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The effects of bacterial endotoxin (LPS) on cardiac function in a medicinal blow fly (*Phaenicia sericata*) and a fruit fly (*Drosophila melanogaster*)

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ABSTRACT

The bacterial endotoxins, lipopolysaccharides (LPS), are known to have direct effects on mammalian heart cells; thus, LPS is likely to have some effects in other cardiac models. *Drosophila melanogaster* was used since it serves as a model for cardiac physiology. Larvae of blow flies (*Phaenicia sericata*) commonly used as therapy for debriding dead tissue, are exposed to high levels of bacterial endotoxins, but their mechanisms of LPS resistance are not entirely understood. Comparative effects of LPS on heart rate (HR) were examined for both *Drosophila* and blowfly larvae. Acute 10-min direct exposure of in situ heart tubes with saline containing 1, 100, and 500 μg/ml LPS from two common bacterial stains (*Pseudomonas aeruginosa* and *Serratia marcescens*) revealed a dose-dependent effect. The effects differed between the two fly models. Larval hearts of *Drosophila* stopped rapidly in low Ca^{2+} containing saline, but the hearts of blow flies appear unaffected for > 30 min. *S. marcescens* increased HR initially in *Drosophila* followed by a reduction for low and high doses, but no change was observed in larvae of blow flies. Whereas *P. aeruginosa* at a high dose decreased HR in larvae of *Drosophila* but increased HR in larvae of blow flies. The goal of this study is to better the understanding in the direct action of LPS on HR. Knowing the acute and direct actions of LPS exposure on HR in different species of larvae may aid in understanding the underlying mechanisms in other animals during septicemia.

1. Introduction

Close to 1.5 million people in the US become hospitalized yearly due to septicemia (CDC Statistics, 2017). The endotoxic effect from bacteria is primarily due to the lipopolysaccharides (LPS) which make up the outer layer of the membrane in non-capsulated bacterial strains (gram negative bacteria) (Hicks and Jia, 2018). LPS is one of the primary activators of the innate immune response by activating cells containing Toll-like receptor 4 (TLR4) known as the CD14/TLR4/MD2 receptor complex in mammals (da Silva Correia et al., 2001; Park and Lee, 2013). The Toll receptors are conserved from primates to insects (Gangloff et al., 2003; Nonaka et al., 2018). However, in insects the peptidoglycan recognition proteins (PGRPs) are the receptors which respond to LPS from gram negative bacteria (Yoshida et al., 1996; Steiner, 2004). These PGRPs activate the Immune Deficient (IMD) signaling cascade (Harris et al., 2018). There is a family of these receptors known in *Drosophila melanogaster* but their expression profiles in different tissues has yet to be fully elucidated (Werner et al., 2000).

Both *Pseudomonas aeruginosa* and *Serratia marcescens* are known to play a role in septicemia in humans and other mammals (Mahlen, 2011; Olexy et al., 1979; Piening et al., 2017; Villalon et al., 2018). *Serratia marcescens* can resist the normal systemic immune response because of the O-antigen of its LPS (Nehme et al., 2007). LPS from *Serratia marcescens* is also known to be more potent than *Pseudomonas aeruginosa* in inducing an immune response in mammals (Kreger et al., 1986; Lorenzon et al., 2002; Luchi and Morrison, 2000). LPS itself has been found to cause septic myocardial dysfunction in mammals due to its effect of sarcoplasmic leak, which decreases the ability of the heart muscles to contract (Celes et al., 2013). Exposure of rodent cardiomyocytes to LPS can result in a decrease of systolic Ca^{2+} transients and myocyte contraction as well as overall sarcoplasmic reticulum Ca^{2+} content (Yang et al., 2018), which may in part be due to decrease L-type Ca(2+) channel current (Hobai et al., 2013). If LPS results in Ca^{2+} dumping from the sarcoplasmic reticulum then a low Ca^{2+} containing bath and exposure to LPS would likely result in a transient increase in heart rate. This possibility was examined in the current study.

This myocardial dysfunction is a major factor in the severity and survival of patients with septicemia (Shankar-Hari et al., 2016; Singer et al., 2016; Jayaprakash et al., 2018). In rodents, infusion of LPS induced bradycardia within a minute (Rameshrad et al., 2015) but it was not established if this effect was directly on the heart or the neural innervation to the heart. Using larval *Drosophila* aids in examining the

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direct action on the heart since the larval heart tube is not innervated. In addition, the larval heart contracts due to Ca^{2+} flux across the plasma membrane as in mammals and must regulate the Ca^{2+} load for maintaining the heartbeat. It is well established that the pacemaker cells in *Drosophila m.* are susceptible to alterations in ionic concentrations in the bathing environment as well as manipulation of internal ion levels (Badre et al., 2005; Desai-Shah et al., 2010; Majeed et al., 2013, 2014; Malloy et al., 2016, 2017; Titlow et al., 2013; Zhu et al., 2016a, 2016b). Thus, this insect species was used to investigate if LPS exposure showed an alteration in HR. The larval heart circulates hemocytes which act as the immune defense system (Myers et al., 2018) but also any LPS present will circulate via the heart and movement of the larvae.

To examine the potential acute effect of LPS on the membrane potential of the myocytes, we performed intracellular recordings while bathing the tissue in a saline with a low concentration of Ca^{2+} to reduce contractions. Recently it was shown that exposure to LPS from *S. marcescens* or *P. aeruginosa* resulted in hyperpolarization of the membrane potential in the body wall muscles of *Drosophila m.* (Cooper and McNabb, 2018) and it appears there is a slight hyperpolarization in cardiac tissue as well. In addition, LPS does not appear to increase an influx of Ca^{2+} within the presynaptic motor nerve terminals since there is no increase in the occurrence of spontaneous fusion of vesicular events (Cooper and McNabb, 2018).

Upon treatment for mild infection or severe septicemia with antibiotics in mammals, there can be an acute surge of LPS from the lysing of the bacteria. This acute response is commonly known as the Jarisch-Herxheimer reaction when providing treatment for sepsis and meningitis (Nau and Eiffert, 2002). Thus, the potential effect on cardiac function from a high concentration of LPS over an acute period is of interest. We examined the effect on heart rate over a wide range in dosage from 1 to 500 μg/ml exposure. This range is enough to cause death in humans, as well as rodent mammal models. The LD50 for mice is 1–25 mg/kg and this was determined to be a 1000-fold to 10,000-fold greater than the dose of LPS that is required to induce severe illness and hypotension in humans (Fink, 2014; Galanos et al., 1979; Luchi and Morrison, 2000; Taveira da Silva et al., 1993). LPS from *S. marcescens* induces a stronger immune response than LPS from *P. aeruginosa* at the same dosage. *Drosophila* serve as a model for addressing many disease states including cardiac dysfunction in humans (Akasaka and Ocorr, 2009; Bellen et al., 2010; Bier and Bodmer, 2004; Cripps and Olson, 2002; Ocorr et al., 2007; Pandey and Nichols, 2011; Wessells and Bodmer, 2004). Since LPS causes arrhythmia in mammals, it is worthy to address the potential physiological effects in this genetically amenable model system for future molecular and pharmacological based studies. Thus, we focused on the LPS from these two bacterial strains to start examining the acute and direct action on the heart, without the influence of an immune response. The in situ heart larval preparations are bathed in saline in which all the immune responsive freely circulating hemocytes are removed.

It is common in humans and other mammals to have a hypercalcemic condition with septicemia (Holowaychuk and Martin, 2007; Vadstrup and Pedersen, 1993); however, the mechanism of how this arises is still an active area of research. There are indications that the rise in procalcitonin (ProCT), the prohormone of calcitonin, particularly for gram negative related infections (Becker et al., 2010) is the key to inducing the response. We are not aware of any study on proteins regulating Ca^{2+} levels in the hemolymph of insects related to LPS exposure. We anticipated the direct effects of LPS on hearts with lowered bathing Ca^{2+} may not demonstrate a significant increase in heart rate. Since bathing Ca^{2+} is essentially zero there would not be an increase rate due to extracellular Ca^{2+} entry but if LPS induced Ca^{2+} release from the sarcoplasmic reticulum then an increase rate would likely be observed. Since LPS did not appear to cause an increase in Ca^{2+} influx in the presynaptic nerve terminal at the neuromuscular junction in the *Drosophila m.,* we anticipated a similar outcome for the heart (Cooper and McNabb, 2018). It has been previously established that 5-HT substantially increases the larval HR in *Drosophila m.* in the cold (10 °C) as well as at 21 °C (Johnson et al., 1997; Zhu et al., 2016a). The increased rate is due to activation of the 5-HT2ADro and/or 5-HT2BDro receptors (Majeed et al., 2014) which targets the PLC-PKC cellular pathway to activate inositol triphosphate (IP3) and diacylglycerol (DAG). The IP3 results in the sarcoplasmic reticulum to release Ca^{2+} into the cytosol and promotes contraction (Majeed et al., 2014). We examined the effects of LPS from *S. marcescens* on HR for *Drosophila m.* in conditions of very low (no Ca^{2+} added to saline), 0.1 mM and 1 mM Ca^{2+} containing saline. To examine the possibility of internal release of Ca^{2+} having an effect on HR during an acute exposure to a low Ca^{2+} bathing solution (0.1 mM), serotonin (5-HT, 1 μM) along with LPS exposure after 2 min of pre-exposure to LPS was examined. The rise in cytoplasmic Ca^{2+} in the *Drosophila m.* with optogenetic approaches has also demonstrated to increase HR (Zhu et al., 2016b) which provides supporting evidence that any potential rise in cytoplasmic Ca^{2+} induced by LPS would relate to an increase in HR.

Comparisons in the effect of LPS on larval blow fly (*Phaenicia sericata*) and *Drosophila m.* are of interests since *Phaenicia s.* larvae grow and survive in LPS enriched environments such as when used for medical and veterinary wound care (Choudhary et al., 2016; Francesconi and Lupi, 2012; Tomberlin et al., 2017) and on animal carcasses (Matuszewski et al., 2014). We initially anticipated LPS would have some direct effects on the hearts for both insect species since Ca^{2+} dynamics is known to be altered in myocytes of mammalian models exposed to LPS (Hobai et al., 2013; Yang et al., 2018) but we were not sure if it would increase or decrease the rate due to time in systole.

Since the native environments for both species (i.e., a rotting fruit or an animal carcass) would potentially have high levels of bacterial exposure, both species were anticipated to show a high resistance to food tainted with LPS. We have no idea on the levels which would be reached in the hemolymph by feeding LPS. Thus, we examined the direct action of LPS on exposed hearts in the larvae instead of feeding larvae LPS.

2. Methods

The common "wild-type" laboratory strain of *D. melanogaster*, Canton S, was used as one model. Only early 3rd instar larvae were used (50–70 h) post hatching. This stage was easily identified and due to the small size of the *Drosophila* larvae (~5 mm) the dissection to exposure the heart tube is relatively easy to obtain without damaging the heart tube. All larvae were maintained at room temperature ~21 °C in vials partially filled with a cornmeal–agar–dextrose–yeast medium. This *Drosophila* strain has been isogenic in the lab for several years and was originally obtained from Bloomington Fly Stock. Larvae of blow flies (*Phaenicia* (=*Lucilia*) *sericata*) were used as a second model. The blow flies were raised in the laboratory on beef liver and the 3rd instar stage was used.

The general larval dissection technique to expose the larval heart tubes has been previously reported (Cooper et al., 2009). In brief, the larvae were dissected ventrally and pinned on four corners. The visceral organs were removed keeping the heart tube intact. This dissection technique was previously used to directly assess pharmacological agents on the heart of *Drosophila* larvae (Desai-Shah et al., 2010; Majeed et al., 2014; Malloy et al., 2016). The dissection time was roughly 3–6 min, and the preparation was allowed to relax while bathed in saline for 3–5 min after dissection. The heart rate was monitored and recorded after the initial dissection, after bathing 30 s in LPS, after 1 min in LPS, after 5 min in LPS, and lastly after 10 min in a saline wash. A modified HL3 saline was used (NaCl 70 mM, KCl 5 mM, $MgCl_2.6H_2O$ 20 mM, $NaHCO_3$ 10 mM, Trehalose 5 mM, sucrose 115 mM, BES 25 mM, and $CaCl_2.2H_2O$ 1 mM, the pH 7.1; de Castro et al., 2014). Pure LPS from *Serratia marcescens* (*S.m.*) and *Pseudomonas aeruginosa* (*P.a.*) as well as the salts for the saline and the modulators were obtained from Sigma Chemical Company. LPS was dissolved in the

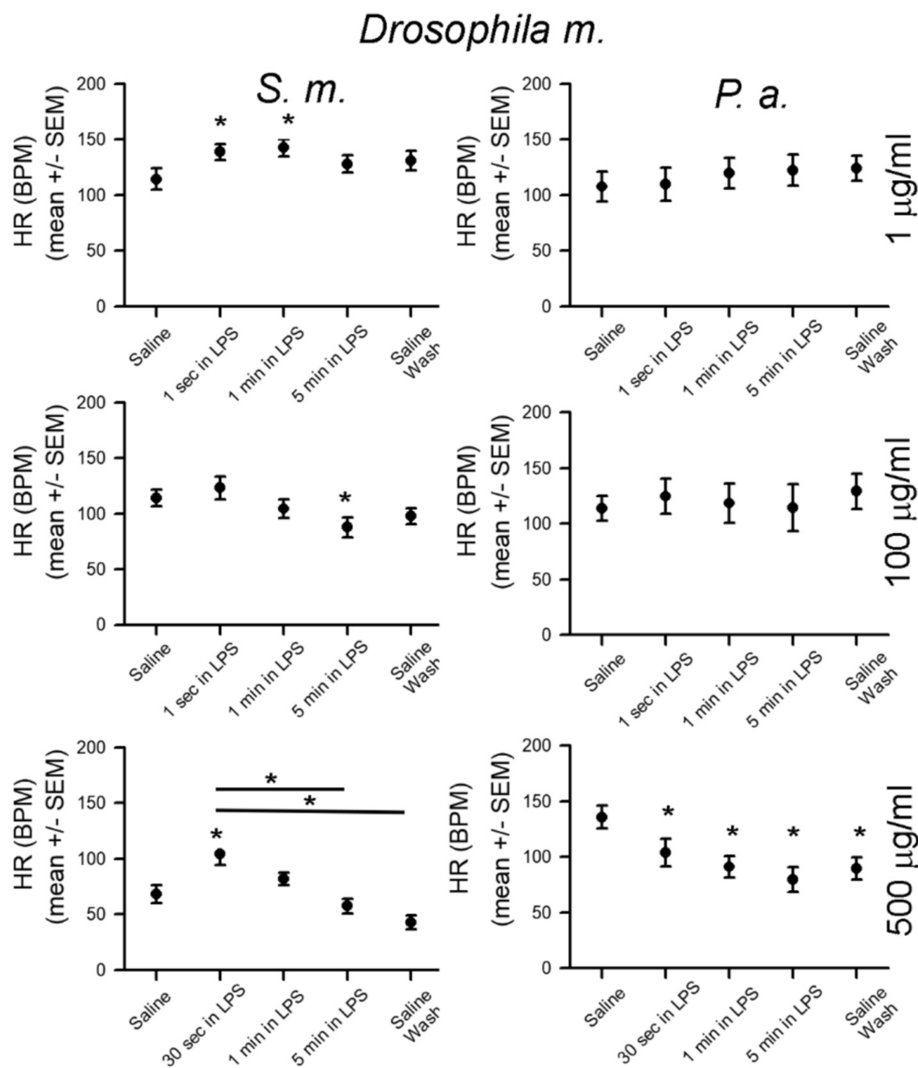

Fig. 1. Heart rates of *Drosophila melanogaster* acutely exposed to LPS from *Serratia marcescens* (*S.m.*) and *Pseudomonas aeruginosa* (*P.a.*). The effect of varied concentrations (A) 1 μg/ml, (B) 100 μg/ml and (C) 500 μg/ml for 12 preparations in each condition are shown (mean ± SEM). Statistical analysis is compared to the previous condition ($P < 0.05$ sign test non-parametric, $N = 12$) unless specified with the bar and a star (* $P \leq 0.05$). Heart rate (HR) is reported as beats per minute (BPM).

physiological saline at 1, 100 and 500 μg/ml concentrations.

After the initial count of HR, the saline was switched to a saline containing the LPS of interest. The rates were made by direct counts by eye for 1 min. Control experiments were performed by exchanging the saline bath with fresh saline and monitoring the HR to control for switching the bathing media and the effect of incubation time. We define a very low Ca^{2+} bath as one in which no calcium is added besides any impurities in other salts. The solution was not a zero Ca^{2+} as we did not use EGTA or BAPTA to chelate any free Ca^{2+} ions. A zero Ca^{2+} bathing solution was not required for these experiments but only a very low Ca^{2+} containing saline to compare the effects between *Drosophila m.* and *Phaenicia s.* larvae and saline with 0.1 mM and 1 mM by adding $CaCl_2$. This procedure is used to examine if the SER is dumping out Ca^{2+} due to the exposure of LPS. By reducing HR with a lower bathing Ca^{2+} concentration any increase in HR would be easier to detect as the rate would be further away from a maximal rate.

To monitor the transmembrane potentials of the myocytes the caudal region of heart was impaled with sharp intracellular electrode (30 to 40 MΩ resistance) filled with 3 M KCl. An Axonclamp 2B (Molecular Devices, Sunnyvale, CA, USA) amplifier and 1 X LU head stage was used. Detailed procedures are shown in video format (Cooper et al., 2009). To reduce the initial contractions of the heart, the saline containing 1 mM Ca^{2+} was exchanged to saline containing very low Ca^{2+}. After the heart stopped beating in about 5 min, the saline was again exchanged to one with very low Ca^{2+} and LPS (500 μg/ml) of *Serratia m.* After monitoring the resting membrane potential for a few minutes, the saline was exchanged to the 1 mM Ca^{2+} saline without LPS.

Electrical pacing of the heart tube was conducted by a fire polished glass focal electrode (approximately 20 μm in diameter) placed over the caudal region of the heart tube and stimulated at 1 Hz with varied voltage to drive the heart to pace (Cooper et al., 2009). The media used inside the suction electrode was drawn from the saline bathing the heart for the experiments of interest. A stimulator (model S9 Grass Instruments, Quincy, Massachusetts, USA) was used at a frequency at 1 to 2 Hz, duration at 0.5 to 1 msec and voltage on the lowest setting and slowly increased until a local contraction could be observed as the saline was being diluted to one with no calcium. While observing the heart's rhythm, the frequency was turned to approximately 2 to 3 Hz, and the voltage slowly increased to ensure the heart was being paced by the stimulation. Voltage did not surpass 20 V as cell damage occurs at higher voltages. Typically, the heart was stimulated between 2 and 8 V, depending on distance and the seal created on the heart. Once a stimulation driven rhythm is established the remaining saline was

exchanged to very low calcium and the suction electrode was flushed and new saline pulled back into the electrode. The seal and stimulation procedure was returned to initial conditions.

To drive the sensory-CNS-motor circuit of 3rd instar *Phaenicia s.* larvae, the same procedure was used as described in Dasari and Cooper (2004). In brief, the larvae were dissected open with a longitudinal cut along the dorsal midline and the internal organs along with the heart were removed. The brain and segmental nerves were left intact to the muscles they innervate and segments the sensory nerves monitor. A fire polished glass electrode was filled with the bathing saline and a segmental nerve was pulled into the tip of the glass electrode in en-passant fashion. This electrode was used to provide stimuli to the segmental nerve until evoked EPSPs could be obtained in a contralateral segment which would occur after the stimulus train was given. This ensured the sensory-CNS-motor circuit was driven to induce the observed EPSPs within the muscle fiber. The bathing saline was exchanged with one containing 5-HT to determine if the circuit activity is altered. This technique works well to examine the effect of modulators on central circuits (Cooper and Neckameyer, 1999; Neckameyer and Cooper, 1998; Dasari et al., 2007; Majeed et al., 2015; Mattingly et al., 2018). Since the heart of the *Phaenicia s.* was not sensitive to 5-HT exposure it was of interest to examine if the CNS was responsive to 5-HT as it is for *Drosophila m.*

For statistical analysis, a sign test was used to compare the difference of HR with exposure to compounds when appropriate. An ANOVA was also used to compare differences among treatments with LPS dosage. This analysis was performed with SigmaStat software. P of ≤ 0.05 is considered statistically significant. Normality Test (Shapiro-Wilk) and Equal Variance Test (Brown-Forsythe) were performed by the software. All Pairwise Multiple Comparison Procedures used the post analysis with a Bonferroni *t*-test.

3. Results

In order to examine the direct effects of LPS on altering heart rates, the acute actions in exposing the larval *Drosophila m.* heart to low (1 μg/ml), medium (100 μg/ml) and a high (500 μg/ml) dosages of purified LPS from two common gram negative strains revealed that *Serratia m.* at a low dosage increased HR after 30 s of exposure at 1 μg/ml ($P < 0.05$ sign test non-parametric, $N = 12$) and remained elevated for the next minute but then returned to basal levels (Fig. 1). However, no consistent trend was observed for 100 μg/ml except after 5 min in LPS as the rate significantly decreased in the preparations compared to the heart rate at 1 min exposure ($P < 0.05$ sign test non-parametric, $N = 12$). With 500 μg/ml exposure the HR rapidly increased and then decreased for each of the subsequent time measurements (Fig. 1; ($P < 0.05$ sign test non-parametric, $N = 12$)). The LPS from *Pseudomonas a.* did not have a consistent effect on HR at 1 μg/ml and 100 μg/ml, but at 500 μg/ml the HR continued to decrease at each time point measured over time even after 10 min with flushing 3 times with untainted saline (Fig. 1; ($P < 0.05$ sign test non-parametric, $N = 12$)).

In examining the HR of the larvae from *Phaenicia s.* it was obvious the rates were innately lower than for larval *Drosophila m.* No significant trends were observed for *Serratia m.* or *Pseudomonas a.* at 1 μg/ml, 100 μg/ml, or 500 μg/ml for *Serratia m.*, but at 500 μg/ml for *Pseudomonas a.* there was an increase in HR for each point measured over time (Fig. 2; $P < 0.05$ sign test non-parametric, $N = 12$).

To control for the effects of exchanging the bathing saline and monitoring the rate over time, the saline was exchanged with saline for the identical paradigm. No significant trends in altering HR occurred (Fig. 3). In an earlier report, it was shown that this physiological saline maintains HR for 10 min without significant changes without saline perfusion of the dish (de Castro et al., 2014; Majeed et al., 2014). If longer times are required it would be good to perfuse the dish as fluid movement of the heart may keep it stimulated and oxygenated (deCastro et al., 2015).

In illustrating the relative changes in HR the percent change for each of the condition illustrates the overall trends and the variation. The percent changes for each condition in the experimental paradigm is compared to the initial rate in saline (Fig. 4).

To examine if the action of LPS was due to the release of internal Ca^{2+} from ER, low external Ca^{2+} saline was used while exposing the hearts to LPS. *Drosophila m.* were quite sensitive to the lowered Ca^{2+} and the hearts stopped beating quickly. However, the larvae of *Phaenicia s.* did not show the same sensitivity (Fig. 5). The heart tubes of the *Drosophila m.* were not able to be electrically stimulated with a focal electrode placed next to the heart while being bathed in the very low Ca^{2+} bath; however, the hearts were able to be locally stimulated in *Phaenicia s.* in the same bathing saline (Supplemental video 1).

Neither species of larvae showed any significant change in HR to LPS while bathed in low Ca^{2+} (0.1 mM Ca^{2+}). In examining if HR is able to increase while bathed in low Ca^{2+} (0.1 mM), the cardiac modulator 5-HT, which rises cytoplasmic Ca^{2+} though an IP3 cascade, was used. The larval *Drosophila m.* rapidly increased HR (Fig. 6A; $P < 0.05$ sign test non-parametric, $N = 6$) while *Phaenicia s.* did not show any significant changes (Fig. 6B). The lack of response by *Phaenicia s.* was unusual since 100 nM LPS from *Serratia m.* produces a large response in larvae of *Drosophila m.* Since it is known that a sensory-CNS-motor nerve circuit is sensitive to 5-HT in *Drosophila m.*, we examined if *Phaenicia s.* would also show a response upon exposure while stimulating the circuit. The circuit was as sensitive for *Phaenicia s.* as also shown in earlier reports for *Drosophila m.* (Fig. 6B2 and B3). Thus, *Phaenicia s.* is responsive to 5-HT in neuronal tissue.

It was recently shown that acute exposure to LPS of *Serratia m.* (500 μg/ml) to *Drosophila m.* body wall muscle produces a substantial rapid hyperpolarization (Cooper and McNabb, 2018). Thus, we examined if the same phenomena would occur for cardiac muscle. A consistent hyperpolarization was observed in six out of six preparations examined with an average 0.9 mV (0.25 ± SEM; $P < 0.05$ sign test non-parametric) (Fig. 7A). To examine if the same saline used on the larvae produced the same responses as in the earlier report, it was examined on a body wall muscle which reproduced the earlier findings (Fig. 7B). Thus, the mechanism of action on the cardiac muscle is likely the same as it is on the body wall muscle but not as pronounced.

4. Discussion

In this study it was demonstrated that direct exposure to LPS from two common strains of gram negative bacteria is able to induce changes on the HR of the fruit fly (*Drosophila m.*) and blowfly (*Phaenicia s.*) larvae. The effects of LPS on HR are dosage and time dependent. In addition, the extracellular $[Ca^{2+}]$ alters the responsiveness to LPS. The HR of *Drosophila m.* is very sensitive to very low extracellular $[Ca^{2+}]$ and cannot be electrical induced to contract; however the blow fly heart continues to function in very low extracellular $[Ca^{2+}]$ and can be electrical stimulated to beat. LPS induces a small transient hyperpolarization in cardiac muscle. The heart of larval *Drosophila m.* remains sensitive to modulation by 5-HT in the presence of exposure to high levels of LPS. This indicates that LPS is not likely depleting internal stores of Ca^{2+} from the ER and that the LPS did not block the 5-HT receptors or the 5-HT activated second messenger cascade.

Specific forms of LPS is produced from different strains of bacteria and each has a varied potency in producing immunological responses. *Serratia m.* and *Pseudomonas a.* are known to be different, with *Serratia m.* inducing a heightened response in fever and cytokines in rodents and humans. The older approach of the rabbit pyrogen test for gram negative bacterial response, indicated that *Serratia m.* and *Pseudomonas a.* both take about 1 × 10ˆ6 organisms/ml to induce a response with *Serratia m.* being slightly more sensitive to induce the response (Devleeschouwer et al., 1985). The very sensitive Limulus amoebocyte lysate (LAL) assay for endotoxin demonstrated that the number of bacterial organisms to induce clotting for *Pseudomonas a.* is higher

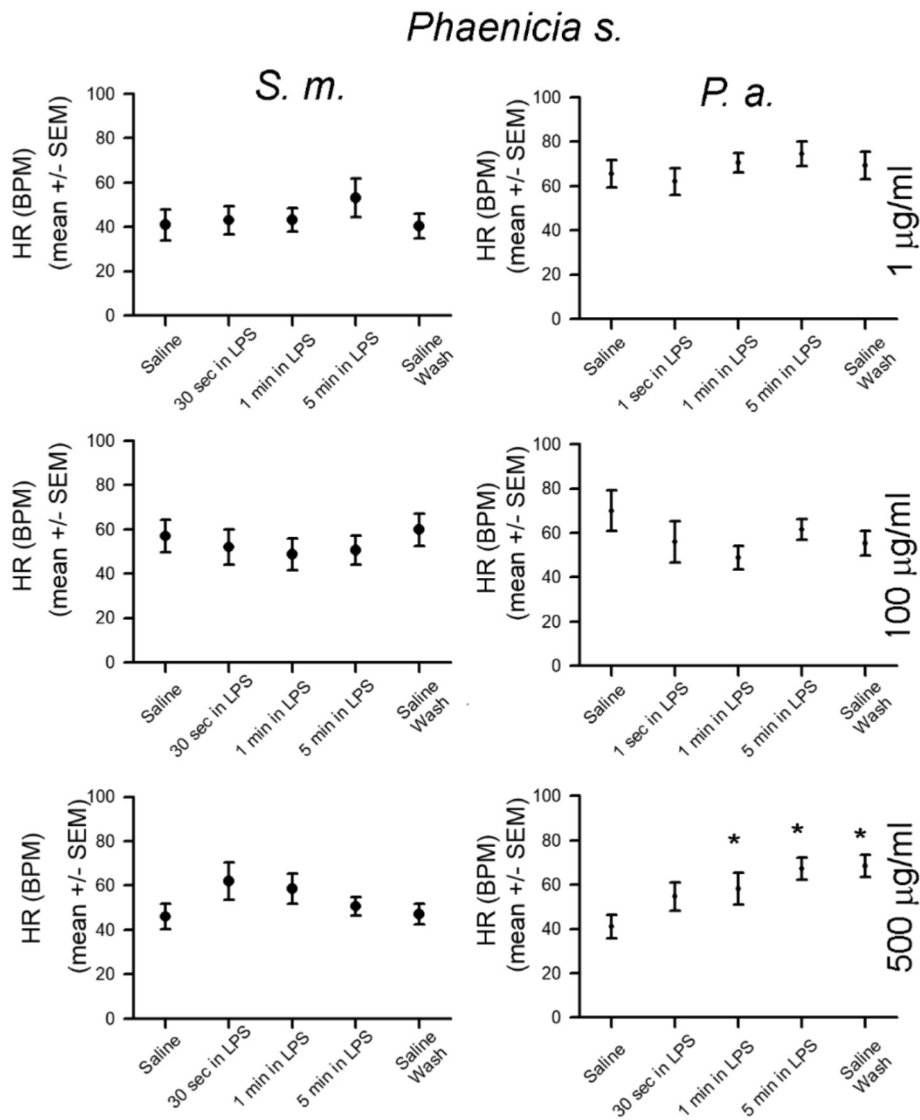

Fig. 2. Heart rates of *Phaenicia sericata* acutely exposed to LPS from *Serratia marcescens* (*S.m.*) and *Pseudomonas aeruginosa* (*P.a.*). The effect of varied concentrations (A) 1 µg/ml, (B) 100 µg/ml and (C) 500 µg/ml for 12 preparations in each condition are shown (mean ± SEM). Statistical analysis is compared to the previous condition ($P < 0.05$ sign test non-parametric, $N = 12$). Heart rate (HR) is reported as beats per minute (BPM).

(1.3 × 10ˆ5) than for *Serratia m.* (8.9 × 10ˆ4) (Devleeschouwer et al., 1985). Humans injected with purified LPS, instead of a bacterial load, also have demonstrated that LPS from *Serratia m.* is more potent than *Pseudomonas a.* in increasing cytokines levels within the blood (Dehus et al., 2006). Even in shrimp (*Palaemon elegans*) LPS injection from *Serratia m.* is more potentate than LPS from *Pseudomonas a.* in reducing the total blood cell count (Lorenzon et al., 2002). Likewise, we demonstrated that LPS has a rapid effect on HR for *Drosophila m.* with as

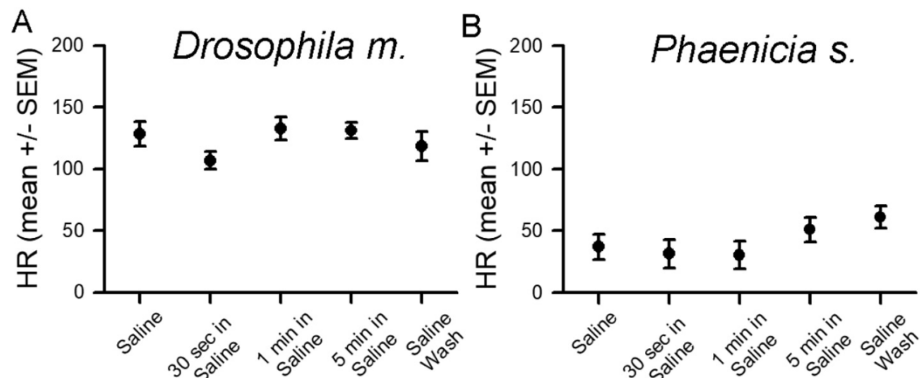

Fig. 3. Heart rates of (A) *Drosophila melanogaster* and (B) *Phaenicia sericata* in saline and for saline exchanges to match the conditions for exposure times to LPS. Six preparations in each condition are shown. Heart rate (HR) is reported as beats per minute (BPM).

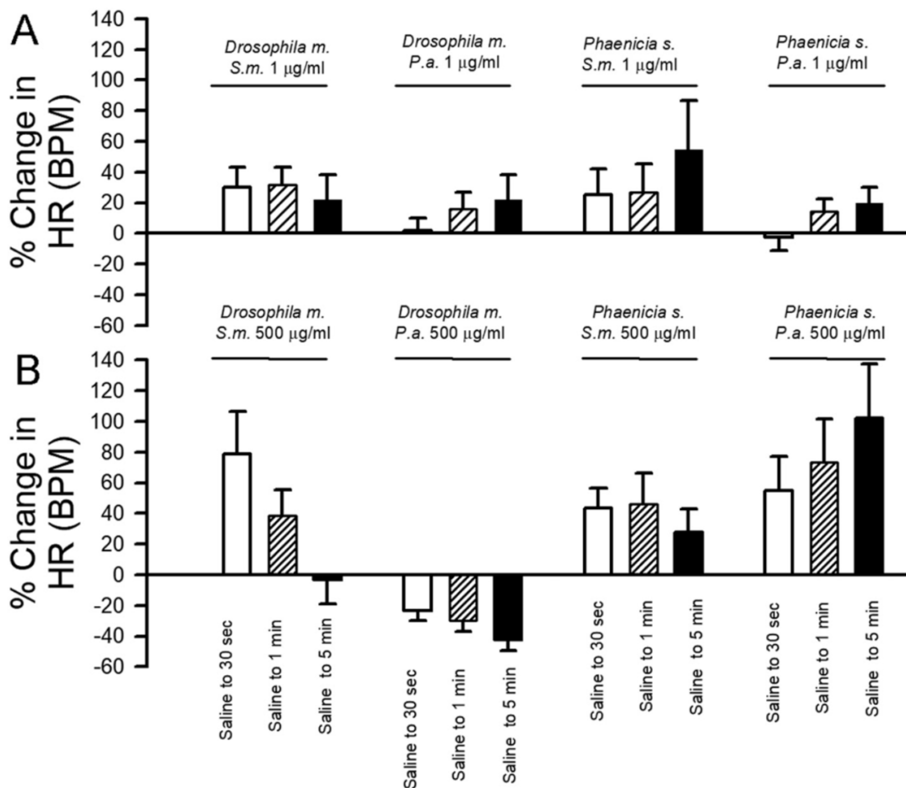

Fig. 4. The average percent change in the *Drosophila melanogaster* and *Phaenicia sericata* preparations to the exposure of LPS from *Serratia marcescens* (*S.m.*) and *Pseudomonas aeruginosa* (*P.a.*). The average percent change (± SEM) for each condition is made to the initial saline condition. (A) The percent changes for the exposure to (A) 1 µg/ml and (B) 500 µg/ml for both forms of LPS are shown. Heart rate (HR) is reported as beats per minute (BPM).

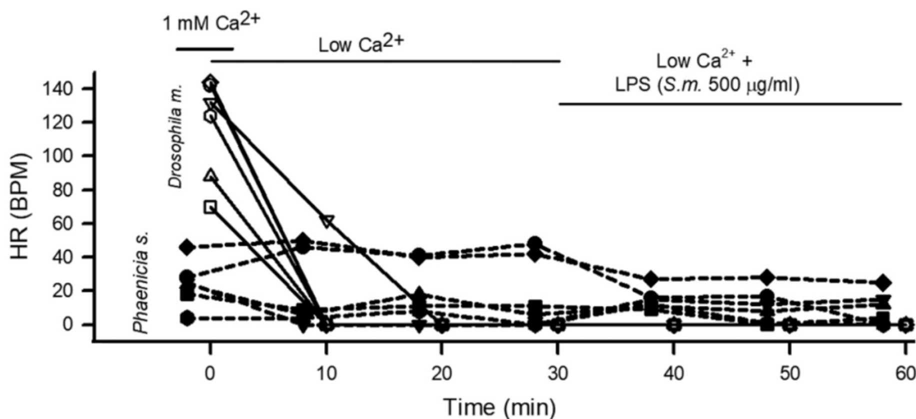

Fig. 5. The effect on heart rate to very low exposure of free Ca^{2+} bathing the hearts of *Drosophila melanogaster* and *Phaenicia sericata*. The heart rate was monitored for 30 min in six preparations for each species in very low Ca^{2+} (essentially zero) and subsequently exposed to LPS from *Serratia marcescens* (*S.m.*) for another 30 min. The dotted traces for *Phaenicia sericata* are offset to the left to avoid overlap with the data points for *Drosophila melanogaster* (solid lines).

little as 1 µg/ml for *Serratia m.* with no effect being observed for *Pseudomonas a.* at 1 µg/ml.

Interestingly, the heart of larval *Drosophila m.* exposure to LPS at 500 µg/ml for *Serratia m.* rapidly increased HR but for *Pseudomonas a.* at 500 µg/ml the HR decreased and continued to do so over the next few minutes. The heart of *Drosophila m.* remained very sensitive to the chronotropic modulator of 5-HT even with prior exposure to 500 µg/ml of LPS from *Serratia m.* suggesting the heart was not dying and that the contractile machinery was not impaired from the acute LPS treatment. Since the heart was sensitive to 5-HT treatments this also implies the 5-HT induced second messenger cascades remained functional.

The HR in larvae of *Phaenicia s.* increased only at 500 µg/ml for LPS from *Pseudomonas a.* and did not consistently show a trend for *Serratia m.* at 500 µg/ml. The heart of *Phaenicia s.* larvae was not responsive to LPS from *Serratia m.* in increasing HR; however, there was a trend to decrease HR within a 5 min exposure. The hearts of *Phaenicia s.* did not show any effect to exposure of 5-HT before or after prior exposure to LPS followed with 5-HT combined with LPS. Thus, the hearts of larval *Phaenicia s.* were not sensitive to the 5-HT modulator. We only examined the late 3rd instar stage to 5-HT, and it is possible that earlier stages or even adults might be sensitive to 5-HT. There might be differences in sensitivity of 5-HT and LPS depending on diet, rearing conditions and age since 5-HT and the immune response are known to be related to environmental conditions (Adamo and Lovett, 2011; Hillyer, 2016; Howick and Lazzaro, 2014; Zhu et al., 2016a, 2016b). The beef liver diet for *Phaenicia s.* is quite different in nutrients than the corn meal diet for *Drosophila m.* (Daniels et al., 1991; Hobson, 1935; Gelman et al., 2000; Yu et al., 2018). A diet with yeast can alter the sleep cycle in *Drosophila* and 5-HT varies with the circadian pattern in *Drosophila* (Catterson et al., 2010; Nall and Sehgal, 2014).

How LPS specifically alters HR by either increasing or decreasing the rate remains to be determined. We do know modulators such as 5-HT, dopamine, octopamine and even acetylcholine can increase HR (Majeed et al., 2013, 2014; Malloy et al., 2016; Titlow et al., 2013; Zhu et al., 2016a). Thus, the CD14/TLR4/MD2 receptor complex or more likely the IMD signaling cascade activated various kinases (Harris et al., 2018; Steiner, 2004). For 5-HT signaling the cascade was shown to be a Gαq G-protein coupled receptor to activate phospholipase Cβ (PLC).

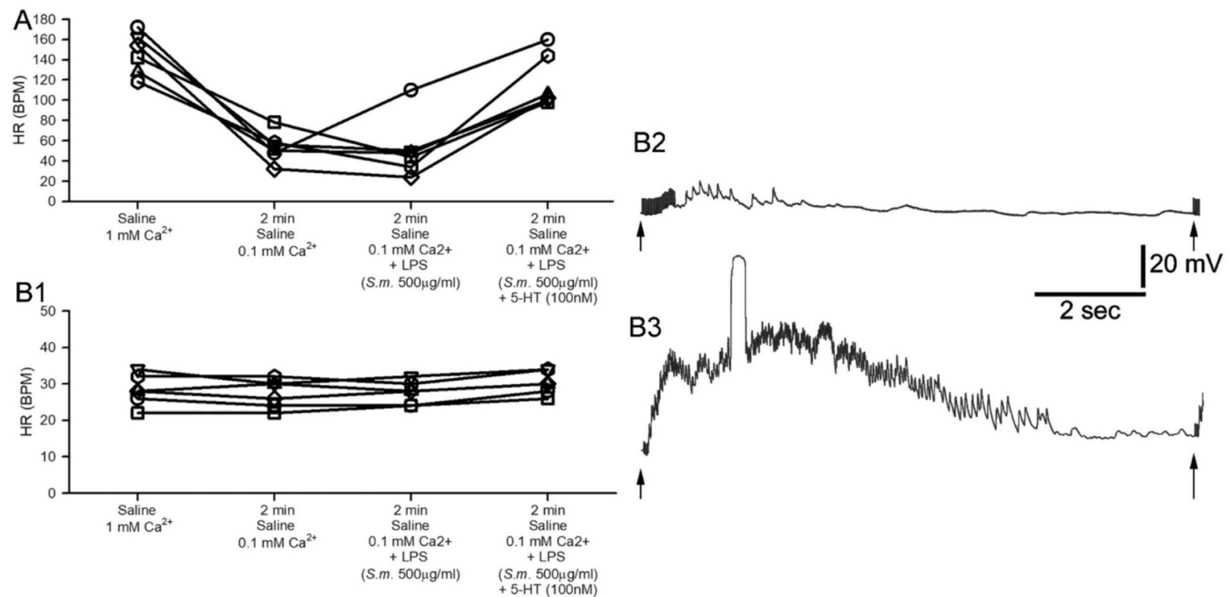

Fig. 6. The effect on heart rate and a sensory-CNS-motor circuit to serotonin. The reduced Ca^{2+} from 1 mM to 0.1 mM followed by bathing in LPS from *Serratia marcescens* (*S.m.*) with 0.1 mM Ca^{2+} and subsequent serotonin (5-HT, 100 nM) for six preparations of (A) *Drosophila melanogaster* and (B) *Phaenicia sericata* are shown. The sensitivity of a driven sensory-CNS-motor circuit was examined for the *Phaenicia sericata* larval with exchange from (B2) saline to (B3) 5-HT (1 μM). A 50 Hz train lasting 600 msec with a 10 s pause between trains of stimuli was used. The frequency of the excitatory postsynaptic potentials (EPSPs) measured in muscle m6 in segment 3 following the stimulus train for saline was substantially enhanced with exposure to 5-HT.

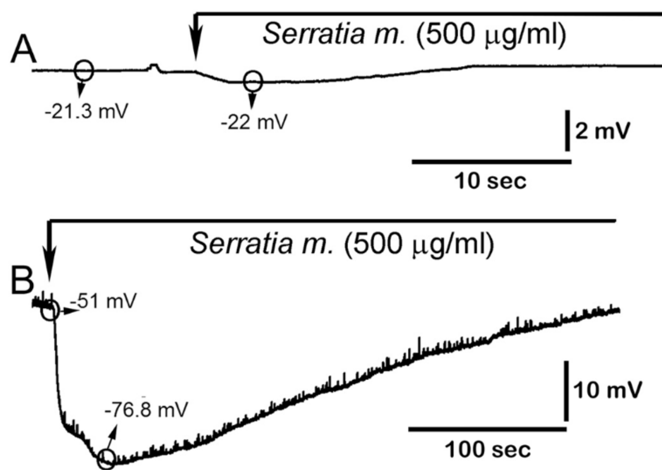

Fig. 7. Intracellular recordings of cardiac and body wall muscles while bathed in low Ca^{2+} for larval *Drosophila melanogaster*. (A) A slight hyperpolarization upon LPS (500 μg/ml) exposure from *Serratia marcescens* occurs for cardiac muscle. (B) A much larger and prolonged hyperpolarization occurs for body wall muscle (m6) in segment 3 of the larval *Drosophila* for the same LPS containing saline as used on the heart. The values are the membrane potentials at the points indicated. The small upward defections in B are spontaneous quantal synaptic potentials as are normally observed in body wall muscles which are neurally innervated.

There, appears to be both nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs) (Malloy et al., 2016) and for dopamine type-1 DA and type-2 DA receptors (Titlow et al., 2013) on *Drosophila m.* Activation of PKC has a dose-dependent stimulatory effect on larval HR (Titlow et al., 2013). It is possible PKC can modulate the larval heart via L-type Ca^{2+} channels as is the case for rat ventricular myocytes (Chen et al., 2012). Since the influx of Ca^{2+} can occur in waves (Gu and Singh, 1995) maybe the timing to measure HR could depend on the cyclic nature of the second messenger cascade, as well as the ability of the cell to regulate the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (Sanyal et al., 2006) and a sodium/calcium exchanger (Desai-Shah et al., 2010). All of these mechanisms that alter internal Ca^{2+} levels are known to have a role on the HR in the larval *Drosophila m.* Could the LPS induce an alteration in the cellular cascade in any of the Ca^{2+} regulator steps and either increase or decrease HR? This remains to be determined along with understanding which type of Toll-like receptors and/or PGRPs are present on the larval heart, or if the LPS response is due to an entirely different mechanism or a combination of factors. We are now examining RNAi expression in the larval heart for a few of the 9 Toll receptors and key proteins known to be involved in the IMD cascade in *Drosophila m.* (Harris et al., 2018; Steiner, 2004).

The rapid acute actions by LPS in the case of *Drosophila m.* larval hearts being bathed in a relatively large saline volume, precludes the likelihood of hormonal action, as in mammals with procalcitonin, in altering external $[Ca^{2+}]$ but more likely effects on ion channels involved with the pacemaker cycle or intracellular regulation of Ca^{2+} loading.

LPS actions in an isolated system without the body to induce an immune response allows one to address the direct effects. However, there may still be cell-cell interactions as an indirect action of LPS which can trigger localized responses. It is known that LPS promotes procalcitonin to be released from many peripheral tissues in mammals besides the parafollicular cells of the thyroid. Procalcitonin can induce Ca^{2+} to be released from internal stores from the ER in adipose cells (Radimerski et al., 2011) and is even be released from neurons altering local glia cell secretion which feeds back on the neuron to alter its function (Thalakoti et al., 2007). However, the heart rate in the study preparations are rapidly altered and are flushed with saline making it unlikely that there are some local paracrine effects. In the intact mammalian system, procalcitonin serves as a biomarker for bacterial infections, particularly for gram-negative types, and can decrease Ca^{2+} levels in the blood (Christ-Crain and Müller, 2005). Thus, there can be indirect effects on cardiac function of LPS in mammals triggered through procalcitonin besides the immune cytokines known to rise systemically (Becker et al., 2010; Christ-Crain and Müller, 2005). It is not known in the intact insect larvae and, as far as we know in any arthropod, if free Ca^{2+} ions in the hemolymph are altered by an infection in the systemic hemolymph.

The lowered external [Ca^{2+}] quickly reduced HR, and the subsequent application of LPS in low external [Ca^{2+}] did not show any further trend in altering HR in *Drosophila m.* larval. This may suggest the cell needs to be loaded with Ca^{2+} or have a rapid HR to produce a sustainable effect on HR by LPS. However, given that 5-HT in the presence and pre-incubation of LPS still produced a large increase in HR with a low (0.1 mM) external [Ca^{2+}] indicates the potential is present for LPS to increase HR if it were to have an action on dumping out Ca^{2+} into the cytosol from the sarcoplasmic/endoplasmic reticulum. The rapid drop of the membrane potential in the body wall muscles of larval *Drosophila m.* when exposed to LPS of *S. m.* would suggest a Cl^- influx or a K^+ efflux from the heart muscle. However, in skeletal muscle of larval *Drosophila* the equilibrium potential for Cl^- is more depolarized than the resting membrane potential (Rose et al., 2007). This may also be the case for the heart muscle. In any case, a hyperpolarization could indicate a slowing of the HR for the cardiac pacemaker. However if a more rapid hyperpolarization occurs reducing the duration of systole and promoting the onset of diastole the rate might even increase. Recording the membrane potential in a contracting and relaxing muscle is a challenge with intracellular electrodes so we used the very low external [Ca^{2+}] to stop the *Drosophila m.* larval hearts in order to record a membrane potential. The approximate 1 mV hyperpolarization with 500 µg/ml of LPS from *S. m.* is transient and is not maintained just as observed for body wall muscles in the larvae. It was not necessary for our experiments to obtain an absolute zero Ca^{2+} in the bath as the larval hearts of *Drosophila m.* stopped in the very low external [Ca^{2+}]. If one needed a zero Ca^{2+} saline for future studies with *Phaenicia s.* using EGTA or BAPTA could be implemented (Markou and Theophilidis, 2000); however, the Mg^{2+} level may also be altered by these chelators. It is not known what causes the hyperpolarization in the body wall muscles of the larvae with LPS. Using low external Ca^{2+} in the bath and even in the presence of incubated TEA and L-NAME to block K^+ channels and NOS production respectively, had no effects on the LPS inducing the rapid transient hyperpolarization (Cooper and McNabb, 2018). The effect on the membrane potential of the cardiac tissue is not likely to be a candidate for altering the HR for more prolonged periods of a few minutes. Thus, this leads us back to the possible mechanism of LPS on internal regulation of cellular cascades or altering ion channel function involved with the pacing of the heart. Possibly future imaging with ion sensitive indicators will help to unmask ionic contributions to the alterations in HR and the small transient hyperpolarization.

The unresponsiveness of *Phaenicia s.* larvae to the 5-HT modulator or lowered external [Ca^{2+}] was not expected as HR in crustaceans (i.e., crabs, crayfish, lobster), other insects (i.e., cockroach) as well as *Drosophila m.* are sensitive to 5-HT (Dasari and Cooper, 2006; Johnson et al., 2002; Listerman et al., 2000). However, a full range of 5-HT effects for *Phaenicia s.* larvae have not been investigated. It is possible that there may be a negative effect at 1 µM for *Phaenicia s.* as biphasic responses are known to occur for octopamine in other insect species (*Apis mellifera* macedonica and *Bactrocera oleae*) (Papaefthimiou and Theophilidis, 2011). In addition, the rapid decrease in HR with lowered free Ca^{2+} in the bath for *Drosophila m.* and inability to locally electrically stimulate the heart tube indicates a quick access of the saline to the heart tube pacemaker cells and contracting cardiac rings of muscle. In speculating why the larvae of *Phaenicia s.* are resistant to the lowered Ca^{2+} bath for as long as 30 min, which is as long as we observed them, either the sarcoplasmic/endoplasmic reticulum can recycle the cytoplasmic Ca^{2+} without it being pumped or exchanged too rapidly to the extracellular side. Considering the heart tube could be locally electrically stimulated with the focal electrode to contract would suggest that Ca^{2+} levels are cycling in the contractile cardiac muscles. Maybe there are multiple layers of cells around the heart tube protecting the pacemaker cells and contractile muscles from rapid changes in the external environment of the hemolymph or bathing solution. The ostia could still have access but maybe the rest of the cells have a protective cellular barrier. Fine anatomical investigation of the heart tube for *Phaenicia s.* larvae would help resolve this issue. As a comparison using the same saline is interesting since larval hearts of *Drosophila m.* stopped but this was not the case for *Phaenicia s.* It is likely using a zero Ca^{2+} bath with chelating compounds the heart for *Phaenicia s.* would slow down and maybe stop as for another insects such as the meal worm (*Tenebrio molitor*) (Markou and Theophilidis, 2000). In addition, depleting the sarcoplasmic/endoplasmic reticulum of Ca^{2+} by using pharmacological agents to block the SERCA pump would address the role of the sarcoplasmic/endoplasmic reticulum in these larvae as has been examined for the larval heart of *Drosophila m.* (Desai-Shah et al., 2010). These additional experiments are beyond the current scope of examining the direct effects of LPS on the larval heart rate but would help dive into more of the differences between the two larval species of *Drosophila m.* and *Phaenicia s.*

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References

Adamo, S.A., Lovett, M.M., 2011. Some like it hot: the effects of climate change on reproduction, immune function and disease resistance in the cricket *Gryllus texensis*. J. Exp. Biol. 214, 1997–2004.

Akasaka, T., Ocorr, K., 2009. Drug discovery through functional screening in the Drosophila heart. Methods Mol. Biol. 577, 235–249. https://doi.org/10.1007/978-1-60761-232-2_18.

Badre, N.H., Martin, M.E., Cooper, R.L., 2005. The physiological and behavioral effects of carbon dioxide on *Drosophila* larvae. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 140, 363–376.

Becker, K.L., Snider, R., Nylen, E.S., 2010. Procalcitonin in sepsis and systemic inflammation: a harmful biomarker and a therapeutic target. British J. Pharmacol. 159 (2), 253–264. https://doi.org/10.1111/j.1476-5381.2009.00433.x.

Bellen, H.J., Tong, C., Tsuda, H., 2010. 100 years of Drosophila research and its impact on vertebrate neuroscience: a history lesson for the future. Nat. Rev. Neurosci. 11 (7), 514–522. https://doi.org/10.1038/nrn2839.

Bier, E., Bodmer, R., 2004. Drosophila, an emerging model for cardiac disease. Gene 342 (1), 1–11.

Catterson, J.H., Knowles-Barley, S., James, K., Heck, M.M., Harmar, A.J., Hartley, P.S., 2010. Dietary modulation of Drosophila sleep-wake behaviour. PLoS One 5 (8), e12062. https://doi.org/10.1371/journal.pone.0012062.

CDC Statistics, 2017. https://www.cdc.gov/nchs/fastats/leading-causes-of-death.htm (Referenced on July 22, 2018).

Celes, M.R., Prado, C.M., Rossi, M.A., 2013. Sepsis: going to the heart of the matter. Pathobiology 80 (2), 70–86. https://doi.org/10.1159/000341640.

Chen, R.X., Liu, F., Li, Y., Liu, G.A., 2012. Neuromedin S increases L-type Ca(2+) channel currents through G(i)α-protein and phospholipase C-dependent novel protein kinase C delta pathway in adult rat ventricular myocytes. Cell. Physiol. Biochem. 30 (3), 618–630. https://doi.org/10.1159/000341443.

Choudhary, V., Choudhary, M., Pandey, S., Chauhan, V.D., Hasnani, J.J., 2016. Maggot

debridement therapy as primary tool to treat chronic wound of animals. Veterinary World 9 (4), 403–409. https://doi.org/10.14202/vetworld.2016.
Christ-Crain, M., Müller, B., 2005. Procalcitonin in –hype, hope, more or less? Swiss Med. Wkly. 135 (31 − 32), 451–460 2005 Aug. 6; bacterial infections.
Cooper, R.L., McNabb, M., 2018. The Effects of a Bacterial Endotoxin LPS on Synaptic Transmission at the Neuromuscular Junction. (In revision).
Cooper, R.L., Neckameyer, W.S., 1999. Dopaminergic neuromodulation of motor neuron activity and neuromuscular function in Drosophila melanogaster. Comp. Biochem. Physiol. B 122, 199–210.
Cooper, A.S., Rymond, K.E., Ward, M.A., Bocook, E.L., Cooper, R.L., 2009. Monitoring heart function in larval Drosophila melanogaster for physiological studies. J. Vis. Exp. 32. http://www.jove.com/index/details.stp?id=1596.
Cripps, R.M., Olson, E.N., 2002. Control of cardiac development by an evolutionarily conserved transcriptional network. Dev. Biol. 246 (1), 14–28.
da Silva Correia, J., Soldau, K., Christen, U., Tobias, P.S., Ulevitch, R.J., 2001. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. J. Biol. Chem. 276 (24), 21129–21135. https://doi.org/10.1074/jbc.M009164200.
Daniels, S., Simkiss, K., Smith, R.H., 1991. A simple larval diet for population studies on the blowfly *Lucilia sericata* (Diptera: Calliphoridae). Med. Vet. Entomol. 5, 283–292.
Dasari, S., Cooper, R.L., 2004. Modulation of sensory to motor circuits by serotonin, octopamine, and dopamine in semi-intact Drosophila larva. Neurosci. Res. 48, 221–227.
Dasari, S., Cooper, R.L., 2006. Direct influence of serotonin on the larval heart of *Drosophila melanogaster*. J. Comp. Physiol. B. 176, 349–357.
Dasari, S., Viele, K., Turner, A.C., Cooper, R.L., 2007. Influence of p-CPA and MDMA on physiology, development and behavior in *Drosophila melanogaster*. Eur. J. Neurosci. 26, 424–438.
de Castro, C., Titlow, J., Majeed, Z.R., Cooper, R.L., 2014. Analysis of various physiological salines for heart rate, CNS function, and synaptic transmission at neuromuscular junctions in Drosophila melanogaster larvae. J. Comp. Physiol. A. 200, 83–92. https://doi.org/10.1007/s00359-013-0864-0.
deCastro, C., Titlow, J., Majeed, Z.R., Vaughn, M., King, K., Cooper, R.L., 2015. Maintaining the drosophila larval heart in situ: Modulators and stretch activated channels. In: The 9th International Congress of Comparative Physiology and Biochemistry. August, pp. 23–28 Kraków, Poland. Abstract book.
Dehus, O., Hartung, T., Hermann, C., 2006. Endotoxin evaluation of eleven lipopolysaccharides by whole blood assay does not always correlate with Limulus amebocyte lysate assay. J. Endotoxin Res. 12 (3), 171–180. https://doi.org/10.1179/096805106X102156.
Desai-Shah, M., Papoy, A.R., Ward, M., Cooper, R.L., 2010. Roles of the sarcoplasmic/endoplasmic reticulum Ca2+-ATPase, plasma membrane Ca2+-ATPase and Na+/Ca2+ exchanger in regulation of heart rate in larval Drosophila. Open Physiol. J. 3, 16–36.
Devleeschouwer, M.J., Cornil, M.F., Dony, J., 1985. Studies on the sensitivity and specificity of the Limulus amebocyte lysate test and rabbit pyrogen assays. Appl. Environ. Microbiol. 50 (6), 1509–1511.
Fink, M.P., 2014. Animal models of sepsis. Virulence 5 (1), 143–153. https://doi.org/10.4161/viru.26083.
Francesconi, F., Lupi, O., 2012. Myiasis. Clin. Microbiol. Rev. 25 (1), 79–105. https://doi.org/10.1128/CMR.00010-11.
Galanos, C., Freudenberg, M.A., Reutter, W., 1979. Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc. Natl. Acad. Sci. USA 76 (11), 5939–5943.
Gangloff, M., Weber, A.N., Gibbard, R.J., Gay, N.J., 2003. Evolutionary relationships, but functional differences, between the Drosophila and human toll-like receptor families. Biochem. Soc. Trans. 31 (Pt 3), 659–663.
Gelman, D.B., Carpenter, J.E., Greany, P.D., 2000. Ecdysteroid levels/profiles of the parasitoid wasp, *Diapetimorpha introita*, reared on its host, *Spodoptera frugiperda* and on an artificial diet. J. Insect Physiol. 46 (4), 457–465.
Gu, G.G., Singh, S., 1995. Pharmacological analysis of heartbeat in Drosophila. J. Neurobiol. 28 (3), 269–280.
Harris, N., Fetter, R.D., Brasier, D.J., Tong, A., Davis, G.W., 2018. Molecular Interface of Neuronal innate immunity, synaptic vesicle stabilization, and presynaptic homeostatic plasticity. Neuron. https://doi.org/10.1016/j.neuron.2018.09.048. (pii: S0896-6273(18)30850-X [Epub ahead of print]).
Hicks, G., Jia, Z., 2018. Structural basis for the lipopolysaccharide export activity of the bacterial lipopolysaccharide transport system. Int. J. Mol. Sci. 19 (9). https://doi.org/10.3390/ijms19092680. (pii: E2680).
Hillyer, J.F., 2016. Insect immunology and hematopoiesis. Dev. Comp. Immunol. 58, 102–118. https://doi.org/10.1016/j.dci.2015.12.006.
Hobai, I.A., Buys, E.S., Morse, J.C., Edgecomb, J., Weiss, E.H., Armoundas, A.A., Hou, X., Khandelwal, A.R., Siwik, D.A., Brouckaert, P., Cohen, R.A., Colucci, W.S., 2013. SERCA Cys674 sulphonylation and inhibition of L-type Ca2+ influx contribute to cardiac dysfunction in endotoxemic mice, independent of cGMP synthesis. Am. J. Physiol. Heart Circ. Physiol. 305 (8), H1189–H1200. https://doi.org/10.1152/ajpheart.00392.2012.
Hobson, R.P., 1935. On a fat-soluble growth factor required by blow-fly larvae. II. Identity of the growth factor with cholesterol. Biochem. J. 29, 2023–2026.
Holowaychuk, M.K., Martin, L.G., 2007. Review of hypocalcemia in septic patients. J. Vet. Emerg. Crit. Care 17, 348–358. https://doi.org/10.1111/j.1476-4431.2007.00246.x.
Howick, V.M., Lazzaro, B.P., 2014. Genotype and diet shape resistance and tolerance across distinct phases of bacterial infection. BMC Evol. Biol. 14 (1), 56. https://doi.org/10.1186/1471-2148-14-56.
Jayaprakash, N., Gajic, O., Frank, R.D., Smischney, N., 2018. Elevated modified shock index in early sepsis is associated with myocardial dysfunction and mortality. J. Crit. Care 43, 30–35. https://doi.org/10.1016/j.jcrc.2017.08.019.
Johnson, E., Ringo, J., Dowse, H., 1997. Modulation of Drosophila heartbeat by neurotransmitters. J. Comp. Physiol. B. 167 (2), 89–97.
Johnson, E., Sherry, T., Ringo, J., Dowse, H., 2002. Modulation of the cardiac pacemaker of Drosophila: cellular mechanisms. J. Comp. Physiol. B. 172 (3), 227–236. https://doi.org/10.1007/s00360-001-0246-8.
Kreger, A.S., Lyerly, D.M., Hazlett, L.D., Berk, R.S., 1986. Immunization against experimental *Pseudomonas aeruginosa* and *Serratia marcescens* keratitis. Vaccination with lipopolysaccharide endotoxins and proteases. Invest. Ophthalmol. Vis. Sci. 27 (6), 932–939.
Listerman, L., Deskins, J., Bradacs, H., Cooper, R.L., 2000. Measures of heart rate during social interactions in crayfish and effects of 5-HT. Comp. Biochem. Physiol A. 125, 251–264.
Lorenzon, S., Pasqual, P., Ferrero, E.A., 2002. Different bacterial lipopolysaccharides as toxicants and stressors in the shrimp *Palaemon elegans*. Fish Shellfish Immunol. 13 (1), 27–45.
Luchi, M., Morrison, D.C., 2000. Comparable endotoxic properties of lipopolysaccharides are manifest in diverse clinical isolates of gram-negative bacteria. Infect. Immun. 68 (4), 1899–1904.
Mahlen, S.D., 2011. Serratia infections: from military experiments to current practice. Clin. Microbiol. Rev. 24 (4), 755–791.
Majeed, Z.R., Nichols, C.D., Cooper, R.L., 2013. 5-HT stimulation of heart rate in Drosophila does not act through cAMP as revealed by pharmacogenetics. J. Appl. Physiol. 115, 1656–1665.
Majeed, Z.R., Stacy, A., Cooper, R.L., 2014. Pharmacological identification of serotonin receptor subtypes on the Drosophila larval heart and aorta. J. Comp. Physiol. B. 184 (2), 205–219.
Majeed, Z.R., Ritter, K., Robinson, J., Blümich, S.L.E., Brailoiu, E., Cooper, R.L., 2015. New insights into the acute actions from a high dosage of fluoxetine on neuronal and cardiac function: Drosophila, crayfish and rodent models. Comp. Biochem. Physiol. C 176–177, 52–61.
Malloy, C., Ritter, K., Robinson, J., Cooper, R.L., 2016. Pharmacological identification of cholinergic receptor subtypes on *Drosophila melanogaster* larval heart. J. Comp. Physiol. B. 186 (1), 45–57.
Malloy, C., Sifers, J., Mikos, A., Samadi, A., Omar, A., Hermanns, C., Cooper, R.L., 2017. Using optogenetics to assess neuroendocrine modulation of heart rate in *Drosophila melanogaster* larvae. J. Comp. Physiol. A. 203 (10), 791–806.
Markou, T., Theophilidis, G., 2000. The pacemaker activity generating the intrinsic myogenic contraction of the dorsal vessel of *Tenebrio molitor* (Coleoptera). J. Exp. Biol. 203, 3471–3483.
Mattingly, M., Weineck, K., Costa, J., Cooper, R.L., 2018. Hyperpolarization by activation of halorhodopsin results in enhanced synaptic transmission: neuromuscular junction and CNS circuit. PLoS One 13 (7), e0200107.
Matuszewski, S., Szafałowicz, M., Grzywacz, A., 2014. Temperature-dependent appearance of forensically useful flies on carcasses. Int. J. Legal Med. 128 (6), 1013–1020. https://doi.org/10.1007/s00414-013-0921-9.
Myers, A.L., Harris, C.M., Choe, K.M., Brennan, C.A., 2018. Inflammatory production of reactive oxygen species by Drosophila hemocytes activates cellular immune defenses. Biochem. Biophys. Res. Commun. https://doi.org/10.1016/j.bbrc.2018.09.126. Oct 3. pii: S0006-291X(18)32060-6 [Epub ahead of print].
Nall, A., Sehgal, A., 2014. Monoamines and sleep in Drosophila. Behav. Neurosci. 128 (3), 264–272. https://doi.org/10.1037/a0036209.
Nau, R., Eiffert, H., 2002. Modulation of release of proinflammatory bacterial compounds by antibacterials: potential impact on course of inflammation and outcome in sepsis and meningitis. Clin. Microbiol. Rev. 15 (1), 95–110.
Neckameyer, W.S., Cooper, R.L., 1998. GABA transporters in *Drosophila melanogaster*: developmental expression, behavior, and physiology. Invertebr. Neurosci. 3 (4), 279–294.
Nehme, N.T., Liégeois, S., Kele, B., Giammarinaro, P., Pradel, E., Hoffmann, J.A., et al., 2007. A model of bacterial intestinal infections in *Drosophila melanogaster*. PLoS Pathog. 3 (11), e173. https://doi.org/10.1371/journal.ppat.0030173.
Nonaka, S., Kawamura, K., Hori, A., Salim, E., Fukushima, K., Nakanishi, Y., Kuraishi, T., 2018. Characterization of Spz5 as a novel ligand for Drosophila Toll-1 receptor. Biochem. Biophys. Res. Commun. https://doi.org/10.1016/j.bbrc.2018.10.096. (pii: S0006-291X(18)32252-6 [Epub ahead of print]).
Ocorr, K., Perrin, L., Lim, H.Y., Qian, L., Wu, X., Bodmer, R., 2007. Genetic control of heart function and aging in Drosophila. Trends Cardiovasc. Med. 17 (5), 177–182.
Olexy, V.M., Bird, T.J., Grieble, H.G., Farrand, S.K., 1979. Hospital isolates of Serratia marcescens transferring ampicillin, carbenicillin, and gentamicin resistance to other gram-negative bacteria including pseudomonas aeruginosa. Antimicrob. Agents Chemother. 15 (1), 93–100.
Pandey, U.B., Nichols, C.D., 2011. Human disease models in *Drosophila melanogaster* and the role of the fly in therapeutic drug discovery. Pharmacol. Rev. 63 (2), 411–436.
Papaefthimiou, C., Theophilidis, G., 2011. Octopamine—a single modulator with double action on the heart of two insect species (*Apis mellifera macedonica* and *Bactrocera oleae*): acceleration vs. inhibition. J. Insect Physiol. 57 (2), 316–325.
Park, B.S., Lee, J.O., 2013. Recognition of lipopolysaccharide pattern by TLR4 complexes. Exp. Mol. Med. 45 (12), e66. https://doi.org/10.1038/emm.2013.97.
Piening, B.C., Geffers, C., Gastmeier, P., Schwab, F., 2017. Pathogen-specific mortality in very low birth weight infants with primary bloodstream infection. PLoS One 12 (6), e0180134. https://doi.org/10.1371/journal.pone.0180134. eCollection 2017.
Radimerski, T.M., Grisouard, J., Timper, K., Zulewski, H., Christ-Crain, M., Keller, U., 2011. Role of calcium in lipopolysaccharide-induced calcitonin gene expression in human adipocytes. Innate Immun. 17 (4), 403–413. https://doi.org/10.1177/1753425910377100.
Rameshrad, M., Maleki-Dizaji, N., Vaez, H., Soraya, H., Nakhlband, A., Garjani, A., 2015. Lipopolysaccharide induced activation of toll like receptor 4 in isolated rat heart

suggests a local immune response in myocardium. Iran. J. Immunol. 12 (2), 104–116 (doi: IJIv12i2A3).
Rose, U., Derst, C., Wanischeck, M., Marinc, C., Walther, C., 2007. Properties and possible function of a hyperpolarisation-activated chloride current in Drosophila. J. Exp. Biol. 210 (14), 2489–2500.
Sanyal, S., Jennings, T., Dowse, H., Ramaswami, M., 2006. Conditional mutations in SERCA, the Sarco-endoplasmic reticulum Ca2+-ATPase, alter heart rate and rhythmicity in Drosophila. J. Comp. Physiol. B. 176 (3), 253–263.
Shankar-Hari, M., Phillips, G.S., Levy, M.L., Seymour, C.W., Liu, V.X., Deutschman, C.S., et al., 2016. Sepsis definitions task F: developing a new definition and assessing new clinical criteria for septic shock: for the third international consensus definitions for sepsis and septic shock (sepsis-3). JAMA 315 (8), 775–787.
Singer, M., Deutschman, C.S., Seymour, C.W., Shankar-Hari, M., Annane, D., Bauer, M., et al., 2016. The third international consensus definitions for sepsis and septic shock (sepsis-3). JAMA 315 (8), 801–810.
Steiner, H., 2004. Peptidoglycan recognition proteins: on and off switches for innate immunity. Immunol. Rev. 198, 83–96.
Taveira da Silva, A.M., Kaulbach, H.C., Chuidian, F.S., Lambert, D.R., Suffredini, A.F., Danner, R.L., 1993. Brief report: shock and multiple-organ dysfunction after self-administration of Salmonella endotoxin. N. Engl. J. Med. 328, 1457–1460. https://doi.org/10.1056/NEJM199305203282005.
Thalakoti, S., Patil, V.V., Damodaram, S., Vause, C.V., Langford, L.E., Freeman, S.E., Durham, P.L., 2007. Neuron-glia signaling in trigeminal ganglion: implications for migraine pathology. Headache 47 (7), 1008–1023.
Titlow, J.S., Rufer, J., King, K., Cooper, R.L., 2013. Pharmacological analysis of dopamine modulation in the *Drosophila melanogaster* larval heart. Physiol. Rep. 1 (2), e00020. https://doi.org/10.1002/phy2.20.
Tomberlin, J.K., Crippen, T.L., Tarone, A.M., Chaudhury, M.F.B., Singh, B., Cammack, J.A., Meise, R.P., 2017. A review of bacterial interactions with blow flies (Diptera: Calliphoridae) of medical, veterinary, and forensic importance. Ann. Entomol. Soc. Am. 110 (1), 19–36. https://doi.org/10.1093/aesa/saw086.
Vadstrup, S., Pedersen, T.F., 1993. Calcium blood changes in septic conditions. Ugeskr. Laeger 155 (48), 3905–3908.
Villalon, N., Farzan, N., Freeman, K., 2018. Rate of bacteremia in the hemodialysis patient presenting to the emergency department with fever: a retrospective chart review. Int J Emerg Med 11 (1), 29. https://doi.org/10.1186/s12245-018-0188-5.
Werner, T., Liu, G., Kang, D., Ekengren, S., Steiner, H., Hultmark, D., 2000. A family of peptidoglycan recognition proteins in the fruit fly *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U. S. A. 97, 13772–13777.
Wessells, R.J., Bodmer, R., 2004. Screening assays for heart function mutants in Drosophila. BioTechniques 37 (1), 58–60.
Yang, J., Zhang, R., Jiang, X., Lv, J., Li, Y., Ye, H., Liu, W., Wang, G., Zhang, C., Zheng, N., Dong, M., Wang, Y., Chen, P., Santosh, K., Jiang, Y., Liu, J., 2018. Toll-like receptor 4-induced ryanodine receptor 2 oxidation and sarcoplasmic reticulum Ca2+ leakage promote cardiac contractile dysfunction in sepsis. J. Biol. Chem. 293 (3), 794–807. https://doi.org/10.1074/jbc.M117.812289.
Yoshida, H., Kinoshita, K., Ashida, M., 1996. Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori*. J. Biol. Chem. 271, 13854–13860.
Yu, S., Zhang, G., Jin, L.H., 2018. A high-sugar diet affects cellular and humoral immune responses in Drosophila. Exp. Cell Res. 368 (2), 215–224. https://doi.org/10.1016/j.yexcr.2018.04.032.
Zhu, Y.-C., Yocum, E., Sifers, J., Uradu, H., Cooper, R.L., 2016a. Modulatory effects on Drosophila larva hearts in room temperature, acute and chronic cold stress. J. Comp. Physiol. B. 186 (7), 829–841.
Zhu, Y.-C., Uradu, H., Majeed, Z.R., Cooper, R.L., 2016b. Optogenetic drive of Drosophila heart rate at different temperatures and Ca2+ concentrations. Phys. Rep. 4 (3), e12695.