The disturbance leg-lift response (DLR): An undescribed behavior in bumble bees

Christopher A Varnon [Corresp., 1] , **Noelle Vallely** [1] , **Charlie Beheler** [1] , **Claudia Coffin** [1]

[1] Department of Psychology, Converse College, Spartanburg, South Carolina, United States

Corresponding Author: Christopher A Varnon
Email address: christopher.varnon@converse.edu

Background. Bumble bees, primarily *Bombus impatiens* and *B. terrestris*, are becoming increasingly popular organisms in behavioral ecology and comparative psychology research. Despite growing use in foraging and appetitive conditioning experiments, little attention has been given to innate antipredator responses and their ability to be altered by experience. In this paper, we discuss a primarily undescribed behavior, the disturbance leg-lift response (DLR). When exposed to a presumably threatening stimulus, bumble bees often react by lifting one or multiple legs. We investigated DLR across two experiments.

Methods. In our first experiment, we investigated the function of DLR as a prerequisite to later conditioning research. We recorded the occurrence and sequence of DLR, biting and stinging in response to an approaching object that was either presented inside a small, clear apparatus containing a bee, or presented directly outside of the subject's apparatus. In our second experiment, we investigated if DLR could be altered by learning and experience in a similar manner to many other well-known bee behaviors. We specifically investigated habituation learning by repeatedly presenting a mild visual stimulus to samples of captive and wild bees.

Results. The results of our first experiment show that DLR and other defensive behaviors occur as a looming object approaches, and that the response is greater when proximity to the object is lower. More importantly, we found that DLR usually occurs first, rarely precedes biting, and often precedes stinging. This suggests that DLR may function as a warning signal that a sting will occur. In our second experiment, we found that DLR can be altered as a function of habituation learning in both captive and wild bees, though the captive sample initially responded more. This suggests that DLR may be a suitable response for many other conditioning experiments.

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6 Christopher A. Varnon[1], Noelle V. Vallely[1], Charlie R. Beheler[1], Claudia A. Coffin[1]

7 [1] Department of Psychology, Converse College, Spartanburg, SC, USA

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9 Corresponding Author:

10 Christopher Varnon[1]

11 580 E Main St, Spartanburg, Spartanburg, SC, USA

12 Email address: Christopher.Varnon@Converse.edu

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21   **Abstract**

22   **Background.** Bumble bees, primarily *Bombus impatiens* and *B. terrestris*, are becoming

23   increasingly popular organisms in behavioral ecology and comparative psychology research.

24   Despite growing use in foraging and appetitive conditioning experiments, little attention has

25   been given to innate antipredator responses and their ability to be altered by experience. In this

26   paper, we discuss a primarily undescribed behavior, the disturbance leg-lift response (DLR).

27   When exposed to a presumably threatening stimulus, bumble bees often react by lifting one or

28   multiple legs. We investigated DLR across two experiments.

29   **Methods.** In our first experiment, we investigated the function of DLR as a prerequisite to later

30   conditioning research. We recorded the occurrence and sequence of DLR, biting and stinging in

31   response to an approaching object that was either presented inside a small, clear apparatus

32   containing a bee, or presented directly outside of the subject's apparatus. In our second

33   experiment, we investigated if DLR could be altered by learning and experience in a similar

34   manner to many other well-known bee behaviors. We specifically investigated habituation

35   learning by repeatedly presenting a mild visual stimulus to samples of captive and wild bees.

36   **Results.** The results of our first experiment show that DLR and other defensive behaviors occur

37   as a looming object approaches, and that the response is greater when proximity to the object is

38   lower. More importantly, we found that DLR usually occurs first, rarely precedes biting, and

39   often precedes stinging. This suggests that DLR may function as a warning signal that a sting

40   will occur. In our second experiment, we found that DLR can be altered as a function of

41   habituation learning in both captive and wild bees, though the captive sample initially responded

42   more. This suggests that DLR may be a suitable response for many other conditioning

43   experiments.

Introduction

44

45   The study of the psychological abilities of bees has become an important research area.

46   Such research provides insights to the valuable and global role of bees in agriculture and in the

47   ecosystem. Additionally, bees are also excellent model organisms for investigating the

48   relationships between complex behavior, ecological demands, and neurophysiology, and are the

49   most researched invertebrate in recent comparative psychology (Varnon, Lang, & Abramson,

50   2018). Psychological research with bees involves a number of topics including color perception

51   (Koethe, Bossems, Dyer, & Luna; 2016), olfactory learning (Riveros & Gronenberg, 2009b),

52   perception of time (Craig, Varnon, Sokolowski, Wells, & Abramson, 2014), conditioned taste

53   aversion (Varnon, Dinges, Black, Wells, & Abramson, 2018), learned helplessness (Dinges,

54   Varnon, Cota, Slykerman, & Abramson, 2017), select and reject stimulus control (Scienza et al.,

55   2019), concept learning (Giurfa, Zhang, Jenett, Menzel, & Srinivasan, 2001), social transmission

56   of learned behaviors (Alem et al., 2016), acquisition and flexibility of foraging skills (Raine &

57   Chittka, 2007; Strang & Sherry, 2014), maximization of resources (Charlton & Houston, 2010),

58   effects of pesticides on learning (Stanley, Smith, & Raine, 2015), alcoholism (Abramson, et al.

59   2006), and the neurophysiology of learning and memory (Hammer & Menzel, 1995; Galizia &

60   Eisenhardt, 2011; Guirfa, 2003).

61   Honey bees (*Apis mellifera*) are currently the most popular species for psychological

62   research, however, bumble bees, primarily *Bombus impatiens* in North America and *B. terrestris*

63   in Europe, have become a popular alternative due to some practical challenges related to

64   maintaining a honey bee laboratory, such as requiring a large outdoor foraging area. While recent

65   psychological research with bumble bees shows promising potential, one area that is notably

66   absent from the bumble bee literature is the study of innate defensive responses, especially in

67   conjunction with learning. For example, in honey bees, sting extension response (SER)

68   conditioning research investigates how restrained bees learn to sting in response to a stimulus

69   associated with shock (Vergoz, Roussel, Sandoz, & Giurfa, 2007; Tedjakumala & Guirfa, 2013).

70   Similar work has also been conducted in other Hymenoptera (e.g., Desment et al., 2017).

71   Unfortunately, there is not yet analogous work with bumble bees. This is surprising given that

72   bumble bees appear to offer a unique and ideal behavior to fulfill this line of research, the

73   disturbance leg-life response.

74   In this paper, we discuss the disturbance leg-lift response (DLR), and its potential use in

75   psychological research. When exposed to a presumably threatening stimulus, bumble bees

76   commonly react by lifting one or multiple legs (see Figure 1). While this behavior is primarily

77   undescribed and we have only found brief mentions in two publications (Djegham, Verhaeghe,

78   & Rasmont, 1994; Free, 1958), it appears to occur in many *Bombus* species worldwide. (Curious

79   readers may perform an online image search for the anthropomorphizations "bumble bee high

80   five" or "bumble bee wave"). In our first experiment, we investigate the temporal relationships

81   between DLR, biting and stinging as an invasive stimulus approaches in order to explore

82   potential functions of the DLR. In our second experiment, we investigate if the DLR is a suitable

83   behavior for conditioning procedures, similar to SER. Specifically, we compare habituation of

84   the DLR across captive and wild samples. Finally, we discuss implications for future research

85   with special considerations for the growing use of *Bombus* species as model organisms.

86   Experiment 1 - The Role of DLR

87   In this experiment, we explore the role of DLR as a reaction to potential danger to

88   establish an understanding of the behavior as a prerequisite to later investigations of DLR

89   conditioning. Many species emit specific responses, like DLR, when threatened. For example,

90   spiders may lift several front legs to reveal fangs (Cloudsley-Thompson, 1995), while hissing

91   cockroaches produce an audible hiss (Hunsinger, Root-Gutteridge, Cusano, & Parks, 2017;

92   Shotton, 2014). Although making distinctions between categories of antipredator responses can

93   be challenging, there are two major categories that could be considered for DLR: the aposematic

94   display, and the pursuit deterrence signal.

95   Conspicuous aposematic displays can signal toxicity or danger to a potential predator.

96   The vibrant colors of poison dart frogs in the family *Dendrobatidae* illustrate a case of honest

97   aposematic signals; the colors indicate that the frog possesses toxic alkaloid compounds (Maan

98   & Cummings, 2012). Similarly, in the southern United States, the bright red banding of coral

99   snakes (*Micrurus euryxanthus, M., fulvius,* and *M. tener*) honestly signals a potent neurotoxic

100 venom. Several species of scarlet snake (*Cemophora sp.*) and kingsnake (*Lampropeltis sp.*) also

101 possess similar conspicuous red banding but lack venom. For the venomless mimic snakes, the

102 aposematic coloration is a dishonest signal (Greene & McDiarmid, 1981).

103   Pursuit deterrence signals can alert a potential predator that it has been detected,

104 communicating vigilance and possibly fitness to the predator (Hasson, 1991). For example,

105 Thomson's gazelles (*Eudorcas thomsonii*) leap vertically into the air, a behavior known as

106 stotting (FitzGibbon & Fanshawe, 1988), while white-tailed deer (*Odocoileus virginianus*) erect

107 their tails to reveal a high contrast white rump when a predator is detected (Bildstein, 1983), and

108 anole lizards (*Anolis sp.*) may signal fitness to potential predators with head bobs, pushups, or

109 dewlap extensions (Leal & Rodríguez-Robles, 1995). While discussions of pursuit deterrence

110 signals typically suggest that they signal the ability to escape, it seems reasonable that they could

111 also signal readiness to use a defense such as venom.

112   It is possible that the DLR of bumble bees functions in an aposematic or venom-based

113   pursuit deterrence role. The stinging response of bumble bees and other Hymenoptera can clearly

114   serve as the foundation for an honest warning signal, and the vibrant color patterns of many bees

115   and wasps are one well-known aposematic display. Not only does the bright coloration lead to

116   predators quickly learning to not consume bumble bees (Brower, Brower, & Westcott, 1960), but

117   this coloration also leads to mimics (Fisher & Tuckerman, 1986; Plowright & Owen, 1980). If

118   DLR functions in either an aposematic or a venom-based pursuit deterrence role, we would

119   expect it to be closely associated with, and precede, stinging. In the following experiment, we

120   investigate this possibility by examining the probability and order of DLR, biting and stinging in

121   response to invasive stimuli. If DLR often precedes, but does not follow, stinging, this would

122   provide the first evidence that DLR is an honest signal of envenomation potential.

Methods

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Subjects

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125   Captive worker bumble bees (*Bombus impatiens*, n = 62) collected from a single, captive-

126   breed "Natupol" bumble bee colony purchased from Koppert Biological Systems Inc. (Howell,

127   MI) were used as subjects. The bees were maintained in the ventilated plastic colony cage (24.5

128   x 21.5 x 12 cm, l x w x h) in which they were shipped. The outer cardboard layer, typically used

129   to shield colonies from outdoor conditions, was removed except for the top piece, which ensured

130   that the hive remained in darkness. The colony was placed on 40-watt intellitemp heating pad

131   (Big Apple Pet Supply; Boca Raton, FL), which maintained a temperature of about 31 ˚C inside

132   the hive. The colony was connected to an adjacent empty colony cage that served as a feeding

133   area through a clear acrylic tube (2.5 cm inner diameter). Two lights (36" Zoo Med Reptisun T5-

134   Ho Terrarium Hood, Zoo Med Laboratories Inc.; San Luis Obispo, CA) were placed

135   approximately 31 cm above the colony. These light fixtures provided a full range of illumination,

136   including ultraviolet (UV) light in the range of 280 – 400 nm. Bumble bees can see UV light in

137   the range of 300 – 400 nm (Skorupski & Chittka, 2010) and naturalistic lighting conditions may

138   be important for their growth and survival (Blacquière, Cornelissen, & Donder, 2007).

139   Lights and heat were automatically turned on at 7 AM and turned off at 7 PM each day to

140   help the bees maintain daily foraging patterns. The laboratory lacked any source of natural light,

141   and all other lights in the laboratory were also turned off by this time. Bees were allowed ad

142   libitum access to food (either a 50% sucrose solution (w/w) or the "Bee-happy" solution

143   provided by Koppert Biological Systems Inc.) in the feeding cage via several paper towel wicks.

144   Water was provided directly in the hive via syringe. A three to one mixture of pollen (Stakich

145   Bee Pollen Powder, Stakich, Inc.; Troy, MI) and pollen substitute (Mann Lake Ultra Bee, Mann

146   Lake LTD.; Hackensack, MN) was made available ad libitum inside the hive.

147   Captive worker bees were collected from the clear acrylic tube and the feeding cage,

148   chilled in a refrigerator around 1.1 C until inactive, then placed in the experimental apparatus.

149   After an experimental session was complete, the bees were chilled, weighed, measured, then

150   marked with an acrylic paint marker between the wings on the thorax before being returned to

151   the colony. Captive bees collected and returned to the hive in this manner were observed alive

152   and healthy up to 8 weeks after participating in an experiment. As Converse College does not

153   require an institutional review for invertebrate research, no specific review was required for the

154   present study.

155 Procedure

156   Subjects were placed in individual apparatuses after being collected. Each apparatus

157 consisted of a clear plastic cube (2.6 x 3 x 2 cm), made from a microscope cover slip container,

158 with two holes (2.55 mm diameter) drilled on opposite sides. The size of the apparatus allowed

159 the bees to freely move but did not permit flight or substantial relocation inside the apparatus.

160 After being placed in the apparatus, subjects were transferred to an experimental room, placed

161 approximately 1.2 m apart, and allowed to acclimate for three hours.

162   Each bee experienced five trials with a 15-minute intertrial interval (ITI) after the

163 acclimation period. Bees were randomly assigned to either an experimental or control group.

164 During trials for the experimental group, a researcher startled the bee by inserting a toothpick

165 approximately halfway (1.3 cm) into the apparatus for 10 seconds through the hole closest to the

166 bee. During this time, it was possible for the bee to physically contact the toothpick. An identical

167 procedure was used for the control bees, except that the toothpick was held outside of the

168 apparatus adjacent to the hole. This group controlled for the general approach of the

169 investigators, as well as the presence of a close object that could not be contacted by the bees.

170   During each trial, several behaviors were scored from video recordings. We recorded

171 both the occurrence of DLR and the number of legs lifted during each DLR. We specifically

172 defined DLR as when one or more legs were lifted above the bee, relative to the bee's position.

173 Legs that were lifted prior to the trial were not considered a DLR; observing the movement

174 during the trial was required to record a DLR. Biting was recorded as any time a bee visibly

175 opened and closed its mandibles during a trial. Often bees made mandible contact with the

176 stimulus, but this was not required. Finally, we recorded stinging any time the bee contacted the

177 stimulus with its abdomen or directed its abdomen toward the stimulus. These abdomen curls are

178   the first component of the sting extension response (Gage, Ahumada, Rivera, Graham, &

179   DeGrandi-Hoffman, 2018). In most cases, the stinger was obvious and contacted the stimulus. In

180   some cases, the activity of the stinger was less clear, but the unusual abdomen curls and contact

181   were easy to observe. These abdomen curls were only observed when stimuli were presented

182   during the experiment or when the bees were handled during collection. We used a broad

183   definition of stinging, relative to DLR and biting, to capture instances where the sting extension

184   could not clearly be observed, or where the bee was not able to physically contact the stimulus

185   from its current location. For each trial, we also recorded the order in which DLR, biting, and

186   stinging occurred.

187   Analysis

188   All analyses were conducted through the StatsModels package (Perktold, Seabold &

189   Taylor, 2018) included in the Anaconda distribution of Python, a free scientific analysis

190   distribution of the Python programming language (Anaconda, 2019; http://www.python.org).

191   Behavior sequences (e.g., DLR then bite, or bite then DLR then sting, etc.) were analyzed with a

192   series of repeated measures logistic regressions via generalized estimating equations (GEE;

193   Hardin & Hilbe, 2003). We used this series of regressions in place of a multinomial logistic

194   regression as GEE controls for repeated measures within subjects. This technique is also less

195   sensitive to the need for many cases per variable than multinomial regression or chi square

196   analyses; an important consideration for our data, as statistical comparisons between commonly

197   and uncommonly observed behavior sequences answer important research questions. We used an

198   interceptless model where groups are treated as two mutually exclusive variables. By default, a

199   logistic regression's parameter estimates and associated p values display a difference from a 50%

200   binary chance level. As our sequence analysis considered 16 possible sequences, we subtracted

201   the log odds of 1/16 from all parameter estimates and confidence intervals, then calculated

202   corresponding p values. Each behavior sequence is therefore tested for statistical difference from

203   chance (1/16) instead of a 1/2 comparison that is arbitrary for this data. Individual parameter

204   estimates were compared directly by creating a z score by dividing the difference between the

205   estimates by the square root of the sum of the squared standard errors of the estimates (Clogg,

206   Petkova, & Haritou, 1995; Paternoster, Brame, Mazerolle, & Piquero, 1998). Even after

207   adjusting parameters by subtracting the log odds of 1/16, the difference between estimates, z

208   score, and p value are still the same as those normally reported by a regression that includes one

209   level of a categorical variable in the intercept.

210   # Results

211   A visual overview of the findings can be seen in Figures 2 and 3, while subsequent

212   sections analyze our primary result, behavior sequence, in detail. Figure 2 shows the percent of

213   trials where a DLR, bite or sting occurred for bees in the experimental and control groups. Bees

214   in the experimental group displayed more behavior. For both groups, DLR was the most

215   common behavior. Bees in the experimental group were more likely to sting than bite, while bees

216   in the control group were more likely to bite than sting. The average number of legs lifted in

217   trials where DLR occurred can be seen in Figure 3. Not only were bees in the experimental group

218   more likely to emit a DLR (Figure 2), but they also lifted more legs on average. Both Figures 2

219   and 3 show little change across trial that would suggest habituation, sensitization, or fatigue.

220   While our analysis in subsequent paragraphs focuses on behavior sequences, we also included

221   our initial exploratory analysis of individual behaviors (without considering their order) in

222   supplementary material that will relate well to Figures 2 and 3.

223   Table 1 shows the percent of trials where a particular behavior sequence occurred. For

224   each trial, the order of DLR, bite and sting were recorded, resulting in 16 possible sequences (no

225   tied rankings were observed). Corresponding statistical analysis that show the log odds of each

226   sequence compared to a 1/16 chance value can be seen in Tables 2 and 3, with Table 2

227   displaying information for the experimental group, and Table 3 displaying information for the

228   control group. We only included the groups as parameters in this analysis given the large number

229   of behavior sequences to be analyzed and the lack of a trial effect in previous graphs. The

230   direction and magnitude of the parameter estimates, as well as the p values, reflect a difference

231   from the chance value. Although we used separate tables due to the large size of a single

232   combined table, the sequence analyses presented on Tables 2 and 3 should be interpreted

233   together.

234   Bees in the experimental group were highly active, only being inactive 3% of the time.

235   DLR was emitted first or by itself around 81% of trials, while biting and stinging rarely occurred

236   first or by themselves. All the DLR-first sequences, except for DLR:Bite, occurred significantly

237   more than chance (p values < 0.000). The least common of the DLR-first sequences was the

238   DLR:Bite sequence, occurring during only 1% of trials, and was the only DLR-first sequence to

239   occur significantly less than chance ($p = 0.017$). Trials with a sting following DLR were much

240   more common (30%), and trials with a bite and sting following DLR (in either order) were also

241   more common (31%). Taken together, this indicates that in the experimental group, DLR is more

242   related to subsequent stings than to subsequent bites. Bees in the control group were inactive

243   during 51% of trials, significantly more than chance ($p < 0.000$). During trials when bees were

244   active, DLR often occurred by itself (31%), or a bite occurred by itself (15%). These were the

245 only behaviors that occurred significantly more than chance (p values < 0.002). Sequences of

246 multiple behaviors were rare.

247   Table 4 shows pairwise comparisons between DLR:Bite and other DLR-first sequences,

248 as well as comparisons between DLR:Sting and other DLR-first sequences for the experimental

249 group. The estimate differences and z scores were calculated from the parameter estimates and

250 standard errors reported in Tables 2 and 3. The pairwise comparisons show that the DLR:Bite

251 sequence occurs significantly less than all other DLR-first sequences (p values < 0.000).

252 Conversely, the DLR:Sting sequence occurs significantly more than DLR:Bite, and other DLR-

253 first sequences (p values < 0.009) except for DLR alone. Though DLR:Sting does occur more

254 than DLR alone, the difference is not significant ($p = 0.064$). Note that for this series for pairwise

255 comparisons, it may be appropriate to use a multiple comparison correction to adjust the

256 significance threshold. For example, the conservative Bonferroni correction would involve

257 dividing the alpha level by the number of comparisons, in this case 0.05 divided by 7 produces a

258 new significance threshold of 0.007, which may affect interpretation of the comparisons between

259 DLR:Sting and DLR-first sequences containing both a sting and a bite. The reader is free to use

260 whichever correction technique they deem appropriate.

261   Taken together, the findings reported in Tables 1 - 4 strongly suggest that DLR is more

262 related to subsequent stinging than it is to subsequent biting for the experimental group. This is

263 also in line with our supplementary analysis of individual behaviors. Ultimately, the fact that

264 DLR often precedes stinging in the experimental group, the group where stinging frequently

265 occurred, indicates DLR may function to signal potential predators that a sting is imminent.

Discussion

266

267   Our findings describe DLR, a primarily undocumented behavior, and show that it often

268   precedes stinging, but rarely precedes biting alone. We also demonstrate that the probability of

269   DLR is sensitive to stimulus intensity, as the increased proximity to the stimulus in the

270   experimental group, compared to the control group, altered behavior. Together, these results

271   suggest that DLR is an honest signal that indicates stinging may occur. DLR may function in

272   either an aposematic or a pursuit deterrence role, and these functions may not be mutually

273   exclusive. Given the already bright coloration of bumble bees, it is possible that DLR serves as a

274   multimodal enhancement of existing aposematic signals, adding a conspicuous posture to vibrant

275   colors (for discussions on multimodal antipredator signals see Ritson-Williams & Paul, 2007;

276   Rowe & Guilford, 1999; and Rowe & Haplin, 2013). If DLR has a pursuit deterrence function, it

277   may signal that the bee is aware of a potential predator and will sting if pursued.

278   While DLR likely signals a sting may occur, DLR can also occur alone. In the control

279   group, bees emit DLR but rarely sting. This likely occurs because the distant stimulus is intense

280   enough to elicit DLR, but does not support stinging. In the wild, if DLR is successful at

281   preventing a potential predator attack, it may occur without subsequent stinging behavior.

282   Therefore, the occurrence of DLR in the control group is consistent with an honest signal

283   interpretation.

284   While our experiment clearly indicates a temporal connection between DLR and stinging,

285   additional research is needed to clarify DLR's specific antipredator function. Such research will

286   need to consider what stimuli and predators elicit DLR, and equally importantly, how predators

287   respond. Field experiments may also study DLR in situ, providing bees with a number of

288   alternative behaviors, including fleeing. Such ecologically valid research may be required to

289   completely determine the function of DLR. For example, if DLR functions strictly as a pursuit

290   deterrence signal, bees may emit a DLR, then flee if a predator approaches, while if DLR has

291   only an aposematic function it may not be related to any antipredator behavior other than

292   stinging.

293   In addition to further clarifying the function of DLR, our initial work facilitates many

294   additional research topics. For example, research may consider how DLR relates to specific

295   stimulus modalities or intensities, and if predators have learned or innate responses to DLR.

296   Research should also consider the extent that DLR occurs in other *Bombus* species, and if it

297   differs across species. Studies of individual differences will likely also be fruitful, especially

298   considering recent literature on the size-dependent behavior in bumble bee workers (e.g. Jandt,

299   Huang, & Dornhaus, 2009; Kodaira, Ohtsuki, Yokoyama, & Kawata, 2009; Raine & Chittka,

300   2008; Riveros & Gronenberg, 2009a; 2009b; Spaethe & Weidenmüller, 2002). Finally, as bees

301   are social animals, future work should also consider social factors, such as if DLR can be elicited

302   by other bees or if DLR affects adjacent bees. It may also be possible that DLR, like a number of

303   other behaviors, is affected by alarm pheromones (e.g., Avalos et. al., 2017; Rossi, N., d'Ettorre,

304   P., & Giurfa, M., 2018). Given that bumble bees possess tarsal glands (Pouvreau, 1991; Schmitt,

305   1990), social odors may even be released during DLR.

Experiment 2 - Habituation of DLR

307   In this experiment, we investigated the possibility that DLR could change as a function of

308   learning. Specifically, we wanted to know if DLR habituates to repeated mild stimuli.

309   Habituation, defined as the diminishing of a response, emotional or physical, to a repeated

310   stimulus (Thompson & Spencer, 1966), is a simple form of learning that can be observed across

311   nearly all species, from planarians (Nicolas, Abramson, & Levin, 2008) to rodents (Geyer &

312   Braff, 1987). Habituation of disturbance responses has also been documented in many species.

313   For example, hissing cockroaches may cease emitting their disturbance hiss in the presence of

314   specific handlers (Davis & Heslop, 2004), rattlesnakes show a reduction in latency and duration

315   of rattling in response to a startling stimulus (Place & Abramson, 2008), and the gill withdrawal

316   reflex of the sea hare *Aplysia* is also known to habituate (Carew, Pinsker, & Kandel, 1972).

317   Studies of habituation are also often the foundation for other procedures, including investigations

318   of mental health (Akdag, Nestor, O'Donnell, Niznikiewicz, Shenton, & McCarley, 2003; Geyer

319   & Braff, 1987; Jaycox, Foa, & Morral, 1998), and neurological processes related to learning and

320   memory (Castellucci & Kandel, 1974; Castellucci, Pinsker, Kupfermann, & Kandel, 1970). If

321   DLR can be altered through habituation learning, this opens new possibilities in behavioral and

322   physiological research with bumble bees.

323   In addition to discovering if DLR can change as a function of learning, we were also

324   interested in differences across populations due to differences we observed in pilot research.

325   Specifically, we compared samples of captive bred to wild caught bumble bees. Given the

326   substantial number of findings on behavioral differences in honey bees due to breed, genetics

327   and environment (e.g., Alaux et al., 2009; Schulz, Haung, & Robinson, 1998; Sheppard, Arias,

328   Grech, & Meixner, 1997; Spivak, 1997; Tautz, Maier, Groh, Rössler, & Brockman, 2003), it is

329   reasonable to investigate if some differences may be found between captive bred and wild caught

330   bumble bees. If DLR and habituation of DLR are observed across samples of both populations,

331   this would also suggest that DLR may be a robust behavior to study in learning experiments.

332   This would be a beneficial comparison considering the use of both wild and captive bees in the

333   literature.

Methods

Subjects

Both captive (n = 64) and wild worker bumble bees (n = 64) were used in this experiment. Captive bees were acquired and maintained in a similar manner as described in experiment 1 with a few exceptions. The captive colony was connected to a screen flight cage (91 x 46 x 46 cm, l x w x h), made from a modified Zoo Med "Reptibreeze" reptile cage via a 16 cm long, clear acrylic tube (2.5 cm inner diameter). In this flight cage, bees fed from plastic dishes. Two lights (one 36" Zoo Med Reptisun T5-Ho Terrarium Hood and one 30-38" Zoo Med Reptisun LED Terrarium Hood) were placed approximately 31 cm above the colony, providing a full range of light.

Captive bees were collected in the flight cage and prepared in a similar manner as described in the previous experiment and were observed alive and healthy up to 4.5 weeks after participating in an experiment. Thirty-two captive bees were collected from one colony in 2018, while the remaining 32 captive bees were collected from a second colony in 2020. Wild worker bees were collected while foraging, primarily on clover and *Abelia*, at the Converse College campus (Spartanburg, SC). Thirty-two wild bees were collected during July 2018, while the remaining 32 wild bees were collected during August and September 2020. Procedures for capturing, chilling, using, and marking wild bees were similar to the procedures for captive bees. Wild bees were released at the capture location, and many immediately returned to foraging. Marked wild bees were observed foraging two weeks after the experiment. As Converse College does not require an institutional review for research with non-threatened invertebrates, no specific review or permits were required for the present study.

356   Captive bees were visibly smaller than their wild counterparts. We recorded head width

357   in a sample of 249 bees; 123 captive and 62 wild bees from 2018 pilot research, as well as the 32

358   captive and 32 wild bees collected for this experiment in 2020. An independent samples t-test

359   revealed that captive bees were significantly smaller that wild bees (mean difference = –0.61

360   mm, t_{247} = –10.727, p < 0.000).

361   Procedure

362   Subjects were placed in individual apparatuses after being collected. Each apparatus

363   consisted of a capsule formed from a clear acrylic tube (4.5 cm long, 2.5 cm inner diameter),

364   with two white plastic caps sealing the tube. Two holes (0.4 cm diameter) were drilled near the

365   center of each cap. Each apparatus was placed approximately half a meter apart, and bees were

366   allowed an acclimation period of 45 minutes.

367   Each bee experienced 10 trials with a seven-minute ITI after the acclimation period was

368   complete. During each trial, a researcher startled the bee by presenting a hand 15 cm above the

369   apparatus, rapidly lowering it to approximately 6 cm above the apparatus, rotating the hand once

370   in a clockwise circular motion, and then withdrawing the hand. As strong stimuli can inhibit

371   habituation or cause sensitization, we used this relatively mild stimulus, compared to those used

372   in the first experiment, to increase the chance that habituation could be observed. The bees'

373   response was recorded during the two-second stimulus presentation and for three seconds after

374   the presentation. DLR was recorded as a binary response and no other behaviors were recorded.

375   Subjects collected in 2020 also experienced three additional trials. On the 11th trial, the bottom

376   of the apparatus was tapped once, out of view of the subject. This trial served as a dishabituation

377   trial to determine if DLR would occur to other stimuli and verify that any decrease in DLR

378   during the preceding 10 habituation trials was not caused by fatigue. The 12th trial was a return

379   to the standard habituation trial. Finally, on the 13th trial, subjects were collected by reaching

380   directly toward the front of the apparatus, visible to the subject, and holding a hand adjacent to,

381   but not touching, the apparatus for the five-second observation period before placing the

382   apparatus in the refrigerator for chilling. All bees, in both 2018 and 2020, were chilled after

383   recollection, marked, and returned to their colony or the collection area.

Analysis

385   We analyzed the probability of DLR across trial using repeated measures logistic

386   regression via GEE. As with previous logistic regressions, we use an interceptless form so that

387   the parameters can be directly compared to a chance value (in this case 50%), then compared

388   them to each other by creating a z score by dividing the difference between the estimates by the

389   square root of the sum of the squared standard errors of the estimates. As initial analysis revealed

390   no significant differences between bees in the 2018 and 2020 collection periods (p values <

391   0.489), and there was no theoretical reason to expect differences, we did not include collection

392   period as a parameter in our main analysis.

Results

394   Figure 4 shows the percent of captive and wild bees emitting DLR across the 10

395   habituation trials in both the 2018 and 2020 collection periods while Table 5 shows

396   corresponding statistical analysis. The captive bees were initially much more likely to respond;

397   nearly 65% of captive bees responded compared to around 35% of wild bees. The analysis shows

398   that the initial probability of response for captive bees was significantly greater than chance ($p =$

399   0.005). Wild bees initially responded less than chance, but not significantly so ($p = 0.157$). A

400   direct comparison of the parameters revealed that captive bees were significantly more likely to

401   initially respond than the wild bees (estimate difference = 0.931, z = 2.899, p = 0.004). The

402   probability of response decreased significantly across trials for both captive and wild bees at a

403   somewhat similar rate (p values < 0.000). Though the captive bees showed a slightly stronger

404   effect, a direct comparison reveals that this difference was not significant (estimate difference =

405   –0.030, z = –0.549, p = 0.583).

406   The final three trials, also shown in Figure 4, were only conducted with subjects collected

407   in 2020. Corresponding analysis comparing the final three trials to trials 1 and 10 can be seen in

408   Table 6. While the original GEE regression is shown in supplemental material (Table S4), it is

409   more useful to interpret the pairwise comparisons shown in Table 6 that were derived from the

410   GEE regression. The 11th, dishabituation trial shows the probability of response returned to

411   nearly a trial 1 level, as does the 13th, collection trial. The analysis shows that for both captive

412   and wild bees, these response levels are statistically indistinct (p values > 0.317). The 12th trial

413   was a return to a standard habituation trial and approximately follows the trends seen in the

414   previous 10 habituation trials. The analysis shows that, although the response level for wild bees

415   on the 12th trial was somewhat higher than might be expected, for both captive and wild bees,

416   these response levels are statistically indistinct (p values > 0.453). Both trial 10, the final

417   habituation trial, and trial 12, the return to habituation trial, showed significantly lower

418   probabilities of response than trial 11, the dishabituation trial, and trial 12, the collection trial (p

419   values < 0.032). As with the previous pairwise comparisons in experiment 1, it may be

420   appropriate to use a multiple comparison correction to adjust the significance threshold. In this

421   case, interpretations of comparisons between the wild bees' probability of response on trial 12

422   may change slightly. Overall, the tendency of bees to perform DLR when stimuli were presented

423   in the 11th and 13th trials suggests that the decrease in DLR during the habituation trials occurred

424   due to habituation learning, not motor fatigue or sensory adaptation.

Discussion

426   In this experiment, we provided the first demonstration of habituation of DLR, as well as

427   documented differences in DLR across samples of captive and wild populations. While both

428   samples showed a similar rate of habituation, the captive bees were initially more likely to

429   perform DLR. This difference in DLR may have occurred for two different reasons. First, the

430   samples of worker bees we collected from captive and wild populations may have differed in

431   role specialization. Bumble bee castes include the reproductive queen and drone castes, as well

432   as the primarily non-reproductive worker caste frequently used in research. Workers may be

433   further specialized. The smaller worker bees are more likely to feed larvae and attend to hive

434   maintenance, while larger workers act as foragers. In bumble bees, role specialization appears to

435   be determined during early development, and research suggests that physical dimensions can

436   predict behavioral performance (Jandt, Huang, & Dornhaus, 2009; Kodaira, Ohtsuki, Yokoyama,

437   & Kawata, 2009; Raine & Chittka, 2008; Riveros & Gronenberg, 2009a; 2009b; Spaethe &

438   Weidenmüller, 2002).

439   The bees sampled from our captive population were significantly smaller than those from

440   our sample of wild bees, suggesting the wild bees were more likely to be foragers. The wild bees

441   were also clearly collected during the act of foraging, further increasing our confidence they fit

442   this role specialization. While our captive bees were also collected in their foraging area, we are

443   less confident this collection method ensures they are true foragers due to one of the challenges

444   of maintaining an indoor bumble bee hive. In laboratory colonies, bumble bee workers may not

445   return to the hive after feeding, and may instead inhabit a flight cage or feeding area. Workers

446   may build clusters of cells near a food dish, store food, and even raise drones. We call this

447   tendency the "lost bee effect." If not carefully managed, this may result in half the colony

448   moving away from the hive within a month (see Figure S1 in the supplemental material).

449   Although not widely published (we have only seen this reported in Jandt & Dornhaus, 2009 and

450   Blacuiére, Cornelissen, & Donders, 2007), the lost bee effect appears to be a common issue.

451   While there is no clear solution, preventative measures include capturing lost bees each day,

452   returning them to the colony, and cleaning the flight cage to remove odors (F. Muth, personal

453   communication, June, 2018); capturing and returning lost bees while also killing repeat offenders

454   (J. Jandt, personal communication, June, 2018); feeding the bees inside the hive and completely

455   preventing access to other areas (W. Gronenberg & A. Riveros, personal communication, June,

456   2018); and providing a smaller antechamber between the hive and the flight cage to encourage

457   lost bees to build closer to the hive (J. Nieh, personal communication, June, 2018). While our

458   observations and the above communications are in regards to the North American *Bombus*

459   *impatiens*, the lost bee effect also occurs in the European *B. terrestris* (L. Chittka, personal

460   communication, August, 2018). Given the tendency of worker bees to become "lost", it may not

461   be possible to ensure that bees collected near food are the larger, foraging-specialized workers.

462   Instead, collected subjects may also consist of smaller bees specialized for brood care and hive

463   maintenance. While a growing body of research demonstrates the usefulness of laboratory

464   colonies, the lost bee effect indicates they may not be thriving, and this may impact their use as

465   model organisms. In our case, the lost bee effect may have made our sample of captive bees less

466   likely to be foragers.

467   A second possible reason for the difference in DLR between captive and wild bees may

468   be the distinct experiences of bees raised indoors compared to those of wild bees. Captive bees

469   were only exposed to stimuli in their hive and flight cage, and ultimately experienced only a

470   small number of stimuli before research. Conversely, wild bees likely contact many stimuli

471   during daily foraging including other insects, birds, pedestrians, and even landscaping

472   equipment. It is possible that exposure to a wide variety of stimuli served to acclimate the wild

473   bees to mild visual stimuli, such as the hand wave used in this experiment.

474   Regardless of the difference in initial rate of DLR, both captive and wild bees showed

475   clear habituation trends, and thus our experiment suggestions expansive opportunities for a new

476   area of non-associative learning research with bumble bees. Future research may consider the

477   principles of habituation and sensitization outlined by Thompson and Spencer (1966), Groves

478   and Thompson (1970), and Rankin et al. (2009). For example, altering the time between stimulus

479   presentations may change the rate of habituation, and placing the animal in an agitated state prior

480   to habituation trials may instead result in sensitization. Future work may also consider exploring

481   classical conditioning or operant conditioning of DLR. This would be a reasonable next step

482   considering the reports of sting extension response (SER) conditioning in honey bees, though the

483   primary author and one reviewer note that SER conditioning may be less robust than the

484   literature suggests. Additionally, various DLR conditioning studies could be used as a basis for

485   research on pesticides, sensory perception, memory, pharmacology, and neurophysiology

486   research, as conditioning research with honey bees has also done for these same topics (e.g.,

487   Abramson, Squire, Sheridan, & Mulder, 2004; Abramson et al., 2006; Faber, Joerges & Menzel,

488   1999; Giurfa et al., 2009; Linader, de Ibrra, Laska, & 2012; Mustard, Dews, Brugato, Dey,

489 Wright, 2012; Varnon, Dinges, Black, Wells, & Abramson, 2018; Vergoz, Roussel, Sandoz &

490 Giurfa, 2007).

Conclusions

492   Our experiments document a primarily undescribed behavior, the disturbance leg-lift

493 response (DLR). We suggest an antipredation role for DLR, show that DLR can change as a

494 function of learning, and outline future considerations for DLR as a behavior of interest for both

495 behavioral ecology and comparative psychology. A growing body of research with bumble bees

496 is indicating they are becoming an important model organism for ecological, behavioral, and

497 physiological research. We hope that our work will stimulate additional research on DLR, and on

498 bumble bees in general. We also hope that special considerations will be given to reporting not

499 only what bumble bees can do, but also what they cannot do. Reporting differences, including

500 deficits, is an important component of research in animal behavior (Avarguès-Weber & Giurfa,

501 2013), and this is especially important for new model organisms.

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References

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506 Abramson, C. I., Squire, J., Sheridan, A., & Mulder Jr., P. G. (2004). The effect of insecticides

507   considered harmless to honey bees (*Apis mellifera*): Proboscis conditioning studies by

508   using the insect growth regulators tebufenozide and diflubenzuron. *Environmental*

509   *Entomology*, *33*(2), 378-388. doi: 10.1603/0046-225X-33.2.378

510 Abramson, C. I., Stone, S. M., Ortez, R. A., Luccardi, A., Vann, K. L., Hanig, K. D., & Rice, J.

511   (2006). The development of an ethanol model using social insects 1: Behavior studies of

512   the honey bee (*Apis mellifera* L.). *Alcoholism Clinical & Experimental Research*, *24*(8),

513   1153-1166. doi: 10.1111/j.1530-0277.2000.tb02078.x

514 Akdag, J. S., Nestor, P. G., O'Donnell, B. F., Niznikiewicz, M. A., Shenton, M. E., & McCarley,

515   R. W. (2003). The startle reflex in schizophrenia: Habituation and personality correlates.

516   *Schizophrenia Research*, *64*, 2-3(15), 165-173. doi: 10.1016/S0920-9964(03)00059-8

517 Alaux, C., Saurabh, S., Hasadsri, L., Hunt, G. J., Guzmán-Novoa, E., DeGrandi-Hoffman, G., …

518   Robinson, G. E. (2009). Honey bee aggression supports a link between gene regulation

519   and behavioral evolution. *Proceedings of the National Academy of Sciences of the United*

520   *States of America*, *103*(36). 15400-15404. doi: 10.1073/pnas.0907043106

521 Alem, S., Perry, C. J., Zhu, X., Loukola, O. J., Ingraham, T., Søvik, E., Chittka, L. (2016)

522   Associative mechanisms allow for social learning and cultural transmission of string

523   pulling in an insect. *PLOS Biology*, *14*(12): e1002589. doi: 10.1371/journal.pbio.1002589

524 Anaconda (2019). Anaconda Software Distribution (Version 4.6.14). [Computer software].

525   Available from http://www.anaconda.com.

526   Avalos, A., Pérez, E., Vallejo, L., Pérez, M., Abramson, C. I., & Giray, T. (2017). Social signals

527   and aversive learning in honey bee drones and workers. *Biology Open*, *6,* 41-49. doi:

528   10.1242/bio.021543

529   Avarguès-Weber, A., & Giurfa, M. (2013). Conceptual learning by miniature brains.

530   *Proceedings of the Royal Society B: Biological Sciences.* doi: 280:2013190.

531   Bildstein, K. L. (1983). Why white-tailed deer flag their tails. *The American Naturalist*, *121*(5),

532   709-715. doi:10.1086/284096.

533   Blacquière, T., Cornelissen, B., & Donder, J. (2007). Bumble bee colony decline in greenhouses

534   with supplemental lighting. *Proceedings of the Netherlands Entomological Society*

535   *Meeting*, *18*, 71-77.

536   Brower, L. P., Brower, J. V. Z., & Westcott, P. W. (1960). Experimental studies of mimicry. 5.

537   The reactions of toads (*Bufo terrestris*) to bumblebees (*Bombus americanorum*) and their

538   robber fly mimics (*Mallophora bomboides*), with a discussion of aggressive mimicry.

539   *The American Naturalist*, *94*(878), 343-355. doi: 10.1086/282137

540   Carew, T. J., Pinsker, H. M., & Kandel, E. R. (1972). Long-term habituation of a defensive

541   withdrawal reflex in *Aplysia*. *Science, 175(4020)*, 451-454. doi:

542   10.1126/science.175.4020.451

543   Castellucci, V. F., & Kandel, E. R. (1974). A Quantal analysis of the synaptic depression

544   underlying habituation of the gill-withdrawal reflex in *Aplysia*. *Proceedings of the*

545   *National Academy of Sciences of the United States of America*, *71*(12), 5004-5008. doi:

546   10.1073/pnas.71.12.5004

547   Castellucci, V., Pinsker, H., Kupfermann, I., & Kandel, E. R. (1970). Neuronal mechanisms of

548   habituation and dishabituation of the gill-withdrawal reflex in *Aplysia*. *Science*, *167*,

549   1747-1748. doi: 10.1126/science.167.3926.1745

550   Charlton, N. L., Houston, A. I. (2010). What currency do bumble bees maximize? *PLoS ONE*

551   *5*(8): e12186. https://doi.org/10.1371/journal.pone.0012186

552   Clogg, C. C., Petkova, E., & Haritou, A. (1995). Statistical methods for comparing regression

553   coefficients between models. *American Journal of Sociology, 100*(5), 1261-1293. doi:

554   10.1086/230638

555   Cloudsley-Thompson, J. L. (1995). A review of the anti-predator devices of spiders. *Bulletin of*

556   *the British Arachnological Society*, 10(3), 81-96.

557   Craig, D. P. A., Varnon, C. A., Sokolowski, M. B. C., Wells, H., & Abramson, C. I. (2014). An

558   assessment of fixed interval timing in free-flying honey bees (*Apis mellifera ligustica*):

559   An analysis of individual performance. *PloS One*, *9*, e101262.

560   doi:10.1371/journal.pone.0101262.

561   Davis, H., & Heslop, E. (2004). Habituation of hissing by Madagascar hissing cockroaches

562   (*Gromphadorhina portentosa*): Evidence of discrimination between humans?

563   *Behavioural Processes, 67(3)*, 539-543. doi: 10.1016/j.beproc.2004.08.003

564   Desmedt, L. Baracchi, D., Devaud, J. Giurfa, M., d'Ettorre, P. (2017). Aversive learning of odor-

565   heat associations in ants. *Journal of Experimental Biology*, *220*, 4661-4668, doi:

566   10.1242/jeb.161737

567   Djegham, Y., Verhaeghe, J.C, & Rasmont, P. (1994). Copulation *of Bombus terrestri*s L.

568   (Hymenoptera: Apidae) in captivity. *Journal of Apicultural Research*, *33*(1), 15-20, doi:

569   10.1080/00218839.1994.11100844

570   Dinges, C. W., Varnon, C. A., Cota, L. D., Slykerman, S. C., & Abramson, C. I. (2017). Studies

571   of learned helplessness in honey bees (*Apis mellifera* L.). *Journal of Experimental*

572   *Psychology: Animal Learning and Cognition*, *43*(2), 147-158. doi: 10.1037/xan0000133.

573   Faber, T., Joerges, J., & Menzel, R. (1999). Associative learning modifies neural representations

574   of odors in the insect brain. *Nature Neuroscience*, *2*(1), 74-78. doi: 10.1038/4576

575   Fisher, R., & Tuckerman, R. (1986). Mimicry of bumble bees and cuckoo bumble bees by

576   carrion beetles (*Coleoptera: Silphidae*). *Journal of the Kansas Entomological Society,*

577   *59*(1), 20-25.

578   FitzGibbon, C. D. & Fanshawe, J. H. (1988). Stotting in Thomson's gazelles: An honest signal of

579   condition. *Behavioral Ecology and Sociobiology*, *23*, 2, 69-74.

580   Free, J. B. (1958). The defence of bumblebee colonies. *Behavior*, *12*(3) 233-242. doi:

581   10.1163/156853957X00128

582   Gage, S. L., Ahumada, F., Rivera, A., Graham, H., & DeGrandi-Hoffman, G. (2018). Smoke

583   conditions affect the release of venom droplet accompanying sting extension in honey

584   bees (Hymenoptera: Apidae). Journal of Insect Science, 18(4), 1-7. doi:

585   10.1093/jisesa/iey073

586   Galizia C. G., Eisenhardt, D. & Giurfa, M. (2011). Honeybee Neurobiology and Behavior: A

587   Tribute to Randolf Menzel. Springer Netherlands. doi: 10.1007/978-94-007-2099-2

588   Geyer, M. A., & Braff, D. L. (1987). Startle habituation and sensorimotor gating in

589   schizophrenia and related animal models. *Schizophrenia Bulletin, 13*(4), 643-668. doi:

590   10.1093/schbul/13.4.643

591   Giurfa, M. (2003). Cognitive neuroethology: Dissecting non-elemental learning in a honeybee

592   brain. *Current Opinion in Neurobio*logy, *13*, 726-735. doi: 10.1016/j.conb.2003.10.015

593 Giurfa, M., Fabre, E., Flaven-Pouchon, J., Groll, H., Oberwallner, B., Vergoz, V., Roussel, E., &
594   Sandoz, J. (2009). Olfactory conditioning of the sting extension reflex in honeybees:
595   Memory dependence on trial number, interstimulus interval, intertrial interval, and
596   protein synthesis. *Learning & Memory*, *16*, 761-765. doi: 10.1101/lm.1603009
597 Giurfa, M., Zhang, S., Jenett, A., Menzel, R., Srinivasan, M. V. (2001). The concepts of
598   'sameness' and 'difference' in an insect. *Nature*, *410*(6831), 930–933.
599   https://doi.org/10.1038/35073582
600 Greene, H. W., & McDiarmid, R. W. (1981). Coral snake mimicry: Does it occur? *Science*,
601   *213*(4513), 1207-1212. doi: 10.1126/science.213.4513.1207
602 Groves, P. M., Thompson, R. F. (1970). Habituation: A dual-process theory. *Psychological*
603   *Review*, *77*, 419-450. doi: 10.1037/h0029810
604 Hammer, M., & Menzel, R. (1995). Learning and memory in the honeybee. *Journal of*
605   *Neuroscience*, *15*(3), 1617-1630. doi: 10.1523/JNEUROSCI.15-03-01617.1995
606 Hardin J., Hilbe J.M. (2003). *Generalized Estimating Equations*. Boca Raton, FL: Chapman &
607   Hall.
608 Hasson, O. (1991). Pursuit-deterrent signals: Communication between prey and predator. *Trends*
609   *in Ecology & Evolution*, *6*(10), 325-329. doi: 10.1016/0169-5347(91)90040-5.
610 Hunsinger, E., Root-Gutteridge, H., Cusano, D. A., & Parks, S. E. (2017). A description of
611   defensive hiss types in the flat horned hissing cockroach (*Aeluropoda insignis*).
612   *Bioacoustics*. doi: 10.1080/09524622.2017.1327371
613 Jandt, J. M., & Dornhaus, A. (2009). Spatial organization and division of labour in the
614   bumblebee *Bombus impatiens*. *Animal Behaviour*, *22*, 641 – 651.
615   doi:10.1016/j.anbehav.20 08.11.019

616   Jandt, J. M., Huang, E., & Dornhaus, A. (2009). Weak specialization of workers inside a bumble

617   bee (*Bombus impatiens*) nest. *Behavioral Ecology and Sociobiology*, *63*(12), 1829–1836.

618   doi: 10.1007/s00265-009-0810-x

619   Jaycox, L. H., Foa, E. B., & Morral, A. R. (1998). Influence of emotional engagement and

620   habituation on exposure therapy for PTSD. *Journal of Consulting and Clinical*

621   *Psychology*, *66*(1), 185-192. doi: 10.1037/0022-006X.66.1.185

622   Kodaira, Y., Ohtsuki, H., Yokoyama, J, & Kawata, M. (2009). Size-dependent foraging gene

623   expression and behavioral caste differentiation in *Bombus ignitus*. *BMC Research Notes*,

624   *2*, 184-189.

625   Koethe, S., Bossems, J., Dyer, A. G., & Luna, K. (2016). Colour is more than hue: Preferences

626   for compiled colour traits in the stingless bees *Melipona mondury* and *M. quadrifasciata*.

627   *Journal of Comparative Physiology A*, *202*, 615–627(2016). doi: 10.1007/s00359-016-

628   1115-y

629   Leal, M., & Rodríguez-Robles, J. A. (1995). Antipredator responses of *Anolis cristatellus*

630   (Sauria: Polychrotidae). *Copeia*, 1995, 155-161.

631   Linader, N., de Ibrra, N. H., & Laska, M. (2012). Olfactory detectability of L-amino acids in the

632   European honeybee (*Apis mellifera*). *Chemical Senses*, *37*(7), 631-638. doi:

633   10.1093/chemse/bjs044.

634   Maan, M., & Cummings, M. (2012). Poison frog colors are honest signals of toxicity,

635   particularly for bird predators. *The American Naturalist, 179*(1), E1-E14. doi:

636   10.1086/663197

637   Mustard, J. A., Dews, L., Brugato, A., Dey, K., & Wright, G. A. (2012). Consumption of an

638   acute dose of caffeine reduces acquisition but not memory in the honey bee. *Behavioral*

639   *Brain Research*, *232*(1), 217-224. doi: 10.1016/j.bbr.2012.04.014

640   Nicolas, C. L., Abramson, C. I., & Levin, M. (2008). Analysis of behavior in the planarian

641   model. In R. B. Raffa (Ed.) *Planaria: A Model for Drug Action and Abuse.* Austin, TX:

642   Landes Bioscience.

643   Paternoster, R., Brame, R., Mazerolle, P., & Piquero, A. (1998). Using the correct statistical test

644   for the equality of regression coefficients. *Criminology*, *36*(4), 859 - 866.

645   Perktold, J., Seabold, S., Taylor, J. (2018). StatsModels (Version 0.9.0). [Computer software].

646   Available from http://www.statsmodels.org.

647   Place, A. J. & Abramson, C. I. (2008). Habituation of the rattle response in western diamondback

648   rattlesnakes, *Crotalus atrox. Copeia, 2008*(4), 835-843. doi: 10.1643/CE-06-246

649   Plowright, R. C., & Owen R. E. (1980). The evolutionary significance of bumble bee color

650   patterns: A mimetic interpretation. *Evolution*, *34*(4), 622-637. doi: 10.2307/2408017

651   Pouvreau, A. (1991). Morphology and histology of tarsal glands in bumble bees of the genera

652   *Bombus*, *Pyrobumbus* and *Megabombus*. *Canadian Journal of Zoology*, *69*(4), 866-872.

653   doi: 10.1139/z91-130

654   Raine, N. E, & Chittka, L. (2007). Pollen foraging: learning a complex motor skill by

655   bumblebees (*Bombus terrestris*). *Naturwissenschaften*, *94*(6), 459-464

656   Raine, N. E., & Chittka, L. (2008). The correlation of learning speed and natural foraging

657   success in bumble-bees. *Proceedings of the Royal Society B*, *275*, 803-808.

658   Rankin, C. H., Abrams, T., Barry, R. J., Bhatnager, S., Claytong, D., Colombo, J., . . .

659   Thompson, R. F. (2009). Habituation revisited: An updated and revised description of the

660   behavioral characteristics of habituation. *Neurobiology of Learning and Memory*, *92*(2):

661   135-138. doi: 10.1016/j.nlm.2008.09.012.

662   Ritson-Williams, R., & Paul, V. J. (2007). Marine benthic invertebrates use multimodal cues for

663   defense against reef fish. *Marine Ecology Progress Series*, *340*(29), 29-39. doi:

664   10.3354/meps340029

665   Riveros, A. J., & Gronenberg, W. (2009a). Learning from learning and memory in bumblebees.

666   *Communicative & Integrative Biology*, *2*(5), 437-440. doi: 10.4161/cib.2.5.9240

667   Riveros, A. J., & Gronenberg, W. (2009b). Olfactory learning and memory in the bumblebee

668   *Bombus occidentalis*. *Naturwissenschaften, 96*: 851-856. doi: 10.1007/s00114-009-0532-

669   y

670   Rossi, N., d'Ettorre, P., Giurfa, M. (2018). Pheromones modulate responsiveness to a noxious

671   stimulus in honey bees. *Journal of Experimental Biology*, 221, 1-10, doi:

672   10.1242/jeb.172270

673   Rowe, C., & Guilford, T. (1999). The evolution of multimodal warning displays. *Evolutionary*

674   *Ecology*, *13*(7-8), 655-671.

675   Rowe, C., & Haplin, C. (2013). Why are warning displays multimodal. *Behavioral Ecology and*

676   *Sociobiology, 67*(9), 1425-1439.

677   Schmitt, U. (1990). Hydrocarbons in tarsal glands of *Bombus terrestris*. *Experientia*, *45*(10)

678   1080-1082.

679   Schulz, D. J., Haung, Z., & Robinson, G. E. (1998). Effects of colony food shortage on

680   behavioral development in honey bees, *45*(5), 295-303.

681

682   Scienza, L., Pinheiro de Carvalho, M., Machado, A., Moreno, A. M., Biscassi, N., & Graças de

683   Souza, D. (2019). Simple discrimination in stingless bees (*Melipona quadrifasciata*):

684   Probing for select– and reject–stimulus control. *Journal of the Experimental Analysis of*

685   *Behavior*, *112*, 1-14. doi: 10.1002/jeab.531

686   Sheppard, W. S., Arias, M. C., Grech, A., & Meixner, M. D. (1997). *Apis mellifera ruttneri*, a

687   new honey bee subspecies from Malta. *Apidologie*, *28*(5), 287-293. doi:

688   10.1051/apido:19970505

689   Shotton, R. (2014). Testing the disturbance hiss of the Madagascar hissing cockroach

690   (*Gromphadorhina portentosa*) as an anti-predatory response. *Bioscience Horizons: The*

691   *International Journal of Student Research, 7*, 1-7. doi: 10.1093/biohorizons/hzu010

692   Skorupski, P., & Chittka, L. (2010). Photoreceptor spectral sensitivity in the bumblebee, *Bombus*

693   *impatiens* (Hymenoptera: Apidae). *PLoS ONE*, *5*(8): e12049. doi:

694   10.1371/journal.pone.0012049

695   Spaethe, J., & Weidenmüller, A., (2002). Size variation and foraging rate in bumblebees

696   (*Bombus terrestris*). *Insectes Sociaux*, *49*(2), 142-146.

697   Spivak, M., (1997). Honey bee hygienic behavior and defense against *Varroa jacobsoni*.

698   *Apidologie*, 27, 245-260, doi: 10.1051/apido:19960407

699   Stanley, D. A., Smith, K. E., & Raine, N. E., (2015). Bumblebee learning and memory is

700   impaired by chronic exposure to a neonicotinoid pesticide. *Scientific Reports*, *5*(16508),

701   1-10. doi: 1038/srep16508

702   Strang, C. G., & Sherry, D. F. (2014). Serial reversal learning in bumblebees (*Bombus*

703   *impatiens*). *Animal Cognition*, *17*, 723-734. doi: 10.1007/s10071-013-0704-1

704

705   Tautz, J., Maier, S., Groh, C., Rössler, W., & Brockman A., (2003). Behavioral performance in

706   adult honey bees is influenced by the temperature experienced during their pupal

707   development. *Proceedings of the National Academy of Sciences of the United States of*

708   *America*, *10*(12), 7343-7347. doi: 10.1073/pnas.1232346100

709   Tedjakumala, S. R., Guirfa, M. (2013). Rules and mechanisms of punishment learning in honey

710   bees: the aversive conditioning of the sting extension response. *Journal of Experimental*

711   *Biology*, *216*: 2985-2997; doi: 10.1242/jeb.086629

712   Thompson, R. F., & Spencer, W. A. (1966). Habituation: A model phenomenon for the study of

713   neuronal substrates of behavior. *Psychological Review, 73*(1), 16-43. doi:

714   10.1037/h0022681

715   Varnon, C. A., Dinges, C. W., Black, T. E., Wells, H., & Abramson, C. I. (2018). Failure to find

716   ethanol-induced conditioned taste aversion in honey bees (*Apis mellifera* L.). *Alcoholism:*

717   *Clinical and Experimental Research*, *42*(7), 1260-1270. doi: 0.1111/acer.13761.

718   Varnon, C. A., Lang, H., & Abramson, C. I. (2018). Automated research in comparative

719   psychology: Limitations and new directions. *International Journal of Comparative*

720   *Psychology*, 31, 1-17.

721   Vergoz, V., Roussel, E., Sandoz, J., & Giurfa, M. (2007). Aversive learning in honeybees

722   revealed by the olfactory conditioning of the sting extension reflex. *PLoS ONE 2*(3):

723   e288. doi: 10.1371/journal.pone.0000288

Figure 1

The disturbance leg-lift response (DLR) of the bumble bee. Artwork by Jennifer Salazar. Original reference photographs by Ivan Mikhaylov.
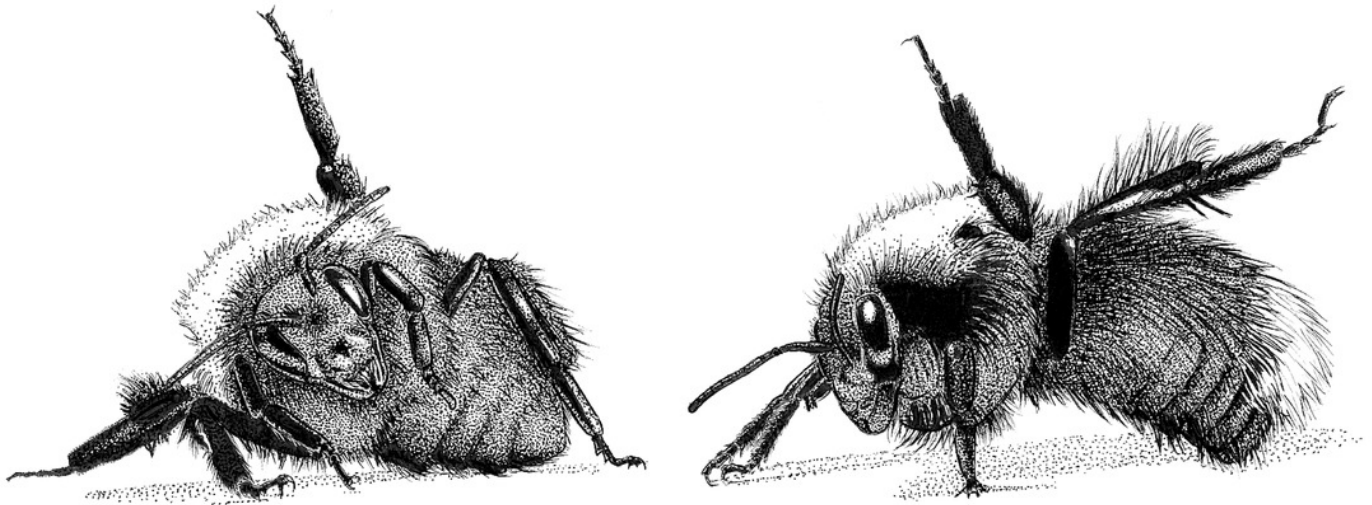
Figure 2

Percent of trials where bees emitted DLR, bite or sting for the experimental group (A) and control group (B). Error bars show standard error of the mean.
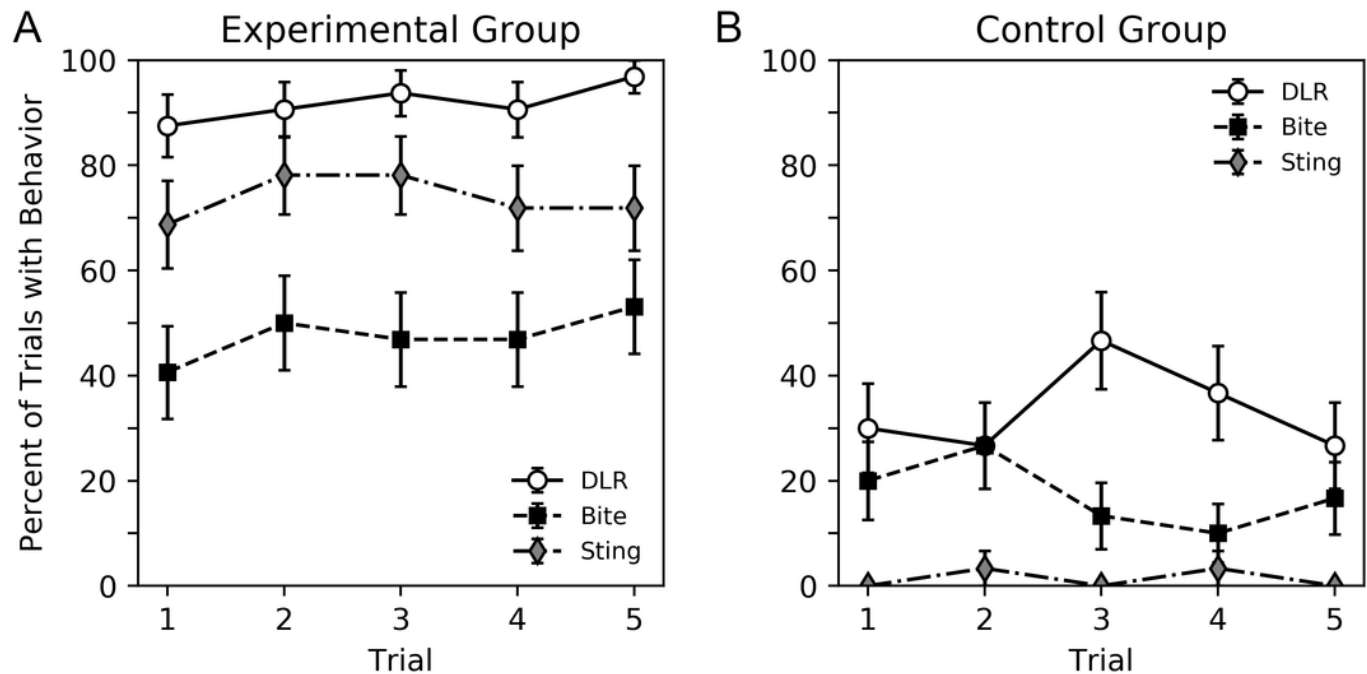
Figure 3

Average number of legs lifted during trials where DLR occurred for the experimental and control groups. Error bars show standard error of the mean.
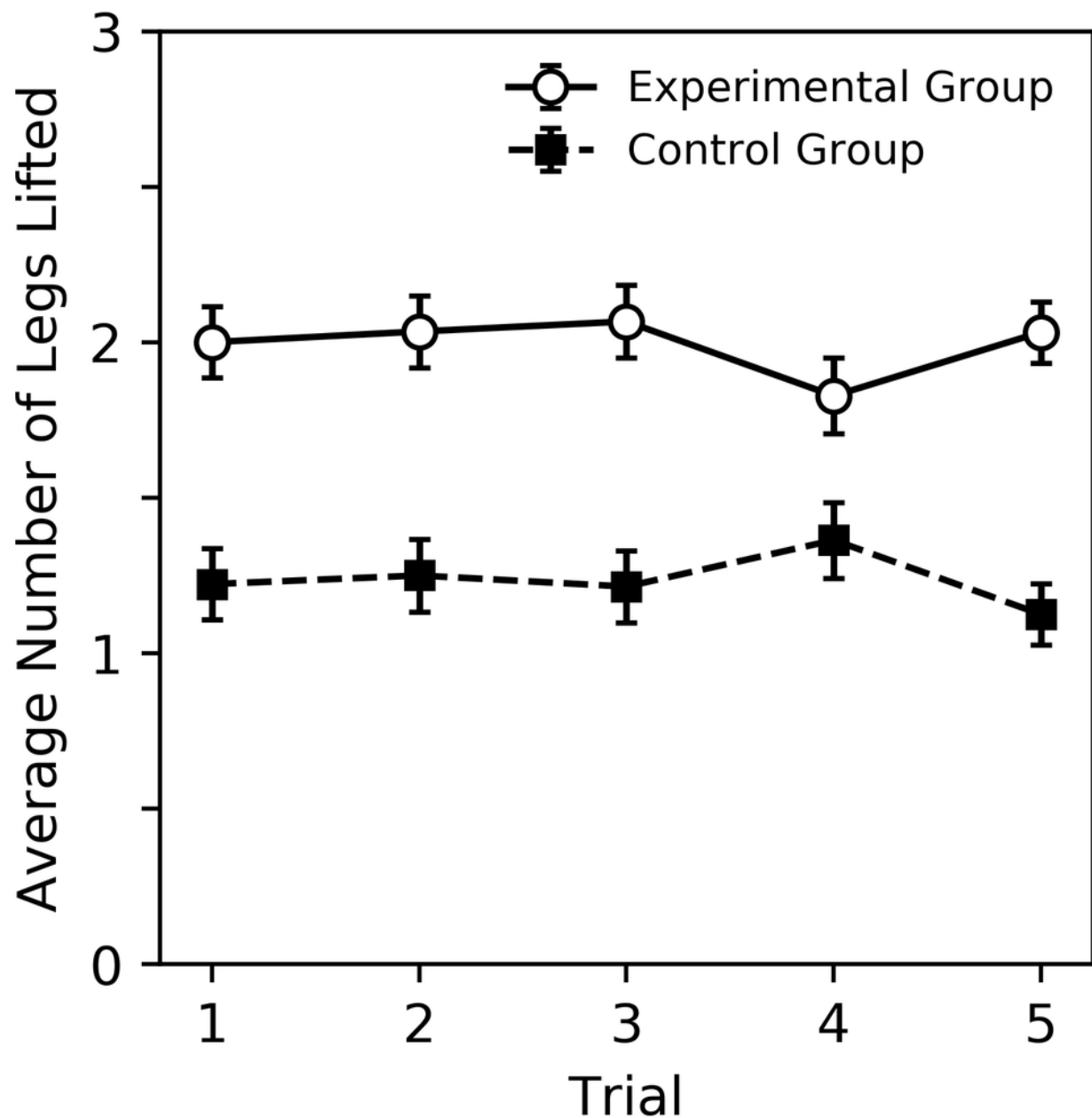
Figure 4

Percent of captive and wild bees emitting DLR across the 10 habituation trials and final three control trials. Sixty-four subjects per sample were used in trials 1 to 10. The final three trials represent 32 subjects per sample. Error bars show standard er

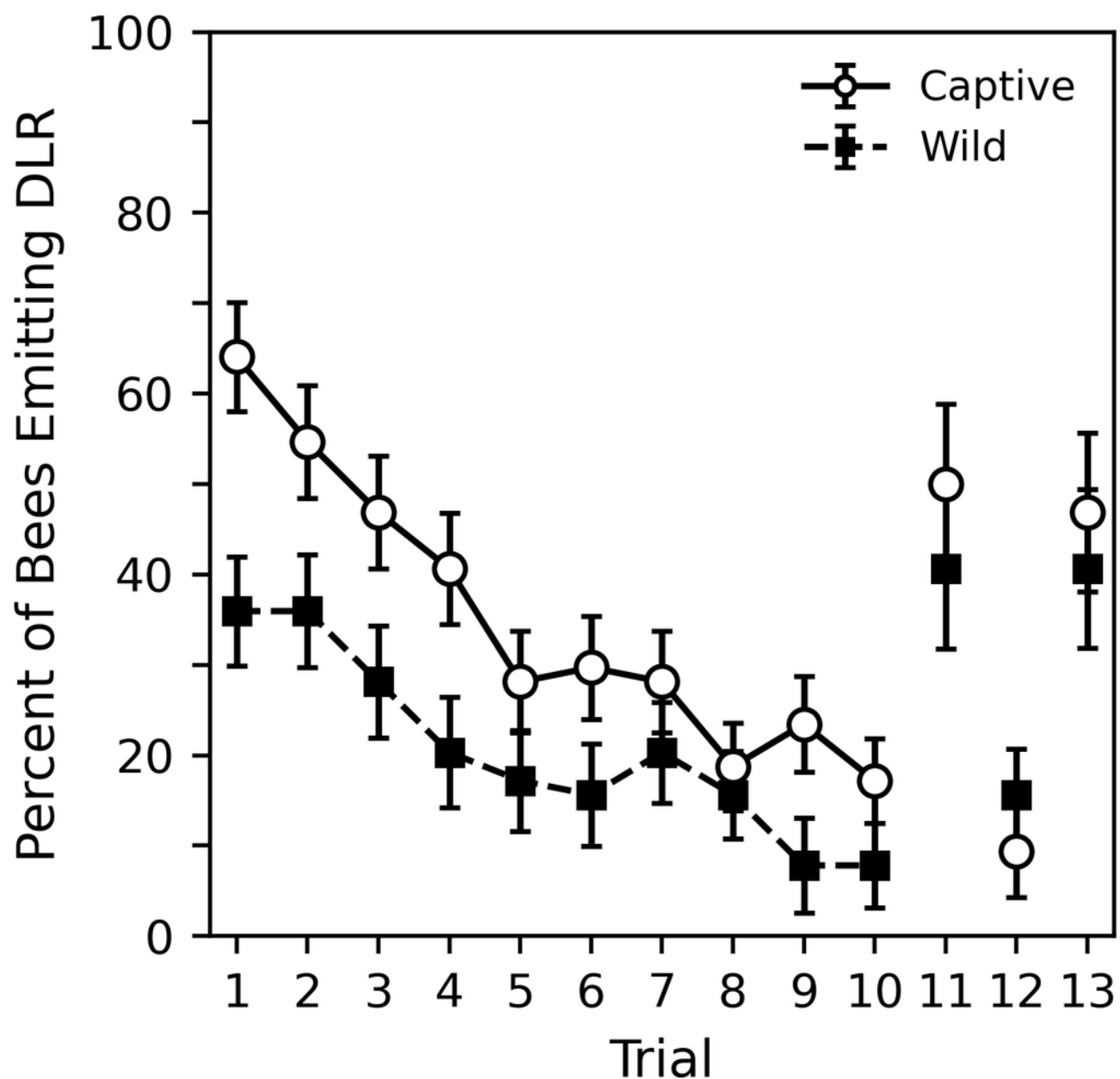**Table 1**(on next page)

Percent of Trials with Behavior Sequence

1   Table 1

Percent of Trials with Behavior Sequence

Behavior Sequence	Experimental	Control
Inactive	3.12	51.33
DLR	18.12	30.67
DLR:Bite	1.25	0.00
DLR:Sting	30.00	0.67
DLR:Bite:Sting	16.25	0.00
DLR:Sting:Bite	15.62	0.00
Bite	2.50	14.67
Bite:DLR	1.25	2.00
Bite:Sting	1.25	0.67
Bite:DLR:Sting	5.62	0.00
Bite:Sting:DLR	2.50	0.00
Sting	0.62	0.00
Sting:DLR	0.62	0.00
Sting:Bite	0.62	0.00
Sting:DLR:Bite	0.00	0.00
Sting:Bite:DLR	0.62	0.00

2

Table 2(on next page)

Experimental Group Sequence Regression

1 Table 2

Experimental Group Sequence Regression

Sequence	Estimate	Standard Error	95% Confidence Intervals		*p* value
Inactive	-0.726	0.424	-1.557	0.105	0.087
DLR	1.200	0.306	0.601	1.799	0.000
DLR:Bite	-1.661	0.693	-3.020	-0.303	0.017
DLR:Sting	1.861	0.183	1.501	2.220	0.000
DLR:Bite:Sting	1.068	0.229	0.619	1.517	0.000
DLR:Sting:Bite	1.022	0.266	0.500	1.543	0.000
Bite	-0.956	0.601	-2.134	0.223	0.112
Bite:DLR	-1.661	0.693	-3.020	-0.303	0.017
Bite:Sting	-1.661	0.693	-3.020	-0.303	0.017
Bite:DLR:Sting	-0.112	0.343	-0.784	0.560	0.744
Bite:Sting:DLR	-0.956	0.601	-2.134	0.223	0.112
Sting	-2.361	0.990	-4.302	-0.420	0.017
Sting:DLR	-2.361	0.990	-4.302	-0.420	0.017
Sting:Bite	-2.361	0.990	-4.302	-0.420	0.017
Sting:DLR:Bite	-16.495	0.177	-16.841	-16.148	0.000
Sting:Bite:DLR	-2.361	0.990	-4.302	-0.420	0.017

2

3

Table 3(on next page)

Control Group Sequence Regression

PeerJ

1   Table 3

Control Group Sequence Regression

Sequence	Estimate	Standard Error	95% Confidence Intervals		*p* value
Inactive	2.761	0.247	2.278	3.245	0.000
DLR	1.892	0.269	1.366	2.419	0.000
DLR:Bite	-17.495	0.183	-17.853	-17.137	0.000
DLR:Sting	-2.296	0.990	-4.236	-0.356	0.020
DLR:Bite:Sting	-17.495	0.183	-17.853	-17.137	0.000
DLR:Sting:Bite	-17.495	0.183	-17.853	-17.137	0.000
Bite	0.947	0.291	0.377	1.518	0.001
Bite:DLR	-1.184	0.559	-2.279	-0.088	0.034
Bite:Sting	-2.296	0.990	-4.236	-0.356	0.020
Bite:DLR:Sting	-17.495	0.183	-17.853	-17.137	0.000
Bite:Sting:DLR	-17.495	0.183	-17.853	-17.137	0.000
Sting	-17.495	0.183	-17.853	-17.137	0.000
Sting:DLR	-17.495	0.183	-17.853	-17.137	0.000
Sting:Bite	-17.495	0.183	-17.853	-17.137	0.000
Sting:DLR:Bite	-16.495	0.183	-16.853	-16.137	0.000
Sting:Bite:DLR	-17.495	0.183	-17.853	-17.137	0.000

2

Table 4(on next page)

Experimental Group DLR-first Pairwise Comparisons

1 Table 4

Experimental Group DLR-first Pairwise Comparisons

Comparison	Estimate Difference	z score	*p* value
DLR:Bite vs. DLR	-2.862	-3.776	0.000
DLR:Bite vs. DLR:Sting	-3.522	-4.911	0.000
DLR:Bite vs. DLR:Bite:Sting	-2.730	-3.738	0.000
DLR:Bite vs. DLR:Sting:Bite	-2.683	-3.613	0.000
DLR:Sting vs. DLR	0.661	1.852	0.064
DLR:Sting vs. DLR:Bite:Sting	0.792	2.700	0.007
DLR:Sting vs. DLR:Sting:Bite	0.839	2.597	0.009

2

Table 5(on next page)

Change in DLR Across Trial

1   Table 5

Change in DLR Across Trial

Parameter	Estimate	Standard Error	95% Confidence Intervals		*p* value
Captive	0.587	0.210	0.177	0.998	0.005
Wild	-0.344	0.243	-0.821	0.132	0.157
Captive * Trial	-0.232	0.035	-0.301	-0.163	0.000
Wild * Trial	-0.202	0.042	-0.284	-0.120	0.000

2

Table 6(on next page)

Pairwise Comparisons of Trials

1   Table 6

2

Pairwise Comparisons of Trials

Comparison	Difference	z score	p value
Captive 1 : Captive 10	2.325	3.609	0.000
Captive 1 : Captive 11	0.379	0.752	0.452
Captive 1 : Captive 12	2.648	3.755	0.000
Captive 1 : Captive 13	0.505	0.999	0.318
Captive 10 : Captive 11	-1.946	-3.036	0.002
Captive 10 : Captive 12	0.323	0.399	0.690
Captive 10 : Captive 13	-1.821	-2.839	0.005
Captive 11 : Captive 12	2.269	3.232	0.001
Captive 11 : Captive 13	0.125	0.250	0.803
Captive 12 : Captive 13	-2.144	-3.052	0.002
Wild 1 : Wild 10	2.325	3.609	0.000
Wild 1 : Wild 11	0.379	0.752	0.452
Wild 1 : Wild 12	2.648	3.755	0.000
Wild 1 : Wild 13	0.505	0.999	0.318
Wild 10 : Wild 11	-1.946	-3.036	0.002
Wild 10 : Wild 12	0.323	0.399	0.690
Wild 10 : Wild 13	-1.821	-2.839	0.005
Wild 11 : Wild 12	2.269	3.232	0.001
Wild 11 : Wild 13	0.125	0.250	0.803
Wild 12 : Wild 13	-2.144	-3.052	0.002

3