



Original Article

What makes a multimodal signal attractive? A preference function approach

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Courtship signals are often complex and include components within and across sensory modalities. Unfortunately, the evidence for how multimodal signals affect female preference functions is still rather limited. This is an important scientific gap because preference function shape can indicate which male traits are under the strongest selection. We modelled how preference function shape can be altered under 4 scenarios of varying signal content, including both redundant and non-redundant signals. The model was tested with the brown-headed cowbird (*Molothrus ater*); we manipulated male song attractiveness and visual display intensity, and assessed female preferences in an audiovisual playback study. We found that the intensity of a visual display can modify how attractive a song is for females. This indicates that the visual and acoustic male signal components are non-redundant and modulate each other. Our study shows a change in the direction of female preference functions for one signalling modality resulting from changes in the attractiveness of the other modality. Overall, our findings suggest that male signals in this species may not be under the typical directional selection documented in other species, but rather selection may favour males that possess a range of different signals that can be used strategically during different social contexts.

Key words: Multimodal signals, *Molothrus ater*, preference functions.

INTRODUCTION

In multimodal signals, 2 or more signal components from different sensory modalities can combine or interact to influence receiver behaviour (Candolin 2003; Hebets and Papaj 2005; Partan and Marler 2005). Multimodal signalling is well described across many animal taxa, but the shape of the preference function (i.e., the pattern of female response with variation in a male signal; Wagner 1998) for multimodal signals has been little studied (Bailey 2011; Taylor et al. 2011; Smith and Evans 2013; Reichert and Höbel 2015; Stange et al. 2016). This is an important gap in animal communication because the shape of the female preference function can indicate the specific characteristics of a male signal component that are under the strongest selection (Brooks et al. 2005; Gerhardt and Brooks 2009). One of the biggest challenges in signal evolution studies has been to determine which components of a complex signal are under selection (Girard et al. 2015; Wilkins et al. 2015).

The separate sensory modalities of a multimodal signal are typically described by their function: they can be 1) redundant (also called a degenerate system in Hebets et al. 2016) where the

components derived from different sensory modalities provide functionally similar signals (i.e., the “back-up” hypothesis), or 2) non-redundant where different components provide functionally different signals (i.e., the “multiple messages” hypothesis) (reviewed in Candolin 2003; Hebets and Papaj 2005; Partan and Marler 2005). This classic framework uses a cue-isolation approach that allows us to establish the potential contribution of each modality to the overall signal (Hebets and Papaj 2005; Partan and Marler 2005). This framework typically considers both signal components as binary variables (e.g., present vs. absent).

Smith and Evans (2013) relaxed the assumption of binary signal components by modelling how simultaneous variation in 2 modalities can affect female preference surface plots for both redundant and non-redundant multimodal signals. Here, we have extended the Smith and Evans’ (2013) model to demonstrate that the signal component in one modality (e.g., intensity of a song) can generate different female preference function shapes across intensity levels of a separate signal component (e.g., intensity of a visual display) (Figure 1). This extended modelling exercise (see details below) was used to develop a set of predictions that we tested empirically.

We assumed that signals are honest and that signal intensity (e.g., higher song rates, more saturated colour displays, greater pheromone concentrations, etc.) indicates higher male quality

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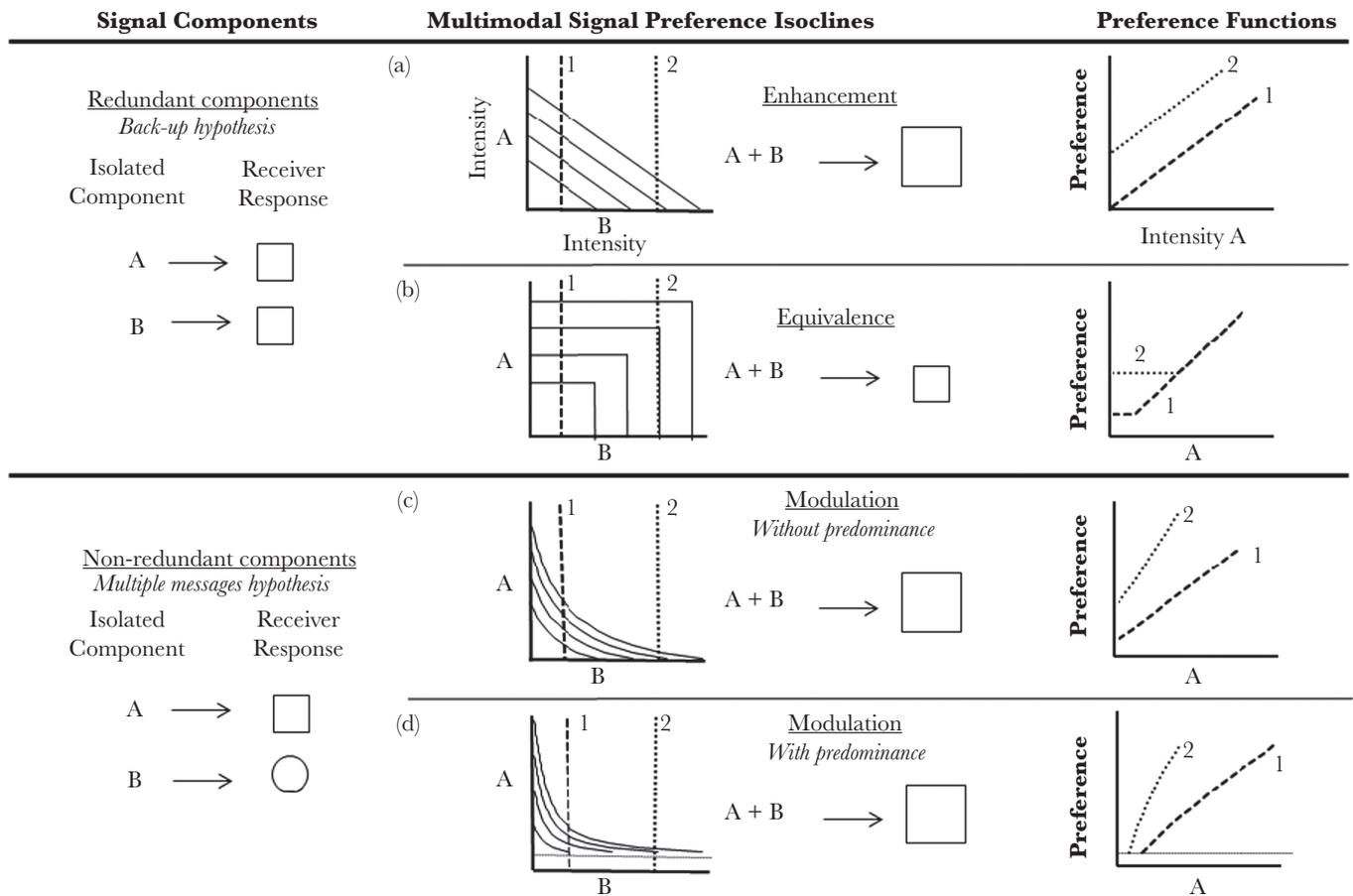


Figure 1

Signal content influences multimodal preference functions. Preference functions for one signal component can be modified by different levels of a second signal component in a different modality in both redundant (a, b) and non-redundant (c, d) multimodal contexts. Female preference isoclines are generated by plotting changes in intensity for signal components A and B. We plot 4 isoclines although they represent an infinite series. From these isoclines we can then derive a preference function in relation to changes in one modality (A) at different intensity levels (1-low, 2-high) in the other modality (B). See text for equations used to generate the functions.

(Zahavi 1975; Searcy and Nowicki 2005). We first generate a series of preference isoclines that illustrate how female preferences can change as a function of intensity in both signalling components (i.e., modality A and modality B; Figure 1). In our figures, a unit increase in signal intensity in one modality is assumed to have the same utility to the female as a unit increase in signal intensity in the alternate modality. From these preference isoclines we can derive a preference function in relation to intensity changes in one modality (i.e., modality A) at 2 different intensity levels in the other modality (i.e., modality B). In order to plot all of our preference functions we fixed intensity at a relatively “low” level of 0.5 and a relatively “high” level of 2.0. All isocline and preference function plots were generated using SAS V 9.3. This approach allowed us to distinguish preference functions resulting from the “back-up” hypothesis of redundant signal components and the “multiple messages” hypothesis of non-redundant signal components (Figure 1).

Redundant signal components elicit similar receiver responses (represented in Figure 1a, b by similarly sized squares as in Partan and Marler 1999) when they are presented in isolation (Candolin 2003; Hebets and Papaj 2005; Partan and Marler 1999; Partan and Marler 2005). The receiver response to the multimodal signal containing these redundant components can be “enhanced”, where the receiver responds more strongly to the multimodal

signal than to either signal component alone (Figure 1a). Here, we show an example of “enhancement” where the components combine additively such that preference (represented by the larger square) is a function of the sum of signal component intensity in both modalities; hereafter labelled A and B (Equation 1) (Partan and Marler 1999).

$$\text{Preference} = f(A + B) \quad (1)$$

In Equation 1 and all subsequent equations, we assume that $f()$ represents a linear relationship between preference and the argument of the function (here $A + B$). The preference isoclines should therefore reflect a linear trade-off between signalling components (Figure 1a). Under this enhancement scenario, when we fix intensity in B, the resulting preference function is a linear function of the level of intensity in A. This illustrates the additive relationship between modalities as it relates to female preference. Regardless of the intensity of B (i.e., high or low) note that the resulting preference functions have the same slope but different intercepts. For this and all subsequent scenarios, the slope of a preference function can be derived based on the relative spacing of the isocline lines at the 2 levels of B; in the “enhancement” condition the relative spacing is the same for level 1 as it is for level 2, thus the slopes of the preference functions are equal.

Redundant signal components can also be “equivalent” to one another; in this scenario, if intensity levels are equal, the receiver responds in the same manner to the multimodal signal as to either signal component alone (Hebets and Papaj 2005; Figure 1b). This is depicted in our model by the isolated signal components and the multimodal signal all depicted by the same sized square (Partan and Marler 1999). However, if one signal component is at a relatively higher intensity level, the receiver responds to the stronger of the 2 signal components irrespective of the strength of the weaker component (Figure 1b). Differences in intensity level could occur due to different propagation properties of the 2 signal modalities across the environment; for example, acoustic signal components may propagate further in a dense, wooden environment than visual signal components (Hebets and Papaj 2005; Smith and Evans 2013).

If intensity in A is greater than in B, preference is a function equal to the intensity in A and vice versa. Therefore,

$$\text{Preference} = f(\max(A, B)) \tag{2}$$

Thus, the resulting preference functions at the 2 levels of B show that preferences for modality A do not increase until the intensity of A is greater than B; after that the preference function is a single line (Figure 1b).

In contrast to redundant signals, non-redundant signal components elicit different receiver responses when each is presented alone, often giving rise to a statistical interaction between the intensity levels of the 2 components (Figure 1c, 1d). These different receiver responses are qualitatively represented by the different shapes in our figure (i.e., one circle, one square) (Partan and Marler 1999). The most common interaction of non-redundant signal components is when one component “modulates” the other so that the response to the multimodal signal is either increased or decreased compared to the response to a single component (Hebets and Papaj 2005). We illustrate 2 examples of modulation in Figure 1: one without predominance (Figure 1c) and one with predominance (Figure 1d). In both of these cases the signal components combine multiplicatively such the presence of A and B together are weighed higher than either in isolation (Figure 1c, d). Modulation without predominance indicates that both signal components in isolation are sufficient for eliciting a female response. On the other hand, modulation with predominance indicates that one signal component alone, but not the other, is sufficient to elicit a female response.

In the modulation without predominance condition (Figure 1c), preference is a function of the sum of intensity in each modality (i.e., A and B) plus the product of intensity in both modalities (i.e., $A \times B$).

$$\text{Preference} = f(A + B + (A * B)) \tag{3}$$

The resulting preference functions derived from these isoclines show an interaction between the modalities (Figure 1c) such that the slope of the preference function should be higher at higher levels of the signal component in the alternative modality.

In the modulation with predominance condition (Figure 1d), a certain threshold of A must be reached before a response is generated. This is similar to mate-choice in female túngara frogs where the male call alone is necessary and sufficient to elicit female phonotaxis while the presentation of the visual stimulus alone is not (Rosenthal et al. 2004). To illustrate this, we set the threshold to $A = 1$. Component B then strengthens this response but the utility of the B asymptotes to zero as the level of A drops to the threshold (designated by the dotted line, Figure 1d). We model this by

discounting the utility of B based on a negative exponential function of A. Preference isoclines were generated by modifying the equation as shown in Equation 4 so that the weighting of B declines as A drops to the threshold. We do this by multiplying signal component B by a coefficient (α) that changes the scaling of component B.

$$\begin{aligned} \text{Preference} &= f(A + B * \alpha + (A * B * \alpha)) \\ \text{where } \alpha &= [2 / (1 + e^{-(A-1)*x})] - 1 \\ x &= \text{scaling parameter, here set at 2} \end{aligned} \tag{4}$$

For both modulation scenarios the relative spacing of the isoclines intersecting line 1 (i.e., the low intensity level of B) is much greater than the relative spacing of isoclines crossing line 2. This translates into the higher level of B having a steeper slope and therefore stronger selection on this trait than the low B intensity level. Note that line 2 is steeper in Figure 1d compared to Figure 1c because the isocline lines converge faster to the asymptote in Figure 1d than Figure 1c. Additionally, the lines generated in the modulation with predominance condition are not linear.

In this study, we asked the question: what makes a signal attractive when we combine different signal components from 2 modalities? We chose the brown headed cowbird (*Molothrus ater*) as a model species for 2 reasons. First, sexual selection on the male display is predicted to be strong in this species (Woolfenden et al. 2002). Cowbirds are obligate brood parasites and females engage in mate-choice for partners (Rothstein et al. 1988; Yokel and Rothstein 1991; Woolfenden et al. 2002). Courting male cowbirds often pair a song with a visual wingspread at a relatively close distance to the female (<1 m) (Rothstein et al. 1988). Females, in turn, give a copulatory solicitation display (CSD) to indicate their willingness to mate (West et al. 1981). Second, the different signal components in the male cowbird multimodal display are well characterized. Female cowbirds prefer the multimodal display more than the song (O’Loughlen and Rothstein 2010a) or visual display presented alone (O’Loughlen and Rothstein 2012). Female cowbirds also seem to prefer low intensity visual displays compared to high intensity displays (O’Loughlen and Rothstein 2012). Moreover, studies suggest that the 2 components of the multimodal signal are non-redundant: the song presented alone is sufficient to elicit a CSD, but females rarely give a CSD to a visual display without a song (O’Loughlen and Rothstein 2012). Together, these findings indicate that the cowbird courtship display may follow the predictions of the modulation with predominance hypothesis of non-redundant signalling where one component (i.e., the visual bow) strengthens the response to another component (i.e., the song) (Partan and Marler 2005).

To test this prediction, we manipulated the attractiveness (i.e., potency) of cowbird perched song and the intensity of visual display simultaneously and measured female preferences to audiovisual playbacks. We measured mate preference with 2 behaviours: 1) CSD duration, where longer duration indicates greater preference (O’Loughlen and Rothstein 2010a; O’Loughlen and Rothstein 2012) and 2) the latency for each female to begin a CSD where shorter latency is a measure of greater mate-preference (Wells and Schwartz 1984; Simmons 1989; Wignall et al. 2014). We tested the predictions about preference function shape of a multimodal signal following the framework outlined in Figure 1. Specifically, from the modulation with predominance hypothesis of non-redundant signals, we predicted that 1) the slope of the preference function should be higher at higher levels of the alternative signal

component modality and 2) preference function shapes may deviate from a linear relationship (Figure 1D).

METHODS

Animal capture and housing

All animal care and experimental procedures were approved by Purdue University's Animal Care and Use Committee (PACUC) Protocol # 1111000151. Between May 2011 and April 2012, 12 male and 10 female cowbirds were wild-caught in decoy traps by the USDA APHIS (Sandusky, OH); these individuals were used in the creation of the experimental stimuli. In May 2013, 42 female cowbirds were captured at the same location for the mate preference experiment. Birds were banded and individually housed at Purdue University in enclosures (64 cm x 40 cm x 64 cm) in single-sex rooms. Birds were provided *ad libitum* access to mixed seed, grit, and water. The lighting schedule followed the natural lighting conditions of West Lafayette, IN (schedule was adjusted weekly and ranged from 14:10 h light:dark in the summer to 10:14 h during the winter).

Male visual display recordings

Additional details regarding the creation of the male video playbacks are described elsewhere (Ronald et al. 2015). Briefly, between May–July 2012, males ($N = 12$) were implanted with either testosterone or a placebo as they were to be used in another experiment not described here. Females caught in 2012 ($N=10$) were used as a stimulus to elicit male displays. Females were implanted with estrogen in order to induce breeding season behaviour. All birds were sedated with a combination of ketamine (40–60 mg/kg) and midazolam (6–8 mg/kg) injected into the breast muscle prior to implantation. Implants were made by packing either 10 mm crystalline testosterone or estrogen (Sigma Chemical Co., St. Louis, MO) into Silastic tubing (outer diameter 1.96 mm) and sealed with Silastic adhesive. Placebo implants were made in the same way but not filled with hormone. After sedation, the implant was placed subcutaneously in a small incision made on the bird's chest. Birds were then allowed to recover for 3 weeks.

During the trials males were randomly placed in an experimental enclosure (64 cm × 40 cm × 64 cm) adjacent to an identical enclosure that contained a randomly selected stimulus female. One side of this enclosure contained a Plexiglas window through which a camera (HD Everio GZ-E10) recorded female-directed wingspreads at 30 fps. Males always displayed head-on to the camera such that the angle of the bird to the camera was always 180 degrees. Trials lasted 30 min between 0600–1600 h. Birds were allowed to rest for 2 h in their home enclosures between subsequent trials. No birds were run more than 4 times in a given day. Trials were repeated every 2 to 3 weeks for the duration of the breeding season.

We selected one video from each of our males based on both the quality of the video (i.e., entire bird in the camera frame, etc.) and the intensity of the visual display (O'Loughlen and Rothstein 2010b). O'Loughlen and Rothstein (2010b) developed a qualitative metric for measuring display intensity (i.e., extent of puffing, wingspread, bow, wing pumping, and tail cocking); this experiment used these established display characteristics to characterize the intensity of the display. Using Adobe Premiere Elements we found the specific video frame that showed the beginning of the display, maximum puffing, maximum wingspread, the deepest part of the bow, and the end of the display. Using the "Snipping Tool" in Windows

2010 we then took a screen-shot of each of these video frames for each of the 12 birds included in the study. Pictures were always the same size and saved as JPEG files. We used the "Measuring Tool" in ImageJ to record the number of pixels for the width of the puffing (i.e., across the widest part of the bird's chest), width of the wingspread (i.e., from wing tip to wing tip), depth of the bow (i.e., from the tip of the bill to the middle of the perch), and height of the tail (i.e., tip of the tail to the middle of the perch). Displays were ordered from lowest to highest intensity and the top 6 were assigned as high intensity and the bottom 6 displays as low intensity.

Male song recordings

Adult male cowbird songs ($N = 12$) were collected by D.W. and taken from a library of songs tested over more than a decade of playback experiments to females: 6 were chosen that reliably produced CSDs (high potency songs) and 6 that rarely produced CSDs (low potency songs). These songs were recorded over 10 breeding seasons from adult male cowbirds captured in Indiana and housed in mixed-sex captive flocks in outdoor aviaries. Recordings were taken at distances less than 0.3 m away from a Sennheiser RF condenser microphone (Sennheiser Electronic Corporation, 1 Enterprise Drive, Old Lyme, CT, USA). All audio recordings were sampled at a rate of 44.8 kHz on a Sony TCD-D10 PRO II DAT recorder (Sony Corporation, 550 Madison Ave., New York, USA) and then digitally converted to 44.1 kHz files and saved as .wav files.

Procedures for measuring song potency are also detailed in King et al. (2003) and West (2006). Briefly, high potency is defined as a song that reliably produces a CSD response within 1 s from the onset of the sound. The procedure for each playback test was to broadcast 6 randomly selected songs to females ($N \geq 10$) housed in 1.3-m³ sound attenuation chambers with one vocalization per trial and each trial separated in time by 90 min, beginning around 0700 h in May and June. Each vocalization was played 5–9 times to each female over the course of the experiment.

After the songs were identified as potent or not potent (i.e., degree to which they consistently elicit female CSDs) (West et al. 1981), we used the noise reduction function in CoolEdit Pro (Version 2) to remove background noise. We then normalized the amplitude of each exemplar to 80%. It is still relatively unknown what spectral components contribute to song potency in cowbirds (but see West et al. 1979), so we quantified the following with Sound Analysis Pro (Version 2011.104): frequency (fundamental, mean, and peak), entropy (i.e., harshness; a measure of the amount of randomness in a sound with harsher songs being more entropic) (Ho et al. 1998; Tchernichovski 2000), and duration of the glugs in phrase 1 (P1), the inter-glug interval, and the final phrase (P2; Supplementary Appendix 1). We used a Praat script ("cross-correlate" in version 5.1.32) (Boersma and Weenink 2009) to generate cross-correlation values to estimate the relative similarity between the 12 different songs. We used multidimensional scaling to plot the relative position of each song in 2D space (see below).

Experimental stimuli

We chose pairs of songs, one potent and one non-potent that were close in multidimensional scaling space, to be paired with high and low intensity visual displays such that we had a balanced design covering the natural range of song/visual display variation, with 3 exemplar videos representing each possible combination of song potency and visual display intensity. Adobe Premiere Pro Software was used to 1) cut the videos to approximately the same length

(4.15 ± 0.22 s), and 2) crop the videos so that only the bird, the background, and the perch were displayed. We added fade-in/fade-out effects so that each video started and ended with a black screen. Adobe Premiere shows the waveform of the original bird's song so that we could align the experimental song with the original song's timing so discrepancies in synchrony would be minimized.

Behavioural mate-preference experiments

Mate-preference trials were conducted from 0700–1300 h between June–Sept 2013. Female cowbirds ($N = 42$) were randomly divided into 7 experimental blocks that underwent the experiment together. On Day 1 for a given block, a blood sample was taken from each bird for hormonal analysis. Birds were then sedated with ketamine (40–60 mg/kg) and midazolam (6–8 mg/kg) injected into the breast muscle and implanted with an estradiol implant (10-mm crystalline estrogen, Sigma Chemical Co., St. Louis, MO, into Silastic tubing, outer diameter 1.96 mm). Estrogen implants induce normal breeding behaviour (e.g., CSDs) in a laboratory setting and are commonly used in cowbirds and other avian species (Hunt and Wingfield 2004; O'Loughlen and Rothstein 2010a; O'Loughlen and Rothstein 2012).

Females recovered for 12 days; on Day 13 females began habituation trials to familiarize the females with the playback procedure so they were not startled by the video stimulus (O'Loughlen and Rothstein 2010a). Habituation trials were conducted from 1400–1700 h and consisted of a randomly selected female being rotated into an experimental enclosure adjacent to a television (Sanyo LCD HD-TV, Model # DP26649). Eight high-flicker light bulbs were used to illuminate the room (Phillips High Energy Advantage F54t5/850/HO/EA). After 25 min she was played a video of a related species, the red-winged blackbird (*Agelaius phoeniceus*) sitting on a perch. She was returned to her home enclosure and another female was randomly selected. Birds were exposed to 3 habituation trials over 3 consecutive days.

The experimental trials were run from Day 16–Day 28. Females were put into the experimental arena in random order and after 25 min one of the 12 experimental videos was randomly chosen and played on a HD-TV connected to a Dell Latitude E6510 laptop running Windows Media Player with an HDMI cable so that playbacks could be controlled from outside the experimental room. Additionally, a Saul Mineroff Field Speaker (Model # SME-AFS) was attached to this laptop so that the audio could be broadcast from a single speaker from directly behind the television. Before each trial, the stimulus video was played in order to ensure the speaker volume was approximately 80 dB 0.3 m from the speaker, approximately the volume of a singing cowbird (Gall et al. 2012). The volume was checked with a Brüel and Kjaer 1613 Precision Sound Level Meter. We also adjusted the width and height of the Windows Media Player screen to centre the video on the television and to ensure that the image of the cowbird was approximately life-size (about 15 cm tall).

Four cameras recorded each trial. One (HD Everio GZ-E10) was connected via a coax cable to a Sony solid-state video monitor (Model # PVJ-510) located outside the test arena so that the experimenter could watch the trial in real-time. The 3 other cameras offered 3 different views of the female: one (Samsung SMX-F40BN) was straight on, one was a PelikanCam bullet camera (TC855) that offered a top-down view, and the last bullet camera was focused on the TV displaying the stimulus video. These 3 cameras were connected to a colour quad splitter (Clover Electronics, Model #QC900), connected to a laptop (Asus Eee PC 1015PEM)

running EZcapTV USB Video Capture Software so that the 3 camera views could be recorded simultaneously. After the video playback we waited 5 min and then the female was returned to her enclosure for 3 h before her next trial. Over the course of the experiment, females completed 2 trials per day for 12 consecutive days. Videos were chosen randomly from the 12 videos with one replacement until all 12 were played twice. On Day 29 another blood sample was taken from each female for hormonal analysis described below.

The latency to begin a CSD and the duration of each CSD were measured using Adobe Premiere Pro software. Following previous work (O'Loughlen and Rothstein 2010a; O'Loughlen and Rothstein 2012), CSD duration was determined as the time the CSD posture began to when the female's tail returned to a position parallel to the ground. Latency to begin a CSD was calculated as the time difference between the beginning of the stimulus presentation and the onset of a CSD. All estimates of duration and latency were coded by an unbiased observer who was blind to the experimental treatment of the videos.

Hormonal Analyses

Blood for hormone analyses was collected within 2 minutes of capture with a heparinized collection tube (RAM Scientific Safe-T-Fill) and centrifuged for 5 min at 300 rpm so that the plasma layer could be separated from the red blood cells. Plasma was stored in a -80 °C freezer until subsequent baseline estrogen analysis. Estradiol concentrations were measured in baseline and post-implantation plasma samples in collaboration with R. Stewart at Indiana University's Center for the Integrated Study of Animal Behavior. Samples were analysed using commercially-obtained ELISA kits for 17- β estradiol (Enzo Life Sciences #900-008) which had been previously validated for other passerine species (Caras et al. 2010; Gall et al. 2013). Additionally, we followed a steroid extraction procedure (Clotfelter et al. 2004; Rosvall et al. 2013) to purify the samples. Briefly, 20 μ L of plasma was combined with 100 μ L water and stored overnight at 4 °C. Samples were extracted twice in diethyl ether, evaporated under nitrogen gas, and reconstituted in 35 μ L of 100% ethanol. Following vortexing, the extract was diluted in 315 μ L of Assay Buffer 3 (Enzo). A preliminary analysis with male cowbird plasma and titrated testosterone determined steroid extraction efficiency using this procedure to be 93.8 ± 4.28 (mean \pm SD; $n = 46$ samples). Extracts were run in duplicate according to the procedures provided with the kit and final readings were read at 405 nm on a BioTek EPOCH plate reader. Final estradiol concentrations were calculated with data reduction software (Gen5 by BioTek) and corrected for plasma starting volume. Serial dilution of pooled cowbird plasma yielded a displacement curve that showed strong parallelism to the standard curve ($r^2 = 0.96$). Intra-assay variability was an average of 2.6% for the high control, and 14.05% for the low control. Inter-assay variability was 16.7% for the high control and 12.5% for the low control ($n = 2$ assays).

Statistical analyses

We first explored whether the propensity to give a CSD was affected by song potency or visual display intensity. Females did not give a CSD in response to the playback in 25% of the trials; these trials were coded in binary such that playbacks that did not result in a CSD were coded as “zero” and playbacks that elicited a CSD were coded as “one”. We used a generalized linear mixed model (Proc GLIMMEX in SAS) with a log link function and Poisson error distribution. Our independent factors included the factor scores used

to quantify the song, the categorical visual display intensity variable, and their interactions. We then ran the model with the visual display quantified using factor scores, the categorical song variable, and their interactions. We also included several covariates related to the female (e.g., bird identity, female body mass, and estrogen concentration after implantation nested within block) and the experimental design (e.g., experimental block, trial day, and trial order: the order in which bird was placed in the experiment on a given day). We considered these covariates because of previous evidence suggesting that female condition (e.g., body mass and hormone levels) could influence her mating preferences (reviewed in Ronald et al. 2012). Similarly, we included block, trial day, and trial order because we expected a female's motivation to mate would decrease over the course of the experiment (White et al. 2006; Ritters et al. 2007).

We then explored how one modality modulates female perception of the other modality by describing one modality in its categorical form (i.e., high or low) and the other on a continuous scale and vice versa. To reduce the dimensionality of the quantitative (i.e., continuous scale) measurements for each signal component, we ran a factor analysis using Proc FACTOR in SAS (version 9.3) with a varimax rotation. We used linear mixed models with Proc MIXED in SAS to analyse how CSD duration and latency is affected by the factor scores used to quantify the song, the categorical visual display intensity variable, and their interactions. For this analysis, we only included the data in which females responded to a playback with a CSD. Then, we then ran the model with the visual display quantified using factor scores, the categorical song variable (i.e., the behavioural classification of high or low potency), and their interactions. We also included factor terms squared to test for patterns of non-linearity. The dependent variables and estrogen concentration were \log_{10} transformed and the residuals were normal after this transformation. We specified an autoregressive covariance structure and the Kenward–Roger method was used to calculate the degrees of freedom. Female identity was included as a repeated factor. For both our models of CSD latency and duration female mass was removed from the model as this model generated the lowest AIC value and the results were qualitatively the same as the full model (see Supplementary Appendix 2). Additionally, in our models for

CSD latency, we also removed the estrogen concentration from being nested within block as this also produced a better fitting model (Supplementary Appendix 2).

For all models, non-significant interactions were removed based on descending F values. To clarify the interpretation of any significant interactions, we ran additional repeated measures Anovas separately for either the high or low potency songs or high or low intensity visual displays. We graphed all significant interactions using the means and standard errors from the predicted values generated by the mixed model. Best fit lines, including the slope and relative intercepts, were also generated using 'solutions' from the Proc MIXED models.

RESULTS

Forty females completed the behavioural trials, but 10 females were removed from the dataset because 1) they never gave a CSD ($N = 4$) or 2) they had insufficient plasma samples for the hormonal assay ($N = 6$).

Factor analysis of song potency and visual display intensity

The first factor explained 31% of the song variation and the second and third factors described an additional 26% and 20%, respectively. In total these 3 factors (eigenvalues >1) described 77% of the song variation (Table 1). Mean frequency of glug 1 and glug 2, and the duration and entropy of glug 2, loaded positively onto factor 1. The entropy of glug 1 loaded negatively on factor 2, while duration of glug 1 and the inter-glug interval (IGI) loaded positively. For factor 3, the duration and entropy of P2 loaded positively, while the mean frequency loaded negatively. For the visual display, the first factor explained 55% of the variation and the second factor explained an additional 27% for a cumulative 82% of the variation explained by our measured traits (Table 1). The time the first wing pump began, the time the song began within the visual display, the extent of puffing, and the width of the wing extension were all positively loaded on factor 1; the height of the tail and the depth of the bill below the perch as well as the overall display duration were positively loaded on factor 2. We used a Discriminant

Table 1
Factor analysis of cowbird song and visual display

Signal component	Signal property	Measure	Factor 1	Factor 2	Factor 3
Song	Glug 1	Duration	0.09	0.84	-0.02
	Glug 1	Mean Frequency	0.72	-0.13	-0.22
	Glug 1	Entropy	0.29	-0.65	0.46
	Inter-glug interval	Duration	0.05	0.94	0.07
	Glug 2	Duration	0.78	0.14	0.05
	Glug 2	Mean Frequency	0.74	0.41	-0.35
	Glug 2	Entropy	0.86	-0.21	0.11
	P2	Duration	0.14	-0.1	0.87
	P2	Mean Frequency	0.33	0.14	-0.92
	P2	Entropy	-0.21	0.5	0.7
	Visual display	Body puffing	Width	0.93	-0.15
Wing extension		Width	0.75	0.28	
Song begins		Time	0.91	0.3	
First wing pump begins		Time	0.92	0.18	
Total display		Duration	0.42	0.69	
Tail height		Height above perch	0.11	0.91	
Bill depth		Depth below perch	-0.01	-0.97	

3Ds significantly explained variation in cowbird song; 2Ds explained variation in the cowbird visual displays. Values here show the magnitude and direction for how each measured variable loaded onto the different dimensions. Bold values show the dimensions with the highest loading scores.

Function Analysis (DFA) using Proc DISCRIM in SAS to ensure these factors described song potency and visual display intensity (Supplementary Appendix 3).

Song potency and visual display intensity and the propensity to initiate a CSD

In 181 of the 720 trials conducted (25%) females did not respond with a CSD. We first modelled whether the propensity to begin a CSD was affected by song described continuously and visual display intensity described categorically; second, we modelled CSD propensity when song was described categorically and visual display intensity was described continuously (Table 2). In both scenarios, neither song nor visual display intensity, or their interactions, altered the likelihood that a female would begin a CSD (Table 2). We did find, however, that female mass significantly affected whether a female would begin a CSD, both when song and visual display were described categorically or continuously and vice versa (Table 2). In both scenarios, females that were larger were also more likely to begin a CSD (Table 2).

Preference functions of the multimodal signal: song properties as a continuous variable

We first modelled the 3 significant dimensions of song with visual display intensity as a categorical variable. Here we saw that females had shorter CSD latencies to a more intense visual display (0.19 ± 0.005 s) than to less intense displays (0.23 ± 0.006 s) ($t_{495} = 6.28, P < 0.001$). Additionally, females began CSDs earlier to songs with higher glug frequency, and higher entropy and longer duration second glugs (i.e., higher values of song factor 1; Table 3). The squared term of song factor 1 was significantly related to CSD duration; generally, females gave longer CSDs to songs higher in song factor 1 but this trend was not linear (Table 3). Female CSD duration and latency were also affected by a significant interaction between song factor 3 and visual display intensity (Table 3). Moreover, CSD latency was affected by one additional interaction

between song factor 2 and visual display intensity (Table 3). We ran separate Repeated Measures Anovas for high- and low-intensity displays to further explore these interactions (see Supplementary Appendix 4).

Our data suggest that females prefer lower values of song factor 2 (i.e., more entropic glug 1, and shorter glug 1 and inter-glug intervals) when these songs are paired with low intensity visual displays. Indeed, females began their CSDs earlier ($F_{1,231} = 10.74, P = 0.001$; Figure 2a) to this display combination. In contrast, when paired with high intensity displays, females did not show a preference for any level of first glug entropy and duration or length of the inter-glug intervals (see Figure 2a; $F_{1,243} = 3.41, P = 0.07$). Visual display intensity also changed the attractiveness of song factor 3 in a non-linear fashion (Table 3, Figure 2b, c). Females gave longer CSDs to higher frequency, less entropic and shorter P2s but only when these songs were paired with a high intensity display ($F_{1,215} = 7.8, P = 0.006$); otherwise, if songs were paired with a low intensity display they showed no preference for variation in the P2 element ($F_{1,208} = 0.03, P = 0.87$) (Table 3, Figure 2b). Similarly, CSD latency increased more gradually when a song high in factor 3 was paired with a highly intense visual display ($F_{1,248} = 8.85, P = 0.003$) compared to those paired with a low intensity visual display ($F_{1,238} = 24.65, P < 0.001$) (Figure 2c). This suggests that high intensity visual displays can change the preference level of a relatively less preferred song.

Preference functions of the multimodal signal: visual display as a continuous variable

The relationship between female CSD duration and visual display factor 1 was significantly non-linear (Table 4); this relationship suggests that females prefer the extremes of this factor, either relatively low or high amounts of body puffing and wing-extension. Moreover, we found a significant interaction between this visual display factor and song potency on CSD latency (Table 4, Figure 2d). Overall, females also preferred the extremes of this trait, beginning their CSDs earlier to the relatively high or low amounts of body puffing and wing extension (Figure 2d); however, these trends also differed depending on the song potency. Females preferred higher degrees of puffing and wing extension if the song had low potency ($F_{1,221} = 6.04, P = 0.02$) but display factor 1 did not affect CSD latency if the song was highly potent ($F_{1,256} = 0.86, P = 0.34$) (see Appendix 4). These results corroborated previous results that females place the greatest emphasis on the song, and preferred songs that are high potency, but that female preferences could be modified depending on the specific characteristics of the visual display.

Females began their CSDs earlier to longer visual display durations and deeper bows (i.e., higher values of visual display factor 2; Table 4). Visual display factor 2 also significantly interacted with song potency to affect CSD duration (Table 4, Figure 2e). Females preferred high potency songs paired with visual displays with high factor 2 scores ($F_{1,240} = 26.34, P < 0.001$) but showed no preference for factor 2 ($F_{1,221} = 1.49, P = 0.22$) when the visual displays were paired with low potency songs (Figure 2e; Table 4; Supplementary Appendix 4). This suggests females preferred a potent song paired with a longer display and deeper bow compared to a shorter display and a shallower bow.

Across all our models, covariates related to the experimental design (e.g., block, trial day, and experimental order) were significant for both CSD duration and latency. Female estrogen

Table 2
Factors influencing the initiation of a CSD

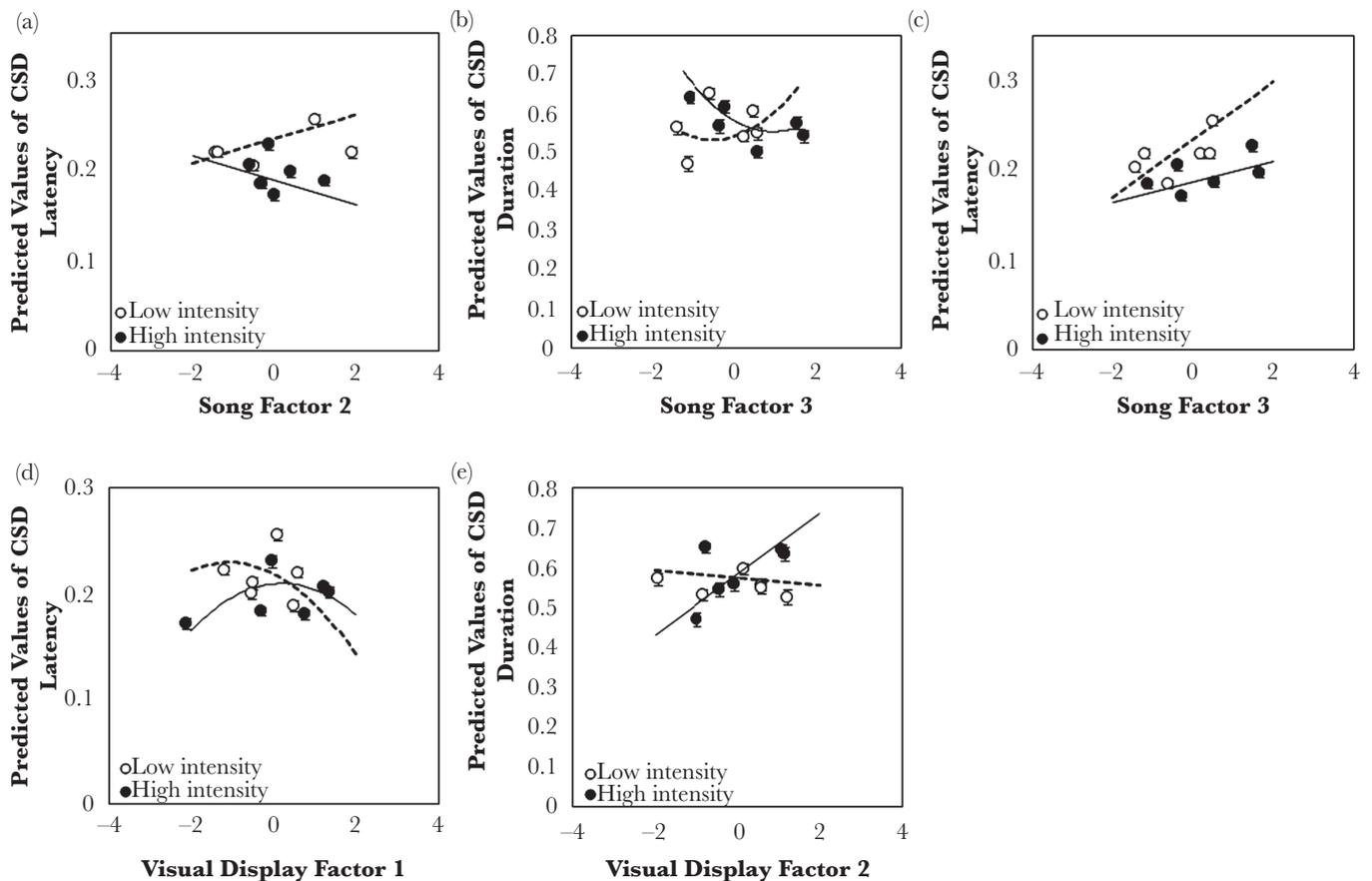
Effect	CSD Initiation	Slope (β)
Song factor 1	$F_{1,683} = 0.12, P = 0.72$	0.02 ± 0.05
Song factor 2	$F_{1,683} = 2.64, P = 0.10$	-0.08 ± 0.05
Song factor 3	$F_{1,683} = 0.02, P = 0.90$	-0.006 ± 0.05
Visual intensity	$F_{1,683} = 0.45, P = 0.50$	
Female mass	$F_{1,683} = 4.3, P = 0.04$	0.04 ± 0.02
Experimental block	$F_{6,683} = 1.12, P = 0.35$	
Trial day	$F_{1,683} = 3.41, P = 0.07$	-0.02 ± 0.01
Experimental order	$F_{1,683} = 0.9, P = 0.34$	-0.01 ± 0.01
Log estrogen (block)	$F_{7,683} = 0.99, P = 0.44$	
Effect	CSD Initiation	Slope (β)
Song potency	$F_{1,684} = 0.12, P = 0.73$	
Visual display factor 1	$F_{1,684} = 0.14, P = 0.71$	0.02 ± 0.05
Visual display factor 2	$F_{1,684} = 0.48, P = 0.49$	0.03 ± 0.05
Female mass	$F_{1,684} = 4.46, P = 0.04$	0.04 ± 0.02
Experimental block	$F_{6,684} = 1.10, P = 0.36$	
Trial day	$F_{1,684} = 3.75, P = 0.05$	-0.02 ± 0.01
Experimental order	$F_{1,684} = 0.96, P = 0.33$	-0.01 ± 0.01
Log estrogen (block)	$F_{7,684} = 0.98, P = 0.45$	

Results from a generalized linear mixed model showing that neither song potency nor visual display intensity affects the likelihood that a female will respond with a CSD. Bold values indicate statistical significance.

Table 3**CSD duration and latency for song factors and categorically described visual display**

Effect	Log CSD Duration	Slope (β)	Log CSD Latency	Slope (β)
Song factor 1				
G1 frequency				
G2 frequency, entropy, duration	$F_{1,440} = 18.4, P < 0.001$	0.03 ± 0.007	$F_{1,465} = 15.98, P < 0.001$	-0.01 ± 0.004
Song factor 1*song factor 1	$F_{1,454} = 11.91, P < 0.001$	0.03 ± 0.01	—	—
Song factor 2				
G1 entropy (-) and duration	$F_{1,464} = 8.92, P = 0.003$	-0.02 ± 0.008	$F_{1,495} = 0.01, P = 0.98$	-0.01 ± 0.008
IGI duration				
Song factor 3				
P2 frequency (-), entropy, duration	$F_{1,458} = 2.10, P = 0.15$	-0.06 ± 0.01	$F_{1,484} = 33.72, P < 0.001$	0.01 ± 0.005
Song factor 3*song factor 3	$F_{1,447} = 8.79, P = 0.003$	0.08 ± 0.02	—	—
Song factor 2*visual intensity	—	—	$F_{1,472} = 8.61, P = 0.004$	HI = -0.01 ± 0.008 LI = 0.01 ± 0.009
Song factor 3*visual intensity	$F_{1,447} = 10.09, P = 0.002$	HI = -0.06 ± 0.01 LI = 0.04 ± 0.03	$F_{1,488} = 6.64, P = 0.01$	HI = 0.01 ± 0.005 LI = 0.03 ± 0.008
Visual intensity	$F_{1,468} = 6.35, P = 0.01$	—	$F_{1,495} = 42.62, P < 0.001$	—
Experimental block	$F_{6,111} = 5.20, P < 0.001$	—	$F_{6,125} = 8.72, P < 0.001$	—
Trial day	$F_{1,308} = 44.39, P < 0.001$	-0.01 ± 0.002	$F_{1,286} = 5.5, P = 0.02$	0.002 ± 0.001
Experimental order	$F_{1,346} = 37.31, P < 0.001$	-0.01 ± 0.002	$F_{1,340} = 11.97, P < 0.001$	0.003 ± 0.0008
Log estrogen	—	—	$F_{1,127} = 12.35, P < 0.001$	-0.06 ± 0.02
Log estrogen (block)	$F_{7,111} = 5.65, P < 0.001$	—	—	—

Statistical model of female mate choice using categorical descriptors of the male visual display and continuous descriptors of the song. Bold values indicate statistical significance. High intensity (HI) and low intensity (LI) visual display slopes are indicated for any significant interactions between visual display intensity and song.

**Figure 2**

Significant interaction effects between song potency and visual display intensity. Interactions between song factor 2 and visual display intensity on CSD latency (a), song factor 3 and display intensity on CSD duration (b) and CSD latency (c), and between visual display factor 1 and song potency on CSD latency (d) and visual display factor 2 and song potency on CSD duration (e). Dashed lines are the predicted functions for low intensity or low potency displays; solid lines are the predicted functions for high intensity or high potency displays. Functions were generated from the solution for fixed effects in Proc MIXED. Standard error bars were generated from the predicted values of the dependent variable.

Table 4
CSD duration and latency for visual display factors and categorically-described song

Effect	Log CSD Duration	Slope (β)	Log CSD Latency	Slope (β)
Visual display factor 1				
Body “puffing”, wing extension, song, and wing pump beginning	$F_{1,449} = 2.31, P = 0.13$	-0.01 ± 0.008	$F_{1,478} = 3.27, P = 0.07$	0.004 ± 0.004
Visual display factor 1* Visual display factor 1	$F_{1,452} = 12.74, P < 0.001$	0.03 ± 0.008	$F_{1,483} = 7.28, P = 0.007$	-0.009 ± 0.003
Visual display factor 2				
Display duration, bow depth	$F_{1,476} = 20.43, P < 0.001$	0.08 ± 0.01	$F_{1,509} = 36.81, P < 0.001$	-0.02 ± 0.004
Song potency	$F_{1,453} = 0.28, P = 0.59$		$F_{1,487} = 1.58, P = 0.21$	
Visual display 2*song potency	$F_{1,446} = 34.13, P < 0.001$	HP: 0.08 ± 0.01 LP: -0.009 ± 0.01	—	—
Visual display 1*song potency	—	—	$F_{1,486} = 9.17, P = 0.003$	HP: 0.003 ± 0.004 LP: -0.02 ± 0.008
Experimental block	$F_{6,108} = 5.01, P < 0.001$		$F_{6,127} = 8.72, P < 0.001$	
Trial day	$F_{1,312} = 42.52, P < 0.001$	-0.01 ± 0.002	$F_{1,288} = 4.7, P = 0.03$	0.002 ± 0.001
Experimental order	$F_{1,345} = 39.29, P < 0.001$	-0.01 ± 0.002	$F_{1,341} = 11.89, P < 0.001$	0.003 ± 0.0008
Log estrogen	—		$F_{1,128} = 12.59, P < 0.001$	-0.06 ± 0.02
Log estrogen (block)	$F_{7,107} = 5.39, P < 0.001$		—	

Statistical model of female mate choice using categorical descriptors of the male song and continuous descriptors of the visual display. Bold values indicate statistical significance. High potency (HP) and low potency (LP) song slopes are indicated for any significant interactions between song potency and visual display intensity.

concentration (nested within a block for our models of CSD duration) was significant for CSD duration and latency (Tables 3 and 4). Females started CSDs earlier and gave longer CSDs at the beginning of the day (i.e., experimental order) and at the beginning of the experiment (i.e., trial day; Tables 3 and 4). This suggests that female motivation to mate decreased as the experiment continued, both within and across days. Estrogen concentrations were typically (in 5 of 7 blocks) positively related with CSD duration and negatively related to the latency to begin a CSD. This indicates that females with higher estrogen levels tended to be more motivated to mate.

DISCUSSION

We found that the intensity of a visual display can modify how attractive a song is for females. In principle, this finding supports our prediction that the visual and acoustic signal components are non-redundant and modulate each another in this species (Candolin 2003; Hebets and Papaj 2005; Partan and Marler 2005). In addition, we found that female preference functions can deviate from linearity. These findings, in combination with past work (O’Loghlen and Rothstein 2010a; O’Loghlen and Rothstein 2012), suggest that cowbird sexual signals follow the modulation with predominance hypothesis. However, in our analysis of the shape of preference functions, we also found that the attractiveness of a visual display and song depends on the quality of the song or visual display that it is paired with. These results are contrary to an implicit assumption in the models tested here: that the attractiveness ranking of a signal component can be defined independently of the other signalling modality.

Our analysis of female preference function shape shows that female preferences for visual display intensity or song potency can change depending on the alternative signalling trait we are examining. Our data show a switch in the direction of female preference functions in a multimodal context. Indeed, we found that females preferred high potency songs paired with longer, deeper bows, but did not show a preference for display intensity if the songs were lower potency. However, when we examined a different feature of

visual display intensity (i.e., higher degrees of puffing and wing extension), females preferred these more intense displays with a lower potency song while this visual display factor did not affect preference if the song was highly potent.

Interestingly, the fact that in some conditions females preferred more intense visual displays goes against previous work in cowbirds (O’Loghlen and Rothstein 2012). This discrepancy may be explained by the fact that we only used female-directed visual displays (of high and low intensity) in the creation of the experimental videos, but previous work (O’Loghlen and Rothstein 2012) used both male-directed (high intensity) and female-directed (low intensity) visual displays. Perhaps female cowbirds can distinguish between male- and female-directed displays independently of the measured intensity of those displays. Overall, these data show that different characteristics of display intensity (amount of wingspread/puffing versus depth of the bow) affected the attractiveness of songs in different ways. This highlights the importance of decomposing a complex multimodal signal to understand how different features can influence female preferences and subsequent selection on mate traits.

This “switching” of mate preferences is reminiscent of mate-choice in female túngara frogs where unattractive signal components (i.e., temporally displaced vocal sac inflation and “whine-chuck” vocalization) generated by a frog robot combined in such a way that the components were “perceptually rescued” to create an attractive multimodal signal (Taylor and Ryan 2013). The Taylor and Ryan (2013) study is inherently different from ours, however, because 1) the multimodal combination of signals the authors used would not be found in nature as vocal sac inflation is “fixed” to the acoustic signal (Higham and Hebets 2013) and 2) the attractiveness of the signal was manipulated by changing the relative timing of the signalling components rather than changing the quality of the signal within a single modality independent of the other modality. Together, however, our findings challenge the way we should think about the honesty of complex signals as signal component values may not combine additively or even multiplicatively, but rather interact in such a way as to change the relative attractiveness of the entire multimodal signal. This raises a very

important question for future research: are signalling quality and honesty correlated across separate sensory modalities?

Evidence in cowbirds suggests that the song and visual display co-evolved via a mechanical trade-off between the 2 signalling components such that the most intense portions of the visual display occur during the silent portions of the song (Cooper and Goller 2004). However, this is not to say that cowbirds have fixed multimodal signals. Males have multiple perched songs that can vary in potency (West et al. 1981) and males can decide when to use their potent songs to reduce the degree of intrasexual aggression they experience (West and King 1980). Moreover, cowbird visual displays are also highly variable in their degree of intensity (O'Loughlen and Rothstein 2010b; O'Loughlen and Rothstein 2012). This variability in signalling, along with our findings on flexible female preferences, suggests that cowbird sexual signals may not be under directional selection as previously suggested (O'Loughlen and Rothstein 2012). Rather, selection may favour males that possess a range of different songs and visual displays that can be used strategically during different social contexts (Freeberg et al. 2012; White et al. 2012). Social and habitat structure are expected to vary across different populations; this may then contribute to differential signal use and the possible formation of dialects within a species. Interestingly, cowbirds are a well-known example of a species with dialects across their native range (Rothstein et al. 1986).

Much of this flexibility in male signals and preferences may have evolved to communicate with a variety of different receivers, males and females alike, which make up the complex social network of this species (Rothstein et al. 1986; White et al. 2012). This is perhaps more evidence supporting the social-complexity hypothesis where males are selected for their ability to respond appropriately in different social conditions rather than the overall quality of their signals per se (Freeberg et al. 2012; White et al. 2012). Indeed, White et al. (2012) found that cowbirds in static versus dynamic social conditions had different relationships between signal use and reproductive success. Males in static groups had a predictable strategy: those who invested more in singing behaviour also achieved high reproductive success. In contrast, males in dynamic social groups did not adapt a particular courtship strategy to attract females (White et al. 2012). Overall, the attractiveness of a multimodal signal may be social-context specific.

Dynamic signals like the cowbird display where the sender has immediate control over the signal are expected to evolve in scenarios where the physical and social environment is changing quickly, while multiple static signals are more likely to evolve when redundant signals are needed across different contexts (Bro-Jørgensen 2010). A recent study on 4 species of *Sceloporus* lizards proposed that variable predation pressure resulted in an evolutionary shift in the use of a static colour display towards the use of a dynamic motion-based head-bob display (Martins et al. 2015). Nevertheless, it is still unknown whether non-redundant multimodal signals are more likely to be comprised of dynamic rather than static components. Determining whether this is the case may help researchers identify which systems are more likely to have inter-signal interactions and potential switches in the strength and direction of female preferences.

We show here that changes in female preference function shape through modulation of one signal component by another can alter the strength of selection acting on both components of the male multimodal signal. This suggests that there are thresholds in signal production and attractiveness below and above which females use alternative signalling modalities to make their mate-choice

decisions. Identifying these thresholds in non-redundant multimodal signals will allow us to predict which modality contains the most reliable signal on mate quality to the receiver. This will be an important step forward towards one of the biggest challenges in signal evolution studies: identifying the components of a complex signal that are under the strongest selection (Girard et al. 2015; Wilkins et al. 2015).

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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Data accessibility: Analyses reported in this article can be reproduced using the data provided by Ronald et al. (2017).

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