Do Allegheny Mountain Dusky Salamanders (*Desmognathus ochrophaeus*) Learn to Associate Non-Predatory Smooth Greensnakes (*Opheodrys vernalis*) with Threat?

by
Emilia Ann Rose Gildemeister

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Honors Committee

Aaron M. Sullivan, PhD Signature: ______________________________

Jamie Potter, PhD Signature: ______________________________

John Rowley, PhD Signature: ______________________________

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Do Allegheny Mountain Dusky Salamanders (*Desmognathus ochrophaeus*) Learn to Associate Non-Predatory Smooth Greensnakes (*Opheodrys vernalis*) with Threat?

Emilia A.R. Gildemeister

Abstract: Learning confers benefits to organisms through increased foraging success, reproductive output, and predator detection. The capacity to recognize and assess predators via chemical stimuli may be especially beneficial for amphibians, whose populations are declining globally due to a variety of environmental challenges. With this study, I attempted to condition Allegheny Mountain Dusky Salamanders (*Desmognathus ochrophaeus*) to associate non-predatory Smooth Greensnakes (*Opheodrys vernalis*) with stimuli from a predation event (i.e., damage-released stimuli). Salamanders were collected from the wooded areas of the campus of Houghton College and randomly assigned to one of six treatment groups: three controls (distilled water, damage-released cues, and aqueous rinses of *O. vernalis*) were used to establish baseline levels of locomotor and foraging activity. Three experimental groups were used to vary the amount of time that salamanders were exposed to the paired chemical stimuli (48 h, 96 h, or 144 h) in an attempt to assess the effect of exposure duration on the learned response. After conditioning, I observed the foraging and locomotor activity of control and conditioned salamanders exposed to kairomones from *O. vernalis* in the laboratory at night. My results show no significant differences in locomotor or foraging behavior between the control and experimental treatments, suggesting a lack of learning by individual *D. ochrophaeus*. This may indicate the inability for associative learning by *D.*

ochrophaeus, which is inconsistent with other studies of amphibian learning, or limitations on the types of associations that can be formed. Alternatively, the lack of pronounced behavioral responses to the damage-released stimuli could partially account for the results of this study.

Keywords: *Desmognathus*, *Opheodrys*, predator-prey, learning, foraging

Introduction

Background

The capacity to learn may lead to increased foraging and reproductive success, along with predator avoidance in many species (Griffin, 2004; Nicol, 2006; Thorhallsdottir et al., 1990). Associative learning in particular has been observed in a number of taxa, including both vertebrates and invertebrates, and may also have fitness benefits (Deyo et al., 1989; Dukas, 1999; Lönnstedt et al., 2012; Mathis et al., 2008; Mirza et al., 2006; Sahley et al., 1981; Walters et al., 1981). It is characterized by linking certain stimuli with a particular response through an association process (Pearce, 2001). A study by Faber et al. (1999) clearly demonstrates properties of associative learning. In this study, honeybees were conditioned in two different odors, one paired with a sucrose reward and the other unpaired or unrewarded. After training, those exposed to the rewarded odor showed an increase in activity and those exposed to the unrewarded odor showed a decrease. This ability to associate certain odors or stimuli with foraging opportunities may be useful in terms of energy conservation and efficiency.

Associative learning also plays an important role in the recognition of new potential threats. Several studies have observed conditioned responses to unfamiliar predators through the use of chemical stimuli (Abudayah and Mathis, 2016; Ferrari and Chivers, 2008; Mirza and Chivers, 2000; Woody and Mathis, 1998), which are often used to assess risk of predation in dark or turbid environments where visual cues cannot be used (Kats and Dill, 1998). There are a variety of chemical stimuli that are used as communication between individuals of the same species and those of different species. For example, damage-released cues are released when tissues are damaged by con- and heterospecifics, disturbance cues are released by prey species under distress, and predator kairomones are chemical stimuli that are either intentionally or unintentionally released by the predator and recognized by the prey (Brönmark and Hansson, 2012; Weldon, 1983; Wisenden, 2000).

The process by which chemical stimuli may facilitate the recognition of novel predators can be seen in adult Eastern red-spotted newts (*Notophthalmus viridescens*). Woody and Mathis (1998) show that newts can be trained to recognize smallmouth bass, an unfamiliar predator, through the pairing of these bass kairomones with damaged-release cues from salamanders. After the conditioning period where newts were exposed to the bass stimulus with either dechlorinated water or a skin extract stimulus, subjects were then tested in the predator cues alone. Individuals that were exposed to dechlorinated water with the predator kairomones served as baseline data to ensure that behavioral responses were due to associative learning, as opposed to a general response to disturbance. The results showed a conditioned avoidance response to the predator cues by those previously exposed to damage-released cues, indicating that newts have the ability to recognize new predators through association. In a similar experiment, hatchery-reared brook trout (*Salvelinus fontinalis*) that had no previous exposure to predators were conditioned with predator kairomones of chain pickerel (*Esox niger*), a known predator, and damage-released and disturbance cues (Mirza and Chivers, 2000). Their response was then measured when exposed to chemical stimuli from chain pickerel alone. Brook trout conditioned in the damage-released signals showed an anti-predator response to the pickerel, but those exposed to the other treatments did not show anti-predator behavior, indicating that associative learning had occurred. The learned response was retained for 10 days beyond the initial measurement, although it showed a reduction over time without reinforcement. An additional study with amphibians that paired predator kairomones and damage-released cues showed that the response of larval toads (*Bufo americanus*) increased with higher concentrations of alarm cues, indicating that the magnitude or severity of exposure may be important in the ability for this behavior to be retained (Mirza et al., 2006).

These studies indicate that a variety of vertebrate ectotherms are capable of associative learning, and highlight the potential for this ability to be useful in a natural setting (i.e., detection of introduced predators). Chivers et al. (2001) show the usefulness of this ability in terms of species introduction by measuring the response of juvenile Pacific treefrogs (*Hyla regilla*) to the introduced bullfrogs (*Rana catesbeiana*), which are a predatory threat to this species. They found that treefrogs from a population syntopic to bullfrogs avoided the kairomones from this predator, whereas those allotopic to bullfrogs did not have an avoidance response. This study shows how predator recognition through association can be applied to natural ecosystems that undergo changes, and how this capability might be valuable in terms of survivorship, especially in the context of invasive species and globalization.

Purpose

The purpose of this study was to assess the ability of Allegheny Mountain Dusky Salamanders (*Desmognathus ochrophaeus*) to recognize a new threat as a result of associative learning. After a training period in which salamanders were exposed to predator cues from Smooth Greensnake (*Opheodrys vernalis*), an insectivore (Gibbs et al., 2007), paired with damage-released alarm cues from conspecifics, salamanders were tested in the chemical cues of the novel predator alone. Both locomotor and foraging behavior were recorded in the final observational period in attempt to detect a learned response, as salamanders have shown to reduce these behaviors in the presence of a predator or damage-released cues from conspecifics (e.g., Johnson and Sullivan, 2014; Sullivan et al., 2001). The goals of this study were to 1) condition salamanders in predator stimuli 2) measure behavior for evidence of a learned response 3) and assess the effect of conditioning duration on the magnitude of response.

Methods and Materials

Collection and maintenance of animals

212 individual *D. ochrophaeus* were collected from wooded areas surrounding the Houghton College campus (Houghton, New York) between 31 August and 11 September 2017 for use in behavioral trials and preparation. To ensure that lack of sexual maturity did not play a role in any behavioral response, the minimum snout-vent-length of salamanders collected (SVL; measured from the snout to the cloacal vent of each salamander) was 3.2 cm, as Keen and Orr (1980) show that females first oviposit at 3.0-

3.4 cm in SVL. All salamanders were housed individually in 15-cm-diameter Petri dishes lined with acid-free paper towels moistened with distilled (DI) water, and housed in a climate-controlled unit at temperatures of 12.8°C (night) and 15.6°C (day) and a seasonal photoperiod (13:11 h light/dark). DI water was added to the substrates as needed, and paper towels were replaced weekly as they became soiled.

Three Smooth Greensnakes (*O. vernalis*) were collected from a sun-exposed area near the *D. ochrophaeus* collection site from 7-17 September 2017 to be used for the collection of snake kairomones. The three snakes were housed together in a glass aquarium with crumpled paper towels for shelter, water, and a heating lamp.

Collection of kairomones from nonpredatory snake

To collect the kairomones from *O. vernalis*, I transferred each snake into a separate clean 4-L beaker, which was covered with cheesecloth for 48 h. During this time, heating lamps were provided for warmth. After 48 h, snakes were transferred into new clean 4-L beaker for another stimulus collection. After two consecutive collections, they were returned to their aquaria for a 48-h rest period before the process was repeated. Beakers were rinsed with 200 mL of DI water, which was then filtered through glass wool and separated into 50-mL vials to be stored in the freezer at -18°C until use. To avoid any confounding variables during the training process or trials, filtrates from each individual *O. vernalis* were labeled and stored in separate vials, but thawed and mixed before use.

Collection of damage-released alarm cues

Damage-released cues were collected from caudal tissue of 32 *D. ochrophaeus*. I induced tail autotomy on each individual by using blunt tweezers to firmly apply pressure 1 cm posterior to the cloacal vent. The total quantity of tail collected had a mass of 3.86 g. In congruence with other studies, a total of 1158 mL of DI water (300 mL of DI water per gram of tissue) was then added to the damaged tissue and blended for two minutes until homogenized (Sullivan et al., 2003). This mixture was filtered through glass wool to remove large tissue and separated into 50-mL vials to be frozen at -18°C until needed.

Bioassay

To test for the capability of associative learning in *D. ochrophaeus,* the remaining 180 individuals were subjected to a conditioning period consisting of three distinct exposures followed by an observational period. To begin, salamanders were randomly sorted into one of six treatment groups (n = 30), including three control groups: DI water, damage-released cues, and *O. vernalis* cues. Salamanders in these control treatments provided a baseline level of locomotor and foraging activity and would allow for the detection of learning or habituation that may occur over time. Each individual experienced three exposures to the appropriate stimulus in the training period, each 48 h, and a final observation in the same stimulus. The remaining three treatment groups participated in a gradient of exposures to predator chemical cues paired with damage-release cued from conspecifics (48 h, 96 h, and 144 h) in attempt to assess the effect of multiple exposures on the magnitude of response. In the observational trial, they were later tested in the chemical cues of *O. vernalis* alone.

Training period

The training period began six days before the final testing period and was composed of three conditioning periods that began every 48 h. In the first conditioning period, individuals were transferred into a clean 15-cm-diameter Petri dish lined with filter paper moistened by 3 mL of the appropriate treatment and 3 mL of DI water, with the exception of individuals exposed to the paired cues, which consisted of 3 mL of cues from *O. vernalis* and 3 mL of damage-released cues (Table 1). They remained on substrates with these treatments for 48 h at which point the second conditioning period began. For second exposure, excess stimulus was removed from each dish and the appropriate treatment was added to the substrate without removal of the salamander. During the conditioning period, two salamanders died from unknown causes: one in the *O. vernalis* treatment and the other in the damage-released treatment. For the final conditioning period, the method described above was repeated using the appropriate treatments for the third exposure. Between each of the exposures, salamanders were stored in the climate-controlled chamber and were not disturbed. Filter papers were not replaced between each of the conditioning periods, but additional applications of the treatments were added to the same substrate, meaning I potentially increased the time of exposure and concentration of each stimulus.

Observational trial

The behavioral assay began 48 h after the final exposure and was performed in a dark laboratory with sufficient red light to examine behavior. Salamanders were randomly assigned a testing order and moved into the laboratory 1 h before the start of observations to adjust to light levels and room temperature. To begin, each salamander was transferred into a clean 15-cm-diameter Petri dish lined with filter paper and moistened with 3 mL of the appropriate stimulus and allowed a two-minute adjustment period. The three control treatments groups were tested in the stimulus that each was trained in, and all three of the experimental groups were tested in the chemical cues of *O. vernalis* alone. After this, five *Drosophila* were added to the dish and the locomotor and foraging activity of each individual were recorded for 10 min. This included the number of steps, latency to strike, number of strikes, and number of captures. The latency to strike in this study was defined as the amount of time elapsed before the first strike at prey. Salamanders that did not forage during the observation period were given a latency to strike of 600 s. Strikes were characterized by rapid movements towards the *Drosophila* prey and strikes that resulted in a successful foraging event were also considered captures. After the 10-min observation, the SVL of each individual was measured and it was returned to its home dish. The day following the final observational period, all animals were returned to their collection site.

Statistical Analyses

Gildemeister et al. (2017) indicate the importance of size (SVL) on locomotor and foraging behavior of *D. ochrophaeus* exposed to kairomones from predatory snakes. As a result, we performed Spearman's rank correlation on SVL and each of the behavioral metrics. I found a significant correlation between SVL and number of steps (r = 0.15; p = 0.047; Figure 1), strikes at prey (r = -0.23; p = 0.018; Figure 2), and successful captures (r = -0.22; p = 0.026; Figure 3). Additionally, I found a marginally significant correlation between SVL and the latency to strike (r = 0.17; p = 0.088; Figure 4). Because SVL is significantly correlated with most of the behaviors that I measured, and the data related to foraging and locomotor behavior did not fit the assumptions of normality, we used the non-parametric generalized linear model (GZLM) to conduct a series of analyses of covariance (ANCOVA) with a loglink function in Statistica (Statsoft, Inc., 2001, version 6.0). The ANCOVA in GZLM generated a Wald statistic that allowed for the concurrent examination of a covariate (i.e. size) while testing for main effects of treatment (i.e. gradient of exposure duration).

Results

My analysis of the locomotor activity (i.e. number of steps taken by test subjects) reveals a significant main effect of treatment but not a significant effect of SVL (Table 2; Figure 5). In addition, latency to strike at prey was significantly different among treatment groups (Table 3; Figure 6), whereas the number of strikes (Figure 7) and captures (Figure 8) show no significant differences between treatment but with a significant covariate of size (Table 3).

Discussion

Our results indicate that foraging may be influenced by the size of the individual, as larger individuals showed reduced foraging behaviors with fewer strikes at prey, fewer captures, and a larger latency to strike. Locomotor activity also appeared to be impacted by the SVL with larger individuals taking more steps. These data are consistent with other studies that show size-dependent behavior in amphibians (Bennett et al., 1989; Gildemeister et al., 2017; Goater et al., 1993; Mathis and Simons, 1994). The reduction of locomotor activity observed in smaller individuals might be the result of increased antipredator behavior as small salamanders are shown to be more vulnerable to predation due to their small body and tail size (Arnold, 1982). Conversely, an increased vulnerability may contribute to higher foraging activity in small salamanders, whereas larger individuals may have more to lose by foraging in a risky way, or by taking strikes at prey without a secure reward. A study by Werner and Anholt (1993) supports this idea through use of a model that reveals the costs associated with risky foraging by individuals with many reserves (i.e., large individuals).

The various treatment groups also had an effect on the latency to strike and number of steps taken by individuals, however treatment did not play a role in the number of strikes and captures of prey. Instead the size of the individuals appeared to be more important to variations in these behaviors. These results are surprising as the various control treatment groups were expected to elicit different behavioral responses to be used as baseline data. The water treatment group was expected to have high locomotor and foraging activity compared to the group tested in damage-released alarm cues, which was expected to show reduced behaviors as a part of an antipredator response as seen in other studies using *D. ochrophaeus* (Johnson and Sullivan, 2014; Lutterschmidt et al., 1994). Along with control treatment groups that did not function as expected, the three experimental treatments showed extreme variation, with those exposed to predator kairomones paired with damage-released alarm cues for 96 hours having reduced foraging activity and increased locomotor activity compared to those exposed for 48 and 144 hours. This pattern could possibly be described as the Goldilocks effect, with either habituation or insufficient association accounting for the lack of consistent behavior, although this possibility is not likely as the unexpected controls indicate the influence of outside factors. Overall the variation in the experimental groups is not indicative of antipredator behavior, suggesting that salamanders did not respond the chemical cues of *O. vernalis*.

The lack of any learned response from *D. ochrophaeus* individuals may suggest the inability for associative learning in this species, however the use of a non-threatening alarm cue may also contribute to the inconsistent results. Because salamanders did not respond to damage-released alarm cues with antipredator behavior in the control treatment group, this stimulus may have been ineffective, meaning that there was no true incentive behind any possible learned behavior. Without a perceived threat when exposed to the snake kairomones, no association would be formed. Along with the use of a potentially inadequate alarm cue, less than half of the salamanders in the 48- and 144-hour exposure treatments and two-thirds of the 96-hour exposure treatment foraged (i.e., struck at prey within the 10-minute testing interval). The significantly smaller sample sizes may contribute to inconsistencies with previously collected data as well as the extreme variation observed in the experimental treatments. There is also the possible influence of habituation due to repetitive exposures to the various stimuli, which may have desensitized the salamanders or caused stress-induced responses, although a study by Mirza and Chivers (2000) using brook trout (*Salvelinus fontinalis*) suggests a sustained response after multiple exposures. Furthermore, a potential impact of DI water on the osmotic balance in *D. ochrophaeus* individuals was realized following the commencement of this study. DI water was used during both the experimental procedures and in the maintenance of salamanders throughout the course of the experiment. Because research suggests a limited osmotic tolerance in amphibians (Gomez-Mestre and Tejedo, 2003; Ruibal, 1962), DI water use may have been the leading cause behind the two salamander deaths that occurred during the conditioning process. A study by Voyles et al. (2007) supports this idea by suggesting that amphibian decline from the disease chytridiomycosis is caused by a disruption of normal epidermal functioning, which creates osmotic imbalance and leads to death. Its use in this study may have also influenced data collection by contributing to a stress-induced response that was then measured during the behavioral assay. In addition, deviations in the expected responses of *D. ochrophaeus* could be caused by seasonal changes in behavior. The observational trials in this study occurred in October, so behavioral responses were likely impacted by the approaching winter. Previous studies support this idea by demonstrating behavioral differences in amphibian species due to seasonal physiological changes (Moore and Jessop, 2003; Moore et al., 2005; Zoeller and Moore, 1986). Future research could repeat this experiment during the summer season, using new methodology to collect the damage-released alarm cues used for the conditioning process.

Conclusion

In this study, I attempted to elicit anti-predator behavior in *D. ochrophaeus* in response to an unknown predator by pairing the kairomones of *O. vernalis* with damage-released alarm cues, showing the capacity for associative learning. This ability could be particularly useful in this salamander species as they are potential prey for many mammals, birds, snakes, and other salamanders because of their small size and lack of noxious skin secretions (Brodie et al., 1979; Gibbs et al., 2007). It could also allow *D. ochrophaeus* to better adjust to a changing environment, which may be a crucial skill in the fluctuation of environments caused by climate change, of which amphibians are particularly vulnerable (Corn, 2005). Although the results of this study indicate the inability for *D. ochrophaeus* to recognize a novel predator as threatening, there were various inconsistencies between the collected data and previous studies, indicating the possible influence of other factors.

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Table 1. The treatments used in the conditioning period and final observational trial for each of the six treatment groups. With the exception of the paired chemical cues, 3 mL of DI water was added with the appropriate treatment. DR cues represent the damage-released stimuli released from injured conspecifics, and *O. vernalis* refers to the predator kairomones from the Smooth Greensnake.

Treatment Group	**1**	**2**	**3**	**4**	**5**	**6**
Exposure 1	DI water	*O. vernalis*	DR cues	*O. vernalis*	*O. vernalis*	*O. vernalis* + DR cues
Exposure 2	DI water	*O. vernalis*	DR cues	*O. vernalis*	*O. vernalis* + DR cues	*O. vernalis* + DR cues
Exposure 3	DI water	*O. vernalis*	DR cues	*O. vernalis* + DR cues	*O. vernalis* + DR cues	*O. vernalis* + DR cues
Trials	DI water	*O. vernalis*	DR cues	*O. vernalis*	*O. vernalis*	*O. vernalis*

Table 2. Result of generalized linear model analysis of covariance to assess the number of steps in Allegheny Mountain dusky salamanders (*Desmognathus ochrophaeus*) individuals exposed to various treatments using snout-vent-length (SVL) as a covariate.

	df	**Wald**	**P**
Intercept	1	30.44	<0.001
SVL	1	3.32	0.069
Treatment	5	17.70	0.003

Table 3. Result of generalized linear model analysis of covariance to test the effect of treatment on the latency to strike, number of strikes at prey, and number of successful captures of prey in foraging *D. ochrophaeus*, using size (SVL) as a covariate.

		Latency to Strike		**No. of Strikes**		**No. of Captures**	
	df	**Wald**	**P**	**Wald**	**P**	**Wald**	**P**
Intercept	1	9.82	0.002	18.78	<0.001	11.17	<0.001
SVL	1	3.51	0.061	6.50	0.011	5.98	0.014
Treatment	5	12.14	0.033	7.54	0.183	5.00	0.416

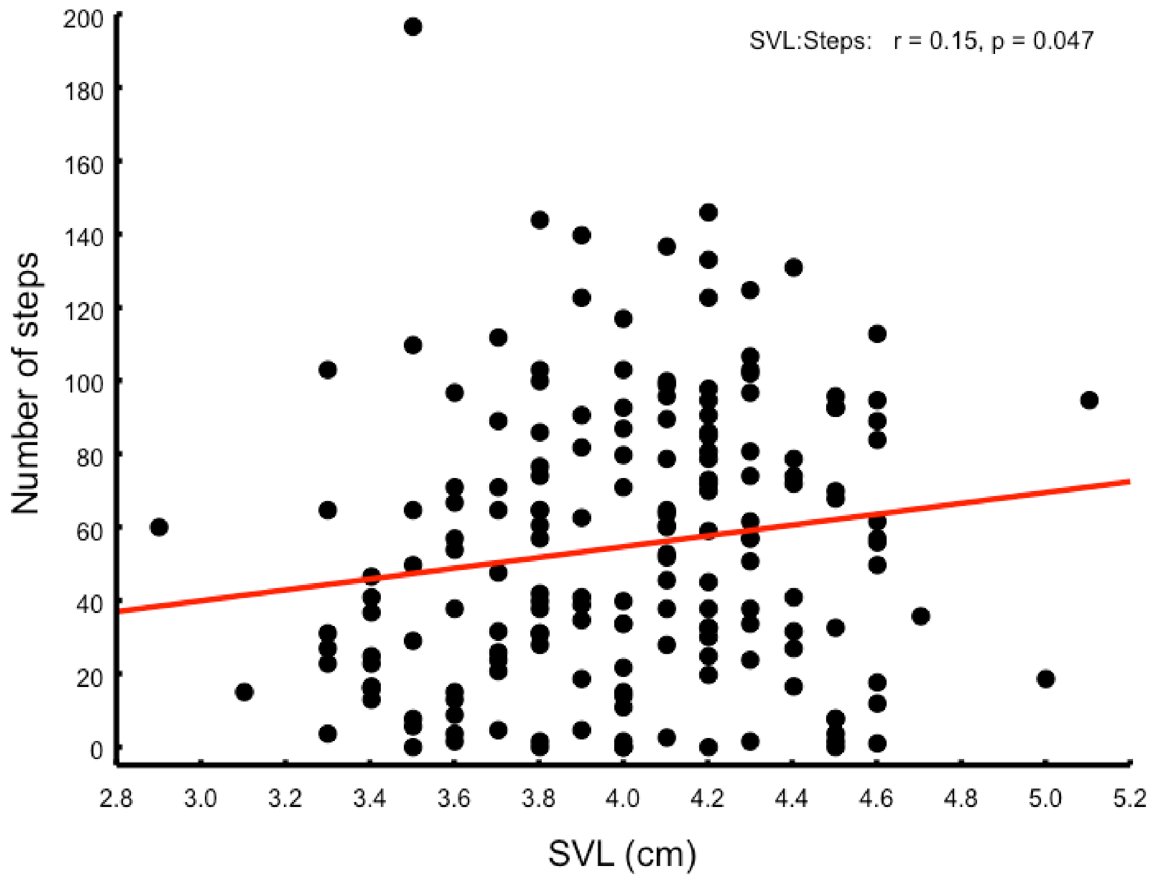

Figure 1. The positive relationship between the number of steps and snout-vent-length (SVL) in *D. ochrophaeus* using a Spearman's rank correlation (r = 0.15; p = 0.047).

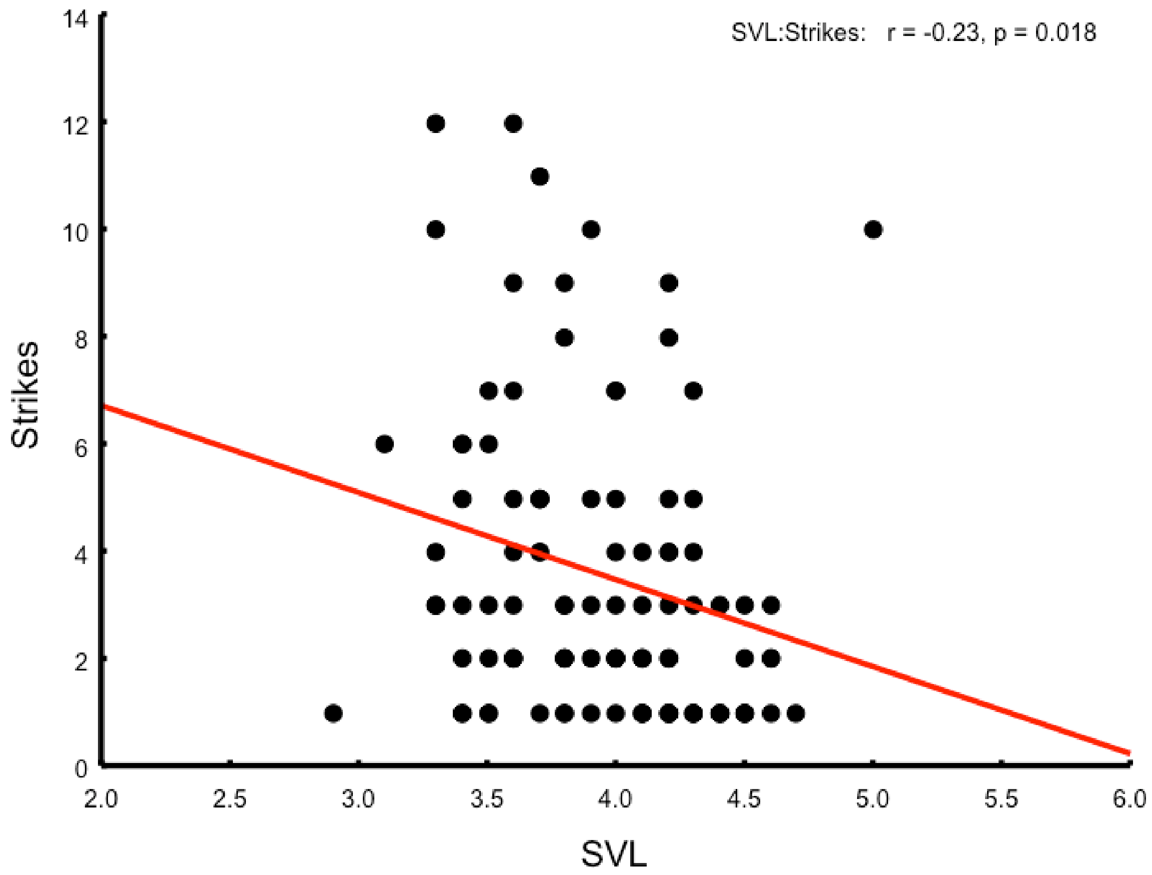

Figure 2. The negative correlation between the number of strikes at *Drosophila* prey and SVL in *D. ochrophaeus* using a Spearman’s rank correlation (r = -0.23; p = 0.018).

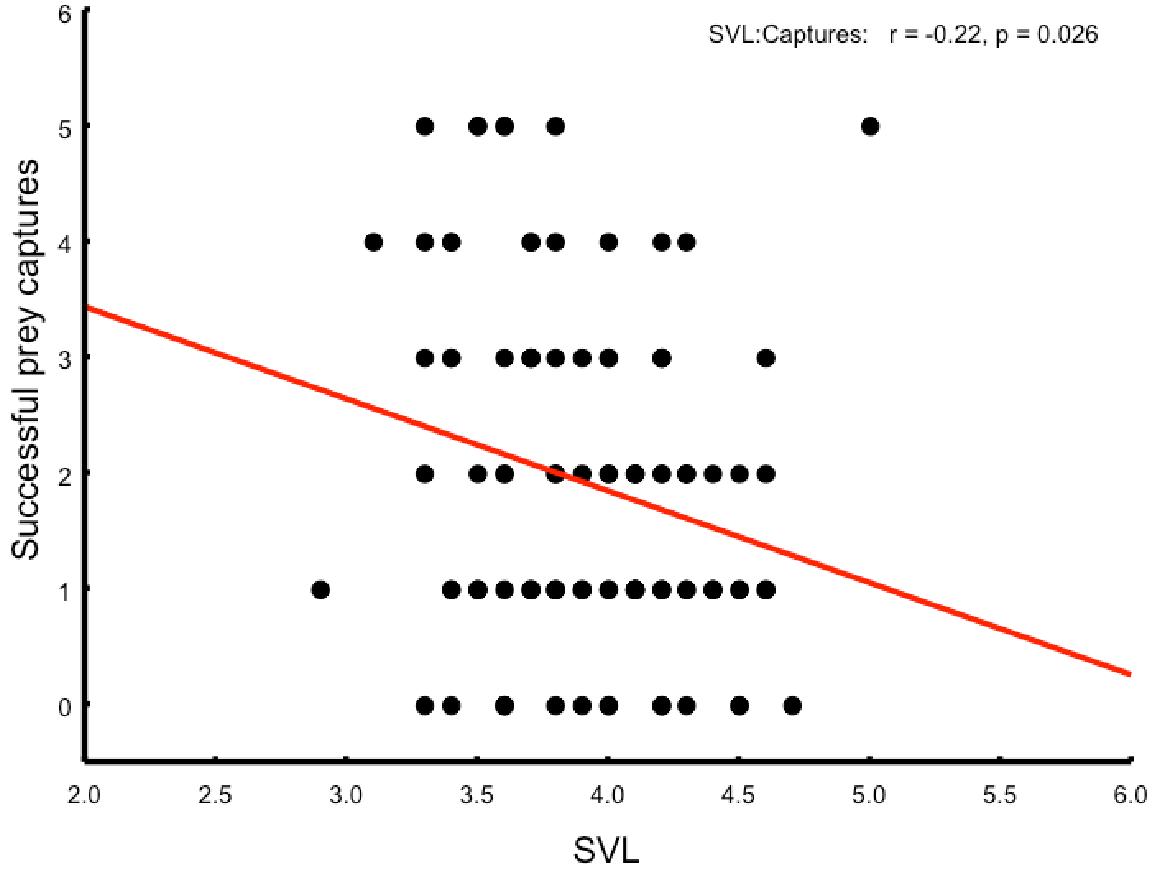

Figure 3. Use of Spearman's rank correlations to demonstrate the significant negative relationship between successful prey captures and SVL in *D. ochrophaeus* (r = -0.22; p = 0.026).

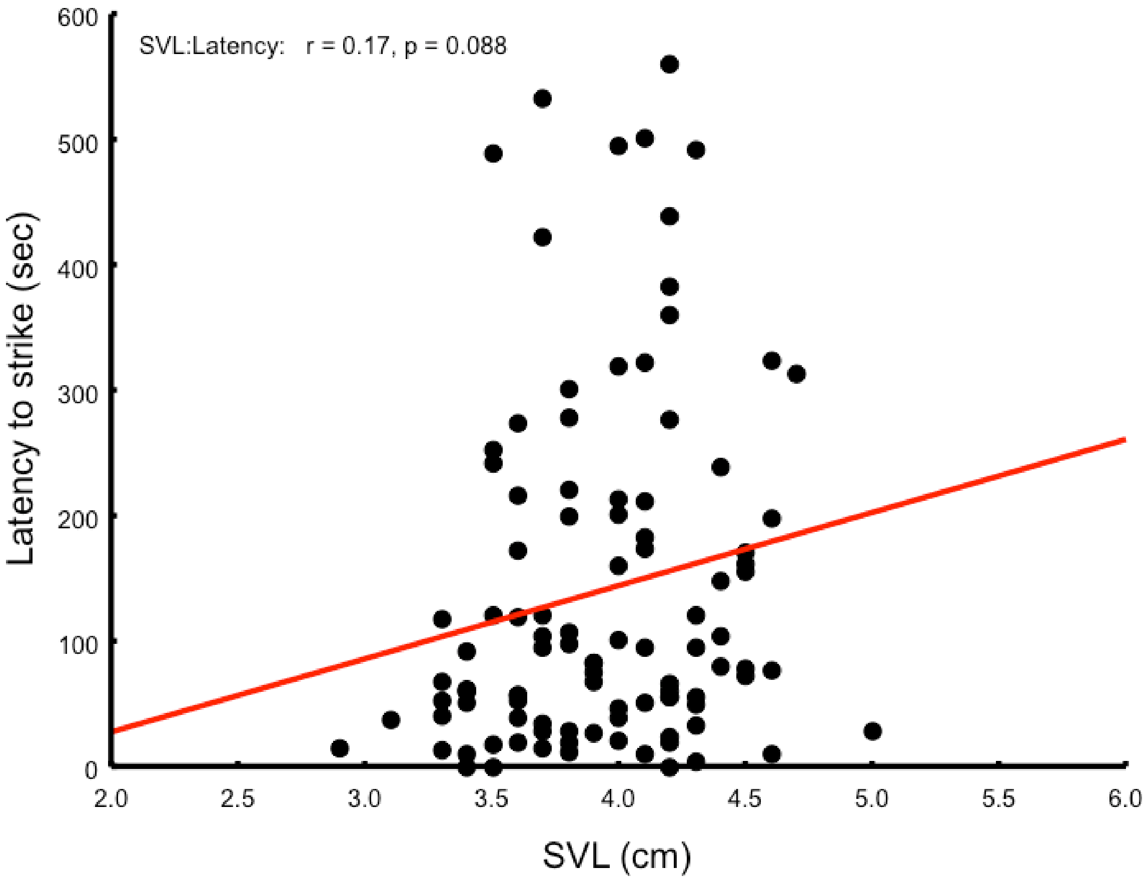

Figure 4. The partially significant positive correlation between the latency to strike (i.e., the time elapsed before the first strike at *Drosophila* prey) and SVL in *D. ochrophaeus* using a Spearman's rank correlation (r = 0.17; p = 0.088).

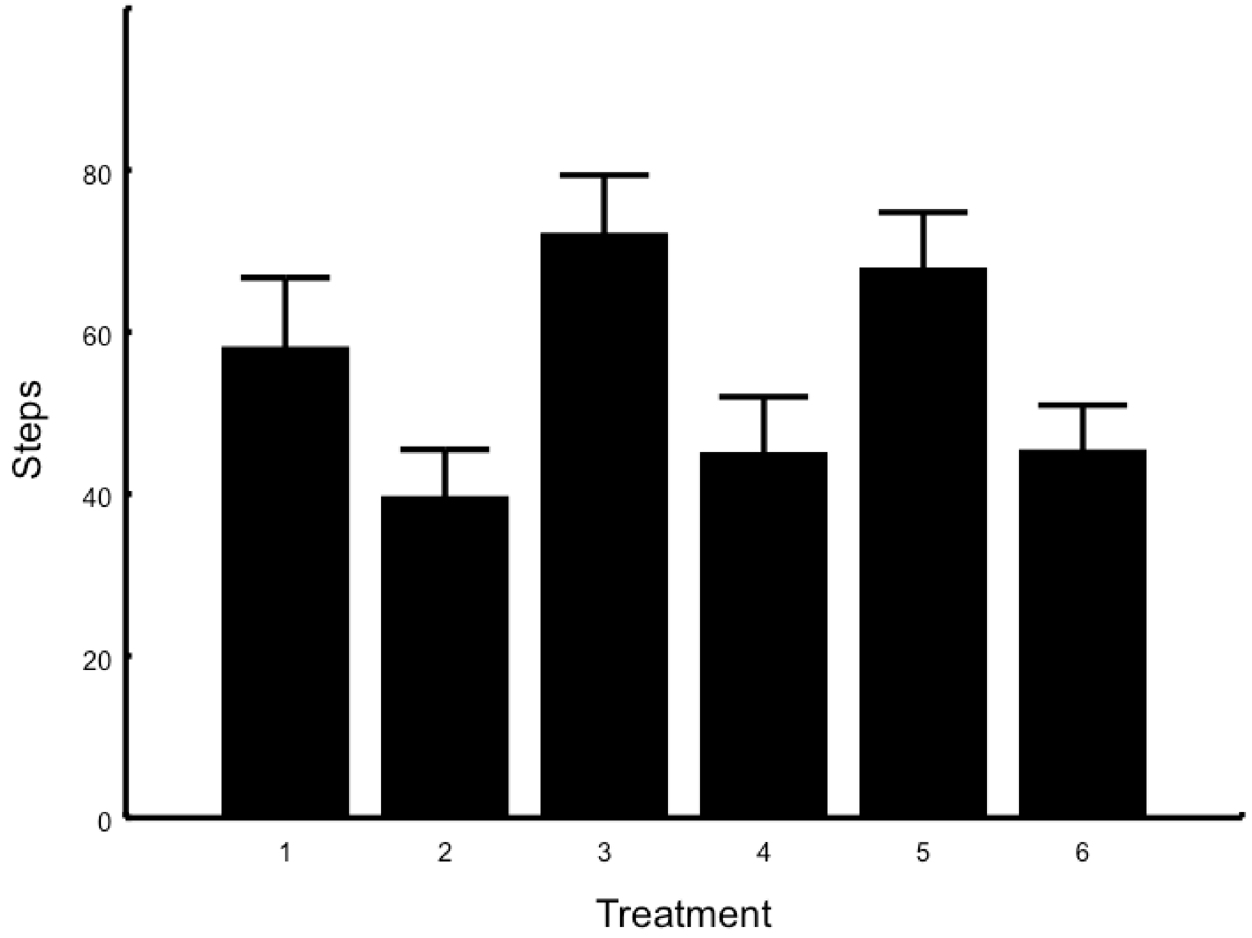

Figure 5. The main effect of treatment on the number of steps in *D. ochrophaeus* using ANCOVA in a generalized linear model analysis with size as a covariate. Treatments 1-3 are control substrates (DI water, *Opheodrys vernalis* stimuli, and damage-released cues respectively) and treatments 4-6 show a gradient of exposure time (48h, 96h, and 144h) to the paired chemical cues of *O. vernalis* stimuli and damage-released cues. A significant effect of treatment (p = 0.003), but no significant effect of SVL (p = 0.069) is observed.

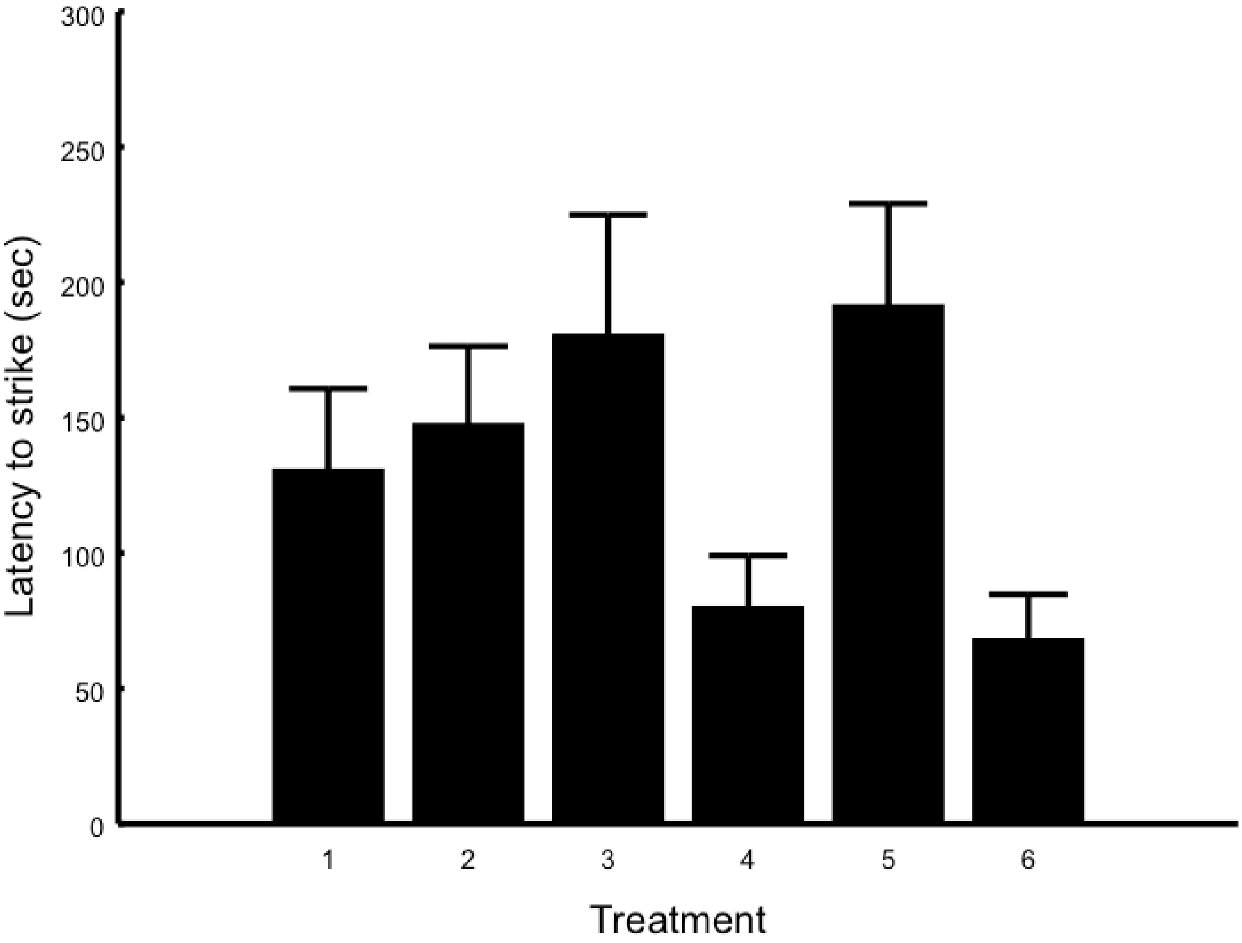

Figure 6. The main effect of treatment on the latency to strike at prey in *D. ochrophaeus* using ANCOVA in a generalized linear model analysis with size as a covariate. Treatments 1-3 are control substrates (DI water, *Opheodrys vernalis* stimuli, and damage-released cues respectively) and treatments 4-6 show a gradient of exposure time (48h, 96h, and 144h) to the paired chemical cues of *O. vernalis* stimuli and damage-released cues. Results showed no significant effect of SVL ($p = 0.061$), but a significant main effect of treatment on the latency to strike ($p = 0.033$).

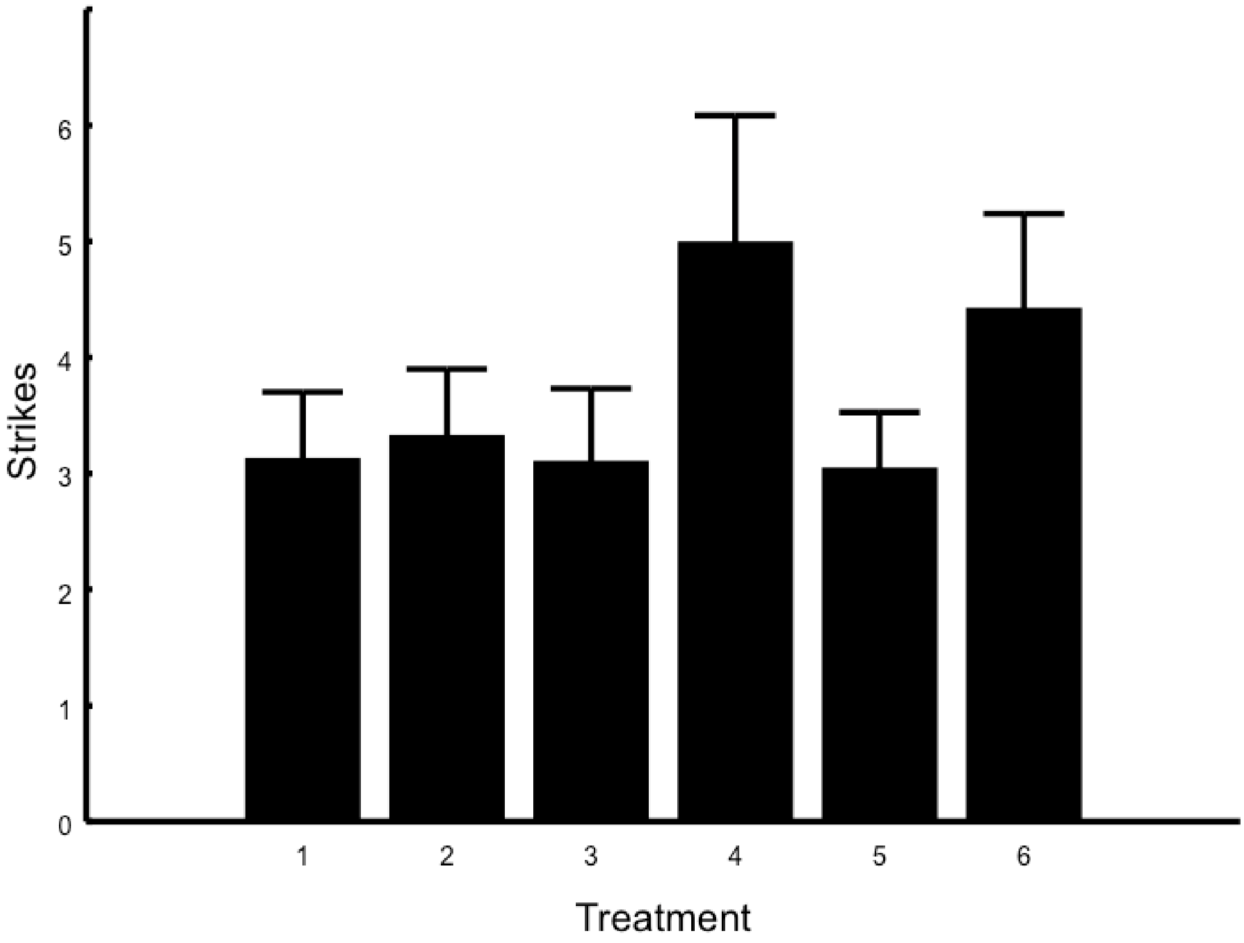

Figure 7. An ANCOVA in a generalized linear model analysis using size as a covariate to test the main effect of treatment on the number of strikes at prey in *D. ochrophaeus*. Treatments 1-3 are control substrates (DI water, *Opheodrys vernalis* stimuli, and damage-released cues respectively) and treatments 4-6 show a gradient of exposure time (48h, 96h, and 144h) to the paired chemical cues of *O. vernalis* stimuli and damage-released cues. No significant difference between treatment groups was observed (p = 0.183), but SVL showed a significant effect on the number of strikes (p = 0.011).

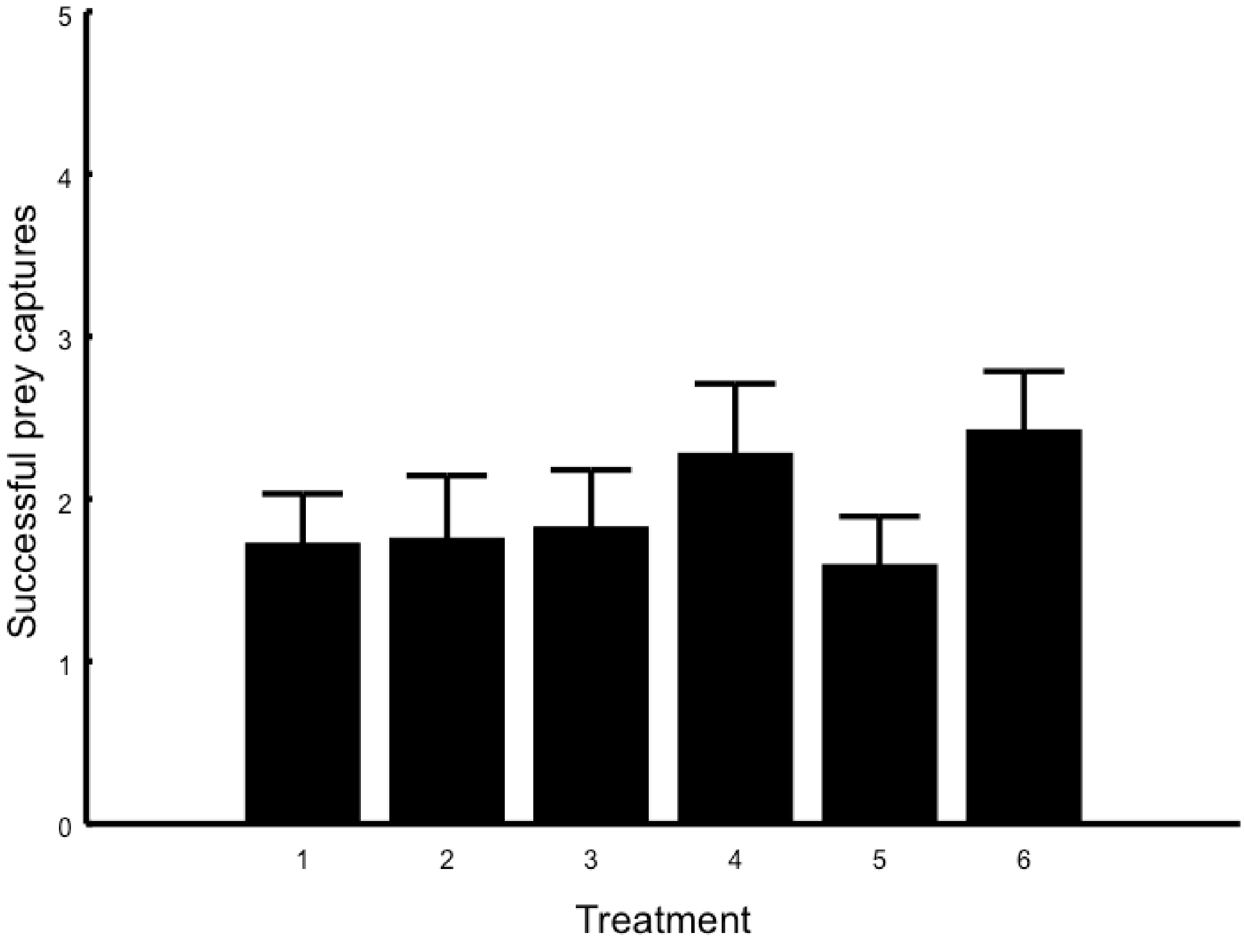

Figure 8. The main effect of treatment on the number of successful prey captures in *D. ochrophaeus* using ANCOVA in a generalized linear model analysis with size as a covariate. Treatments 1-3 are control substrates (DI water, *Opheodrys vernalis* stimuli, and damage-released cues respectively) and treatments 4-6 show a gradient of exposure time (48h, 96h, and 144h) to the paired chemical cues of *O. vernalis* stimuli and damage-released cues. Although treatment showed no main effect on the number of captures (p = 0.416), there was a significant covariate of size (SVL)(p = 0.014).