1 **Behavioural changes in aposematic *Heliconius melpomene* butterflies in response to**

2 **their predatory bird calls**

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9 Running title: Behavioural change in response to predatory bird calls in *Heliconius*

10 *melpomene* butterfly

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Abstract:

Prey-predator interactions have resulted in the evolution of many anti-predatory traits. One of them is the ability for prey to listen to predators and avoid them. Although prey anti-predatory behavioural responses to predator auditory cues are well described in a wide range of taxa, studies on whether butterflies change their behaviours in response to their predatory calls are lacking. *Heliconius* butterflies are unpalatable and form Müllerian mimicry rings as morphological defence strategies against their bird predators. Like many other butterflies in the *Nymphalidae* family, *Heliconius* butterflies possess auditory organs, which are hypothesized to have evolved to assist with predator detection. Here we test whether *Heliconius melpomene* change their behaviour in response to their predatory bird calls by observing the behaviour of male and female *H. m. plessini* exposed to calls of *Heliconius* avian predators: rufous-tailed jacamar, migratory Eastern kingbird, and resident tropical kingbird. We also exposed them to the calls of the toco toucan, a frugivorous bird as a control bird call, and an amplified greenhouse background noise as a noise control. We found that individuals changed their behaviour in response to jacamar calls only. Males increased their walking and fluttering behaviour, while females did not change their behaviour during the playback of the jacamar call. Intersexual behaviours like courtship, copulation, and abdomen lifting did not change in response to bird calls. Our findings suggest that despite having primary predatory defences like toxicity and being in a mimicry ring, *H. m. plessini* butterflies changed their behaviour in response to predator calls. Furthermore, this response was predator specific, as *H. m.*

44 *plesseni* did not respond to either the Eastern kingbird or the tropic kingbird calls. This

45 suggests that *Heliconius* butterflies may be able to differentiate predatory calls, and

46 potentially the birds associated with those calls.

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48 Key words: auditory cues, Lepidoptera, aposematism, toxic, neotropics, bird

49 vocalizations

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51 **Highlights:**

52 1. Many prey animals change their behaviour in response to their predator's calls.

53 2. Whether butterflies alter behaviour in response to bird predator calls is unknown.

54 3. We show that *Heliconius melpomene* change behaviour in response to jacamar calls.

55 4. Males increased walking and fluttering, but did not alter courting behaviour.

56 5. *H. melpomene* did not respond to predatory Eastern kingbird or tropical kingbird calls.

57

58 **Introduction:**

59   Predation is a ubiquitous interspecific interaction in almost all ecosystems and can

60 be a strong evolutionary force for the emergence and selection of prey anti-predatory

61 strategies that increase survival (Lind & Cresswell, 2005). Anti-predatory strategies are

62 widespread in prey animals and can be morphological or behavioural. Morphological

63 strategies include aposematism, chemical toxicity, and crypsis (Rojas et al., 2019; Vallin

64 et al., 2006), while behavioural anti-predatory strategies include active evasion of

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65   predatory attacks, and behaviours that decrease detection (Palmer & Packer, 2021). Anti-

66   predatory strategies can also be a combination of both morphological and behavioural

67   strategies such as the deimatic displays in mountain katydid *Acripeza reticulata* and

68   swallowtail butterflies (Olofsson et al., 2012; Umbers & Mappes, 2015).

69   The most common anti-predatory strategies are behavioural responses to detection

70   and active attacks by predators. These behavioural responses help prey escape predation,

71   either in the absence of morphological defences, or as a combination with morphological

72   defences, and can be highly variable across species, within species, and between sexes

73   (Apfelbach et al., 2005; Lind & Cresswell, 2005). Some species, such as desert isopods

74   (*Hemilepistus reaumuri*), freeze and retreat inside their burrows upon smelling their

75   predator's scent (Zaguri & Hawlena, 2020); while others, such as male tree lizards

76   (*Urosaurus ornatus*) actively escape by fleeing after detecting their predators (Thaker et

77   al., 2009). Anti-predatory behaviours can also differ within species in response to

78   different predators, as illustrated by red squirrels (*Tamiasciurus hudsonicus*), which have

79   different alarm calls for avian predators and ground predators (Greene & Meagher, 1998).

80   Anti-predatory behavioural responses can also be sex-specific, either due to the inherent

81   sex-specific differences in physiology and behaviour, or due to the increased vulnerability

82   of predation during intraspecific sexual behaviours (Curlis et al., 2016; Edomwande &

83   Barbosa, 2020; Lea & Blumstein, 2011; Sitvarin & Rypstra, 2012; Wormington &

84   Juliano, 2014). Both males and females are known to alter their courtship and mating

85   behaviours under predation risk (Acharya & McNeil, 1998; Torsekar et al., 2019). In wolf

86 spider (*Schizocosa ocreata*), males cease courtship behaviour after detecting predatory

87 birds' calls and take longer to return to courting compared to non-threatening control

88 sounds (Lohrey et al., 2009), while female túngara frogs (*Physalaemus pustulosus*)

89 approach calling males more cautiously when exposed to bat wingbeat sounds (Bernal et

90 al., 2007). The cost of predation during intraspecific sexual behaviours such as courtship

91 and copulation is high, forcing individuals to switch from sexually oriented behaviours to

92 survival behaviours.

93 Detecting and recognizing predatory cues are necessary for active predator

94 avoidance behaviours, and these cues can either be visual, chemical, vibrational, or

95 auditory. Auditory cues play a vital role in predator avoidance by prey animals, from

96 invertebrates (Faure & Hoy, 2000; Jacobs et al., 2008; Lohrey et al., 2009; Prakash et al.,

97 2021; Rosen et al., 2009; Triblehorn et al., 2008) to vertebrates (Bernal et al., 2007;

98 Cantwell & Forrest, 2013; Deecke et al., 2002). In Lepidoptera (moths and butterflies),

99 anti-predatory behaviours in moths to predators' auditory cues have been extensively

100 studied under various ecological contexts. Moths have evolved hearing to detect bat

101 echolocation calls and avoid bat predation by performing aerial manoeuvrers and

102 jamming echolocation calls (Conner & Corcoran, 2012). Both male and female moths

103 also reduce sexual activity under bat predation pressure (Acharya & McNeil, 1998;

104 Edomwande & Barbosa, 2020).

105 While moths are particularly well known for their hearing ability and anti-predator

106 behaviours, butterflies, their day-flying relatives, are also known to have auditory organs,

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107 which may be sensitive to predator sounds (Lane et al., 2008). In particular, many species

108 in the family *Nymphalidae* possess auditory organs on their wings, such as the blue

109 morpho *Morpho peleides* (Lane et al., 2008; Lucas et al., 2009; Mikhail et al., 2018),

110 common wood nymph *Cercyonis pegala* (Sun et al., 2018), the owl butterfly *Caligo*

111 *eurilochus* (Lucas et al., 2014), butterflies from the genus *Erebia* (Ribarič & Gogala,

112 1996), and *Heliconius* butterflies (Swihart, 1967). However, unlike moths, it is generally

113 unknown whether butterflies that possess auditory organs change their behaviour in

114 response to their predator's vocalizations. In this study, we used a butterfly from the

115 genus *Heliconius* to test whether these butterflies change their behaviour in response to

116 their predator's vocalizations.

117   *Heliconius* butterflies (Family Nymphalidae), found in North, Central, and South

118 America, are toxic, unpalatable, display aposematic colouration, form Müllerian mimicry

119 rings, and roost communally to avoid bird and bat predation (Engler-Chaouat & Gilbert,

120 2007; Finkbeiner et al., 2012; Mallet & Gilbert, 1995; Pinheiro De Castro et al., 2019).

121 Despite these anti-predatory strategies, *Heliconius* butterflies are vulnerable to predation

122 by specialist bird predators, as well as by naïve generalist predatory birds; and their

123 mortality is higher when young birds are learning which butterfly species are toxic and

124 should be avoided (Chai, 1986; Langham, 2004, 2006; Pinheiro, 1996; Pinheiro & Cintra,

125 2017). Hence, it may be evolutionarily advantageous for *Heliconius* butterflies to detect

126 the presence of their bird predators and change their behaviours to reduce detection,

127 despite having multiple anti-predatory strategies. One possible way these butterflies

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128   could detect the presence of their bird predators is by using avian vocalization cues,

129   which are often species specific (Lane et al., 2008; Lucas et al., 2009; Mikhail et al.,

130   2018). In *Heliconius* butterflies, hearing organs located at the base of the hindwing with

131   peak sensitivity between 0.5 to 4 KHz at 70-90 dB pressure have been described (Swihart

132   1967). However, the hypothesis that *Heliconius* butterflies change their behaviour in

133   response to their predatory birds' vocalizations has never been tested.

134   In this study, we tested whether *Heliconius melpomene plessini* butterflies change

135   their behaviour in response to the vocalizations of their known bird predators. We first

136   tested butterfly response to the vocalizations of two predatory birds with disparate calls

137   as well as the vocalization of a frugivorous bird, to assess whether *H. m. plesseni*

138   butterflies respond to both predator bird calls and calls of non-predatory birds. After

139   answering that question, we then tested the response of *H. m. plessini* butterflies to

140   predators that differ in annual patterns of predation (year-round resident or migratory), to

141   assess whether strength of *H. m. plesseni* response is associated with degree of annual

142   avian predator exposure. During both these experiments, we also tested whether

143   intraspecific sexual behaviours like male courtship and female acceptance/rejection

144   behaviours changed in response to *H. m. plessini's* bird predatory calls.

145

146   **Materials and Methods**

147   ***Study species husbandry***

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148   *Heliconius melpomene* (Order: *Lepidoptera*, Family: *Nymphalidae*), is native to

149   Central and South America. The subspecies *H. m. plessini* is found in the mountainous

150   forests of Ecuador and Peru in South America (Hines et al., 2011). Live pupae of *H. m.*

151   *plessini* were shipped from Ecodecision Heliconius Works in Quito, Ecuador to the

152   University of Arkansas Biology greenhouse facility in Fayetteville AR, USA, where they

153   were maintained at an average temperature of 27°C, average relative humidity of 70%

154   and a 13:11 hour L:D cycle, to mimic summer tropical conditions. All pupae were hung

155   and housed in mesh BioQuip cages (34.29 x 34.29 x 60.96 cm, Rancho Dominguez, CA,

156   U.S.A.) until their eclosion in the greenhouse facility. Newly eclosed individuals were

157   sexed and tagged with a unique number with a silver metallic permanent marker

158   (SHARPIE 39108PP) and placed in sex-specific mesh BioQuip cages (60.96 x 60.96 x

159   142.24 cm) with *ad libitum* BIRDS choice butterfly nectar (Birdschoice, Chilton, WI,

160   USA) and pollen from *Lantana spp* flowers. Marking butterflies with a marker does not

161   have long term effects on their behaviour and lifespan (Gall, 1984). Female *H. m. plessini*

162   were housed with females of two other subspecies, *H. m. malleti* and *H. m. rosina* while

163   male *H. m. plessini* were housed on their own. Both the male and female cages were

164   visually isolated from the opposite sex and had no more than 15 individuals in each sex

165   specific cage at any point in time.

166

167   ***Bird calls and control treatments***

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168   We used the calls of four different bird species during our experiments: three

169   *Heliconius* predators and one frugivore as a control species. Our predatory bird species

170   were the rufous-tailed jacamar (*Galbula ruficauda*), Eastern kingbird (*Tyrannus*

171   *tyrannus*), and tropical kingbird (*Tyrannus melancholicus*) (Pinheiro, 1996, 2011;

172   Pinheiro & Cintra, 2017). We used the non-predatory toco toucan (*Ramphastos toco*) call

173   to test if *H. m. plessini* respond to bird calls in general, and amplified greenhouse

174   background noise as a random noise control. We chose toco toucan as our control bird

175   call because it is a non-predatory frugivorous bird found in the same habitat as our focal

176   butterflies and has a naturally loud call. Playback recordings of the four bird calls

177   (rufous-tailed jacamar, Eastern kingbird, tropical kingbird and toco toucan) with minimal

178   disturbance from background animals were downloaded from Xeno-Canto (Xeno-Canto

179   Foundation; www.xeno-canto.org) (for sonograms of all calls see Supplementary Figure

180   2). These bird calls were characterized as 'songs' in the original files uploaded on Xeno-

181   Canto. All the bird calls contain elements within previously reported *Heliconius* hearing

182   frequency 1-4 KHz (Swihart, 1967), though the main components of the kingbird calls'

183   are just outside that range at 5 KHz (Supplementary Figure 2).

184   The University of Arkansas butterfly facility has constant and continuous noise

185   generated by fans and misters which were measured at 65 dB near the behavioural watch

186   cage using an android sound meter application (Sound Meter-Decibel and noise Meter).

187   To account for any butterfly behavioural responses to this background noise, or to loud

188   noises in general, we recorded the greenhouse noise using the android voice recorder

189   application (Voice Recorder, version 3 (42.0)) and used this recording in behavioural

190   assays as a greenhouse background noise control. During the behavioural assays, the calls

191   of rufous-tailed jacamar (76 dB), Eastern kingbird (79 dB), tropical kingbird (80 dB),

192   toco toucan (80 dB) and the greenhouse background noise control (77 dB) were played at

193   10-15 dB louder than the actual greenhouse background noise. Bird calls in forests are

194   always against a naturally generated background noise (by other animals; leaves rustling,

195   waterfalls, and streams). While our constant greenhouse background noise is admittedly

196   different from that of a forest, the presence of background noise broadly emulates such

197   sounds generated in the forest. All calls were standardized to one minute long .mp3 files.

198

199   *Behavioural Assays*

200   All behavioural assays were conducted between 11:00 AM and 2:00 PM, when *H.*

201   *melpomene* are most active in our greenhouse (Rather et al., 2022). We conducted

202   behavioural assays using 3-15-day-old males and females in a large behavioural cage

203   (60.96 x 60.96 x 142.24 cm). In each assay, we used one male and one female and

204   acclimated them in the behavioural cage for 15 minutes with a JBL$_®$ Flip 4 portable blue-

205   tooth speaker (Harman) and a *Lantana spp.* plant. We used both a male and a female in

206   our behavioural assay to determine whether predatory bird calls had an effect on

207   intersexual behaviours (*courtship, abdomen lifting, copulation and, sitting near*) in

208   addition to any other types of behaviour (*wing fluttering, antennae wiggling, basking,*

209   *flying, resting, walking*). After a 15-minute acclimation period, we recorded all the

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210 behaviours performed by the two individuals in the assay for 15 minutes prior to any

211 playback calls. We then played one of the bird calls or the control greenhouse background

212 noise using a JBL$_®$ Flip 4 portable blue-tooth speaker from the observer's phone (Google

213 Pixel), placed inside the behavioural cage for 1 minute and recorded the behaviours of the

214 two individuals during the playback of the call/background noise. After the playback, we

215 recorded the behaviours of the two individuals for an additional 14 minutes

216 (Supplementary Figure 1). We recorded the frequency of *fluttering* and *antenna wiggle*

217 behaviours and the frequency and duration of *basking, flying, resting, walking, courtship,*

218 *copulation, abdomen lifting,* and *sitting near each other* behaviours throughout the entire

219 30-minute observational period.

220 We defined behaviours for *H. m. plessini* as follows: *fluttering*- opening and closing

221 of wings either while resting or walking; *antenna wiggle*- movement of antennae at 45°

222 angle in any direction (Robertson et al., 2020); *basking*- individuals sitting with wings

223 partially or fully open; *flying*- movement from one point to another in the air using rapid

224 wing flaps; *resting*- individuals sitting with wings fully closed (Rather et al. 2022);

225 *walking*- movement from one point to another along the substrate using the legs;

226 *courtship*- sequences of behaviours where males hover, land and rapidly flap their wings

227 next to females, and bend their abdomen to initiate copulation (Klein & De Araújo,

228 2010); *copulation*- where both male and female are mating; *abdomen lifting*- raising the

229 abdomen at an angle from the normal resting body axis, usually performed by females as

230 a courtship rejection behaviour (Chouteau et al., 2017); *sitting near each other*- where

231 both individuals are resting or basking within one wingspan from each other (Robertson

232 et al. 2020).

233   We used Spectator Go (BIOBSERVE, Fort Lee, NJ, USA) software on an Apple

234 iPad (1st generation) to manually record the frequency and duration of behaviours

235 performed by the two individuals during the assay. This software enables the observer to

236 record user defined behaviours in real time, separately for the two individuals, without

237 instantly visualizing quantities during the recording, and has been used in previous

238 studies to observe and record butterfly behaviours (Rather et al., 2022; Robertson et al.,

239 2020; Westerman et al., 2014). To reduce observer bias, only one observer recorded all

240 the behaviours in this study. We did not use a video camera to record behaviours as some

241 butterfly inter-individual interactions are minute and nuanced happening at a close range,

242 while others occupy the full three-dimensional flight area of the cage, and simultaneously

243 capturing both of these types of behaviours is challenging for a stationary camera, but

244 relatively straightforward for a trained human observer. Within each experiment, we

245 tested each male-female pair with all calls with at least 24 hours between each call assay,

246 and randomized the order of calls for each pair. If either of the butterflies in the pair died

247 between the assays, then those pairs were eliminated from being tested for the remaining

248 calls.

249

250 ***Experiment 1: Do H. m. plesseni butterflies behaviourally respond to predator bird***

251 ***calls***

252   To test whether *H. m. plessini* butterflies respond to their avian predator calls or to

253   other birds or loud random noises in general, we subjected the butterflies to four call

254   treatments in this experiment: rufous-tailed jacamar (N=22 pairs), Eastern kingbirds

255   (N=22 pairs), toco toucan (N=22 pairs) and greenhouse background noise control (N=18

256   pairs) using the behavioural assay described above with the calls randomized. We

257   conducted Experiment 1 from February 2019 to March 2020.

258

259   ***Experiment 2: Does predator residence status influence butterfly response to bird call***

260   Due to the results of Experiment 1 (see below), we conducted a follow up

261   experiment to test whether predator residence status (migratory or present year-round)

262   influenced likelihood of *H. m. plessini* butterflies changing their behaviour in response to

263   predator call. For this experiment, we used the calls of the resident tropical kingbird and

264   the migratory Eastern kingbird, as they have vocalizations in the same auditory

265   frequencies, and are more closely related than the Eastern kingbird and rufous-tailed

266   jacamar. We subjected butterflies to three call treatments: resident tropical kingbird

267   (N=23 pairs), migratory Eastern kingbird (N=22 pairs) and the control greenhouse

268   background noise control (N=25 pairs) using the same behavioural assay as Experiment

269   1, as described above. We conducted Experiment 2 from August to December 2021. We

270   conducted the same statistical analyses for both Experiment 1 and Experiment 2, albeit

271   separately.

272

273 ***Statistical analyses***

274   We downloaded the data from Spectator Go software and converted them into .csv

275 files. Each file consisted of approximately 15 minutes of data, and each assay had four

276 files (15 minutes before, and during plus after call for male and female separately). Each

277 bout of behaviour was recorded separately by the software for the 10 behaviours

278 described above. A *de novo* python code (supplementary material 2) was written to add

279 each bout of a behaviour and provide the total time spent performing that particular

280 behaviour. This way, we got the total time spent by an individual butterfly performing

281 behaviours for the whole assay. Further, we manually extracted the behavioural states

282 before and after the start and end of calls, as well as extracted the behaviours performed a

283 minute before, during and after the calls. We performed three separate analyses for each

284 experiment: behavioural state change between before and after the start and end of calls;

285 short term (1 minute) changes in behaviours between before, during, and after calls; and

286 long term (14 minutes) changes in behaviour before and after calls.

287   To determine whether butterflies changed their behavioural state in response to bird

288 call, we compared the behaviours performed across three time points of an assay: 1)

289 before vs after the start of call; 2) before vs after the end of call; and 3) before start vs

290 after end of the call. We used generalized linear mixed models (GLMM) with change in

291 behaviour between the above time points (yes or no) as the response variable, treatment

292 (calls), and sex (male or female) as fixed predictor variables, and the order of calls as a

293 random predictor variable. We later used a pairwise Fisher's test to determine if the

294   proportion of individuals that changed their behaviours were similar or different between

295   the treatments (bird calls and noise control).

296   To test if the frequency and duration of short-term behaviours changed during and

297   after a call compared to before a call, we extracted the frequency of *fluttering* and

298   *antenna wiggle* and duration of the other eight behaviours for the minute before, minute

299   during, and minute after the call. We performed Principal Component Analysis (PCA) for

300   the behavioural data during these three minutes, to identify the correlation between

301   different behaviours and identify new composite behavioural variables. We removed

302   *abdomen lifting* from the male data set and *courtship* from the female data set as males

303   and females respectively did not perform these behaviours. We fit a linear mixed model

304   (LMMs), followed by an ANOVA, with treatment (bird call), state (before, during, and

305   after call) and their interaction as fixed predictor variables, the order of the calls as a

306   random predictor variable, and the first three principal components as the response

307   variables. Further, we performed a Tukey HSD test to determine the pairwise differences

308   between different combinations of treatment (bird call) and state (before, during, and

309   after call). Later, we tested whether male *courtship, sitting near each other,* female

310   *abdomen lifting*, *copulation* behaviours changed in response to bird calls by fitting

311   LMMs followed by an ANOVA, with the same predictor variables. We ran these models

312   for males and females separately, as males and females performed different behaviours.

313   We also performed these analyses separately for experiments 1 and 2.

15

314   Next, to test if there was a prolonged long term response of butterfly behaviour to

315   the bird calls, we extracted the frequency of *fluttering* and *antenna wiggle* and duration of

316   the other eight behaviours for the 14 minutes before the call and the 14 minutes after the

317   call, and performed a PCA for these 28 minutes, again removing *abdomen lifting*

318   behaviour from male data set and *courtship* behaviour from the female data set. We fit

319   LMM, followed by an ANOVA, with treatment (bird call), state (before, and after call),

320   and their interaction as the fixed predictor variables, the order of the calls as a random

321   predictor variable, and the first three principal components as the response variables for

322   each sex. Further, we performed a Tukey HSD test to determine the pairwise differences

323   between different combinations of treatment (bird call) and state (before, and after call).

324   We also tested whether male *courtship, sitting near,* female *abdomen lifting*, *copulation*

325   behaviours changed in response to bird calls by fitting LMMs followed by an ANOVA,

326   with the same predictor variables. We again ran these models for both males and females

327   separately, and performed these analyses separately for experiments 1 and 2.

328   All statistical analyses were run using R version 4.3.0 (R Core Team, 2023). All

329   plots were generated using *ggplot2* (Wickham, 2016) package.

330

331   **Ethical Note**

332   All butterflies used in this study were maintained in climate-controlled greenhouse

333   conditions similar to those of their natural habitat, as stated in the U.S. Department of

334   Agriculture, Animal and Plant Health Inspection Service permits P526P-17-00343 and

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335   P526P-20-00417. Before and after the assays, all butterflies were maintained in cages

336   with *ad libitum* food (nectar and flowering *Lantana spp.* plants for pollen). After the

337   assays, they were moved to breeding cages with *ad libitum* food, where they were kept

338   until natural death. No butterflies were sacrificed for the purpose of this study.

339

340   **Experiment 1 Results:**

341   ***H. m. plessini* immediately changed their behavioural state in response to the rufous-**

342   **tailed jacamar call**

343   *H. m. plessini* butterflies immediately changed their behavioural state when the

344   rufous-tailed jacamar call started ($\chi2= 16.03$, $p<0.01$; Supplementary Figure 3A;

345   Supplementary Table 1, 2), when the jacamar call stopped ($\chi2= 17.47$, $p<0.001$;

346   Supplementary Figure 4A; Supplementary Table 3, 4), and when compared between

347   before the call started versus after the call ended ($\chi2= 27.12$, $p<0.001$, Table 1, 2, Figure

348   1A). They did not significantly change their behavioural state in response to any other

349   bird call, or in response to the noise control (Supplementary Table 1, 2, 3, 4; Table 1, 2).

350   We did not find an effect of sex on the change in behavioural state when the calls started,

351   when the calls stopped, or when compared between before the calls started versus after

352   the calls ended nor was there an effect of call order on butterfly response (Supplementary

353   Table 1, 3; Table 1).

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355 ***H. m. plessini* males increased their walking and fluttering behaviour during the**

356 **playback of the rufous-tailed jacamar call**:

357   When combining the behavioural data for the 3 minutes before, during, and after

358 each call in a PCA for each sex, male PC2 values were higher during the rufous-tailed

359 jacamar call compared to before and after the rufous-tailed jacamar call, and compared to

360 before, during, and after the Eastern kingbird, toco toucan, and greenhouse background

361 noise (ANOVA, $F= 2.336$, $Df= 6$, $p= 0.0328$; Figure 2C; Table 3; Supplementary Table 7;

362 see Supplementary Table 5 for PCA loadings). There was no effect of any of the bird calls

363 or greenhouse background noise control on male PC1 (Figure 2A; Table 3;

364 Supplementary Table 7, 8), male PC3 (Table 3; Supplementary Table 7), female PC1

365 (Figure 2B; Table 3; Supplementary Table 7; see Supplementary Table 6 for PCA

366 loadings), female PC2 (Figure 2D; Table 2; Supplementary Table 7), or female PC3

367 (Table 2; Supplementary Table 7).

368

369 ***H. m. plessini* males and females had no long-term changes in behaviour in response**

370 **to calls**:

371   When combining the behavioural data for the 14 minutes before and after each call

372 in a PCA for each sex, there was no effect of any of the bird calls or greenhouse

373 background noise control on male PC1 (Supplementary Figure 5A; Supplementary Table

374 10, 11; see Supplementary Table 8 for PCA loadings), male PC2 (Supplementary Figure

375 5C; Supplementary Table 10, 11), male PC3 (Supplementary Table 10, 11); female PC1

376   (Supplementary Figure 5B; Supplementary Table 10, 11; see Supplementary Table 9 for

377   PCA loadings), female PC2 (Supplementary Figure 5D; Supplementary Table 10, 11), or

378   female PC3 (Supplementary Table 10, 11).

379

380   **No effect of predatory bird calls on *H. m. plessini* intersexual behaviours**

381   Male *courtship, sitting near each other,* female *abdomen lifting, copulation*

382   behaviours did not have short-term or long-term changes in response to any bird calls

383   (Table 3; Supplementary Table 10).

384

385   **<u>Experiment 2 results:</u>**

386   While there are a number of hypotheses as to why *H. m. plessini* did not change

387   their behaviour in response to the migratory Eastern kingbird calls, but did change their

388   behaviours in response to the resident jacamar calls, two we found particularly interesting

389   were 1) that jacamars are year round residents while Eastern kingbirds are migratory; and

390   2) jacamars and Eastern kingbirds have different call frequencies (Hz ranges). To test the

391   hypothesis that residence status is driving *H. m. plesseni* behavioural response while

392   holding call frequency (Hz) constant, we then tested whether *H. m. plessini* butterflies

393   changed their behaviour in response to the resident tropical kingbird call compared to the

394   migratory Eastern kingbird call in Experiment 2, as these two kingbird species have

395   vocalizations in the same auditory frequency ranges (Supplementary Figure 2).

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19

Residence status of kingbirds did not change *H. m. plessini* behavioural state:

We found that *H. m. plessini* butterflies did not change their behavioural state when either of the resident or migratory kingbird calls or greenhouse background noise started (Supplementary Figure 3B; Supplementary Table 12, 13), when either of the kingbird calls or greenhouse background noise stopped (Supplementary Figure 4B, Supplementary Table 14, 15), and when compared between before the kingbird calls started versus after the kingbird calls ended, as well as between before the start and after the end of greenhouse background noise (Figure 1B; Supplementary Table 16, 17). We did not find an effect of sex on the change in behavioural state when calls started, when the calls stopped, and when compared between before the calls started versus after the calls ended nor was there an effect of call order on butterfly response (Supplementary Table 12, 14, 16).

Residence status of kingbirds did not change short-term *H. m. plessini* behaviours:

When combining the behavioural data for the 3 minutes before, during, and after each call in a PCA for each sex, there was no effect of any kingbird calls or greenhouse background noise control on male PC1 values (Figure 3A; Supplementary Table 20, 21; see Supplementary Table 18 for PCA loadings), on male PC2 (Figure 3C; Supplementary Table 20, 21), male PC3 (Supplementary Table 20, 21), female PC1 (Figure 3B; Supplementary Table 20, 21; see Supplementary Table 19 for PCA loadings), female PC2

417   (Figure 3D; Supplementary Table 20, 21), and female PC3 (Supplementary Table 20, 21)

418   values.

419

420   **Residence status of kingbirds did not change long-term *H. m. plessini* behaviours:**

421   When combining the behavioural data for the 14 minutes before and after each call

422   in a PCA for each sex, there was no effect of any of the kingbird calls or greenhouse

423   background noise control on male PC1 (Supplementary Figure 6A; Supplementary Table

424   24, 25; see Supplementary Table 22 for PCA loadings), male PC2 (Supplementary Figure

425   6C; Supplementary Table 24, 25), male PC3 (Supplementary Table 24, 25); female PC1

426   (Supplementary Figure 6B; Supplementary Table 24, 25; see Supplementary Table 23 for

427   PCA loadings), female PC2 (Supplementary Figure 6D, Supplementary Table 24, 25), or

428   female PC3 (Supplementary Table 24, 25).

429

430   **No effect of predatory kingbird calls on *H. m. plessini* intersexual behaviours**

431   Male *courtship, sitting near each other,* female *abdomen lifting, copulation*

432   behaviours did not have short-term or long-term changes in response to any bird calls

433   (Supplementary Table 20, 24).

434

435   **Discussion:**

436   *Heliconius melpomene plessini* butterflies changed their behaviour in response to

437   predatory rufous-tailed jacamar calls but did not change their behaviour in response to

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438   predatory Eastern kingbird or tropical kingbird calls. We found a sex-specific difference

439   in behaviour, where males, but not females, increased their fluttering and walking

440   behaviours during the playback of the rufous-tailed jacamar calls. The observed

441   behavioural changes in response to rufous-tailed jacamar calls are short-term and do not

442   persist over an extended duration of time.

443   A major finding of this study is that toxic, unpalatable, and aposematic *Heliconius*

444   *melpomene plessini* butterfly changed their behaviour in response to the predatory rufous-

445   tailed jacamar calls. Contrary to our expectations, *H. m. plessini* butterflies did not

446   change their behaviour in response to either the Eastern kingbird or tropical kingbird

447   calls. Two non-mutually exclusive hypotheses can be postulated to explain these results:

448   1) There may be reduced predation pressure from both the Eastern and tropical kingbirds

449   compared to rufous-tailed jacamar, which has led to an evolved behavioural response to

450   the rufous-tailed jacamar but not to two kingbird species, and/or 2) *H. m. plessini* may be

451   incapable of hearing the Eastern and tropical kingbird calls. Since Eastern kingbirds are

452   migratory and tropical kingbirds are year-round residents, we had hypothesized that, if

453   *H.m. plessini* could hear the Eastern and tropical kingbird calls, they might respond to the

454   resident tropical kingbird due to their year-round presence, but not the migratory Eastern

455   kingbird. Tropical kingbird calls are similar in frequency to the calls of Eastern kingbird

456   (Supplementary Figure 2). However, we found that *H. m. plessini* butterflies did not

457   change their behaviour in response to either of the kingbird calls, suggesting that between

22

458   kingbird species variation in predation pressure was not sufficient to induce *H. m.*

459   *plesseni* variation in response to Eastern and tropical kingbird calls.

460   *H. m. plessini* may be under reduced predation pressure from kingbirds relative to

461   rufous-tailed jacamars. While the rufous-tailed jacamar is a year-round resident of *H. m.*

462   *plesseni's* habitat, the Eastern kingbird is migratory and is not present during half of the

463   year in South America, where *H. m. plessini* is found. Eastern kingbird is also frugivorous

464   during their migration over Central and South America (Blake & Loiselle, 1992; Morton,

465   1971). While this does not explain the lack of response to the tropical kingbird, an

466   additional possibility is that *Heliconius melpomene* may be differentially palatable for

467   rufous tailed jacamars and tropical kingbirds. Future studies should explore whether there

468   is variability in toxicity across different subspecies of *H. melpomene,* or variability in

469   predator sensitivity to *Heliconius* toxicity. Although there is no current support for this

470   hypothesis in *Heliconius,* the aposematic striped skunks (*Mephitis mephitis*) perform anti-

471   predatory behaviour in response to the calls of the great horned owl (*Bubo virginianus*)

472   from which they are not chemically defended, but not in response to the calls of the

473   coyote (*Canis latrans*), from which they are chemically defended (Fisher & Stankowich,

474   2018). Moreover, we found that *H. m. plessini* did not change their behaviour in response

475   to the frugivorous control toco toucan bird call, despite the toucan calls being in the range

476   of *Heliconius* hearing, which may indicate that *Heliconius* butterflies are capable of

477   differentiating between predatory and non-predatory bird calls.

23

478   An alternative hypothesis is that *H. m. plessini* butterflies may not be capable of

479   detecting kingbird calls but are able to detect the rufous tailed jacamar calls. Rufous

480   tailed jacamar calls have a peak frequency below 4 kHz (Mikhail et al. 2018;

481   Supplementary Figure 2), whereas both the Eastern and tropical kingbirds have a peak

482   call frequency above 4 kHz (Supplementary Figure 2). Previous electrophysiological tests

483   of the auditory organ in *H. erato* found that *H. erato* butterflies have the best hearing

484   capabilities below 4 kHz at 70-90 dB power (Swihart, 1967). Any calls with frequencies

485   above 4 kHz will require a higher decibel power to hear, which may be the case with the

486   kingbird calls, as their peak call frequency is between 5-8 kHz. Similar trends have been

487   observed in the blue morpho (*Morpho peleides*), and common wood nymph (*Cercyonis*

488   *pegala*) butterflies, where a higher decibel power is required for higher frequency calls to

489   elicit a response, and that these butterflies are tuned to hear sounds below 5 kHz

490   (Fournier et al., 2013; Mikhail et al., 2018; Sun et al., 2018). Future studies in *Heliconius*

491   can test this hypothesis by recording the butterfly responses to reduced frequency (below

492   4 kHz) kingbird calls and enhanced frequency (above 5kHz) rufous-tailed jacamar calls,

493   and observe whether *H. m. plesseni* butterflies behaviourally respond to the altered

494   kingbird and jacamar calls. We also found that *H. m. plessini* did not change their

495   behaviour in response to the toco toucan calls and greenhouse background noise despite

496   their calls being below 4 KHz, suggesting that *H. m. plessini* are able to distinguish

497   between bird calls within their hearing range.

498   Our study is testing the hypothesis that *Heliconius* change their behaviours in

499   response to predatory bird calls. Although an auditory organ has not yet been described in

500   *Heliconius melpomene*, the auditory organ is described in a closely related butterfly

501   *Heliconius erato* (Swihart, 1967). Here we do provide evidence that *H. m. plesssini*

502   changed their behaviour after hearing their predatory rufous-tailed jacamar calls. Future

503   work can explore the presence of a morphological hearing structure in *Heliconius*

504   *melpomene plessini* and their electrophysiological range like that performed in other

505   butterflies (Lane et al. 2008; Lucas et al. 2009; Mikhail et al. 2018), to enhance our

506   understanding of the physiological mechanisms *H. m. plesseni* may be using to facilitate

507   their response to the rufous-tailed jacamar.

508   We found that males, but not females, changed their behaviour in response to the

509   rufous-tailed jacamar calls. This male-specific response to predators is similar to that

510   found in other species, and may reflect sexual dimorphic predation pressures. Previous

511   studies in wolf spiders (*Pardosa milvina*) have found that males, but not females, used a

512   predatory chemical cue experience to decrease predation from a live predator (Sitvarin

513   and Rypstra, 2012). Similarly, male yellow-billed marmots (*Marmota flaviventris*)

514   decreased foraging followed by a playback of alarm calls (Lea and Blumstein, 2011). The

515   sex-specific differences observed in the response of *H. m. plessini* might reflect

516   differences in predation pressures between the sexes. Male *Heliconius* butterflies in the

517   wild spend greater time flying in the middle of the forest canopy, and mostly near their

518   larval/food plants in search of females or for foraging whereas female *Heliconius* spend

25

519   time fluttering near the understory in search of host plants for egg-laying (Mallet and

520   Gilbert, 1995). Jacamars and kingbirds are "aerial hawking" predators that catch insects

521   in flight (Fitzpatrick, 1980), and flying male butterflies might be at greater risk of

522   predation. This may be the reason for increased fluttering and walking during the jacamar

523   calls. Moreover, Swihart observed fast wing flutters in *H. erato* when he exposed them to

524   loudspeaker generated sound (Swihart, 1967), indicating that butterflies may have an

525   innate wing fluttering response to sound cues. Similar results have been found in *Erebia*

526   butterflies, where they flutter in response to sound (Ribaric and Gogala 1996) and in the

527   peacock butterflies (*Inachis io*) where they walk and flutter to avoid rodent predation

528   during winter hibernation (Olofsson et al., 2011). In *Heliconius*, fluttering may advertise

529   aposematic colouration and could reinforce the birds' learned behaviour to avoid brightly

530   coloured butterflies (Langham, 2006). Similar to the mimicry of aposematic colours

531   among *Heliconius* species*,* there is also evidence of locomotor mimicry in the flight of

532   unpalatable *Heliconius*, including flight measures associated with response to jacamars

533   (Chai & Srygley, 1990; Srygley, 1994). Future studies of the responses in *H. melpomene,*

534   their model *H. erato* and other species of the same aposematic mimicry rings could

535   inform us if certain predators have influenced the evolution of mimetic behavioural

536   responses.

537   Palatability experiments with jacamars have found that experienced birds sight-

538   reject flying *Heliconius* butterflies (Pinheiro & Campos, 2019). Therefore, flying,

539   fluttering and walking behaviours could be advantageous under different ecological

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540   contexts (for example bird predator community and experience) as an immediate

541   response to predator's presence, which may be another reason why we did not see the

542   behavioural changes over a long-term (14 minutes) period. Future studies could look at

543   the advantages of these behaviours under different ecological contexts such as

544   microhabitats (Dell'Aglio et al., 2022), as well as test the behavioural responses of the

545   butterflies using other predatory birds.

546

547   **Conclusions:**

548   We found that unpalatable and brightly coloured *Heliconius melpomene plessini*

549   butterflies respond and change their behaviour during the playback of the rufous-tailed

550   jacamar call. This change in behaviour is sex-specific, where males, but not females,

551   increase their walking and fluttering behaviour over a short time-frame. Males reverted

552   back to their original behaviour after the call ended. *H. m. plessini* did not change their

553   behaviour in response to the two kingbird and the toco toucan calls. Our study opens

554   avenues for future research in the field of butterfly auditory anti-predatory behaviour

555   response, its mechanistic underpinnings and ecological and evolutionary consequences,

556   especially in the context of mimicry.

557

27

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564

Data Availability:

566 Analyses reported in this article can be reproduced using the data provided by the authors

567 in Dryad (link: XXXX)

568

Supplementary Materials:

570 Supplementary material 1: a PDF file containing all supplementary figures and tables

571 (supplementary_material_1.pdf)

572 Supplementary material 2: *De novo* python code to extract behavioural data from

573 SPECTATOR GO! Software files (supplementary_material_2.txt)

574 Supplementary material 3: An excel file containing the results of linear mixed models for

575 (supplementary_material_3.xlsx)

576

581

582 **CRediT author contributions:**

583 SP- Conceptualization, Methodology, Investigation, Data curation, Formal analysis,

584 Funding acquisition, Writing- original draft, Writing- review and editing; MD- Data

585 curation, Software, Writing- review and editing; ELW- Conceptualization, Methodology,

586 Supervision, Resources, Funding acquisition, Writing- original draft, Writing- review and

587 editing.

588

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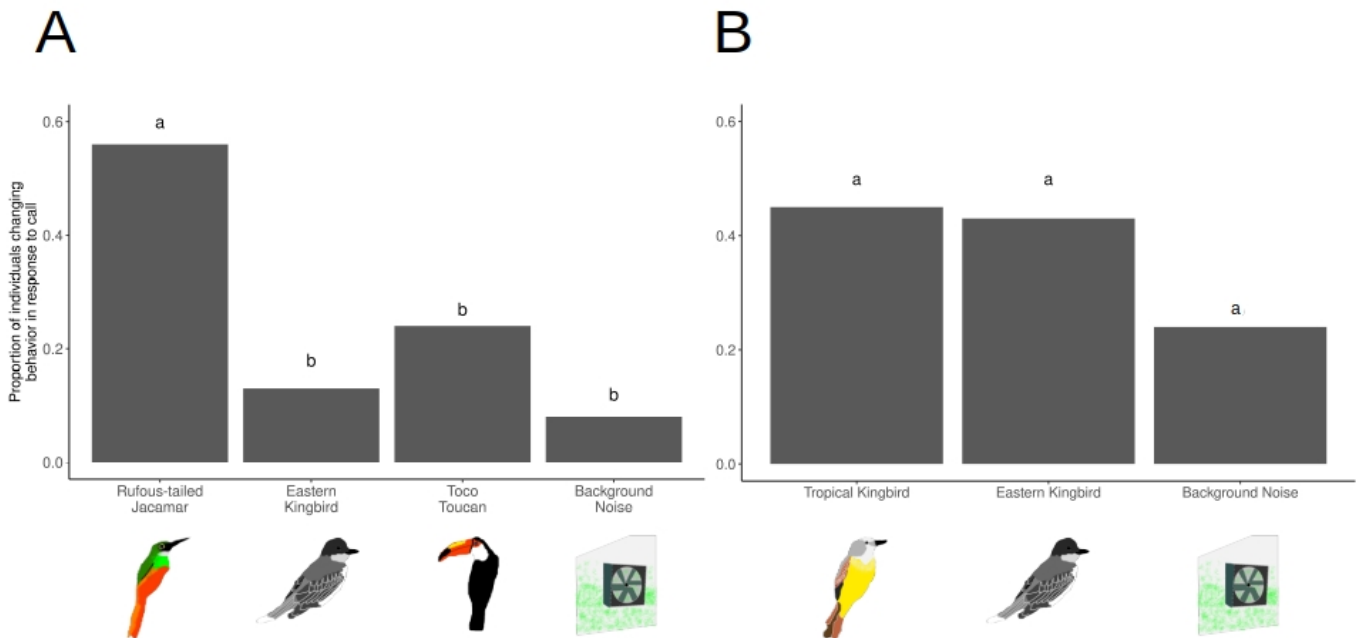592

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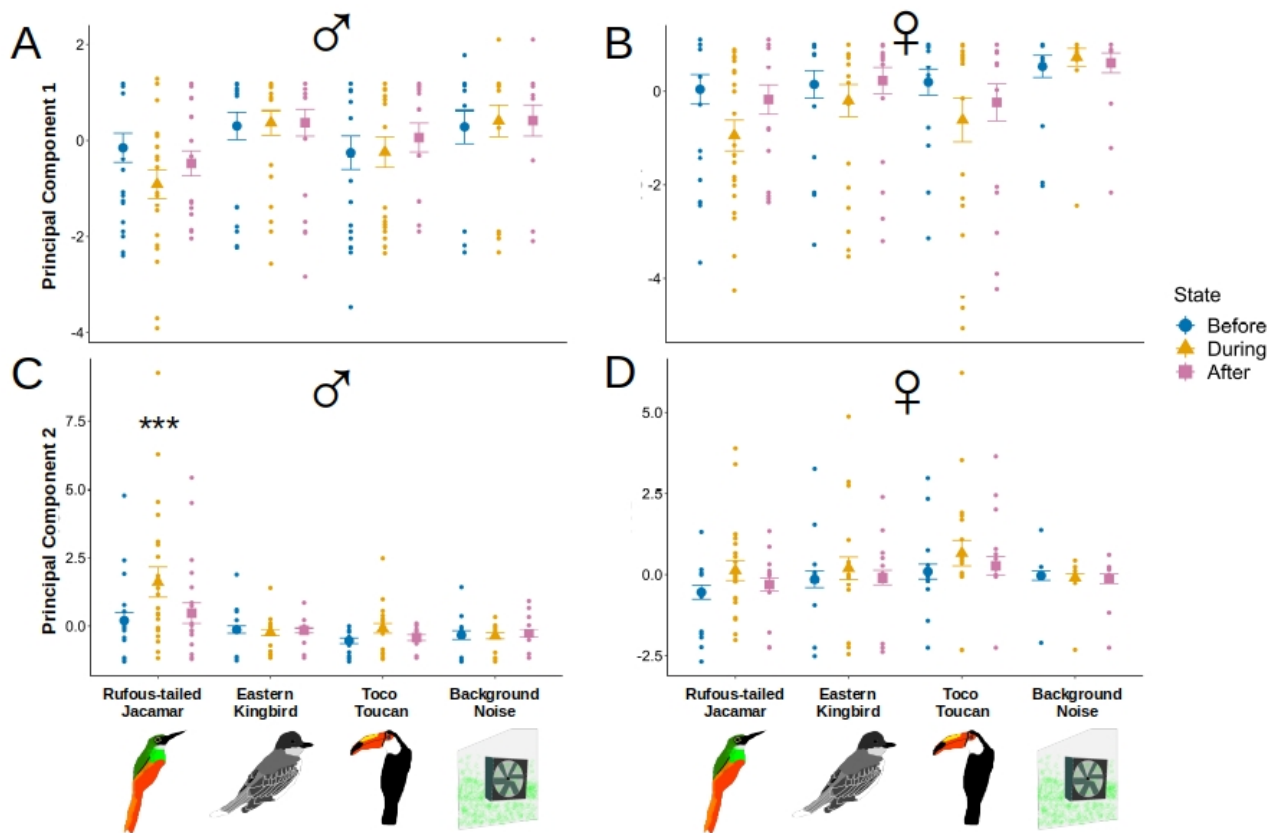
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Figures:

Figure 1: Proportion of *H. m. plessini* individuals changing behaviour in response to calls (between before start and after end of calls) for A) experiment 1; B) experiment 2; Different letters on each bars indicate statistical significance at $p < 0.05$.

Figure 2: Mean ± SE of principal component variables for male and female *H. m. plessini* for a minute before, during and after calls. A) PC 1 in males for experiment 1; B) PC 1 in females for experiment 1; C) PC 2 in males for experiment 1; D) PC 2 in females for experiment 1. *** indicates significance with p<0.0001.
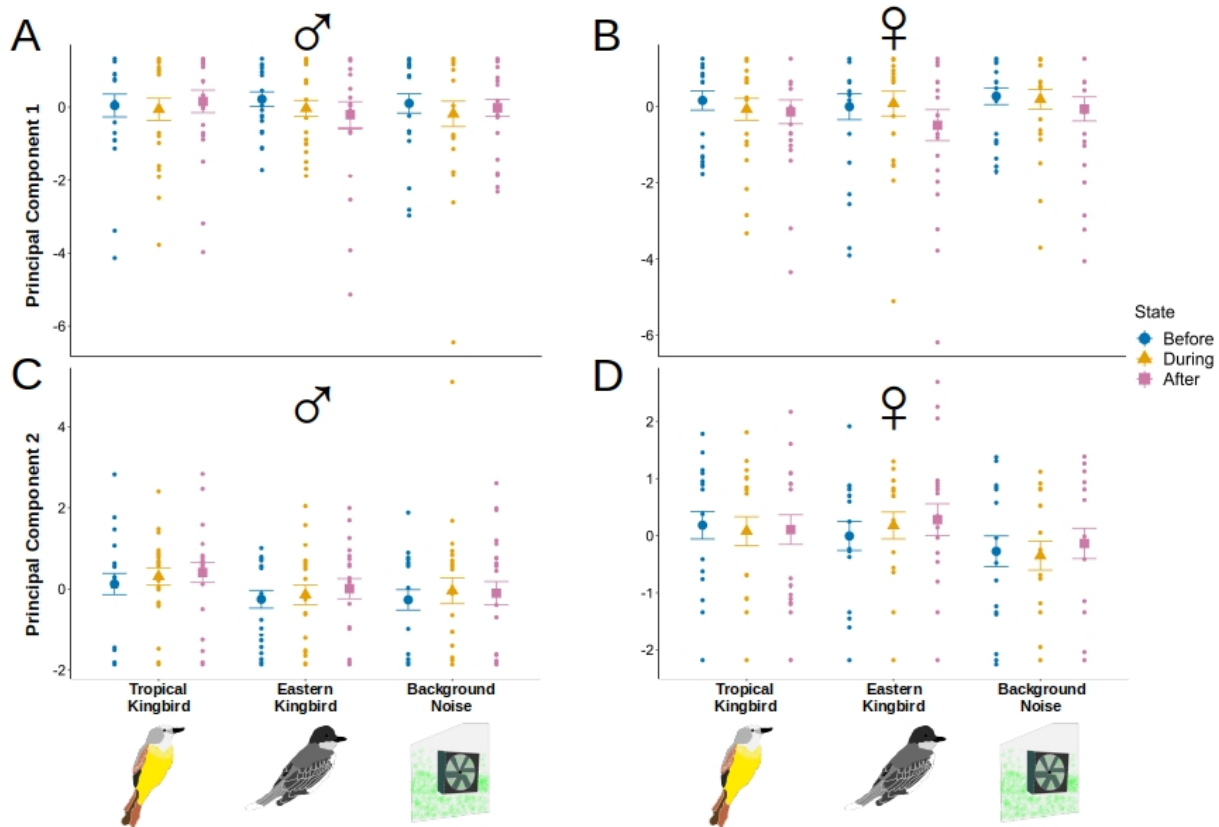
Figure 3: Mean ± SE of principal component variables for male and female *H. m. plessini* for a minute before, during and after calls. A) PC 1 in males for experiment 2; B) PC 1 in females for experiment 2; C) PC 2 in males for experiment 2; D) PC 2 in females for experiment 2. None of them are significantly different from each other.

621 **Tables:**
622
623 **Table 1: GLMM results on the effect of treatment (calls) and sex on proportion of**
624 **butterflies changing their behaviour in response to calls. p<0.05 are bolded**
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Fixed effect	Estimate	SE	z value	Pr (>|z|)
Intercept	**-2.55**	**0.64**	**-3.98**	**<0.0001**
Treatment (Rufous-tailed Jacamar)	**2.67**	**0.67**	**3.96**	**<0.0001**
Treatment (Eastern Kingbird)	0.5	0.74	0.67	0.640
Treatment (Toco Toucan)	1.24	0.69	1.78	0.777
Sex (male)	0.29	0.38	0.76	0.44
Random effect				
Order (Intercept)	1.7e-15	4.1e-8		

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Table 2: Pairwise differences in the proportion of individuals changing their behavioural state in response to calls in experiment 1. p<0.05 are bolded

ANOVA Type II Wald Chisquare test

Treatment: $\chi2 = 27.12$; df = 3; p-value < 0.0001

Sex: $\chi2 = 0.577$; df = 1; p-value = 0.44

Pairwise comparisons between treatment

Group 1	Group 2	p-value	Adj. p-value
Rufous-tailed Jacamar	**Eastern Kingbird**	**0.00002**	**0.0001**
Rufous-tailed Jacamar	**Toucan**	**0.002**	**0.01**
Rufous-tailed Jacamar	**Greenhouse noise**	**0.000005**	**0.00003**
Eastern Kingbird	Toco Toucan	0.28	1
Eastern Kingbird	Greenhouse noise	0.72	1
Toco Toucan	Greenhouse noise	0.07	0.47

659 Table 3: Effect of treatment (rufous-tailed jacamar, Eastern kingbird, toco toucan
660 and greenhouse background noise calls), state (one minute before, during and after
661 call) and their interaction on male and female PC1 and PC2 in experiment 1. p<0.05
662 are bolded
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♂	AIC	Df	F value	Pr (>F)	♀	AIC	Df	F value	Pr (>F)
PC1	884				PC1	910			
Treatment		**3**	**5.887**	**0.0006**	**Treatment**		**3**	**5.090**	**0.001**
State		2	0.447	0.640	State		2	2.794	0.063
Treatment*State		6	0.540	0.777	Treatment*State		6	0.732	0.624
PC2	781				PC2	805			
Treatment		**3**	**13.900**	**2.2e-08**	**Treatment**		**3**	**2.759**	**0.043**
State		**2**	**3.649**	**0.0275**	State		2	2.498	0.084
Treatment*State		**6**	**2.336**	**0.0328**	Treatment*State		6	0.373	0.896
PC3	787				PC3	745			
Treatment		3	1.432	0.234	Treatment		3	2.017	0.112
State		2	0.856	0.426	State		2	1.071	0.344
Treatment*State		6	0.697	0.652	Treatment*State		6	0.577	0.748
Courtship	239				Copulation	1692			
Treatment		3	0.984	0.401	**Treatment**		**3**	**2.765**	**0.042**
State		2	0.999	0.370	State		2	0	1
Treatment*State		6	0.984	0.437	Treatment*State		6	0	1
Sitting near other	1659				Abdomen lift				
Treatment		**3**	**3.500**	**0.016**	Treatment		3	0.922	0.431
State		2	0.011	0.988	State		2	0.997	0.370
Treatment*State		6	0.003	1	Treatment*State		6	0.922	0.480

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666   **Supplementary Material 1**

667   **Behavioural changes in aposematic *Heliconius melpomene* butterflies in response to**

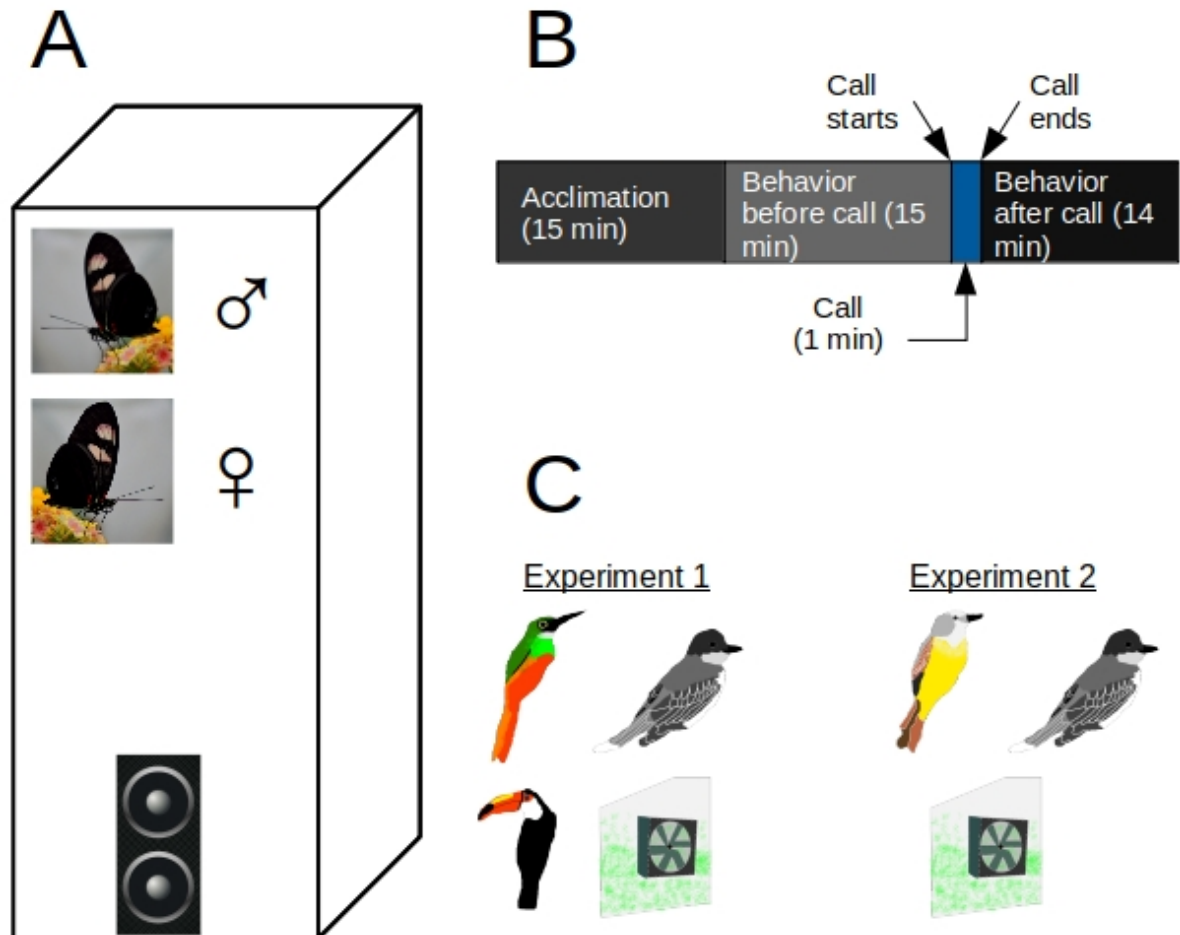668   **their predatory bird calls**

669   **<u>Table of Content</u>**

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Supplementary Figure 1: Experimental design. A) 3-15-day male and female *H. m. plessini* butterflies were subjected in an experimental cage with a blue tooth speaker and a *Lantana spp.* plant during each experimental assay. B) The timeline of each assay conducted where the butterflies were acclimated for 15 minutes and their behaviours recorded for the next 30 minutes. During the 16th minute, a call was randomly played for a minute. C) The calls used in the two experiments in this study. Clockwise from top left in experiment 1: rufous-tailed jacamar, Eastern kingbird, greenhouse background noise, and toco toucan. Clockwise from top left in experiment 2: tropical kingbird, Eastern kingbird, and greenhouse background noise.

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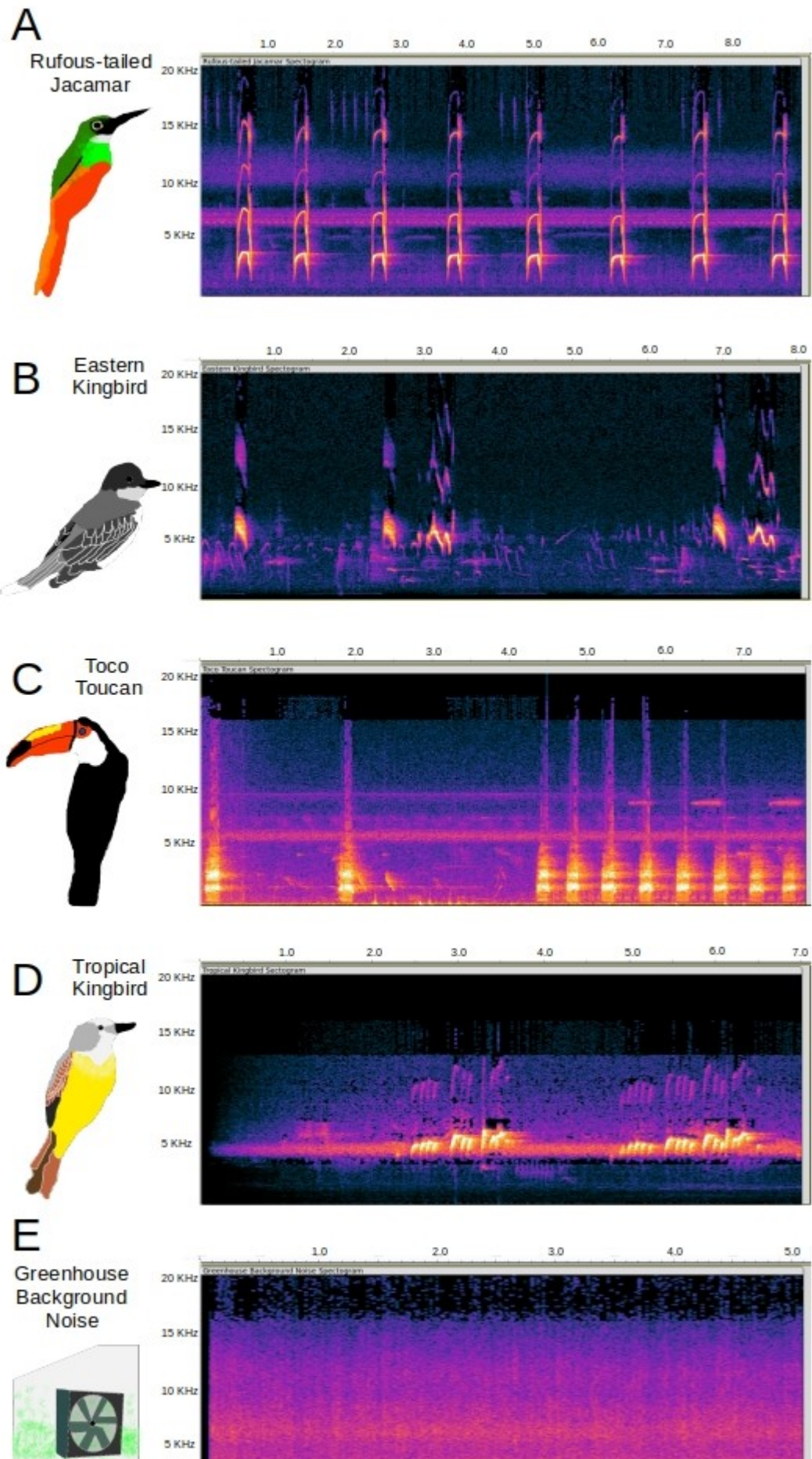
Supplementary Figure 2: Spectograms of the calls used during this study A) rufous-tailed jacamar; B) Eastern kingbird; C) toco toucan; D) tropical kingbird; E) greenhouse background noise.

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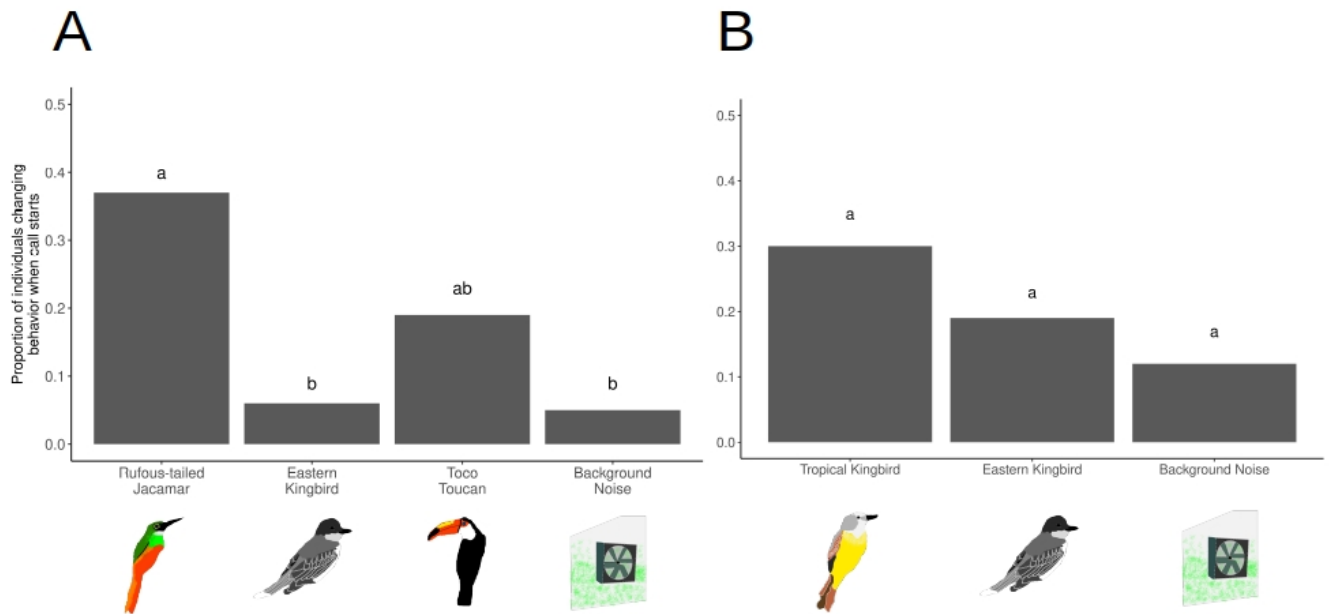
Supplementary Figure 3: Proportion of *H. m. plessini* individuals changing behaviour in response to the start of the calls (between before start and after start of calls) for A) experiment 1; B) experiment 2; Different letters on each bars indicate statistical significance at p<0.05.

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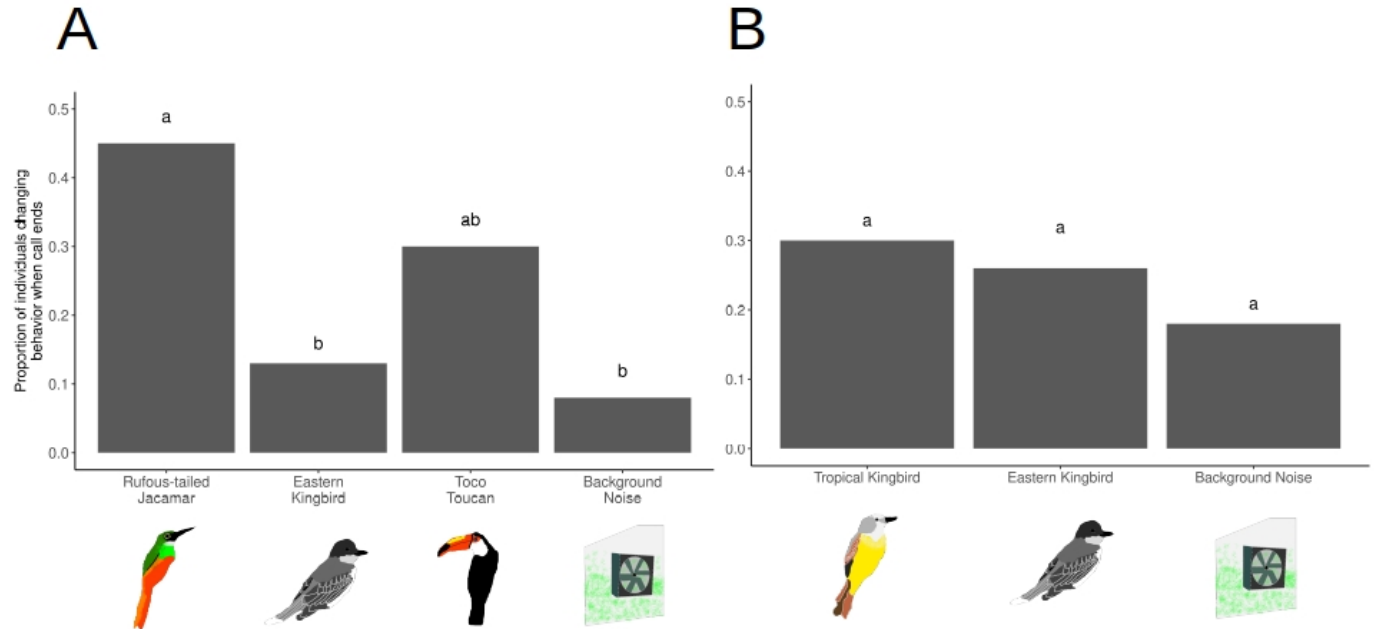
Supplementary Figure 4: Proportion of *H. m. plessini* individuals changing behaviour in response to the end of the calls (between before end and after end of calls) for A) experiment 1; B) experiment 2; Different letters on each bars indicate statistical significance at p<0.05.

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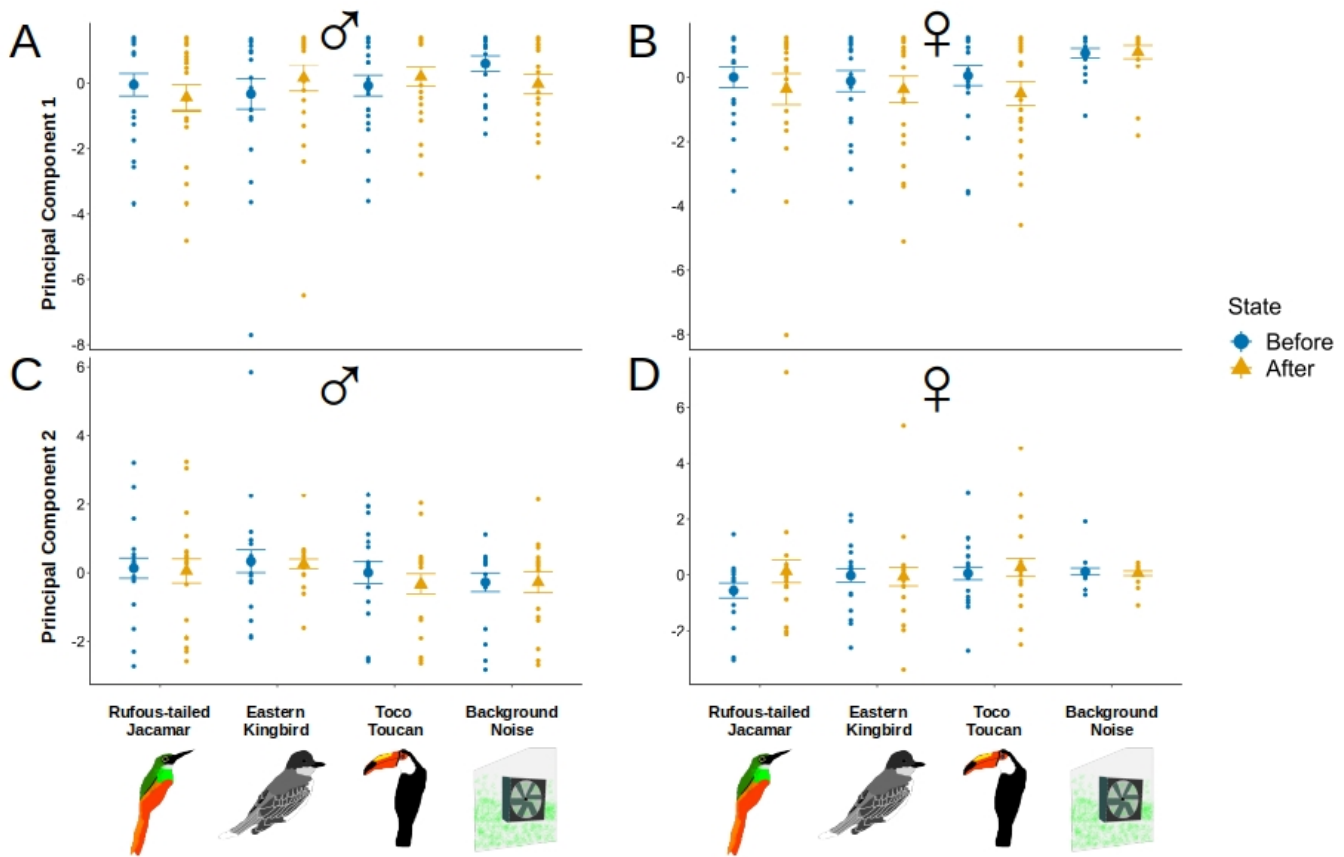
Supplementary Figure 5: Mean ± SE of principal component variables for male and female *H. m. plessini* for 14 minutes before, and after calls. A) PC 1 in males for experiment 1; B) PC 1 in females for experiment 1; C) PC 2 in males for experiment 1; D) PC 2 in females for experiment 1. None of them are significantly different from each other.

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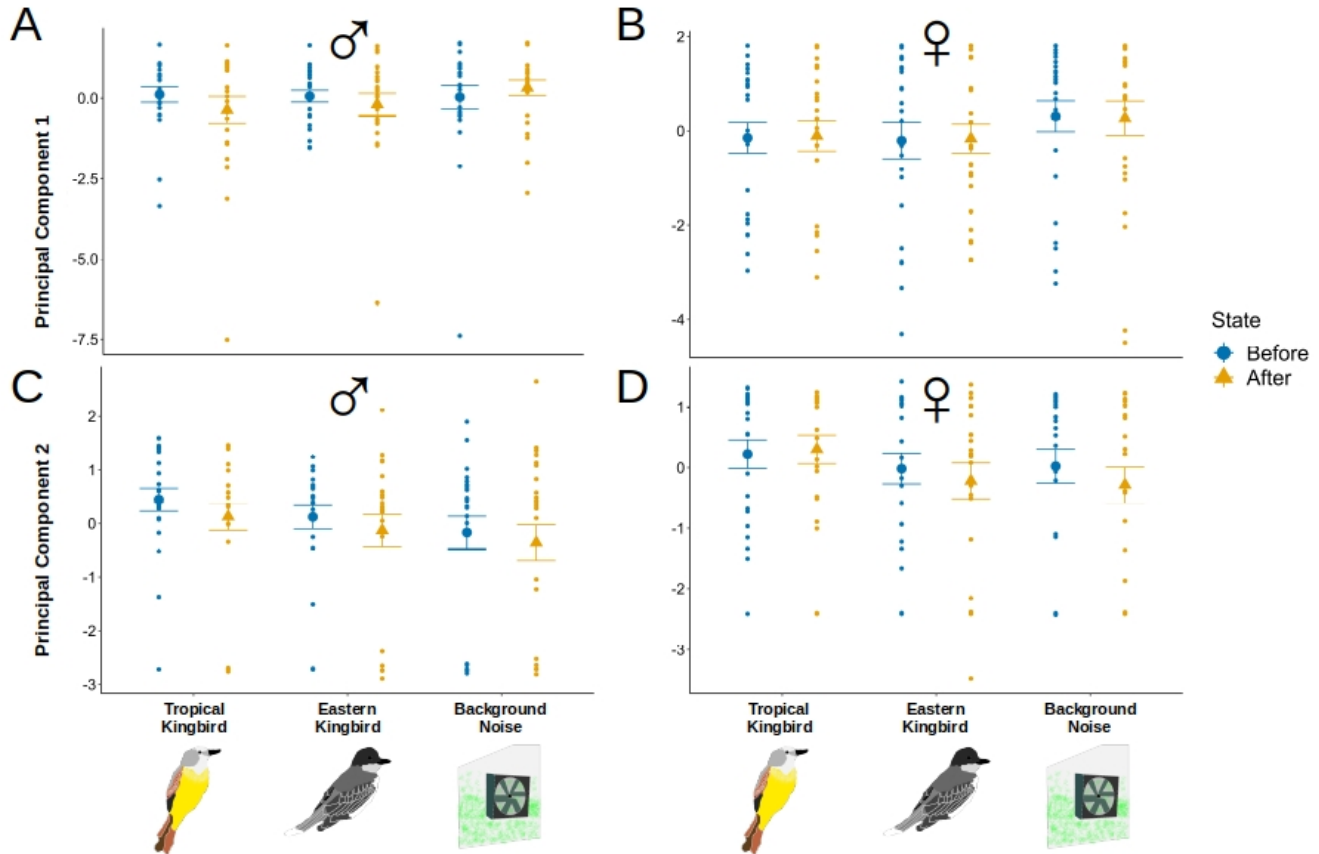
Supplementary Figure 6: Mean ± SE of principal component variables for male and female *H. m. plessini* for 14 minutes before, and after calls. A) PC 1 in males for experiment 2; B) PC 1 in females for experiment 2; C) PC 2 in males for experiment 2; D) PC 2 in females for experiment 2. None of them are significantly different from each other.

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Supplementary Table 1: GLMM results on the effect of treatment (calls) and sex on proportion of butterflies changing their behaviour at the start of calls in experiment 1. p<0.05 are bolded

Fixed effect	Estimate	SE	z value	Pr (>|z|)
Intercept	**-2.97**	**0.76**	**-3.88**	**<0.001**
Treatment (Rufous-tailed Jacamar)	**2.30**	**0.78**	**2.91**	**<0.001**
Treatment (Eastern Kingbird)	0.17	0.94	0.18	0.856
Treatment (Toco Toucan)	1.42	0.81	1.73	0.082
Sex (male)	0.26	0.42	0.62	0.529
Random effect				
Order (Intercept)	0	0		

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Supplementary Table 2: Pairwise differences in the proportion of individuals changing their behavioural state in at the start of calls in experiment 1. p<0.05 are bolded

ANOVA Type II Wald Chisquare test

Treatment: $\chi 2 = 16.03$; df = 3; p-value < 0.01

Sex: $\chi 2 = 0.396$; df = 1; p-value = 0.529

Pairwise comparisons between treatment

Group 1	Group 2	p-value	Adj. p-value
Rufous-tailed Jacamar	**Eastern Kingbird**	**0.0007**	**0.004**
Rufous-tailed Jacamar	Toucan	0.104	0.624
Rufous-tailed Jacamar	**Greenhouse noise**	**0.001**	**0.006**
Eastern Kingbird	Toco Toucan	0.11	0.7
Eastern Kingbird	Greenhouse noise	1	1
Toco Toucan	Greenhouse noise	0.10	0.61

810 **Supplementary Table 3: GLMM results on the effect of treatment (calls) and sex on**
811 **proportion of butterflies changing their behaviour at the end of calls in experiment**
812 **1. p<0.05 are bolded**
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Fixed effect	Estimate	SE	z value	Pr (>|z|)
Intercept	**-2.46**	**0.64**	**-3.79**	**<0.001**
Treatment (Rufous-tailed Jacamar)	**2.22**	**0.67**	**3.30**	**<0.001**
Treatment (Eastern Kingbird)	0.44	0.75	0.59	0.554
Treatment (Toco Toucan)	**1.62**	**0.69**	**2.34**	**<0.05**
Sex (male)	0.13	0.37	0.37	0.709
Random effect				
Order (Intercept)	0.06	0.25		

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839 **Supplementary Table 4: Pairwise differences in the proportion of individuals**
840 **changing their behavioural state in at the end of calls in experiment 1. p<0.05 are**
841 **bolded**
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ANOVA Type II Wald Chisquare test

Treatment: $\chi2 = 17.47$; df = 3; p-value < 0.001

Sex: $\chi2 = 0.139$; df = 1; p-value = 0.709

Pairwise comparisons between treatment

Group 1	Group 2	p-value	Adj. p-value
Rufous-tailed Jacamar	**Eastern Kingbird**	**0.001**	**0.006**
Rufous-tailed Jacamar	Toucan	0.197	1
Rufous-tailed Jacamar	**Greenhouse noise**	**0.0002**	**0.001**
Eastern Kingbird	Toco Toucan	0.07	0.451
Eastern Kingbird	Greenhouse noise	0.724	1
Toco Toucan	Greenhouse noise	0.015	0.093

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Supplementary Table 5: Loadings of each behaviour in Principal Component (PC) composite variables for males in a minute before, during, and after calls in experiment 1

Behaviour	PC1	PC2	PC3
Rest	0.673	0.112	0.115
Fly	0.170	0.100	0.689
Bask	0.602	0.294	0.200
Flutter	0.195	0.674	0.088
Court	0.053	0.015	0.600
Copulate	0.095	0.254	0.009
Walk	0.169	0.602	0.140
Antenna wiggle	0.253	0.080	0.281
Sitting near each other	0.108	0.025	0.061
% Variance explained	22.28	17.05	14.43
% Total variance explained	22.28	39.33	53.77

Supplementary Table 6: Loadings of each behaviour in Principal Component (PC) composite variables for females in a minute before, during and after calls in experiment 1

Behaviour	PC1	PC2	PC3
Rest	0.605	0.269	0.208
Fly	0.193	0.236	0.576
Bask	0.490	0.493	0.136
Flutter	0.362	0.577	0.016
Copulate	0.066	0.162	0.583
Walk	0.355	0.512	0.149
Antenna wiggle	0.304	0.071	0.491
Lifting abdomen	0.028	0.030	0.037
% Variance explained	28.18	17.83	13.77
% Total variance explained	28.18	46.02	59.79

900 **Supplementary Table 7: ANOVA Post-hoc test results with PC as dependent**
901 **variables and the treatments (calls), state (before, during, and after) and their**
902 **interaction as response variables for males and females in a minute before, during,**
903 **and after calls in experiment 1. EK= Eastern kingbird; RJ=Rufous-tailed jacamar;**
904 **TT=Toco toucan; GN=Greenhouse background noise; Difference=pairwise**
905 **difference; lwr=lower range; upr=upper range; padj= adjusted p-value.**

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| | PC1 | | | | PC1 | | | |
Treatment	Difference	lwr	upr	padj	Difference	lwr	upr	padj
EK-RJ	**0.861**	**0.235**	**1.487**	**0.002**	0.418	-0.240	1.077	0.357
TT-RJ	0.368	-0.264	1.001	0.434	0.141	-0.525	0.807	0.947
GN-RJ	**0.882**	**0.223**	**1.542**	**0.003**	**0.982**	**0.287**	**1.676**	**0.001**
TT-EK	-0.492	-1.125	0.140	0.185	-0.276	-0.943	0.389	0.705
GN-EK	0.021	-0.638	0.680	0.999	0.564	-0.130	1.258	0.155
GN-TT	0.513	-0.512	1.180	0.192	**0.840**	**0.138**	**1.542**	**0.011**
State								
During-Before	-0.153	-0.661	0.355	0.757	-0.516	-1.051	0.019	0.061
After-Before	0.040	-0.468	0.548	0.981	-0.129	-0.665	0.406	0.836
After-During	0.193	-0.315	0.701	0.643	0.386	-0.149	0.922	0.206
	PC2				PC2			
Treatment	Difference	lwr	upr	padj	Difference	lwr	upr	padj
EK-RJ	**-0.934**	**-1.443**	**-0.426**	**0.00002**	0.226	-0.307	0.761	0.690
TT-RJ	**-1.108**	**-1.622**	**-0.594**	**0.000004**	**0.583**	**0.042**	**1.124**	**0.028**
GN-RJ	**-1.074**	**-1.610**	**-0.538**	**0.000002**	0.154	-0.408	0.717	0.893
TT-EK	-0.173	-0.688	0.340	0.818	0.356	-0.183	0.897	0.322
GN-EK	-0.139	-0.675	0.396	0.906	-0.072	-0.635	0.490	0.987
GN-TT	0.033	-0.507	0.575	0.998	-0.429	-0.998	0.140	0.209
State								
During-Before	**0.454**	**0.040**	**0.867**	**0.027**	0.396	-0.037	0.830	0.081
After-Before	0.111	-0.301	0.524	0.799	0.102	-0.332	0.536	0.844
After-During	-0.342	-0.755	0.070	0.125	-0.294	-0.728	0.140	0.248

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Supplementary Table 8: Loadings of each behaviour in Principal Component (PC) composite variables for males in 14 minutes before and after calls in experiment 1

Behaviour	PC1	PC2	PC3
Rest	0.465	0.402	0.242
Fly	0.276	0.407	0.350
Bask	0.334	0.575	0.147
Flutter	0.443	0.378	0.235
Court	0.117	0.282	0.599
Copulate	0.006	0.093	0.039
Walk	0.489	0.275	0.321
Antenna wiggle	0.377	0.182	0.240
Sitting near each other	0.047	0.016	0.467
% Variance explained	29.23	19.82	15.88
% Total variance explained	29.23	49.06	64.94

Supplementary Table 9: Loadings of each behaviour in Principal Component (PC) composite variables for females in 14 minutes before and after calls in experiment 1

Behaviour	PC1	PC2	PC3
Rest	0.548	0.285	0.204
Fly	0.320	0.272	0.104
Bask	0.469	0.416	0.153
Flutter	0.388	0.537	0.026
Copulate	0.045	0.136	0.842
Walk	0.376	0.446	0.051
Antenna wiggle	0.262	0.332	0.381
Sit near each other	0.041	0.087	0.246
Lifting abdomen	0.100	0.215	0.068
% Variance explained	28.99	17.63	12.24
% Total variance explained	28.99	46.63	58.87

998 **Supplementary Table 10: Effect of treatment (Rufous-tailed Jacamar, Eastern**
999 **Kingbird, Toco Toucan and Greenhouse background noise calls), state (before call**
1000 **and after call) and their interaction on 14 minute behaviours before and after call,**
1001 **and male PC1, PC2, PC3,** *courtship, copulation, sit near* **and female PC1, PC2, PC3,**
1002 *abdomen lifting* **behaviours in experiment 1.**
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♂	AIC	Df	F value	Pr (>F)	♀	AIC	Df	F value	Pr (>F)
PC1	644				PC1	635			
Treatment		3	0.734	0.533	**Treatment**		**3**	**3.615**	**0.014**
State		1	0.014	0.907	State		1	1.488	0.224
Treatment*State		3	1.068	0.364	Treatment*State		3	0.236	0.871
PC2	580				PC2	560			
Treatment		3	1.482	0.222	Treatment		3	0.731	0.535
State		1	0.380	0.538	State		1	0.155	0.284
Treatment*State		3	0.133	0.952	Treatment*State		3	0.770	0.512
PC3	542				PC3	500			
Treatment		3	1.639	0.183	Treatment		3	1.142	0.334
State		1	0.664	0.416	State		1	0.498	0.481
Treatment*State		3	0.062	0.980	Treatment*State		3	0.433	0.730
Courtship	1606				Copulation	1983			
Treatment		3	1.313	0.272	Treatment		3	1.967	0.121
State		1	1.933	0.166	State		1	0	1
Treatment*State		3	0.243	0.866	Treatment*State		3	0	1
Sitting near other	2027				Abdomen lift	1676			
Treatment		3	0.953	0.417	Treatment		3	0.613	0.608
State		1	0.264	0.608	State		1	0.621	0.432
Treatment*State		3	0.221	0.882	Treatment*State		3	0.979	0.404

Supplementary Table 11: ANOVA post-hoc test results with PC as dependent variables and the treatments (calls), state (before and after) and their interaction as response variables for males and females in 14 minutes before and after calls in experiment 1. EK= Eastern kingbird; RJ=Rufous-tailed jacamar; TT=Toco toucan; GN=Greenhouse background noise; Difference= pairwise difference; lwr=lower range; upr=upper range; padj= adjusted p-value.

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| | PC1 | | | | PC1 | | | |
Treatment	Difference	lwr	upr	padj	Difference	lwr	upr	padj
EK-RJ	0.160	-0.755	1.076	0.968	0.064	-0.956	0.826	0.997
TT-RJ	0.305	-0.610	1.221	0.821	-0.043	-0.934	0.848	0.999
GN-RJ	0.528	-0.435	1.492	0.486	**0.948**	**0.010**	**1.887**	**0.046**
TT-EK	0.145	-0.759	1.050	0.975	0.021	-0.859	0.902	0.999
GN-EK	0.367	-0.586	1.321	0.748	**1.013**	**0.084**	**1.942**	**0.026**
GN-TT	0.222	-0.731	1.176	0.929	**0.992**	**0.063**	**1.920**	**0.031**
State								
After-Before	-0.029	-0.530	0.471	0.907	-0.301	-0.789	0.186	0.224
	PC2				PC2			
Treatment	Difference	lwr	upr	padj	Difference	lwr	upr	padj
EK-RJ	0.202	-0.552	0.956	0.898	0.177	-0.533	0.887	0.916
TT-RJ	-0.250	-1.005	0.503	0.823	**0.382**	**-0.328**	**1.093**	**0.502**
GN-RJ	-0.370	-1.165	0.423	0.620	0.305	-0.443	1.053	0.714
TT-EK	-0.452	-1.198	0.292	0.394	0.205	-0.497	0.907	0.872
GN-EK	-0.572	-1.358	0.213	0.235	0.128	-0.612	0.868	0.969
GN-TT	-0.119	-0.905	0.666	0.978	-0.077	-0.817	0.663	0.993
State								
After-Before	-0.128	-0.542	0.284	0.538	0.211	-0.177	0.600	0.284

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Supplementary Table 12: GLMM results on the effect of treatment (calls) and sex on proportion of butterflies changing their behaviour at the start of calls in experiment 2. p<0.05 are bolded

Fixed effect	Estimate	SE	z value	Pr (>|z|)
Intercept	**-2.03**	**0.48**	**-4.18**	**<0.0001**
Treatment (Eastern Kingbird)	0.57	0.57	1.01	0.312
Treatment (Tropical Kingbird)	**1.16**	**0.54**	**2.15**	**<0.05**
Sex (male)	0.08	0.42	0.21	0.832
Random effect				
Order (Intercept)	6.9e-15	8.3e-8		

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Supplementary Table 13: Pairwise differences in the proportion of butterflies changing their behavioural state in response to the start of calls in experiment 2

ANOVA Type II Wald Chisquare test

Treatment: $\chi2 = 4.807$; df = 2; p-value = 0.09

Sex: $\chi2 = 0.044$; df = 1; p-value = 0.832

Pairwise comparisons between treatment

Group 1	Group 2	p-value	Adj. p-value
Tropical Kingbird	Eastern Kingbird	0.336	1
Tropical Kingbird	Greenhouse noise	0.042	0.128
Eastern Kingbird	Greenhouse noise	0.402	1

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1064 **Supplementary Table 14: GLMM results of the effect of treatment (calls) and sex on**
1065 **proportion of butterflies changing their behaviour at the end of calls in experiment**
1066 **2. p<0.05 are bolded**
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Fixed effect	Estimate	SE	z value	Pr (>|z|)
Intercept	**-1.55**	**0.41**	**-3.70**	**<0.001**
Treatment (Eastern Kingbird)	0.47	0.49	0.95	0.340
Treatment (Tropical Kingbird)	0.68	0.48	1.41	0.157
Sex (male)	0.07	0.39	0.19	0.844
Random effect				
Order (Intercept)	0	0		

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1095 **Supplementary table 15: Pairwise differences in the proportion of males and females**
1096 **changing their behavioural state in response to the end of calls in experiment 2**
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ANOVA Type II Wald Chisquare test

Treatment: $\chi 2 = 2.037$; df = 2; p-value = 0.361

Sex: $\chi 2 = 0.038$; df = 1; p-value = 0.844

Pairwise comparisons between treatment

Group 1	Group 2	p-value	Adj. p-value
Tropical Kingbird	Eastern Kingbird	0.817	1
Tropical Kingbird	Greenhouse noise	0.231	0.693
Eastern Kingbird	Greenhouse noise	0.459	1

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1114 **Supplementary Table 16: GLMM results of the effect of treatment (calls) and sex on**
1115 **proportion of butterflies changing their behaviour in response to calls in experiment**
1116 **2. p<0.05 are bolded**

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Fixed effect	Estimate	SE	z value	Pr (>|z|)
Intercept	**-1.31**	**0.38**	**-3.43**	**<0.001**
Treatment (Eastern Kingbird)	**0.89**	**0.44**	**2.00**	**0.044**
Treatment (Tropical Kingbird)	**0.98**	**0.44**	**2.20**	**0.027**
Sex (male)	0.31	0.35	0.88	0.376
Random effect				
Order (Intercept)	0	0		

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1145 **Supplementary table 17: Pairwise differences in the proportion of individuals**
1146 **changing their behavioural state in response to the calls in experiment 2**
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ANOVA Type II Wald Chisquare test

Treatment: $\chi2 = 5.756$; df $= 2$; p-value $= 0.056$

Sex: $\chi2 = 0.783$; df $= 1$; p-value $= 0.376$

Pairwise comparisons between treatment

Group 1	Group 2	p-value	Adj. p-value
Tropical Kingbird	Eastern Kingbird	1	1
Tropical Kingbird	Greenhouse noise	0.032	0.096
Eastern Kingbird	Greenhouse noise	0.052	0.158

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Supplementary Table 18: Loadings of each behaviour in Principal Component (PC) composite variables for males in a minute before, during and after calls in experiment 2

Behaviour	PC1	PC2	PC3
Rest	0.537	0.514	0.139
Fly	0.292	0.017	0.413
Bask	0.274	0.401	0.400
Flutter	0.485	0.438	0.224
Court	0.258	0.026	0.332
Copulate	0.033	0.463	0.622
Walk	0.493	0.400	0.249
Antenna wiggle	0.056	0.074	0.194
% Variance explained	23.84	19.50	16.99
% Total variance explained	23.84	43.35	60.34

Supplementary Table 19: Loadings of each behaviour in Principal Component (PC) composite variables for females in a minute before, during and after calls in experiment 2

Behaviour	PC1	PC2	PC3
Rest	0.427	0.609	0.087
Fly	0.277	0.017	0.141
Bask	0.365	0.288	0.556
Flutter	0.506	0.314	0.319
Copulate	0.121	0.598	0.550
Walk	0.478	0.275	0.420
Antenna wiggle	0.307	0.045	0.278
Lifting abdomen	0.106	0.099	0.045
% Variance explained	27.03	19.39	15.16
% Total variance explained	27.03	46.42	61.58

Supplementary Table 20: Effect of treatment (Tropical Kingbird, Eastern Kingbird, and Greenhouse background noise calls), state (before, during, and after call) and their interaction on male PC1, PC2, PC3, *courtship, sit near* and female PC1, PC2, PC3, *copulation*, *abdomen lifting* behaviours in experiment 2. p<0.05 bolded. For male *sit near* behaviour, there were zero occurrences.

♂	AIC	Df	F value	Pr (>F)	♀	AIC	Df	F value	Pr (>F)
PC1	760				PC1	784			
Treatment		2	0.062	0.940	Treatment		2	0.599	0.550
State		2	0.440	0.645	State		2	1.249	0.289
Treatment*State		4	0.249	0.910	Treatment*State		4	0.172	0.952
PC2	713				PC2	712			
Treatment		2	2.531	0.082	Treatment		2	2.361	0.096
State		2	0.667	0.514	State		2	0.207	0.813
Treatment*State		4	0.048	0.995	Treatment*State		4	0.167	0.954
PC3	683				PC3	661			
Treatment		**2**	**3.157**	**0.044**	Treatment		2	1.075	0.343
State		2	0.015	0.985	State		2	0.385	0.681
Treatment*State		4	0.205	0.935	Treatment*State		4	0.453	0.770
Courtship	1021				Copulation	1929			
Treatment		2	2.064	0.130	**Treatment**		**2**	**3.413**	**0.034**
State		2	0.292	0.747	State		2	0	1
Treatment*State		4	0.731	0.572	Treatment*State		4	0	1
Sitting near other	NA				Abdomen lift	1034			
Treatment		2	0	0	Treatment		2	1.279	0.280
State		2	0	0	State		2	1.588	0.207
Treatment*State		4	0	0	Treatment*State		4	1.292	0.274

Supplementary Table 21: ANOVA post-hoc test results with PC as dependent variables and the treatments (calls), state (before, during, and after) and their interaction as response variables for males and females in a minute before, during, and after calls in experiment 2. EK= Eastern kingbird; TK=Tropical kingbird; GN=Greenhouse background noise; Difference= pairwise difference; lwr=lower range; upr=upper range; padj= adjusted p-value.

	♂				♀			
	PC1				PC1			
Treatment	Difference	lwr	upr	padj	Difference	lwr	upr	padj
EK-TK	-0.055	-0.620	0.508	0.970	-0.120	-0.717	0.477	0.883
GN-TK	-0.080	-0.633	0.472	0.936	0.150	-0.435	0.735	0.817
GN-EK	-0.025	-0.578	0.527	0.993	0.270	-0.315	0.856	0.520
State								
During-Before	-0.213	-0.773	0.339	0.627	-0.078	-0.667	0.511	0.947
After-Before	-0.144	-0.701	0.411	0.812	-0.373	-0.962	0.215	0.294
After-During	0.072	-0.483	0.629	0.949	-0.295	-0.884	0.293	0.463
	PC2				PC2			
Treatment	Difference	lwr	upr	padj	Difference	lwr	upr	padj
EK-TK	-0.409	-0.914	0.095	0.136	0.028	-0.475	0.533	0.990
GN-TK	-0.417	-0.912	0.077	0.116	-0.376	-0.870	0.118	0.173
GN-EK	-0.007	-0.502	0.486	0.999	-0.404	-0.899	0.089	0.131
State								
During-Before	0.175	-0.322	0.673	0.684	-0.002	-0.499	0.495	0.999
After-Before	0.234	-0.263	0.731	0.508	0.116	-0.381	0.613	0.845
After-During	0.058	-0.438	0.556	0.957	0.118	-0.379	0.615	0.840

Supplementary Table 22: Loadings of each behaviour in Principal Component (PC) composite variables for males in 14 minutes before and after calls in experiment 2

Behaviour	PC1	PC2	PC3
Rest	0.013	0.435	0.374
Fly	0.325	0.102	0.225
Bask	0.047	0.233	0.631
Flutter	0.480	0.052	0.378
Court	0.538	0.207	0.190
Copulate	0.244	0.636	0.039
Walk	0.262	0.203	0.401
Antenna wiggle	0.014	0.455	0.217
Sit near each other	0.491	0.212	0.141
% Variance explained	24.60	20.21	17.82
% Total variance explained	24.60	44.81	62.64

Supplementary Table 23: Loadings of each behaviour in Principal Component (PC) composite variables for females in 14 minutes before and after calls in experiment 2

Behaviour	PC1	PC2	PC3
Rest	0.210	0.708	0.151
Fly	0.350	0.029	0.221
Bask	0.430	0.234	0.262
Flutter	0.424	0.180	0.462
Copulate	0.278	0.603	0.319
Walk	0.452	0.090	0.429
Antenna wiggle	0.355	0.062	0.360
Sit near each other	0.078	0.175	0.468
Lifting abdomen	0.227	0.047	0.097
% Variance explained	29.96	18.61	13.99
% Total variance explained	29.96	48.58	62.58

Supplementary Table 24: Effect of treatment (Rufous-tailed Jacamar, Eastern Kingbird, Toco Toucan and Greenhouse background noise calls), state (before call and after call) and their interaction on 14 minute behaviors before and after call, and male PC1, PC2, PC3, *courtship, copulation, sit near* and female PC1, PC2, PC3, *abdomen lifting* behaviours in experiment 2.

♂	AIC	Df	F value	Pr (>F)	♀	AIC	Df	F value	Pr (>F)
PC1	527				PC1	555			
Treatment		2	0.558	0.574	Treatment		2	1.184	0.309
State		1	0.288	0.592	State		1	0.002	0.961
Treatment*State		2	0.840	0.434	Treatment*State		2	0.010	0.990
PC2	497				PC2	486			
Treatment		2	1.979	0.142	Treatment		2	1.363	0.259
State		1	1.212	0.273	State		1	0.463	0.497
Treatment*State		2	0.025	0.975	Treatment*State		2	0.280	0.756
PC3	483				PC3	443			
Treatment		2	0.009	0.991	Treatment		2	2.640	0.075
State		1	0.060	0.808	State		1	0.645	0.423
Treatment*State		2	0.391	0.677	Treatment*State		2	0.573	0.565
Courtship	1436				Copulation	2028			
Treatment		2	0.492	0.612	Treatment		2	2.821	0.063
State		1	0.332	0.565	State		1	0.838	0.361
Treatment*State		2	0.829	0.439	Treatment*State		2	0.112	0.894
Sitting near other	1148				Abdomen lift	1456			
Treatment		2	0.115	0.891	Treatment		2	0.101	0.904
State		1	1.041	0.309	State		1	0.002	0.966
Treatment*State		2	0.268	0.765	Treatment*State		2	0.554	0.576

Supplementary Table 25: ANOVA post-hoc test results with PC as dependent variables and the treatments (calls), state (before and after) and their interaction as response variables for males and females in 14 minutes before, during, and after calls in experiment 2. EK= Eastern kingbird; TK=Tropical kingbird; GN=Greenhouse background noise; Difference= pairwise difference; lwr=lower range; upr=upper range; padj= adjusted p-value.

| | ♂ | | | | ♀ | | | |
| | PC1 | | | | PC1 | | | |
Treatment	Difference	lwr	upr	padj	Difference	lwr	upr	padj
EK-TK	0.059	-0.683	0.802	0.980	-0.057	-0.879	0.763	0.984
GN-TK	0.304	-0.423	1.032	0.584	0.418	-0.387	1.223	0.437
GN-EK	0.244	-0.483	0.972	0.705	0.476	-0.329	1.281	0.343
State								
After-Before	-0.135	-0.634	0.363	0.592	0.013	-0.583	0.565	0.961
	PC2				PC2			
Treatment	Difference	lwr	upr	padj	Difference	lwr	upr	padj
EK-TK	-0.290	-0.958	0.378	0.560	-0.379	-1.024	0.264	0.345
GN-TK	-0.549	-1.204	0.105	0.118	-0.391	-1.023	0.239	0.308
GN-EK	-0.259	-0.914	0.395	0.615	-0.012	-0.643	0.619	0.998
State								
After-Before	-0.249	-0.698	0.199	0.272	-0.149	-0.582	0.284	0.497

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