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Research Article
Investigation Regarding the Physiological Effects of Zinc on *Drosophila* and Crawfish Cardiac, Neural, Synaptic and Behavioral Processes

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Abstract

Background and Objective: Zinc (Zn^{2+}) is essential for physiological function but excessive zinc can cause disruption to physiological processes. This study examined the impact of acute zinc overexposure on various facets of zinc exposure in both *Drosophila* and crawfish, as well as the role of zinc in the mechanism of neurological processes. **Materials and Methods:** *Drosophila* larvae development/survival was examined with feeding various $ZnCl_2$ concentrations (N = 100 and t-Test). The $ZnCl_2$ crawfish injections were observed (N = 6 and t-Test). Effects of $ZnCl_2$ (0.1, 1 mM) at both animals' synaptic transmission at the neuromuscular junction were observed (N = 6 per concentration and paired t-Test). **Results:** Increased dietary Zn^{2+} delayed development, decreased survival/survival time, and impaired normal *Drosophila* behavior. Acute Zn^{2+} exposure eliminated the response to sensory stimulation of crawfish, but increased activity in the muscle receptor organ. Zinc exposure also decreased heart rate (sometimes to elimination) in both *Drosophila* and crawfish. Excessive zinc exposure also drastically reduced excitatory junction potentials of both *Drosophila* and crawfish, though this activity was recovered upon removal of the zinc exposure. **Conclusion:** Overall, acute zinc overexposure largely disrupted neurological and cardiac function, behavior, development and survival of *Drosophila* and crawfish.

Key words: Behavior, crayfish, development, insect, motor, physiology, sensory, zinc

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Zinc is essential for plants and animals, mainly as a co-factor for physiological enzymes[1-3]. However, while pathological disorders caused by dietary zinc deficiency are rare, overdoses have also have occurred. Stress responses (e.g., under military conditions) can trigger intense metabolic and mental demands, as well as immune challenges, which affect zinc requirements[4,5]. Zinc miners face frequent exposure, as airborne particulates are common in the workplace[6-8]. Additionally, though treatments are yet imperfect[8].

Zinc's effects may depend on exposure method and compound form; for example, zinc sulfate's effects may differ from zinc chloride's. Zinc aggregates in the brain, as associated enzymes and transporters occur throughout neural tissue[9,10]. Excess zinc has also been linked to Alzheimer's and Parkinson's, albeit controversially[11-13].

Free zinc occupies the interior regions of the central nervous system for integration of sensory function[10]. However, the effects of excessive zinc on primary sensory neuron physiology are not fully researched, nor are the mechanisms behind sensory transduction. Zinc sulfate has a pathological role in dampening smell for mammals with olfactory epithelium, including humans[14-16], but this has not been examined for zinc chloride.

Given the wide range of stretch-activated channel (SAC) subtypes in existence, the effects of free Zn^{2+} on each individual SAC subtype are not fully catalogued. The SAC subtypes within arthropod proprioceptive sensory neurons, as in the crustacean muscle receptor organ (MRO), have not yet been pharmacologically or molecularly described. It is, thus, unsurprising that the effects of acute zinc exposure on sensory transduction and neuronal biophysical properties in other animal models are not yet understood.

Divalent Zn^{2+} blocks mammalian T-type Ca^{2+} channels[17]. It also reduces transmission at chemical synapses, likely by blocking evoked presynaptic Ca^{2+} entry responsible for vesicular fusion at the neuromuscular junction (NMJ); in mice, both nerve-evoked response and spontaneous quantal events were dampened[18]. However, these effects may vary by model, as squid (*Torpedo*) featured no restriction of evoked vesicle movement at nerve-electroplate junctions, blocked acetylcholine release and calcium accumulation in the stimulated presynaptic terminal[19]. Parducz *et al.*[19] proposed an effect on vesicle pore transmission, contrasting observations of blocked evoked transmission and decreased spontaneous quantal events at the mouse NMJ[20,21]. Literature searches regarding zinc at *Drosophila* or crawfish NMJs were unsuccessful.

Excessive zinc alters cardiac function. In isolated guinea pig hearts, bradycardia occurred upon exposures of 7.5 µM and 15 µM. Exposure at 30 µM was toxic, causing, within 15 min, a first-degree atrioventricular block, idioventricular rhythms, atrial and ventricular extrasystoles, and asystolia[22]. Few reports discussed the effects of excessive zinc on mammalian cardiovascular function and almost none on invertebrates generally.

Research into zinc's developmental effects largely focuses on dietary deficiency, rather than overexposure[23,24]. Since zinc is commonly found in over-the-counter supplements and cold/flu remedies, excessive consumption may well occur in both adults and children[25]. Additionally, zinc can be transferred from mother to child via breast milk[24]. Zinc supplementation was also connected to reduced mortality from severe pneumonia during the COVID-19 pandemic[26].

Environmental zinc exposure also warrants studying. Exposure of this sort generally requires gastrointestinal transport mechanisms or respiratory structures (i.e., gills) to enter an organism. However, a substance does not need to reach systemic circulation to act and metals can impede compound transport, block ion channels and affect digestion[27-30].

Investigating zinc overexposure can provide interesting insight into the physiological effects of chronic zinc exposure at lower levels. Thus, this investigation sought to comprehensively fill research gaps by investigating the effects of acute zinc exposure on (a) Heart, NMJ and survival/development of *Drosophila* and crawfish, (b) *Drosophila* behavior and (c) Crawfish MRO.

MATERIALS AND METHODS

Study area: This research was conducted at the University of Kentucky in Lexington, Kentucky, USA, between January and May, 2023.

Animals: All fruit fly assays (behavioral and physiological) utilized 550 *Drosophila melanogaster*, Canton S (CS) flies, initially sourced from Bloomington *Drosophila* Stock Center (BDSC) and subsequently kept isogenic in the lab for several years within partially filled vials of cornmeal-agar-dextrose-yeast medium.

Crawfish studies were conducted using red swamp crawfish (*Procambarus clarkii*) purchased for lab use from a supermarket in Lexington, Kentucky, USA upon delivery from an Atlanta, Georgia, USA distribution center. During the experiments, 30 crawfish of length 6-10 cm and weight 12.5-25 g were housed independently in plastic containers of

33 cm by 28 cm by 23 cm, containing aerated water at a depth of about 10 to 15 cm and a temperature of 20-21°C. The water was exchanged weekly to maintain salinity levels of 25-26 ppt and O_2 levels of 7.4-7.6 mg L^{-1}, they were also fed weekly with dry fish food. Since all were between molts and treated in the same manner, variables were controlled as well as can be expected for this generalized investigation.

Most of the methods used herein parallel those described in Pankau *et al.*[29], which assessed the effects of Mn^{2+} in the same manner. The slight differences between the protocols are described below.

Survival and developmental studies of *Drosophila melanogaster*: To investigate life cycle alterations upon zinc exposure, pupation time was examined in first and third-instar *Drosophila* larvae that had received food containing 2.5 or 5 or 10 or 15 mM $ZnCl_2$. The moist food was placed at the bottom of ten larvae vials 21°C. Ten larvae were placed in each vial. Time passed, allowing the larvae to develop into pupae (which were counted each day). After several days, the food was removed and the entire setup was scrutinized for dead larvae.

Adult survival was examined similarly. Adults were collected three days after emerging from their pupa. Ten vials were produced using the same conditions and concentrations as the larvae above and each vial contained ten adults. As such, each of the six experimental groups used 100 adults. Survival was monitored for 16 days, with all conditions replicated across the ten trials. The adults were switched to new vials containing equivalent food (i.e., with the same concentrations of $ZnCl_2$) on the seventh day to avoid confusion with newly formed adults. These are similar to procedures published previously[31,32].

Behavioral assays of *Drosophila melanogaster*: Larval locomotive ability was measured using a dissecting microscope (PZMIII Stereo Zoom Trinocular Microscope, World Precision Instrument, 2x zoom, 10x eyepiece), which allowed for counting the number of body wall contractions per minute. Second-instar larvae were used. The $ZnCl_2$ was dissolved in 1 mL of water to reach concentrations of 0 mM (control), 5, 30, 40 and 80 mM. One milliliter of each solution was combined with one gram of corn meal (~1 mL in volume) to produce tainted food at the final concentrations of the study: 0, 2.5, 5, 10, 15, 20 and 40 mM. Once 24 hrs had passed, the third instars were placed on apple-juice agar Petri dishes (1% agar, 8.5-9 cm diameter). These larvae remained in the dish for a minute to acclimatize before assessment began. Then, a microscope at room temperature (21-22°C), and lightly illuminated environment was used to count body wall movements (BWMs) per 1 min.

These larvae were then immediately used to assess larval feeding behavior. Just after BWMs were counted, the larvae were transferred to a small (5.5 cm diameter) Petri dish containing the solution produced by mixing a few dried yeast granules with water. The larvae were left alone for one minute, after which mouth hook movements (MHMs) were counted per one minute[31,32].

Heart rate measures in larval *Drosophila*: A detailed description of the procedure used to dissect an early third instar for exposure of the larval heart is provided in video format in Cooper *et al.*[33]. Briefly, however, the larvae were dissected ventrally, with pins placed on the four corners to expose the heart tube. The preparation was then bathed in physiological saline[34,35]. A modified HL3 saline was used to maintain the *in situ* hearts and body wall muscles (NaCl 70 mM, KCl 5 mM, $MgCl_2 \cdot 6H_2O$ 20 mM, $NaHCO_3$ 10 mM, Trehalose 5 mM, sucrose 115 mM, BES 25 mM and $CaCl_2 \cdot 2H_2O$ 1 mM, pH 7.1[34]. In the experimental groups, varying amounts of $ZnCl_2$ were dissolved in this saline to produce solutions of differing concentrations, though the pH was maintained at 7.1. Each concentration of $ZnCl_2$ (0.05, 0.1, 0.5, 2.5 and 5 mM) was examined across twelve larvae. The caudal region of the heart tube[34] (Fig. 1) was observed for 15 sec and the number of heartbeats was counted, the resulting number was then quadrupled (i.e., converted to beats per minute) for analysis. This rate was first obtained during initial saline exposure for use as a baseline, after which the bathing solution was removed and replaced with a solution containing the compound of interest. Heart rate was taken immediately and then after 2 min of exposure had elapsed. The solution of interest was then removed and replaced with fresh saline, whereupon heart rate was taken both immediately and after another 2 min.

Crawfish cardiac function and survival: The recording procedure has been described in textual and video format[36]. Measures of cardiac activity in the intact crawfish were obtained by placing recording wires under the carapace just above the heart. Insulated stainless steel wires (diameter 0.005 inches, 0.008 inches with coating, A-M Systems, Carlsborg, WA) were used, though a flame was used to burn off a small section of insulation from either end such that a good connection could be established with the recorders. These were placed through thin holes such that the wires spanned the heart, as this allowed for accurate impedance measurements (UFI, model 2991)[37]. The carapace holes were

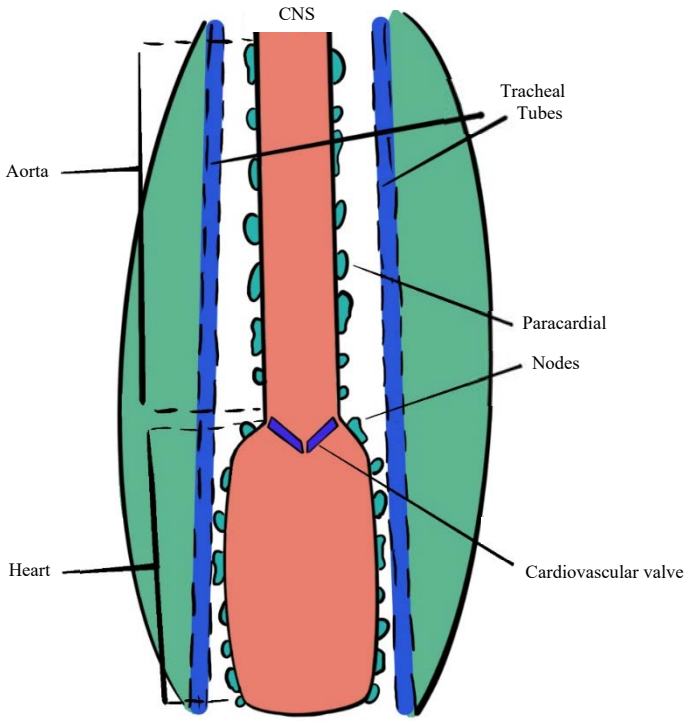

Fig. 1: Dissected larva used for heart rate measurement

Heartbeats were observed through a dissecting microscope and counted manually, both before and after exposure to $ZnCl_2$. The heart rate was measured from the preparation's caudal end, near the bifurcation point of the two tracheal tubes (Illustration made by Alaina Taul)

kept to approximately the size of the wires, ideally by the precision of the scalpel, or by the light application of clay to seal any unpreventable gaps. Only a short portion of the wire (1-2 mm) was inserted, as to use more would run the risk of harming the heart.

Once the wire was in place, glue (cyanoacrylate ester) and accelerator (HobbyTown USA, Lexington, Kentucky) were applied, fixing it into position for the purpose of obtaining good readings. A second layer of adhesive via resin and a resin hardener (HobbyTown five-minute Quick-Cure epoxy of hardener and of resin, Bob Smith Industries Atascadero, California, USA) was also applied, to ensure the security of the wire. These adhesives were chosen as they do not become as hot as other quick-hardening resins can. A PowerLab/4SP interface (AD instruments) was connected to the impedance detector (which was responsible for measuring the dynamic resistance between the cardiac recording wires); this instrument was also calibrated with the PowerLab Chart software version 7.1 (AD Instruments, Australia). As a 1 kHz acquisition rate was used and the heart rate was calculated by counting the number of beats directly observed across thirty seconds, which was then doubled (i.e., transformed into beats per minute).

The $ZnCl_2$ was injected into the crawfish's abdomen by passing a needle through the articulating membrane on the ventral side. Observational studies were also made on the effects of injecting the different doses. Control experiments were run by injecting saline alone to account for cardiac excitation that could be caused by handling the animals and/or injecting foreign matter into the animals' systems. Systemic levels of $ZnCl_2$ were calculated based on dilution with hemolymph from a stock concentration of 50 mM, with the amount of hemolymph estimated from the animal's weight and the assumption that it comprises approximately 30% of a crawfish's total weight[38,39]. Given the crawfish's open circulatory system, the compound was thus carried towards the heart, where it bathed the cardiac ganglion and muscle. Each concentration was observed across multiple preparations.

Steps also had to be taken to assess whether the neurogenic heart still responded to sensory stimulation. Given the crawfish physiology, activating the central nervous system would, in turn, alter neural input to the cardiac ganglion responsible for cardiac innervation[39]. The heart rate was thus monitored for 20 min pre-injection, after which a glass rod was used to forcefully tap the crawfish telson and elicit a tail-flip

response. The recording was maintained for a further 10-20 min, at which point the crawfish received the treatment-either the saline injection or one with $ZnCl_2$-while the heart rate was still being recorded. After another twenty minutes had passed, the telson was tapped again and the results were recorded. The effects of the telson tap were also monitored periodically over the 24 or 48 hrs after the injection. This protocol allowed for an assessment of how the injections affected the crawfish's survival over a period of days.

Environmental exposure: The water in the crawfish holding tanks was modified to hold varying concentrations of zinc and allow for the examination of zinc's effects on crawfish survival and behavior. Crawfish were placed into individual containers containing water, into which $ZnCl_2$ had been dissolved at concentrations of 2.5, 5 and 10 mM. Crawfish were fed on the second day of this new housing arrangement via a small number of fish food pellets within the tainted water.

Crawfish muscle receptor organ: The procedures for dissection, displacement, and electrophysiological recordings used over the course of this project have been described previously[40-42] and in video format[43]. The MRO is known to be analogous to the vertebrate muscle spindle. To restate in brief, however, the dissected crawfish abdomen was placed in a Sylgard-lined dish filled with crawfish saline. This saline was a modified Van Harreveld's solution (in mM: 205 NaCl, 5.3 KCl, 13.5 $CaCl_2$ $2H_2O$, 2.45 $MgCl_2$ $6H_2O$ and 5 HEPES adjusted to pH 7.4). An insect dissecting pin was used as a marker of the displacement range to keep movements consistent and comparable. Suction electrodes made from glass pipettes fitted with plastic tips were used to record extracellular signals from the cut nerves and the details of making these suction electrodes are provided by Baierlein *et al.*[44]. A P-15 amplifier (Grass Instruments, Astro-Med West Warwick, Rhode Island, USA) in conjunction with a PowerLab/4s A/D converter and Lab Chart 7 software (ADI Instruments, Colorado Springs, CO, USA) was used to record signals at a 10 or 20 kHz sampling rate.

The MRO was manipulated using a wooden dowel, transitioning from a relaxed position to a stretched one (as marked by the aforementioned dissecting pins) over the course of one second, being held in place for just over ten seconds and then being moved back to its starting position. Each displacement was marked on the computer recording file for later analysis. Displacements were observed first in saline before the bathing medium was removed and replaced with a solution of interest (whether fresh saline, as a control or $ZnCl_2$ solutions of 0.1, 1 or 10 mM). Effects were observed both immediately and after five minutes of exposure had elapsed. Finally, the solution was removed once more and replaced with fresh saline as a washout. Three displacements, or trials, were performed under each condition. All chemical compounds were obtained from Sigma Aldrich (St. Louis, Missouri, USA). Analysis of the electrical signals from the MRO was carried out by measuring the number of spikes within the first ten seconds of displacing the organ to the stretched position (such that the count included both the spikes from the initial movement and those from the static stretched position). An average of the activity from the three trials at each time point was calculated.

Neuromuscular junctions of larval *Drosophila* and crawfish: Resting membrane potential (RMP), evoked excitatory junction potentials (EJPs) and spontaneous quantal events (mEJPs) were observed to determine the response of each to zinc exposure, both in *Drosophila* and in crawfish preparations.

Each organism was observed under normal conditions (i.e., in physiological saline). Then, the bathing medium was removed and replaced with a solution of interest, whether a saline control or a solution containing zinc. This was then removed and the preparation was flushed (whether with fly or crawfish saline, accordingly) and observed once more, providing insight into the viability of each preparation after cessation of zinc exposure.

To study the effects on *Drosophila*, third-instar larvae were used. Dissections were carried out in fly saline until a saline-filled suction electrode contained a cut segmental nerve. The saline used was the same as that described above for monitoring *Drosophila* cardiac activity. This nerve was then stimulated using an S88 Stimulator (Astro-Med, Inc., Grass Co., West Warwick, Rhode Island, USA), at 0.5 Hz. Monitoring of the larval body wall muscle (m6) transmembrane potentials was carried out using a 3M KCl-containing sharp, intracellular electrode (30 to 40 MegaOhm resistance), which was positioned to impale the fiber (Fig. 2). An Axoclamp 2B (Molecular Devices, Sunnyvale, California, USA) amplifier and 1 X LU head stage were used.

The crawfish opener muscle has been historically used for studying synaptic transmission[45]. The use of the preparation's most distal muscle fibers ensures consistency among preparations[46]. The dissection procedure necessary for exposure and selective stimulation of the opener muscle's excitatory motor neuron is described in both textual and visual format[45]. Briefly, however, a crawfish saline comprising a modified Van Harreveld's solution (which itself represents, in mmol L^{-1}: 205 NaCl, 5.3 KCl, 13.5$CaCl_2$·$2H_2O$, 2.45 $MgCl_2$·$6H_2O$, 10 glucose, 0.5 HEPES adjusted to pH 7.4) was used to sustain a dissected crawfish preparation. The

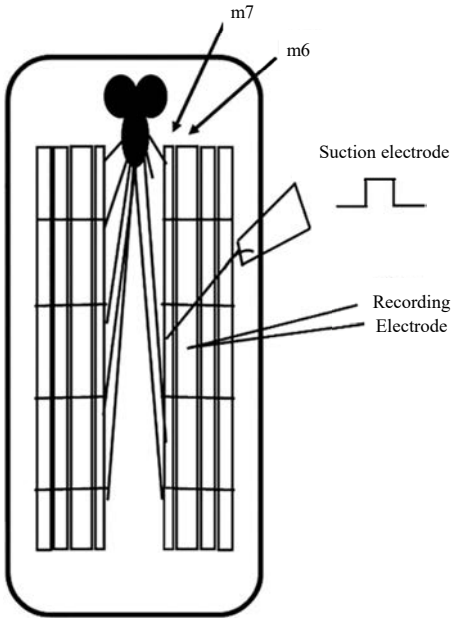

Fig. 2: A schematic depiction of a third-instar larval dissection

This recording arrangement was used to examine the m6 muscle fiber and, specifically, the evoked and spontaneous synaptic excitatory junction potentials at that fiber. A suction electrode was used, filled with saline and containing a cut segmental nerve. The fit is fairly tight for the nerve at the opening of the suction electrode. The intracellular electrode is filled with 3M KCl

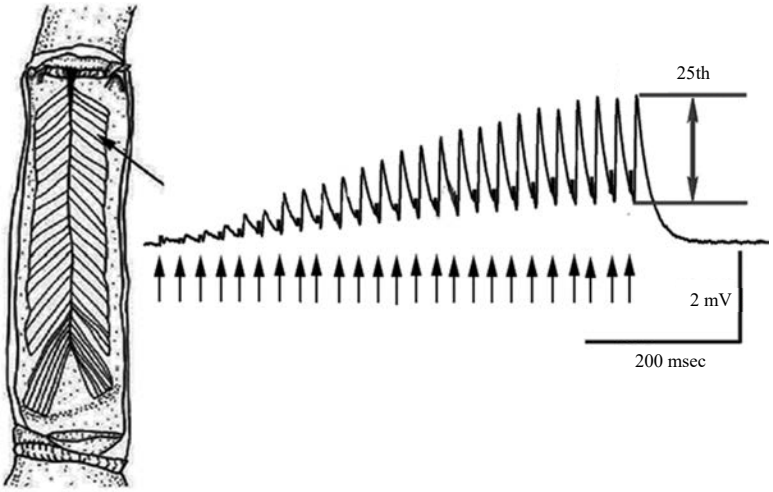

Fig. 3: A diagram of the crawfish opener muscle on a walking leg

Facilitated synaptic response are shown across 25 stimuli, indicated by arrows, through a trace of excitatory junction potentials taken from distal muscle fibers

excitatory and inhibitor neurons were isolated, allowing for stimulation in the meropodite segment at 40 Hz trains separated by ten seconds (Fig. 3). An AxoClamp 2B (Axon Instruments, USA) was used to record the responses, whereupon they were converted using a PowerLab, 4SP (ADInstruments, USA). Analysis was later conducted at a 10 or 20 kHz sampling rate using LabChart 7.0 (ADInstruments, Colorado Springs, Colorado, USA).

Statistical methods: Across the investigation as a whole, the following tests were used. When normality was to be assumed, this assumption was verified with a Shapiro-Wilks Test. This was also used to signal whether the most appropriate subsequent test should be a t-Test, paired-t-Test or Wilcoxon Signed Rank Test. When necessary, ANOVA or Bonferroni *post-hoc* t-Tests were used.

Drosophila survival experiments made use of Weibull regression, as this allowed modeling of survival curves by solution concentration (control, 2.5, 5, 10 and 15 mM). To determine whether significant differences occurred across concentrations, the survival curves were put through ANOVA testing, while the pairwise *post-hoc* differences across the concentrations were determined through the use of Bonferroni correction.

The difference among treatments (n = 100 in each group, control, 2.5, 15, 20 and 40 mM) in behavioral studies were determined with ANOVA testing. Subsequently, Bonferroni *post-hoc* t-Tests were performed if the difference was significant ($p<0.05$), as this allowed the determination of whether the treatments were significantly different on a pairwise level.

Investigation into the larval *Drosophila* heart rate resulted in data that were not normally distributed (Shapiro-Wilks, $p<0.05$), as the data result from an initial saline treatment, followed by a substitution for either fresh saline or $ZnSO_4$ and then a return to fresh saline. Because of this, new variables had to be created: "Pre" and "post". "Pre" was used to represent the initial exposure to saline, without application of the experimental solution. "Post", on the other hand, referred to the initial exposure without the saline washout at the end. By using these variables, the Shapiro-Wilks Test was successfully passed ($p<0.05$) and so a two-way repeated measures ANOVA was used to evaluate the differences in heart rate caused by the $ZnCl_2$ solution, given factors of dose level (n = 6 for each concentration, 0.05, 0.1, 0.5, 2.5 and 5 mM) and time ("pre", "post"). A significant two-way interaction was found. This was then followed with a one-way ANOVA (such that dose level, treatment and time were held constant) as a way of examining the effects of dose alterations. If the result was found to be significant, *post-hoc* analysis was done using the Bonferroni adjustment method.

For studies involving both crawfish and *Drosophila*: If the Shapiro-Wilks Test was passed, a paired t-Test was used, otherwise, a Wilcoxon Signed Rank Test was utilized to compare sensitivity.

All studies used a 0.05 significance level.

RESULTS

Survival and developmental studies of *Drosophila melanogaster*. The first-instar stage larvae placed in food tainted with $ZnCl_2$ were monitored until pupation, with pupae marked on the vials daily. The time taken for each pupa to emerge as adult was also noted. Survival during the following five days of adulthood was also monitored, with the larvae remaining in the same vial and food. The initial ten first-instar larvae are indicated on the graphs as green dots, which are connected to the first pupa in a vial by a dotted line (Fig. 4). Pupa formation is marked with red dots on the graph, while adults are represented in blue (Fig. 4). Each of the ten vials-containing ten first instars per concentration-is represented by an individual line (Fig. 4a control, 4b 2.5 mM, 4c 5 mM, 4d 10 mM and 4e 15 mM).

The observations were made once a day, so any transitions from third instar to pupa or pupa to adult were made within a 24 hrs time frame. This allowed for determining the overall effects of time on pupation and duration of pupation, as well as survival throughout the instar stages and pupation. One of the ten control vials only had nine pupae form, but the other vials all produced ten pupae, which emerged as adults over the following five days. The vials with 2.5 mM $ZnCl_2$ showed a similar pattern in survival and development, but concentrations at 5 mM and greater resulted in a decrease in the number of pupae forming and adults emerging. The time to pupation increased for 5 and 10 mM and survival was observed to be less successful as well, with some pupae forming but failing to emerge as adults. For the 15 mM concentration, results were even more drastic; after six days, the vials had declined to the point that, rather than ten vials of ten larvae each (for a total of 100 larvae), two vials were observed to contain only three larvae and one had only a single larva present. By the seventh day, all larvae were dead. Since no pupae or adults formed at 15 mM concentrations, the red and blue dots are marked as negative values. Dead larvae were observed to turn brown/black and shrivel, while dead pupae turned black.

Since higher concentrations of $ZnCl_2$ saw many of the larvae dying during the transition from first instar to pupae, a subsequent investigation was performed. Ten early third instars (within three days or 72 hrs of hatching) were placed into each of the ten vials, along with another batch of tainted food (Fig. 5a (control), 5b (5 mM), 5c (10 mM) and 5d (15 mM)).

Since no differences were observed for 2.5 mM in the earlier investigation using first instars, the new experiment started at 5 mM and saw a greater decrease in both the formation and survival of pupae (Fig. 5). The vials containing 15 mM bore pupae that died and resulted in very few adults emerging. Those adults that did emerge all died within three days.

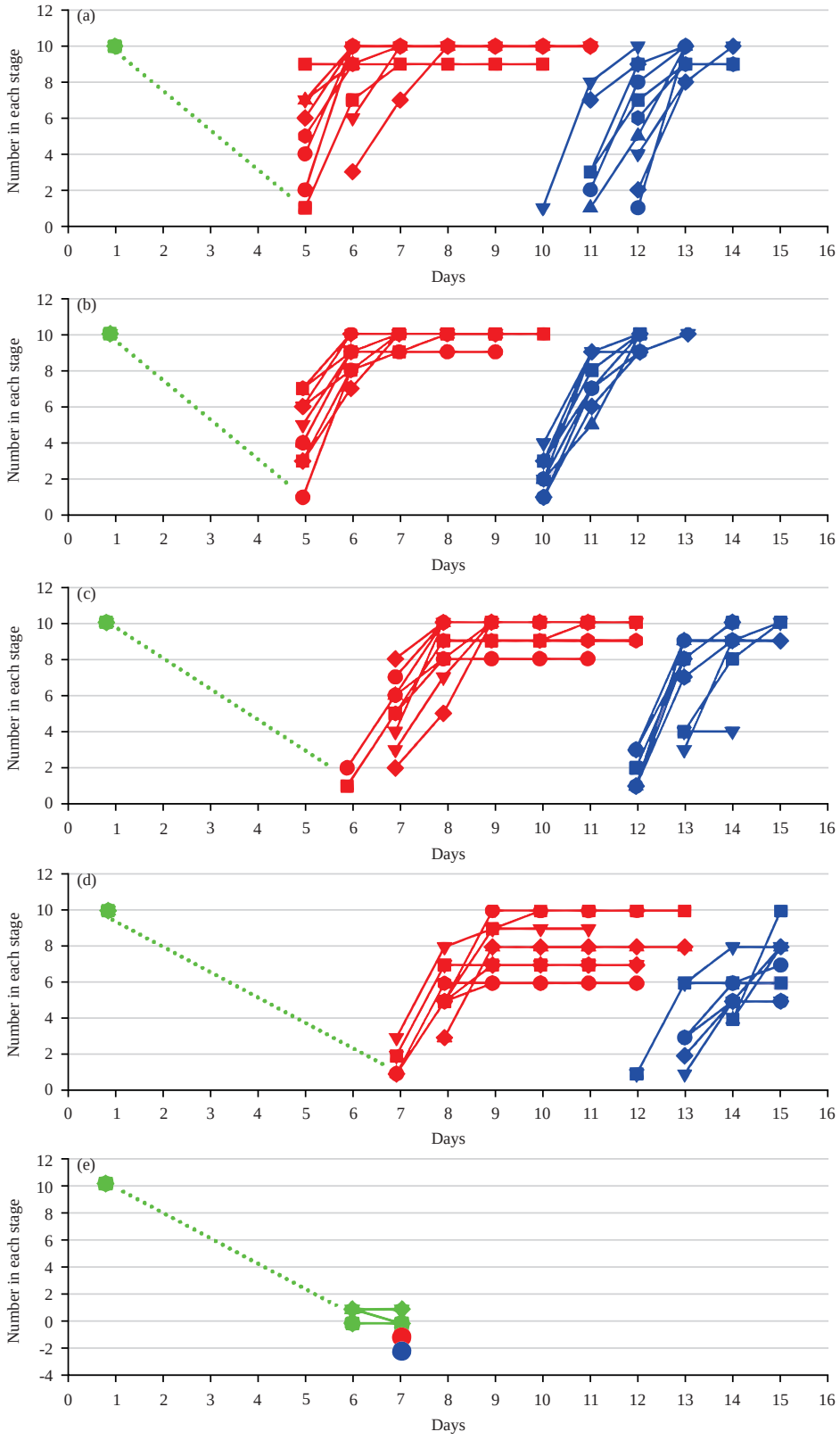

Fig. 4(a-e): Effect of dietary $ZnCl_2$ on larval development, (a) Control, (b) 2.5 mM, (c) 5 mM, (d) 10 mM and (e)15 mM

The first instars (green) were fed tainted food containing various concentrations of $ZnCl_2$. The number of resulting pupae (red) and adults (blue) were counted over time in days for each concentration. Ten vials of ten larvae each were used per concentration for the first-instar larvae

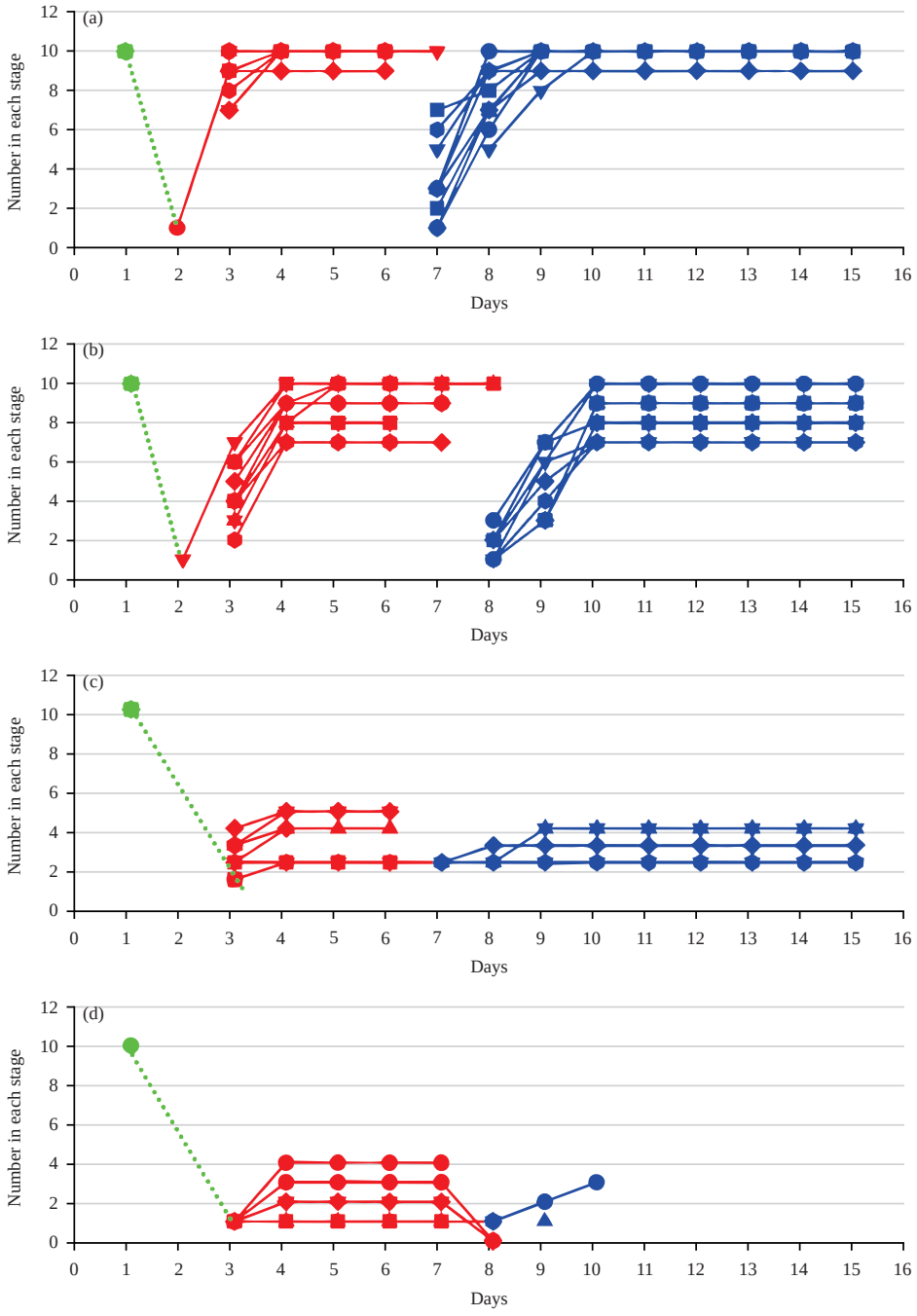

Fig. 5(a-d): Effects of dietary $ZnCl_2$ on the development of larvae, (a) Control, (b) 5 mM, (c) 10 mM and (d) 15 mM

Early third instars (green) were fed tainted food containing various concentrations of $ZnCl_2$. The number of resulting pupae (red) and adults (blue) were counted over time for each concentration. Ten vials were used for each concentration for the third-instar larvae

Upon examination of dietary $ZnCl_2$, the higher concentrations of $ZnCl_2$ were found to be toxic (Fig. 6). The adults were observed for 16 days (384 hours). There was a significant effect on concentration level (ANOVA, $p<0.05$). When comparing the survival curves pairwise, the 15 mM $ZnCl_2$ proved to be highly toxic and significantly different from each other concentration (Bonferroni adjusted $p<0.05$). The adults in every vial comprised a mix of males and females. Eggs, instars, and pupae were observed, so the experimental adults had to be moved to new vials containing the same food conditions, albeit freshly made. This switching of adults to new vials may have produced additional stress as the adults

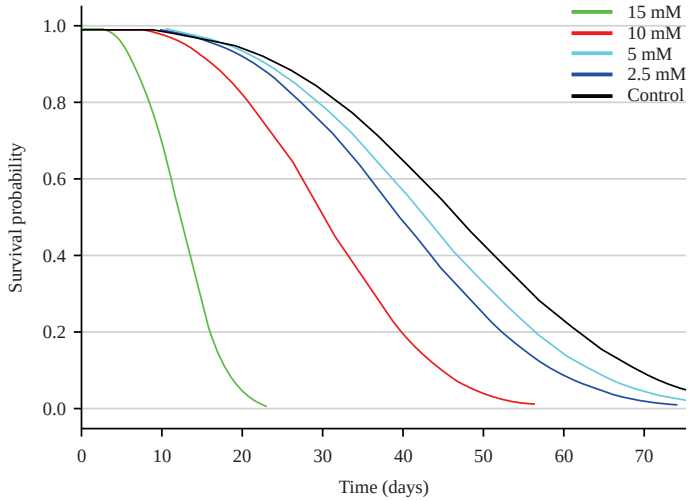

Fig. 6: Survival of adult *Drosophila* exposed to dietary $ZnCl_2$ was examined using food containing zinc concentrations of 2.5, 5, 10 and 15 mM

Ten vials of ten two-day-old adults were tested. *Drosophila* were fed tainted food and their survival was examined over days

adjusted to new environments. Some adults also stuck to the vial or the food, which could have stemmed from the humidity and/or $ZnCl_2$-caused lethargy, so it is hard to determine if their demise was due to the $ZnCl_2$ or simply the effects of being stuck.

Larval behavioral assays: Two standard behaviors were used for the assessment of health and physiological function in larval *Drosophila*: Body wall movements and mouth hook movements. A fly's locomotion rate and its eating rate are quite different, as eating movements occur at about twice the rapidity. Because of this, subtle effects on integration of the central nervous system, synaptic transmission, and/or skeletal muscle are generally more prominently observed by studying changes in the eating assay. This was indeed the case here, as the effects of $ZnCl_2$-tainted food on early third instars for 24 hrs prior to conduction of the assays were far more notable when studying eating motions. The food tainted to concentrations of 20 or 40 mM $ZnCl_2$ resulted in reduced body wall movements compared to that of the control larvae (Fig. 7a, ANOVA, $N = 20$ and $p<0.05$). The rate of the mouth hook movements was even depressed at 15 mM using the same comparison (Fig. 7b, ANOVA, $N = 20$ and $p<0.05$).

Heart rate measures in larval *Drosophila*: The larval heart rate is known to be very sensitive to the concentration of external Ca^{2+} and was found to be affected by the presence of saline containing Mn^{2+} in previous studies, so it was expected that the presence of Zn^{2+} might also strongly affect the rate. No significant effects were noted for 0.05 or 0.1 mM $ZnCl_2$, as there was a lot of variation observed, some preparations showed an increase in cardiac rate, while others saw a decrease (Fig. 8a-b). However, a concentration of 0.5 mM or larger resulted in a rapid decrease of cardiac rates (Fig. 8c-e, Sign tests due to zero values, $N = 6$ and $p<0.05$). Exposure to 2.5 or 5 mM $ZnCl_2$ had such strong effects on the heart that, in many cases, even multiple flushes of fresh saline across the heart did not prompt the beating to pick up again (Fig. 8d-e). The cardiac modulator serotonin (5-HT, 100 μM) was used to determine whether the heartbeat might be regained even after hearts stopped beating in the presence of $ZnCl_2$, in the majority of cases the heartbeat did not pick back up.

Crawfish cardiac function and survival: While assessing the effects of $ZnCl_2$ on crawfish survival and physiology, multiple different investigations were performed. Unlike the myogenic nature of the larval *Drosophila* heart, the crawfish heart is neurogenic and thus requires neural input for the maintenance of its beating. Additionally, the change in the rate due to environmental disturbances-be that a tap on the tank, a pebble dropping nearby or a physical touch to the crawfish telson or head-can result in a pause of the rate, followed by an acceleration analogous to that noted in the mammalian autonomic nervous system response. Thus, the response to environmental stimuli provides information about the central integration of sensory cues and cardiac neural control, as well as synaptic function throughout the circuit and

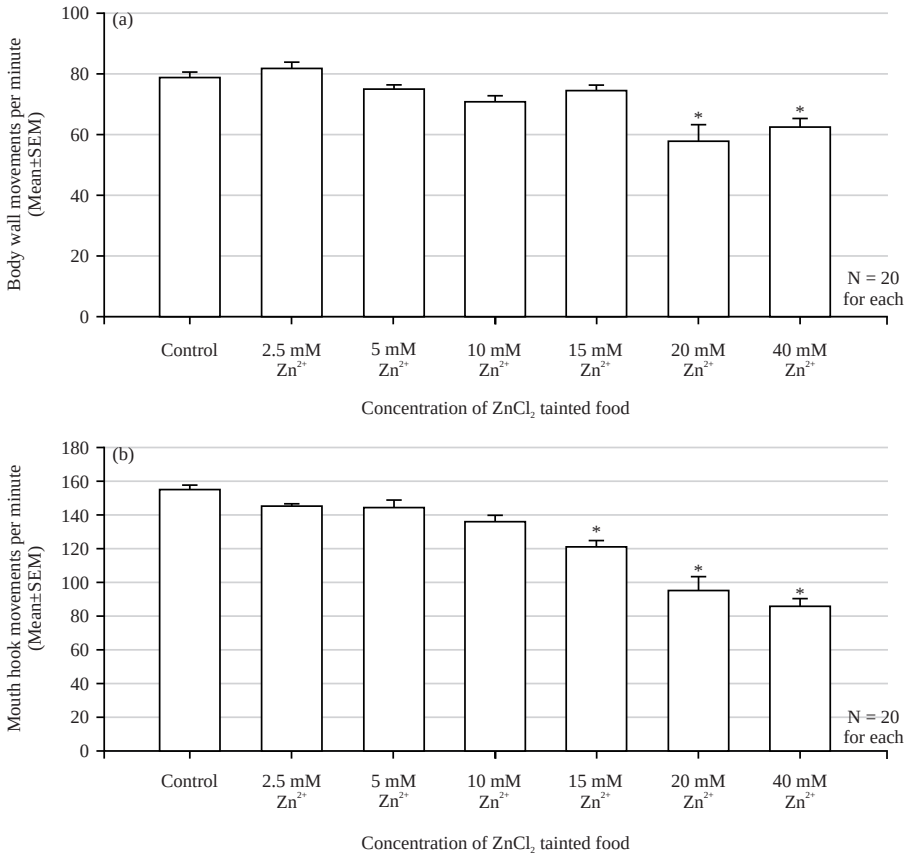

Fig. 7(a-b): Behavioral measures of third-instar larvae after 24 hrs of exposure to food at various concentrations of $ZnCl_2$, (a) Average number of body wall movements per minute for each concentration of $ZnCl_2$ tainted food and (b) Average number of mouth hook movements per minute for each concentration of $ZnCl_2$ tainted food

Individual larvae were fed, placed on an apple-juice-agar plate for observation of body-wall movements and then moved to a yeast solution to assess mouth hook movements, *p<0.05 two- way ANOVA compared to control and N = 20

directly on the heart. In order to ensure the correct dosage within the animal, $ZnCl_2$ was provided by injection. Thus, handling the crawfish and injecting either a control (a volume of plain saline) or an experimental solution (saline containing $ZnCl_2$) was invasive, resulting in an increase in heart rate for some time. Assessment of a telson tap by glass rod both before and 20 min after the injection provided insight into the effects of injected $ZnCl_2$. As shown in Fig. 9a, the telson tap prior to injection resulted in an obvious cardiac pause. The injection and handling of the animal cause a significant alteration in rate (Fig. 9b). However, after only 2 min of exposure to 10 mM $ZnCl_2$, the heartbeat was almost entirely absent and a tap on the telson resulted in merely a small deflection in the trace. No rhythm was present; thus, the small deflection was likely just an artifact of moving the animal and the wires with the telson tap.

The circulating concentrations considering hemolymph dilution and tail tap responsiveness, both before and after the 20 min post-injection mark, are presented in Table 1. The injected crawfish were all held for two weeks, fed weekly and allowed to move freely around their individual holding tanks. The survival rate of these animals was assessed, noting whether they survived for a week or two, or if death occurred within a few minutes or hours, as was noted for the 5 and 10 mM estimated hemolymph concentrations (Table 2).

In addition to $ZnCl_2$ injections, the effects of $ZnCl_2$ exposure from the surrounding environment (i.e., the aquarium water) on survival were also assessed. In this paradigm, the $ZnCl_2$ could be ingested through the gastrointestinal tract or absorbed/transported across the gills. The animals were fed on the second day in the individual aquariums with two small pellets of shrimp food. The higher

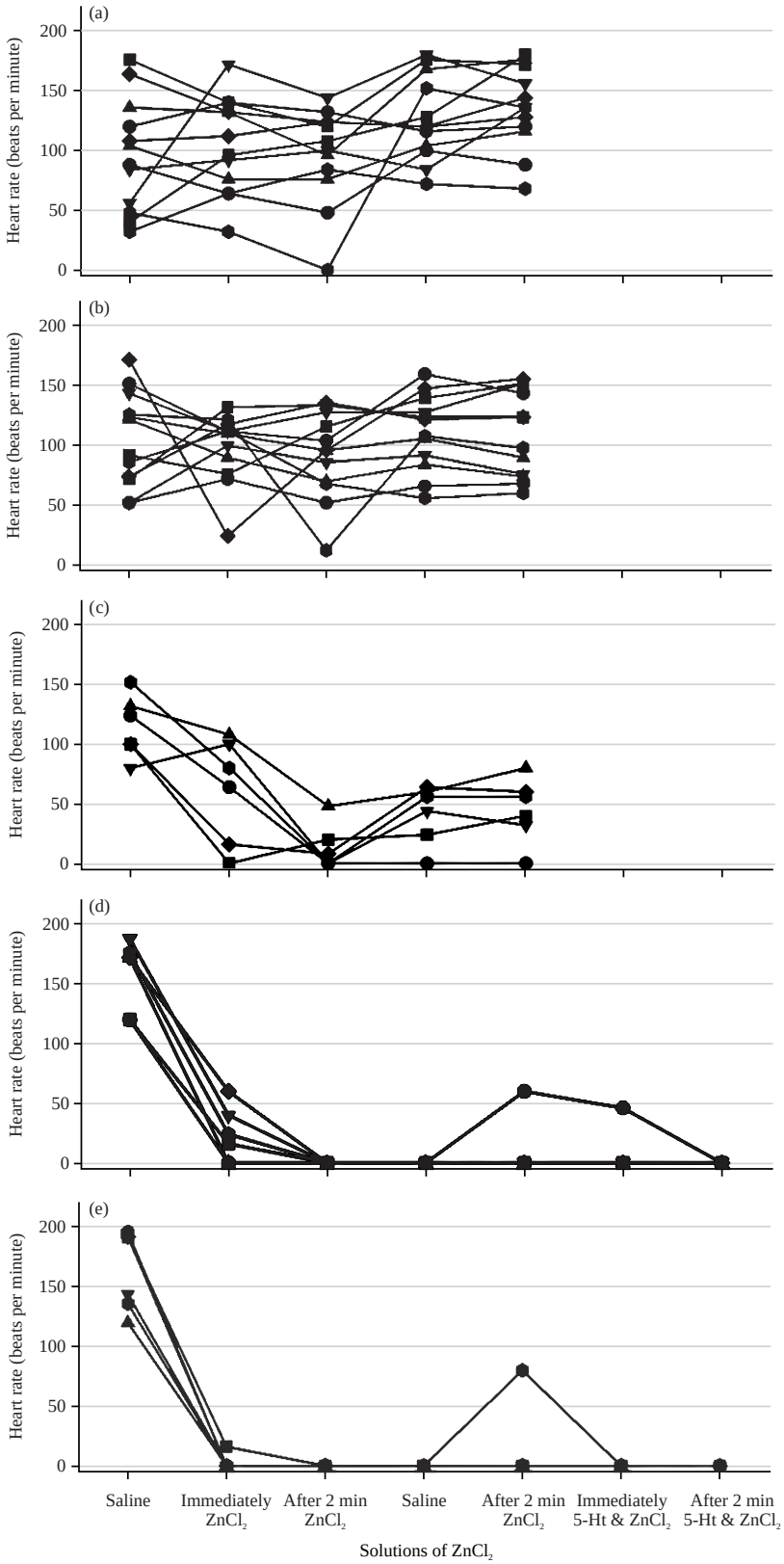

Fig. 8(a-e): Heart rate upon exposure to various concentrations of $ZnCl_2$, (a) 0.05 mM, (b) 0.1 mM, (c) 0.5mM, (d) 2.5 mM and (e) 5 mM

Protocol for changing the solutions is shown on the X-axis. Considering 2.5 mM and 5 mM $ZnCl_2$ resulted in cessation of the heartbeat, the enhancing cardiac modulator serotonin (5-HT) was exposed to the hearts while in the presence of $ZnCl_2$, Each line represents an individual larva

Table 1: Responsiveness and changes in heart rate of crawfish injected with $ZnCl_2$

Concentration	Pre-injection response to telson tap	Heart rate	Post-injection response to telson tap	Heart rate
0.5 mM	+	↑	-	↓
0.5 mM	+	↑	-	↓
0.5 mM	+	↑	+	↑
0.5 mM	+	↑	+	↑
0.5 mM	+	↑	-	↓
0.5 mM	+	↑	+	↑
0.5 mM	+	↑	-	↓
1.0 mM	+	↑	-	↓
1.0 mM	+	↑	-	↓
1.0 mM	+	↑	-	↑
1.0 mM	+	↑	-	↑
1.0 mM	+	↑	-	↓
1.0 mM	+	↑	-	↑
5.0 mM	+	↑	+	↑
5.0 mM	+	↑	+	↑
5.0 mM	+	↑	+	↑
5.0 mM	+	↑	-	↓
Average				
0.5 mM	+	↑	+	↑
1.0 mM	+	↑	-	↓
5.0 mM	+	↑	-	↑

+: Crawfish was responsive and the beating paused, -: No significant change due to a lack of response, ↑: Increase in heart rate and ↓: Decrease in heart rate

Table 2: Survival of crawfish injected with $ZnCl_2$

Concentration	Dead?	Survived?
Saline (control)	None	All after 1 week
0.5 mM	None	All after 1 week
1.0 mM	Within 1 week	None
2.5 mM	Within 2 weeks	None
5.0 mM	Within 1 hr	None
10 mM	10-20 min	None

n = 6 for each concentration

Table 3: Survival of crawfish exposed to environmental $ZnCl_2$.

Concentration	Death	Survived?
1.0 mM	None	All after 2 weeks
2.5 mM	Within 2 weeks	None
5.0 mM	Within 1 week	None
10 mM	Within 1 week	None

n = 6 for each concentration

concentrations of 5 and 10 mM killed the animals within a single week, while 2.5 mM exposure resulted in death only after two weeks. The 1 mM-exposed animals survived for both weeks of observation and were not subsequently monitored (Table 3).

Crawfish muscle receptor organ: Assessing the direct effects of $ZnCl_2$ on sensory neurons utilized the MRO of the crawfish abdomen as a model. When the muscles of the MRO are stretched, the transduction of mechanical stimuli results in the opening of stretch-activated ion channels within sensory endings embedded in the muscle, thus leading to depolarization of the sensory ending. The induced action potentials resulting from this procedure were monitored in the axons close to the MRO (Fig. 10a). Exposure to $ZnCl_2$ at 0.1 or 1 mM concentrations (Fig. 10b) unexpectedly increased MRO responsiveness to joint movements. Flushing the preparation with fresh saline generally decreased the activity (Fig. 10c). There was a significant effect in increased activity at both 0.1 and 1 mM (Paired t-Test, N = 6 for each concentration and $p<0.05$). Individual preparations were used for each concentration (0.1 and 1 mM) (Fig. 11a-b). The percent change from saline to $ZnCl_2$ was not statistically significant, due to the high variability among preparations (Fig. 11c, t-Test, N = 6 and $p>0.05$ unpaired).

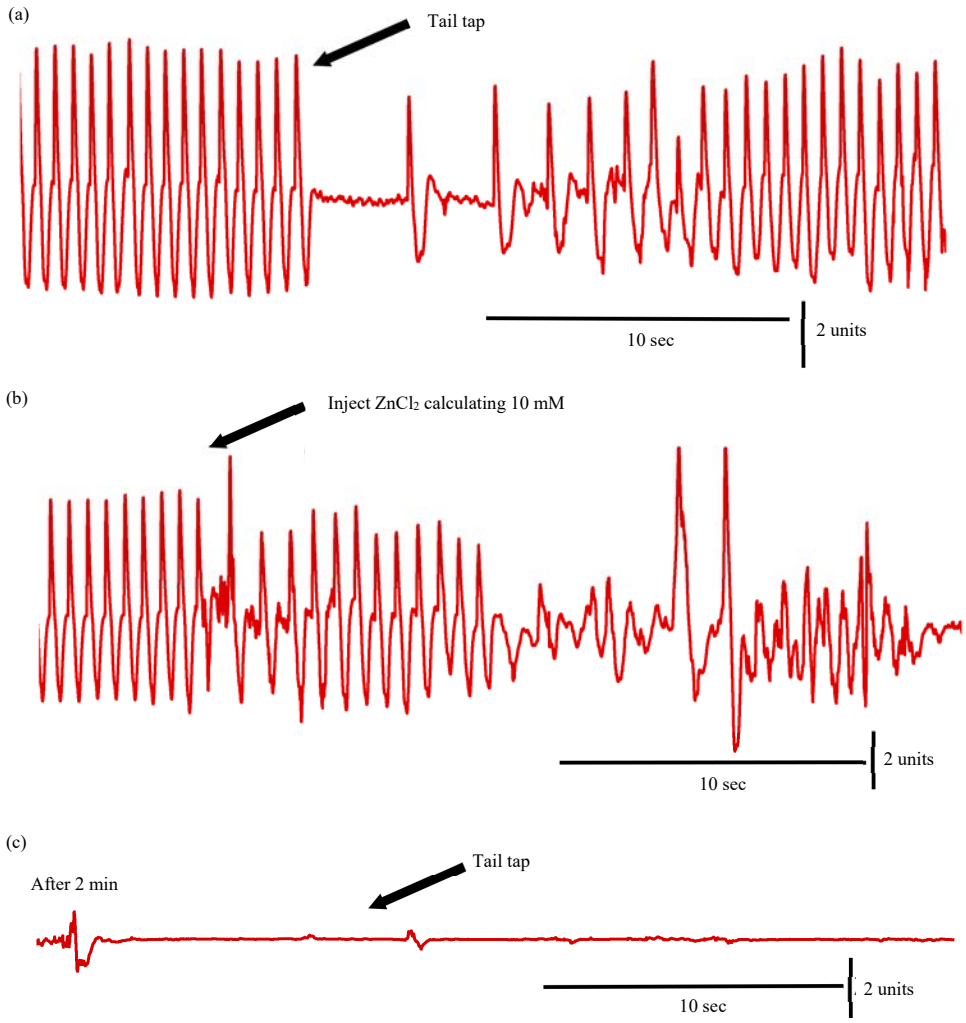

Fig. 9(a-c): A representation of the effects of $ZnCl_2$ injection into the hemolymph on the crawfish heart rate and response to sensory stimuli, (a) Upon tapping the crawfish telson, the heart rate paused before speeding up, (b) Injecting the animal with saline or $ZnCl_2$ caused an alteration in heart rate as the animal was picked up and injected and (c) Within 2 min of $ZnCl_2$ (10 mM) injection, the heart rate was affected and sensory stimulation no longer resulted in noticeable cardiac changes

Neuromuscular junctions of larval *Drosophila* and crawfish: Synaptic efficacy at the *Drosophila* and crawfish neuromuscular junctions (NMJs) is known to be sensitive to extracellular concentrations of Ca^{2+}. Thus, it was expected that $ZnCl_2$ would depress evoked synaptic responses in both types of NMJ. Exposure to 0.1 mM $ZnCl_2$ at the larval *Drosophila* NMJ rapidly reduced the amplitude of the excitatory junction potentials (EJPs) and a representative preparation of this observation is shown in Fig. 12. Note that spontaneous quantal events are present both when only saline is present and during $ZnCl_2$ exposure (Fig. 12b and c). Flushing the preparations with fresh saline allowed the EJPs to recover (Fig. 12d). As a result, $ZnCl_2$ does not seem to reduce EJP amplitudes by blocking the postsynaptic glutamate receptors on the muscle. Application of 1 mM $ZnCl_2$, however, prominently reduced the evoked amplitude of the EJPs (Fig. 13a-b). These trends for 0.1 and 1 mM were found to be significant and 1 mM induced a more pronounced effect ($p<0.05$, paired t-Test and $N = 6$ for each concentration, individual trails). The amplitudes with 1 mM exposure would, in most cases, be no larger than the baseline (Fig. 13c), with a complete reduction in evoked responses. Flushing the

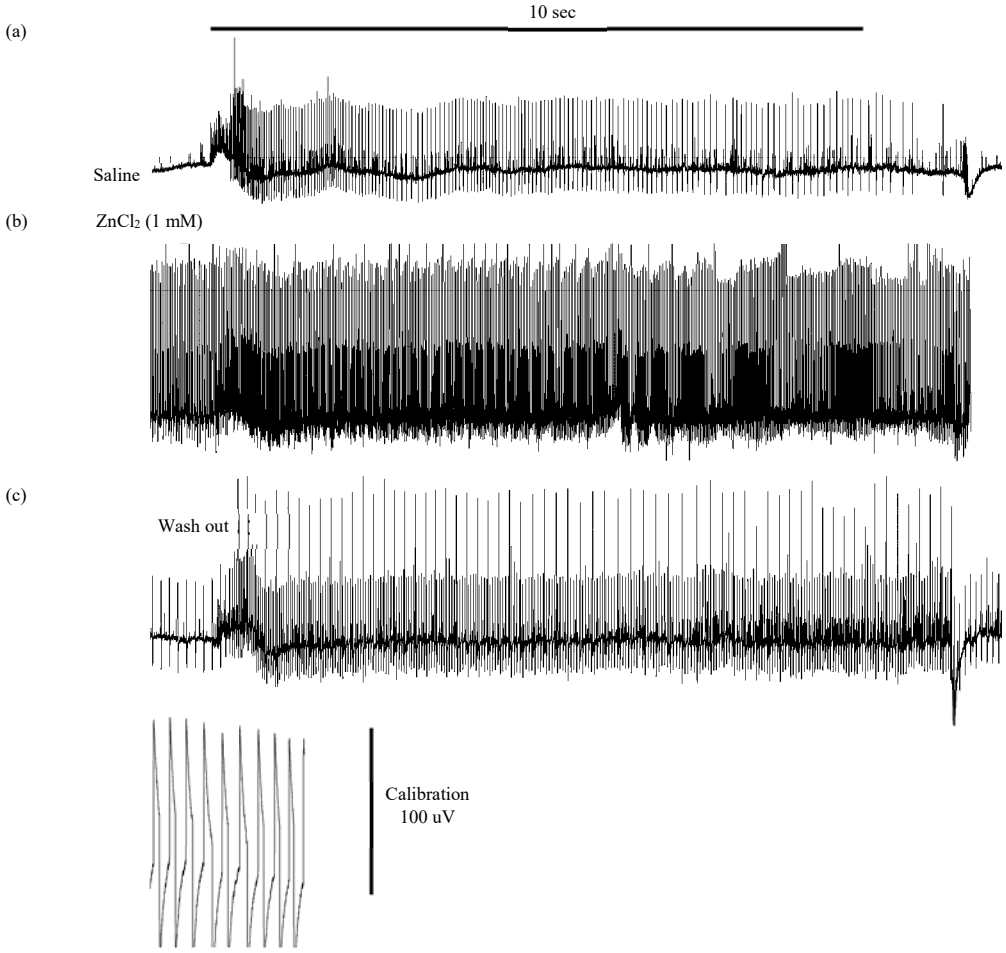

Fig. 10(a-c): Representative recordings of the sensory nerve activity from the muscle receptor organ during displacements (a) Before, (b) During and (c) After (via washout) exposure to 1 mM $ZnCl_2$
A calibration pulse is shown to illustrate the general amplitude of the responses obtained

preparation with fresh saline would allow recovery of the evoked events (Fig. 13d). The spontaneous quantal events also appeared greatly reduced in amplitude; however, the muscle was depolarized as much as 10 mV during 1 mM $ZnCl_2$ exposure. Thus, the driving gradient for the ionotropic glutamate receptors was reduced, further decreasing the amplitude of the spontaneous quantal events and evoked EJPs.

The effects of $ZnCl_2$ exposure on synaptic transmission at the crawfish NMJ mirrored those at the larval *Drosophila* NMJ. Representative preparations are shown in Fig. 14 and 15. Note that, upon examining the first EJP within each train, one can observe a small amplitude with saline or washout but neither 0.1 mM nor 1 mM exposure to $ZnCl_2$ showed EJP amplitudes above baseline. The opener NMJ of the crawfish is a lower-output NMJ than that of larval *Drosophila*. Thus, the NMJ requires the induction of short-term facilitation to increase the amplitudes of EJPs for analysis, as was accomplished here with the use of 25 stimuli at 40 Hz. As 0.1 mM $ZnCl_2$ substantially reduced the evoked amplitude, while 1 mM almost eliminated it entirely. These observed trends for both 0.1 and 1 mM $ZnCl_2$ were found to be significant, though 1 mM induced a more pronounced effect ($p<0.05$, Paired t-Test and $N = 6$ for each concentration, individual trails).

The effects of the 0.1 mM on the amplitudes of the EJPs were relatively rapid, as were those for 1 mM, although the amplitude decrease was more subtle, this is shown in Fig. 16, where the amplitude of each 25th EJP is plotted over time. The rapidity of the effects upon exposure to or removal (with washing via fresh saline) of the $ZnCl_2$ is also highlighted over time (Fig. 16).

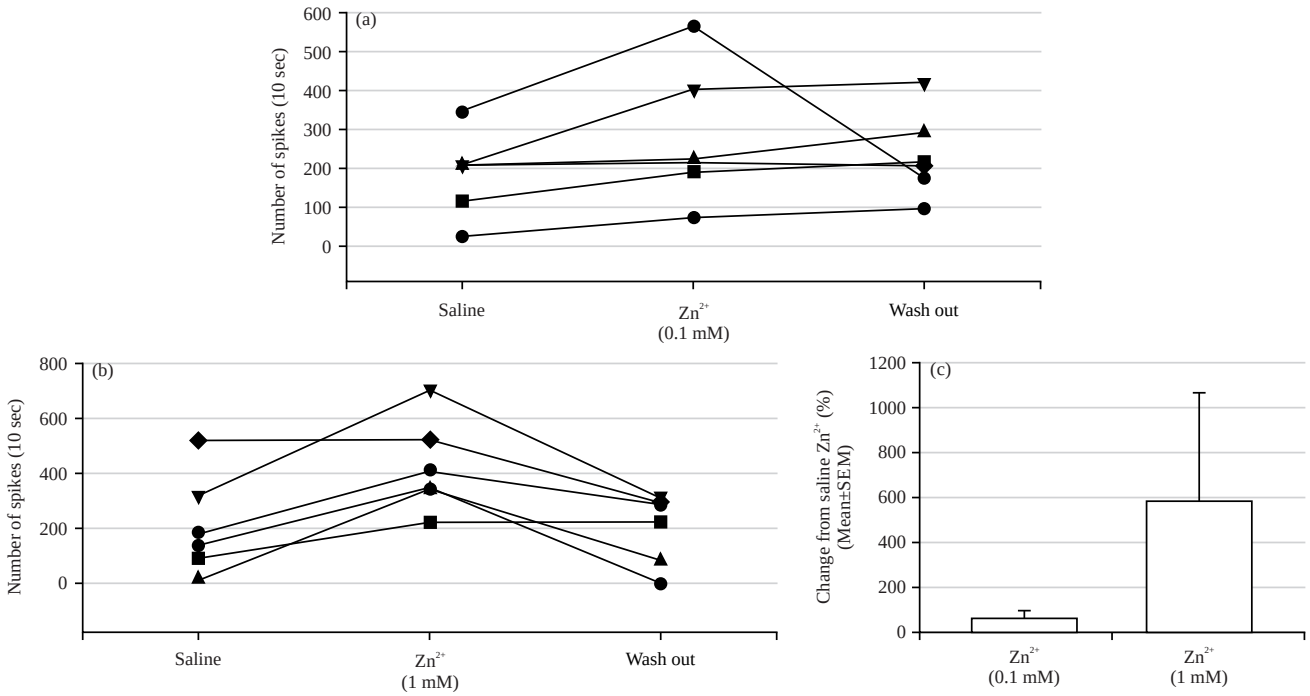

Fig. 11(a-b): Average number of spikes obtained from the MRO nerve during the ten seconds of joint displacement, from before, during, and after exposure to $ZnCl_2$. The response for individual preparations at (a) 0.1 mM and (b) 1.0 mM are shown. The values represent averages of the activity of all three trials in each condition and (c) percent change from saline to Zn^{2+} was large for 1 mM but not statistically significant from 0.1 mM due to the large variability among preparations

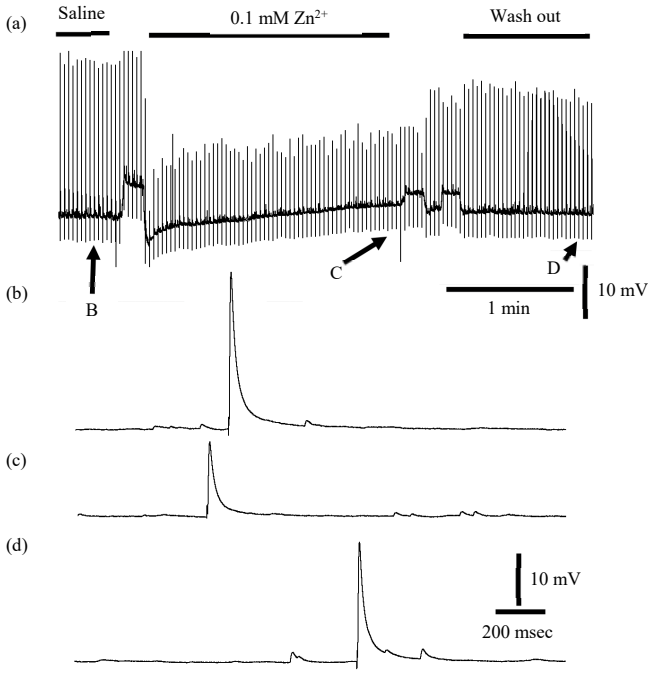

Fig. 12(a-d): (a) Response of evoked excitatory junction potentials (EJPs) and spontaneous quantal events at the neuromuscular junction (NMJ) of early third-instar larval *Drosophila* to the exposure of $ZnCl_2$ (0.1 mM) and (b-d) enlargements of sections within the overall trace shown in A

EJP was smaller upon exposure to $ZnCl_2$ and that spontaneous quantal events were present in all conditions

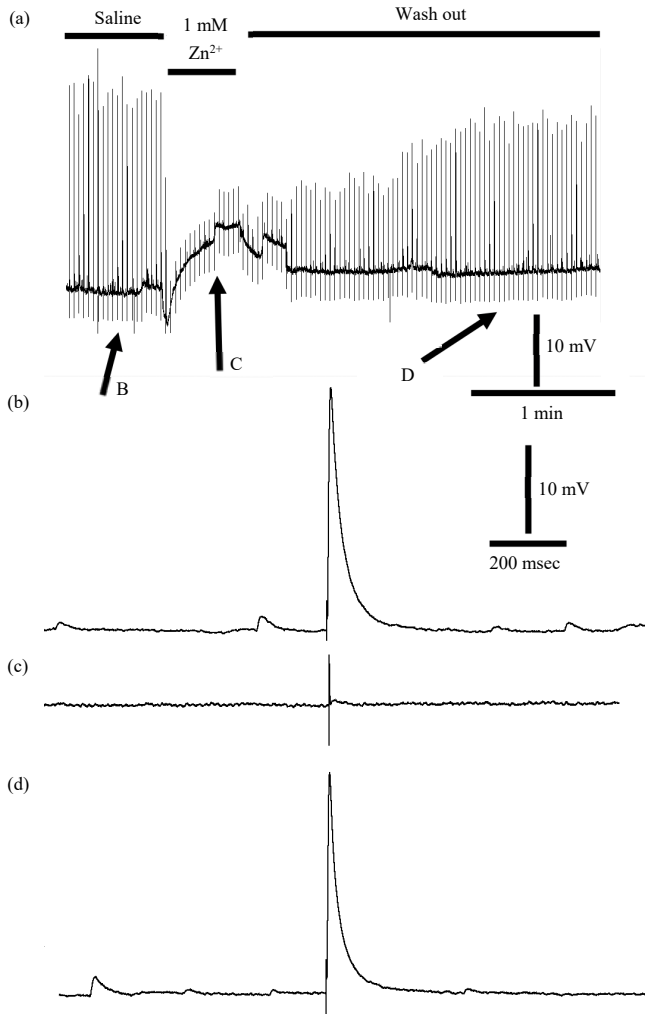

Fig. 13(a-d): (a) A representative response of evoked excitatory junction potentials (EJPs) and spontaneous quantal events at the neuromuscular junction (NMJ) of early third-instar larval *Drosophila* upon exposure to $ZnCl_2$ (1.0 mM) and (b-d) enlargements of sections within the overall trace shown in A

EJP was smaller upon exposure to $ZnCl_2$ and that spontaneous quantal events were not present during this concentration of $ZnCl_2$ exposure

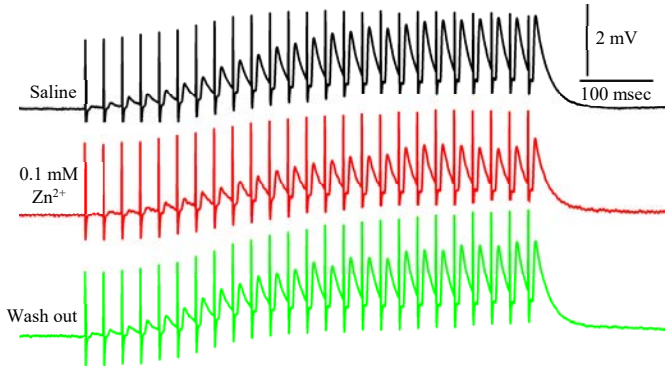

Fig. 14: Representative responses of synaptic transmission in the crawfish opener muscle before, during, and after exposure to $ZnCl_2$ (0.1 mM)

A 25-stimulus train at 40Hz was provided to the excitatory motor neuron. The excitatory junction potentials were slightly reduced in amplitude during $ZnCl_2$ exposure. The stimulus artifacts illustrate each stimulus

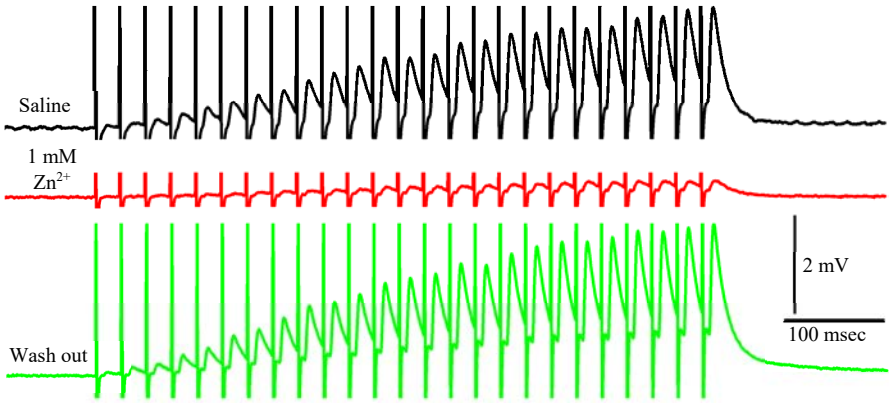

Fig. 15: Representative responses of synaptic transmission in the crawfish opener muscle before, during and after exposure to $ZnCl_2$ (1.0 mM)

A 25-stimulus train at 40 Hz was provided to the excitatory motor neuron. The excitatory junction potentials were greatly reduced in amplitude during $ZnCl_2$ exposure. The stimulus artifacts illustrate each stimulus

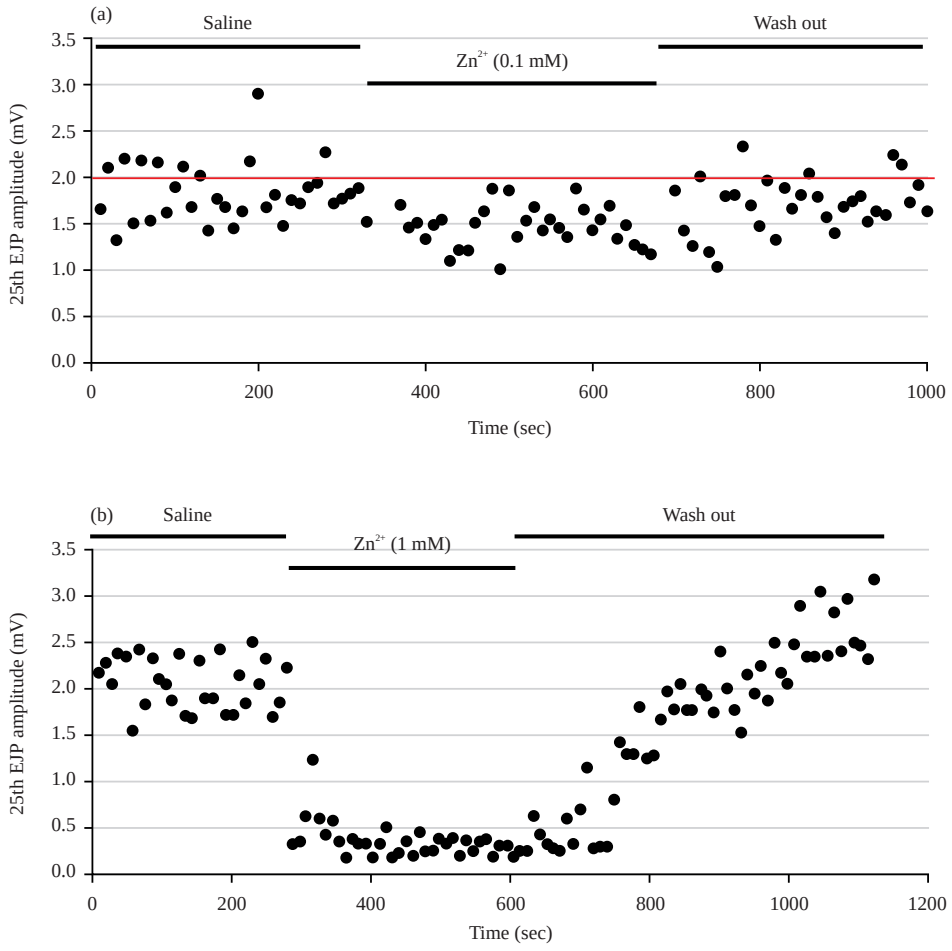

Fig. 16(a-b): Representative responses showing the effects of (a) 0.1 mM $ZnCl_2$ and (b) 1mM $ZnCl_2$ on the EJP amplitude of the 25th response in a 25-stimulus train at 40Hz

EJP amplitudes were rapidly reduced during 1.0 mM $ZnCl_2$ exposure and were slow to recover upon removal of the $ZnCl_2$. The lines at the top of the graph describe the preparation environment, while the dots show the amplitude value of the 25th EJP amplitude in mV

DISCUSSION

This investigation focused on using two invertebrate species to assess the acute effects of environmental Zn^{2+} exposure, as well as the direct effects of Zn^{2+} on the physiological function of various tissues. Both *Drosophila melanogaster* and crawfish serve as model invertebrate organisms for their respective environments, providing insight into the potential effects of acute Zn^{2+} exposure on other organisms. To understand more about the mechanistic actions of Zn^{2+}, direct measurements of sensory, cardiac, and NMJ function upon exposure might provide further details about the potential biological actions of Zn^{2+} exposure on an overall organism.

Acute exposures at the concentrations used in these experiments are higher than what these animals would likely encounter in their native environments, but these results provide useful information nonetheless. The effects highlighted here give insight into how Zn^{2+} acts, as well as how lower concentrations might have subtle effects upon prolonged exposure. Additionally, some situations might well result in organisms (including humans) being exposed to high concentrations of zinc; for example, zinc ore mining operations, industrial exposure (such as smelting) and overconsumption of dietary supplements are all examples of such methods of exposure. Indeed, zinc ranks as the fourth most mined ore in terms of tonnage mined worldwide[47]. Coal mining, as well as other metals like lead and copper more generally, can also lead to high levels of zinc exposure, as the metals are often found in close proximity to one another before being concentrated during industrial refining processes[48-52]. High concentrations of zinc are also used in research, such as that directly with pure $ZnCl_2$ (Sigma-Aldrich), and in galvanization processes, where pure $ZnCl_2$ is used to produce protective coatings[53,54]. Cases of toxicity, even to the point of death, have been recorded in humans exposed to zinc through various media since the 1940s[55,56]. Even today, people are still intentionally exposed to $ZnCl_2$ in training exercises with white smoke, such as those experienced by firefighters[56,57]. For these reasons, it is practical to understand how concentrations higher than those found diluted in nature might affect organisms and their physiological processes should exposure occur.

Additionally, these results provide insight into realms of research that haven't yet been fully explored. The model fish species for ecotoxicology and environmental research is the marine medaka, *Oryzias melastigma*[58]. This species has been used to address zinc toxicity for both early life and adult stages in freshwater, brackish water, and sea water[59]. Interestingly, higher toxicity was observed in freshwater than marine or brackish water, which may stem from the formation of a zinc precipitate and subsequent reduction of free zinc ions. The mayfly insect, *Neocloeon triangulifer*, has been used to observe zinc toxicity through dietary exposure in an aqueous environment and a ratio of 81 μg dry food mass to 10 μg L^{-1} water volume led to toxicity. However, dietary zinc toxicity in *Drosophila* had not yet been investigated. This, as well as many other physiological effects of zinc, bore more investigation than had previously been done.

Survival and developmental studies of *Drosophila melanogaster*: It is unsurprising that the effects of dietary Zn^{2+} exposure were more pronounced for *Drosophila* larvae in the first-instar larva to pupa-adult stages than they were for *Drosophila* only exposed from early third-instar to pupa-adult; after all, accumulation in the tissues is likely and physiological effects could be progressive over time. However, it bears mentioning that homeostatic regulation in the zinc transporter systems of the gastrointestinal tract could have occurred, thus decreasing the amount of zinc absorbed and upregulating the excretion processes if higher than normal zinc levels occurred in the hemolymph. The Zn^{2+} transporters and their expression in the gastrointestinal tract of *Drosophila* have been investigated[60,61].

Such compensations may also occur for adults over time. Biphasic responses in survival and tissues could be observed by comparing compensation in lower concentrations to that in higher concentrations, as damage to tissues at a higher exposure might not allow for physiological acclimation. In this experiment, dietary Zn^{2+} delayed the development of first-instar larvae at higher concentrations, with more death observed during development. Similar effects were observed during pupation and as adults. Enhancements in larval development were observed at 2.5 mM, as compared to the controls or higher concentrations. Further investigation into the measurement of Zn^{2+} levels in the hemolymph over time would help address gastrointestinal absorption and excretion rates, especially if examined in both larvae and adults. The internal zinc concentration in zinc-treated animals can be measured directly by spectroscopy or molecular probes, but this equipment was not available to us for this study.

Behavioral assays: The larval body-wall movements (or crawling behavior) and the rapid mouth-hook movements associated with eating are both common metrics of an organism's coordination in locomotion, muscle regulation, and neural circuit processing[62].

These behaviors require the integration of sensory activity within the central nervous system, coordinated motor neuron activity and functional synaptic transmission in the neural connections and NMJs. Late second-instar to early third-instar larvae were observed to, within just 24 hrs of dietary Zn^{2+} ingestion, have altered behaviors in a dose-dependent manner. Given the rapidity of mouth-hook movements, it is not surprising that these movements exhibited larger physiological effects. The coordination of the segmental movements was not assessed in this experiment, but it is known to be affected by defects in neural circuit activity[63,64]. Future studies could address the more subtle effects of Zn^{2+} on this coordination. The mouth-hook movement assay involves the coordination of various muscles[65], at 140 to 160 rhythmic movements per minute (compared to the 60 to 80 movements carried out by the body wall muscles); mouth-hook movements involve more rapid synaptic transmission within each synapse of the neural circuit, so slight effects exhibited at each synaptic junction would have a larger effect than more slowly utilized synapses, this allows more recovery time for synaptic priming and Ca^{2+} handling. The physiological response to Zn^{2+} observed at exposed body wall muscles suggests that Zn^{2+} is likely blocking presynaptic voltage gated Ca^{2+} channels. Such actions are also likely to occur at each synaptic connection, not just the NMJs directly involved with mouth-hook movements and body wall locomotion.

Larval *Drosophila* heart: The cardiac and skeletal muscles of larval *Drosophila* contract primarily due to Ca^{2+} entry from extracellular fluid[66-68]. The Zn^{2+} could be blocking these voltage-gated channels on the muscles, resulting in reduced skeletal and cardiac muscle function. As with the rate of mouth-hook movements, the *Drosophila* heart rate is high, at around 140 to 160 beats per minute, thus, changes in Ca^{2+} dynamics caused by Zn^{2+} influx or efflux would cause rapid alterations in the contraction rate. Since the larval heart is not innervated by neurons at the early third-instar stage, this experiment could provide insight into the direct actions of Zn^{2+} on cardiac tissue. Acute action at 0.1 mM Zn^{2+} wasn't consistent, but 0.5 mM Zn^{2+} resulted in a rapid decrease in cardiac activity. The heart slowed down upon increasing exposure to $ZnCl_2$. Exposures of 2.5 mM and above saw very little recovery after only 2 min of contact, even with the removal of the $ZnCl_2$. Additionally, the heart under these conditions resulted in low sensitivity to 5-HT, even after removal of the Zn^{2+}-tainted saline, despite the heart normally being very sensitive to exogenously applied 5-HT[68,69]. An exposure to a 5-HT at 100 µM would normally increase the rate by 30 to 40% from baseline. Thus, the Zn^{2+} either continued to block the voltage-gated Ca^{2+} channels on the heart even after exposure was discontinued or that exposure damaged the tissues.

Crawfish cardiac function and survival: Measuring heart rate and behavioral responses upon stimulation of crawfish neural circuitry allows assessment of whether these functions are connected to circulating Zn^{2+} levels in the hemolymph. Unlike the larval *Drosophila* heart, the crawfish heartbeat is neurogenic; thus, one would expect to observe similar effects in each, with decreased heart rate observed in conjunction with the organism being lethargic and/or unresponsive to sensory stimuli. However, at injections of high Zn^{2+} concentration (estimated at circulating levels of 10 mM), the crawfish tucked its telson under the body and fell into catatonia; while the heartbeat was still detectable, this indicates that, while the animal was paralyzed, the heart continued to function. In some cases, the crawfish appeared dead, lying on its side without the tucked telson and in a more flaccid state (without leg or cheliped movement when touched) and yet the heart rate was still able to be recorded. Thus, it could be stated that, in a Zn^{2+}-induced state of either contracted or flaccid paralysis, the heart continued to beat, without significant alterations in heart rate upon sensory stimulus. However, with these high concentrations, the crawfish died within two hours, while some lost cardiac activity only two minutes after injection. It is possible that Zn^{2+} chelated and bound to circulating proteins differently in crawfish.

Crawfish muscle receptor organ: Primary sensory neurons are devoid of synaptic activity, allowing for assessment of how neural activity is affected by Zn^{2+} exposure without synaptic involvement, however, just as the presynaptic voltage-gated Ca^{2+} channels at synapses may be blocked, the stretch-activated channels (SACs) in sensory endings might be similarly affected. If this is the case, sensory transduction may not be as effective at recruiting the neuron, resulting in fewer action potentials for recording. Even though the specific mechanisms responsible for any Zn^{2+}-provoked alterations in MRO nerve activity may not be identified, the preparation still allows for the assessment of zinc's potential effects on primary sensory function. Initially, it was expected that Zn^{2+} would decrease neural activity by blocking the sensory ending SACs, with joint displacement responses decreasing as the Zn^{2+} exposure increases; however, the opposite occurred and

there was an increase in neural activity. Since neural activity increases upon Zn^{2+} exposure, both upon displacement and while at rest, it is likely that SACs in the sensory ending are not blocked by the Zn^{2+}. Additionally, these observed results could be a result of enhanced SAC deformation stimulating the sensory ending, since these endings are buried within the muscle fibers they monitor and Zn^{2+} exposure might result in enhanced muscle stiffness or a lower threshold for the axons. If so, this would produce a more depolarized state, spontaneous neural activity, and enhanced sensitivity to joint displacement, which lines up with the results reported here.

Recording from an isolated section of nerve without stimulable sensory endings and recording the compound action potentials both before and during Zn^{2+} exposure would provide insight into the precise mechanism behind the heightened neural activity observed. Indeed, if it were possible to record directly over the SACs using tight patch electrodes (which has yet to be done in this preparation), channel opening time and threshold could be observed. The possibility that the recordings detected the pulling of MRO-associated skeletal muscle on the sensory endings is high, since recording from the larval *Drosophila* muscle while stimulating the motor nerve at 0.5 Hz produced (in one preparation of six) spontaneous excitatory junction potentials (EJP) upon exposure to 1 mM $ZnCl_2$. However, recording from the crawfish opener muscle in the walking leg during exposure to $ZnCl_2$ (0.1 mM and 1 mM) resulted in no observed spontaneous EJP activity.

Assuming that the loss of evoked synaptic transmission upon zinc exposure is caused by Zn^{2+} blocking voltage-gated Ca^{2+} channels in the motor neuron presynaptic terminals, it would be logical to assume that the Ca^{2+} channels along the nerve would also be blocked and that Zn^{2+} would replace some Ca^{2+} from the surface of the plasma membrane as the number of zinc ions increase. The observation that neuronal activity can be enhanced with lowered extracellular $[Ca^{2+}]$[70,71] may also explain some of the responses observed with the crawfish MRO sensory nerve. It was recently demonstrated that the sensory nerves of a marine crab exhibited increased electrical activity and spontaneously induced action potentials when Ca^{2+} was removed from the bathing medium or replaced with Ba^{2+} [72]. This was supported by the fact that reducing extracellular $[Ca^{2+}]$ removed some of the partial blockage of voltage-gated Na^+ channels observed in other preparations[73-76]. A small influx of Ca^{2+} can activate calcium-activated potassium channels ($K_{(Ca)}$) and help maintain a more negative membrane potential; similar activation is observed even if the Ca^{2+} leak is low provided that calcium enters during evoked activity[76]. It is known that other ions, like Ba^{2+}, do not activate the $K_{(Ca)}$ channel to the same extent as Ca^{2+}. After an action potential occurs, a membrane may be unable to fully repolarize due to reduced activation of the $K_{(Ca)}$ channels; if this is the case, the axon would be closer to threshold for voltage-gated Na^+ channels. These mechanisms may explain why sensory neurons are hyperactive with the presence of Gd^{3+}, which blocks Ca^{2+} channels[40,77]. This culminates in the following idea: If Zn^{2+} displaced the actions of Ca^{2+} and resulted in a reduced block of Na^+ channels or reduced function of $K_{(Ca)}$ channels, a nerve would more easily reach action potential threshold and may even produce them spontaneously.

Neuromuscular junctions of larval *Drosophila* and crawfish: The observed effects of Zn^{2+} on evoked release at the NMJs of larval *Drosophila* and crawfish are likely explained by the blockage of presynaptic voltage-gated Ca^{2+} channels, which would reduce Ca^{2+}-driven vesicular fusion and transmission[78,79]. Both preparations saw substantially reduced evoked synaptic transmission upon exposure to 0.1 mM and 1 mM $ZnCl_2$, both also recovered well after the solutions were flushed away. This indicates that the Zn^{2+} is not strongly bound to these preparations' channels. As previously discussed, flushing the larval heart preparations did not result in good recovery, indicating that the heart tissue was likely damaged by the Zn^{2+} exposure or that the Zn^{2+} remained bound to the Ca^{2+} channels present. The use of Ca^{2+} indicators in cardiac myocytes, presynaptic motor nerve terminals, and the axons of the MRO would help verify whether or not the primary actions of Zn^{2+} on these preparations are carried out through altered Ca^{2+} dynamics.

CONCLUSION

Over the course of this investigation, experimentation with both *Drosophila* and crawfish provided valuable insight into the effects of acute zinc exposure on models for mammalian physiology. Zinc (Zn^{2+}) was found to impair behavior in *Drosophila* organisms and to increase the number of activity spikes observed at the crawfish muscle receptor organ (MRO). It was also found to delay development and diminish survival in both, as well as to decrease heart rate (sometimes to cessation, with variable success upon washout) and excitatory junction potentials (which recovered upon removal of zinc). These observations could very well be useful to future investigation into zinc as regards its effects and applications.

SIGNIFICANCE STATEMENT

Given the wide variety of possibilities for zinc exposure, it is prudent to know how overexposure would affect animals. These experiments represent a comprehensive survey of how model organisms respond in conditions of excess zinc exposure to allow for further research. In crawfish and fruit flies, zinc can block synaptic transmission, alter sensory function and delay both development and survival. It appears that zinc affects voltage-gated ion channels such as Ca^{2+} and Na^{+} channels. This is a significant study for understanding the physiological effects of zinc on model organisms such that zinc's actions on other organisms may be assessed.

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