The disturbance leg-lift response (DLR): An undescribed behavior in bumble bees (#50899)

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

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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 describe an undocumented 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 investigate 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 greater is 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.

The Disturbance Leg-Lift Response (DLR): An Undescribed Behavior in Bumble Bees

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Abstract

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 describe an undocumented 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.

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Introduction

The study of the psychological abilities of bees has become an important research area. Such research provides insights to the valuable and global role of bees in agriculture and in the ecosystem. Additionally, bees are also excellent model organisms for investigating the relationships between complex behavior, ecological demands, and neurophysiology, and are the most researched invertebrate in recent comparative psychology (Varnon, Lang, & Abramson, 2018). Psychological research with bees involves a number of topics including perception of time (Craig, Varnon, Sokolowski, Wells, & Abramson, 2014), conditioned taste aversion (Varnon, Dinges, Black, Wells, & Abramson, 2018), learned helplessness (Dinges, Varnon, Cota, Slykerman, & Abramson, 2017), select and reject stimulus control (Scienza et al., 2019), concept learning (Giurfa, Zhang, Jenett, Menzel, & Srinivasan, 2001), social transmission of learned behaviors (Alem et al., 2016), acquisition and flexibility of foraging skills (Raine & Chittka, 2007; Strang & Sherry, 2014), effects of pesticides on learning (Stanley, Smith, & Raine, 2015), and the neurophysiology of memory (Hammer & Menzel, 1995).

Honey bees (*Apis mellifera*) are currently the most popular species, however, bumble bees, primarily *Bombus impatiens* in North America and *B. terrestris* in Europe, have recently become a popular alternative due to some challenges related to maintaining a honey bee laboratory. While recent psychological research with bumble bees shows promising potential, one area that is notably absent from the bumble bee literature is the study of innate defensive responses, especially in conjunction with learning. For example, in honey bees, sting extension response (SER) conditioning research investigates how restrained bees learn to sting in response to a stimulus associated with shock (Vergoz, Roussel, Sandoz, & Giurfa, 2007; Tedjakumala & Guirfa, 2013). Unfortunately, there is not yet analogous work with bumble bees. This is surprising given that bumble bees appear to offer a unique and ideal behavior to fulfill this line of research, the disturbance leg-life response.

In this paper, we discuss the disturbance leg-lift response (DLR), a previously undocumented behavior, and its potential in psychological research. When exposed to a presumably threatening stimulus, bumble bees commonly react by lifting one or multiple legs (see Figure 1). This behavior appears to occur in many *Bombus* species worldwide. (Curious readers may perform an online image search for the anthropomorphizations "bumble bee high five" or "bumble bee wave.") In our first experiment, we investigate the temporal relationships between DLR, biting and stinging as an invasive stimulus approaches in order to explore potential functions of the DLR. In our second experiment, we investigate if the DLR is a suitable behavior for conditioning procedures, similar to SER. Specifically, we compare habituation of the DLR across captive and wild samples. Finally, we discuss implications for future research with special considerations for the new and growing use of *Bombus* species as model organisms.

Experiment 1 - The Role of DLR

In this experiment, we explore the role of DLR as a reaction to potential danger to establish an understanding of the behavior as a prerequisite to later investigations of DLR conditioning. Many species emit specific responses, like DLR, when threatened. For example, spiders may lift several front legs to reveal fangs (Cloudsley-Thompson, 1995), while hissing cockroaches produce an audible hiss (Hunsinger, Root-Gutteridge, Cusano, & Parks, 2017; Shotton, 2014). Although making distinctions between categories of antipredator responses can be challenging, there are two major categories that could be considered for DLR: the aposematic display, and the pursuit deterrence signal.

Conspicuous aposematic displays can signal toxicity or danger to a potential predator. The vibrant colors of poison dart frogs in the family *Dendrobatidae* illustrate a case of honest aposematic signals; the colors indicate that the frog possesses toxic alkaloid compounds (Maan & Cummings, 2012). Similarly, in the southern United States, the bright red banding of coral snakes (*Micrurus euryxanthus, M., fulvius,* and *M. tener*) honestly signals a potent neurotoxic venom. Several species of scarlet snake (*Cemophora sp.*) and kingsnake (*Lampropeltis sp.*) also possess similar conspicuous red banding but lack venom. For the venomless mimic snakes, the aposematic coloration is a dishonest signal (Greene & McDiarmid, 1981).

Pursuit deterrence signals can alert a potential predator that it has been detected, communicating vigilance and possibly fitness to the predator (Hasson, 1991). For example, Thomson's gazelles (*Eudorcas thomsonii*) leap vertically into the air, a behavior known as stotting (FitzGibbon & Fanshawe, 1988), while white-tailed deer (*Odocoileus virginianus*) erect their tails to reveal a high contrast white rump when a predator is detected (Bildstein, 1983), and anole lizards (*Anolis sp*.) may signal fitness to potential predators with head bobs, pushups, or dewlap extensions (Leal & Rodríguez-Robles, 1995). While discussions of pursuit deterrence signals typically suggest that they signal the ability to escape, it is also possible they signal readiness to use some other defense, such as venom.

It is possible that the DLR of bumble bees functions in an aposematic or venom-based pursuit deterrence role. The stinging response of bumble bees and other Hymenoptera can clearly serve as the foundation for an honest warning signal, and the vibrant color patterns of many bees and wasps are one well-known aposematic display. Not only does the bright coloration lead to predators quickly learning to not consume bumble bees (Brower, Brower, & Westcott, 1960), but this coloration also leads to mimics (Fisher & Tuckerman, 1986; Plowright & Owen, 1980). If

DLR functions in either an aposematic or a venom-based pursuit deterrence role, we would expect it to be closely associated with, and precede, stinging. In the following experiment, we investigate this possibility by examining the probability and order of DLR, biting and stinging in response to invasive stimuli. If DLR often precedes, but does not follow, stinging, this would provide the first evidence that DLR is an honest signal of envenomation potential.

Methods

Subjects

Captive worker bumble bees (*Bombus impatiens*, n = 62) collected from a single, captive-breed "Natupol" bumble bee colony purchased from Koppert Biological Systems Inc. (Howell, MI) were used as subjects. The bees were maintained in the ventilated plastic colony cage (24.5 x 21.5 x 12 cm, l x w x h) in which they were shipped. The outer cardboard layer, typically used to shield colonies from outdoor conditions, was removed except for the top piece, which ensured that the hive remained in darkness. The colony was placed on 40-watt intellitemp heating pad (Big Apple Pet Supply; Boca Raton, FL), which maintained a temperature of about 31 ˚C inside the hive. The colony was connected to an adjacent empty colony cage that served as a feeding area through a clear acrylic tube (2.5 cm inner diameter). Two lights (36" Zoo Med Reptisun T5-Ho Terrarium Hood, Zoo Med Laboratories Inc.; San Luis Obispo, CA) were placed approximately 31 cm above the colony. These light fixtures provided a full range of illumination, including ultraviolet (UV) light in the range of 280 – 400 nm. Bumble bees can see UV light in the range of 300 – 400 nm (Skorupski & Chittka, 2010) and naturalistic lighting conditions may be important for their growth and survival (Blacquière, Cornelissen, & Donder, 2007).

Lights and heat were automatically turned on at 7 AM and turned off at 7 PM each day to help the bees maintain daily foraging patterns. The laboratory lacked any source of natural light, and all other lights in the laboratory were also turned off by this time. Bees were allowed ad libitum access to food (either a 50% sucrose solution (w/w) or the "Bee-happy" solution provided by Koppert Biological Systems Inc.) in the feeding cage via several paper towel wicks. Water was provided directly in the hive via syringe. A three to one mixture of pollen (Stakich Bee Pollen Powder, Stakich, Inc.; Troy, MI) and pollen substitute (Mann Lake Ultra Bee, Mann Lake LTD.; Hackensack, MN) was made available ad libitum inside the hive.

Captive worker bees were collected from the clear acrylic tube and the feeding cage, chilled in a refrigerator around 1.1 C until inactive, then placed in the experimental apparatus. After an experimental session was complete, the bees were chilled, weighed, measured, then marked with an acrylic paint marker between the wings on the thorax before being returned to the colony. Captive bees collected and returned to the hive in this manner were observed alive and healthy up to 8 weeks after participating in an experiment. As Converse College does not require an institutional review for invertebrate research, no specific review was required for the present study.

Procedure

Subjects were placed in individual apparatuses after being collected. Each apparatus consisted of a clear plastic cube (2.6 x 3 x 2 cm), made from a microscope cover slip container, with two holes (2.55 mm diameter) drilled on opposite sides. The size of the apparatus allowed the bees to freely move but did not permit flight or substantial relocation inside the apparatus. After being placed in the apparatus, subjects were transferred to an experimental room, placed approximately 1.2 m apart, and allowed to acclimate for three hours.

Each bee experienced five trials with a 15-minute intertrial interval (ITI) after the acclimation period. Bees were randomly assigned to either an experimental or control group. During trials for the experimental group, a researcher startled the bee by inserting a toothpick approximately halfway (1.3 mm) into the apparatus for 10 seconds through the hole closest to the bee. During this time, it was possible for the bee to physically contact the toothpick. An identical procedure was used for the control bees, except that the toothpick was held outside of the apparatus adjacent to the hole. This group controlled for the general approach of the investigators, as well as the presence of a close object that could not be contacted by the bees.

During each trial, several behaviors were scored from video recordings. We recorded both the occurrence of DLR and the number of legs lifted during each DLR. We specifically defined DLR as when one or more legs were lifted above the bee, relative to the bee's position. Legs that were lifted prior to the trial were not considered a DLR; observing the movement during the trial was required to record a DLR. Biting was recorded as any time a bee visibly opened and closed its mandibles during a trial. Often bees made mandible contact with the stimulus, but this was not required. Finally, we recorded stinging any time the bee contacted or directed its abdomen toward the stimulus. These abdomen curls are the first component of the sting extension response (Gage, Ahumada, Rivera, Graham, & DeGrandi-Hoffman, 2018). In most cases, the stinger was obvious and contacted the stimulus. In some cases, the activity of the stinger was less clear, but the unusual abdomen curls and contact were easy to observe. We used a broad definition of stinging, relative to DLR and biting, to capture instances where the sting extension could not clearly be observed, or where the bee was not able to physically contact the stimulus from its current location. The abdomen curls we observed did not occur in any other context. For each trial, we also recorded the order in which DLR, biting, and stinging occurred.

Analysis

All analyses were conducted through the StatsModels package (Perktold, Seabold & Taylor, 2018) included in the Anaconda distribution of Python, a free scientific analysis distribution of the Python programming language (Anaconda, 2019; http://www.python.org). Code is available on request. Behavior sequences (e.g., DLR then bite, or bite then DLR then sting, etc.) were analyzed with a series of repeated measures logistic regressions via generalized estimating equations (GEE; Hardin & Hilbe, 2003). We used this series of regressions in place of a multinomial logistic regression as GEE controls for repeated measures within subjects. This technique is also less sensitive to the need for many cases per variable than multinomial regression or chi square analyses; an important consideration for our data, as statistical comparisons between commonly and uncommonly observed behavior sequences answer important research questions. We used an interceptless model where groups are treated as two mutually exclusive variables. By default, a logistic regression's parameter estimates and associated *p* values display a difference from a 50% binary chance level. As our sequence analysis considered 16 possible sequences, we subtracted the log odds of 1/16 from all parameter estimates and confidence intervals, then calculated corresponding *p* values. Each behavior sequence is therefore tested for statistical difference from chance (1/16) instead of a 1/2 comparison that is arbitrary for this data. Individual parameter estimates were compared directly by creating a z score by dividing the difference between the estimates by the square root of the sum of the squared standard errors of the estimates (Clogg, Petkova, & Haritou, 1995; Paternoster, Brame, Mazerolle, & Piquero, 1998). Even after adjusting parameters by subtracting the log odds of 1/16, the difference between estimates, z score, and *p* value are still the same as those normally reported by a regression that includes one level of a categorical variable in the intercept.

Results

Figure 2 shows the percent of trials where a DLR, bite or sting occurred for bees in the experimental and control groups. Bees in the experimental group displayed more behavior. For both groups, DLR was the most common behavior. Bees in the experimental group were more likely to sting than bite, while bees in the control group were more likely to bite than sting. The average number of legs lifted in trials where DLR occurred can be seen in Figure 3. Not only were bees in the experimental group more likely to emit a DLR (Figure 2), but they also lifted more legs on average. This suggests both probability and topography of DLR change as a function of stimulus intensity. Both Figures 2 and 3 show little change across trial that would suggest habituation, sensitization, or fatigue. While our analysis in subsequent paragraphs will focus primarily on behavior sequences, we also included our initial exploratory analysis of individual behaviors in supplementary material that will relate well to Figures 2 and 3.

Table 1 shows the percent of trials where a particular behavior sequence occurred. For each trial, the order of DLR, bite and sting were recorded, resulting in 16 possible sequences (no tied rankings were observed). Bees in the experimental group were highly active, only being inactive 3% of the time. DLR was emitted first or by itself around 81% of trials, while biting and stinging rarely occurred first or by themselves. Note that the least common of the DLR-first sequences was the DLR:Bite sequence, occurring during only 1% of trials. Trials with a sting following DLR were much more common (30%), and trials with a bite and sting following DLR (in either order) were also more common (31%). Bees in the control group were only active during 49% of trials. During these trials, DLR often occurred by itself (31%), or a bite occurred by itself (15%). Sequences of multiple behaviors were rare. Taken together, this indicates that in the experimental group, DLR is more related to subsequent stings than to subsequent bites.

Tables 2 and 3 shows the result of a series of logistic regressions of behavior sequence, with Table 2 displaying information for the experimental group, and Table 3 displaying information for the control group. Although we used separate tables due to the large size of a single combined table, the sequence analyses presented on Tables 2 and 3 should be interpreted together. We only included the groups as parameters in this analysis given the large number of behavior sequences to be analyzed, and the lack of a trial effect in previous graphs. For each behavior sequence, the analysis shows the log odds of that sequence compared to a 1/16 chance value. The direction and magnitude of the parameter estimates, as well as the *p* values reflect a difference from the chance value.

The analysis shows that most behavior sequences occur significantly less than chance, with a few exceptions. For the experimental group, all the DLR-first sequences, except for DLR:Bite, occurred significantly more than chance. DLR:Bite was the only DLR-first sequence to occur significantly less than chance. Inactivity, Bite, Bite:DLR:Sting, and Bite:Sting:DLR occurred near the chance level for the experimental group. For the control group, inactivity, DLR, and Bite occurred significantly more than chance.

Table 4 shows pairwise comparisons between DLR:Bite and other DLR-first sequences, as well as comparisons between DLR:Sting and other DLR-first sequences for the experimental group. The estimate differences and z scores were calculated from the parameter estimates and standard errors reported in Tables 2 and 3. The pairwise comparisons show that the DLR:Bite sequence occurs significantly less than all other DLR-first sequences. Conversely, the DLR:Sting sequence occurs significantly more than DLR:Bite, and other DLR-first sequences except for DLR. Though DLR:Sting does occur more than DLR, the difference is not significant (p = 0.064). Note that for this series for pairwise comparisons, it may be appropriate to use a multiple comparison correction to adjust the significance threshold. For example, the conservative Bonferroni correction would involve dividing the alpha level by the number of comparisons, in this case 0.05 divided by 7 produces a new significance threshold of 0.007. The reader is free to use whichever correction technique they deem appropriate.

Taken together, the findings reported in Tables 1 - 4 strongly suggest that DLR is more related to subsequent stinging than it is to subsequent biting for the experimental group. This is also in line with our supplementary analysis of individual behaviors. Ultimately, the fact that DLR often precedes stinging in the experimental group, the group where stinging frequently occurred, indicates DLR may function to signal potential predators that a sting is imminent.

Discussion

Our findings describe DLR, an undescribed behavior, and show that it often precedes stinging, but rarely precedes biting alone. We also demonstrate that the probability and topography of DLR is sensitive to stimulus intensity, changing as stimuli become closer across groups. Together, these results suggest that DLR is an honest signal that indicates stinging may occur. DLR may function in either an aposematic or a pursuit deterrence role, and these functions may not be mutually exclusive. Given the already bright coloration of bumble bees, it is possible that DLR serves as a multimodal enhancement of existing aposematic signals, adding a conspicuous posture to vibrant colors (for discussions on multimodal antipredator signals see Ritson-Williams & Paul, 2007; Rowe & Guilford, 1999; and Rowe & Haplin, 2013). If DLR has a pursuit deterrence function, it may signal that the bee is aware of a potential predator and will sting if pursued.

While DLR likely signals a sting may occur, DLR can also occur alone. In the control group, bees emit DLR but rarely sting. This likely occurs because the distant stimulus is intense enough to elicit DLR, but does not support stinging. In the wild, if DLR is successful at preventing a potential predator attack, it may occur without subsequent stinging behavior. Therefore, the occurrence of DLR in the control group is consistent with an honest signal interpretation.

While our experiment ~~clearly~~ indicates a temporal connection between DLR and stinging, additional research is needed to clarify DLR's specific antipredator function. Such research will need to consider what stimuli and predators elicit DLR, and equally importantly, how predators respond. Field experiments may also study DLR in situ, providing bees with a number of alternative behaviors, including fleeing. Such ecologically valid research may be required to completely determine the function of DLR. For example, if DLR functions strictly as a pursuit deterrence signal, bees may emit a DLR, then flee if a predator approaches, while if DLR has only an aposematic function it may not be related to any antipredator behavior other than stinging.

In addition to further clarifying the function of DLR, our initial work facilitates many additional research topics. For example, research may consider how DLR relates to specific stimulus modalities or intensities, and if predators have learned or innate response to DLR. Research should also consider the extent that DLR occurs in other *Bombus* species, and if it differs across species. Studies of individual differences will likely also be fruitful, especially considering recent literature on the size-dependent behavior in bumble bee workers (e.g. Jandt, Huang, & Dornhaus, 2009; Kodaira, Ohtsuki, Yokoyama, & Kawata, 2009; Raine & Chittka, 2008; Riveros & Gronenberg, 2009a; 2009b; Spaethe & Weidenmüller, 2002). Finally, as bees are social animals, future work should also consider social factors, such as if DLR can be elicited by other bees or if DLR affects adjacent bees. It may also be possible that DLR is affected by alarm pheromones. Given that bumble bees possess tarsal glands (Pouvreau, 1991; Schmitt, 1990), social odors may even be released during DLR.

Experiment 2 - Habituation of DLR

In this experiment, we investigated the possibility that DLR could change as a function of learning. Specifically, we wanted to know if DLR habituates to repeated mild stimuli. Habituation, defined as the diminishing of a response, emotional or physical, to a repeated stimulus (Thompson & Spencer, 1966), is a simple form of learning that can be observed across nearly all species, from planarians (Nicolas, Abramson, & Levin, 2008) to rodents (Geyer & Braff, 1987). Habituation of disturbance responses has also been documented in many species. For example, hissing cockroaches may cease emitting their disturbance hiss in the presence of specific handlers (Davis & Heslop, 2004), rattlesnakes show a reduction in latency and duration of rattling in response to a startling stimulus (Place & Abramson, 2008), and the gill withdrawal reflex of the sea hare *Aplysia* is also known to habituate (Carew, Pinsker, & Kandel, 1972). Studies of habituation are also often the foundation for other procedures, including investigations of mental health (Akdag, Nestor, O'Donnell, Niznikiewicz, Shenton, & McCarley, 2003; Geyer & Braff, 1987; Jaycox, Foa, & Morral, 1998), and neurological processes related to learning and memory (Castellucci & Kandel, 1974; Castellucci, Pinsker, Kupfermann, & Kandel, 1970). If

DLR can be altered through habituation learning, this opens new possibilities in behavioral and physiological research with bumble bees.

In addition to discovering if DLR can change as a function of learning, we were also interested in differences across populations due to differences we observed in pilot research. Specifically, we compared samples of captive bred to wild caught bumble bees. Given the substantial number of findings on behavioral differences in honey bees due to breed, genetics and environment (e.g., Alaux et al., 2009; Schulz, Haung, & Robinson, 1998; Sheppard, Arias, Grech, & Meixner, 1997; Spivak, 1997; Tautz, Maier, Groh, Rössler, & Brockman, 2003), it is reasonable to assume that some differences may be found between captive bred and wild caught bumble bees. If DLR and habituation of DLR are observed across both samples, this would also suggest that DLR may be a robust behavior to study in learning experiments. This would be a beneficial comparison considering the use of both wild and captive bees in the literature.

Methods

Subjects

Both captive (n = 32) and wild worker bumble bees (n = 32) 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 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. Wild worker bees were collected while foraging, primarily on clover and *Abelia*, at the Converse College campus (Spartanburg, SC) during July and August. 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. Many bees immediately returned to foraging. Marked wild bees were observed foraging 2 weeks after the experiment.

Captive bees were visibly smaller than their wild counterparts. We sampled both populations (130 captive bees, 62 wild bees) and recorded head width. An independent sample *t*-tests revealed that captive bees were significantly smaller (mean difference = –0.67 mm, $t_{190} = -9.255$, $p < 0.000$). For this experiment, we were not able to collect physical measurements for specific subjects. 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.

Procedure

Subjects were placed in individual apparatuses after being collected. Each apparatus consisted of a capsule formed from a clear acrylic tube (4.5 cm long, 2.5 cm inner diameter), with two white plastic caps sealing the tube. Two holes (0.4 cm diameter) were drilled near the center of each cap. Each apparatus was placed approximately half a meter apart, and bees were allowed an acclimation period of 45 minutes.

Each bee experienced 10 trials with a seven-minute ITI after the acclimation period was complete. During each trial, a researcher startled the bee by presenting a hand 15 cm above the apparatus, rapidly lowering it to approximately 6 cm above the apparatus, rotating the hand once in a clockwise circular motion, and then withdrawing the hand. As strong stimuli can inhibit habituation or cause sensitization, we used this relatively mild stimulus, compared to those used in the first experiment, to increase the chance that habituation could be observed. The bees' response was recorded during the two-second stimulus presentation and for three seconds after the presentation. DLR was recorded as a binary response and no other behaviors were recorded. After the final trial, the bees were recollected, still inside their apparatuses, and chilled in preparation for being marked and returned to the colony or collection area. The act of recollection served as a dishabituating stimulus, and we recorded general locomotor activity to determine if a decrease in DLR was due to habituation or leg fatigue.

Analysis

We analyzed the probability of DLR across trial using repeated measures logistic regression via GEE. As with previous logistic regressions, we use an interceptless form so that the parameters can be directly compared to a chance value (in this case 50%), then compared them to each other by creating a z score by dividing the difference between the estimates by the square root of the sum of the squared standard errors of the estimates.

Results

Figure 4 shows the percent of captive and wild bees emitting DLR across the 10 habituation trials. The captive bees were initially much more likely to respond; nearly 70% of captive bees responded compared to around 35% of wild bees. The probability of response decreases for both captive and wild bees as a function of trial at a somewhat similar rate. When the bees were recollected after the final, all bees were observed walking and engaging in normal locomotion and we informally observed around half of the bees emitting DLR as they were moved, suggesting that DLR decreased due to habituation, not leg fatigue.

Table 5 shows a logistic regression for the probability of DLR. The analysis shows that the initial probability of response for captive bees was significantly greater than chance (50%). Wild bees initially responded less than chance, but not significantly so. A direct comparison of the parameters revealed that captive bees were significantly more likely to initially respond than the wild bees (estimate difference = 1.301, z = 2.691, *p* = 0.007). Both captive and wild bees showed small, but significant decreases in probability of DLR as trial increased. Though the captive bees showed a slightly stronger effect, a direct comparison reveals that this difference was not significant (estimate difference = –0.046, z = –0.567, *p* = 0.571). The findings of this analysis strongly support what can be seen in Figure 4; captive bees are more likely to respond, but both populations show a decrease in response that indicates habituation learning.

Discussion

In this ~~we~~ experiment, we provided the first demonstration of habituation of DLR, as well as documented differences in DLR across captive and wild samples. While both samples showed a similar rate of habituation, the captive bees were initially more likely to perform DLR. This difference in DLR may have occurred for two different reasons. First, the samples of worker bees we collected from captive and wild populations may have differed in role specialization. Bumble bee castes include the reproductive queen and drone castes, as well as the primarily non-reproductive worker caste frequently used in research. Workers may be further specialized. The smaller worker bees are more likely to feed larvae and attend to hive maintenance, while larger workers act as foragers. In bumble bees, role specialization appears to be determined during early development, and research suggests that physical dimensions can predict behavioral performance (Jandt, Huang, & Dornhaus, 2009; Kodaira, Ohtsuki, Yokoyama, & Kawata, 2009; Raine & Chittka, 2008; Riveros & Gronenberg, 2009a; 2009b; Spaethe & Weidenmüller, 2002). The bees sampled from our captive population were significantly smaller than our sample of wild bees, suggesting the wild bees were more likely to be foragers. Additionally, wild bees were collected during the act of foraging, further increasing our confidence they fit this role specialization. The likely distinction in the specialization of the captive and wild bees may have contributed to the initial difference in DLR.

A second possible reason for the difference in DLR between captive and wild bees may be the distinct experiences of bees raised indoors compared to those of wild bees. Captive bees were only exposed to stimuli in their hive and flight cage, and ultimately experienced only a small number of stimuli before research. Conversely, wild bees likely contact many stimuli during daily foraging including other insects, birds, pedestrians, and even landscaping equipment. It is possible that exposure to a wide variety of stimuli served to acclimate the wild bees to mild visual stimuli, such as the hand wave used in this experiment.

Regardless of the difference in initial rate of DLR, both captive and wild bees showed clear habituation trends, and thus our experiment suggest~~ion~~s expansive opportunities for a new area of non-associative learning research with bumble bees. Future research may consider the principles of habituation and sensitization outlined by Thompson and Spencer (1966), Groves and Thompson, (1970) and Rankin et al.~~,~~ (2009). For example, altering the time between stimulus presentations may change the rate of habituation, and placing the animal in an agitated state prior to habituation trials may instead result in sensitization. Future work may also consider exploring classical conditioning or operant conditioning of DLR. This would be a very reasonable next step considering the success of sting extension response (SER) conditioning procedures in honey bees. Additionally, various DLR conditioning studies could be used as a basis for research on pesticides, sensory perception, memory, pharmacology, and neurophysiology research, as conditioning research with honey bees has also done for these same topics (e.g., Abramson, Squire, Sheridan, & Mulder, 2004; Abramson et al., 2006; Faber, Joerges & Menzel, 1999; Giurfa et al., 2009; Linader, de Ibrra, Laska, & 2012; Mustard, Dews, Brugato, Dey, Wright, 2012; Varnon, Dinges, Black, Wells, & Abramson, 2018; Vergoz, Roussel, Sandoz & Giurfa, 2007).

Conclusions

Our experiments document an undescribed behavior, the disturbance leg-lift response (DLR). We suggest an antipredation role for DLR, show that DLR can change as a function of learning, and outline future considerations for DLR as a behavior of interest for both behavioral ecology and comparative psychology. Research with bumble bees is quickly indicating they are becoming an important new model organism for ecological, behavioral, and physiological research. We hope that our work will stimulate additional research on DLR, and on bumble bees in general. We also hope that special considerations will be given to reporting not only what bumble bees can do, but also what they cannot do. Reporting differences, including deficits, is an important component of research in animal behavior (Avarguès-Weber & Giurfa, 2013), and this is especially important for new model organisms.

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Figure 1

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

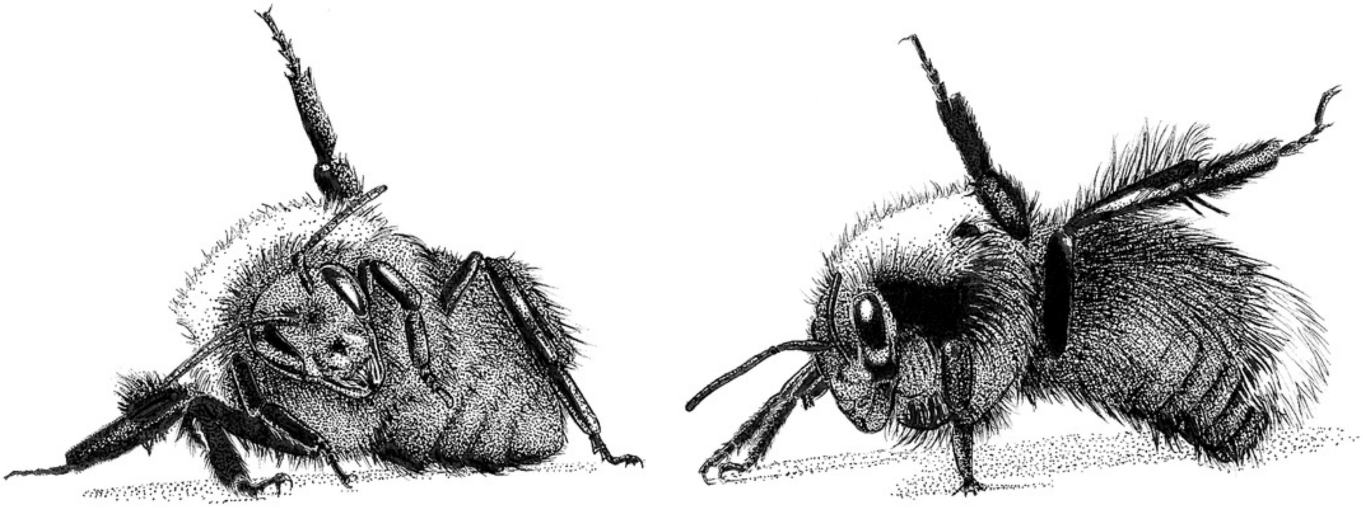

Figure 2

Percent of trials where bees emitted DLR, bite or sting for the experimental and control groups. Error bars show standard error of the mean.

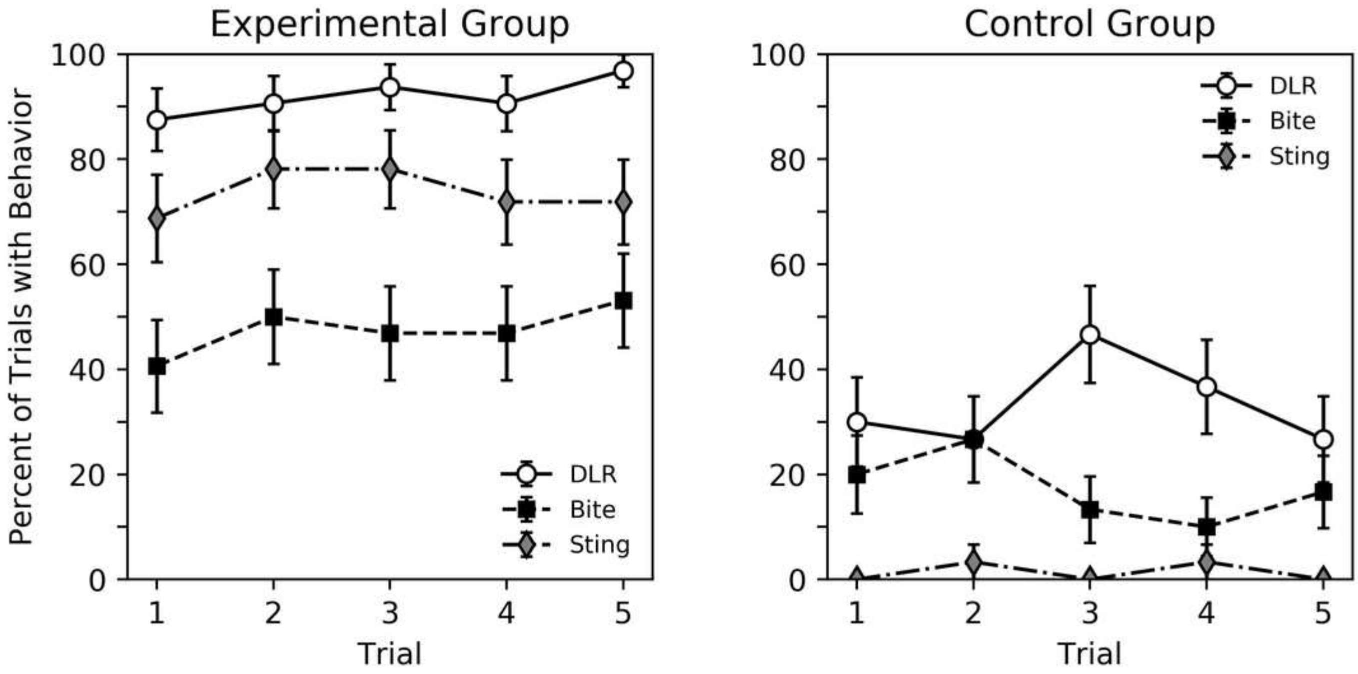

Figure 3

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

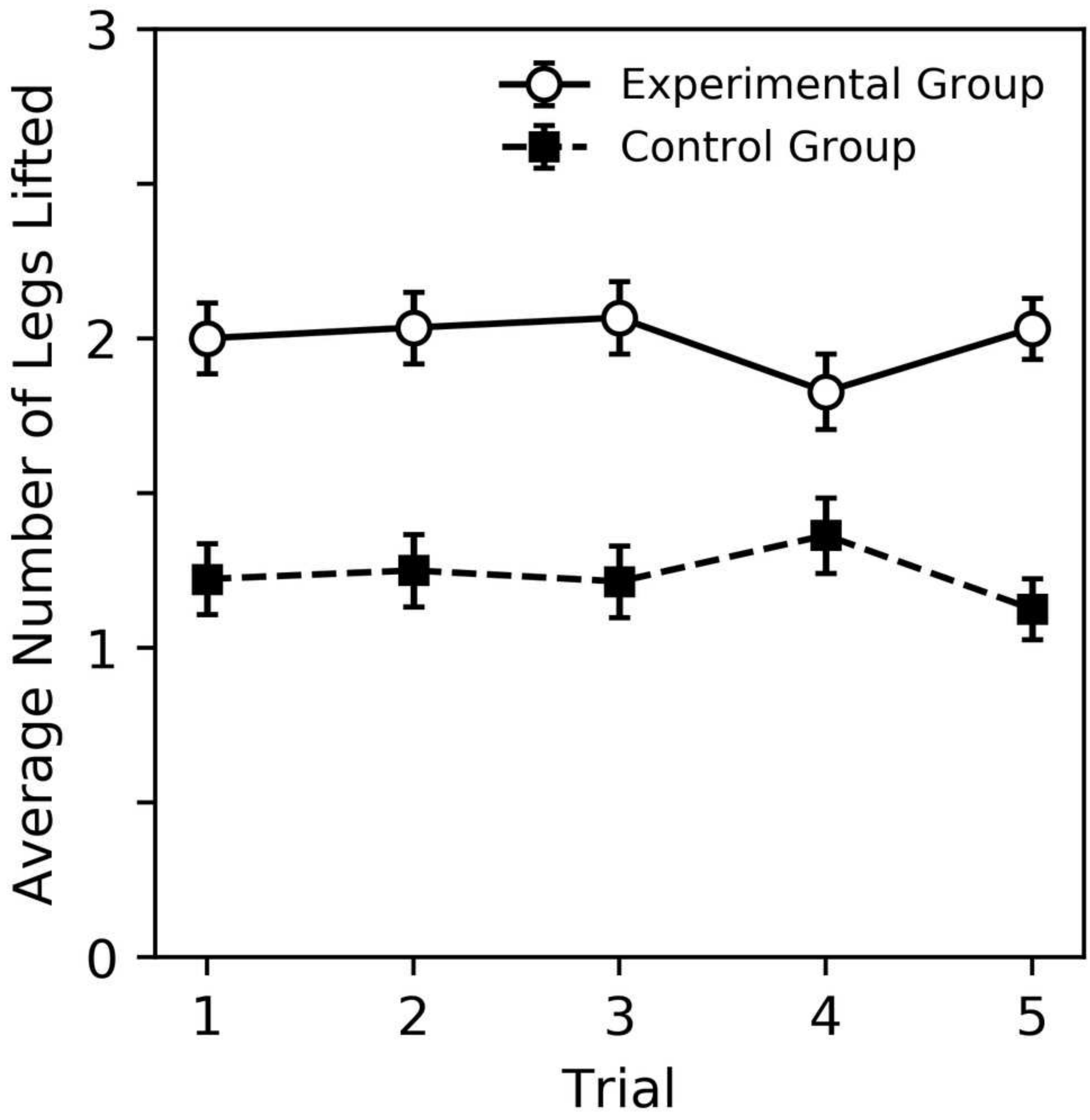

Figure 4

Percent of captive and wild bees emitting DLR across the 10 habituation trials. Error bars show standard error of the mean.

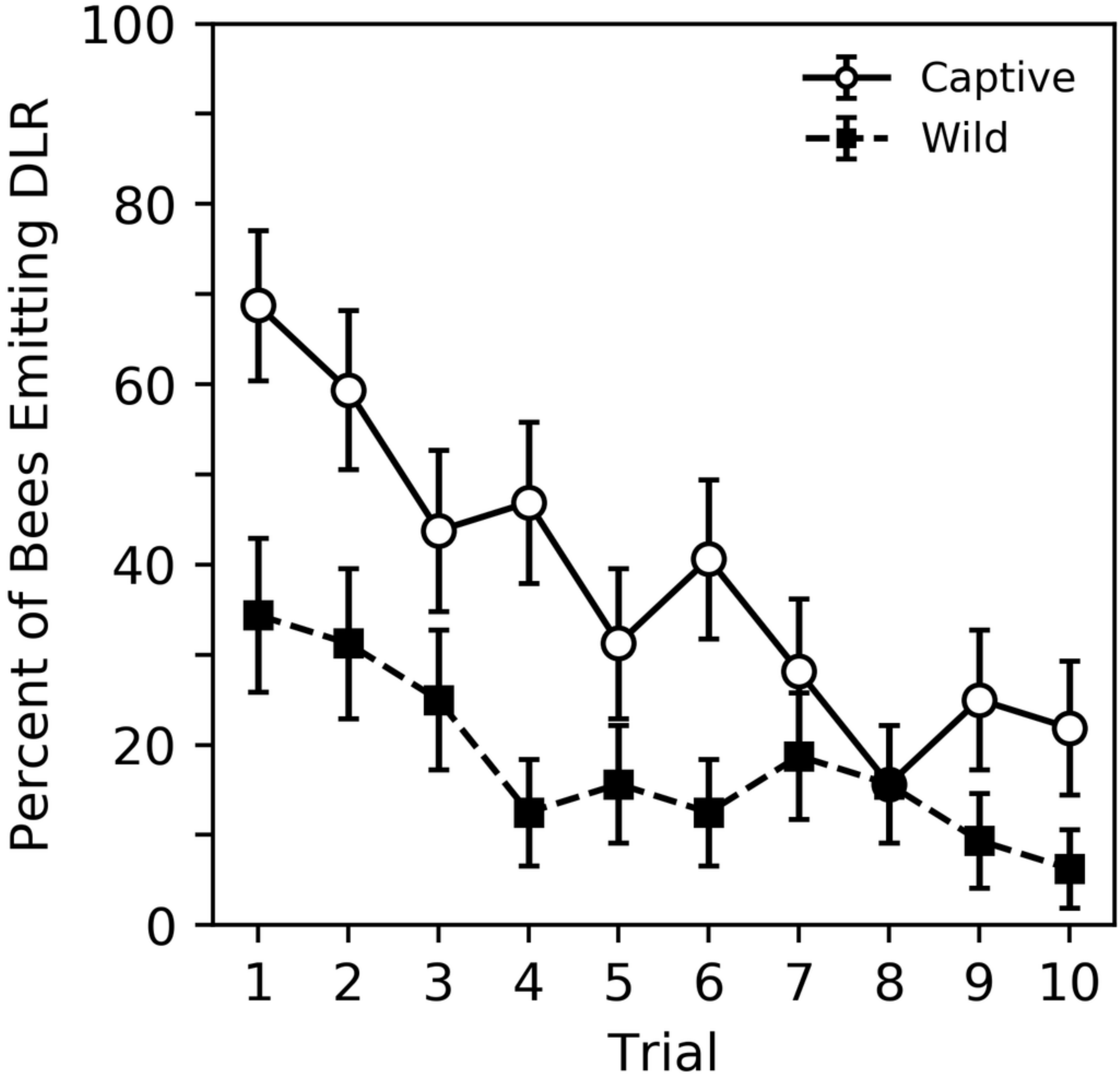

Table 1(on next page)

Percent of Trials with Behavior Sequence

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

Table 2(on next page)

Experimental Group Sequence Regression

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

Table 3(on next page)

Control Group Sequence Regression

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

Table 4(on next page)

Experimental Group DLR-first Pairwise Comparisons

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

Table 5(on next page)

Change in DLR Across Trial

Table 5

Change in DLR Across Trial

Parameter	Estimate	Standard Error	95% Confidence Intervals		*p* value
Captive	0.729	0.304	0.134	1.324	0.016
Wild	-0.572	0.376	-1.310	0.166	0.129
Captive * Trial	-0.233	0.042	-0.316	-0.150	0.000
Wild * Trial	-0.188	0.068	-0.321	-0.054	0.006