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Article

Paralyzing effects of CO_2 and hypothermia on Madagascar hissing and dubia cockroaches

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SUMMARY

Cockroaches are often used as experimental subjects in a diverse range of biological studies. It can be necessary to temporarily immobilize an individual cockroach for measuring or marking purposes; in such cases, hypothermia or CO_2 can serve as paralyzers. Immersion in cold water is a commonly used technique: a cockroach, when put into cold water, starts to lose the ability to move and respond to stimuli over time. However, this method requires a relatively long time to paralyze a cockroach and leads to a slow recovery. We aimed to find a more ethical and efficient way to temporarily paralyze a cockroach by comparing the results of two methods: one that uses hypothermia as a paralyzer and an analogous method that uses high concentrations of CO_2 instead. Cockroaches, like most insects, have a trachea system, which allows the CO_2 gas to easily penetrate their breathing system. Thus, changes caused by CO_2 manifest in an insect's body quicker than the effect of cold. We hypothesized that the paralyzing effect of CO_2 would be more time-efficient than that of hypothermia. Our results support this hypothesis given that the time required for immobilization using CO_2 was decreased compared to that using cold water. This research aims to contribute to the usages of laboratory animal ethics, especially cockroaches.

INTRODUCTION

Cockroaches are commonly used as laboratory animals due to their larger size compared to the majority of insects, intersection with daily human lives, high resistance to gel bait insecticides (including fipronil, clothianidin, indoxacarb, emamectin benzoate, and hydramethylnon), and easy maintenance (1-3). In particular, Madagascar hissing cockroaches (*Gromphadorhina portentosa*) and dubia cockroaches (*Blaptica dubia*) are species commonly cultivated by humans as food for larger exotic animals or pets. The large size of *G. portentosa* as well as the accessibility of purchasing *B. dubia* are often beneficial for many experiments.

Descriptive research requires larger testing samples for higher data accuracy. Experiments that use animals often require the preparation of each single insect for measuring, transporting, marking, or chemical application. The process of immobilization can be unnecessarily long-lasting and cause side effects, such as loss of orientation, that can be avoided. We investigated the time management and use of cockroaches in a wide range of biological research by assessing different immobilization methods: low temperature and CO_2.

One of the methods used to immobilize a cockroach is exposure to cold temperatures - around 0°C (4). Cold temperatures slow their biological processes, including the circulation of body liquids, which reduces the transportation of oxygen and results in decreased activity (4). Despite leading to full immobilization, this method can be time-consuming and cause temporary side effects, such as an inability to navigate the space, right themselves, and react to outer stimuli (5). These downsides motivate search of an analogous way to paralyze cockroaches. A previous study looked at paralyzing cockroaches with CO_2, which relied on decreased oxygen levels caused by the displacement of oxygen CO_2 (6). Cockroaches exposed to a 100% CO_2 environment became completely paralyzed for up to 3 days and required up to 5 days to regain the ability to normally move (6).

We hypothesized that CO_2 would induce paralysis faster than hypothermia in cockroaches. We tested this by exposing roaches to pure CO_2 environment and submerging them into ice-cold water, measuring the time it took for them to stop moving completely as well as the time until visible recovery from the state of immobilization. On average, the CO_2 method resulted in decreased time for immobilization as well as decreased recovery time. Future studies are needed to determine the long-lasting effects, the efficiency difference between two methods in different species and animals.

RESULTS

Time Required for Immobilization

We measured the time it took for full immobilization to occur when *G. portentosa* and *B. dubia* were exposed to 0.6°C water or a 100% CO_2 environment. When the cockroaches became unresponsive, we recorded the "time before full immobilization" and when they became completely still, we recorded the "immobilization time".

Immobilization of the *G. portentosa* cockroaches with cold water took 3:36 ± 1:13 minutes vs. 1:05 ± 0:28 minutes when CO_2 was used (n=15, **Table 1, 2**). The same hypothermia method on *B. dubia* took 3:56 ± 1.:35 minutes and the CO_2 method took 1:08 ± 0:28 minutes (n=10, **Table 3, 4**). While exposed to CO_2, both species showed a sudden increase in moving intensity, followed by rhythmical contractions and curling up on their backs. A comparison of the two methods showed that the CO_2 method's time required for immobilization was 69.6% less for *G. portentosa* and 71.2% less for *B. dubia* than the hypothermia method (**Figure 1**).

However, some specimens (2 out of 15 in the *G. portentosa* group, and 2 out of 10 in the *B. dubia* group) showed an increased resistance to CO_2 and did not fall under our requirements of complete immobilization. Because cockroaches with higher CO_2 resistance did not stop

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Exp №	Mass (g)	Time before full immobilization (m:s)	Recovery time (m:s)	Sex
1A	7.20	4:28	14:20	f
2A	6.27	2:51	15:50	m
3A	8.80	1:28	0:10	m
4A	7.50	5:38	10:45	f
5A	5.00	5:12	17:50	m
6A	8.13	2:56	10:22	f
7A	6.50	4:10	11:09	m
8A	7.45	1:54	13:12	f
9A	5.48	2:30	7:20	f
10A	4.90	5:05	5:50	f
11A	6.74	4:05	15:06	m
12A	8.03	3:33	12:20	m
13A	6.25	2:46	8:15	f
14A	7.23	3:19	6:40	f
15A	8.05	4:09	11:05	f
Average	**6.90**	**3:36**	**10:40**	
Standard deviation		**1:13**	**4:31**	
Average for f	**6.91**	**3:38**	**9:45**	
Average for m	**6.89**	**3:33**	**12:04**	

Table 1: Paralyzing effect of hypothermia on *Gromphadorhina portentosa*. Mass, time before full immobilization, recovery time, and sex for each *G. portentosa* cockroach submerged in 0.6 *°C water* (n=15). Average and standard deviation, along with average time by sex are also given.

Exp №	Mass g	Time before full immobilization m:s	Recovery time m:s	sex
1B	6.07	0:47	1:15	m
2B	6.06	1:32	1:35	f
3B	7.65	1:12	1:50	f
4B	5.93	1:09	0:50	f
5B	4.62	1:50	1:51	m
6B	7.91	0:33	0:36	m
7B	6.90	0:44	1:24	f
8B	5.23	0:49	3:44	m
9B	4.98	0:36	3:47	m
10B	7.27	1:04	1:02	f
11B	6.98	0:54	2:19	f
12B	4.88	0:57	3:09	m
13B	5.50	2:08	1:10	f
14B	7.70	-	-	f
15B	5.05	-	-	f
Average	**6.18**	**1:05**	**1:53**	
Average for f	**6.61**	**1:14**	**1:27**	
Average for m	**5.62**	**0:55**	**2:23**	
Standard deviation		**0:28**	**1:03**	

Table 2: Paralyzing effect of CO_2 on *Gromphadorhina portentosa*. Values stated in the table: mass, time before full immobilization, recovery time, sex, average values, standard deviation. Cockroaches were put in a 235-mL glass tank with a 100 % CO_2 environment at room temperature (21 *°C*) (n=15). Specimen with CO_2 resistance were not included in average time calculations due to and inability to record complete immobilization. g – grams; m:s – minutes:seconds; f – female; m – male.

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Exp №	Mass g	Time before full immobilization m:s	Recovery time m:s	sex
1C	2.09	2:00	2:10	f
2C	1.77	3:10	6:30	f
3C	2.60	4:40	6:50	f
4C	1.61	3:12	8:15	f
5C	1.65	3:11	4:20	f
6C	1.80	3:52	6:58	m
7C	1.50	6:13	5:13	f
8C	1.90	7:04	3:10	m
9C	2.40	3:15	9:08	f
10C	1.40	2:50	3:43	m
Average	**1.87**	**3:56**	**5:37**	
Average for f	**1.95**	**3:40**	**6:03**	
Average for m	**1.70**	**4:35**	**4:37**	
Standard deviation		**1:35**	**2:16**	

Table 3: Paralyzing effect of hypothermia on *Blaptica dubia*. Values stated in the table: mass, time before full immobilization, recovery time, sex, average values, standard deviation. Cockroaches were submerged in 0.6 °*C water* (n=10). g – grams; m:s – minutes:seconds; f – female; m – male.

Exp №	Mass g	Time before full immobilization m:s	Recovery time m:s	sex
1D	1.55	1:27	2:23	f
2D	1.8	0:53	0:30	f
3D	1.14	2:00	0:59	f
4D	1.86	1:31	1:10	m
5D	1.78	0:56	0:25	m
6D	1.59	1:02	3:00	f
7D	1.32	0:38	0:45	m
8D	1.24	0:39	0:20	f
9D	1.8	-	-	f
10D	1.55	-	-	f
Average	**1.54**	**1:08**	**1:11**	
Average for f	**1.46**	**1:12**	**1:26**	
Average for m	**1.65**	**1:01**	**0:46**	
Standard deviation		**0:28**	**0:58**	

Table 4: Paralyzing effect of CO_2 on *Blaptica dubia*. Values stated in the table: mass, time before full immobilization, recovery time, sex, average values, standard deviation. Cockroaches were put in a 235-mL glass tank with a 100 % CO_2 environment at room temperature (21 °*C*) (n=10). Specimen with CO_2 resistance were not included in average time calculations due to and inability to record complete immobilization. g – grams; m:s – minutes:seconds; f – female; m – male.

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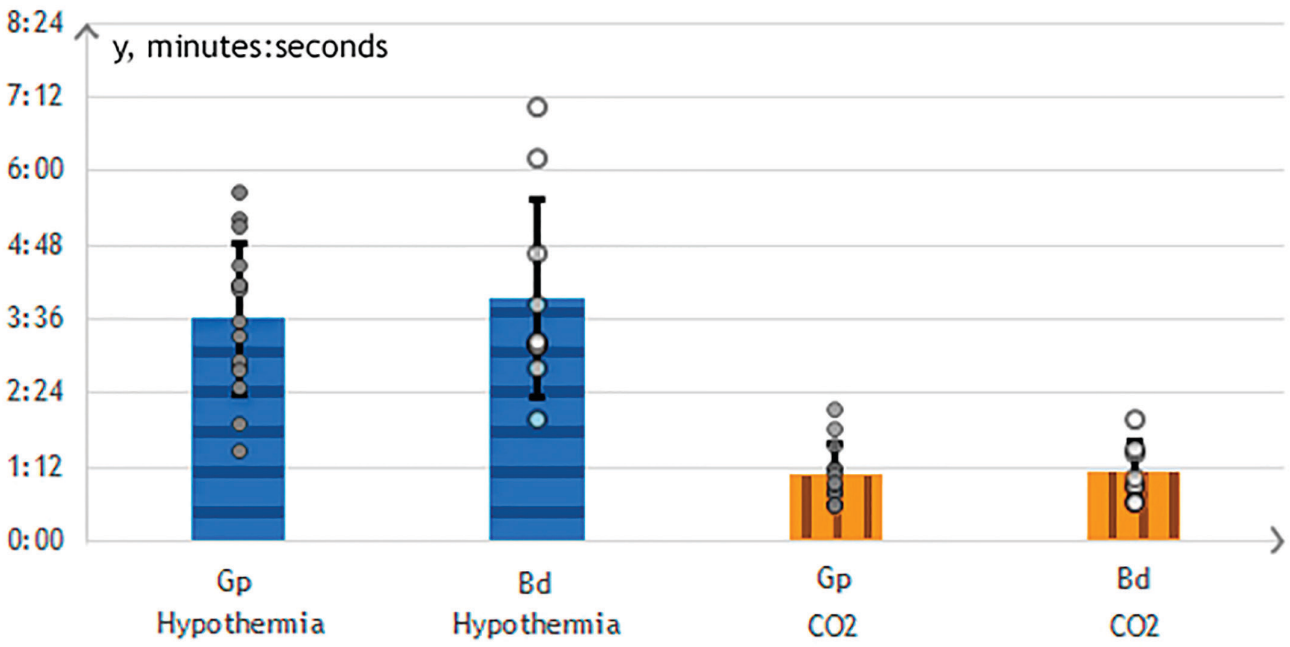

Figure 1: Time required for full immobilization with CO_2 and hypothermia. *Gromphadorhina portentosa* (Gp; n=15) and *Blaptica dubia* (Bd, n = 10) cockroaches were submerged in approximately 0.6 °*C water then later exposed to a 100%* CO_2 environment on a different day. The time it took cockroaches to reach full immobilization (e.g., no response to external stimuli) was recorded. CO_2 resistant specimen not included. Data shown as mean ± SD, with individual data points shown.

contracting even after 5 minutes in a 100% CO_2 environment, it was not possible to precisely measure the time before full immobilization and, therefore, recovery time. Thus, they were not included in the average period calculations.

Recovery Time

Recovery time refers to the time frame from the extraction of the cockroach from the CO_2 tank or 0.6°C water tank to the restoration of its movement abilities, which includes responsiveness to light or touch and an ability to firmly hold onto the surface (**Figure 2**). The recovery time for *G. portentosa* was 10:40 ±4:31 minutes after hypothermia and 1:53 ± 1:03 minutes after CO_2 (n=15, **Table 1, 2**). The recovery time for *B. dubia* was 5:37 ± 2:16 minutes after hypothermia and 1:11 ± 0:58 minutes after CO_2 (n=10, **Table 3, 4**). The periods of recovery with the CO_2 method were decreased by 8:47 minutes (80.7%) for *G. portentosa* and 4:26 minutes (82.3%) for *B. dubia* was on average.

Deviation Between Sexes and Species

We also looked at any sex-specific differences by comparing male vs. female time required for immobilization. We saw that CO_2 method required about 8-10% more time for immobilization in male cockroaches in both species (**Table 5**). However, no sex-specific difference in recovery time was seen for either species.

Additionally, we looked at the impact of weight, as *G. portentosa* cockroaches used in the experiment averaged 6.54 grams and *B. dubia* averaged 1.71 grams (**Table 1-4**). Considering the results, the weight of a cockroach did not appear to be a crucial factor for the immobilization time. However, the recovery time was shorter for the *B. dubia* species, which are significantly lighter compared to *G. portentosa* (**Table 6**). To better understand the results, a two-tailed t-test has been performed for all four groups (**Table 6**).

DISCUSSION

Our results indicate that immobilizing *G. portentosa* and *B. dubia* cockroaches using CO_2 as a paralyzer is more time-efficient than using low temperatures. In some experiments, longer but not complete immobilization can benefit scientists. For example, longer immobilization would be helpful when wanting to showcase an insect's anatomy or to take precise measurements of the insect. However, in many cases a long-lasting immobilization effect is not required such as when marking, weighting, or amputating. The side effects (loss of special orientation, slowness, and unresponsiveness to light) should be considered when choosing an immobilization method and a short-term immobilization performed whenever possible.

The data collected showed a decrease in the time required for immobilization using CO_2. The average decrease for both species was 70.4% in the time until full immobilization and 80.6% in recovery time. The average recovery period decreased by 82.3% for *G. portentosa* when using the CO_2 method instead of hypothermia (**Table 6**). The analogous decrease for *B. dubia* was 78.8%, which reduced the period of the cockroaches' contractions and unresponsiveness to outer stimuli, observed during recovery time (**Table 6**).

Despite the overall higher efficiency, it is important to mention that the CO_2 method had exceptions. As mentioned, 2 out of 15 cockroaches in the *G. portentosa* group and 2 out of 10 cockroaches in the *B. dubia* group did not reach full immobilization. Since the full immobilization was not observed, the time of recovery could not be noted either.

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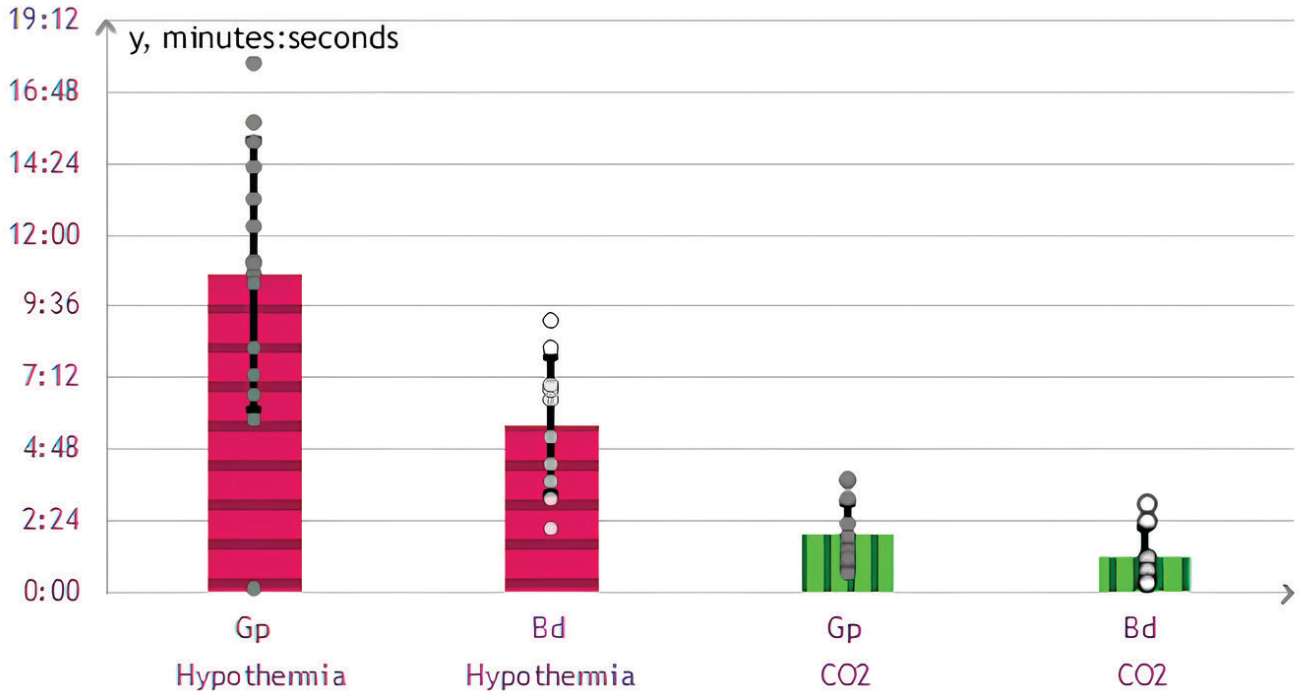

Figure 2: Recovery time for CO_2 and hypothermia. *Gromphadorhina portentosa* (Gp, n=15) and *Blaptica dubia* (Bd, n=10) cockroaches were submerged in approximately 0.6 °C water then later exposed to a 100 % CO_2 environment on a different day. CO_2 resistant specimen not included. The time between the moment of full immobilization and visible recovery of cockroaches from immobile state (e.g., response to touch, ability to move) was recorded. Data shown as mean ± SD, with individual data points shown.

Despite this, cockroaches with increased resistance were affected by CO_2 after the average immobilization time. Their movement activity greatly decreased, and the cockroaches did not respond to stimuli, such as bright light or touch. Even though this uncertainty in measuring makes consideration of these results ambiguous, an observed short-term decrease in movement can still be used for the same purposes as the short-term full immobilization. The reasons behind the resistance to CO_2 are still unknown and require further investigation in future experiments.

During the experiments, we observed other interesting behaviors. After the recovery time from exposure to ice water had been noted, cockroaches were still significantly less active and did not avoid light for periods of up to several hours. These behaviors were not observed before the exposure to hypothermia in both groups. These behaviors might be a consequence of the paralyzing process and could indicate a longer period of nervous system paralysis or some other unpredicted behavior.

Another observation made during the experiments with CO_2 was that cockroaches, specifically *G. portentosa*, tended to defecate when exposed to high levels of CO_2. Such behavior was only observed after the exposure to CO_2 and not the cold. This might indicate an attempt of the insect's body to get rid of the toxic gas or a high level of stress; however, such conclusion requires more research.

One item that may have impacted our results is that the weight of cockroaches from the same groups varied due to the measuring error caused by the excessive water on the insects after the immersion in ice water. In addition, experiments were conducted on different dates over two months. However, it is worth mentioning that the research shows a great advantage of the CO_2 paralyzing method in terms of time, but the long-lasting effect on cockroaches' body, nervous system, and reproductive system is yet to be observed (**Figure 1, 2**). Discovering more time-efficient techniques for working with lab insects makes it easier for researchers to manage their time and focus on what is important. It also reduces the time

	Time required for full immobilization m:s:ms		Recovery time m:s; sidems	
Female Gp	2:24	**65.78%**	8:18	**85.12%**
Male Gp	2:38	**74.04%**	9:41	**80.16%**
Female Bd	2:28	**67.20%**	4:37	**76.25%**
Male Bd	3:34	**77.60%**	3:50	**83.15%**

Table 5: Reduced immobilization time for different sexes and species with the use of CO_2 as a paralyzer instead of hypothermia (in minutes and percentage). m:s – minutes:seconds; Gp – *Gromphadorhina portentosa*; Bd – *Blaptica dubia*.

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	Gromphadorhina portentosa				Blaptica dubia			
	Time before full immobilization m:s		Recovery time m:s %		Time before full immobilization m:s		Recovery time m:s	
Hypothermia	3:36		10:40		3:56		5:37	
CO_2	1:05		1:53		1:08		1:11	
Reduced time	2:30	**69.59%**	8:47	**82.33%**	2:48	**71.17%**	4:26	**78.83%**
two-tail t-test	2.29929E-07		3.05827E-07		0.000194046		0.000103352	

Table 6: Average values of time reduced with the use of CO_2 method instead of hypothermia in minutes and percentage. Average time before full immobilization and recovery time for *Gromphadorhina portentosa* cockroaches (n=15) and *Blaptica dubia cockroaches (n=10)* after submersion in 0.6 °*C* and exposure to 100 % CO_2 environment; time reduced with the use of CO_2 method in minutes and percentage; a two-tailed *t*-test comparing hypothermia and CO_2 methods. m:s – minutes: seconds

frame of animal suffering the effects of immobilization, making the process relatively less stressful for the cockroaches.

MATERIALS AND METHODS

Hypothermia Method

Cockroaches (*G. portentosa* and *B. dubia*) were obtained in a pet store and selected at random. Each cockroach was exposed to both hypothermia and CO_2 methods. One by one, cockroaches were submerged in ice water at a temperature of 0.6°C (measured with Digital Thermometer TP-101) until full immobilization.

CO_2 Method

For the CO_2 method, two glass tanks were connected with a silicone tube (5 mm in diameter). One of the tanks was filled with 100% baking soda ($NaHCO_3$) and a 10% vinegar (CH_3COOH) solution in the proportion of 3.2 g/28.5 mL. A cockroach was placed in the second 235-mL tank. Right after immobilization, each insect was weighed on high-precision scales (MH-500 Pocket Scale). Then cockroaches were left at room temperature 21 °C (measured with Digital Thermometer TP-101) until they were able to tightly grab onto the surface and move freely.

The time of complete immobilization was noted when no visible movement was detected and a cockroach did not respond to light or touch. The recovery time was noted when the cockroaches regained the ability to move freely and respond to tactile stimuli by grabbing on an object. The time of full immobilization, recovery time, weight and sex were noted and compiled into the tables. Cockroaches with increased CO_2 resistance were excluded from the mean calculation due to the lack of data on complete immobilization and recovery time.

Statistical Analysis

A two-tailed *t*-test was performed including recovery/immobilization time using hypothermia and recovery/immobilization time using gas (a=0.05). Standard deviation was also added to better see the deviation from average time values. Excel 2016 was used to do all the calculation in this article.

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REFERENCES

1. Sellner, Michael, and Wicht, Jordan. "How many American Homes have Pests?". United States® Census Bureau. April 21, 2021. Accessed August 3, 2024. census.gov/library/stories/2021/04/how-many-american-homes-have-pests.html
2. Lee, Shao-Hung, *et al.* "Reduced Susceptibility Towards Commercial Bait Insecticides in Field German Cockroach (*Blattodea: Ectobiidae*) Populations From California." *Journal of Economic Entomology*, vol. 115, no. 1, Feb. 2022, pp. 259–265. https://doi.org/10.1093/jee/toab244.
3. "Cockroaches – pest control". Health.vic. Accessed July 29, 2024.health.vic.gov.au/environmental-health/cockroaches-pest-control
4. HarvardX. "Fundamentals of Neuroscience, Part 1: The Electrical Properties of the Neuron". MCB80.1x, DIY Lab, "Recording and Stimulating a Nerve". Accessed July 30, 2024. harvardonline.harvard.edu/course/fundamentals-neuroscience-part-1-electrical-properties-neuron
5. Bradt, David, *et al.* "American Cockroach Response to Cold Temperatures" *BioOne: Society of Southwestern Entomologists,* vol. 43, no. 2, June, 2018, pp. 335-342, https://doi.org/10.3958/059.043.0205.
6. Gannon, B, *et al.* "Effect of carbon dioxide on the Oriental *cockroach Blatta orientalis*." *Medical and Veterinary Entomology,* vol. 15, no. 1, 2001, pp. 68-72, https://doi.org/10.1046/j.1365-2915.2001.00277.x.