1 **Title**

2 Arousal from death feigning by vibrational stimuli: comparison of *Tribolium*

3 species

4

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21 **Running head:** Arousal from death feigning

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Abstract

Death feigning (or tonic immobility) is an effective antipredator strategy. However, prolonged immobility on the ground increases the risk of being parasitized or eaten by predators, and thus, insects must rouse themselves when appropriate stimulation is provided. Very few studies on the effect of stimulus intensity on arousal from death feigning have been conducted. A previous study using *Tribolium castaneum* showed an existing threshold for the intensity of the stimulus that causes arousal from death feigning. Whether there are differences between species in the threshold for arousal is an interesting question. In the present study, we therefore compared the effect of stimulus strength on arousal from death feigning in two closely related species, namely, *T. confusum* and *T. freemani*, which are established strains that have been artificially selected for longer duration of death feigning. Also, part of the study was to determine whether there was a positive association between intensity of stimulus needed to rouse and the duration of death feigning. We discuss why there is a difference in the strength of the stimulus needed for arousal from death feigning among *Tribolium* species, for which we included the data for *T. castaneum* from a previous study.

Introduction

Death-feigning behaviors (sometimes called tonic immobility or thanatosis) have been observed across many animal taxa (e.g., Miyatake et al. 2004, 2009, Cassill et al. 2008, Rogers and Simpson 2014, Ruxton et al. 2018, Humphreys and Ruxton 2018, Skelhorn 2018). Death-feigning behaviors are thought to be an adaptive defense against predators (Miyatake et al. 2004, 2009, Honma et al. 2006, Ohno and Miyatake 2007), a tactic to avoid sexual harassment (Khelifa 2017), a strategy to avoid individuals of conspecific species (Cassill et al. 2008), or as a predator strategy to approach and attack prey without moving (McKaye 1981).

To date, a large proportion of research has been concerned with the stimuli and mechanisms that induce death-feigning behaviors across animal species, including physical touch (Miyatake 2001, Hozumi and Miyatake 2005, Gregory and Gregory 2006), predatory attacks (Miyatake et al. 2004) and vibration (Acheampong and Mitchell 1997). For example, death feigning (more precisely, freezing behavior) in Colorado potato beetles (*Leptinotarsa decemlineata*) was induced by dropping weights on the leaves where the beetles were resting, or by acoustic vibration, and the strength of the vibrations to cause immobility were quantified (Acheampong and Mitchell 1997).

However, studies investigating the stimulation that causes arousal from death feigning in animals is limited. Prolonged immobility increases an organism's risk of being parasitized or eaten by other predators which may rely on other cues such as scent. Thus, insects must rouse themselves when appropriate stimulation is provided. To our knowledge, there has been only two studies focusing on arousal from death feigning. Firstly, a report of a grasshopper trapped in the mouth of a frog

70 being aroused after being spat out (Honma et al. 2006), and secondly, a study on

71 *Tribolium castaneum* (Herbst) that demonstrated a threshold of stimulation

72 required to rouse from death feigning (Miyatake et al. 2019).

73   From a physiological perspective, a death feigning individual can be said to be in a

74 state of suspended immobility. It has been shown that respiration rate decreases

75 markedly during death feigning, while the heart rate is doubled, compared with that

76 in the resting state (Nishino and Sakai, 1996). In a nocturnal weevil species, *Cylas*

77 *formicarius*, adults feign death for a longer duration during the day compared to

78 during the night when they are most active i.e. they have a greater depth of death

79 feigning during the day (Miyatake 2001). Previous studies of death-feigning

80 behavior have suggested one hypothesis: a positive relationship between the depth

81 of death feigning and the difficulty of arousing from this behavior (Miyatake 2001;

82 Miyatake et al. 2019). In a recent study, the segregation in the duration of death

83 feigning for a F2 population of *T. castaneum* was analyzed by performing crossing

84 experiments between strains selected for shorter and longer durations of death

85 feigning (Miyatake et al. 2019). Indeed, a positive genetic correlation between

86 amplitude level for arousal and duration of death feigning was found (Miyatake et al.

87 2019).

88   There is substantial variation in the depth of death feigning in natural (field)

89 populations, which poses a challenge with respect to quantifying the strength of a

90 stimulus required for an insect to rouse from this behavior (see Miyatake et al. 2019).

91 The response to a stimulus can be accurately measured by using populations

92 selected to feign death for a longer duration, and thus, fixed for a depth of death

93 feigning. Recently, the strength of vibrational stimuli to rouse beetles from death

94   feigning was quantified using a population of *T. castaneum* selected for a long

95   duration of death feigning (Miyatake et al. 2019). A positive genetic correlation

96   between the intensity of arousal stimuli and the duration of death-feigning was

97   found (Miyatake et al. 2019). In addition to *T. castaneum*, populations with long

98   death feigning behavior have been established by selection in two additional, closely

99   related, *Tribolium* species: *T. confusum* (Nakayama et al. 2010) and *T. freemani*

100   (Konishi et al. 2020). These populations provide an ideal opportunity to examine the

101   relationship between the intensity of arousal stimulus, and the duration of death

102   feigning in two additional, same genus species. Understanding the relationship

103   between these two traits across species allows interspecific comparisons, and allows

104   us to determine the generality of this relationship across species in light of ecological

105   and evolutionary perspective.

106   In the present study, we thus, determine the intensity of the vibrational stimuli

107   required to rouse *T. confusum* and *T. freemani* from death feigning. To this end, we

108   use two populations of *T. confusum* and *T. freemani* which have been selected for a

109   long duration of death feigning to allow measurement of the intensity of vibrational

110   stimuli needed to rouse from this behavior (see Nakayama et al. 2010; Konishi et al.

111   2020). In addition, we determine whether the intensity of the vibrational stimuli

112   required to rouse the beetles from death feigning is positively correlated with the

113   beetle's depth of death feigning, i.e., duration of death feigning. From previous

114   studies, the average duration of death feigning in the strain selected for long

115   duration in *T. confusum* (ca 350 sec; Nakayama et al. 2010) has been found to be nine

116   times greater than that of *T. freemani* (ca 3200 sec; Konishi et al. 2020). On the other

117   hand, the death-feigning durations of the long strains were more than 10,000 sec in

118   *T. castaneum* (Matsumura and Miyatake 2018).

119   We predict that *T. confusum* which feigns death for a longer duration, will need a

120   greater strength of vibrational stimuli to rouse from death feigning compared to *T.*

121   *freemani*. Lastly, we include data from a previous study on *T. castaneum* to examine

122   how the strength of vibrational stimuli compares across the three species of

123   *Tribolium* beetle (Miyatake et al. 2019).

124

125   **Materials and Methods**

126   *Test of death feigning*

127   One day prior to testing the duration of death feigning, each beetle was placed in

128   a separate well of a 48-well tissue culture plate (Falcon, Becton Dickinson and

129   Company, Franklin Lakes, NJ, USA), thereby avoiding disturbance of this behavior by

130   other beetles (Miyatake 2001). The following day, each beetle was gently picked up

131   and turned over onto a white china saucer (14 cm diameter, 1.5 cm deep). Death

132   feigning was induced by touching the abdomen with a wooden stick. If the beetle did

133   not feign death, this process was repeated up to a maximum of three times. When

134   the beetle feigned death, the duration (from touching to detection of first movement)

135   of death feigning of the beetle was recorded with a stopwatch. If the beetle failed to

136   respond to all three stimuli, its duration of death feigning was recorded as zero.

137

138   *Insects and artificial selection*

139   We used strains of *T. confusum* and *T. freemani* that were artificially selected for

140   longer duration of death feigning. For *T. confusum*, the protocol of the artificial

141   selection experiment was described by Nakayama et al. (2010). The beetles were

142 reared on a mixture of whole meal (Nisshin, Tokyo, Japan) enriched with brewer's

143 yeast (Asahi Beer, Tokyo, Japan) in a chamber (Sanyo, Tokyo, Japan) maintained at

144 25 °C for *T. castaneum* and 27 °C for *T. confusum* under a 16 L:8 D photoperiod (lights

145 on at 07:00, lights off at 23:00). They were sexed based on distinct morphological

146 characteristics of the abdomen in the pupal stage and stored in single-sex groups in

147 a Petri dish (90 mm in diameter, 15 mm in height) for up to 1 day until the

148 experiment, and then the experiment was conducted.

149   Here, we describe only the selection methods for longer duration of death

150 feigning, although the selection experiments have been conducted not only for

151 longer but also shorter durations in previous research (Nakayama et al. 2010,

152 Konishi et al. 2020).

153   For *T. confusum*, 100 virgin males and 100 virgin females (7-14 days old) were

154 randomly collected from the stock culture and the duration of death feigning was

155 measured for each individual via the methods described above (Nakayama et al.

156 2010). Males and females (10 each) with the longest duration of death feigning were

157 selected to propagate the long-duration strains (L-strains). The selected males and

158 females were put together in a plastic container (70 mm diameter × 25 mm height)

159 with rearing medium (20 g), and females were allowed to lay eggs for one week.

160 Larvae were allowed to develop until they reached the pupa stage, at which point

161 they were sexed based on distinct morphological characters of the abdomen. The

162 pupae were then stored in single-sex groups in a dish (90 mm diameter × 15 mm

163 height). Fourteen days after emergence, 100 male and 100 female adults were

164 randomly collected, and their duration of death feigning was measured again. This

165 procedure was repeated for each generation. Three replicate lines for the L strains,

166 all initiated at the same time, were produced (see Nakayama et al. 2010 for details).

167 Three selection replicates for the long strains (L-A, L-B and L-C) initiated at the same

168 time were tested and maintained in the chamber. The selection regimes were

169 continued for more than 57 generations until 2016 for each line, and then, each line

170 was maintained without the selection regimes, i.e., relaxed selection, in the

171 laboratory (K. Matsumura unpublished data).

172   For *T. freemani*, the protocol of the artificial selection experiment was described

173 in Konishi et al. (2020). Briefly, 50 males and 50 females were collected from the

174 stock culture, and their death-feigning behavior was observed (F0 generation). The

175 observation method of death feigning and selection protocol were the same as in *T.*

176 *confusum*, with the exception of selection intensities (weaker than *T. confusum*:

177 10/50). At the same time, we produced three replicate lines for the L strains

178 following the same method described above (i.e., L1, L2, and L3). The selection

179 regimes were continued for more than 10 generations for each line (see Konishi et

180 al. 2020).

181

182 *Measurement of vibrational stimuli*

183   A beetle was gently placed at the center of the bottom of a Petri dish (90 mm in

184 diameter, 15 mm in height) that had a vibrator (Vp408, Acouve Laboratory Inc.,

185 Tokyo, Japan) attached to the bottom. Each beetle was poked with a stick, which

186 caused tonic immobility. To determine the amplitude of the vibrational stimulus

187 required to arouse individuals from death feigning, we conveyed vibrational stimuli

188 to the Petri dish by generating a continuous sine wave made by a sound editorial

189 system (Audacity 2.05, Softonic, Barcelona, Spain) as an input signal and amplifying

190 the wave by a digital amplifier (Lepy LP-2020A, Kyohritsu Electronic Industry Co.,

191 Ltd., Osaka, Japan). Only one Petri dish was used throughout the present experiment.

192 Four carrier frequencies, that is, 25 Hz, 40 Hz, 50 Hz, and 60 Hz, were provided to

193 death-feigning beetles to give different strengths of vibration. We also provided a

194 100 Hz (0.5 signal) to some beetles of the long strains.

195 Because the actual amplitude of the vibration stimulus depends on the frequency

196 characteristics of the vibrator and the substrate, we measured and identified vertical

197 displacement while vibrating the Petri dish using a triangular displacement meter

198 (LK-G155A, Keyence Corp., Osaka, Japan). As a result of the measurement, each

199 vibration in the vertical direction of the Petri dish was identified as follows: 25 Hz:

200 input signal, 0.3 → 0.03 mm, 0.5 → 0.08 mm, 40 Hz: 0.1 → 0.07 mm, 0.3 → 0.14 mm,

201 0.5 → 0.21 mm, 50 Hz: 0.1 →0.18 mm, 0.3 →0.34 mm, 0.5 → 0.38 mm, and 60 Hz: 0.1

202 → 0.12 mm, 0.3 → 0.48 mm, 0.5 →0.53 mm. We also measured vibration of 100 Hz

203 0.5 as 0.08 mm.

204 The experimental protocol was as follows. First, we provided vibrational stimuli

205 to a death-feigning beetle for 5 sec, and then, we observed the beetle's mobility for

206 3 sec. Next, we provided stronger vibration to the beetle and then observed the

207 beetle for 3 sec. After repeating this protocol, if the beetle woke up from death

208 feigning, we recorded the strength of vibration and considered the sensitivity value

209 for arousal from death feigning. Each individual was gradually subjected to

210 increasing vibration frequencies, and the amplitude which each individual was

211 aroused from death feigning was recorded.

212

213 *Statistics*

214   For comparisons of amplitude, nested ANOVA was used. Species, sex, and the

215   interaction between species and sex were used as explanatory variables, and the

216   replicate line of selection was nested within species. To compare the amplitudes

217   required for the three species, nested ANOVA with the same model was used. Tukey's

218   HSD test was used as the post hoc test. JMP version 12.2 (SAS Institute Inc., 2015)

219   statistical software was used for these analyses.

220

221   **Results**

222   Figure 1 shows the effects of amplitude on arousal in *T. confusum* beetles. In both

223   males and females, the proportion of individuals aroused from death feigning

224   increased as the intensity of the vibration increased (Figure 1a). A histogram of the

225   arousal rate (%) for each amplitude is shown in Figure 1 (b).

226   Figure 2 shows the effects of amplitude on arousal in *T. freemani* beetles. In both

227   males and females, the proportion of individuals aroused from death feigning

228   increased as the intensity of the vibration increased (Figure 2a). A histogram of the

229   arousing rate (%) for each amplitude is shown in Figure 2 (b) for *T. freemani*.

230   Mean values of arousal amplitude (with SEs) for *T. confusum* and *T. freemani* are

231   shown in Figure 3. Table 1 shows the results of nested ANOVA for arousal amplitude

232   of the two beetle species, namely, *T. confusum* and *T. freemani*. *T. freemani* beetles

233   required a significantly stronger amplitude (approximately 1.5 times greater) for

234   arousal from death feigning than *T. confusum* beetles (Figure 3, Table 1). Replicate

235   lines nested within the selection regime showed significant effects on the amplitude

236   (Table 1). There was a significant effect of sex on the amplitude—females required

237   a stronger amplitude for arousal from immobility (Figure 3)—and there was no

238   significant effect of the interaction between beetle species and sex (Table 1).

239

240   **Discussion**

241   In the present study, we found for the first time that the intensity of the stimulus that

242   causes arousal from death feigning varies among *Tribolium* species. Death feigning

243   is an important behavioral strategy adopted by animals for survival. It has been

244   hypothesised that the intensity of vibrational stimuli needed to arouse from death

245   feigning is positively correlated to the depth of death feigning i.e. duration of death

246   feigning (Miyatake 2001, Miyatake at al. 2019). In this study, we determined the

247   intensity of vibrational stimuli needed to arouse *T. freemani* and *T. confusum* from

248   death feigning which are known to differ in their duration of death feigning. We

249   found that in line with this prediction, *T. freemani*, which feigns death for longer,

250   required a higher average intensity of vibrational stimuli to arouse from death

251   feigning compared to *T. confusum*.

252   The mean and SE values of the durations of death feigning were 3158.9 0 ± 89.16

253   sec and 348.98 ± 26.65 sec in *T. freemani* (Konishi et al. 2020) and *T. confusum*

254   (Nakayama et al. 2010), respectively. In *T. confusum*, there was a period of

255   approximately 3 years (approximately 10 generations) of relaxed selection, which

256   may have further shortened the duration of death feigning (see Matsumura and

257   Miyatake 2018). The duration of death feigning of the base population of *T.*

258   *freemani* used in the selection experiment was approximately 500 sec (Konishi et al.

259   2020), which was longer than that of the base population of *T. confusum*

260   (approximately 1- sec) (Nakayama et al. 2010).

261   The present study found that females required significantly stronger stimuli for

11

262 arousal from death feigning than males in the two *Tribolium* species (Table 1).

263 However, no difference in death feigning duration between males and females has

264 been reported in *T. freemani* (Konishi et al. 2020), *T. confusum* (Matsumura et al.

265 2017) and *T. castaneum* (Matsumura et al. 2017). This means that our hypothesis

266 does not hold for males vs. females, although it is known that females are larger than

267 males in *T. cataneum* (Sokoloff 1977).

268 What would the comparison look like if we added the data of stimuli for arousal

269 from death feigning obtained in a previous study using *T. castaneum* (Miyatake et al.

270 2019) to the present data for the two species *T. confusum* and *T. freemani*, and

271 compared them? Figure 4 shows a comparative analysis of the three species of

272 *Tribolium* beetles, including data on the arousing time of *T. castaneum* (Miyatake et

273 al. 2019: Electronic supplementary material 1_XLSX 15 kb), which was measured

274 using the same method used in the present study. The results of the statistical

275 analysis are shown in Table 2. A multiple post hoc comparison (Tukey-HSD test)

276 showed that *T. freemani* required significantly greater vibrational intensity for

277 arousal than *T. castaneum* and *T. confusum* (Figure 4). The death feigning duration of

278 the base population of *T. castaneum* was about 5 sec (Miyatake et al. 2004; Konishi

279 et al. 2020), which is approximately the same length as that of *T. confusum*.

280 Incidentally, the results of the analysis showed no species-sex interaction (Table 2),

281 so the males and females are shown together in Figure 4.

282 Why does the intensity of the stimulus required for arousal differ among the three

283 species, and why does *T. freemani* require a stronger vibrational stimulus? One

284 possible explanation for arousal difference is that the three species share different

285 predators of which vary in size. However, all three of these species are cereal storage

286   pests and infest flour (Sokoloff 1977; Suzuki and Nakakita H 1991). Hence, it seems

287   unlikely that the predators of these three species would differ as they are likely to

288   encounter the same predators in their habitats. However, predators (of the same

289   species) are likely to vary in their size. Larger-sized predators may attack the largest

290   of the three species (*T. freemani*), while smaller predators may attack the other two

291   smaller *Tribolium* species resulting in these differences.

292   Secondly, differences in the size of the base population in each species, i.e.,

293   bottlenecks, may have contributed to the differences in the vibration level for

294   arousal. In *T. freemani*, the base population of the selection experiment was derived

295   from approximately 20 individuals collected from the field in 2014 (Konishi et al.

296   2020). However, the base population size of *T. castaneum* and *T. confusum* is

297   unknown (T. Miyatake, unpublished). Thus, the possibility of a bottleneck cannot be

298   verified in the present experiment. It may be possible to verify this if we can

299   experiment with different population sizes. However, as previously discussed,

300   variation in the duration of death feigning in natural populations presents a

301   challenge to verify this.

302   Thirdly, the history of predation pressure could differ among the three species.

303   The strain of *T. freemani* using in this present study is wild in origin. While the strains

304   of *T. confusum* and *T. castaneum* are of laboratory origin. These species may have

305   therefore, been exposed to a predator-free environment for a longer period of time

306   compared to *T. freemani*. This difference in history with respect to predatory

307   pressure may be the cause of the different arousal times.

308   Finally, body size may be a factor contributing to the difference in arousal intensity.

309   Compared to *T. castaneum* (body length: 3.0-4.0 mm) and *T. confusum* (body length:

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310 3.0-4.0 mm), *T. freemani* has a long body, approximately 5 mm (Konishi et al. 2020).

311 Different body sizes may suggest that even in the same habitat, *T. freemani* may not

312 share the same predators as *T. castaneum* or *T. confusum*. Thus, the magnitude of the

313 vibrational stimulus required for arousing from death feigning may be greater in *T.*

314 *freemani*. However, no studies have compared the differences among the predators

315 of these three species in the field. It will be necessary to investigate predators in the

316 field and the relationship between the intensity of amplitude and arousal brought

317 about by the approach of different predators to the beetles.

318 In summary, there were interspecies differences in the intensity of the stimulus

319 for arousal from death feigning. A comparison of the three species showed that the

320 intensity of the stimulus required for arousing was higher in species with greater

321 depth of death feigning, i.e., species with longer duration of death feigning. In the

322 future, comparative validation of death-feigning-related behavioral traits using

323 additional species, taking into account phylogenetic relationships, principles of

324 animal personality and related physiology aspects (see Kiyotake et al. 2014, Krams

325 et al. 2013, 2018, Nishi et al. 2010) is needed.

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410   *freemani* HINTON. In: Yushima K, Kamano S, Tamaki Y (eds) Rearing methods of

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414

415   **Table 1**. Results of nested ANOVA for the arousal amplitude of two beetle species: *T.*

416   *confusum* and *T. freemani*.

Factor	d.f.	F	p
Species	1	49.70	< 0.0001
Replicate line	4	13.16	< 0.0001
Sex	1	5.22	0.0229
Species*sex	1	1.50	0.2218

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419

420   **Table 2**. Results of nested ANOVA for the arousal amplitude of three beetle species: *T.*

421   *castaneum, T. confusum* and *T. freemani*.

Factor	d.f.	F	p
Species	2	32.16	< 0.0001
Replicate line	5	12.02	< 0.0001
Sex	1	3.84	0.0506
Species*sex	2	1.01	0.3640

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424

425   Figure legends

426

427   **Figure 1** Relationships between amplitude (mm) and arousing rate in *T. confusum*

428   beetles. Sample sizes of males and females were 84 and 90, respectively. Each

429   amplitude (mm) of vibration in the vertical direction of the Petri dish was identified

430   in the Materials and Methods.

431

432   **Figure 2** Relationships between amplitude (mm) and arousing rate in *T. freemani*.

433   Sample sizes of males and females were 115 and 109, respectively. Each amplitude

434   (mm) of vibration in the vertical direction of the Petri dish was identified in the

435   Materials and Methods.

436

437   **Figure 3** Arousal amplitudes for *T. confusum* and *T. freemani* beetles. Black and

438   white indicate male and female, respectively. Graphs show mean amplitude to

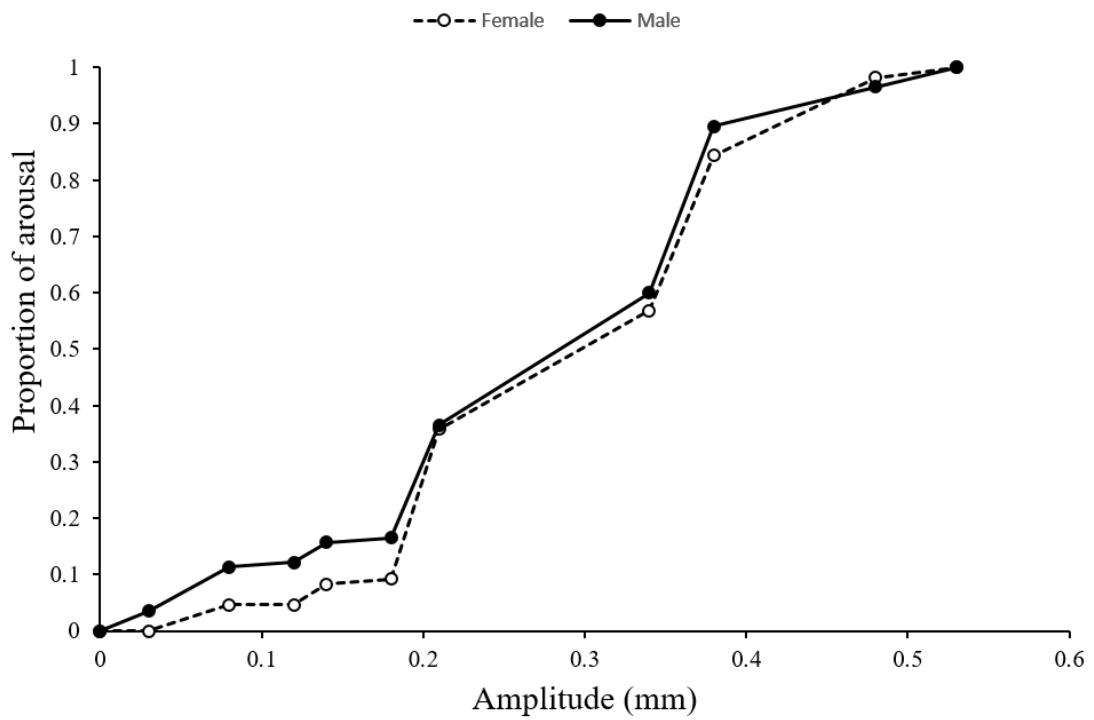439   arouse from death feigning. Error bars show the SE.

440

441   **Figure 4** Arousal amplitudes for beetles of each species. Graphs show mean

442   amplitude to arouse from death feigning. Error bars show the SE. There are

443   significant differences between the data indicated by different letters (Tukey's HSD

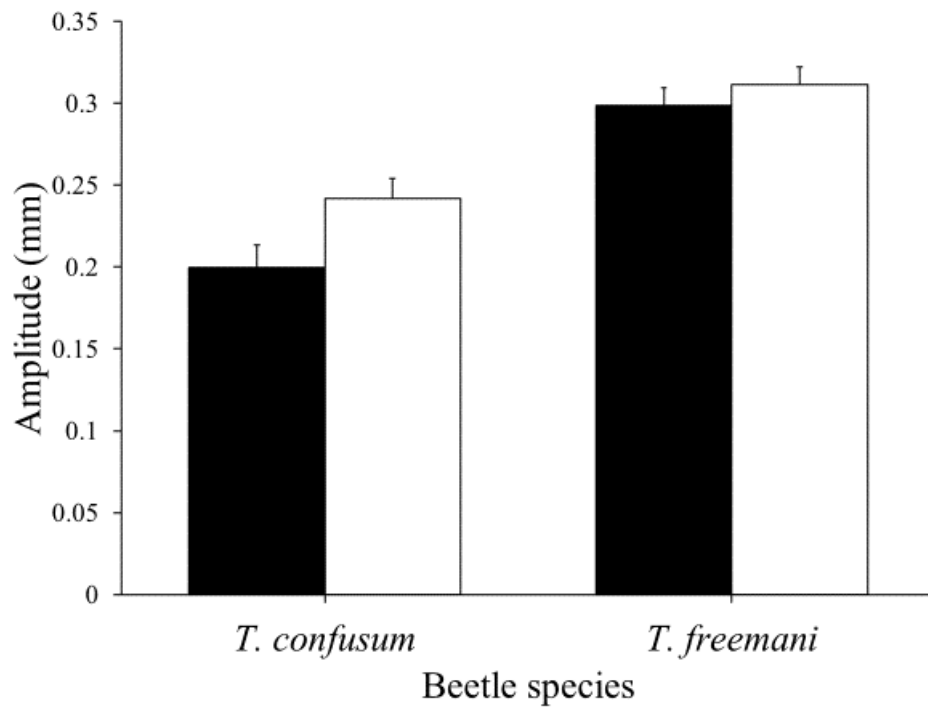444   test: $\alpha=0.05$).

445
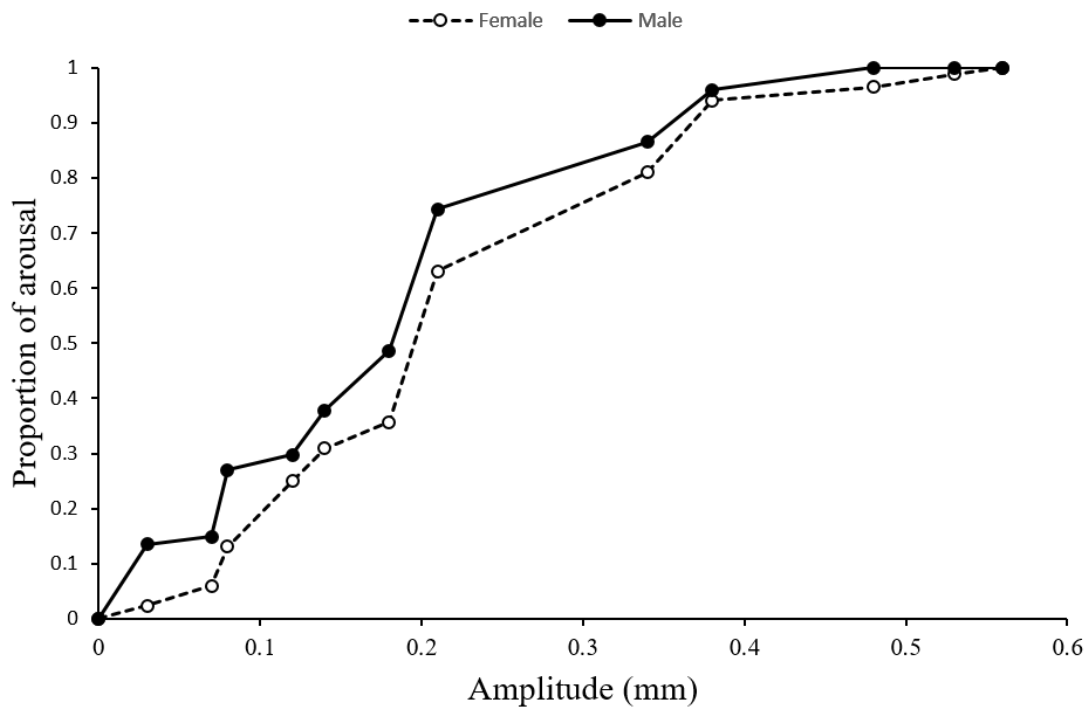
446
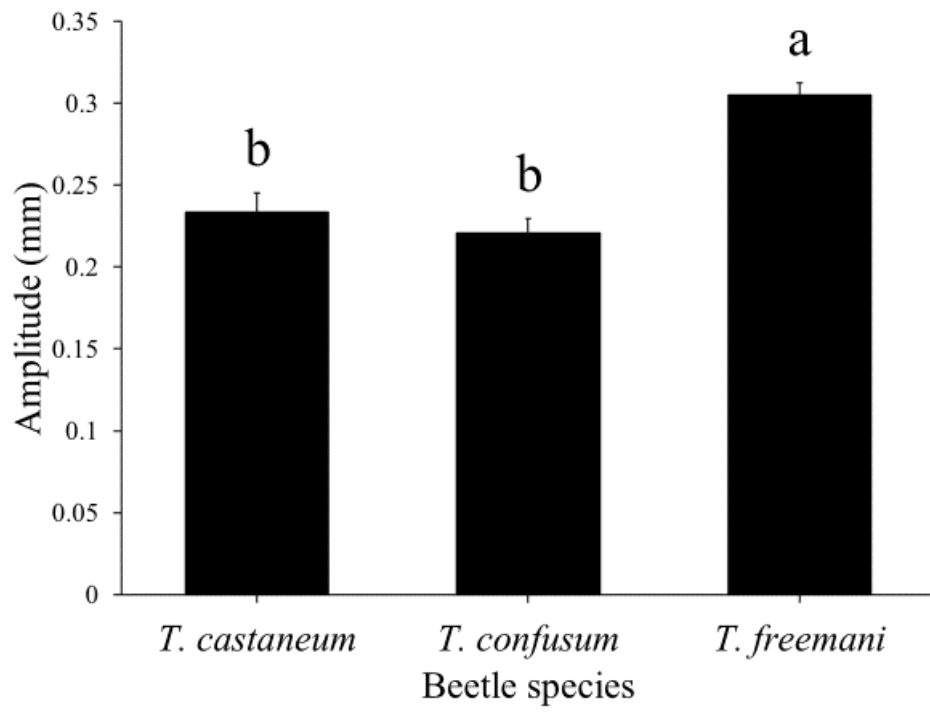447   Figure 1

448
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451   Figure 2

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Figure 3

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460   Figure 4