



The effects of Gram-positive and Gram-negative bacterial toxins (LTA & LPS) on cardiac function in *Drosophila melanogaster* larvae

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ABSTRACT

The effects of Gram negative and positive bacterial sepsis depend on the type of toxins released, such as lipopolysaccharides (LPS) or lipoteichoic acid (LTA). Previous studies show LPS to rapidly hyperpolarize larval *Drosophila* skeletal muscle, followed by desensitization and return to baseline. In larvae, heart rate increased then decreased with exposure to LPS. However, responses to LTA, as well as the combination of LTA and LPS, on the larval *Drosophila* heart have not been previously examined. This study examined the effects of LTA and a cocktail of LTA and LPS on heart rate. The combined effects were examined by first treating with either LTA or LPS only, and then with the cocktail. The results showed a rapid increase in heart rate upon LTA application, followed by a gradual decline over time. When applying LTA followed by the cocktail, an increase in the rate occurred. However, if LPS was applied before the cocktail, the rate continued declining. These responses indicate the receptors or cellular cascades responsible for controlling heart rate within seconds and the rapid desensitization are affected by LTA or LPS and a combination of the two. The mechanisms for rapid changes which are not regulated by gene expression by exposure to LTA or LPS or associated bacterial peptidoglycans have yet to be identified in cardiac tissues of any organism.

1. Introduction

Sepsis is estimated to kill 11 million people each year and is primarily caused by bacteria (World Health Organization, 2020). Each year, around 1.7 million adults in the United States develop sepsis; in 2022, 350,000 of these adults died during their hospitalization (CDC Statistics, 2022). The immune responses resulting from exposure to bacteria vary according to which type of bacterial strain is involved and which substances are released from bacteria. Lipopolysaccharides (LPS) from Gram-negative bacteria and lipoteichoic acid (LTA) from Gram-positive bacteria are key toxins (also referred to as endotoxins) that induce the immune response. Most bacterial sepsis research focuses primarily on the response of tissues and gene regulated immune responses. In mammals, the LPS receptor is complex and positioned on the surface of tissues. This is a Toll-like receptor 4 (TLR4) with associated proteins referred to as CD14/TLR4/MD2 complex (Yoshida et al., 1996; Steiner, 2004). The TLR4 protein is noted to be conserved from insects to mammals (Anderson et al., 1985; Levin and Malik, 2017; Poltorak et al., 1998; Tauszig et al., 2000). The receptor for LTA in mammals is referred

to as a Toll-like receptor 2 (TLR2) (Schwandner et al., 1999; Takeuchi et al., 1999a,b). The forms of LTA receptors have been subdivided into Type I (one) and Type II (two) based on their structure and inhibitors which block the synthesis of LTA receptors (Mitchell et al., 2007; Fischer, 1994; Schneewind and Missiakas, 2014; Hong et al., 2014). The TLR2 and TLR4 receptors are on the cell surface (Imler and Hoffmann, 2001) and the downstream actions of these receptors are known (Imler and Hoffmann, 2001). However, only scant research is available regarding the electrophysiological properties of tissues following acute, direct actions of toxins by LTA or LPS on the membrane potential of cells or on ion channels which can alter function of pacemaker cells.

Many studies have focused on the effects of LPS and LTA; however, it is now known that commercially obtained samples of these compounds in isolation also contain other peptidoglycans due to isolation procedures. Fragmented forms of LPS and LTA are present in samples due to degradation during the isolation procedures (Hong et al., 2014; Hardy and White, 2001; Hirschfeld et al., 2000; Gao et al., 2001; Morath et al., 2001; Morath et al., 2002). Ultra-pure forms of LPS are known to reveal different results than from commercially obtained samples for the few

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studies in which ultra-pure samples were able to be obtained (Kaneko et al., 2004; Ochoa-Cortes et al., 2010). It is known that Gram negative bacteria (i.e. *Bordetella pertussis*) also releases a toxin referred to as repeats-in-toxin (RTX) (Linhartova et al., 2010). This RTX toxin affects adenylate cyclase and results in a rapid K^+ efflux from cells (i.e., sheep erythrocytes and Jurkat cells, a human T cell leukemia) (Gray et al., 1998). Different types of bacteria release various forms of RTX toxins, and not all have been investigated for their actions (Hertle, 2000). Commercially obtained LPS may also contain RTX toxin, as well as other peptidoglycans, in addition to LPS, resulting in the observed effects assumed to be more related to LPS (Ballinger-Boone et al., 2020). It is of interest to know that commercial lipoteichoic acid preparations may have some LPS contamination (Morath et al., 2002). However, during sepsis in an intact organism, the organism would be exposed to all of these factors associated with bacteria. Thus, use of commercially obtained LPS/LTA serves a purpose in examining the potential effects of the bacteria and all of its components, but one should be aware that the observed effects may not be due solely to the LPS or LTA compounds.

It is interesting to note that both the *toll* genes and initial immunological relationship to bacterial infection with Toll receptors were first described in *Drosophila* (Anderson et al., 1985; Imler and Hoffmann, 2001; Belvin and Anderson, 1996; Lemaitre et al., 1996). Examining the effects of cardiac function with bacterial infections in insects has been primarily investigated in mosquitoes and *Drosophila*. Whole bacterial injection in mosquitoes of various strains (*Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, and *Staphylococcus epidermidis*) reduced heart rate over a period of days (Estévez-Lao et al., 2020). In mosquitoes the mechanism of action to reduce heart rate is correlated with the production of nitric oxide (NO) by peristial hemocytes. This was substantiated by inducing inhibition of NO production and demonstrating that bacterial-induced decrease in heart rate did not occur (Estévez-Lao et al., 2020). Additionally, it was demonstrated by the fact that nitric oxide from injected chicken lysozymes also resulted in a depressed heart rate. As in *Drosophila* reducing the levels of nitric oxide through dietary cobinamide, an effective NO scavenger, or direct injections improved survival of flies injected with LPS from *E. coli* (Procópio Pinheiro et al., 2020). The reduction in heart rate measured after 4 h post-injection with LPS injection was blocked in adult *Drosophila* with the NO scavenger (Procópio Pinheiro et al., 2020). The reduction in heart rate over days with LPS or whole bacterial exposure can likely be explained by high-tend NO production; however, the rapid action within seconds of hyperpolarizing body wall muscle of larval *Drosophila* with exposure to LPS is not induced by NO as L-NAME (NOS inhibitor) did not block the response (Procópio Pinheiro et al., 2020). The effects of LPS on body wall muscle is also not due to activation of a Cl^- current (Stanley et al., 2019) but maybe due to a transiently activated K^+ current (Stanley et al., 2019). However, it was also demonstrated in the Vietnamese stick insect, *Baculum extrudentatum*, that exposure to NO by application of 6-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-hexanamine (MAHMA-NONOate) led to dose-dependent decrease in heart rate and could be inhibited by L-NAME within minutes (da Silva et al., 2012). It must be acknowledged that NO does not depress heart rates in all insects, as it caused a dose-dependent increase in heart rate within minutes with NO donors in locust (Bullerjahn et al., 2006). In the locust, it appears neurons innervating the heart release NO with electrical activity.

In the intact organisms the hemocytes likely are responsible for the production of NO to alter cardiac function in insects (Estévez-Lao et al., 2020; Procópio Pinheiro et al., 2020; da Silva et al., 2012; Yan et al., 2022). In dissected larvae with a saline flush and bathing in saline, the hemolymph is replaced along with freely floating cellular components, such as hemocytes and lysozymes. Lysozymes are generally in the gut of *Drosophila* (Daffre et al., 1994) and could be released during dissection to expose the heart tube in larvae; however, the saline rinsing and flushing though the heart tube would greatly reduce such immune interactions for the *in situ* dissected preparations. Sessile haemocytes are likely present in and on the intact larval heart tube; however after

rinsing and flushing, it would be interesting to know how many sessile haemocytes would still be present to release NO and, thus, have an effect on the pacing heart. We assume LPS/LTA may well have a direct action on ion channels which rapidly alter membrane potential; thus, heart rate is altered similarly to the rapid (within a second) effects noted on body wall muscles of larval *Drosophila* (Stanley et al., 2019).

The immune response in *Drosophila* for Gram-positive bacteria is mediated via the Toll cascade and in time leads to the production of antimicrobial peptides (AMPs) which is mediated via immune deficiency (IMD) pathway (Hetru and Hoffmann, 2009; Aggarwal and Silverman, 2008). The review by Bangham et al., (Bangham et al., 2006) nicely highlights the mechanism by which Gram-positive bacteria mediates the cellular response through the Spaetzle-Toll receptor complex to activate the genomic response to produce AMPs and that Gram-negative bacteria mediates the cellular response through the PGRP-LE/PGRP-LC receptors and the Imd cascade through the NF- κ B factor. Relish for the genomic response to produce AMPs. However, the use of RNAi expression for PGRP-LC and PGRP-LE did not alter the acute responses to LPS on the larval *Drosophila* body wall muscles (Ballinger-Boone et al., 2020). When using whole bacteria or lysed bacteria for physiological assays, there are other factors besides LPS and LTA to consider, such as repeats-in-toxin (RTX), which can make pores in the membrane of cells. In addition, commercially available LPS may have such contaminants as RTX, lipoproteins, glutamate, and even adenosine (Hardy and White, 2001; Hirschfeld et al., 2000; Ochoa-Cortes et al., 2010; Linhartov et al., 2010; Gray et al., 1998; Hertle, 2000). Ultrapure forms of LPS produce different responses from commercially obtained forms (Hardy and White, 2001; Hirschfeld et al., 2000; Ochoa-Cortes et al., 2010). Thus, it is hard to assess the absolute effects directly attributed to LPS or LTA when commercially obtained.

Since it is known that LPS rapidly hyperpolarizes skeletal muscle of larval *Drosophila* and crayfish (Saelinger et al., 2019) as well as rapidly depressing synaptic transmission at the neuromuscular junction (NMJ) of larval *Drosophila* (Cooper et al., 2019; Vacassenno et al., 2023) but enhance synaptic transmission at the NMJ of crayfish (Saelinger et al., 2019), it is assumed the response to LPS maybe indeed be non-genomic and target ion channels. These acute responses are too immediate and rapid for gene regulation of protein synthesis to be affecting membrane potential in such a way. In the skeletal muscle of *Drosophila*, the hyperpolarization response appears to be a transient activation of two-pore domain potassium (K2p) channels (Greenhalgh et al., 2021). The TWIK-1, TASK-1, and tandem-pore acid-sensing K^+ (TASK-3) channels are sensitive to protonation in low pH. Since larval body wall muscle depolarizes with low pH and the heart increases in rate with slightly lower pHs, acid-sensitive channels such as those of the K2p family may be present in these tissues.

K2p channels are known to be present ubiquitously throughout the plant and animal kingdoms, with a number of subtypes. These K^+ channels are responsible for maintaining the resting membrane potential of cells. Within organisms, their expression profile, their specific characterization yielded by their subtype and their density varies by cell within a given tissue. There may even be multiple subtypes within a given cell. Subsets sensitive to acid appear to be present in the skeletal muscle of larval *Drosophila*, since the resting membrane potential is very sensitive to pH (Cooper and Krall, 2022). The antagonist to these particular K2p channels, doxapram, also leads to depolarization of the skeletal muscle (Cooper and Krall, 2022). This is important when one considers the larval *Drosophila* heartbeat is also very sensitive to pH, as its rate increases in a lower than a pH of 7.4 and maintains a rapid rate at pH 7.0 (De Castro et al., 2014). It appears that LPS transiently activates a K2p channel, resulting in hyperpolarization in skeletal muscle of the larval *Drosophila*. Within 2 to 3 min, the muscle desensitizes to LPS, causing the membrane potential to return to its initial values. The rapid onset and desensitization of the responses to LPS on skeletal muscle is also a phenomenon common to the larval *Drosophila* heart (Anyagaligbo et al., 2019; Ista et al., 2020). Thus, it is of interest to investigate

whether LTA demonstrates similar actions as LPS in alteration of the heart rate of larval *Drosophila*.

The K2p channel is referred to as the ORK channel in *Drosophila* (Buckingham et al., 2005; Lalevee et al., 2006; Goldstein et al., 1996). The *kcnk* \emptyset gene codes for a K2p channel present in neuromuscular junctions (NMJs). NMJs are composed of a variety of cell types and ion channels (Zilberberg et al., 2000; Zilberberg et al., 2001; Ilan and Goldstein, 2001; Adams et al., 2000). There are 11 known *kcnk* \emptyset genes in *Drosophila* (Littleton and Ganetzky, 2000; Niederbichler et al., 2006). The pharmacological and physiological profiles of K2p channel subtypes in *Drosophila* have yet to be investigated (Buckingham et al., 2005). One of the ORK (or K2p) channels expressed in the pupal heart is known to promote a diastolic state and the tissue-specific knockdown promotes a faster heartbeat (Lalevee et al., 2006), implying that the myocytes may be more depolarized. However, because the heart still beats in this knockdown line, there are likely other K2p receptor subtypes present on the heart acting to maintain membrane potential.

Past studies of LPS actions on the larval *Drosophila* heart were conducted *in situ* in a minimal physiological saline. Similarly, direct actions of isolated, cultured cardiac myocytes from rodents showed a direct effect by LPS in depression of sarcomere contraction (Mutig et al., 2013). However, the action of LTA in rodents resulted in increased contractile strength, with an increase in both the transient Ca^{2+} concentration and the Ca^{2+} decay (Zila et al., 2015). The effects of LTA were exaggerated with reduced extracellular Ca^{2+} concentrations. The effects of LPS and LTA were examined after at least one hour of incubation (Mutig et al., 2013; Zila et al., 2015). It would be of interest to know if the effects would also occur within minutes of exposure. In rodents, an infusion of LPS induced an increase in heart rate (Buckingham et al., 2005) as well as bradycardia within a minute (Rameshrad et al., 2015), but it was not established if this effect was directly on the heart or on the heart's neural innervation. The effect of LPS in dysfunction of the rodent heart may, in part, be due to decreased L-type Ca^{2+} channel current (Hobai et al., 2013).

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). The *Drosophila* heart continues to serve as a proof of concept for examining cardiac function in mammals (Taghli-Lamalle et al., 2016; Bier and Bodmer, 2004; Cammarato et al., 2011; Ocorr et al., 2007; Wolf et al., 2006; Perrin and Röder, 2016; Ugur et al., 2016). Since the larval heart in *Drosophila melanogaster* is myogenic, it serves as a useful model for related disorders known in mammals (Frankenreiter et al., 2017; Imlach et al., 2010; Lai et al., 2014; Patel et al., 2018; Pineda et al., 2021). In addition, *Drosophila* served a vital role in addressing the actions of the immune response induced by LPS, which later led to a better understanding of the mechanisms of action in mammals (Poltorak et al., 1998; Tauszig et al., 2000). The dissected larvae's isolated heart tube allows one to investigate the direct actions of compounds on the heart independent from an immune response of the intact larvae. The hemolymph of insects and blood of mammals have peptidoglycan recognition proteins (PGRPs). The PGRPs are key for innate immunity in both insects and vertebrates (Dziarski and Gupta, 2006). Removing the compounding variability of the systemic immune system by removing the hemolymph/blood and flushing the tissue with saline, as well as removing other organs, allows one to investigate both direct and acute actions within a few seconds. This provides the opportunity to address other responses independent of the systemic induced response in an intact organism. Cytokines can act in an "autocrine" fashion, and it is possible that the body wall muscle or heart cells can induce cytokines and activate themselves; however, a dissected preparation flushed with saline at a much larger volume than the larval's hemolymph volume would minimize such effects. In addition, the response to LPS or LTA is immediate when either exposed to body wall muscles or the heart, which suggests a direct action on these tissues. This study adds significantly to what is currently known about LTA toxicity in intact organisms, as direct actions were investigated

within seconds. In addition, the combined action of LPS and LTA, as well as the other compounds in the bacterial extracts, address the actions that might take place in bacterial septicemia induced by a mix of Gram-positive and -negative bacteria.

Given that no studies have addressed the effects of LTA on cardiac physiology in larvae *Drosophila*, it was of interest to us to determine whether similar responses would result from LTA exposure as was previously observed after LPS exposure, or if an opposing response occurs for LTA as compared to LPS effects reported for rodent cardiac myocytes. In addition, only a few studies address the combined effects of LTA and LPS on physiological functions in animal models in general. Finney et al., (Finney et al., 2012) examined the effect on cytokine production by LPS and LTA alone and in combination in mice. The cocktail of LPS and LTA produced a larger production of the cytokine IL10 than LTA or LPS alone. Thus, there is precedent enough to suggest that the cocktail might have an enhanced effect on cardiac function in *Drosophila*; it would be of interest to know whether application of one or the other toxin might have an altered effect if added while the other endotoxin is already in a state of desensitization.

2. Results

The heartbeat transiently increased with exposure to LTA at a high concentration of 500 $\mu\text{g}/\text{mL}$ but not at a lower concentration of 200 $\mu\text{g}/\text{mL}$ (Fig. 1A1 and B1). The increase occurred within the first full minute of exposure and then the rate gradually decreased. An average heart rate among preparations shows a similar trend but, due to the wide variation between individual preparations, the average response to the various conditions is not as representative of the effects (Fig. 1A2 and B2).

The initial differences in heart rate between preparations were normalized to examine the effects of LTA exposure. Observation of percent change from the initial rate in saline to that in LTA and then to the rates after exposure over time for each preparation provides some insight into the trends (Fig. 2). The average increase in percent change for initial exposure to LTA at 200 $\mu\text{g}/\text{mL}$ was 8% whereas, at 500 $\mu\text{g}/\text{mL}$, the percent change was 12%. The rapid increase in heart rate following exposure to 500 $\mu\text{g}/\text{mL}$ LTA significantly decreased over a short time interval (Fig. 2).

Previously, it was shown that LPS from *Pseudomonas aeruginosa* or *Serratia marcescens* increased heart rate in larval *Drosophila* at 500 $\mu\text{g}/\text{mL}$ (Daffre et al., 1994). Because of this, as well as the fact that our data showed an increased heart rate following acute LTA exposure, it was of interest to test whether an initial exposure to LTA or LPS followed by a cocktail of the two endotoxins would show additive or antagonizing effects. To account for the rapid decline in the effects of both LPS or LTA, the cocktail was added within 30 s of initial endotoxin exposure. The LTA and cocktail progression resulted in increased heart rate as compared to saline and the 30 s of exposure to the cocktail (Fig. 3A1). The heart rate decreased over time as compared to the peak rate during exposure to the cocktail. With initial exposure to LPS being followed by the LTA-LPS cocktail, only the initial exposure to LPS showed a significant increase in heart rate (Fig. 3B1). Over time the rates decreased compared to the peak rate. The average rates of all preparations illustrate the general trends, but the high variation minimizes the effects among individual preparations (Figure 3 A2 and B2).

To better view the effects for individual preparations, a percent change compared to initial values obtained in saline was determined for each condition over time for both the LTA and cocktail progression, as well as the LPS and cocktail one (Fig. 4 A and B, respectively). Comparing the percent change between saline to LTA and saline to LPS to the rate after subsequent exposure to the cocktail over time showed that a significant decrease in rate did not occur until after 5 min for LTA (Fig. 4A). The increase associated with LPS alone rapidly decreased upon cocktail exposure (Fig. 4B).

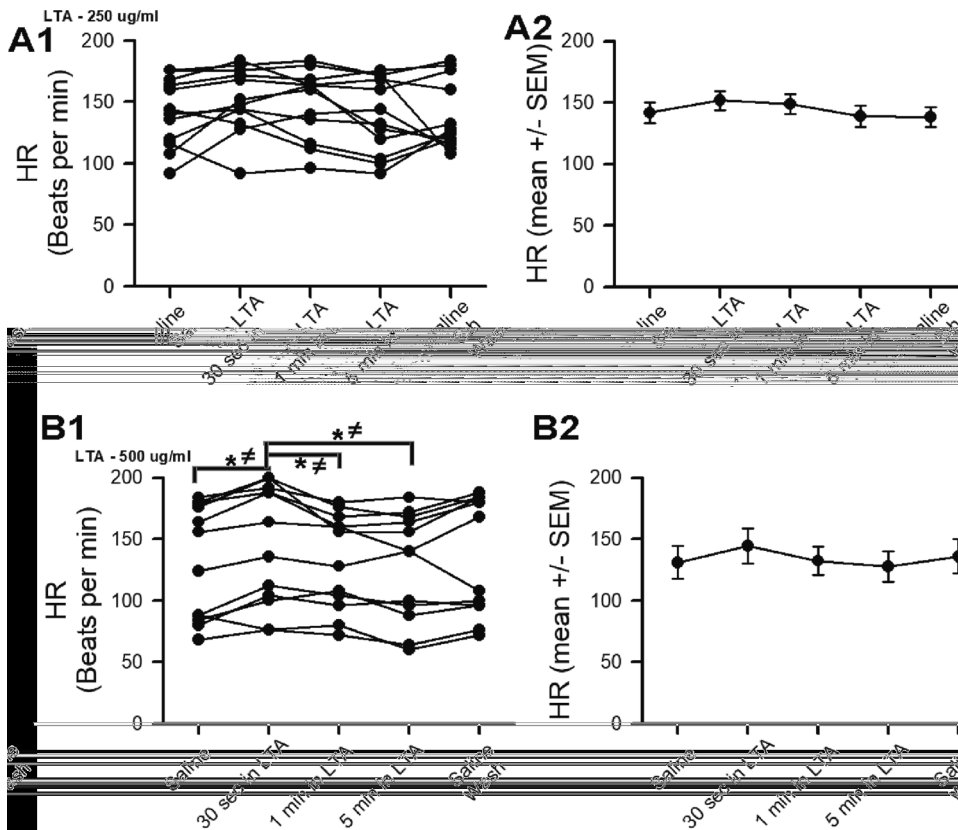


Fig. 1. The effect of lipoteichoic acid (LTA) from Gram-positive bacteria *Staphylococcus aureus* on the heart rate (HR). (A1) The HR of 12 individuals over time with exposure to LTA at 250 µg/mL are shown. (A2) The mean and standard error mean of the values are shown in A1 for each condition over time. (B1) The HR of 12 individuals over time with exposure to LTA at 500 µg/mL are shown. (B2) The mean and standard error mean of the values are shown in B1 for each condition over time. There is no significant differences between the groups for 250 µg/mL by a one-way repeated measures analysis of variance (N = 12). However, a paired *t*-test between saline and 30 s exposure to LTA indicated a two-tailed P-value = 0.0925 and a one-tailed P-value = 0.0462. The 500 µg/mL exposure of LTA was significant for the groups of 30 s to saline as well as to 1 min and 5 min exposures as indicated by the star (*) P < 0.05 one-way repeated measures analysis of variance (normality test of a Shapiro-Wilk and equal variance test of a Brown-Forsythe) with a post-hoc Bonferroni *T*-test. A two-way repeated measures analysis of variance with with a post-hoc Bonferroni *T*-test for significant difference is shown as ≠. The saline wash is not considered in statistical analysis.

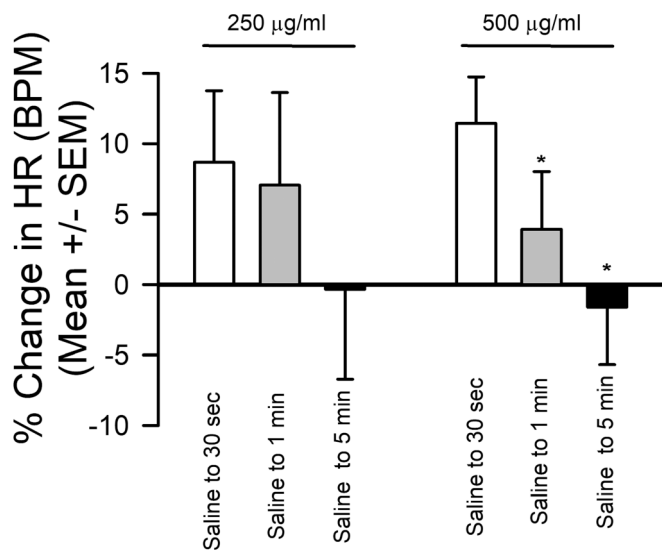


Fig. 2. The percent change in the heart rate (HR) with respect to the initial rate in saline alone within individuals over time for exposure to LTA at 250 µg/mL or 500 µg/mL. A percent change for each individual larvae was determined and an average change for the group was determined. Different groups of larvae were used for the effects of 250 µg/mL or 500 µg/mL of lipoteichoic acid LTA. Since the rates decreased over time it was expected the percent changes to decrease. (N = 12; star (*) P < 0.05 one way repeated measures analysis of variance (normality test of a Shapiro-Wilk and equal variance test of a Brown-Forsythe) with a post-hoc Bonferroni *T*-test.

3. Discussion

While it has been previously shown that LPS did have a transient effect in increasing heart rate, LTA's effects on the larval *Drosophila* heart had not yet been investigated (Anyagaligbo et al., 2019). In mammals it appears that LPS is mediated via TL4 receptor complex, and LTA is mediated via a TL2 receptor (Takeuchi et al., 1999a). However, reports of potential rapid effects within seconds of exposure to either LTA or LPS in mammals are not fully addressed; neither are the effects in mixtures of bacterial endotoxins. In *Drosophila*, it appears that the immune response to LPS is mediated via IMD pathway. The IMD cascade is activated by PGRP-LE and PGRP-LC surface receptors (Martin et al., 2018); however, the rapid effects on membrane potential of skeletal muscle does not appear to utilize the IMD receptors (Ballinger-Boone et al., 2020) and, instead, seem to use a subset of K2p channels (Cooper and Krall, 2022). The rapid response within 1 to 2 s, as observed on LPS-exposed skeletal muscle, would likely be mediated via ion channels instead of altered protein expression. As far as we are aware, the acute effects of LTA on membrane potential have yet to be examined with skeletal muscle in mammals as well as *Drosophila*. The responses to Gram-positive bacteria in invertebrates are mediated via Spaetzle and a Toll cascade (Lemaitre et al., 1996; Bangham et al., 2006; Valanne et al., 2011; Valanne et al., 2010) but the cascade has yet to be linked to ion channels that could rapidly alter membrane potential and impact the heart rate. Toll-like receptors interact directly with microbial components, making them bona fide pattern recognition receptors. In insects, microbial detection leads to the cleavage of Spaetzle, which then binds Toll and activates the pathway. Thus, Toll receptors are not pattern recognition receptors and are subject to different evolutionary pressures in mammals and insects. In summary, from a functional perspective, Toll-like and Toll receptors function differently in humans and insects.

Further analysis into the prolonged exposure of LTA on cardiac function within the *Drosophila* model may provide insight into the type of cellular responses carried out in specific pathways, as well as into the

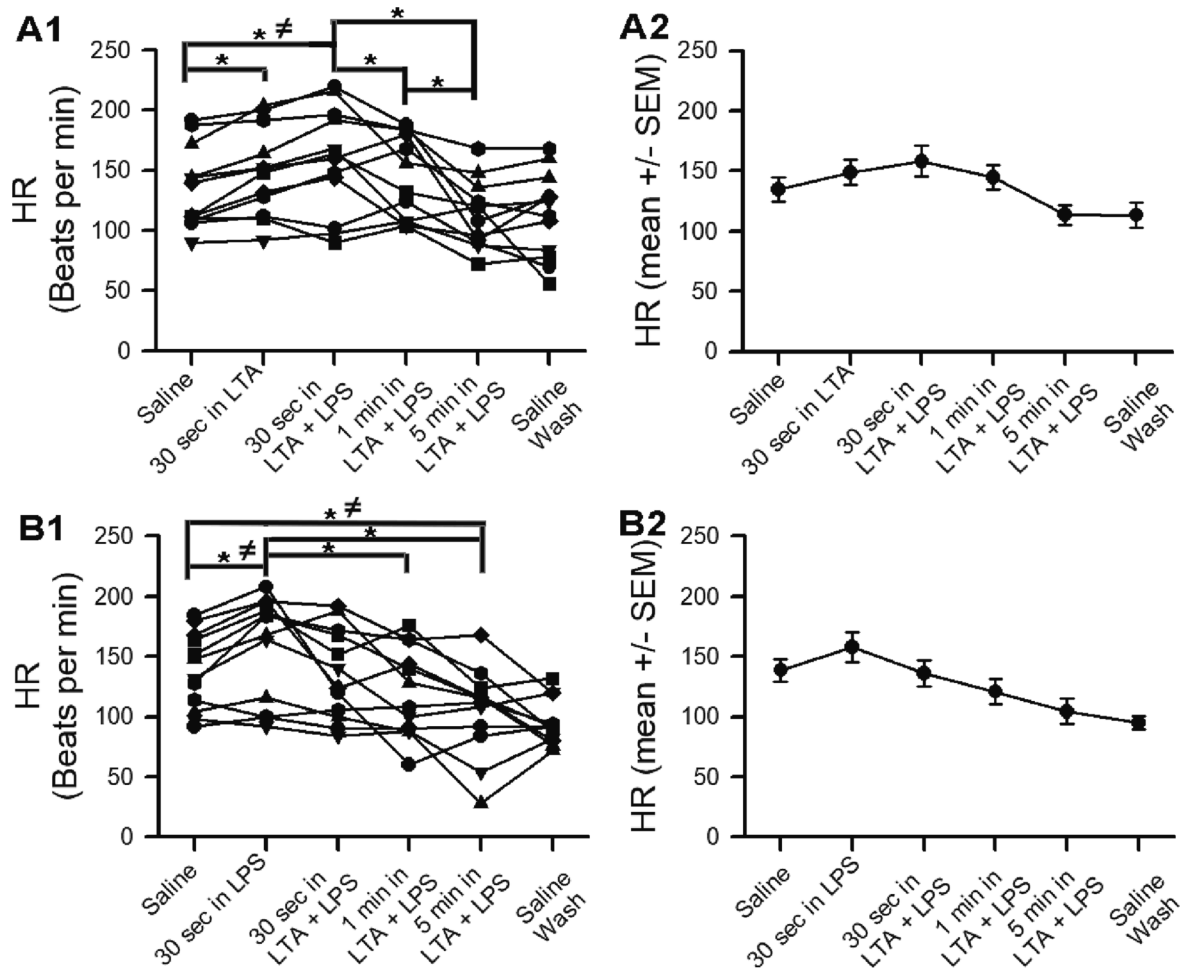


Fig. 3. The heart rate of larval *Drosophila* while acutely exposed to either lipopolysaccharides (LPS), from Gram-negative bacteria, and lipoteichoic acid (LTA), from Gram-positive bacteria, endotoxin alone or in combination. (A1) The larval *in situ* hearts were exposed to saline and, subsequently, to LTA (500 µg/mL) followed by a cocktail of LTA and LPS (both at 500 µg/mL) over time, and finally a flush with fresh saline. (A2) A mean (+/-SEM) of the individual preparations shown in A1. (B1) The *in situ* larval hearts were exposed to saline and, subsequently, to LPS (500 µg/mL), followed by a cocktail of LTA and LPS (both at 500 µg/mL) over time, and finally a flush with fresh saline. (B2) A mean (+/-SEM) of the individual preparations shown in A1. The star (*) P < 0.05 one-way repeated measures analysis of variance (normality test of a Shapiro-Wilk and equal variance test of a Brown-Forsythe) with a Bonferroni *t*-test. A two-way repeated measures analysis of variance with with a post-hoc Bonferroni *T*-test for significant difference is shown as #. The saline wash is not considered in statistical analysis.

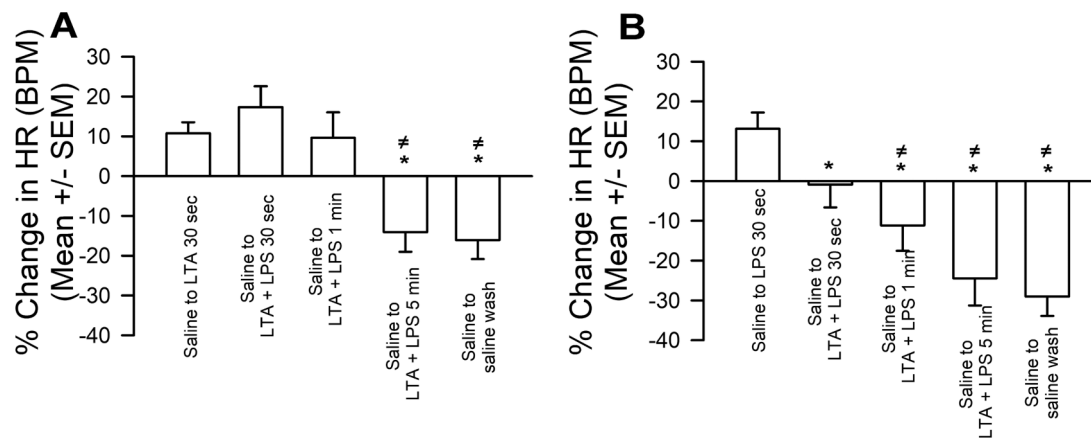


Fig. 4. The percent change in the heart rate (HR) with respect to the initial rate in saline alone within individuals over time for exposure to either lipopolysaccharides (LPS), or lipoteichoic acid (LTA) alone or in combination. A percent change for each individual larvae was determined and an average change for the conditions shown as in Fig. 3. The raw data for determining the percent changes are shown in Fig. 3. Star (*) P < 0.05 one-way repeated measures analysis of variance with with a post-hoc Bonferroni *T*-test. A two-way repeated measures analysis of variance with with a post-hoc Bonferroni *T*-test for significant difference is shown as #. All compared to group of salines to the 30 s of exposure to LTA (A) or LPS (B).

question of whether the Spaetzle and Toll cascades have a function in cardiac tissue. Perhaps LTA's transient rapid effects are mediated by additional pathways yet to be discovered for LTA in *Drosophila* and, potentially, in mammals.

In rodents, LTA was shown to reduce sarcomere shortening with myocytes *in vitro*, but *in vivo* studies did not indicate substantial hemodynamic changes of the sort that would be correlative to the *in vitro* observations. However, LTA still resulted in inflammatory responses in whole animals over time (Boehm et al., 2013). Many studies address the isolated effects of one strain of LPS or LTA, but few address a cocktail of LPS strains and combinations of LPS and LTA. It is worthy to note that mice did show an enhanced elevation in cytokine IL10 to the combined response as compared to the individual exposures to LTA or LPS (Finney et al., 2012). A synergistic effect in the immune responses by LTA and LPS have also been shown in bovine epithelial cells (Wu et al., 2020). Cocktails of different LPS or LTA strains alone would be of interest, as well as combinations of LPS and LTA strains to determine acute actions and prolonged responses within both intact organisms and isolated tissues and cells. In the studies herein, high concentrations of LPS and LTA were used. The 250 µg/mL of LTA did not produce any rapid effects on heart rate. However, examining effects over a longer period might indeed produce alterations. This study addresses the rapid acute effects within seconds to a few minutes. Studies using cell culture of human and murine cells have used *S. aureus*-LTA at 50 µg/mL for examining effect over 24 h (Zadeh et al., 2012). Concentrations in the range from 0.01 to a 100 µg/mL have been used to address the effect of LTA on T84 intestinal epithelial cells (Cabral et al., 2013). Bone cells (i.e., MC3T3-E1) were treated with 100 µg/mL of LTA for 3 days to assay viability and osteogenic induction (Ishihata et al., 2022). The 5 times higher concentration used in this study was chosen to accentuate any of the potential actions of LTA since the 250 µg/mL did not produce any notable effects on the larval heart rate. Even rodents are quite different in responses as compared to humans in relation to septic actions. A range of 1 to 500 µg/mL exposure is enough to cause death in humans, as well as rodent models. The LD50 for mice is 1–25 mg/kg and this is 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; Luchi and Morrison, 2000; Taveira da Silva et al., 1993).

The transient responses in the altered heart rate of larval *Drosophila* suggest that the receptors or cellular cascades that mediate the response rapidly desensitize as the tissue attempts to regain a normal function. It is also not yet established if a homeostatic mechanism drives the response to return to the condition it had been in prior to the endotoxin exposure. A homeostatic mechanism in systemic regulation is unlikely due to the preparations being *in situ*, bathed in a saline free of circulating hormones and peptides, and featuring no neural innervation. The nature of the larval myogenic heart is comparable to the mammalian heart in that pacemaker cells divide the rate such that different regions of the heart tube have their own intrinsic rate (Majeed et al., 2014). However, the pacemaker cells in the caudal region override the more slowly paced regions of the heart tube. The pacing of the heart is correlated with the extracellular concentration of free Ca²⁺ (Desai-Shah et al., 2010). Thus, intracellular Ca²⁺ regulation is key and is dependent on various cellular processes in the larval heart just as it is for hearts in mammals (Hove-Madsen et al., 2004; Morgan, 1991). Attempts have been made to unravel the contribution of these processes to regulation of the Ca²⁺ dynamics within the larval heart for pacing (Majeed et al., 2013). The plasmalemmal Na⁺/Ca²⁺ exchanger (NCX), the Ca²⁺-ATPase (PMCA) as well as the Sarcoplasmic/Endoplasmic Reticulum Ca²⁺-ATPase (SERCA) on the endoplasmic reticulum in cells of the larval heart all have some role, with both the NCX and PMCA playing a dominant role in regulating the heart rate (Valanne et al., 2011). Thus, a rapid disturbance of the intracellular Ca²⁺ concentration by LPS or LTA could be compensated for quickly by the NCX, PMCA or even the SERCA. It is known that cAMP does not appear to have a large role in altering larval heart rate through serotonin modulation (Majeed et al., 2013), but it does appear that IP3

may be the mediator by actions on the SER (Majeed et al., 2014; Dasari and Cooper, 2006). It would be interesting to examine if the recovery of the hyperpolarized membrane potential, while exposed to LPS or LTA, would be prolonged were the NCX or PMCA were compromised.

It is apparent that the physiological saline is not optimal for the heart since the heart rate generally decreases in dissected larvae as compared to intact larvae (Desai-Shah et al., 2010). The *Drosophila* larval heart is myogenic and does not have neural innervation in the early 3rd instar, so one can rule out any direct neural influence (which may not be the case in other insect preparations investigating the effects of compounds on cardiac function). It is known that the heart rate in many insects, including larval *Drosophila*, is modulated by biogenic amines and peptides (Hillyer, 2018; Dasari and Cooper, 2006). The effect of the dissection, and possibly the effects of basic saline producing such varied heart rates among individuals, is surprising, as the same saline performs extremely well for skeletal NMJs of *Drosophila* larvae (De Castro et al., 2014). The numerous peptides and biogenic amines in the hemolymph may influence the inotropic and chronotropic nature of the heart as observed in intact preparations. However, the modulators and peptides introduce uncontrolled variability when assessing compounds directly on the heart as stress of a restrained intact larvae or even the stress of an injection into larvae may release modulators into the hemolymph. It is known that optogenetically stimulating 5-HT, dopamine or octopamine containing neurons in intact larvae affects heart rate (Malloy et al., 2017).

It is of interest to know if the rate and degree of the acute effects of LPS or LTA are dependent on temperature, or even dependent on the ionic composition of the saline bath, particularly regarding the concentration of Ca²⁺. Sepsis is known to alter free Ca²⁺ homeostasis; thus, understanding endotoxin effects in varied Ca²⁺ concentrations is needed (Steinhorn et al., 1990; Zaloga, 2000). We are now addressing whether internal Ca²⁺ stores within the sarcoplasmic reticulum have a role in the rapid response of the cardiac system to LPS and LTA. It is too early to propose a model yet of how LPS and LTA may be regulating the pacemaker cells until one can provide Ca²⁺ imaging and/or electrophysiological recordings of the myocytes.

4. Materials and methods

Drosophila melanogaster, Canton S (CS) flies were used in all physiological assays. This strain has been isogenic in the lab for several years and was originally obtained from Bloomington *Drosophila* Stock Center (BDSC).

4.1. Dissection and procedures

The same procedures for dissecting the *Drosophila* larvae and exposing the larval heart were used as described in Anyagaligbo et al., (Anyagaligbo et al., 2019) and shown in video format (Cooper et al., (JoVE) 2009). The heart in the larval *Drosophila* is composed of a dorsal vessel, also referred to as the heart tube, which runs from the most caudal region of the larvae to the base of the brain. The heart tube is divided into the posterior heart and the anterior aorta (Rizki and Wright, 1978). Only the most caudal region of the heart was used to determine the heart rate. Since the larval heart is very susceptible to biogenic amines and peptides that could vary in the hemolymph, the dissected preparation was thoroughly flushed with saline prior to measuring the heart rate.

Only early 3rd instars were used, which were still within the corneal food and did not yet occupy the sides of the vials. Standard saline is HL3 saline (in mM): 1.0 CaCl₂·2H₂O, 70 NaCl, 20 MgCl₂, 5 KCl, 10 NaHCO₃, 5 trehalose, 115 sucrose, 25 5 N, N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) at pH of 7.1 (Anyagaligbo et al., 2019; Stewart et al., 1994). The water used for saline was deionized and freshly filtered via a Milli-Q® IQ water purification system which produces ultrapure water (MilliporeSigma, Burlington, MA, USA).

Exchanges in saline bathing media are shown within the figures. The pharmacological agents used were pure LPS from *Serratia marcescens* (Sigma-Aldrich, St. Louis, MO, USA) and lipoteichoic acid (LTA) from Gram-positive bacteria *Staphylococcus aureus* (InvivoGen, San Diego, CA, USA). LPS and LTA were dissolved in the physiological saline at concentrations specified in the Results. The salts for the saline were obtained from Sigma Chemical Company. The 500 µg/mL was used to match the concentration of the LPS used. The LPS concentration used in this study was used to match the concentrations from earlier studies so direct comparisons in results could be made to the previous findings. We could not find any reports of the concentration of LTA in the GI contents of insects in general for reference.

4.2. Statistical methods

Normality was examined to validate statistical assumptions. Repeated measures of an ANOVA and T-tests were used and stated when used in the Results section. Analysis of Variance in pairwise multiple comparison a post-hoc Bonferroni T-test was used. Significant difference is $p < 0.05$.

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CRediT authorship contribution statement

Kaitlyn E. Brock: Conceptualization, Methodology, Software, Data curation, Writing – original draft, Visualization, Investigation, Supervision, Software, Validation, Writing – review & editing. **Elizabeth R. Elliott:** Conceptualization, Methodology, Software, Data curation, Writing – original draft, Visualization, Investigation, Supervision, Software, Validation, Writing – review & editing. **Maya O. Abul-Khoudoud:** Conceptualization, Methodology, Software, Data curation, Writing – original draft, Visualization, Investigation, Supervision, Software, Validation, Writing – review & editing. **Robin L. Cooper:** Conceptualization, Methodology, Software, Data curation, Writing – original draft, Visualization, Investigation, Supervision, Software, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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