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TECHNICAL NOTE

How to take an ant's pulse: a procedure for non-destructively monitoring baseline and stimulated heart rate in Formicidae

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Abstract

Ants, like other insects, have a heart that pumps hemolymph rhythmically. We designed an apparatus and procedure to non-destructively monitor the rate of cardiac contractions in ants, using a modified light microscope and infrared light. This allowed us to obtain the first baseline heart rate data on three ant species, collected in Georgia, USA. We next describe how ant heart rates can be stimulated, for comparing cardiac reactions among colonies, or for assessing stress responses. The procedures described here may be useful for other researchers interested in ant physiology.

Introduction

Small animals, by their diminutive nature, pose intrinsic challenges to certain forms of scientific investigation, especially regarding physiological experimentation or measurement. This limits the breadth of research questions and scientific opportunities within certain taxonomic groups. One useful physiological metric is the measurement of cardiac output, or heart rate. In many small model invertebrates, scientists have successfully developed specialized tools and applications to monitor and record these contractions (Présing & Véró, 1983; Depledge & Andersen, 1990; Schapker et al., 2002; Calosi et al., 2003; Bradu et al., 2009; Cooper et al., 2009; Fink et al., 2009; Davis, 2020). The development of these approaches has provided information on invertebrate physiological responses to a variety of anthropogenic or natural stimuli. For example, cardiac output in marine mussels becomes elevated when the animals are physically attacked by a predator (Rovero et al., 1999). Heart rate irregularities were observed in snails during physical jostling by researchers (Renwrantz & Spielvogel, 2011). Early researchers showed how insecticides tend to depress heart rates in cockroaches (Orser & Brown, 1951). Cardiac reactions to stimulus can be used to monitor the effects of pollutants in marine bivalves (Kholodkevich et al., 2017). Heart rates can be used to estimate the energetic cost of fighting in crabs (Rovero et al., 2000). And finally, heart rates of naïve monarch caterpillars rise when exposed to loud traffic noise (Davis et al., 2018). Collectively, this small but growing body of work highlights how studies of cardiac function in small invertebrates can advance scientific knowledge across a variety of disciplines, including animal behavior, ecotoxicology, and physiological ecology.

Here, we describe a low-cost procedure that allows for non-destructive monitoring of heart rate (HR) in small ants (2–3 mm). Ants, like other invertebrates, have a central blood vessel under the dorsal surface that pumps hemolymph, and serves as the heart. Our study details how the pumping of this vessel can be visualized in real-time on live ants. In addition, we show a procedure for instigating a controlled physical stimulation which can allow for comparisons of the magnitude of HR change, and/or how long the recovery period is.

Description of microscopy and heart rate measurement

The procedure can be conducted with any older style light microscope that uses a mirror to direct light through the stage, plus any low-cost microscope camera (fitted either to the ocular or to a trinocular head of the microscope) that can be easily taken apart. Also needed is a battery-operated infrared flashlight, glass microscope slides, and

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some clear packing tape. The procedure requires that ants be first secured (live) on a standard microscope slide, so that they remain immobile throughout the measurement. A simple and low-cost approach is to use strips of clear packing tape, which hold down the animals, yet allow for their visualization. This approach has the advantage of being rapid, which allows measurement of ants soon after human contact. If done gently, it is non-lethal, and the ant can be released if needed for future experiments. Here, the ants in question – *Solenopsis invicta* Buren (fire ant), *Linepithema humile* (Mayr) (Argentine ant), and *Prenolepis imparis* (Say) (winter ant), three species of locally common ants in north Georgia, USA – were kept in Petri dishes next to the observer. The following steps describe the procedure. The observer lets one ant out on the table, then using a strip of tape, gently presses the tape to the ant to pick it up. The tape and ant are pressed to a microscope slide (without pressing on the ant, Figure 1A), so the ant is rendered immobile on the slide. The slide is placed on the stage of a microscope for viewing the heart (below). With practice this first step can be completed within 10 s, so the first HR reading can be obtained within 20 s of contacting the ant. A video of this step is in the online supplemental material.

On the microscope we view the ant using a 4× or 10× objective (depending on the size of the species), and use the stage XY positioning controls to focus on the dorsal surface of the gaster (Figure 1B). In insects and other invertebrates, the dorsal blood vessel, which runs longitudinally within the body, acts as the 'heart', pumping

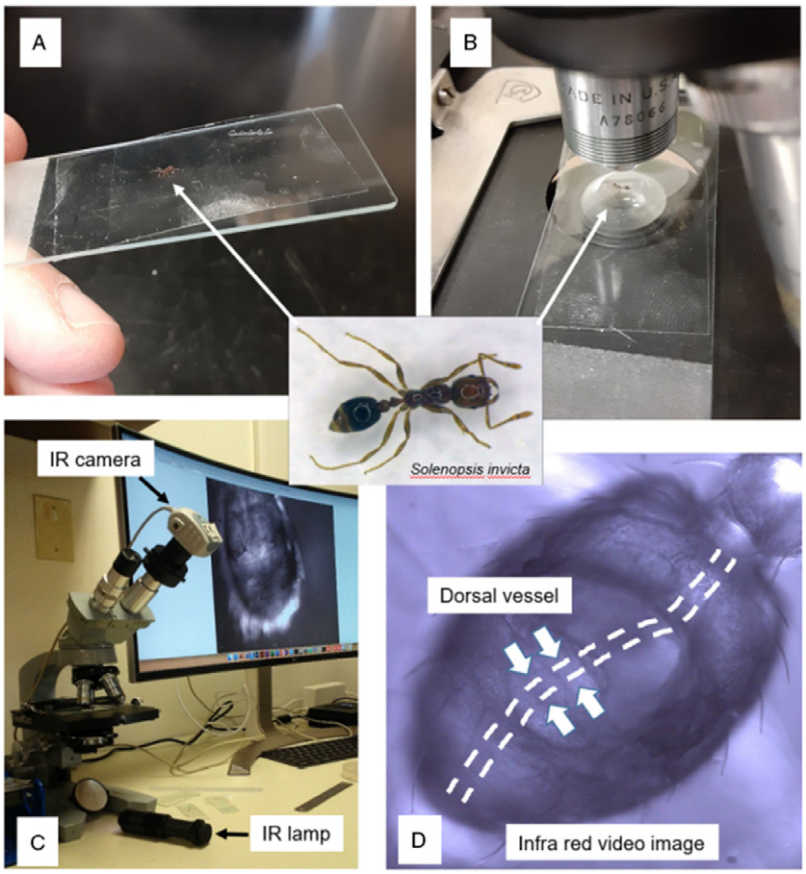

Figure 1 Photographs of the procedure for examining dorsal vessel (heart) contractions of ants. (A) The ant (*Solenopsis invicta* pictured) is 'taped' to a microscope slide with transparent tape. This maintains the ant motionless (center image), which is necessary to view the vessel contractions. (B) The slide is placed under 40× magnification of a light microscope. (C) A modified digital camera (with the infrared filter removed) mounts to the microscope. Light from an infrared lamp (IR flashlight) is directed upward through the ant via a mirror. (D) After adjusting the exposure of the video software, carefully positioning the light beam, and adjusting the focus, the user can see interior structures of the gaster, including the dorsal vessel contractions. When finished, the tape is removed and the ant released. Videos of the procedure are provided in supplemental files. [Colour figure can be viewed at wileyonlinelibrary.com]

rhythmically to distribute hemolymph to body regions and/or muscle tissues (Jones, 1964). Although this vessel runs most of the length of the ant, its pumping is most pronounced in the gaster.

We use an older model microscope with a simple adjustable mirror under the condenser (Figure 1C), which allows us to shine an infrared lamp (an IR 'flashlight') upward through the ant. Infrared lighting provides a superior view of the interior of the gaster, and it even allows for internal-imaging of darker colored ants. Near-infrared lighting has also been used to study the internal development of fly pupae (Moran & Parker, 2016). Since infrared cannot be seen with the naked eye, the microscope is equipped with a digital microscope camera (Motic Moticam, San Antonio, TX, USA), which has had its internal infrared filter removed. Such filters are standard internal components of most cameras, but can be removed by disassembling the camera; the filter is typically a thin, translucent plastic panel (red in appearance) that covers the lens (AK Davis, pers. obs.). Once removed, and the camera reassembled, the camera can detect infrared light. Using this 'IR' camera, and by fine-tuning the contrast settings of the camera software, the real-time image of the *interior* of the ant gaster can be viewed on a computer monitor (Figure 1D), almost like an x-ray image. A video of dorsal vessel contractions in *S. invicta* is available in the supplemental material.

Once the measurement of heart rate is complete, the tape can be removed from the glass slide and the ant pulled off (with soft forceps). Researchers may need to experiment with different brands of tape to find one with the right amount of stickiness, and that does not damage the ants. For some species with especially soft bodies, it may not be possible to recover the ant without harm.

Cardiac reactions to the procedure

Whereas the procedure described is effective for allowing real-time visualization of cardiac movement in ants, an obvious question is whether the ant heart rate is affected by the handling and manipulations of the animals. With this in mind, we recorded the time course of cardiac responses (to the procedure itself) of *S. invicta*, *L. humile*, and *P. imparis* (Figure 2). One author examined 10 or 11 individuals of each, which had all been collected on university grounds near our laboratory in Athens, GA (or on our office floor, in the case of the Argentine ants). They were brought to the laboratory and maintained in Petri dishes on the day of testing. Here we specifically sought to document the cardiac changes (if any) that might occur over the first 5 min of this procedure, and whether these reactions differ between species. At the beginning of the

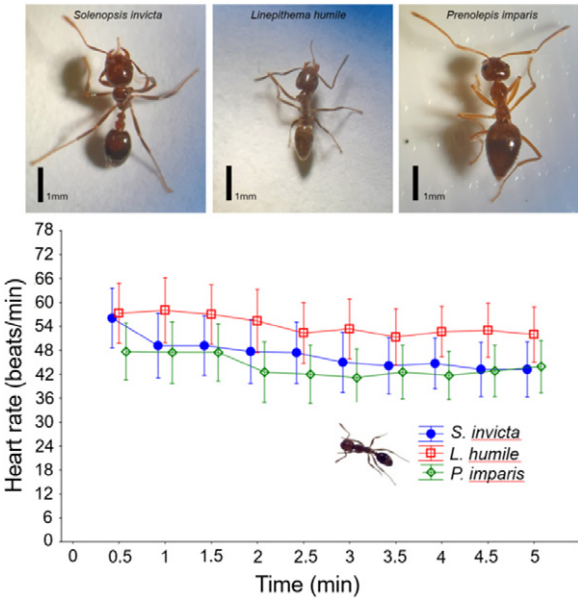

Figure 2 Average (+ 95% confidence intervals) heart rates of three ant species at 30-s intervals during 5 min of monitoring using the infrared procedure. For these trials we evaluated *Solenopsis invicta* (n = 10), *Linepithema humile* (n = 10), and *Prenolepis imparis* (n = 11). Ants had been collected earlier during the day and held in plastic containers with their colony members. A goal here was to monitor how the ants 'react' to the procedure, including the taping and restraint. Heart rate readings all started within 20 s of initial contact with the ant (removal from their container with forceps). [Colour figure can be viewed at wileyonlinelibrary.com]

trial, each ant was removed from its Petri dish and quickly fixed to the microscope slide, then viewed on our microscope immediately to record HR (i.e., all within 20 s of contact). Then, we recorded HR at 30-s intervals for the next 5 min while the ant remained in place.

The data obtained from these trials (heart rates of 31 ants, measured 10× per ant) were approximately normally distributed based on visual inspection of histograms. We used repeated-measures ANOVA to compare HR between species and over the 5-min time period. The average initial HR of all 31 ants was 53.5 beats per min (Table 1). Heart rates did not differ between species ($F_{2,28} = 2.77$, $P = 0.079$), although winter ants tended to have a low HR, whereas HR of Argentine ants tended to be higher. There was significant variation in HR over time ($F_{18,252} = 8.98$, $P<0.0001$). All three ant species displayed a similar pattern of gradual decline in contraction frequency (Figure 2). The magnitude of the decline depended on the species; contraction frequency of fire ants declined by 23% (Table 1), whereas the other species displayed more moderate declines under 10%.

Table 1 Summary of heart rate (HR) measurements of three ant species, at the initial measurement (within seconds of initial contact), and after 5 min of being immobilized on a microscope slide

Species	n	Mean (± SD) initial HR (no. beats/min)	Mean (± SD) HR after 5 min	% change
Solenopsis invicta	10	56.1 ± 11.4	43.2 ± 9.2	−23.0
Linepithema humile	10	57.3 ± 10.1	52.0 ± 12.3	−9.3
Prenolepis imparis	11	47.7 ± 12.8	43.9 ± 10.2	−8.0
All three species	31	53.5 ± 12.0	46.3 ± 11.0	−13.5

To ensure that the declining HRs were not due to the ants suffocating (or otherwise being harmed), we conducted a longer term trial, using a separate collection of fire ants. Ten additionally collected specimens were examined for 90 min, using the procedure above to measure HR (immobilization, microscope viewing). One of us recorded the HR of each ant at 10-min intervals. From the pattern obtained (Figure 3), it is clear that after an initial decline in HR over the first 20 min, a steady recovery follows. Importantly, the hearts all continued to beat for the duration of the 90-min trials. This fact alone indicates that the procedure itself is not harmful (though it is affecting the HR).

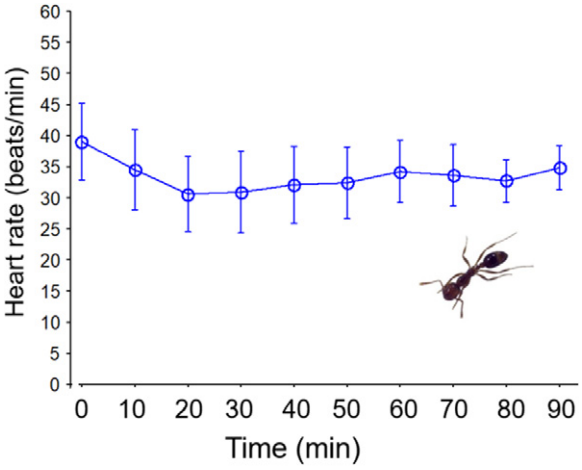

Figure 3 F Average (+ 95% confidence intervals; n = 10) heart rates of *Solenopsis invicta* at 10-min intervals during 90 min of monitoring, to determine whether ants are harmed (suffocated) by being restrained on a microscope slide. All ants survived the procedure. [Colour figure can be viewed at wileyonlinelibrary.com]

Mechanical agitation procedure

As a secondary part of this project, we sought to evaluate how this heart rate monitoring procedure could be used to track rapid changes in HR, such as might occur after forced physical activity. We designed a simple apparatus that allowed for standardized 'physical agitation' of individual ants (Figure 4). This device consists of an open-faced plastic container attached to a slowly rotating mechanical drum (rotating at ca. 20 rpm), in which the insect is placed for a standardized amount of time. Similar rotating drums have been successfully employed for assessing acute physiological changes in other insects, as it causes jostling and physical agitation (Davenport & Evans, 1984; Davis et al., 2017). Note, however, that this device and 'agitation' procedure is useful because of its applicability and repeatability at instigating a controlled physiological reaction, but it does not replicate any known scenario in nature that ants would face.

With the device used here, a single ant can be placed in an Eppendorf tube (or any small container) and the container is then deposited in the tumbler. The tumbler device is positioned at an angle so the 'ant container' does not fall out during the rotating. We exposed ants to this stimulus

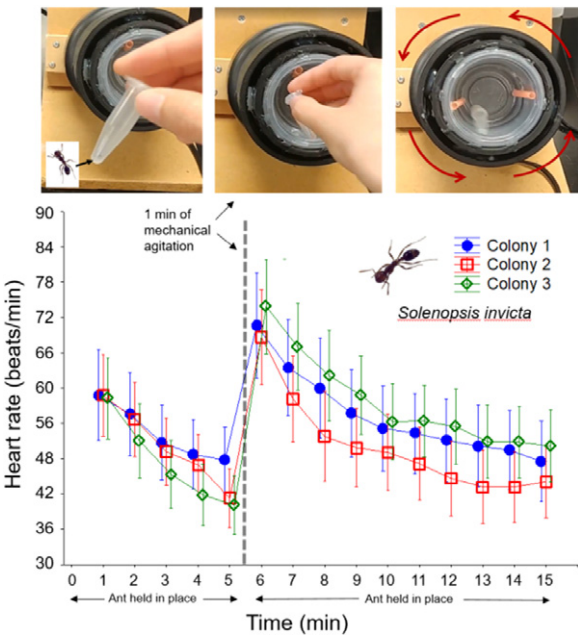

Figure 4 The mechanical agitation procedure (top) and average (+ 95% confidence intervals) heart rates (HR; bottom) of three colonies of *Solenopsis invicta* in response to the agitation. After 5 min of HR monitoring, we placed each ant in an Eppendorf tube which is then placed in the container where it 'tumbles' for 1 min. [Colour figure can be viewed at wileyonlinelibrary.com]

for 1 min; thus, the ant experiences physical jostling and forced movement during this time, as the Eppendorf tube gently tumbles. This device was also designed with the ease of transitioning in mind; thus, ants can be placed in the device for the required time, and then quickly removed and returned to the microscope slide for further heart rate monitoring. This timing is important for evaluating the immediate HR reaction to the stimulus. With practice, our observer was able to complete the transfer of ants to and/or from the microscope to the device within 30 s. A video of this procedure is supplied in the supplemental material.

We used 93 fire ants for this part of the project, which were collected from each of three colonies near our laboratory (n = 25, 33, and 35 ants). The ants were collected by scooping up nest material (plus ants) from the colonies in a plastic container, and brought to the laboratory. We conducted these trials over the span of 3 weeks, where each testing day, different collections were made from these same three colonies. The trials were conducted as follows: an ant from one of the collections was picked up and secured on a microscope slide, using the clear tape method outlined above, then immediately placed on our IR microscope to begin HR measurement. The time from initial contact to recording was less than 30 s (see video in supplemental files). We recorded the ant's heart rate every minute for 5 min. Next, we removed the ant from the microscope slide and placed it in the rotating agitation device for 1 min. The ant was then immediately returned to the microscope for HR monitoring. We continued recording HR each minute thereafter for 10 additional minutes. Our intention here was to capture the response (or 'recovery') of the ant heart rate following the physical stimulation.

At the end of this test, we had 15 serial heart rate readings for each ant. Preliminary inspection of these data indicated they were normally distributed. To determine how HR changed over the course of the trials, we used repeated-measures ANOVA, where the 15 HR readings were the repeated response variable, and colony was the predictor. Results of this analysis revealed no main effect of colony on fire ant HR ($F_{2,84} = 0.949$, $P = 0.43$), but significant variation due to time ($F_{14,1176} = 35.91$, $P<0.0001$) and, importantly, a significant time*colony interaction ($F_{28,1176} = 2.00$, $P = 0.0016$). These results indicate that fire ant HR changed during the course of the trials, which is clear also from plotting the mean HR over the 15 time points (Figure 4). Furthermore, there were notable differences between colonies in the magnitude of the changes. For example, ants from colony 3 displayed a larger reaction to the physical stimulus; comparing HR from just prior to immediately after the stimulus, we calculated a 110% increase, on average, in those 31 ants. Meanwhile, ants in colony 1 showed a 59% elevation in HR. These results, although stemming only from three colonies, highlight the potential of this procedure for evaluating and comparing baseline and 'stimulated' heart rates of different ant colonies.

Concluding remarks

Evaluating heart rates of insect species, and especially how they change in response to stimuli, can be useful for a variety of research purposes (Kholodkevich et al., 2008, 2017; Davis et al., 2018). Additionally, heart rates can be used as a proxy for metabolic rate in invertebrates (Bruning et al., 2013), or for evaluating physiological effects of temperature variations (Zhu et al., 2016). The dearth of research into such questions using ants is likely due to the lack of methodological options available. The procedures and devices described here should prove useful for conducting such experiments involving (non-destructive) monitoring of heart rates of these or other Formicidae species.

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Data Availability Statement

Data from this project will be made available in supplemental material upon publication.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Ant heartbeat under IR light.mp4.

Data S2. Procedure for ant placement and removal.mp4.

Data S3. Procedure for mechanical agitation.mp4.