by repeated exposure to females by assessing if it was consistent over the duration of the experiment. We investigated male behaviour over repeated encounters with the same female (repetition number), as well as over a larger time scale, between the two experimental blocks (separated by a week-long pause). None of the male behaviour changed across the three weeks (repetition number) during which they encountered the same females. For instance, element 1 duration was significantly different between males ($F_{9.243} = 185.85$, $P < 0.001$) but did not differ between repetition number ($F_{2.250} = 0.65$, $P = 0.51$) (Figure 4.A.). However, some aspects of the courtship structure changed between the two experimental blocks (one block corresponds to the three-week period during which a bird was tested with the same group of opposite sex birds). There were changes in the acoustic temporal structure (Pillai's trace = 0.17, $F_{8,244} = 6.46$, $P < 0.001$), visual structure (Pillai's trace = 0.07, $F_{4,168} = 3.01$, $P = 0.01$), but not acoustic spectral structure (Pillai's trace = 0.01, $F_{4,248} = 1.03$, $P = 0.39$) or in the synchronization between the acoustic and the visual components of the courtship (Pillai's trace = 0.03, $F_{4,168} = 1.28$, $P = 0.28$). One way analysis of variance revealed that the call rate was higher ($F_{1,251} = 10.51$, $P = 0.001$, Figure 4.B) and the duration of the element 2 of the call longer ($F_{1,251} = 9.70$, $P = 0.002$) during the second block. Courtship duration was shorter in the second block ($F_{1,251} = 7.81$, $P = 0.005$), as well as coo-duration ($F_{1,251} = 13.79$, $P < 0.001$) and duration of courtship bouts ($F_{1,251} = 16.35$, $P < 0.001$). Regarding variables characterizing visual courtship, the time the bird spends in the up position of the bow was shorter in the second block compared to the first block ($F_{1,251} = 13.79$, $P = 0.002$).

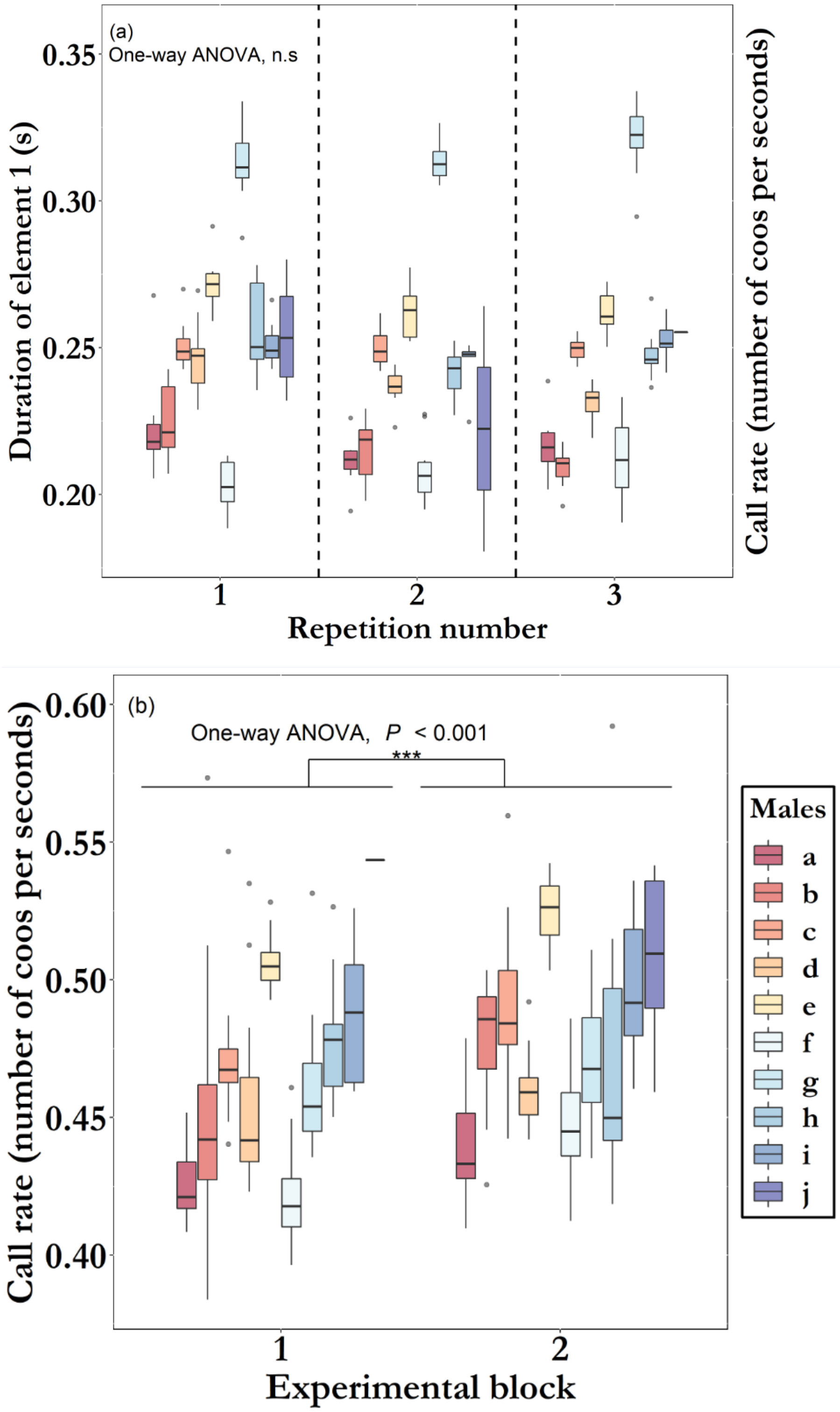

Figure 4. Changes in male behaviour over the course of the experiment. (a) Box-plot of the individual duration of the first element of the coo-call in the three sessions (repetition number) in which males encountered the same female. Values are averaged over experimental blocks. Results of the ANOVA testing the difference between repetition numbers are displayed (b) Box-plot of the individual call rate during the two experimental blocks. Values are averaged over repetition number. Boxplots' horizontal lines display the median, lower and upper quartiles. Whiskers represent values within 1.5 times the interquartile range and dots are outliers. Results of the ANOVA testing the difference between experimental blocks are displayed.

Link between male courtship traits and female response

After discarding highly correlated courtship variables to deal with multicollinearity issues, we defined the following as predictor variables: difference between start of descent movement and call part 1 (synchronization), bowing amplitude, total courtship duration, average bout duration, ascent time, descent time, up time, down time, e1 median f0, e2 median f0, e1 duration, e2 duration, inter-coo duration and call rate (Table A1). Several of these variables impacted tail quivering in females (full-null model comparison: likelihood ratio test (LRT): $\chi^2_{14} = 24.04$, $P = 0.04$). The effect size of this model for the fixed effects was $R^2 = 0.24$ and for the whole model including random effects $R^2 = 0.69$, meaning that individuals' identities were responsible for a large proportion of variation in tail quivering. In particular, the variability between individual females was higher (1.480, Table 4) compared with inter-individual variability for males (2.17e-11) or dyad (2.34e-8). The median fundamental frequency of element 2 of the call was negatively associated with tail quivering behaviour ($\chi^2_1 = 5.806$, $P = 0.016$; Figure 5, Table 4). Courtship structure was also associated with female tail quivering events, with the number of tail quivering events being higher for high values of total courtship duration ($\chi^2_1 = 11.873$, $P < 0.001$) and low values of courtship bout duration ($\chi^2_1 = 4.117$, $P = 0.042$).

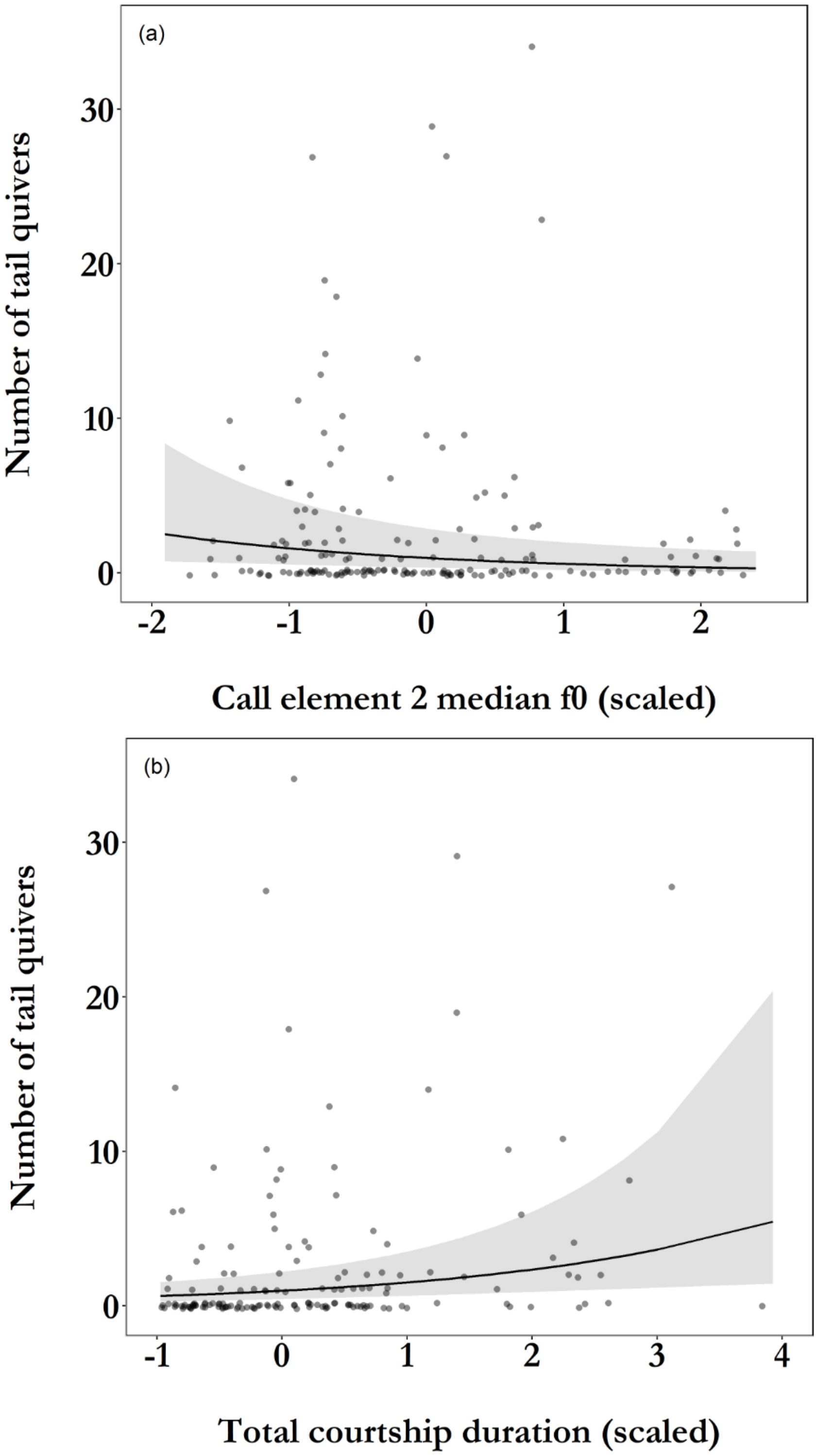

Figure 5. Raw data of the fixed effects with regression line predictions and 95% confidence intervals for the model investigating the effect of male courtship variables on female number of tail quivering events. (a) Plot of number of tail quivering events vs. median fundamental frequency of element 2 of the coo-call. (b) Plot of number of tail quivering events vs. total courtship duration.

All other full-null models testing the effects of male courtship variables on female response were not significant. This concerns the proportion of time females spent next to the male (χ^2_{14} = 11.87, *P* = 0.61), number of preening events (χ^2_{14} = 17.42, *P* = 0.23), approach attempts (χ^2_{14} = 18.68, *P* = 0.17), steps (χ^2_{14} = 20.96, *P* = 0.08) and pecking events (χ^2_{14} = 21.34, *P* = 0.09).

Table 4. Result of the GLMM predicting the number of tail quivers.

Fixed effects	**Estimate**	**Std Error**	**z**	***P***
(Intercept)	-0.067	0.557	-0.121	0.904
Bout duration	-0.334	0.170	-1.968	**0.042**
Total courtship duration	0.436	0.142	3.071	**<0.001**
Coo rate	0.059	0.257	0.230	0.936
E1 duration	-0.250	0.168	-1.484	0.271
E2 duration	0.191	0.234	0.818	0.276
E1 median f0	0.208	0.237	0.876	0.273
E2 median f0	-0.502	0.201	-2.492	**0.016**
Inter-coo duration	-0.321	0.253	-1.265	0.152
Up time	0.193	0.227	0.854	0.452
Descent time	0.082	0.180	0.454	0.568
Ascent time	-0.208	0.178	-1.168	0.198
Down time	-0.207	0.259	-0.800	0.336
Synchronization	0.081	0.152	0.533	0.561
Bowing amplitude	0.330	0.230	1.435	0.174
RN 2	0.294	0.398	0.740	0.460
RN 3	-0.141	0.413	-0.342	0.732
EB 2	-0.355	0.667	-0.532	0.595
Displaying box	-0.038	0.285	-0.132	0.895
RN 2 * EP 2	0.158	0.684	0.231	0.817
RN 3 * RN 2	0.966	0.659	1.467	0.142
Random effects	**Variance**			
Male	2.17e-11			
Female	1.480			
Dyad	2.34e-08			

Male courtship variables are fixed effects. Repetition number (RN), experimental block (EB) and displaying box are used as controls in the models. Random effects included female and male identity, as well as the male-female dyad. Estimates are from the full models and are z-transformed (scaled). P-values are from a likelihood ratio test of a reduced model lacking this specific effect compared with the full model.

Effect of repeated encounters and male identity

Number of steps was linked to repetition number (repeated encounters with the same individual) (full-null model comparison, likelihood ratio test: $\chi^2_5 = 11.91$, $P = 0.035$), as was the number of approaches ($\chi^2_5 = 15.92$, $P = 0.007$). In particular, the interaction term between repetition number and experimental block was significant in explaining the number of female approaches ($\chi^2_2 = 9.25$, $P = 0.009$), with females approaching the male less often during the third encounter with the same male of the second experimental block (ie. the very last test session of the experiment). Steps number was linked to experimental block ($\chi^2_2 = 9.53$, $P = 0.002$). It was smaller during the second (58.32 ± 85.08, $N = 147$, range 0-435) than during the first experimental block (100.68 ± 124.48, $N = 148$, range 0-529) but did not vary depending on the repetition number ($\chi^2_2 = 0.12$, $P = 0.93$).

When testing the effects of male identity, we had to take into account the inherent asymmetry in the experimental design, in that males from group C met females during the experimental block 1 whereas males of group D met females during block 2. Additionally, some female behaviours (number of steps and approaches) were found to differ between experimental blocks (see above). We therefore conducted separate analyses for each experimental block, each containing five individual males, i.e. males C in block 1 and males D in block 2. For C males, we found that male identity was linked to preening behaviour (full-null model comparison, likelihood ratio test: $\chi^2_4 = 12.35$, $P = 0.01$, Figure 6) and pecking number ($\chi^2_4 = 9.63$, $P = 0.04$). For group D males, male identity was associated with the number of approaches ($\chi^2_4 = 15.75$, $P = 0.003$), preening behaviour ($\chi^2_4 = 13.17$, $P = 0.01$, Figure 6) and steps number ($\chi^2_4 = 11.24$, $P = 0.02$). The link between male identity and female behaviour remained the same whether the dataset we used in our model comprises all the sessions or only the sessions where males were courting.

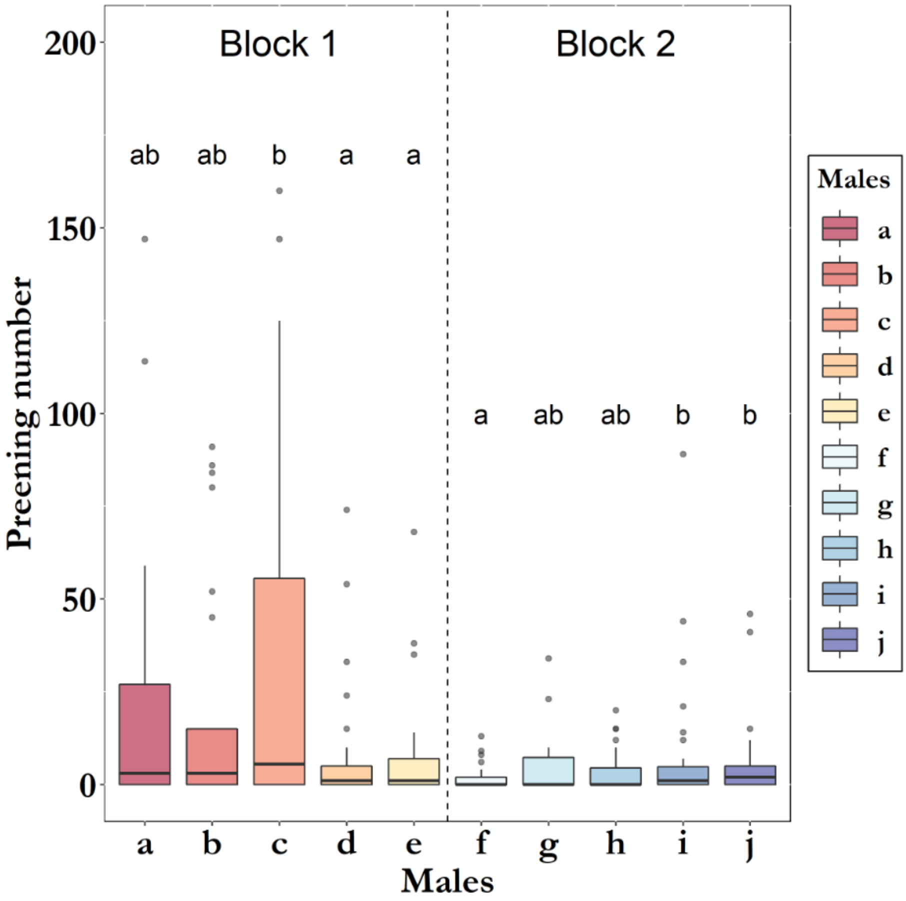

Figure 6. Box plot of female preening events depending on individual males. Significance letters are extracted from post-hoc tests run after generalized linear mixed models using a negative binomial distribution. Analyses were performed separately for the two male groups (ie. the two female experimental blocks). Boxplots' horizontal lines display the median, lower and upper quartiles. Whiskers represent values within 1.5 times the interquartile range and dots are outliers.

Discussion

The aim of this study was to describe and quantify how variation in the structure and synchronization of components of male ring dove courtship was associated with variation in patterns and strength of female behavioural response. Rather than investigating female choice for specific characters, we studied whether different male signals would be associated with different female behavioural responses. We hypothesized that different types of female response (that would be overlooked if only mating choice or time spent with a focal male was considered) could relate to different information contained in the male multimodal signal. Overall, tail quivering and number of steps were distinctive responses of females that changed depending on presence of male courtship. Male courtship showed individual differences in most components, both acoustic and visual. Both male and female behaviour changed across the experimental period. The fundamental frequency of the call, number of courtship bouts and the total duration of the courtship were related to tail quivering, which turned out to be the only variable of female behaviour that was associated with male courtship traits. Finally, we found that female behaviour varied depending on which male they encountered.

When investigating female response to male presence, we found that number of tail quivers, steps and pecks were higher when females interacted with a male. Steps and tail quivers were also more frequent during encounters with a courting male than in the presence of a non-courting male. Female activity was therefore positively influenced by male presence as well as male courtship. In pigeons (*Columbia livia*), changes in females general activity and number of steps is linked to female choice and female sexual interest (Partan et al., 2005), and in *Drosophila sp*, female movement is needed for the male to keep courting (Tompkins et al., 1982), demonstrating a role of female activity for the maintenance of courtship interaction. Additionally, we found that females quivered their tail exclusively when a male was present, and the number of events was much higher during encounters with a courting male. Rapid shaking of the tail is a sign of sexual stimulation and male acceptance in zebra finches (Witte, 2006) and sparrows (O'loghlen & Beecher, 1996). In birds of paradise, wing fluttering seems to be linked to mutual courtship (Scholes et al., 2017). In ring doves, the copulation solicitation display that appears after several days of courtship interaction is sometimes also associated with wing fluttering (Zenone, 1979). Thus, our results strongly support the notion that tail quivering is a reliable indicator of female sexual response in ring doves.

The analyzed courtship variables were similar to the ones already described in the dove literature (Davis, 1970; Fusani et al., 1997). We found that males greatly differed from each other regarding all of the courtship variables investigated (acoustic structure, spectral, visual and audio-visual synchronisation variables). A previous study (Fusani et al., 1997) did not find differences in the temporal structure of the bowing movements. The discrepancy is probably due to differences in statistical power, as in the present study we studied a larger number of individuals and number of courtship events. Our results are consistent with findings in other species where males show high inter-individual and small intra-individual variation regarding courtship variables (Jouventin et al., 1979; Abs & Jeismann, 1988; Jiguet & Bretagnolle, 2001). When investigating the role of specific courtship variables, we found that the only female behaviour to be influenced by variation in male courtship variables was the number of tail quivering events. We found that courtship total duration was positively associated with numbers of tail quivers while mean bout duration had a negative impact. This seems to indicate that females were more stimulated by many shorter bouts than by one long courtship bout. In doves, the bow-call courtship is structurally very similar to the aggressive display males use in agonistic contexts (Lovari & Hutchison, 1976). A long, uninterrupted bow-call display could therefore be perceived as more aggressive than attactive and might decrease the level of interest or stimulation in the female, a phenomenon already known from other species (Bastock, 1967; Patricelli et al., 2002; Ophir et al., 2005; but see Borgia & Coleman, 2000 for the opposite effect).

The other variable that had an effect on tail quivering was the median fundamental frequency of the second element of the call: a lower pitched call triggered more tail quivers. As the median frequency depends on both the maximum and minimum frequency of the call, this variable captures how high-pitched the call was on average. In birds, preference for higher or lower pitched vocalizations is found in a number of species (Cardoso, 2012). For instance, female Japanese barn swallows (*Hirundo rustica*) seem to prefer high-frequency male calls (Hasegawa & Arai, 2015*)*, and the same is true in rock sparrows *(Petronia petronia,* Nemeth & al., 2012). On the other hand, Miyasaki & Wass (2003) found that females were more likely to respond to low pitched calls in little penguins *(Eudyptula minor*). In the grey partridge (*Perdrix perdrix*), females also seem to prefer males with lower formant frequency (Beani & Dessì-Fulgheri, 1995). Bird song, and especially the spectral properties of vocalizations, can carry honest signals of male physical traits that are associated with mate quality (i.e. size or age) and can be used by females in a partner choice context (Gil & Gahr, 2002). One of the commonly invoked mechanisms is the action of androgens on sound frequency production. Indeed, female selection for androgen-dependent traits was documented in several species (Fusani et al., 1997; Peters et al., 2000; Hagelin & Ligon, 2001). In particular, lower fundamental frequency calls are associated with higher androgen levels in birds (Cynx et al., 2005), and low frequency calls can therefore be used by females as an indicator of male quality (Fusani et al. 1994; Beani & Dessì-Fulgheri, 1995). However, the link between androgen levels and male quality is not unequivocal, as high androgen levels may also affect the immune system (Roberts et al., 2004). Circulating androgens at a given time can affect songs parameters, such as call duration (Fusani et al., 1994), call fundamental frequency (Cynx et al., 2005) or call rate (Beani et al., 2000). Basal testosterone levels during development can also alter the fundamental frequency of vocalizations by impacting trachea and syrinx morphology as is the case in Grey partridges (*Perdix perdix*) (Beani et al., 1995). A link between fundamental frequency and trachea morphology exists in many species, with animals having larger vocal cords usually producing lower frequency calls or songs (Riede & Brown, 2013). This could also be true for doves, as in this species administration of exogenous androgen does not affect the fundamental frequency of calls nor other aspects of courtship like duration or structure (Feder et al., 1977; O'Connel et al., 1981; Fusani & Hutchison 2002). In our study call fundamental frequencies were not affected by experimental factors and remained constant for each male across the experiment, suggesting that they are unlikely to depend on contingent external conditions or internal physiological factors. Rather, call fundamental frequency in doves seems to be a marker of androgen levels during development. However, we cannot exclude that call fundamental frequency could also be linked to morphological features such as body size or weight, as it is the case in New world doves (Tubaro & Malher (1998)). For instance in the males used for the experiment, the e2 median frequency was positively correlated with P8 feather length (128.7 millimeters ± 2.07 (mean ± SD)), a common proxy measure of body size in birds, but not with the tarsus (29.1 ± 0.93) or the wing length (172.5 ± 4.26) (Figure A5). Temporal structure and spectral frequency of calls are linked to individual identity in some species (Grunst et al., 2017), including doves (Hutchison et al., 1997). Like most courtship variables, the fundamental frequency of the second call element was highly variable between males and had relatively small intra-individual variation, making it a potential marker for male identity.

Additionally to the variables with low intra-individual variation mentioned above, our results show that females were influenced by courtship characteristics that can change over time. For example, as already stated, females preferred longer courtship separated into smaller bouts. These features were not consistent for each male, as total courtship duration and bout duration were shorter during the second experimental block. Courtship call rate was also influenced by repeated encounters, as it was higher at the end of experimental testing than at the beginning. The correlation values between different courtship elements show that this higher rate was due to shortening of both the duration of the second call element and of the time spent in the up position of bows. Courtship duration can be linked to physical condition (Simon, 1988; Bertram & Rook, 2012) and therefore was proposed as a way of displaying current mate quality (Seymour & Sozou, 2009). The decrease in courtship duration toward the end of the experiment could indicate increasing fatigue as testing continued. For example, similar patterns exist in the Ruffed Grouse (*Bonasa umbellus*), where number and rate of displays declined over time in some males, which was suggested to be due to fatigue (Déaux et al. 2019). By preferring long overall courtship duration, females might therefore favor high performing males. Similarly to courtship duration, display rate is associated with vigor and condition in many different clades (Mowles & Jepson, 2015; Pellitteri-

Rosa et al., 2011; Mowles et al., 2017; Takeshita et al., 2018; Dunning et al., 2020). In doves, the fact that courtship length decreased over multiple testing sessions, whereas courtship rate increased might indicate that males perform an energetic trade-off between courtship vigor and courtship time. Such a mechanism occurs in the golden-collared manakin (*Manacus vitellinus*), where Tobiansky et al., (2020) recently demonstrated the role of androgen action on male muscles in mediating the trade-off between endurance and speed during courtship.

The time a female spends next to the male is a variable often used as a proxy for female choice and female interest (William, 1998; Witte et al., 2006; Dougherty, 2020). In our study, however, it was not influenced by male courtship variables. It is possible that our experimental setup was not optimal for measuring sexual interest using the distance between male and female. The apparatus is relatively small and movements within the test box might be too limited to reflect the extent of female interest. Other aspects of female behaviour that were not affected by variation in male behavior were general activity and self-preening. However, although these behavioural patterns are linked to sexual interest in some species (Tinbergen, 1952; Cheng, 1973; Partan, 2005), they could also simply be displacement behaviour and reflect discomfort (Delius, 1988; Maestripieri et al., 1992; Laurence et al., 2012). Regarding approach attempts, our results suggest that this variable does not reflect sexual interest in doves. Although the difference was not significant, females attempted to enter the opposite box more often in the control condition (where no male was present) than when a male was present. This seems to indicate that these behaviours do not reflect female sexual or social interest in doves, but rather arousal or excitement.

Female response varied depending on the identity of the male within the same experimental block. Approach behaviour, general activity (steps and pecks) and preening, differed depending on which male they encountered. It is not clear however what exactly is responsible for these differences. Female response could depend on physical characteristics, such as the male's size or plumage colors, factors assessed by females in some species in the context of sexual interactions (Hill, 2006), even if this is less likely to occur in a monomorphic and monogamous species like the ring dove (Kirkpatrick et al., 1990). Additionally, other aspects of male behaviour which we did not consider could be the source of this female behavioural variation, such as call frequency modulation (Drăgănoiu et al., 2002) or call sound pressure level (Ritschard et al., 2010). We measured courtship variables that have been shown previously to influence female response, however, some non-investigated courtship variables (like call modulation or velocity of movement for instance), as well as physical characteristics like body size, might also have an impact on female response.

Some male variables we suspected to have an impact on female behaviour were not associated with differences in females' response. For instance, females might have been expected to respond differently to different bowing amplitude, as it is the case in the fiddler crab (*Austruca perplexa*), where Murai & Backwell (2006) described a female choice for display structure and claw movement amplitude during courtship. Also, temporal synchronization between different modalities and the extent to which individuals manage to coordinate two (or more) different signals in time is linked to female preference in some species. In túngara frogs (*Physalaemus pustulosus*) for example, females prefer synchronous multimodal courtship signals (Taylor et al., 2011), and the same is true for the brush-legged wolf spider (*Schizocosa ocreata*) (Kozak & Uetz, 2016). Although in our study we did not find any effect of movement amplitude, multimodal synchronization or other courtship variables on female dove's response, this does not mean that those variables are meaningless. The multiple message hypothesis states that different elements of a complex signal can have different impacts on a receiver. Here, we exclusively studied immediate female behavioural responses, but it is likely that variation of courtship variables affects other aspects of female response. For instance, female physiological state can be affected by courtship in arthropods (Rybak et al., 2002), reptiles (Crews, 1975; Kelso & Martin, 2008), and doves (Cheng et al., 1998). In doves, we know that females' physiological state is strongly courtship-dependent, as females show higher neuroendocrine response (measured by oviduct size) when presented with multisensory courtship compared to auditory only (Friedmann, 1977). In the present study we did not measure the physiological response to courtship, however, we would expect that some courtship elements would affect female physiological response the same way call fundamental frequency or courtship duration affected female behavioural response. For example, it is not clear why female behaviour was influenced by the fundamental frequency of the second call element and not by that of the first one. The duration of the call could play a role, as the second element was on average almost 5 times longer than the first element. However, it is also possible that the fundamental frequency of the first element impacted an aspect of female response we did not investigate. It could be a behaviour that we did not consider, or even a physiological response in the form of hormonal changes influencing ovarian development (Crews, 1975; Friedmann, 1977).

Assessing the extent to which females vary regarding their response to courtship by measuring individual preference variation was beyond the scope of this study. Nevertheless, we showed that even in a very controlled experimental setup, females greatly differed from each other regarding the range and intensity of their responses to courtship, and also changed their behaviour over time. In fact, a large proportion of response variation to courtship parameters was even due to female identity. Females were housed together in aviaries during the experiment and their last visual interaction with males was weeks before the experiment started. However, the large female individual variation seems to suggest that they were not all in the same reproductive state or equally motivated during this experiment, some of them appearing to be more sexually receptive than others. The fact that females showed fewer approach attempts as well as less general activity toward the end of the experiment, might indicate that their behaviour was also impacted by repeated exposures to courtship. Whether this was due to the potential aggressive messages contained in repeated courtship signals, or simply habituation or disinterest is unknown. However, it is important to note that despite the large inter-individual variability, patterns regarding exposure to courtship and variation in courtship variables were consistent between females. For example, females that showed the greatest number of tail quivers in front of a courting male also quivered their tail even when the male in front of them was not courting. This suggests that more than the absolute frequency of sexual behaviours displayed towards a male, the relative increase of sexual behavioural events compared to the individual female's pre-courtship state might be more informative when studying the female response to courtship.

To our knowledge, this study is one of the first to investigate on such a fine scale the influence of small, natural variation in a large number of male courtship elements on different female behavioural responses. By analyzing in parallel the effects of component signals, we identified female behavioural responses to acoustic spectral elements and courtship structure (courtship length and number of courtship bouts, both likely markers of courtship effort) on female response. Additionally, investigating a wide range of female behavioural responses allowed us to assess possible targets of male courtship variables that would potentially be overlooked if taking into account only her final mate choice. We showed that females might be interested in markers of courtship effort, as well as in specific individual males. Ring doves are monogamous, and it is also highly likely that courtship is not only used by females to make a mating decision, but also to optimize pair matching and reinforce an existing pair bond over several reproduction episodes. Ultimately, the final pairing and mating decision in doves might depend not only on several messages contained in the bow-call display, but also on courtship elements present at later stages of the interaction which we did not explore here. Our results remain correlational, and manipulative studies are needed to specifically test how variation in male behavioural variables affects female response. Progress in audio-visual technology now allows creation and display of high quality video stimuli and these playback techniques can be particularly relevant in the context of sexual communication. Future studies should focus on creating modified courtship stimuli and presenting them to females. This will allow an assessment of how females respond to specific, controlled variation in targeted male courtship variables.

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Chapter 3. Audio-visual synchronization of multimodal courtship signals affects female sexual behaviour in the ring dove

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Audio-visual synchronization of multimodal courtship signals affects female sexual response in the ring dove

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Abstract

Multimodal signals (i.e. occurring in more than one sensory modality) are thought to carry information which is not present when individual channels are assessed in isolation. To understand the function of the ring dove's (Streptopelia risoria) multimodal courtship, we used audio-visual playback of male displays to investigate female response to stimuli differing in their audiovisual synchronization timing. From natural courtship recordings, we created a shifted stimulus where audio was shifted relative to video by a fixed value, and an asynchronous stimulus where calls were moved randomly along the visual channel. We presented three female groups with the same stimulus type for seven days. We recorded their behaviour and assessed pre- and post-test blood estradiol concentration. We found that females behaviorally responded more often to playback than to control, and that playback exposure increased estradiol levels, confirming that this technique can be efficiently used to study doves' sexual communication. Additionally, chasing behaviour (indicating sexual stimulation) increased over experimental days only in the synchronous condition, suggesting an effect of multimodal synchronization on female response. This stresses the importance of signal configuration in multimodal communication, as additional information is likely to be contained in the temporal association between modalities.

Keywords

elaborate display; ring dove; multisensory playback; artificial stimulus; temporal synchronization

Introduction

To communicate, animals use signals occurring in a variety of sensory modalities. Visual, acoustic, vibratory or olfactory signals are used to transmit information, and are sometimes emitted concomitantly within a short temporal window. This leads to the production of complex multi-component signals, referred to as "multisensory" or "multimodal" [1,2]. Recently, theoretical and empirical studies have attempted to explain the prevalence and adaptive advantages of these complex signals over unimodal signals. The usual approach to study composite signals has been to independently investigate the role of each component. Separate signals can carry separate pieces of information (multi-message hypothesis) or improve signal efficiency by enhancing physical transmission in the environment and act as a back-up (redundant signal hypothesis) [3–5].These mechanisms are not mutually exclusive [6,7].

Another important aspect is the configuration of the signal itself. Individual modalities can interact and enhance or suppress their respective effects [2]. In some cases, new information can emerge from their association [8] so that multimodal signals are "more than the sum of their parts" [2,9–12]. For example, certain prey defensive warning signals are aversive to predators only when visual and chemical signals are presented together [8,13].

Multimodal signaling implies spatial and temporal coupling [9], and for the receiver, multisensory integration is typically facilitated by spatial and temporal coincidence [15–17], although this is not necessarily the case [18]. Many examples exist of signals from multiple modalities belonging to the same display undergoing a high level of temporal synchronization [14]. Much work has been done on birds' courtship behaviour, as these displays are diverse and often very multimodal. In the broad-tailed hummingbird (*Selasphorus platycercus*), male courtship dives show an impressive consistency in their audiovisual synchronization timing [19]. Similarly, in the Montezuma oropendola (*Psarocolius montezuma*), two elements of the visual courtship display (bow and wing spread) are temporally synchronized with two elements of the auditory courtship display (loudest note and lowest peak frequency, respectively) [20]. In superb lyrebirds (*Menura novaehollandiae*), males synchronize specific songs with specific visual display types [21]. Finally, in zebra finches (*Taeniopygia guttata*), the courtship dance is strongly temporally associated with specific parts of the auditory display [22].

Several authors examined the advantage of producing synchronized multisensory signals rather than emitting them sequentially [2,23,24]. The "by-product" hypothesis proposes that one part of the display directly causes another, making them necessarily synchronized [25], as in frogs where visible vocal sac inflation is a by-product of acoustic communication [26], or human speech where lip movements are necessary for speech production [8]. Multimodal synchronization could also be due to mechanical constraints. For example, in brown-headed cowbirds (*Molothrus ater*), it has been proposed that males time the visual part of their display to occur during silence to avoid any influence of movement on sound production, thereby mechanically maximizing both signals [27] Multimodal temporal synchronization could also serve to improve signal efficiency by transmitting information in a shorter time [19]. These hypotheses all imply a cost reduction of the display [27]. Finally, in the specific case of courtship behaviour, temporal synchronization could be an indicator of individual quality as it reflects motor performance and neuromuscular ability. Examples include the complex synchronized displays in Montezuma oropendola [20], golden-collared manakin (*Manacus vitellinus* [28]) or zebra finch [29]. In addition, coordinating several displays can be cognitively challenging and therefore also under sexual selection [21].

One way of investigating the importance of the association between modalities is to experimentally disassociate them by spatial separation or temporal desynchronization [9]. For instance, a robotic male túngara frog (*Physalaemus pustulosus*) has been used to present females with different temporal combinations of visual (inflated vocal sac) and auditory (whine and chuck) courtship signals, showing that female response was reduced when calls and sac inflation were asynchronous [30]. Another study in the same species showed that females did not prefer a synchronized over a unimodal signal, but would strongly reject an asynchronous one [31]. Finally, in the wolf spider (*Schizocosa ocreata*), temporal asynchrony of visual and vibratory courtship components negatively influenced female receptivity [32]. The technical difficulties implicit in this type of study has limited investigations to a restricted number of species. In addition, no study has yet investigated the influence of courtship synchronization on physiological responses. Presentation of single-channel signals during courtship can trigger physiological changes, in terms of sex steroid concentration for instance [33–35]. In the ring dove (*Streptopelia risoria*), females show greater reproductive tract development when exposed to full male courtship compared to vocalizations only [34]. It is therefore likely that the configuration of the signal itself not only impacts female behavioural response and choice, but also female physiological responses.

Playback experiments are a powerful tool to study multimodal displays [36,37] as they allow targeted modification of specific aspects of interest. In birds, auditory stimuli have been successfully used across social contexts in laboratory [38,39] and field studies [40,41]. The high temporal resolution of bird compared to human vision [42] has so far limited the use of visual stimuli. Nowadays, high-speed recording and display devices allow efficient testing of how variation in display parameters affects receiver response, although limitations still persist, namely regarding colour and polarization perception [43]. Multimodal playback has been used successfully in fowls (*Gallus gallus*) [44] and black capped chickadees [45], among others. In Columbiformes, Shimizu [46] successfully triggered courtship behaviour in male pigeons (*Columbia livia*) by presenting them with female videos. More recently, female pigeons responded with natural sexual behaviour to audio-visual stimuli of courting males, and the response was stronger to multimodal compared to unimodal playback [47]. Again in pigeons, motion quality of displayed videos influenced receiver response [48], with video frame rate found to be critical for revealing differences in birds' behavioural responses to different stimulus material.

Male ring doves perform audio-visual courtship, bending repeatedly toward the ground facing the female (ie. the bow), while vocalizing a synchronized bow-call. To assess the role of multimodal synchronization and signal configuration, we presented females with playback of manipulated courtship differing in temporal synchronization between acoustic and visual channels. The temporal interval between body movements and vocalizations was either unchanged ('synchronized'), shifted by a fixed amount ('shifted'), or randomized ('asynchronous'). A novelty of our study was that we analyzed both behavioural and physiological endpoints to assess whether female response was impacted by the temporal structure of male multimodal courtship. In female ring doves, blood estradiol (E2) levels increase after exposure to a courting male [49], and the neuroendocrine response triggering the development of the reproductive tract depends on which courtship modalities they are exposed to [34].

We found that females were more behaviorally active when presented with audio-visual courtship playback versus a control video. Most investigated female behaviours increased over the testing phase. We did not find an effect of synchronization treatment on non-sexual behaviour. However, we found a significant increase in a sexual behaviour, chasing, between the first and the last experimental days only in the synchronized condition. Moreover, E2 plasma concentration at the end of the playback experiment was higher than before testing and differed depending on stimulus type. These results indicate that playback of male courtship is a viable method for investigating female response in ring doves. Although we did not find evidence that audio-visual display synchronization influences females' immediate response to courtship, it seems that signal structure is important to trigger female sexual behaviour and physiological receptivity prior to mating.

Materials and Methods

Study species

Ring doves were obtained from breeders in Austria and France and were at least one year old. We housed the 24 females in outdoor aviaries (3.6 x 3.0 x 2.7 meters) separated from males 5 weeks prior to testing and transferred them indoors into individual cages (50 x 38 x 60 cm) 3 days before testing, where they remained during the whole experiment. This is a standard pre-experimental procedure in this species to limit the effects of social interaction on sexual receptivity [50,51]. The light regime was 14D:10N. Birds had access to seed mix, grit, and water ad libitum. Prior to the experiment, doves underwent a habituation period of several weeks with regular handling and exposure to the experimental setup.

Experimental apparatus

The experimental apparatus was composed of two compartments (each 50 x 50 x 50cm) separated by a net, one containing a screen and a speaker, the other containing the tested female (see electronic supplementary material (ESM) for detailed specification of playback setup, figure *s*1). A camera recorded a side view of the female at 60 frames per second, and a microphone recorded vocalizations. After placing a female in the setup, the screen displayed an empty setup and she was allowed to habituate. We started audio and video recording of the female and then started playback stimulus display. The testing session lasted for 15 minutes, after which recording was stopped and the female returned to her individual cage.

Stimulus manipulation

See ESM for a description of playback acquisition and display. For each of the original courtship recordings, we first denoised them using the spectral noise gating algorithm from Audacity® [52] . We then annotated the timing of the bow (Loopy, http://loopb.io, loopbio gmbh, Vienna, Austria) and the bow-call (PRAAT, v.6.0.26) in order to estimate the natural variation in temporal offset between the starts of bows and bow-calls. We used these values to determine the amount of desynchronization to apply to modified stimuli (see ESM). We created three stimulus treatments by varying the auditory track to change auditory and visual signal synchronization [31,32,53] (figure 1). Using Matlab (Mathworks, Natick, MA), each bow-call was extracted from the source track using the annotations mentioned above. For the synchronous stimulus, no change in timing was made and bow-calls were placed at the same onsets in the new audio track (figure 1). For shifted courtship, each bow-call was placed -0.32s earlier in the new audio track. In the asynchronous courtship condition, the relative onset of each bow-call was randomly drawn from a normal distribution with $\mu = 0$ and SD = 0.25s (with the constraint subsequent bow-calls did not overlap). Thus, for both shifted and asynchronous treatment we manipulated the naturally occurring variation in but the spectral and temporal structure of the calls and the total number of bow-calls were unchanged for the three stimulus treatments. Finally, we normalized audio of all stimuli to the same peak amplitude. Videos of the three stimulus type can be found in ESM *s*12, *s*13 and *s*14.

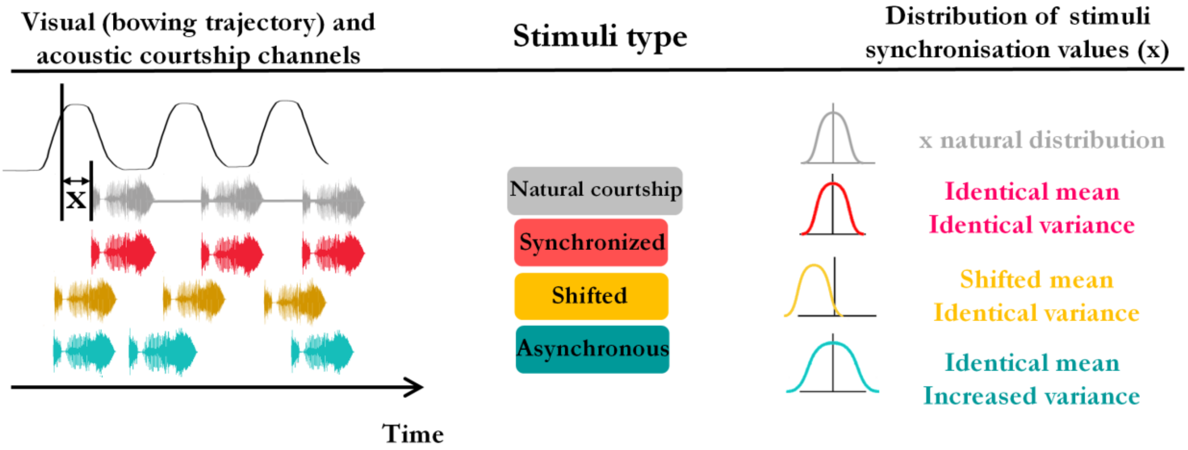

Figure 1. Stimulus creation. Bow-calls were isolated from natural courtship recordings (grey) and placed into new auditory tracks to create modified stimuli. We used unaltered timing for the synchronized stimulus (blue), which has a natural distribution of audiovisual synchronization timing as depicted on the right. We shifted the start of each bow-call by a fixed value to create the shifted stimulus (yellow), shifting the mean but conserving the synchronization variance. For the asynchronous stimulus (orange), the mean of the distribution was maintained, while the variance increased.

Experimental design

We assigned each female to a treatment group (synchronized, shifted or asynchronous; 8 females per group) and to a stimulus male (1 or 2; 4 females per male per treatment group) (figure s2). Each female was exposed to the stimulus during 15 min playback sessions on each of 7 consecutive days [54,55]. During this 15 minutes playback, only 8 minutes displayed a courting male, as playback courtship videos (each ~30 s duration) were interspersed with a view of an empty testing compartment that served as control videos for behavioural analysis (see ESM for more detail). Because of time constraints that prevented testing all birds in parallel, we tested half of each group (12 females, 4 females of each group) in the second week of August 2019 (group a), and half in the third week of August 2019 (group b).

Video analysis

Using Loopy, we coded three female behaviours previously associated with sexual behaviour in doves and other bird species: tail quivering [56–58], preening [47,57] and chasing. In doves, chasing has not previously been studied in females, but in males this behavior, which is typically displayed before the bow-call display, is known to be an important component of sexual behavior [49,50]. The bird adopts a characteristic horizontal body posture while walking, feathers of the back are often erected and there is sometimes an approach toward the other individual and a specific call. We also coded the number of steps to obtain a proxy for overall locomotor activity. For every behaviour, we recorded its duration but also the time and frame number at which it occurred during the 15 minutes testing. As we knew the times when courtship or control videos were played, we could assign behavioural occurrences to either period. If a behaviour bridged two periods, it was assigned to the first one.

Estradiol assays

One day before the first and one day after the last exposure to playback, we took a blood sample from each female. Samples were centrifuged for 5 minutes at 2000 rpm, then plasma was pipetted into Eppendorf tubes and stored at -20°C until assay. Estradiol plasma concentrations were quantified in duplicate using commercial enzyme-linked immunosorbent assay (ELISA) kits (RE52041; IBL, Hamburg, Germany). We conducted validations to eliminate the interference of the dove plasma matrix that caused a shifting problem (see ESM). Sensitivity was 3.6 pg/mL and 5.81 pg/ml for the first and second assay, respectively. Intra-assay CV% of duplicates was below 5% (mean 0.9%). Inter-assay CV% of the kit control and of an extracted plasma control pool were 4.74% and 0.61%, respectively.

Statistical analysis

We performed all statistical analysis using R [59]. For all female behaviours, number of events and overall duration were highly correlated ($r > 0.90$, $p < 0.001$). Therefore, we used only counts for further analysis. For every model, we used a full-null model comparison approach with a likelihood ratio test to investigate the effect of fixed factors [60]. The null model lacked the respective fixed effects or interaction, but random effects and controls remained.

We used generalized linear mixed models to assess if female behaviour differed depending on whether the playback displayed courtship or empty setup (courtship playback present vs. absent). We fitted models using the glmmTMB function and a negative binomial distribution to model number of female behaviour events (preening, steps, chasing, tail quivers) and to account for overdispersion. Full models included female identity nested into test session as a random effect, playback content (courtship playback present vs. absent) and experimental days (1-7) as fixed effects and experimental group (a/b), stimulus male (1/2) and female origin (France/Austria) as controls.

We also used glmmTMB to model the effect of stimulus type (synchronized, shifted, asynchronous) on female behavioural response. We assumed that female behaviour during control videos was influenced by the preceding stimulus and therefore used the full dataset comprising female behaviour during the whole 15 minute session. Full models included female identity as random effect, interaction between stimulus type and experimental day as fixed effect, and experimental group, stimulus male, and female origin as controls.

We used linear mixed models (lmer function) and linear models (lm function) to investigate whether E2 concentration was influenced by stimulus type or sampling phase (pre-exposure/post-exposure). Full models comprised female identity as random factor, stimulus type and sampling phase as fixed effects, and experimental group, female origin and stimulus male as controls.

We used Spearman correlation tests [61] to investigate the association between number of behavioural events on the first day and pre-testing E2 blood concentration, and the association between the number of behavioural events on the last day and post-test E2 concentration.

Results

I – Female response to audio-visual playback

Playback content had an effect on occurrence of preening (χ^2_1 = 109.3, $p < 0.001$), steps (χ^2_1 = 38.206, $p < 0.001$), chasing (χ^2_1 = 55.118, $p < 0.001$) and tail quivering behaviours (χ^2_1 = 25.21, $p < 0.001$). For every behaviour, event number was on average significantly higher when the courtship playback was present than when courtship playback was absent (table 1, figure *s*3, table s4).

Table 1. Number of behavioural events displayed by females and mean (SD) per female depending on playback content and χ2 test statistics from the full-null model comparisons.

	Empty setup		**Courtship**		
	Total	**Mean (SD)**	**Total**	**Mean (SD)**	**χ2 test statistics**
Chasing	135	0.80 (2.83)	552	3.28 (7.28)	55.11*
Preening	8671	51.61 (49.46)	18880	112.38 (91.35)	109.3*
Steps	18409	109.57 (107.79)	22666	134.91 (127.05)	38.206*
Tail quivers	138	0.82 (1.41)	341	2.02 (3.12)	25.21*

*: p<0.001

Experimental day significantly affected occurrence of preening (χ^2_6 = 53.261, $p < 0.001$), steps (χ^2_6 = 18.179, $p = 0.005$) and chasing (χ^2_6 = 24.477, $p < 0.001$), with number of behavioural events increasing over experimental days. No difference between days was found for tail quivers occurrence (χ^2_6 = 7.25, $p = 0.29$).

II – Effect of courtship synchronization on female behavioural response

Although females preened more often in the synchronized condition than in shifted and asynchronous conditions (table s5), the effect of audio-visual timing was not significant (χ^2_2 = 3.10, $p < 0.21$). Stimulus type had no effects on number of steps (χ^2_2 = 0.52, $p = 0.76$), tail quivers (χ^2_{14} = 17.33, $p = 0.23$), or chasing behaviour (χ^2_2 = 1.80, $p = 0.40$), although similarly to preening, females displayed the most steps and chasing in the synchronous condition. Experimental day impacted preening (χ^2_6 = 49.00, $p < 0.001$), steps (χ^2_6 = 22.03, $p < 0.001$) and chasing (χ^2_{14} = 43.33, $p < 0.001$), with females displaying more of these behaviours as the experimental days passed. However, day did not impact the tail quivering behaviour (χ^2_{14} = 8.84, $p = 0.18$).

The interaction between experimental day and stimulus type was not significant for preening (χ^2_{12} = 7.38, $p = 0.83$), steps (χ^2_{12} = 14.86, $p = 0.24$) or tail quivers (χ^2_{12} = 14.55, $p = 0.26$), but was significant for chasing behaviour (χ^2_{12} = 21.11, $p = 0.04$). Post-hoc tests and pairwise comparisons revealed that chasing significantly increased during the experimental period only in the synchronized condition (figure 2, table *s*6, *s*7).

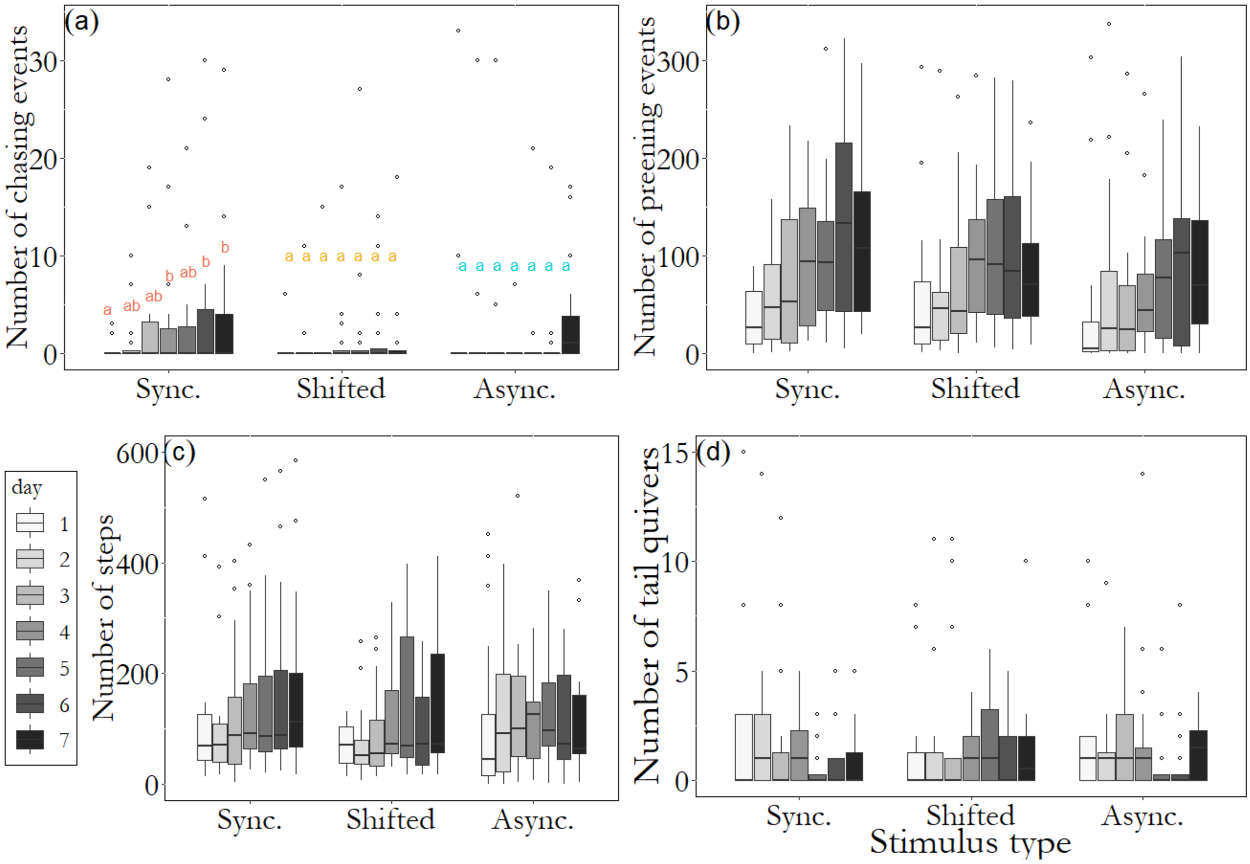

Figure 2. Boxplots (median, lower and upper quartiles, values within 1.5 times the interquartile range and outliers) of behavioural occurrences displayed by females, grouped by stimulus type and day of experiment. There was no significant difference between conditions for any behaviour. We displayed significance letters between experimental days for chasing only, as for this behaviour we found a significant interaction between stimulus type and days.

III – Effect of courtship synchronization on female physiological response

E2 plasma concentration ranged from non-detectable (below 5.2pg/ml) to 31.98 pg/mL (12.17±8.53, mean ± SD) for pre-exposure blood sampling, and from non-detectable to 41.88 pg/mL (17.92±11.03) for post-exposure blood sampling. Difference in E2 levels between pre and post-test ranged from -8.52 to 23.44 (5.74±7.51, positive differences reflecting higher post-test concentration), and percentage increase ranged from -28% to 286% (+59% ±72). E2 plasma concentration was significantly increased after the playback experiment ($\chi^2_1 = 12.05$, $p < 0.001$, figure 3, table *s*8, s9).

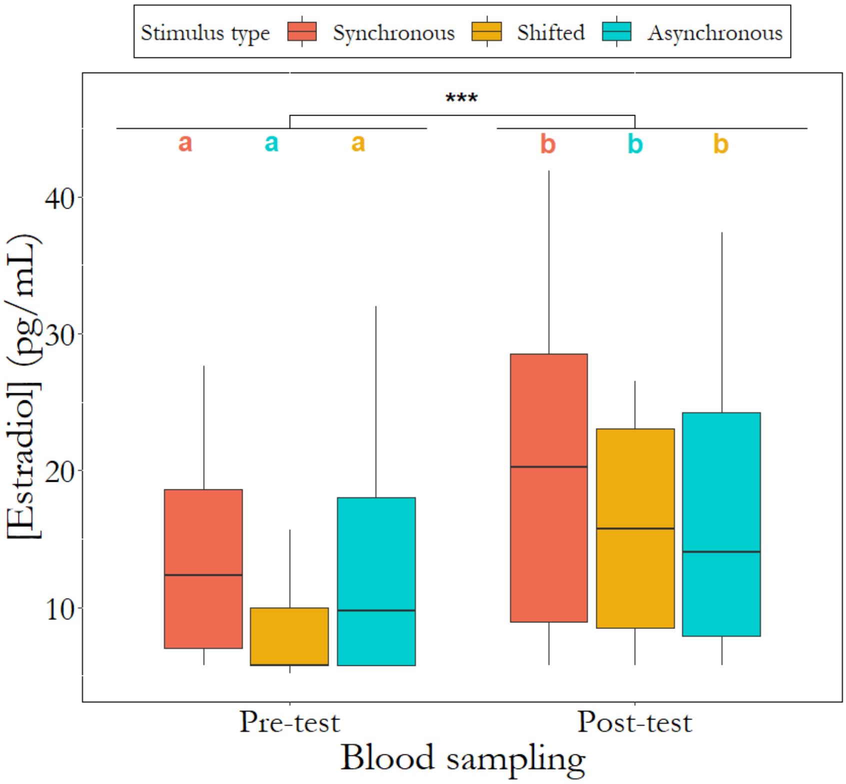

Figure 3. Boxplots (median, lower and upper quartiles and values within 1.5 times the interquartile range) of E2 concentration (pg/mL) of females grouped by pre- and post-exposure and stimulus types. Stars indicate significant differences between pre- and post-exposure E2 concentration and letters indicate lack of differences between stimulus types within blood sampling phase.

Although plasma E2 concentration was higher in the synchronous condition and stimulus type influenced E2 concentration ($\chi^2_2 = 6.45$, $p = 0.03$), post-hoc pairwise comparison tests between stimuli types were not significant (table *s*10). There was no interaction between stimulus type and sampling status ($\chi^2_2 = 1.35$, p = 0.50). Stimulus male did not influence E2 concentration ($\chi^2_1 = 0.15$, $p = 0.69$), but females from group 2 (tested one week after females from group 1) had lower E2 values than females from group 1 ($\chi^2_1 = 37.37$, $p < 0.001$). However, percentage increase between pre and post-exposure concentration did not differ between groups ($F = 2.26$, $p = 0.14$), condition ($F = 1.48$, $p = 0.25$) or stimulus male ($F = 0.005$, $p = 0.94$).

The only significant correlation between E2 concentration and behaviour was between post-exposure concentration and tail quivers number on the seventh day ($r = -0.52$, $p = 0.008$), with females showing higher number of tail quivers on the last experimental day having lower E2 concentration (table *s*11).

Discussion

We investigated behavioural and physiological responses of female ring doves to playback stimuli of male courtship differing in the synchronization between the visual and acoustic components of the bowing display. In addition, we examined how female behaviour changed over the seven consecutive experimental days during playback of courtship stimuli or of control videos showing a silent and empty cage. We found that females displayed the behaviours of interest more often during presentation of audio-visual courtship stimuli than control videos. We also showed that sexual and non-sexual behaviours increased as the experiment progressed. We additionally documented an increase in E2 concentration at the end of the experiment compared to pre-experimental values, reflecting female sexual stimulation. Finally, although we could not convincingly show that manipulation of audio-visual synchronization affected all aspects of female behavioural response, we found that only those females presented with the unaltered, synchronous stimulus significantly increased their chasing behaviour across the experimental period. Additionally, stimulus type also influenced plasma E2. This suggests that the natural variation present in audio-visual synchronization of courtship plays a role in female evaluation of male courtship.

Ring dove courtship is a dynamic phenomenon, with behaviour and physiological responses of males and females developing through different stages [50]. Before testing, females were separated from males to bring them into a basal stimulation state. In females, E2 production significantly increases after a few days of courtship interaction [62], which was confirmed by our E2 results. Females' behavioral responses to male bow-calls [63,64] and their subsequent shift to nest-oriented behaviour after a few days of courtship [57] are also well documented. This shift could explain the correlation patterns we found between E2 levels and number of tail quivers. Indeed, if tail quiver is a sign of sexual interest displayed in the first phase of the courtship interaction, we could expect a negative correlation of this behaviour with post-exposure E2 concentration, which was measured on the subsequent day. Additionally to physiological stimulation, our results show that audiovisual playback increased females' sexual and non-sexual behavioural responses, as steps, preening, and chasing increased over experimental days. This sensitization effect (increased response after repeated exposure to courtship) has already been documented in doves and canaries [56,57].

We designed this study to examine the importance of multimodal synchronization in courtship. The amount of courtship (number of bows and bow-calls) in both modalities was the same for all three synchronization conditions. A similar response to the synchronous and the shifted condition in contrast to the asynchronous condition would therefore indicate that more than the average relative audio-visual timing, repetition rate and variance in timing of the multimodal courtship are important for females. In contrast, a similar response to the two modified stimuli compared to the synchronous one could indicate a negative effect of a signal whose parts are too strongly desynchronized. Our results suggest the latter is the case. Consistent shifting of the acoustic track (shifted condition), as well as randomly placing calls along the bowing sequence (asynchronous condition), negatively affected female sexual response, but not non-sexual response. The number of chasing events increased from the beginning to the end of the experiment only in females presented with synchronized stimuli, although the overall number was not significantly influenced by stimulus type (although numerically greater in the synchronized condition, table 2). These behavioural results, in addition to the moderate effect of stimulus type on plasma E2 concentration (where the lack of post-hoc significant differences is possibly due to an overall weak effect or a small sample size), therefore provide support to the hypothesis that multimodal synchronization and signal configuration of courtship influence female response. Some information triggering an increase of chasing and plasma E2 appears to be present in naturally synchronous stimuli, but not in modified videos.

Signal configuration, and particularly multimodal synchronization, is likely to play an important role in sexual interactions, and our results add to existing data suggesting that signal configuration itself is a target of inter- and intra-sexual selection. Our results are in line with work on courtship synchronization in wolf spiders [32] and túngara frogs [31], where females were more attracted to males displaying synchronized, natural courtship and rejected shifted or asynchronous stimuli. Although ring dove courtship is less complex and elaborated than the displays of some tropical birds, the capacity to synchronize multimodal courtship could reflect good motor and neural control and might positively affect female sexual response [20,28]. Whether the by-product hypothesis or the mechanical constraint hypothesis are relevant here is unknown. Male doves can produce calls without simultaneously performing the bow (perch-call and nest-call [65]). In doves, we know that esophagus inflation amplifies calls [66,67], and we cannot exclude that bowing further enhances this inflation. The visual bowing component could thereby increase call amplitude, and high sound pressure level is more attractive for females in many bird species [68,69].

A previous behavioural study by our group showed that tail quiver occurrence in female ring doves was associated with lower fundamental frequency calls but had no relation to average audio-visual synchronization of courtship [64]. Our present results indicate that manipulations of audiovisual synchronization also do not affect tail quiver behaviour. Rather, the increased number of tail quivers during playback is probably linked to the acoustic part of the courtship alone. In doves, females' own calls are physiologically self-stimulating and increase E2 levels [63]. As female and male calls are extremely similar, it is possible that the acoustic part of male courtship is responsible for triggering partial hormonal changes [34]. However, the higher plasma E2 of females in the synchronous stimulus condition (even though this effect would need to be confirmed in further experiments) supports the notion that audio-visual synchronization enhances sexual stimulation of females. We would need to include a unimodal acoustic stimulus in a similar experiment to formally test this hypothesis. In túngara frogs, females preferred asynchronous multimodal signals over unimodal signals [30] and this could be true for doves as well.

The moderate effects on plasma E2 levels, where we found an effect of stimulus type but did not obtain significant post-hoc differences between stimuli, suggest that our stimulus manipulations were not sufficiently large to yield strong differences in the hormonal response between stimulus types. Indeed, E2 concentrations in this experiment were lower than previous studies where average peak values of 85 pg/mL were documented for females paired with a male for several days [62]. It is possible that 8 minutes of active courtship a day for 7 days were not enough to fully stimulate females [70], or that the post-exposure blood sampling missed the typically short E2 peak. Moreover, it is possible that the non-interactive nature of playback negatively impacted female sexual stimulation, and that physical interaction with a live male for the same amount of time would have triggered higher hormonal changes and stronger differences between displays differing in the extent of audio-visual synchronization.

Remarkably, tail quivering was the only behaviour that occurred consistently over experimental days, suggesting that it is an immediate response to courtship in ring doves, showing momentary interest of a female. In contrast, chasing behaviour seems to reflect increased sexual stimulation over a longer time scale, meaning courtship likely triggers both immediate and delayed behavioural responses. Our results also showed a peculiar increase in chasing on the 7th day in the asynchronous condition. It could be that asynchronous stimuli need a longer period of exposure to trigger sexual response [34]. At this time, we do not have a convincing interpretation for this unexpected aspect of the study.

This study is to our knowledge the first to show that female doves respond to audiovisual playback of courtship displays. The higher E2 concentration at the end of the experiment, and increased behavioural response across testing days and during courtship compared to control playback, are strong evidence that females responded to conspecific courtship in the presented videos. In birds, acoustic-only playback has been used extensively to successfully trigger responses. However, the higher flicker fusion rate of avian visual systems [42], as well as differences between avian and human color and polarized light perception [43] makes it challenging to create natural-looking visual stimuli for birds. As visual and auditory stimuli were systematically displayed together, the increased behavioural and hormonal response at the end of the experimental period could be due solely to the acoustic part of the playback. However, visual stimuli have been used successfully in pigeons [47], a closely related species, to trigger natural responses to courtship, and the temporal quality of the video directly influenced the duration of sexual behaviour displayed by test subjects [48]. We are therefore confident that our videos, with even higher temporal fidelity (120 frames/second), triggered natural responses in doves. Additionally, when males were presented with silent videos of a female dove, they regularly displayed courtship (personal observation). Finally, and most importantly, females from different experimental groups reacted differently (stronger chasing behaviour), confirming that both visual and acoustic stimuli were assessed and integrated by females.

This study provides important advances in our understanding of the importance of the configuration of multimodal signals. Audiovisual playback was used for the first time to study how targeted variation in male behaviour can influence both female sexual behaviour and physiological state. The demonstrated success of the playback technique opens up many possibilities for future studies. Here, we targeted the temporal association between sensory channels, but the spatial configuration of the signal is likely to be important and could also be investigated using multimodal playback. More importantly, our results support the hypothesis that multicomponent signals are "more than the sum of their parts", and that specific information is contained in the way signals are built and temporally structured. The nature of this integrated information is still to be determined and further quantitative studies comparing responses to differently manipulated stimuli need to be performed. In the long term, our aim is to understand if the integration of multiple signals in multimodal courtship is associated with an evaluation of the whole display by the receiver that relies on mechanisms that go beyond the evaluation of single components. This approach, initially used in controlled laboratory conditions and for species that show relatively simple courtship, could later be extended to the elaborate and bizarre displays of many species of arthropods and vertebrates.

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Ethics

This work was approved by the local ethics committee of the Faculty of Life Sciences, University of Vienna, and by the national committee of the Austrian Federal Ministry of Education, Science and Research (BMWFW permit 66.006/0042-WF/V/3b/2017). This study adheres to the ASAB/ABS Guidelines for the Use of Animals in Research and the ARRIVE Guidelines.

Data accessibility

All relevant data are available from Phaidra, the University of Vienna data repository (https://phaidra.univie.ac.at/o:1126739)

Competing interests

We declare we have no competing interests.

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GENERAL DISCUSSION

The main goal of the studies presented in this dissertation was to quantify variation in male courtship parameters and their effects on female responses, with the hypothesis that different female responses can reveal different signal functions. I first performed an extensive review of studies on multimodal courtship displays in order to highlight gaps in the literature and identify new research directions (Chapter 1). Then, I used two complementary approaches and assessed both the correlation (Chapter 2) and the causation (Chapter 3) between a number of variables describing both visual and vocal components of male courtship and a number of female behavioural and physiological responses.

Signal evolution theory states that signals are beneficial for the sender in terms of fitness. In the context of sexual selection, the aim of signals sent during courtship should ultimately be to increase the reproductive success of the individual performing the display. In many studies investigating the evolution of sexual signals, female choice or female preference are used as measures of male reproductive success or courtship efficiency. In this thesis, however, I did not study female choice or female preference but I focused on a wide array of female responses, both behavioural and physiological. This approach cannot be used to study mate choice directly, as it has the risk of using potentially misleading proxies for female preference. However, it also has the advantage of giving the possibility to investigate potential signal functions that would remain undetected otherwise.

The ring dove is a monogamous species, and once a pair is formed, the two individuals can undergo several reproduction episodes together over many years. However, both males and females continue to perform courtship prior to each reproduction episode. This case is not isolated, as mutual courtship is often observed in monogamous species with strong durable pair bonds (Wachtmeister, 2001; Jones & Hunter, 1993; Nolan et al., 2010). This suggests that the role of courtship in socially monogamous species is not limited to favor female choice and female preference. Specifically in the ring dove, additional commonly invoked mechanisms include the reinforcement of pair bonds or the physiological stimulation of the female prior to copulation (Wachtmeister, 2001). In this thesis, by adopting a comprehensive investigation of the effect of different males' courtship parameters, we could shed light on different roles of male ring dove courtship display.

First, we could demonstrate that quantitative variation of different male courtship parameters affected distinct female behaviours. We showed that specific call frequencies and courtship durations influenced female immediate sexual behaviour (i.e. tail quivers, Chapter 2), while another courtship parameter, audio-visual synchronization, seemed to be important in increasing female sexual stimulation over several days (i.e. chasing behaviour, Chapter 3). Call frequency and courtship audio-visual configuration therefore probably have evolved to carry different messages as they trigger different types of female responses, both immediate and on a longer term. Still regarding long-term effects of courtship, I confirmed results from previous studies (Friedman, 1977; Korenbrot et al., 1974; Cheng, 1974) showing that female circulating estradiol concentration was positively impacted by repeated exposure to courtship (Chapter 3). Maybe more importantly, I also demonstrated that the extent of temporal association between auditory and visual signals has an effect on female physiological response. These findings support the fact that transmitting several pieces of information is at least one of the driving forces behind the evolution of the ring dove's courtship display. Finally, despite the challenge of creating natural looking audio-visual stimuli, I also documented that artificial audio-visual stimuli efficiently triggered hormonal and behavioural changes in female ring doves.

The finding that courtship triggers both immediate and delayed responses in females is not surprising. The female role in courtship has long been considered to be mostly passive and a tremendous male bias exists in the literature regarding sexual signaling (Staub et al. 2020). However, evidence is accumulating that female behaviour during interactions can greatly influence male courtship. For instance, male mice (*Mus musculus*) adjust their courtship in response to female behaviour (Ronald et al., 2020), by varying parameters of ultrasonic courtship calls depending on female presence and females' estrous phase (Hanson & Hurley, 2012). In ring doves, females possess a large behavioural repertoire in response to courtship, and it is likely that males use information contained in female immediate response. In particular, females displayed tail quivers exclusively in front of a male, and to a much higher extent when males courted. We showed that tail quivers were linked to lower call frequency, a possible indication of male quality (Chapter 2). It is therefore possible that females would signal their interest in a male through this behaviour. Female interest for male courtship, for example in the form of copulation solicitation displays, is observed in many bird species (Nagle et al., 1993; Fernández & Mermoz, 2003; Anderson, 2009; Amy et al., 2015). However, to date we do not know if the display of tail quivers by females reciprocally influences male behaviour. In contrast, we also showed that other behaviours (i.e. chasing or preening) were not displayed as an immediate response to courtship, but increased progressively over several days while the courtship presented to females remained the same. Those behaviours therefore are more likely to be linked to the internal state of the female, reflecting their increasing internal excitation state due to repeated exposures to courtship. This was confirmed by the hormonal results, as female plasma E2 concentration, linked to female sexual stimulation state, increased over the course of the experiment as well.

Taken together, those results suggest that specific variation of courtship parameters and the temporal structure of the display have different effects on female response, and therefore are likely to have different functions. Our results are also consistent with the concept that multimodal signals are "more than the sum of their parts" and that multimodal courtship probably has an added value compared to unimodal signals. Indeed, the configuration of the signal itself seem to have a role in female stimulation on the long term, with naturally synchronized stimuli triggering more chasing behaviour in females and higher plasma E2 concentration. If call frequency seems to influence female interest and maybe female choice, courtship audio-visual synchronization seems to affect female general sexual stimulation or arousal, necessary prior to mating. Those results have important implications regarding the need to adopt an integrative view when investigating multimodal signals. Indeed, without specifically investigating courtship audio-visual synchronization, the increased female sexual stimulation we documented could have been attributed to variation in the visual or the acoustic courtship, or their very presence.

Limitations of the studies and suggestions for further research

Despite these promising results, several questions still lack a definitive answer. First, whether the function of carrying multiple messages is the only selective pressure behind the evolution of the ring dove's courtship display remains unknown. It is possible that some of the courtship parameters have redundant functions. I postulated that the temporal synchronization between acoustic and visual displays could reflect neuromuscular coordination. However, I cannot exclude that some parameters of the call redundantly carry the same information. In Bengalese finches (*Lonchura striata*), females prefer song characteristics that are more challenging from a neuromuscular point of view (Dunning et al., 2020). One way of answering these questions would be to investigate female behaviour in response to unimodal and multimodal playback stimuli. Various playback experiments showed an increased response to multimodal compared to unimodal playback in hens (*Gallus gallus*) (Evans & Marler, 1991), black capped chickadees (*Poecile atricapillus*) (Baker et al., 1996), pigeons (*Columbia livia*) (Partan et al., 2005) and in wolf spiders (*Schizocosa ocreata*) (Kozak & Uetz, 2019). Given the very robust female response to the playback experiment I report here, this potential redundancy between acoustic and visual component could be investigated by comparing females' response to unimodal and multimodal stimuli. The physiological response of female doves is higher when exposed to the full multimodal display than when exposed to the acoustic channel of the courtship alone (Friedmann, 1977). However, we do not know if there are similar differences regarding effects on female behaviour, or to what extent females respond to the visual courtship alone. It is very unlikely that the acoustic part of the courtship alone triggers substantial physiological changes in females as this could be highly maladaptive (Cheng, 2008).

Indeed, this would mean that females would be stimulated by male coos directed to others females, and undergo costly anatomical changes leading to ovulation, which would be useless without a partner. In Chapter 2, I showed that female behaviour was not impacted by variation in courtship parameters that I investigated for the visual signal, but only by acoustic and structural components. However, it is possible that the presence of visual courtship itself modulates the female response through mechanisms related to the receiver psychology hypothesis or the back-up signal hypothesis. In domesticated ring doves, courtship mostly occurs in short range and during one-to-one interactions. The role of visual display is unlikely to be that of improving signal transmission in the environment but movements could serve to attract female attention or enhance the call properties for instance.

When it comes to investigating the effects of artificial stimuli on female response, playback methods are not the only available tools. Actually, several studies pointed out issues linked to perception of artificial visual signals displayed on a monitor (Rosenthal, 1999; Cuthill et al., 2000; Oliveira et al., 2000), making the use of audio-visual playback problematic in some cases. Video playbacks further have the disadvantage of being difficult to use outside a laboratory setup, which is important as some species do not necessary display natural behaviour under artificial conditions or cannot be kept in captivity at all. One alternative technique that has been used successfully in the last years, especially in the context of studying multisensory displays, is the use of robotic animals. In the field, receiver responses to signals coming from robotic models have been investigated successfully in several frog species (Narins et al., 2003; Taylor et al. 2008), satin bowerbird (*Ptilonorhynchus violaceus*) (Patricelli et al., 2002, Patricelli et al., 2006), eastern grey squirrels (*Sciurus carolinensis*) (Partan et al., 2009) and several lizard species (Martins et al., 2005; Ruiz et al. 2010). This technique could also be considered in captive conditions in doves to increase the quality of the stimulus by presenting a 3D model which cannot be effectively reproduced by a screen, and tackle limitations linked to display rate or image polarization for instance. The use of robots could also be an efficient technique to start investigating multimodal signals composed of more than two sensory modalities by creating more natural looking stimuli. For instance, in ring doves, tactile signals have importance in the courtship context, as mutual preening occurs in the later phase of the interaction (Miller & Miller, 1958). One could imagine studying tactile signals using these types of techniques.

Courtship as a male unilateral communicative behaviour is a very simplified view that has been challenged over the past years (Staub et al., 2020). Indeed, females often have a very active role, and courtship displays are in fact typically interactive. Male courtship is often influenced by female behaviour, sexual receptivity and physiological state (Kelso & Verrell, 2002; Parker & Mason, 2012). A few studies have investigated the interactive nature of sexual interaction using highly challenging experimental designs. For example, Ware et al. (2017) used a closed-loop teleprompter system to present an audio-visual courtship playback to pigeons and showed that they behaved interactively. In field conditions, Patricelli et al., (2006) used robotic females to investigate male courtship adjustment to female response and showed that males adapted their courtship intensity when females were unreceptive. Even though the exact function of female signaling in the ring dove sexual interaction remains unknown, the studies presented in this thesis showed that female signals involved in courtship interaction are worth considering. Indeed, very distinct behaviours were displayed in response to distinct courtship signals, suggesting they are involved in the courtship interaction. One way of investigating these questions would be to perform analysis on a finer time scale, such a sequence analyses, to accurately associate female behavioural response to specific male behaviour. Mutual courtship has been shown to be important for the success of the reproductive episode in Java sparrows (*Lonchura oryzivora*), where Soma & Iwama (2017) observed a higher mating success when both females and males perform dances.

Sometimes, signals produced by females during courtship interactions are even multimodal. For instance in mice, males adapt their courtship to females, and females send multimodal signals in response to courtship (Ronald et al., 2020). Female multimodal behaviour has also been studied in canaries (Amy et al., 2015) and salamanders (Staub et al., 2020). In blue-capped cordon-bleu, multimodal courtship occurs in both sexes, and is promoted by the presence of an audience in both sexes while unimodal display are suppressed by the audience, meaning that multimodal mutual courtship is probably used to signal the mating status to other cordon bleus (Ota et al., 2015). In the same study, however, the authors did not find assortative mating based on courtship parameters. This is also an aspect that could be studied by broadening the view we have on courtship interaction as assortative mating occurs often in relation to physical characteristics, for example with males courting females of their own size (Shine et al. 2003, Shine et al. 2001). In order to fully understand the function of multimodality in the courtship context, we need to extend our knowledge of signals sent by both parties of the interaction, and reshape our view that females are purely acting as receivers, as they are also an active agent in the sexual interaction.

Not only we do need to extend our studies to both partners of the courtship interaction, we also need to broaden the scale of study, in term of which phase of the courtship is studied. As mentioned in the introduction, courtship in the ring dove is a dynamic phenomenon that develops over the course of several days. In my thesis, I exclusively investigated the function of bow-call display parameters. However, the later stages of courtship are highly likely to have different functions, as the behaviour displayed by both males and females is different. Investigation of the whole courtship interaction, from the first to the last stage directly preceding copulation is important in order to understand all of the forces at play in the evolution of the ring dove mating signals.

Finally, even though in both experiments presented in this thesis I mostly analyzed behavioural responses averaged across repeated interactions or groups, I showed that a consistent inter-individual variability exists in both male courtship parameters and female responses to them. Investigating individual preferences to courtship is particularly relevant in monogamous species like the ring dove, as often individuals show strong preferences that persist over several years after the first reproduction episode (Morris & Erickson, 1971). This was not possible in our case as the previous mating history of individuals used in the experiments were unknown. However, this is an aspect that deserves more attention in future studies, as female inter-individual mating preferences have been proposed as one explanation for the evolution of multimodal signals (Mitoyen et al., 2019, Ronald et al., 2018).

Concluding remarks

The evolution of communication signals depends on a multitude of factors we are just starting to understand. In this thesis, I performed one of the first comprehensive studies of a multimodal courtship display by investigating variation and differential effects of component signals and their configuration on receiver response. I showed that by precisely quantifying male multimodal displays and performing an integrated analysis of both male courtship and female response we can reduce the risk of overlooking possible functions and potential selective pressures participating in building complex communication signals. Additionally, in spite of having ignored colours or polarization information in this work, and despite the lack of interactivity, I validated the use of audio-visual playback in the ring dove to study both female physiological and behavioural responses. I showed that this technique can be used efficiently to study multimodal communication in this species, opening the door to further exciting investigations of the functions of courtship components and courtship configuration.

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APPENDIX

A. Supplementary material and appendix from Chapter 2

Supplementary material

Video S1. Example of a male audio-visual courtship behaviour recorded in the experimental setup.

Video S2. Result of an automatic annotation of the male visual courtship. Additionally to the eyes, the beak tip and the two feet were tracked.

Appendix

Table A1. Summary statistics of male courtship variables of interest.

Courtship parameters	***N***	**Mean**	**SD**	**Min**	**Max**	**Median**
Call: temporal structure						
Courtship duration (s)	253	79.13	52.98	5.52	288.95	66.82
Courtship bout duration (s)	253	38.60	29.40	5.52	288.95	31.25
Number of coos	253	35	24	3	136	30
Number of bouts	253	3	2	1	14	2
Coo rate (coo per second)	253	0.46	0.03	0.38	0.54	0.46
e1 duration (s)	253	0.25	0.03	0.18	0.34	0.24
e2 duration (s)	253	1.16	0.10	0.83	1.51	1.14
Intra-coo duration (s)	253	0.12	0.07	0.00	0.33	0.11
Inter-coo duration (s)	253	0.72	0.13	0.37	1.39	0.72
Call: spectral structure						
e1 maximum f0	253	640.15	35.50	559.47	717.05	635.99
e1 median f0	253	549.29	29.64	472.79	618.22	549.88
e1 minimum f0	253	458.42	32.06	372.23	523.23	454.71
e2 maximum f0	253	597.85	36.03	532.53	681.89	584.51
e2 median f0	253	468.17	45.76	380.89	580.94	464.63
e2 minimum f0	253	338.48	67.52	184.56	483.86	335.51
Bow						
Bowing amplitude (in pixel)	205	200.72	49.02	80.79	294.29	210.33
Up time (s)	204	0.566	0.149	0.167	1.054	0.545
Down time (s)	204	0.694	0.167	0.229	1.176	0.695
Ascent time (s)	204	0.404	0.067	0.229	0.584	0.405
Descent time (s)	204	0.460	0.079	0.254	0.757	0.449
Audio-visual synchronization (s)						
Onset of e1 - Onset of descent movement	174	0.144	0.204.76	-0.404	0.785	0.183
Offset of e1 - Offset of descent movement	174	-0.070	0.223.97	-0.540	0.953	-0.011
Onset of e2 - Onset of ascent movement	174	-0.610	0.257.71	-0.998	0.907	-0.615
Offset of e2 - Offset of ascent movement	174	0.027	0.269.33	-0.683	0.930	0.051

Table A2. ANOVA for every courtship variable testing differences between males.

	Male					Error		
Variable	**Sum sq**	**Df**	**Mean Sq**	**F value**	***P***	**Sum sq**	**Df**	**Mean Sq**
CD total duration	237559.8	9	26395.54	13.68973	<0.001	468534.	243	1928.12
Calls number	10786.39	9	1198.488	8.423826	<0.001	34572.4	243	142.273
Bouts number	164.2352	9	18.24835	5.298321	<0.001	836.934	243	3.44417
Call rate	0.16424	9	0.018249	24.35559	<0.001	0.18207	243	0.00074
e1 duration	0.242555	9	0.02695	185.848	<0.001	0.03523	243	0.00014
e1 duration	1.46	9	0.16222	37.3515	<0.001	1.05537	243	0.00434
intra-call duration	0.991311	9	0.11014	174.711	<0.001	0.15319	243	0.00063
inter-call duration	2.361099	9	0.26234	30.4728	<0.001	2.09201	243	0.00860
e1 max f0	286971.5	9	31885.7	272.380	<0.001	28446.3	243	117.063
e1 min f0	206139	9	22904.3	96.4782	<0.001	57689.2	243	237.404
e1 median f0	196664.8	9	21851.6	207.563	<0.001	25582.3	243	105.277
e2 max f0	294478.7	9	32719.8	247.886	<0.001	32074.9	243	131.995
e2 min f0	885575.3	9	98397.2	93.0224	<0.001	257040.	243	1057.78
e2 median f0	450261.3	9	50029.0	160.631	<0.001	75682.8	243	311.452
Bowing amplitude	240737.1	9	26748.5	20.9103	<0.001	249444.	195	1279.20
Up duration	2.334278	9	0.25936	22.8204	<0.001	2.20489	194	0.01136
Descent duration	0.195819	9	0.02175	3.91015	<0.001	1.07949	194	0.00556
Down duration	3.13594	9	0.34843	28.9389	<0.001	2.33584	194	0.01204
Ascent duration	0.43572	9	0.04841	23.8108	<0.001	0.39444	194	0.00203
Sync 1	1.275967	9	0.14177	3.88949	<0.001	5.97789	164	0.03645
Sync 2	1.012865	9	0.11254	2.40770	<0.001	7.66567	164	0.04674
Sync 3	3.536867	9	0.39298	8.10390	<0.001	7.95290	164	0.04849
Sync 4	4.705035	9	0.52278	12.084	<0.001	7.09519	164	0.04326

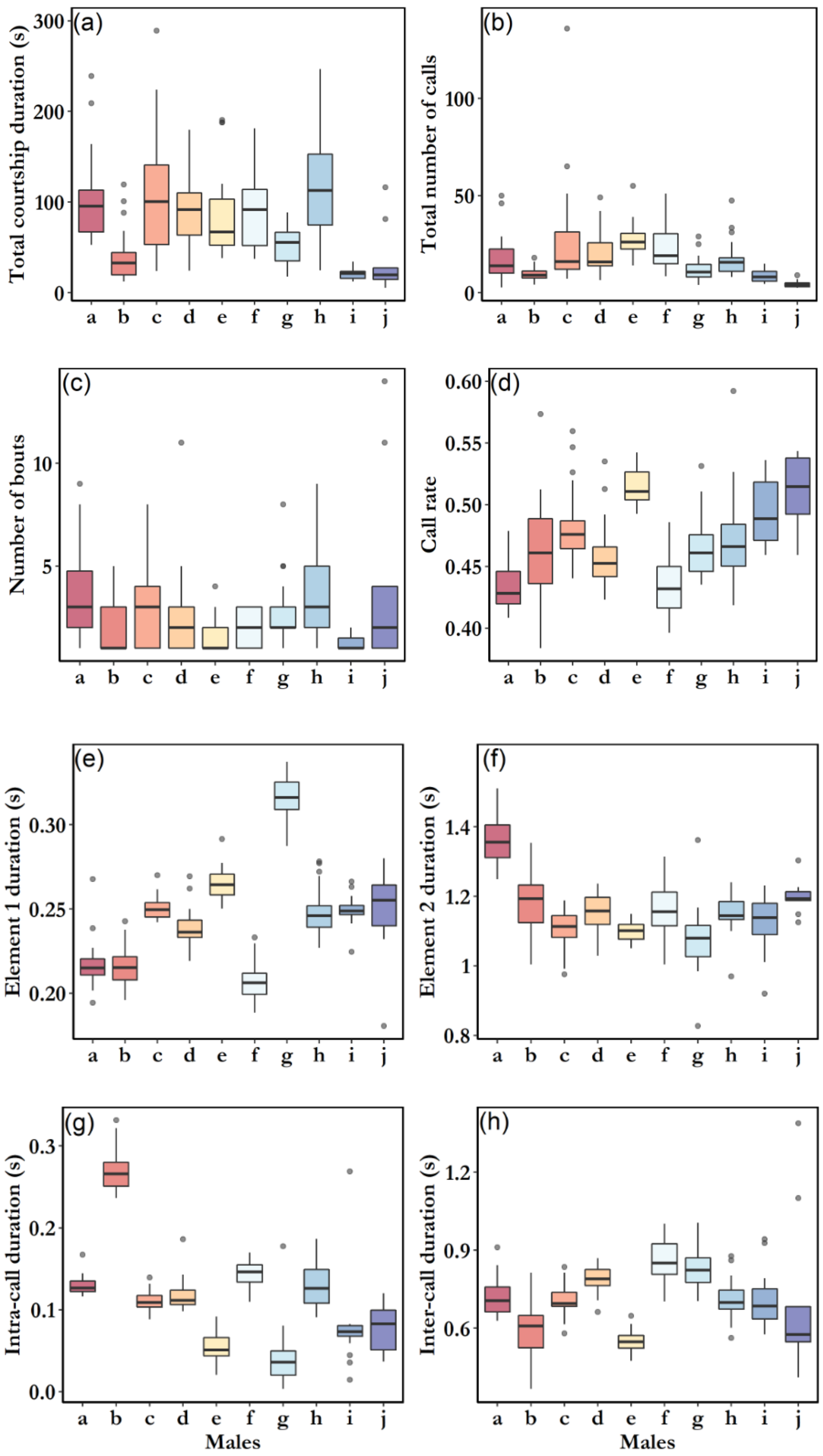

Figure A1. Box-plots representing inter and intra-male variability for courtship structure variables: (a) total courtship duration, (b) total number of calls, (c) number of courtship bouts, (d) call rate, (e) call element 1 duration, (f) call element 2 duration, (g) intra-call duration, (h) inter-call duration. Boxplots' horizontal lines display the median, lower and upper quartiles. Whiskers represent values within 1.5 times the interquartile range and dots are outliers.

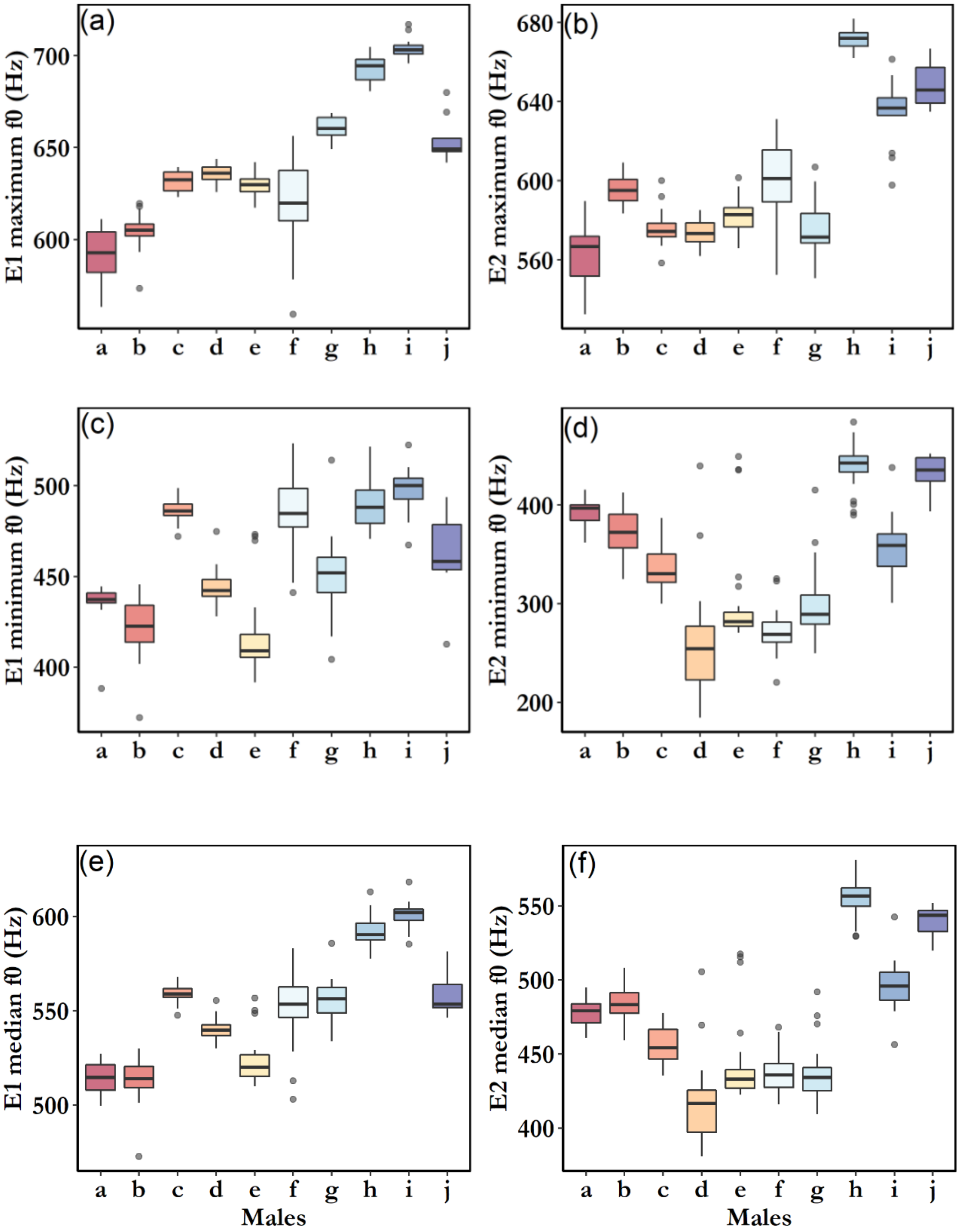

Figure A2. Box-plots representing inter and intra-male variability for spectral acoustic courtship variables: (a) element 1 maximum fundamental frequency, (b) element 2 maximum fundamental frequency, (c) element 1 minimum fundamental frequency, (d) element 2 minimum fundamental frequency, (e) element 1 median fundamental frequency, (f) element 2 median fundamental frequency. Boxplots' horizontal lines display the median, lower and upper quartiles. Whiskers represent values within 1.5 times the interquartile range and dots are outliers.

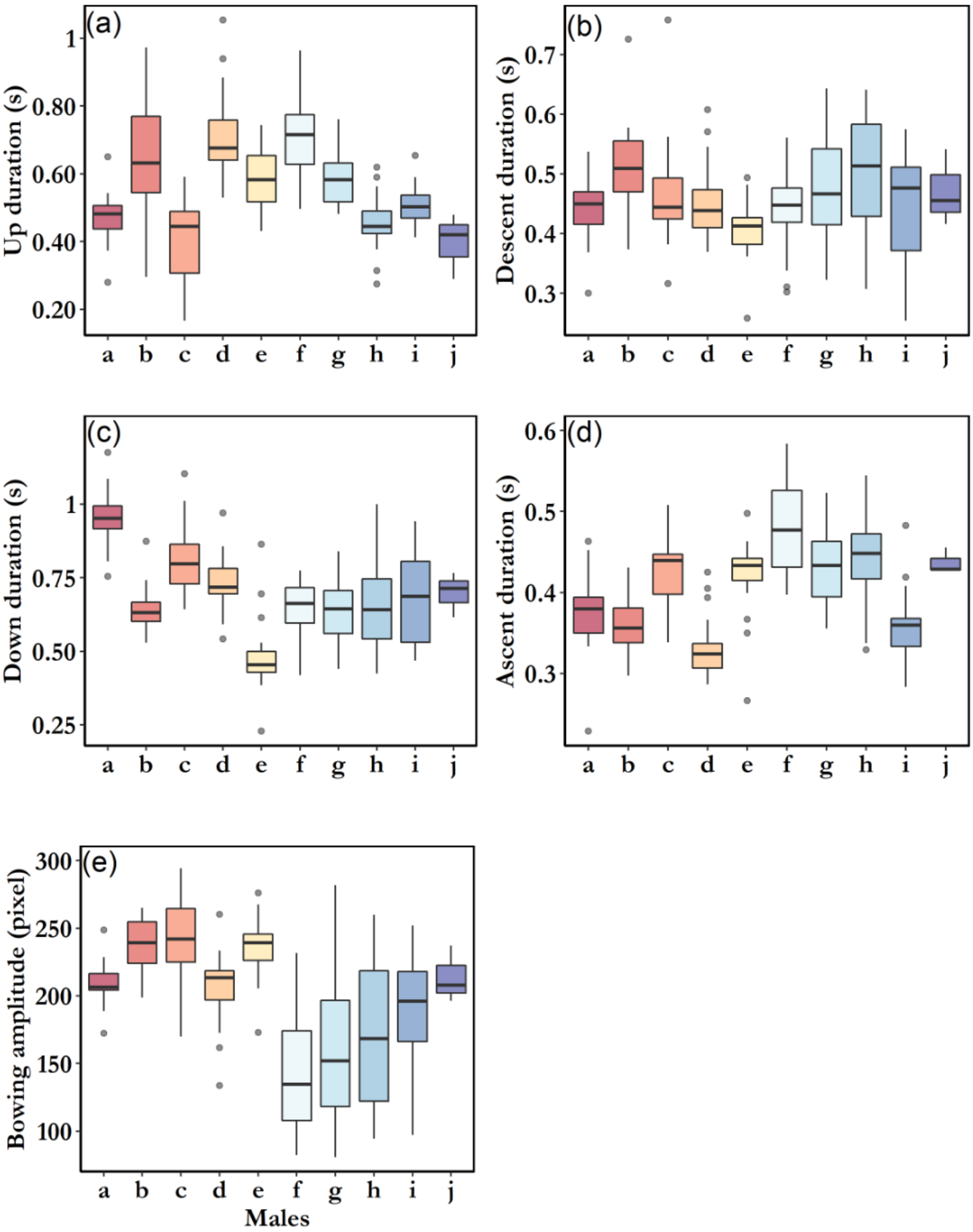

Figure A3. Box-plots representing inter and intra-male variability for visual courtship variables: (a) bowing up duration, (b) bowing descent duration, (c) bowing down duration, (d) bowing ascent duration, (e) bowing amplitude. Boxplots' horizontal lines display the median, lower and upper quartiles. Whiskers represent values within 1.5 times the interquartile range and dots are outliers.

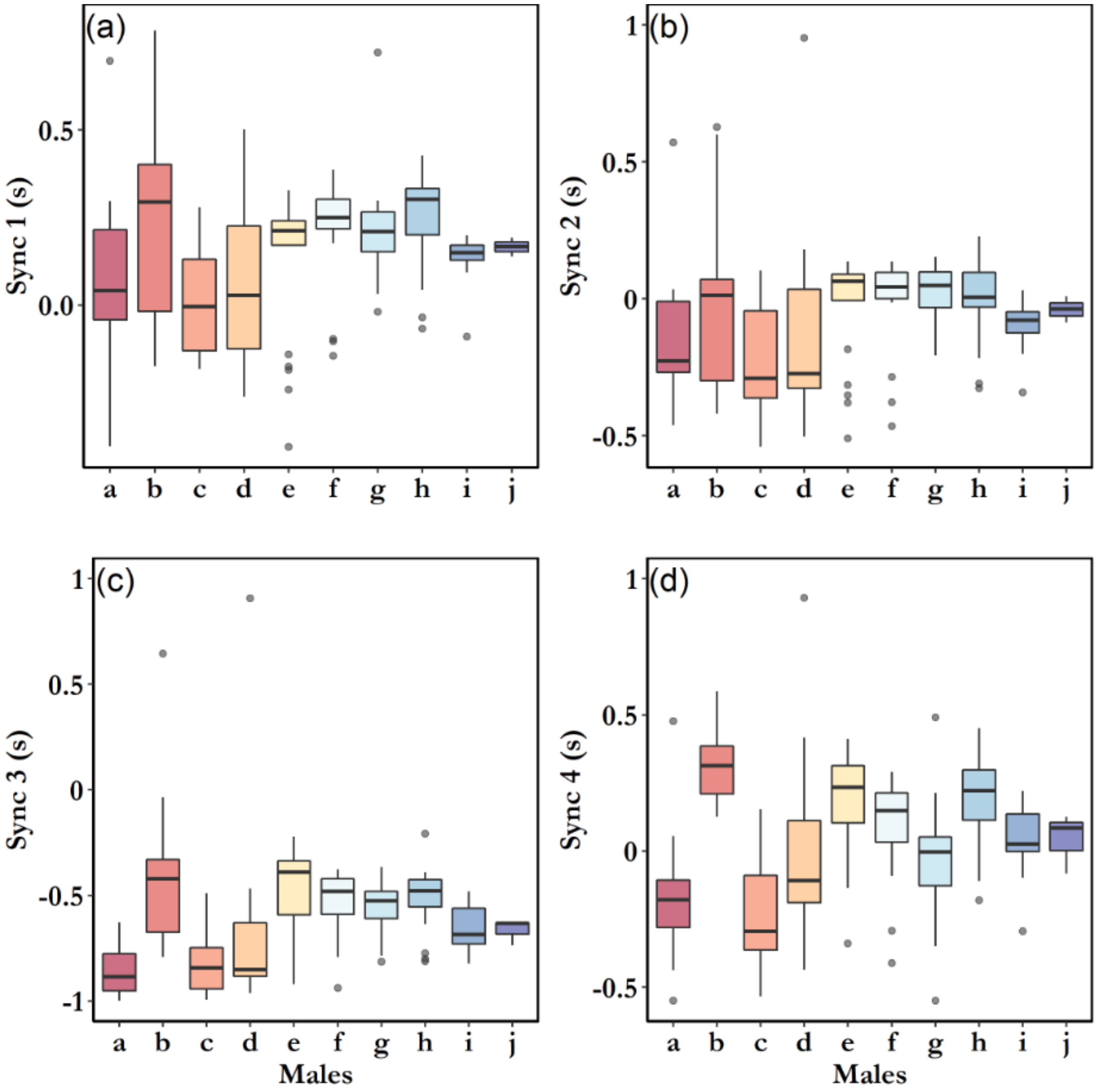

Figure A4. Box-plots representing inter and intra-male variability for courtship audio-visual synchronization variables: (a) difference between onset of e1 and onset of descent movement, (b) difference between offset of e1 and offset of descent movement, (c) difference between onset of e2 and onset of ascent movement, (d) difference between offset of e2 and offset of ascent movement. Boxplots' horizontal lines display the median, lower and upper quartiles. Whiskers represent values within 1.5 times the interquartile range and dots are outliers.

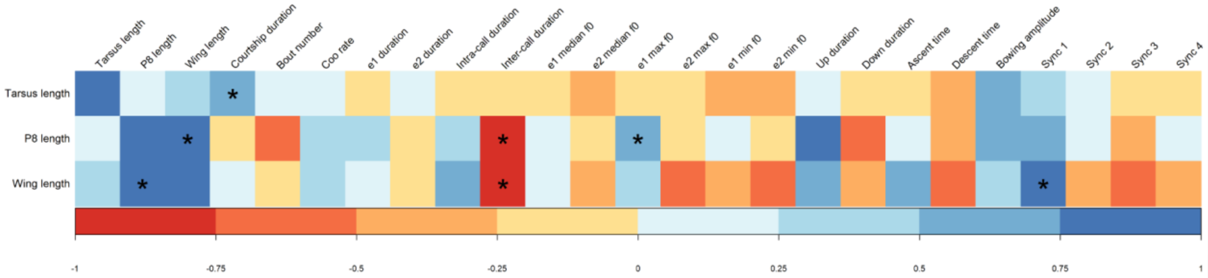

Figure A5. Figure A5. Correlation matrix between body size measurements from 9 of the males, and their courtship audio-visual parameters. Stars indicate a significant correlation between two variables (Spearman correlation test, P < 0.05).

B. Supplementary material from Chapter 3

This document contains:

- Methods supplementary information (I)
- Result supplementary information (II)
- Figure s1, s2, s3
- Table s4-s11
- Legend of the supplementary videos s12, s13, s14 (III)
- Supplementary information references (IV)

I - Methods additional information

A) Experimental apparatus and playback display

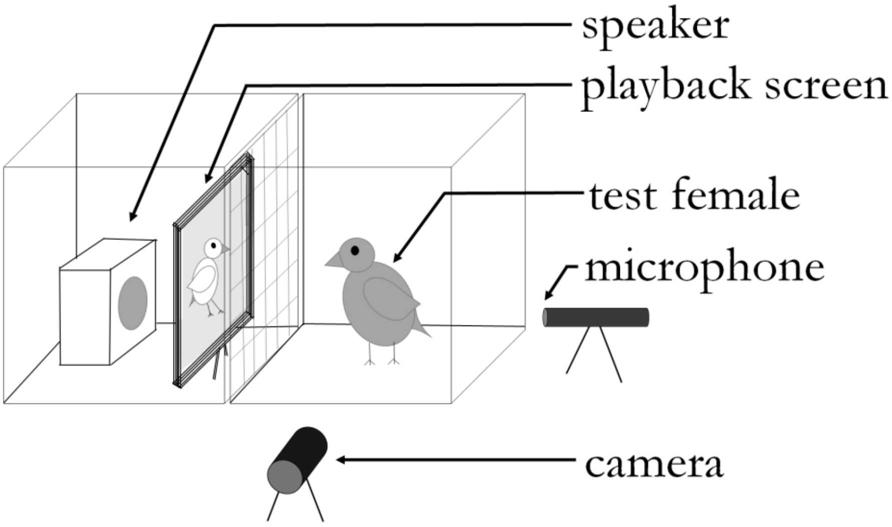

Figure s1. Experimental setup used to present audio-visual playback stimuli to female ring doves

List and technical specification of experimental setup, experimental devices and software

- Playback screen (ASUS VG248QE, refresh rate 120 Hz)
- Speaker (Fostex FE108ez).
- Camera (Basler acA1920-155uc, recording at 60fps second)
- Microphone (Sennheiser ME66 K6, directional head with battery powered K66 module)

Display of playback was controlled by custom written software using Psychophysics Toolbox [1–3], running on Octave and Ubuntu (v.18.04 LTS), run on a standard gaming computer (graphics card: AMD Radeon Pro WX 4100, 4GB GDDR5). The same computer was used to control video

(Motif Video Recording System, loopbio gmbh, Vienna, Austria) and acoustic (Audacity, [4]) recording. We used acoustic foam on the walls and fabric curtains around the experimental setup for optimal acoustic recordings. We lit the testing compartment with LED lights above the compartment and behind the camera to limit shadow, both powered by DC power supplies to eliminate flicker.

Playback videos were displayed on a portion of the screen, and the rest of the monitor and its frame was hidden using a black cardboard aperture. The screen was located approximately 22 cm from the net between the testing compartments, with the distance chosen to allow the male to look of natural size when a female was viewing from a position close to the net.

To allow for smooth playback, audio files were pre-loaded into audio buffers before each experiment run. Videos were pre-loaded and were displayed frame-by-frame, taking advantage of Psychophysics Toolbox precise timing of graphics card operations. During the first frame of a playback video, the corresponding audio playback was scheduled and started playing. To ensure that audio and video playback began synchronously, we used a self-made hardware device to objectively measure visual and auditory onsets (based on the StimSync device described in [5]) of simple stimuli presented using the same method that was later used for the playback stimuli. Audio stimulus onset was one frame before video, and we then compensated for this in the playback script used for the experiment to remove any delay between modalities.

Recording room temperature ranged between 25°C and 28°C.

The stimulus recording setup was the same as for testing except that a female was placed behind the camera in a small cage to elicit male courtship and record it from the female's perspective. We recorded male courtship video at 120 frames per second and audio at 48kHz. We attempted to record courtship from each of the test males twice a day, and obtained courtship recordings of sufficient quality from two males. During recordings, we measured sound amplitude using a sound pressure level meter (Voltcraft 329, Conrad Electronic SE) to calibrate volume of acoustic playback during later testing.

B) Stimulus manipulation

Each bow-call was extracted from the source track using the annotations mentioned in the main article, plus a buffer of 10 ms before and 25 ms after the annotated times. A 15 ms sine ramp was applied to the end of each audio extract to avoid audible offset artifacts. In order to decide which values to use for stimuli modifications, we estimated the natural variation found in our stimulus males by quantifying the temporal offset between start of bows and start of bow-calls from all courtship recordings we obtained from the two stimulus males (i.e. more than the final stimulus material used). We excluded outliers (< -0.8s and > 0.2s) based on histograms of both birds' data from the natural variation in . Male A had an offset of -0.2753 s (M ± 0.1393 s SD, *n* =133) between bow and bow-call of his display, whereas male B had a mean offset of -0.3620 s (± 0.1274 s, *n* =109).

To create the final stimuli, we selected four courtship bouts from each male and a recording of the empty setup to serve as control. Duration of courtship bouts ranged between 30 and 31 seconds i.e. 13 - 14 bow-call displays. Each session contained courtship video separated by empty control videos alternating short (2-4 s, each duration selected from a uniform distribution) and long (57 s) control bouts. The courtship videos were presented in random order to females, resulting in 15 min sessions containing 8 min of active bow-call courtship display. The order of presentation of courtship videos was randomized, with the constraint that the same video could not appear twice in a row.

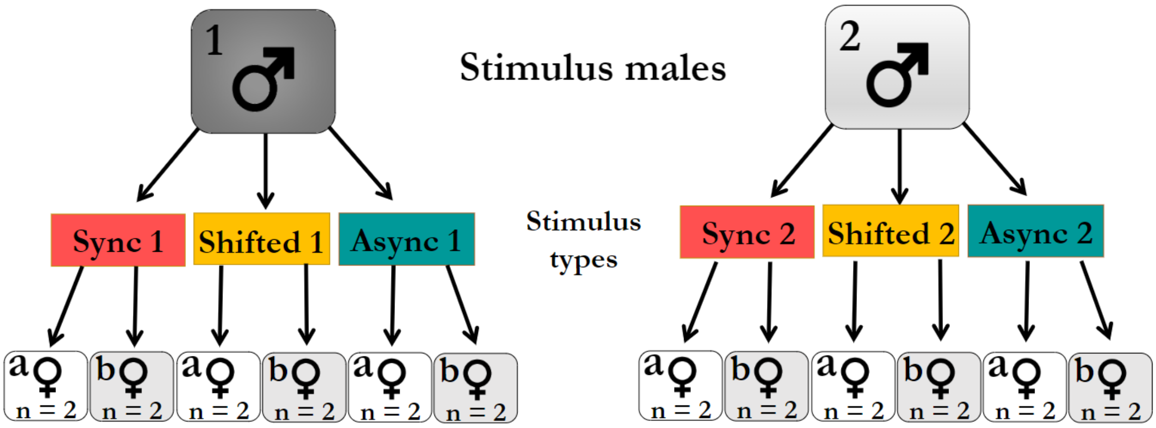

Figure s2. Experimental design. We used courtship from each stimulus male (1 and 2) to create the stimuli. For each male (male 1 and male 2), we recorded and extracted four courtship bouts of 30 seconds. We then used those bouts to create three different stimulus types as in Figure 1 of the main text. A synchronous, a shifted and an asynchronous stimulus were created for each male, resulting in 6 unique stimulus types, each containing 4 videos. We created two female experimental groups (a and b), each containing 12 females. Females from group a were tested in the 2nd week of August 2020 and females from group b in the 3rd week of the same month. We randomly assigned 2 females from each group to each of the 6 available stimuli. We ran all playback sessions between 8:00 and 13:00. Every day, we pseudo-randomized the order of testing so that every female was tested in every possible time slot during the experiments.

C) Hormone assays

We obtained a satisfying parallelism by extracting the samples as following before performing the ELISA assay:
Plasma (50-100ul) was brought up to a total volume of 400ul with ddH2O, vortexed and then loaded on an ISOLUTE® SLE+ 400 uL column (Biotage Sweden AB). Extraction was performed following manufacturer's instructions. After initial pressure samples were soaked for 5min. Then columns were loaded three times with 500 ul Ethyleneacetate and let it flow under gravity for 5 min. Ethylenacetate gave us the best results in recovery (98%). Elution was collected in a glass tube, dried down under a N2 stream at 40°C and resuspended with 60 ul Standard 0 (provided by IBL). After two hours on a shaker at RT, samples were immediately analyzed in duplicates on two plates. The sensitivity of the assays were 3.6 pg/mL and 5.81 pg/ml, respectively. Intra assay CV% of duplicates was below 5% (mean 0.9%). Interassay CV% of assay control and of an extracted plasma control pool was 4.74% and 0.61%, respectively. Data were corrected for dilutions.

D) Statistical analysis

We used the glmmTMB function, from the glmmTMB packages [6] to run generalized linear models testing for the effect of playback status, and stimulus type on female behavioural response. We used the lmer function from the lme4 packages [7] to fit linear mixed models investigating the effect of stimulus type on E2 plasma concentration. We used the performance package [8] to check model assumptions, and the emmeans from the emmeans packages [9] and the cld function from the multcomp package [10] to compute estimated marginal means and perform post-hoc tests. We extracted marginal and conditional R² using the tab_model function from the package sjPlot [11]. To extract p values and Analysis of Deviance Table for fixed factors from glmmTMB models, we used Anova.glmmTMB from the glmmTMB package [6]. We used the anova function from the stats package [12] and the lmerTest package [13] to extract analysis of deviance table from lmer models. We used tab_model function from the sjPlot package [11] to obtain marginal and condition R².

II- Results additional information

A) Female response to audio-visual playback

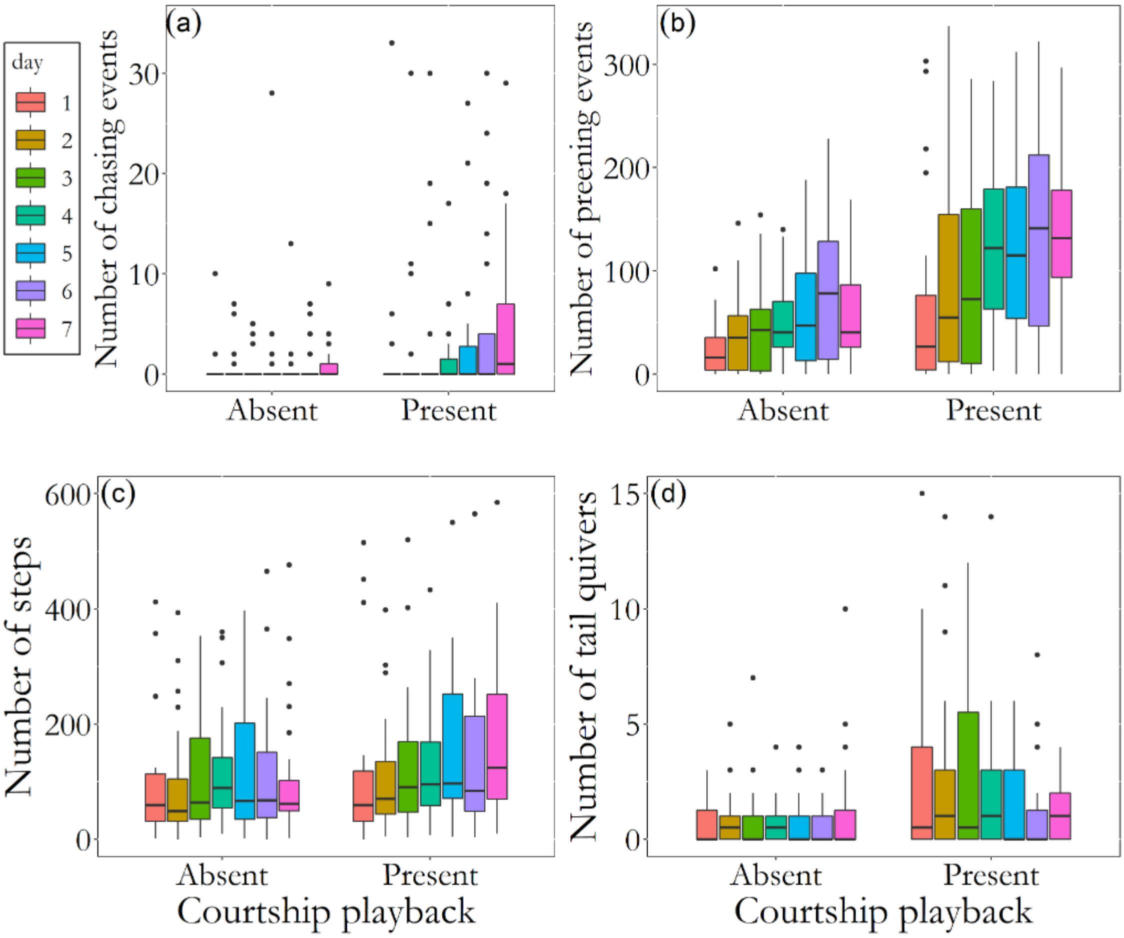

Figure s3. Boxplots of behavioural occurrences displayed by females, grouped by playback content and day of experiment. Playing back courtship stimuli resulted in more behavioural events, and the response increased across the experimental period.

Table s4. Results of generalized linear models (with estimates, standard error (SE), z and p-values) investigating the effect of playback content (PB), as well as analyses of deviance table (with Chi-square value, degrees of freedom). Results are presented for all the investigated female number of behavioural events: (a) Chasing, (b) Preening, (c) Steps, (d) Tail quivers.

(a) Chasing

	Estimate	SE	z	p-value
(Intercept)	-4.8703	1.4569	-3.343	0.000829
PB_courtship	1.4258	0.1662	8.577	<0.001
day2	0.5021	0.5655	0.888	0.374567
day3	0.9131	0.5539	1.649	0.099236
day4	1.0162	0.5575	1.823	0.068338
day5	1.2474	0.5518	2.261	0.023787
day6	1.5614	0.5466	2.857	0.004283
day7	2.1605	0.5902	3.661	0.000251
group_b	0.1595	1.2348	0.129	0.897242
male_2	-1.3621	1.2392	-1.099	0.271677
origin_France	2.0321	1.2675	1.603	0.108896

Marginal $R^2 = 0.208$
Conditional $R^2 = 0.788$

Analysis of deviance table

	Chisq	Df	p-value
PB	73.5667	1	< 0.001
day	17.1165	6	0.008864
group	0.0167	1	0.897242
male	1.2083	1	0.271677
origin	2.5702	1	0.108896

(b) Preening

	Estimate	SE	z	p-value
(Intercept)	2.62144	0.37518	6.987	< 0.001
PB_courtship	0.76407	0.06112	12.501	< 0.001
day2	0.37907	0.16688	2.272	0.02311
day3	0.46759	0.1651	2.832	0.00462
day4	0.82805	0.1617	5.121	< 0.001
day5	0.86383	0.16026	5.39	< 0.001
day6	0.98206	0.1584	6.2	< 0.001
day7	0.92366	0.1616	5.716	< 0.001
group_b	0.56696	0.33493	1.693	0.0905
male_2	-0.2625	0.33912	-0.774	0.43891
origin_France	0.3525	0.33948	1.038	0.2991

Marginal $R^2 = 0.273$
Conditional $R^2 = 0.784$

Analysis of deviance table

	Chisq	Df	p-value
PB	156.286	1	< 0.001
day	60.5574	6	< 0.001
group	2.8655	1	0.0905
male	0.5991	1	0.4389
origin	1.0782	1	0.2991

(c) Steps

	Estimate	SE	z	p-value
(Intercept)	3.84386	0.33866	11.35	< 0.001
PB_courtship	0.2116	0.0325	6.512	< 0.001
day2	0.04912	0.13906	0.353	0.7239
day3	0.20772	0.13775	1.508	0.13156
day4	0.3987	0.1367	2.917	0.00354
day5	0.40875	0.13632	2.999	0.00271
day6	0.29235	0.13714	2.132	0.03302
day7	0.40211	0.13657	2.944	0.00324
group_b	0.27302	0.30975	0.881	0.37808
male_2	-0.48182	0.314	-1.534	0.12492
origin_France	0.6132	0.31405	1.953	0.05087

Marginal R² = 0.222
Conditional R² = 0.884

Analysis of deviance table

	Chisq	Df	p-value
PB	42.3999	1	< 0.001
day	18.8319	6	0.004457
group	0.7769	1	0.378082
male	2.3545	1	0.124924
origin	3.8125	1	0.05087

(d) Tail quivers

	Estimate	SE	z	p-value
(Intercept)	0.07308	0.3903	0.187	0.8515
PB_courtship	0.78268	0.15283	5.121	< 0.001
day2	0.09482	0.29032	0.327	0.744
day3	0.02056	0.2952	0.07	0.9445
day4	0.01889	0.296	0.064	0.9491
day5	-0.41106	0.31755	-1.294	0.1955
day6	-0.55884	0.32641	-1.712	0.0869
day7	-0.17063	0.30536	-0.559	0.5763
group_b	-0.40636	0.30735	-1.322	0.1861
male_2	-0.0571	0.30914	-0.185	0.8535
origin_France	-0.32544	0.30973	-1.051	0.2934

Marginal R² = 0.130
Conditional R² = 0.426

Analysis of deviance table

	Chisq	Df	p-value
PB	26.2279	1	< 0.001
day	6.9781	6	0.3229
group	1.748	1	0.1861
male	0.0341	1	0.8535
origin	1.104	1	0.2934

Table s5. Total number of behavioural events and mean (SD) per female displayed by females for each of the three stimulus types.

	Synchronized		**Shifted**		**Asynchronous**	
	Total	**Mean (SD)**	**Total**	**Mean (SD)**	**Total**	**Mean (SD)**
Chasing	312	5.57 (11.18)	152	2.71 (6.42)	223	3.98 (9.79)
Preening	10221	182.51 (136.03)	9422	168.25 (116.44)	7908	141.21 (130.80)
Steps number	15705	280.44 (277.07)	11644	207.92 (183.35)	13726	245.10 (210.78)
Tail quivers	135	2.41 (3.76)	150	2.67 (3.70)	124	2.21 (2.95)

Table s6. Results of generalized linear models (with estimates, standard error (SE), z and p-values) investigating the effect of stimulus type (stimulus A: synchronized, B: shifted and C: asynchronous), as well as analyses of deviance table (with Chi-square value, degrees of freedom (Df) and p-values). Results are presented for all the investigated female number of behavioural events: (a) Chasing, (b) Preening, (c) Steps, (d) Tail quivers)

(a) Chasing

	Estimate	SE	z	p-value
(Intercept)	-3.5505	1.7108	-2.075	0.037951
stimulus_B	-0.2226	1.8725	-0.119	0.905358
stimulus_C	2.164	1.6966	1.275	0.202136
day2	1.9116	1.1125	1.718	0.085762
day3	2.8478	1.0466	2.721	0.006508
day4	3.1371	1.0355	3.03	0.002448
day5	2.8731	1.0456	2.748	0.005997
day6	3.3225	1.0317	3.221	0.00128
day7	3.2498	1.0298	3.156	0.001601
group_b	0.1204	1.13	0.107	0.915123
male_2	-1.3297	1.1364	-1.17	0.241956
originFrance	2.0059	1.1466	1.749	0.080235
stimulus_B:day2	-1.3056	1.4601	-0.894	0.371216
stimulus_C:day2	-2.0802	1.2176	-1.708	0.087556
stimulus_B:day3	-2.1209	1.3984	-1.517	0.129369
stimulus_C:day3	-3.043	1.1591	-2.625	0.008658
stimulus_B:day4	-1.7289	1.3296	-1.3	0.193479
stimulus_C:day4	-4.7457	1.2902	-3.678	0.000235
stimulus_B:day5	-1.1021	1.3226	-0.833	0.404677
stimulus_C:day5	-3.3349	1.1717	-2.846	0.004426
stimulus_B:day6	-1.8303	1.3264	-1.38	0.167621
stimulus_C:day6	-3.8118	1.1607	-3.284	0.001023
stimulus_B:day7	-1.7747	1.3193	-1.345	0.178572
stimulus_C:day7	-3.2397	1.1376	-2.848	0.004401

Marginal R^2 = 0.260
Conditional R^2 = 0.777

Analysis of deviance table

	Chisq	Df	p-value
stimulus type	1.7352	2	0.41995
day	7.4854	6	0.27828
group	0.0114	1	0.91512
male	1.3692	1	0.24196
origin	3.0601	1	0.08023
stimulus:day	24.0713	12	0.01989

(b) Preening

	Estimate	SE	z	p-value
(Intercept)	3.7812	0.4572	8.27	< 0.001
stimulus_B	0.451	0.4857	0.929	0.353067
stimulus_C	-0.3334	0.5111	-0.652	0.51426
day2	0.4757	0.3235	1.47	0.141465
day3	0.8109	0.3048	2.66	0.007809
day4	1.1315	0.2921	3.874	0.000107
day5	1.1528	0.2921	3.946	7.94E-05
day6	1.3266	0.2879	4.609	4.05E-06
day7	1.2588	0.2891	4.354	1.34E-05
group_b	0.5364	0.3085	1.739	0.082092
male_2	-0.2591	0.3122	-0.83	0.406585
originFrance	0.3655	0.3127	1.169	0.242477
stimulus_B:day2	-0.4251	0.4329	-0.982	0.326144
stimulus_C:day2	0.1445	0.4566	0.316	0.75168
stimulus_B:day3	-0.5321	0.413	-1.288	0.197639
stimulus_C:day3	-0.3537	0.4501	-0.786	0.432024
stimulus_B:day4	-0.4914	0.392	-1.254	0.209949
stimulus_C:day4	-0.4404	0.4357	-1.011	0.312094
stimulus_B:day5	-0.5013	0.3917	-1.28	0.200617
stimulus_C:day5	-0.3197	0.4327	-0.739	0.460102
stimulus_B:day6	-0.6789	0.3873	-1.753	0.079603
stimulus_C:day6	-0.3587	0.4239	-0.846	0.397498
stimulus_B:day7	-0.7761	0.3932	-1.974	0.048424
stimulus_C:day7	-0.4994	0.4385	-1.139	0.254793

Marginal $R^2 = 0.283$
Conditional $R^2 = 0.735$

Analysis of deviance table

	Chisq	Df	p-value
stimulus type	3.1541	2	0.20658
day	48.8426	6	< 0.001
group	3.023	1	0.08209
male	0.6888	1	0.40659
origin	1.3661	1	0.24248
stimulus:day	7.7741	12	0.80252

(c) Steps

	Estimate	SE	z	p-value
(Intercept)	4.98337	0.37633	13.242	<0.001
stimulus_B	-0.48399	0.41175	-1.175	0.2398
stimulus_C	-0.21718	0.39714	-0.547	0.5845
day2	-0.22805	0.22405	-1.018	0.3087
day3	0.12906	0.20757	0.622	0.5341
day4	0.31001	0.20034	1.547	0.1218
day5	0.3056	0.20098	1.521	0.1284
day6	0.41365	0.19637	2.107	0.0352
day7	0.46046	0.19455	2.367	0.0179
group_b	0.2122	0.27647	0.768	0.4427
male_2	-0.46387	0.28045	-1.654	0.0981
originFrance	0.56243	0.28033	2.006	0.0448
stimulus_B:day2	0.31957	0.34524	0.926	0.3546
stimulus_C:day2	0.52451	0.31062	1.689	0.0913
stimulus_B:day3	0.27638	0.32352	0.854	0.3929
stimulus_C:day3	0.27685	0.29754	0.93	0.3521
stimulus_B:day4	0.3468	0.31083	1.116	0.2645
stimulus_C:day4	-0.0685	0.29781	-0.23	0.8181
stimulus_B:day5	0.48042	0.30981	1.551	0.121
stimulus_C:day5	0.03835	0.2949	0.13	0.8965
stimulus_B:day6	0.08634	0.31315	0.276	0.7828
stimulus_C:day6	-0.22463	0.2958	-0.759	0.4476
stimulus_B:day7	0.24234	0.30692	0.79	0.4298
stimulus_C:day7	-0.26434	0.29663	-0.891	0.3728

Marginal R^2 =
0.264
Conditional R^2 = 0.760

Analysis of deviance table

	Chisq	Df	p-value
stimulus type	0.4862	2	0.784191
day	23.0457	6	0.000781
group	0.5891	1	0.442749
male	2.7357	1	0.098128
origin	4.0254	1	0.04482
stimulus:day	16.3108	12	0.177414

(d) Tail quivers

	Estimate	SE	z	p-value
(Intercept)	1.551792	0.478991	3.24	0.0012
stimulus_B	-0.6397	0.641896	-0.997	0.31897
stimulus_C	0.215102	0.529628	0.406	0.68464
day2	0.162427	0.423551	0.383	0.70136
day3	-0.34338	0.500902	-0.686	0.49301
day4	-0.19349	0.457295	-0.423	0.6722
day5	-1.37221	0.639353	-2.146	0.03185
day6	-0.80975	0.5438	-1.489	0.13647
day7	-1.17357	0.622985	-1.884	0.05959
group_b	-0.39648	0.311197	-1.274	0.20265
male_2	-0.0555	0.313077	-0.177	0.85929
originFrance	-0.32028	0.313063	-1.023	0.30629
stimulus_B:day2	-0.00072	0.747423	-0.001	0.99923
stimulus_C:day2	-0.49126	0.603383	-0.814	0.41554
stimulus_B:day3	0.446952	0.822589	0.543	0.58689
stimulus_C:day3	0.161426	0.652801	0.247	0.80469
stimulus_B:day4	0.455073	0.740523	0.615	0.53887
stimulus_C:day4	-0.18734	0.643541	-0.291	0.77097
stimulus_B:day5	2.159052	0.836015	2.583	0.00981
stimulus_C:day5	0.326968	0.829502	0.394	0.69345
stimulus_B:day6	0.75496	0.833709	0.906	0.36518
stimulus_C:day6	-0.6967	0.889251	-0.783	0.43335
stimulus_B:day7	1.331864	0.862339	1.544	0.12247
stimulus_C:day7	0.908569	0.751289	1.209	0.22653

Marginal R^2 = 0.1771
Conditional R^2 = 0.409

Analysis of deviance table

	Chisq	Df	p-value
stimulus type	0.2387	2	0.8875
day	6.9783	6	0.3229
group	1.6232	1	0.2026
male	0.0314	1	0.8593
origin	1.0466	1	0.3063
stimulus:day	16.1962	12	0.1824

Table s7. Tukey post-hoc test comparing difference in chasing behaviour between days, for each of the stimulus type. (a) Estimated marginal means with their standard errors, lower and upper confidence intervals for days and stimulus type. Results are averaged over the levels of group, male and origin (b). Result of the pairwise comparisons with estimates, degrees of freedom (df), t-value and p-values.

(a)

Stimulus: Synchronized

day	emmean	SE	lower.CL	upper.CL
1	-3.1522	1.402	-5.92	-0.381
2	-1.2406	1.072	-3.36	0.879
3	-0.3044	1.005	-2.29	1.683
4	-0.0151	0.993	-1.98	1.949
5	-0.279	1.004	-2.26	1.705
6	0.1703	0.99	-1.79	2.128
7	0.0976	0.991	-1.86	2.056

Stimulus: Shifted

day	emmean	SE	lower.CL	upper.CL
1	-3.3748	1.384	-6.11	-0.639
2	-2.7689	1.3	-5.34	-0.198
3	-2.6478	1.287	-5.19	-0.105
4	-1.9666	1.223	-4.38	0.452
5	-1.6038	1.205	-3.99	0.778
6	-1.8826	1.221	-4.3	0.531
7	-1.8997	1.22	-4.31	0.512

Stimulus: Asynchronous

day	emmean	SE	lower.CL	upper.CL
1	-0.9882	1.048	-3.06	1.083
2	-1.1568	1.057	-3.25	0.933
3	-1.1834	1.059	-3.28	0.91
4	-2.5968	1.214	-5	-0.198
5	-1.4499	1.078	-3.58	0.681
6	-1.4775	1.078	-3.61	0.653
7	-0.9781	1.072	-3.1	1.141

(b)

Stimulus: Synchronized

Contrast	estimate	df	t.ratio	p.value
1-2	-1.9116	142	-1.718	0.6051
1-3	-2.8478	142	-2.721	0.1005
1-4	-3.1371	142	-3.03	**0.045**
1-5	-2.8731	142	-2.748	0.0941
1-6	-3.3225	142	-3.221	**0.026**
1-7	-3.2498	142	-3.156	**0.0314**
2-3	-0.9362	142	-1.65	0.6504
2-4	-1.2255	142	-2.261	0.2704
2-5	-0.9616	142	-1.714	0.6079
2-6	-1.4109	142	-2.617	0.1286
2-7	-1.3382	142	-2.494	0.1692
3-4	-0.2893	142	-0.735	0.9901
3-5	-0.0253	142	-0.06	1
3-6	-0.4747	142	-1.254	0.8714
3-7	-0.402	142	-1.058	0.939
4-5	0.264	142	0.679	0.9935
4-6	-0.1854	142	-0.529	0.9984
4-7	-0.1127	142	-0.323	0.9999
5-6	-0.4493	142	-1.183	0.8994
5-7	-0.3766	142	-0.996	0.9541
6-7	0.0727	142	0.217	1

Stimulus: Shifted

Contrast	estimate	df	t.ratio	p.value
1-2	-0.6059	142	-0.637	0.9954
1-3	-0.727	142	-0.779	0.9866
1-4	-1.4082	142	-1.683	0.6285
1-5	-1.771	142	-2.174	0.3164
1-6	-1.4922	142	-1.782	0.5627
1-7	-1.4751	142	-1.79	0.5571
2-3	-0.121	142	-0.149	1
2-4	-0.8023	142	-1.137	0.9154
2-5	-1.1651	142	-1.716	0.6066
2-6	-0.8863	142	-1.252	0.872
2-7	-0.8692	142	-1.256	0.8705
3-4	-0.6812	142	-0.999	0.9534
3-5	-1.0441	142	-1.596	0.6854
3-6	-0.7652	142	-1.118	0.9216
3-7	-0.7482	142	-1.12	0.9211
4-5	-0.3628	142	-0.723	0.991
4-6	-0.084	142	-0.157	1

4-7	-0.0669	142	-0.129	1
5-6	0.2788	142	0.564	0.9977
5-7	0.2959	142	0.614	0.9963
6-7	0.0171	142	0.033	1

Stimulus: Asynchronous

Contrast	estimate	df	t.ratio	p.value
1-2	0.1686	142	0.341	0.9999
1-3	0.1952	142	0.392	0.9997
1-4	1.6086	142	2.114	0.3503
1-5	0.4617	142	0.892	0.9733
1-6	0.4893	142	0.94	0.9654
1-7	-0.0101	142	-0.022	1
2-3	0.0266	142	0.051	1
2-4	1.44	142	1.862	0.509
2-5	0.2931	142	0.547	0.998
2-6	0.3207	142	0.595	0.9969
2-7	-0.1787	142	-0.37	0.9998
3-4	1.4134	142	1.822	0.5352
3-5	0.2665	142	0.494	0.9989
3-6	0.2941	142	0.543	0.9981
3-7	-0.2053	142	-0.422	0.9996
4-5	-1.1469	142	-1.461	0.7671
4-6	-1.1193	142	-1.422	0.789
4-7	-1.6187	142	-2.172	0.3174
5-6	0.0276	142	0.05	1
5-7	-0.4718	142	-0.954	0.9628
6-7	-0.4994	142	-1	0.9533

B) Effect of courtship synchronization on female physiological response

Table s8. Mean (standard deviation) of E2 plasma concentration (pg/ml) depending on stimulus type and treatment.

	Pre-test	Post-test
Synchronous	14.07 (8.58)	20.45 (13.24)
Shifted	8.31 (4.10)	15.77 (8.62)
Asynchronous	14.14 (11.08)	17.53 (11.72)

Table s9. (a) Results of linear mixed models (with estimates, standard error (SE), degrees of freedom (df), t and p-values) investigating the effect of stimulus type (A: synchronous, B: shifted, C: asynchronous) on E2 plasma concentration. (b) Analyses of deviance table (Sum- and mean-square values, degrees of freedom, F values and p-values).

(a)

	Estimate	SE	df	t-value	p-value
(Intercept)	28.4125	2.4867	21.7904	11.426	< 0.001
stimulus_B	-5.22	2.2905	18	-2.279	0.03509
stimulus_C	-1.4262	2.2905	18	-0.623	0.5413
treatment_pretest	-5.7421	1.5331	23	-3.745	0.00106
group_b	-15.3562	1.8702	18	-8.211	< 0.001
male_2	-0.6494	1.8967	18	-0.342	0.73604
originFrance	-0.5486	1.8967	18	-0.289	0.77569

Marginal $R^2 = 0.678$
Conditional $R^2 = 0.741$

(b)

Analysis of Variance table type III (Satterthwaite's method)

	Sum-sq	Mean sq	df	Fvalue	p-value
stimulus type	156.53	78.26	18	2.775	0.089032
treatment	395.66	395.66	23	14.0286	0.001056
group	1901.57	1901.57	18	67.4228	< 0.001
male	3.31	3.31	18	0.1172	0.736044
origin	2.36	2.36	18	0.0837	0.775686

Table s10 Tukey post-hoc test test comparing difference in E2 plasma concentration between stimulus types (A: synchronous, B: shifted, C: asynchronous) (a) Estimated marginal means with their standard errors, lower and upper confidence intervals for stimulus type. Results are averaged over the levels of treatment, group, male and origin. (b) Result of the pairwise comparisons with estimates, degrees of freedom (df), t-value and p-values.

(a)

stimulus	emmean	SE	df	lower.CL	upper.CL
A	17.3	1.62	18	13	21.5
B	12	1.62	18	7.78	16.3
C	15.8	1.62	18	11.58	20.1

(b)

contrast	estimate	SE	df	t.ratio	p.value
A-B	5.22	2.29	18	2.279	0.0848
A-C	1.43	2.29	18	0.623	0.8097
B-C	-3.79	2.29	18	-1.656	0.2488

Table s11. Correlation tables with r (corr coefficient) and p-values with the corrplot function (XX packages), between (a) pre-test E2 plasma concentration and behaviour displayed the first day, and (b) post-test E2 plasma concentration and behaviour displayed the last day.

(a)

r

	Tail quivers	Preening	Steps	Chasing	Pre [E2]
Tail quivers	1	0.18	0.11	-0.16	0.1
Preening	0.18	1	0.19	0.4	-0.35
Steps	0.11	0.19	1	0.36	-0.09
Chasing	-0.16	0.4	0.36	1	-0.07
Pre [E2]	0.1	-0.35	-0.09	-0.07	1

P

	Tail quivers	Preening	Steps	Chasing	Pre [E2]
Tail quivers		0.4056	0.6036	0.4615	0.6446
Preening	0.4056		0.3738	0.0531	0.0936
Steps	0.6036	0.3738		0.0799	0.6795
Chasing	0.4615	0.0531	0.0799		0.7401
Pre [E2]	0.6446	0.0936	0.6795	0.7401	

(b).

r

	Tail quivers	Preening	Steps	Chasing	Post [E2]
Tail quivers	1	-0.27	0.43	0.37	-0.52
Preening	-0.27	1	-0.15	-0.04	-0.05
Steps	0.43	-0.15	1	0.82	0.04
Chasing	0.37	-0.04	0.82	1	0.04
Post [E2]	-0.52	-0.05	0.04	0.04	1

P

	Tail quivers	Preening	Steps	Chasing	Post [E2]
Tail quivers		0.1962	0.0341	0.075	0.0085
Preening	0.1962		0.4841	0.8484	0.8063
Steps	0.0341	0.4841		0	0.8649
Chasing	0.075	0.8484	0		0.8612
Post [E2]	0.0085	0.8063	0.8649	0.8612	

III. Supplementary video information

Legend of supplementary videos:

Video s12. Sample video of the synchronous courtship stimulus.

Video s13. Sample video of the shifted courtship stimulus.

Video s14. Sample video of the asynchronous courtship stimulus.

IV. Supplementary material references

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Abstract

Multimodal signals composed of different sensory modalities are used in a variety of contexts. Despite their prevalence, questions regarding their function in animal communication remain unanswered. Multimodal courtship displays are particularly interesting, as they are often very conspicuous and costlier to produce than simpler unimodal signals. In an extensive literature review, I summarized current known functions of multimodal courtships and suggested further aspects to consider during empirical investigations. Using the ring dove (*Streptopelia risoria*), the main goal of this PhD was to gain knowledge on the role of different modalities and the structure of their multimodal courtship. To do so, I assessed how variation of different male courtship parameters impacted females' behavioural and physiological response. I used cutting-edge technology to record and perform integrated analyses of courtship modalities, and multimodal playback techniques. In my first experiment, I recorded live interactions between individuals and showed that lower call fundamental frequency and long courtships containing short bouts triggered an immediate behavioural response linked to sexual interest in females. My second experiment used artificial stimuli to show that courtship signal structure matters, as synchronization timing between acoustic and visual channels impacted female behavioural response, probably by stimulating females prior to copulation. Finally, female blood estradiol concentration increased following playback, demonstrating that artificial stimuli also triggered physiological changes in females. This PhD work is one of the first attempts to perform a comprehensive study of a wide array of multimodal display parameters and associate them with female response. Taken together, my findings demonstrate that parameters of ring dove courtship influence different female responses, suggesting that they carry varied information and possess distinct functions. This contributes to our understanding of the evolution and function of multimodal displays, while stressing the need to adopt an integrative approach, investigating both variation in signal modalities and signal structure.

Zusammenfassung

Multimodale Signale aus verschiedenen Sinnesmodalitäten werden in vielen unterschiedlichen Kontexten verwendet. Trotz ihrer Prävalenz bleiben Fragen bezüglich ihrer Funktion in der Tierkommunikation unbeantwortet. Multimodales Balzverhalten ist von besonderem Interesse, da es oft auffälliger und aufwendiger zu produzieren ist als einfachere, unimodale Signale. In einem ausführlichen Literaturüberblick fasse ich die derzeit bekannten Funktionen multimodaler Balzverhalten zusammen und schlage weitere Aspekte vor, die bei empirischen Untersuchungen berücksichtigt werden sollten. Hauptziel dieser Doktorarbeit war es, Erkenntnisse über die Rolle verschiedener Modalitäten und die Struktur des multimodalen Balzes zu gewinnen. Als Untersuchungsobjekt diente die Lachtaube (*Streptopelia risoria*). Ich untersuchte, wie die Variation verschiedener männlicher Balzparameter das Verhalten und die physiologische Reaktion der Weibchen beeinflusst. Ich verwendete modernste Technologien zur Aufzeichnung und Durchführung integrierter Feinanalyse der Balzmodalitäten, sowie multimodale Wiedergabetechniken. Durch die Videoaufnahme von Live-Interaktionen zwischen Individuen konnte ich im ersten Experiment zeigen, dass eine niedrigere Grundfrequenz der Laute und lange Balzdauer unterteilt in kurze Periode eine direkte Verhaltensreaktion auslösten, die das sexuelle Interesse der Weibchen vermittelt. Im zweiten Experiment zeigte ich mit manipulierten Stimuli, dass die Struktur des Balzsignals wichtig ist, da die audiovisuelle Synchronisationszeit das Verhalten des Weibchens beeinflusst, wahrscheinlich durch die Anregung von Weibchen vor der Kopulation. Schließlich erhöhte sich die Estradiolkonzentration in weiblichen Blutproben nach der Playback-Exposition, was zeigt, dass Playback Stimuli auch physiologische Veränderungen bei den Weibchen auslösten. Diese Doktorarbeit war einer der ersten Versuche, eine umfassende Studie über eine breite Palette multimodaler Balzparameter durchzuführen und diese mit dem weiblichen Verhalten in Verbindung zu bringen. Zusammengenommen zeigen meine Ergebnisse, dass Parameter des Lachtaubenbalzverhaltens unterschiedliche weibliche Reaktionen beeinflussen, was darauf hindeutet, dass sie verschiedene Informationen tragen und daher unterschiedliche Funktionen haben. Dies trägt zu unserem Verständnis der Entwicklung und Funktion multimodaler Verhalten bei und unterstreicht gleichzeitig die Notwendigkeit eines integrativen Ansatzes, bei dem sowohl die Variation der Signalmodalitäten als auch der Signalstruktur untersucht werden sollte.