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Research article

Examining the effect of iron (ferric) on physiological processes: Invertebrate models

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ABSTRACT

Iron is a common and essential element for maintaining life in bacteria, plants and animals and is found in soil, fresh waters and marine waters; however, over exposure is toxic to organisms. Iron is used in electron transport complexes within mitochondria as well as a co-factor in many essential proteins. It is also established that iron accumulation in the central nervous system in mammals is associated with various neurological disorders. Ample studies have investigated the long-term effects of iron overload in the nervous system. However, its acute effects in nervous tissue and additional organ systems warrant further studies. This study investigates the effects of iron overload on development, behavior, survival, cardiac function, and glutamatergic synaptic transmission in the *Drosophila melanogaster*. Additionally, physiological responses in crayfish were examined following Fe^{3+} exposure. Fe^{3+} reduced neuronal excitability in proprioceptive neurons in a crayfish model. Thus, Fe^{3+} may block stretch activated channels (SACs) as well as voltage-gated Na^{+} channels. Exposure also rapidly reduces synaptic transmission but does not block ionotropic glutamatergic receptors, suggesting a blockage of pre-synaptic voltage-gated Ca^{2+} channels in both crustacean and *Drosophila* models. The effects are partly reversible with acute exposure, indicating the cells are not rapidly damaged. This study is relevant in demonstrating the effects of Fe^{3+} on various physiological functions in different organisms in order to further understand the acute and long-term consequences of overload.

1. Introduction

Iron is found in various forms in nature. While iron occurs in many oxidation states such as +2, +3, +4, and +6, it is commonly found in the +2 and +3 forms. Iron is essential for survival in many organisms, from the range of bacteria to humans (Frawley and Fang, 2014; Lasocki et al., 2014). Deficiencies in iron are associated with a number of health conditions in humans and numerous other organisms (Abbaspour et al., 2014; Gisslen et al., 2023). Likewise, iron overload is also involved in the manifestation of many pathological conditions in humans and other animals (Joerling and Doll, 2019; Gu et al., 2023). A military perspective of iron overexposure stems from the battlefield's overabundance of potentially toxic hazards including heavy metals. Metal-containing particulates in smoke from destroyed vehicles, open-air burn pits and environmental dust presents one possible mode of internalization of heavy metals (Kalinich and Kasper, 2016). Once iron is obtained systemically, excess amounts are not excreted in urine or feces, but rather accumulate in mammals (D'Mello and Kindy, 2020). However, blood loss, sweat, and cell loss from turnover in the intestinal epithelium, skin, and urinary tract are means of iron loss (Gorman, 2023; Hentze et al., 2010; Nichol et al., 2002). In mammals, iron is known to accumulate in specific regions of the central nervous system (CNS) and has been linked to dysfunction associated with Parkinson's disease and other neurological disorders (Huenchuguala and Segura-Aguilar, 2023; Snyder and Connor, 2009). Like mammals, insects obtain iron through diet and absorb it through the intestine by transport. The hemolymph then transports iron into cells via similar proteins such as transferrin and the storage protein ferritin (Gutierrez et al., 2013; see review by Nichol et al., 2002). Iron is also noted to be stored in fat bodies and intestinal tissue within insects (Nichol et al., 2002; Tang and Zhou, 2013). Iron excretion within invertebrates has not been fully investigated and was reviewed in studies primarily associated with the *Drosophila* model

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(Tang and Zhou, 2013).

In mammals, iron obtained by dietary consumption is transported across the gastrointestinal tract and into the blood. Iron accumulation occurs in plants, insects, crustaceans, and mammals, all of which are regularly consumed by humans. Thus, exposure to iron from dietary accumulation, contamination, or therapeutic use creates the potential for bioamplification throughout the food chain. Additionally, as balanced iron levels are necessary for optimal physiological function, iron is therapeutically administered in the form of oral supplements or injections when dietary intake is insufficient or in the case of gastrointestinal malabsorption (Aggett, 2012; Gisslen et al., 2023; Murray-Kolbe and Beard, 2010; Peroni et al., 2023).

Since iron is readily obtained from over the counter as supplements in the Fe^{2+} and Fe^{3+} forms, it is not surprising that cases of iron overload leading to direct cellular damage, and even death, exist (Dixon et al., 2012; Ríos-Silva et al., 2023; Yang and Stockwell, 2008). Excess iron is a pathological condition referred to as ferroptosis and has received increased attention in recent clinical studies (Dixon et al., 2012; Zhang et al., 2023a, 2023b). The effects of ferroptosis are related to the production of free radicals which can lead to cellular damage (Zhang et al., 2023a, 2023b). In investigating the effects of ferroptosis on biological systems, the effects of iron are experimentally performed with iron chloride ($FeCl_3$) or ferric ammonium citrate.

Given excessive iron has a plethora of effects on animals and results in physiological dysfunction of various tissues, it is of interest to assess a number of mechanistic effects to explain the whole-body impact of iron on an organism. Dietary consumption is the primary route of iron exposure. Transport within the circulatory system, via hemolymph or blood, involves a carrier protein to deliver or expose tissues to iron. In mammals, iron is incorporated into hemoglobin, myoglobin and cells, providing an additional means of extracellular exposure in the case of tissue injury and/or lysing of erythrocytes. Thus, neurons in the periphery and CNS, as well as skeletal and cardiac muscle, can be directly exposed to free iron. It is now known that with hemorrhagic stroke in humans, removal of blood present on the neural tissue reduces the extent of neuronal damage in comparison to waiting for the dissolution of the blood. This neuronal damage is likely from the release of hemoglobin and iron inside cells (Almutairi et al., 2019; Dabbagh Ohadi et al., 2023; Han et al., 2023). Likewise, addressing iron overload in the circulatory system by the use of systemic chelators reduces ferroptosis-induced tissue damage (Fang et al., 2019; Farr and Xiong, 2021). Thus, investigating acute iron-exposure in tissues may help to establish the window of time in which recovery is optimized.

The common fruit fly (*Drosophila melanogaster*) and red swamp crayfish (*Procambarus clarkii*) were chosen as model animals in this study to address the effects of excess iron exposure. These two model systems, along with other invertebrates, allow relatively rapid and inexpensive preparations to assess and compare the physiological effects of compounds on various tissues in addition to survival and development of the intact animal (Brock et al., 2023; Elliott et al., 2023a, 2023b; Mann et al., 2023; Pankau et al., 2022; Stanley et al., 2020).

2. Methods

The methodology used in these investigations parallels the same procedures as detailed in previous studies from our research group examining the effects of Mn^{2+} and Zn^{2+} exposure (Elliott et al., 2023a, 2023b; Pankau et al., 2022; Pankau and Cooper, 2022). Thus, the procedures are replicated in this report for standardized comparison. The effects of Fe^{3+} (ferric ammonium citrate and ferric chloride) on sensory neurons in marine crabs were presented previously and are not presented in this paper herein; however, the effects of ferric ammonium citrate on the crayfish MRO abdominal proprioceptor follows a similar procedure as detailed for the marine crab (Wagers et al., 2023). Thus, procedures are redundant from previous reports from the same research group.

Iron chloride ($FeCl_3$) or ferric ammonium citrate is commonly used in experimental studies. However, $FeCl_3$ in water is acidic as compared to ferric ammonium citrate. Thus, the effects of pH or excessive buffering must also be accounted for when using $FeCl_3$ (Wagers et al., 2023). Ferric ammonium citrate is more soluble in physiological saline than $FeCl_3$ at high concentrations, resulting in its widespread use in experimental studies investigating the effects of iron in a physiological context (Che et al., 2021; Irrsack et al., 2021; Zeng et al., 2021). Furthermore, $FeCl_3$ precipitates in a pH range of 7.4, creating difficulty in assessing the bioavailability of the compound under physiological conditions. Ferric ammonium citrate, however, maintains a soluble iron (III) complex (Hider and Kong, 2010; Neilands, 1995; Sánchez et al., 2017; Wagers et al., 2023).

2.1. Animals

Two different animal models were used in this study: fruit flies (*Drosophila melanogaster*) and red swamp crayfish (*Procambarus clarkii*). The same animal models and rearing conditions as used in Pankau et al. (2022) were used in the current study. In brief, Canton S (CS) flies were used in all behavioral and physiological assays. The CS *Drosophila melanogaster* strain has remained isogenic in the laboratory and was originally obtained from Bloomington Drosophila Stock Center (BDSC). All animals were maintained in vials partially filled with a cornmeal-agar-dextrose-yeast medium. The *Drosophila melanogaster* model provides a means to address the effects of dietary iron on development and survival for larvae as well as adults. In addition, acute exposure on the cardiac system and synaptic transmission at neuromuscular junction (NMJ) were examined. The larval heart tube in *Drosophila* is used to directly count the heart beats and assess the effects of iron on heart rate without the confounding factor of neural innervation. The larval heart is exclusively myogenic and can be assessed in situ while bathing in a physiological saline to eliminate the effects of circulating modulators on the heart. Likewise, the larval NMJs do not produce action potentials on the muscle fibers; thus, allowing insight on pre- and post-synaptic quantal release and responses as well as effects on membrane potential of the muscle fibers (Jan and Jan, 1976; Kurdyak et al., 1994).

Red swamp crayfish were obtained from Kyle LeBlanc Crawfish Farms (302 Saint Peter St., Raceland LA 70394, USA) and delivered to Lexington, KY, USA. Throughout the study, midsized crayfish measuring 6–10 cm in body length and 15.5–30 g in body weight were used. Each animal was housed in individual, standardized plastic containers with dry fish food exchanged weekly and aerated water (20–21 °C). This model also allows easy access to investigate synaptic transmission at the NMJs since the opener muscle in the walking legs is non-spiking. In addition, the proprioceptive muscle receptor organ provides access to primary sensory neurons to monitor changes in function due to iron exposure. The survival of the animal after directly injecting iron into the open circulatory system was assessed while also monitoring changes in the heart rate. Heart rate as a measure of cardiac function, serves as a monitor of physiological activity for continuous monitoring and repeated measurements on individuals over a short timeframe.

2.2. Drosophila development and survival

To determine the effect of Fe^{3+} (ferric ammonium citrate) on development in larval *D. melanogaster*, the eggs were captured after a 30 min pre-pulse of egg laying at 21 °C on an apple juice agar dish. This was performed to clear the females of stored eggs in the ovipositor. The adults were allowed access to a new apple juice agar dish for 4 h. This dish was then removed and maintained for 48–54 h for 2nd instars to appear. Ten 2nd instars were placed in a vial of food containing a mixture of standard fly food and Fe^{3+}. This procedure was repeated for a total of ten vials, each containing ten larvae. When pupa formed they were marked and numbered on the side of the vial. This occurred in 6 h intervals in the day or 12 h intervals overnight (night to morning). The

number of pupae formed and the number which enclosed to form adults was recorded. Thus, the number of larvae and pupae that died could be determined. Adults left in the vials were also followed for 5 days to determine the effects on Fe^{3+} exposure from instar, pupa, and adult stages. Three different concentrations of Fe-citrate were used. The final concentrations after dilution in the food were 0.145 mM, 0.25 mM and 5 mM, obtained by using a 0.5 g of cornmeal food and mixing with a 10 mM stock of Fe^{3+} and water. One gram of food was assumed to be equivalent to 1 mL of solution. Additionally, adults (not previously exposed to Fe^{3+}) which had enclosed within 24 h were also used to examine the effects of consuming Fe^{3+} in the cornmeal diet. Only the 5 mM Fe^{3+} tainted food was used to examine overload on adults. Ten adults were placed into each of the ten vials for assessment. Controls for the larvae and adults were conducted in parallel with plan cornmeal food and water added. Ten larvae and ten adults were used for each vial for ten vials for analysis. A chi-squared test was used at each level (2nd instar and adult). For 2nd instar, since all levels of Fe^{3+} were compared, post-hoc chi-squared tests were used with the chisq.post.hoc function in R and significant differences were based on a Bonferroni corrected *p*-value. At the 3rd instar, a Fisher's Exact test needed to be used due to the assumptions of a chi-squared test being violated.

2.3. Drosophila behaviors

Two assays to assess neural function and motor command were utilized for larvae that consumed Fe^{3+} – tainted food (0.145 mM, 0.25 mM and 5 mM) for 24 h from 2nd instar to early 3rd instar ($n = 15$ larvae in each vial): body wall movements (BWMs) during locomotion and mouth hook movements (MHMs) during eating. BWMs were assessed by placing larvae on a plate of apple-juice agar (1 % agar) Petri dishes (about 8.5–9 cm diameter) and visually recording the number of contractions per minute with the use of a dissecting microscope. The larvae were left inside the dish for 1 min to acclimate to the environment. Then BWMs were counted for 1 min in a lightly illuminated environment at room temperature (21–22 °C). Afterwards, the larvae were used to assess larval feeding behavior after being transferred to a small Petri dish (5.5 cm diameter) containing a yeast solution consisting of several dried yeast granules mixed with water. The larvae were left undisturbed for 1 min before counting MHMs for 1 min. An ANOVA with normality test (Shapiro-Wilk), equal variance test (Brown-Forsythe) and multiple comparisons versus control group (Holm-Sidak method) was used to compare controls versus subjects fed Fe^{3+}.

2.4. Larval Drosophila heart

A detailed description for exposing larval hearts in early 3rd instars is provided in Cooper et al. (2009). The procedures followed are as described in Pankau et al. (2022). In brief, the larvae were dissected ventrally and pinned on four corners to expose the heart tube. The preparation was then bathed in physiological saline (de Castro et al., 2014). 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; Stewart et al., 1994). In the experimental groups, varying amounts of Fe^{3+} was dissolved in this saline. The pH of the saline was maintained at 7.1. Concentrations of 5 mM and 10 mM were examined for 12 larvae. The heart rate was measured in the caudal region of the heart tube in a 30 s period by visually counting the beats with a dissecting microscope. The values were converted to beats per minute for graphical analysis. The heart rate was first obtained during the initial saline exposure before the bathing solution was exchanged to one containing Fe^{3+}. This was followed by flushing out the compound with fresh saline three times and then waiting 1 min before recording data of the heart rate after the Fe^{3+} was removed. A paired *t*-test if Shapiro-Wilks test passed, or a Wilcoxon Signed Rank test in which ties were treated as averages of the ranks, was used to compare the differences in responses before and during exposure to Fe^{3+}.

2.5. Larval Drosophila neuromuscular junction

The larval dissections were performed as described in Cooper et al. (1995a). In brief, third instar larvae were dissected in physiological saline. A longitudinal mid-dorsal incision was made, and the edges were pinned so the preparation was spread out on a glass slide in the preparation dish as originally described for studies of the leech nervous system (Muller et al., 1981). Internal organs were carefully removed to expose the body wall muscles, particularly the ventral longitudinal muscles of segment 4. The electrical recordings were obtained from the prominent longitudinal m6 muscle. Intracellular recordings were made with microelectrodes filled with 3 M KCl (30–60 MΩ). The segmental nerves were cut and sucked into a suction electrode, which was filled with saline and stimulated (Fig. 1). The segmental nerves were stimulated at 0.5 Hz (S88 Stimulator, Astro-Med, Inc., Grass Co., WestWarwick, RI, USA). The responses were recorded with a 1 X LU head stage and an Axoclamp 2A amplifier followed by a converter PowerLab, 4SP (ADInstruments, USA) and analyzed with LabChart 7.0 (ADInstruments, Colorado Springs, CO, USA) on a computer at a 10 or 20 kHz sampling rate. The effects of Fe^{3+}on the membrane potential evoked excitatory junction potentials (EJPs) and spontaneous quantal events (mEJPs) were examined by changing the bathing media to saline containing Fe^{3+}. This was followed by flushing the preparation with standard fly saline to determine viability after exposure. A paired *t*-test, if Shapiro-Wilks test passed, or a Wilcoxon Signed Rank test was used to compare the differences in responses before and during exposure to Fe^{3+}.

2.6. Crayfish muscle receptor organ

The dissection procedure is described in video format (Leksrisawat et al., 2010). The muscle receptor neurons of the crayfish abdominal

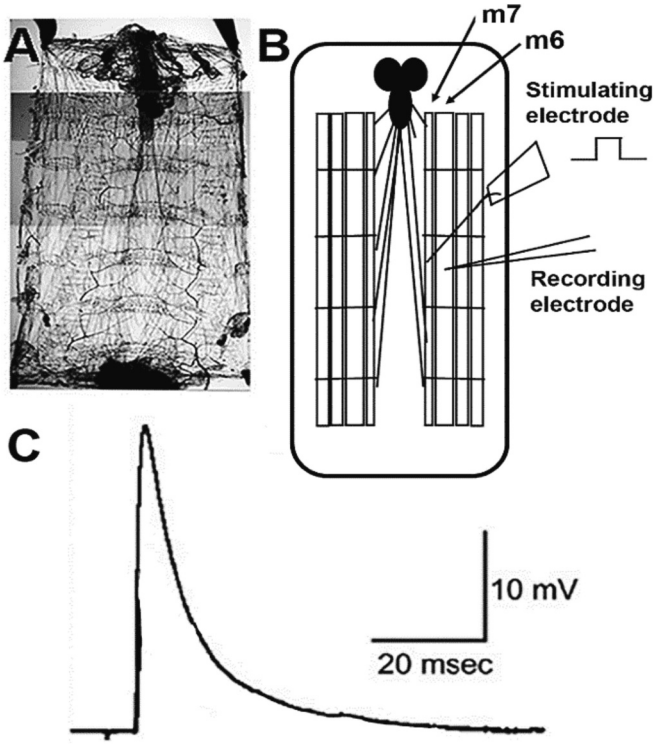

Fig. 1. The larval *Drosophila* preparation for recording synaptic responses at the neuromuscular junctions. (A) A 3rd instar larvae pinned open and (B) schematically illustrated where a segmental nerve is stimulated to evoke (C) EJPs in m6 muscle fibers.

proprioceptor are fully exposed to the bathing saline and produce spikes measured with extracellular recording upon bending of the abdominal joint. As the segmental nerve only measures the two neurons, one sensitive to dynamic movement and one sensitive to static movement, fewer variations in the amplitudes of spikes are recorded. The two sensory neurons have their sensory endings embedded within a single associated muscle fiber for each neuron. The muscle fibers span the joint in which they monitor, and they reside directly under the dorsal cuticle. With the joint being moved rapidly in 1 s to a set position and held for 10 s, both types of neurons are able to be monitored. Abdomen joints not involved in recordings were pinned in a Sylgard-lined dish and covered with crayfish saline. The MRO nerve was then exposed and pulled into a suction electrode for recording. Suction electrodes made from glass pipettes fitted with plastic tips were used to record extracellular signals from the cut nerves (details of making the suction electrodes is provided in Baierlein et al., 2011). A P-15 amplifier (Grass Instruments) in conjunction with a PowerLab/4s A/D converter and Lab Chart 7 software (ADI Instruments, Colorado Springs, CO, USA) obtained the signals to be recorded on a computer at a 10 or 20 kHz sampling rate. The neural activity is readily determined from noise when the MRO is not stretched and held still.

During the experiment, the joint of interest was moved in a 1 s time frame, held in that position for 10 s, and then moved back to the starting position. An insect dissecting pin was used as a reference for the maximum displacement range for consistency and each displacement was marked on the computer recording file. The joint was moved back to its starting position and allowed to rest for 10 s. The movements were repeated 3 times for three trials with identical rates and degrees of movement while exposed to saline only. After the bathing media was switched to one with the compound to be examined, the paradigm was repeated. The bath was exchanged again to saline by rinsing the bath twice to remove the Fe^{3+} being tested previously. The movements were again repeated three times in the fresh saline. The procedure was then followed with a higher concentration of Fe^{3+} and rinsed again. A separate set of experiments were conducted in which the preparations were only exposed to 20 mM Fe^{3+} and incubated for 20 min prior to moving the joint and collecting the neural activity associated with the movements. The number of spikes recorded over the 10 s from the start of the movement for each of the three movements in each condition was used for analysis. In addition, an average number of spikes of the three displacements in each condition were also determined for graphical purposes. A percent change among the preparations was also used for analysis to normalize differences among preparations in the number of spikes initially obtained in saline. Concentrations of 10 mM and 20 mM Fe-citrate were examined. A paired *t*-test (if Shapiro-Wilks test passed) or a Wilcoxon Signed Rank test was used to compare the differences in responses before and during exposure to Fe^{3+}.

2.7. Crayfish neuromuscular junction

The dissection and recording procedure is described in textual and video format (Cooper and Cooper, 2009). In brief, the most distal muscle fibers in the preparation were used for consistency among preparations (Fig. 2; Cooper et al., 1995b). The excitatory neuron was isolated from the inhibitor neuron and was then stimulated in the meropodite segment. The stimulation paradigm consisted of providing a train of 25 pulses at 40 Hz with10s between trains. Responses were recorded with an AxoClamp 2B (Axon Instruments, USA), converted with a PowerLab, 4SP (ADInstruments, USA), and analyzed with LabChart 7.0 (ADInstruments, Colorado Springs, CO, USA) on a computer at a 10 or 20 kHz sampling rate. Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mmol/L: 205 NaCl; 5.3 KCl; 13.5$CaCl_2 \cdot 2H_2O$; 2.45 $MgCl_2 \cdot 6H_2O$; 10 glucose; 0.5 HEPES adjusted to pH 7.4). For assessing the effect on synaptic transmission, Fe^{3+} at 5 mM and 10 mM was used. A paired *t*-test, if Shapiro-Wilks test passed, or a Wilcoxon Signed Rank test was used to compare before and during exposure to Fe^{3+}.

2.8. Crayfish cardiac function and survival

The recording procedure is described in textual and video format (Bierbower and Cooper, 2009). The rate of the heartbeat in intact crayfish was obtained by placing recording wires under the carapace over the heart. The wires consisted of insulated stainless steel wires (diameter 0.005 in./0.008 in. with coating; A-M Systems, Carlsburg, WA). The insulation was burned off the ends with a flame to provide a good connection with the recording devices. Small holes, approximately the thickness of the wires, cause minimal loss of hemolymph and a higher probability of the wires remaining in place during fixation. The insulated steel wires were placed into the carapace, spanning the heart to facilitate an accurate impedance measure (UFI, model 2991; Listerman et al., 2000). To eliminate the risk of damaging the heart, special

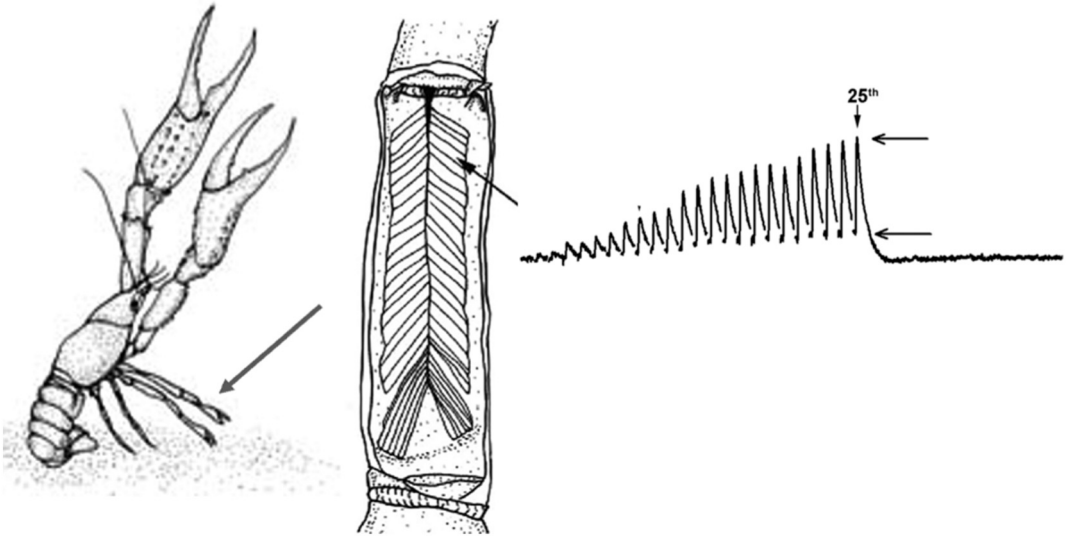

Fig. 2. The walking leg of the crayfish houses the opener muscle used to address the effects of ferric ammonium citrate on synaptic transmission in a crustacean model. Schematic of opener muscle as seen from a ventral view. Excitatory junction potentials were obtained from the distal muscle fibers. The excitatory motor neuron was stimulated at 40 Hz for 25 stimuli. The amplitude of the 25th EJP in the train was used for quantitative purposes.

attention was made to insert only a short portion of wire (1–2 mm). After placing the wire in the optimal position, the fixation was ensured via a small drop of glue (cyanoacrylate ester) and accelerator (HobbyTown USA, Lexington, KY). The impedance detector, measuring the dynamic resistance between the two wires, was linked to a PowerLab/4SP interface (AD Instruments) and calibrated with the PowerLab Chart software version 7.1 (AD Instruments, Australia). The acquisition rate was set at 1 kHz. The calculation of the heart rate (HR) was accomplished by direct counts of each beat over 1 min intervals and reported as beats per minute (BPM).

Fe^{3+} was injected into the crayfish in the abdomen by passing a needle through the articulating membrane on the ventral side. Observational studies were also made on the effects of injecting different doses. Sham experiments consisting of saline injections were conducted in order to account for excitation of the HR caused by the handling and injection of the animals. Systemic levels of Fe^{3+} were calculated based on dilution in the hemolymph from a stock concentration of 50 mM. The amount of hemolymph was estimated on the basis of animal weight assuming that 30 % of an animal's weight is hemolymph (Gleeson and Zubkoff, 1977; Guirguis and Wilkens, 1995). The total volume was established for the appropriate volume of stock to be injected. Fe^{3+} was diluted in physiological saline. Since the animals have an open circulatory system, the compounds are carried towards the heart where it bathes the cardiac ganglion and muscle. Experiments were carried out using several animals for each concentration.

To assess if the neurogenic heart was responding to sensory stimuli, activating the central nervous system which altered neural input to the cardiac ganglion, the telson of the crayfish was forcefully tapped with a glass rod to elicit a tail flip response. The recording was maintained for 10 to 20 min longer followed by saline injections or Fe^{3+} while recording the heartbeats. After 20 min the telson was forcibly tapped again to note the effects on the rate of the heartbeat. The effects of the telson tap were monitored periodically over 24 to 48 h after the injection. This injection protocol also provided an assessment on the survival of the crayfish over a period of days as a result of the injections.

3. Results

3.1. Drosophila development and survival

The effect of dietary Fe^{3+} on the survival of larvae, pupa, and adults was dependent on concentration. The larvae were introduced to iron-tainted food of various concentrations (0.145 mM, 0.25 mM and 5 mM) from beginning the 2nd instar stage. The low concentration had no effect on survival as compared to controls (Fig. 3). However, the mid-level (0.25 mM) and high (5 mM) concentrations resulted in the death of larvae and pupa (Fig. 3). For the 2nd instar stage, a significant difference in the development between the levels of Fe^{3+} from low to mid or to high ($p < 0.0001$) exists. With the chi-square post-hoc analysis, there was a significant difference between the low and middle concentration of Fe^{3+}, between the low and high concentration, between middle and the controls, and the high concentration and control group (all adjusted $p < 0.0001$). Several adults that enclosed had died in the high concentration (Fig. 3). It is likely the pupa were unhealthy from the prolonged consumption during the larvae period. Thus, it was of interest to learn the consequences of initially exposing larvae to Fe^{3+} in the 3rd instar stage as well as adult stage to examine survival.

Initial exposure to Fe^{3+} in the early 3rd instar and assessing larval, pupal, and adult stages was examined only for the high concentration of Fe^{3+} (5 mM). When exposed to food for the 24 h during the later part of the early 3rd instar and during the late 3rd instar before pupation occurred, a number of the 3rd instar larvae died (Fig. 4). A number of pupae did not enclose and died within the pupal case. It is not known if they were too weak to break out of the pupa case or if they died during the metamorphosis. Of the pupa which did enclose, the adults were exposed to the iron-tainted food. After 5 days, there were several dead adults and several alive (Fig. 4).

To better determine the effect of consuming Fe^{3+} specifically for the adults without prior exposure, adults within 24 h of eclosion were exposed to food containing Fe^{3+} (5 mM). After 7 days ~40 % of the flies died with the iron-tainted food. In the control vials, one adult died which may have resulted from entrapment in the food (Fig. 5).

3.2. Drosophila behaviors

Standard behavioral assays of larval *Drosophila* are indexing body wall crawling on an apple juice agar plate to promote crawling and mouth hook movements while eating. To determine how consuming Fe^{3+}-tainted food affected the larvae, these behavioral assays were performed. The larvae were placed in the tainted food (0.145 mM, 0.25 mM and 5 mM; 15 larvae placed in each vial) or controls while in the 2nd instar stage and 24 h later were assessed for the behaviors. Comparisons

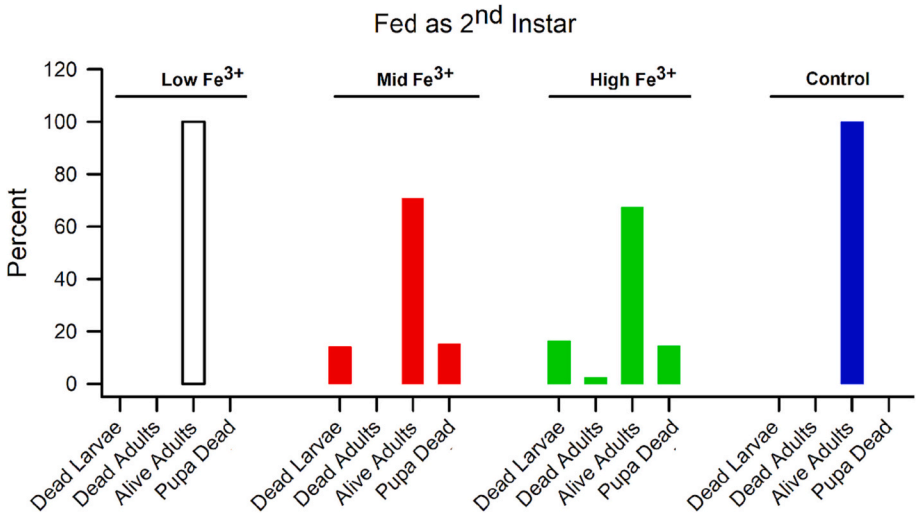

Fig. 3. The effect of dietary Fe^{3+} (ferric ammonium citrate) on *Drosophila* larval and adult survival. The larvae were fed Fe^{3+} starting from the second instar either 0.145 mM (Low), 0.25 mM (Mid) and 5 mM (High) and monitored through development to pupation and adulthood. The Fe^{3+} was mixed with standard cornmeal food. The low concentration had no significant effect on survival to adulthood. However, both Mid and High resulted in more death as larvae and pupa. The Mid and High were not significantly different in the overall effects (chi-square post-hoc analysis). 10 larvae in 10 different vials for each condition were used: thus, $n = 100$ for each paradigm.

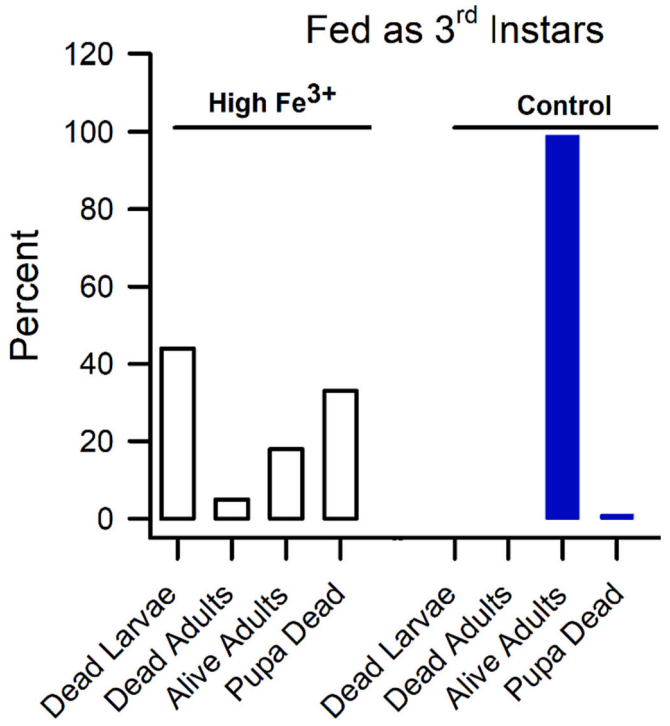

Fig. 4. The effect of dietary Fe^{3+} (ferric ammonium citrate) on survival of *Drosophila* larval from 3rd instar to adulthood. The larvae were fed Fe^{3+} starting from the 3rd instar 5 mM (High) and monitored through development to pupation and adulthood. The Fe^{3+} was mixed with standard cornmeal food. The larvae and pupa as well as adults had an increase in death rate compared to control (chi-square post-hoc analysis). 10 larvae in 10 different vials for each condition were used; thus, n = 100 for each condition.

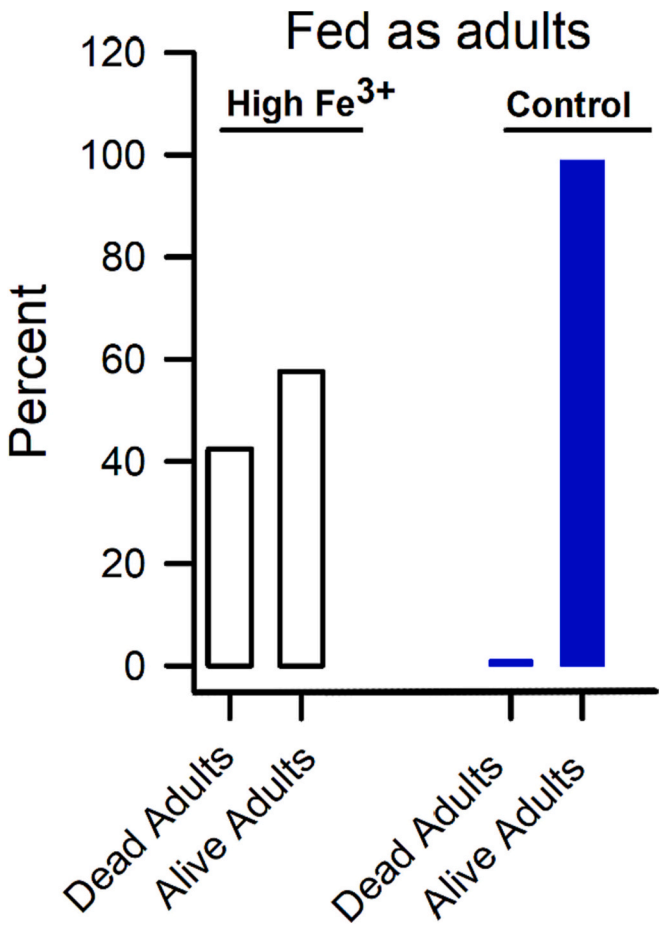

Fig. 5. The effect of dietary Fe^{3+} (ferric ammonium citrate) on survival of adult Drosophila. The young adults, within 24 h of eclosion, were fed Fe^{3+} 5 mM (High) and monitored for 7 days. The Fe^{3+} was mixed with standard cornmeal food. The adults had an increase in death rate compared to controls (chi-square post-hoc analysis). 10 adults in 10 different vials for each condition were used; thus, n = 100 for each condition.

to control larvae which were fed the standard food without the addition ferric ammonium citrate were made. (Fig. 6: * $P < 0.050$). The 0.145 mM and 0.5 mM were significantly different from control but not the 0.25 mM for body wall movements (Fig. 6A). Whereas only the 0.5 mM was significantly different compared to control for mouth hook movements (Fig. 6B).

3.3. Larval Drosophila heart

The effect of direct exposure of Fe^{3+} on the heart tube of the early 3rd instar was assayed by measuring the rate of the heartbeat for 1 min in normal saline and for 1 min in saline containing 5 mM or 10 mM Fe^{3+}. After rinsing the dish with fresh saline two times, the rate was determined again over 1 min. Significant differences for both concentrations ($p < 0.05$) occurred without differences in the responses between concentrations (Fig. 7).

3.4. Larval Drosophila neuromuscular junction

The effect of Fe^{3+} on synaptic transmission at the larval *Drosophila* neuromuscular junction (NMJ) was directly assessed by changing the bathing saline containing Fe^{3+}. To assess a dose-response effect, a low concentration was first examined followed by removal of the Fe^{3+}. Subsequently, exposure to a higher concentration was performed and again followed with a washout of the Fe^{3+}. The response for a representative preparation exposed to 10 mM and then 20 mM ferric ammonium citrate is depicted in Fig. 8. Since 20 mM Fe^{3+} was relatively high concentration with a large depolarization on the membrane potential, a lower concentration was used for assessing the effect on synaptic transmission (5 mM and 10 mM). The resting membrane potential depolarized in a concentration-dependent manner with exposure to the Fe^{3+} (Fig. 9A). The decrease in the excitatory junction potentials (EJPs) was also concentration dependent (Fig. 9B). The exposure to 10 mM was greater than for the 5 mM exposure for an effect on the resting membrane potential as well as a reduction in the amplitude of the EJPs (non-paired *t*-test; $p < 0.05$; $N = 7$).

3.5. Crayfish muscle receptor organ

As sensory nerve activity of the proprioceptive organ (PD organ), in a marine crab was shown to be depressed by ferric ammonium citrate (Wagers et al., 2023), it was expected that the freshwater crayfish proprioceptive organ, the muscle receptor organ (MRO), would also be depressed. The effects of both 10 mM and 20 mM Fe^{3+} were examined in series with flushing of fresh saline between exposures. The saline flushing relieved the depression caused by exposure to the 10 mM. The exposure to 20 mM resulted in a rapid depression of neural activity when moving the segment to cause the MRO to respond (Fig. 10). Both 10 and 20 mM significantly depressed evoked activity in the sensory neurons ($n = 8$ for 10 mM and $n = 7$ for 20 mM; $P < 0.05$ non-parametric Wilcoxon rank sum test. Since the data is not normally distributed when the activity decreases to zero the non-parametric statistical test was most appropriate).

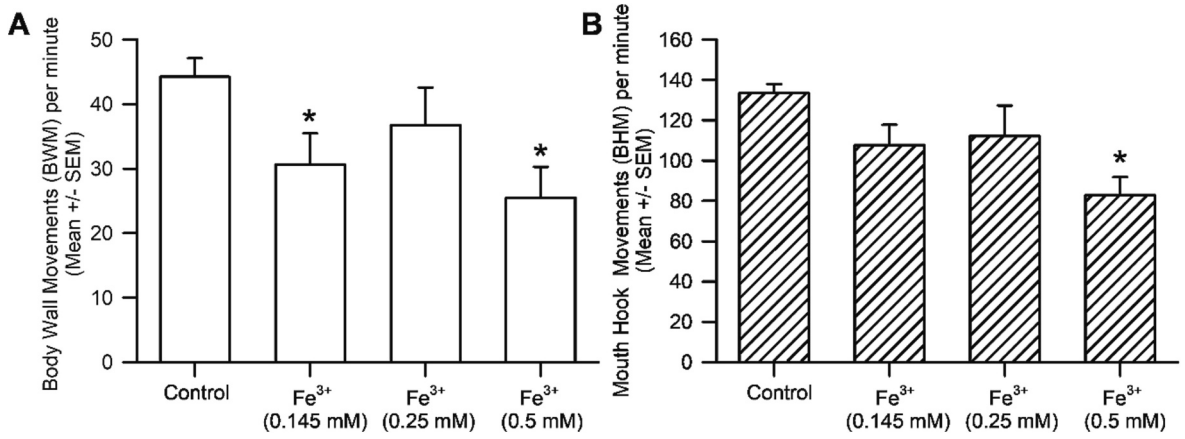

Fig. 6. Behavioral analysis of larvae after 24 h of consumption of iron at various concentrations. Second instar larvae were placed in food with 0.145 mM or 0.25 mM or 5 mM of ferric ammonium citrate. After 24 h (A) body wall movements (BWM) as full locomotive contractions and (B) mouth hook movements (MHM) were determined. Comparisons to control larvae which were fed the standard food without the addition ferric ammonium citrate were made by analysis of variance with normality test (Shapiro-Wilk), equal variance test (Brown-Forsythe) and multiple comparisons versus control group (Holm-Sidak method) * $P < 0.050$; $n = 15$ for each condition. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.6. Crayfish neuromuscular junction

As for the larval *Drosophila* NMJ mentioned above, it was expected that synaptic transmission would be depressed by exposure to Fe^{3+}. 5 mM and 10 mM greatly depressed synaptic transmission at the crayfish NMJ (Fig. 11A). Although, the resting membrane potential of the muscle was not as greatly affected in depolarization as the larval muscle (Fig. 11B). The resting membrane potential of the crayfish muscle was not significantly affected by either 5 or 10 mM ferric ammonium citrate. However, the EJPs were significantly affected and showed a dose-dependent effect with 10 mM, completely stopping evoked responses in some cases. (Fig. 11C; $p < 0.05$ for both 5 and 10 mM before and during exposure and between 5 and 10 mM; non-parametric Wilcoxon rank sum test; $n = 6$).

3.7. Crayfish cardiac function and survival

The effect of ferric ammonium citrate on the rate of the crayfish heartbeat was assayed with intact animals as the heart rate could be monitored well with freely moving crayfish within a holding container (Listerman et al., 2000). The injections of concentrated ferric ammonium citrate were estimated to the circulating concentrations of 5 mM, 7.5 mM, and 10 mM. These concentrations were used to record the effect on basal heart activity and response to mechanical stimulus. Saline injections of similar volumes used for the 5 mM were used as a control for the effects of handlining and injection. The volume used was larger for the 5 mM than the 10 mM. Thus, a control volume of saline for effects of volume change in the hemolymph was matched to the larger more intrusive effect.

Comparisons to the responses of the control saline injection revealed no significant differences with 5 mM or 7.5 mM or 10 mM circulating concentrations of Fe^{3+} (Fig. 12). All crayfish exhibited an increase in heart rate with physical stimulation in the form of a tap on the telson soon after injections and following a period of time to facilitate relaxation (Fig. 12). Since each crayfish had an intrinsically different heart rate in the basal state, a percent change in heart rate for each measurement was determined. Comparisons of pre-injection tail tap, 2 min post-injection tail tap and 24 h post-injection tail tap showed significant differences for 5 mM to saline ($P < 0.05$, $N = 6$) and to 10 mM ($P < 0.001$, $N = 4$), as well as 7.5 mM to saline ($P < 0.05$, $N = 6$) and to 10 mM ($P < 0.05$, $N = 6$) in the average percent change in heart rate (Fig. 13).

4. Discussion

4.1. The advantages of behavioral and physiological assays

The use of behavioral assays provides insight into the overall health of an animal, as well as its ability to respond to stimuli. However, the underlying cause resulting in the behavioral changes remains to be determined. Thus, larval *Drosophila* BWMs and MHMs serve as a first pass in detecting subtle effects in the overall status of the animal but do not provide information as to where specific disturbances occur along the sensory-CNS-motor-muscle path. Likewise, responses to stimuli which cause a change in heart rate in crayfish, can be due to a variety of physiological factors. This is particularly the case for the neurogenic regulation of the heartbeat in crayfish. Thus, the approaches in this study were also to assess the direct effects on the saline-exposed, myogenic heart of larval *Drosophila* to assess the consequence of Fe^{3+} without neural input or stress factors released into the hemolymph. In addition, measuring primary sensory neurons of the MRO provides a more direct measure of how sensory neurons are affected. Likewise, the isolated NMJs and skeletal muscles of the larval *Drosophila* and crayfish provide measures in pre- and post-synaptic function, as well as membrane properties of muscle directly. Such direct measures aid in deciphering the impact on behaviors of the intact animals. How the additive, physiological effects of the tissues cumulatively impact the behavior of animals is difficult to predict.

4.2. Review of results

In summary, initial exposure to dietary iron resulted in a larger death toll for early 3rd instars than 2nd instar larval *Drosophila*. However, data for early 3rd instars and adults yielded similar results, with a death rate of approximately 40 % for food tainted at 5 mM. The larval behavioral assays of BWMs and MHMs from 2nd to early 3rd instars demonstrated a trend with small effects at low concentrations (0.145 mM or 0.25 mM), while 5 mM Fe^{3+} substantially reduced the rate of movement. In the NMJs of larval *Drosophila* and crayfish, the number of evoked vesicle fusion events was reduced in the presynaptic nerve terminal. Furthermore, the amplitude of the evoked EJPs was reduced while the spontaneous quantal events remained unchanged. Such results suggest that postsynaptic, quisqualate ionotropic glutamate receptors were not compromised nor the ability of vesicles to fuse and release their contents from the presynaptic terminal. The depolarizing effect of ferric ammonium citrate on the resting membrane potential of the muscle fibers was

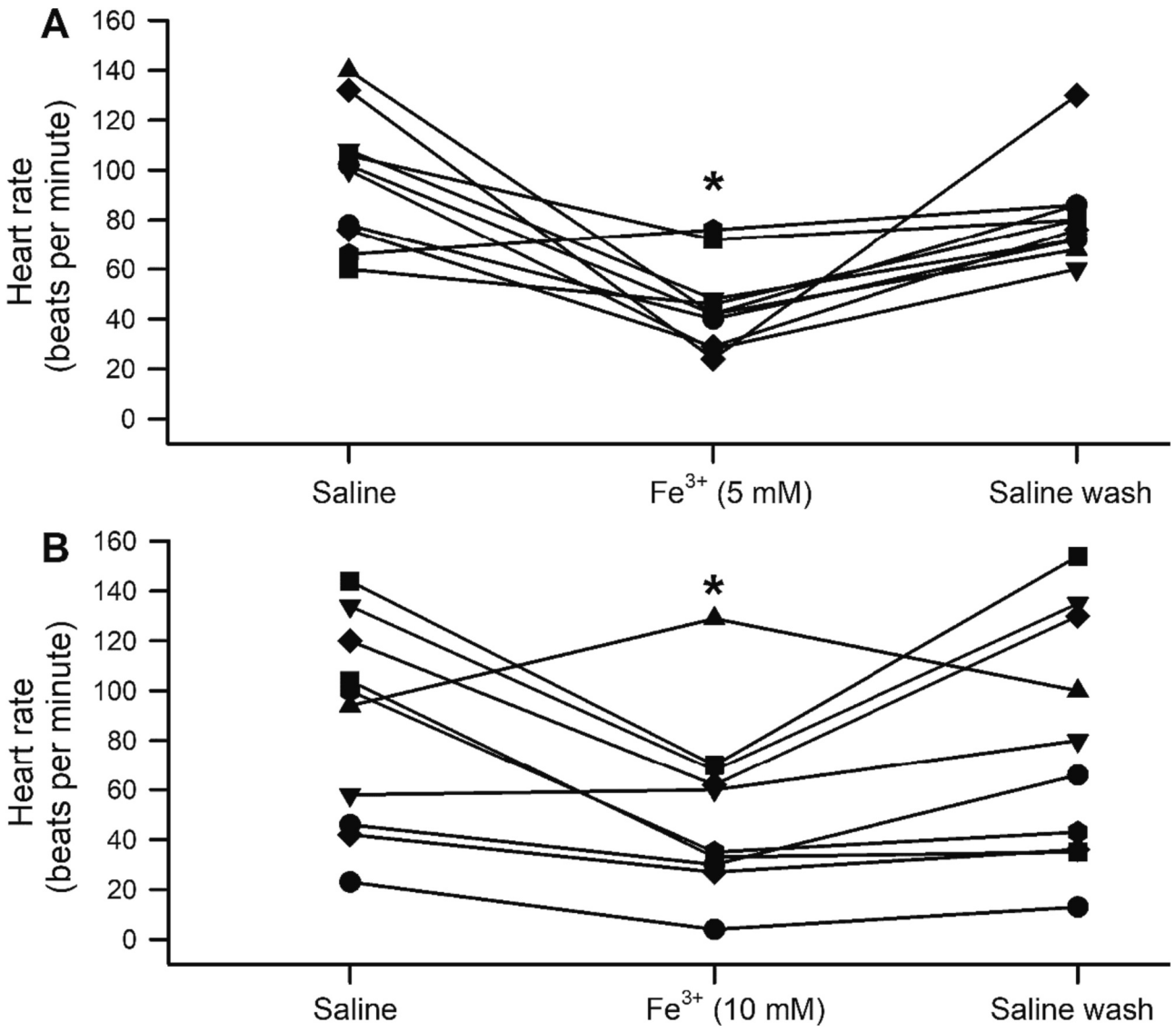

Fig. 7. The effect of acute exposure to iron on the in situ 3rd instar larval heart. The in situ larval heart tube was exposed to ferric ammonium citrate at (A) 5 mM or (B) 10 mM followed by exchanging the bathing solution to fresh saline without ferric ammonium citrate. The rate was determined for 1 min during exposure to ferric ammonium citrate and after 2 bath exchanges to fresh saline. A paired *t*-test from initial saline to ferric ammonium citrate with normality test (Shapiro-Wilk) revealed significant differences for both concentrations (*, $p < 0.05$) without differences in the responses between concentrations. $n = 10$ for each concentration.

much greater for the *Drosophila* muscle than for the crayfish muscle, despite maintaining the pH of the saline for both preparations. Activity of the primary sensory neurons in the MRO of the crayfish was rapidly reduced by exposure to 10 mM Fe^{3+} and silenced at 20 mM. As for cardiac responses, the heart rate of the larval *Drosophila* hearts exposed in situ was rapidly reduced by Fe^{3+} at 5 and 10 mM. With respect to the intact crayfish preparations, systemic injections of both 5 mM and 7.5 mM with tail touch stimulation minutes after injection were shown to significantly increase the average heart rate with 7.5 mM remaining significantly elevated >24 h later. Interestingly, 10 mM reduced heart rate compared to both 5 and 7.5 mM with tail touch stimulation but did not significantly reduce the effects of sensory stimulation on the animal in a neural response to increase in heart rate. As shown with 5 mM and to some extent 7.5 mM, the initial increase in heart rate indicates elevated levels of ferric ammonium citrate causes an increase in metabolic activity of the heart at cellular levels. This increase at lower levels is most likely due to the shock of toxicants but gradually recovers followed by decrease in heart rate. The apparent difference may lie in the physiological response of 7.5 mM exposure where crayfish did not drop back to pre-experimental heart rates and this may be an adaptive response due to the metal uptake or direct toxicosis by the metal. Crayfish and *Drosophila* NMJs, exposed larval *Drosophila* hearts, and crayfish MRO sensory neurons were able to recover activity to some extent following Fe3+ exposure, indicating that the tissues of interest were not irreparably damaged during acute overload. Given that acute exposure was sufficient to reduce physiological function in the tissues examined, prolonged exposure would likely be detrimental to the survival of intact animals. Long-term, direct tissue exposure was not addressed in this paper beyond the developmental studies for intact larval and adult *Drosophila*. Similar physiological and developmental studies as described herein were also performed with Zn^{2+} and Mn^{2+} (Elliott et al., 2023a, 2023b; Pankau et al., 2022; Pankau and Cooper, 2022).

4.3. *Drosophila* developmental studies

With respect to the *Drosophila* developmental studies, dietary Fe^{3+} exposure allows one to address the general environmental effects of food sources but does not provide direct insight to the systemic

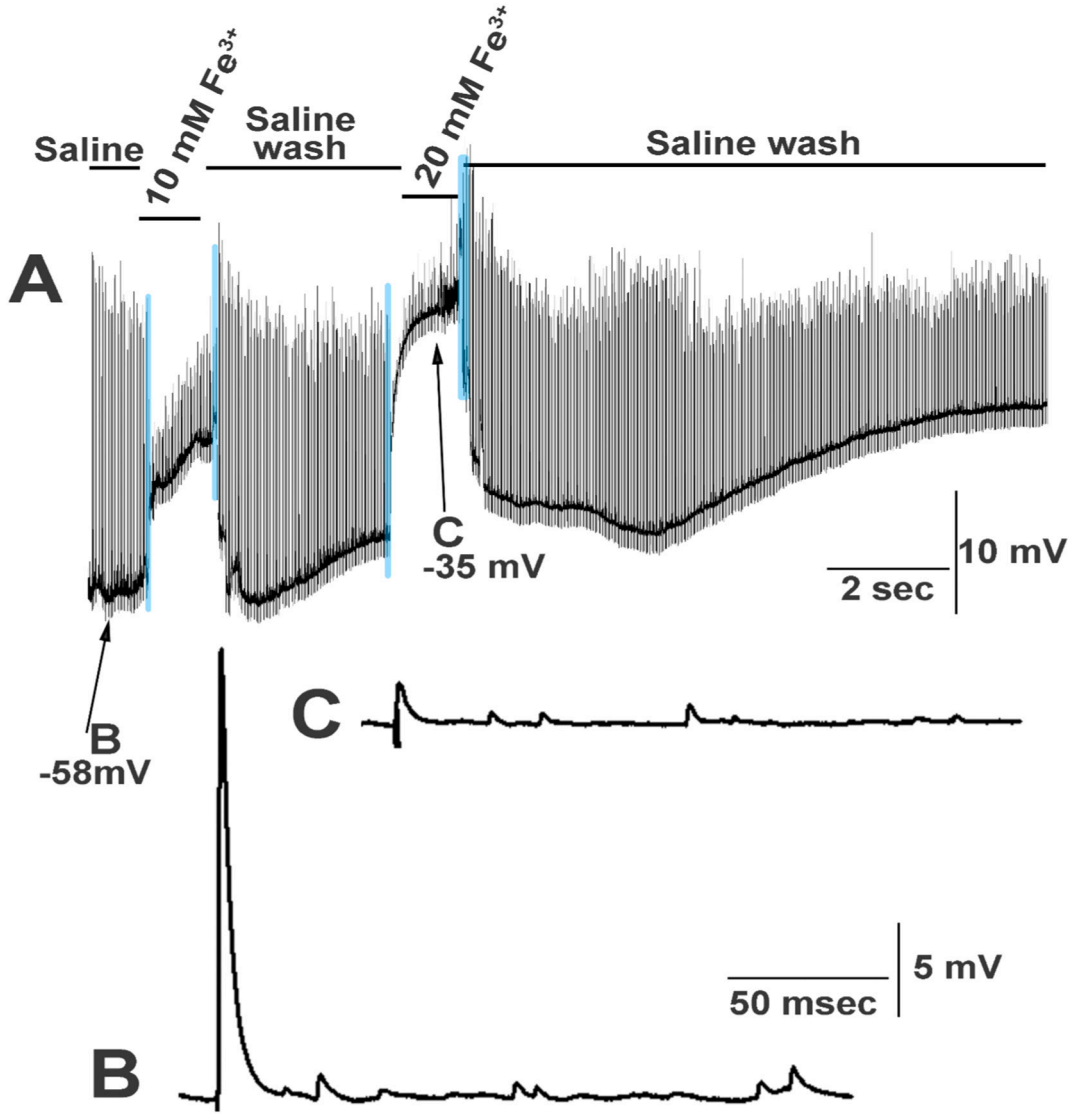

Fig. 8. The effect of ferric ammonium citrate on membrane potential and evoked excitatory junction potentials (EJPs) at the larval *Drosophila* neuromuscular junction for a representative preparation. (A) The segmental nerve was stimulated at a rate of 0.5 Hz. Ferric ammonium citrate (10 mM) rapidly depolarized the membrane and depressed evoked synaptic transmission. With the bath exchange back to fresh saline rapidly hyperpolarized back to the initial resting membrane potential and fully relieved the EJP amplitudes. Exposure to a higher concentration of ferric ammonium citrate (20 mM) rapidly depolarized the membrane and depressed evoked synaptic transmission to a greater extent than 5 mM. Enlarged segment to highlight the amplitude of the EJP and the spontaneous quantal events (B) before exposure and (C) during exposure of ferric ammonium citrate at 20 mM. The light blue rectangles shadow the traces where the bathing solution is changed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

concentrations of Fe^{3+}. Intestinal transport is a limiting factor to the extent of systemic Fe^{3+} present. Furthermore, oxidation state as well as chemical formulation, such as $FeCl_3$ versus ferric ammonium citrate, may produce different effects on the intestinal lining. As far as we are aware, there are no studies addressing differences in uptake nor the effects on the intestinal lining for these forms of iron. How much free Fe3+ is present in the systemic circulation, as compared to bound by specific transporters and additional proteins, remains an open question.

Although, physiological measures were not performed in adults in this study, the shortened life span for adults consuming Fe^{3+} is undoubtedly due to a combination of physiological issues, from cardiovascular, neural, and altered synaptic transmission in the CNS as well as at NMJs as shown for the larvae. Adult *Drosophila* exposed to much lower concentrations of Fe^{3+} (as $FeSO_4$ - $7H_2O$; 5, 10, 20 mg/L) in earlier studies also showed shortened life span (Begum et al., 2018). Due to behavioral deficits in adult locomotive behaviors, there are effects on the physiological processes even at these lower concentrations (Begum et al., 2018).

The rational for the greater survival rate in 2nd instars, compared to 3rd instars or adults at the same concentration, may be explained by developmental differences in intestinal Fe^{3+} uptake and transport (Mandilaras et al., 2013). The intestinal tract 3rd instar maybe more developed and take up more Fe^{3+} than in the case for 2nd instar larvae. Such developmental differences may involve the expression of transporter proteins that allow Fe^{3+} to travel from the intestines to, and between, other tissues. However, high levels of dietary Fe^{3+}, just as well as for $ZnCl_2$, $MnSO_4$ and $MnCl_2$, is acutely toxic to larvae (Elliott et al., 2023a; Pankau et al., 2022; Pankau and Cooper, 2022). However, $MnSO_4$ and $MnCl_2$ resulted in a higher death rate for 1st instars as compared to 2nd and 3rd for the same concentrations. Adults exposed to dietary $MnSO_4$ and $MnCl_2$ (15 mM or 30 mM) had a high percentage of deaths. Likewise, adults exposed to $ZnCl_2$ (15 mM) proved to be highly toxic (Elliott et al., 2023a). The exact mechanism to account death with these elements in the diet is not known. Perhaps it is damage to the

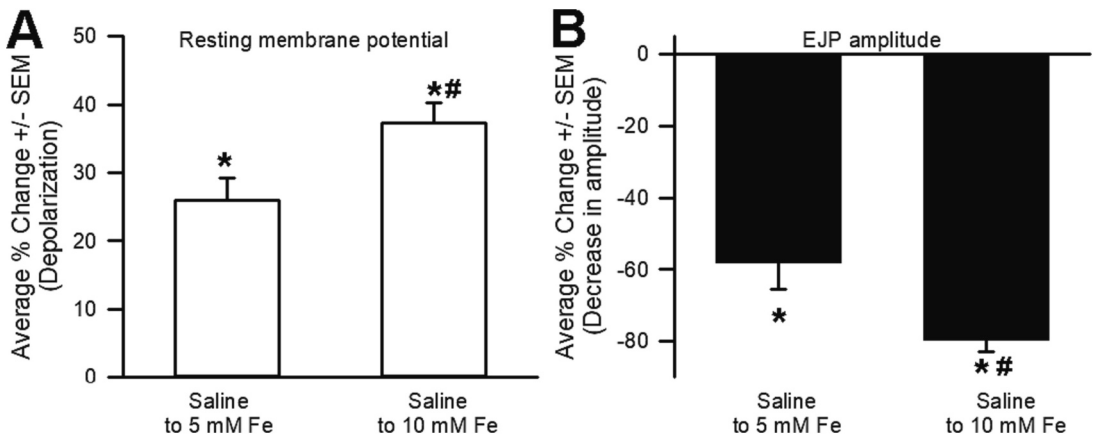

Fig. 9. The average effect of ferric ammonium citrate on membrane potential and evoked EJPs for 5 mM and 10 mM ferric ammonium citrate. Percent changes from the initial values in saline prior to and during exposure to ferric ammonium citrate. Percent change was used for comparisons due to the variation in initial values. (A) The exposure to 5 mM and 10 mM ferric ammonium citrate significantly depolarized the membrane potential (paired *t*-test from initial saline to ferric ammonium citrate with normality test by Shapiro-Wilk; $p < 0.05$; $N = 7$; *). The exposure to 10 mM was greater than for the 5 mM exposure (non-paired *t*-test; $p < 0.05$; #). (B) The reduction in the EJP amplitude was also significant for both 5 mM and 10 mM exposure to ferric ammonium citrate (paired *t*-test from initial saline to ferric ammonium citrate with normality test by Shapiro-Wilk; $p < 0.05$; $N = 7$; *). Also, the exposure to 10 mM was greater than for the 5 mM exposure (non-paired *t*-test; $p < 0.05$; #).

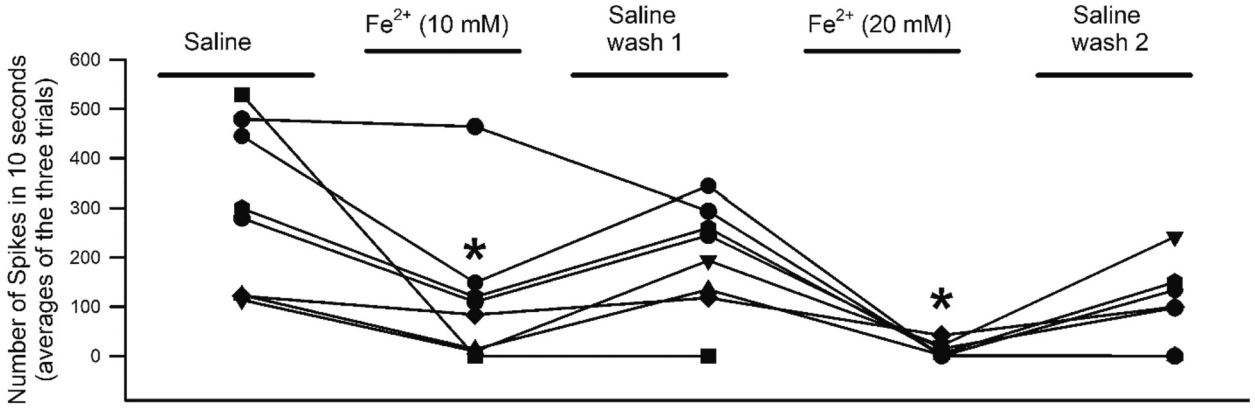

Fig. 10. The effect of ferric ammonium citrate on stimulated sensory nerve activity of the muscle receptor organ of crayfish. (A) The number of spikes measured in a 10 s window in which the abdominal joint was moved from the starting position within 1 s to the set position and held for 9 s. Each line represents a different preparation. Data was collected for three saline trials, three trials after switching the bathing media to ferric ammonium citrate (10 mM), three trials after changing the bath to fresh saline and three trials following ferric ammonium citrate (20 mM) exposure. The final exchange was performed to rinse the preparation two times with fresh saline and to replace the bath with saline. The joint movements were then repeated three more times. Each movement was separated by a minimum of 10 s while the joint was held in a relaxed position. The number of spikes in each of the three trails was averaged and graphed. There is a significant decrease for both 10 mM and 20 mM exposure to ferric ammonium citrate compared to the prior activity in saline (*,$P < 0.05$; non-parametric Wilcoxon rank sum test). ($n = 8$ for 10 mM; $n = 7$ for 20 mM).

intestinal tract, hormonal imbalance or effects on CNS and peripheral systems such as NMJ and skeletal muscle. The lack of a heartbeat is not likely to have been a key factor due to the fact that larvae can live without the development of the heart tube (Stutt et al., 2022). The cause of death in the larvae and adult is likely a combination of dysfunctional tissues for Fe^{3+}as well as Mn^{2+} and Zn^{2+}.

4.4. *Drosophila heart tubes, the crayfish MRO and heart, ion channel inhibition*

Even with direct tissue recordings, the cellular mechanism of Fe^{3+} overload remains to be addressed. However, it appears that the ionic form of Fe^{3+} blocks or compromises Ca^{2+} channels in the hearts of larval *Drosophila* and crayfish MRO preparations. Voltage-gated Ca^{2+} channels on the plasma membrane of larval *Drosophila* hearts regulate the rhythm and strength of contraction (Desai-Shah et al., 2010; Johnson et al., 1997; Wei and Salkoff, 1986). However, if the stretch-activated ion channels are blocked or compromised by Fe^{3+}, or the channels allowing electrical condition along the nerves, nerve activity could be depressed as shown in this study. Studies utilizing the isolated leg nerve of the proprioceptive organ in marine crab noted decreased nerve activity and slowed electrical conduction upon exposure to Fe^{3+} (20 mM) (Wagers et al., 2023). Furthermore, 30 mM $MnSO_4$ and $MnCl_2$ were shown to depress activity and slow the electrical condition in isolated nerves (Pankau et al., 2022). Patch clamp recordings of the channels, or the use of intracellular ion indicators to determine if ion influx occurs in the presence of extracellular Mn^{2+} or Fe^{3+}, can be utilized in the future to resolve this question. With respect to Fe^{3+} exposure and slowed nerve conduction, it is known that Ca^{2+} can partially block voltage-gated Na^{+} channels (Armstrong and Cota, 1999; Narahashi et al., 1976); thus, if Fe^{3+} behaved like Ca^{2+} and contributed to additionally blocking Na^{+} channels, an additive effect could occur in reducing and slowing electrical conduction.

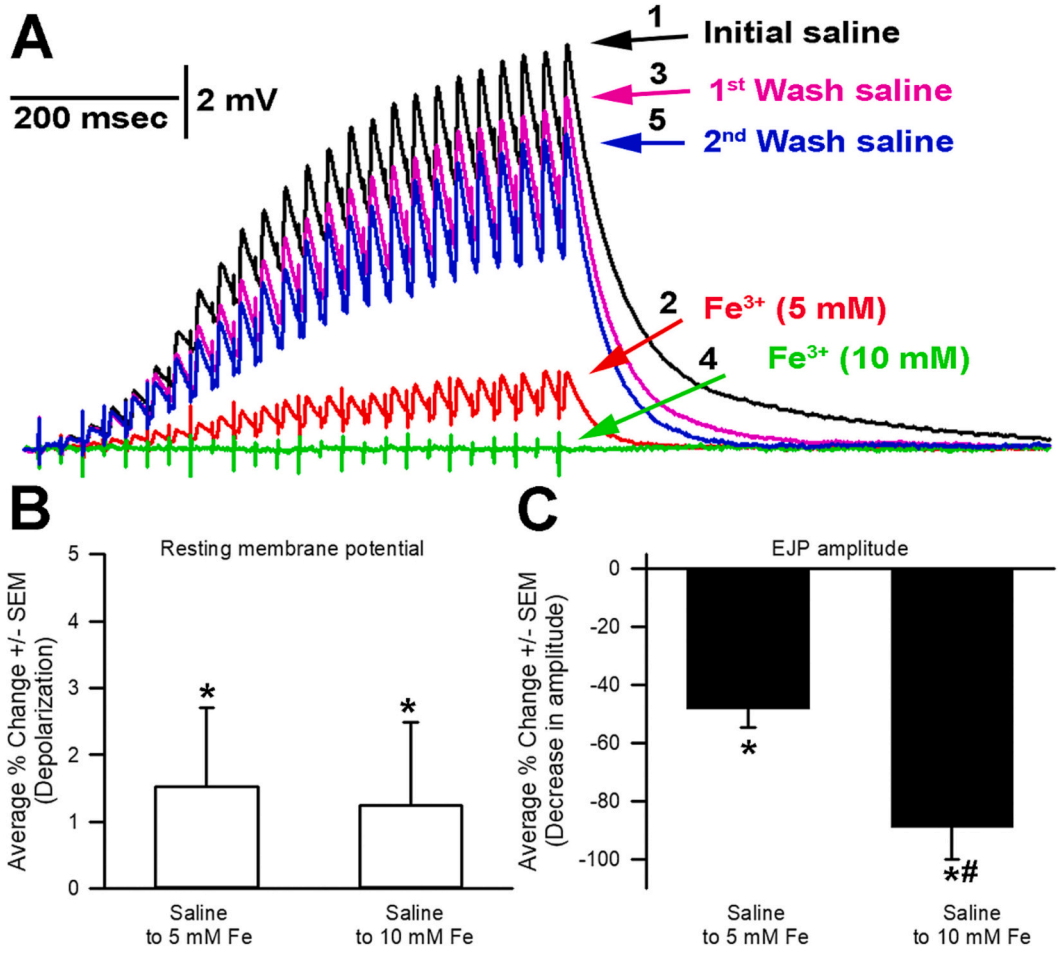

Fig. 11. The effect of ferric ammonium citrate on membrane potential and evoked excitatory junction potentials (EJPs) at the neuromuscular junction of the opener muscle in the walking leg of crayfish. (A) The motor nerve was stimulated at a rate of 40 Hz with 25 pulses. Ferric ammonium citrate (5 mM) rapidly depolarized the depressed evoked synaptic transmission. With the bath exchange back to fresh saline the EJP amplitudes were revived. Upon exposure a subsequent higher concentration of ferric ammonium citrate (10 mM) evoked synaptic transmission was depressed to a greater extent than 5 mM. (B) The exposure to 5 mM and 10 mM ferric ammonium citrate did not significantly depolarize the membrane potential (paired *t*-test from initial saline to ferric ammonium citrate with normality test by Shapiro-Wilk; $p > 0.05$; $N = 6$). However, a Sign test did illustrate a consistent significant depolarization upon exposure to 5 or 10 mM but the changes were only 1 to 5 mV ($P < 0.05$; Wilcoxon rank sum test; *). In normalizing the variation in the initial values with determining a mean percent change illustrated small changes. (C) The depression in the EJP amplitude was significant for both the 5 and 10 mM ($p < 0.05$ for both 5 and 10 mM before and during exposure and between 5 and 10 mM; non-parametric Wilcoxon rank sum test; n = 6; *). The mean percent change was greater for 10 mM than 5 mM (non-paired *t*-test; $p < 0.05$; #).

4.5. Ca^{2+} channel inhibition at the crayfish and Drosophila NMJ

As the amplitude of the evoked synaptic responses at the larval *Drosophila* and crayfish NMJ were reduced with Fe^{3+} exposure, and spontaneous quantal responses could still be observed, postsynaptic glutamate receptors were not blocked by Fe^{3+}. Thus, it is likely that voltage-gated Ca^{2+} channels within the presynaptic terminal were proportionally blocked as the concentration of Fe^{3+} increased. Such predictions remain to be examined, potentially with the use of Ca^{2+} indicators. Utilizing the same preparations from the present paper, previous studies investigating Zn^{2+} and Mn^{2+} yielded similar results (Pankau et al., 2023a; Elliott et al., 2023a). In additional studies, blocking Ca^{2+} channels has been shown to reduce cellular damage by Fe^{3+} (Bostanci and Bagirici, 2013). In cardiomyocytes, ferrous iron (Fe^{2+}) is taken up though L-type Ca^{2+} channels specifically when excess Fe^{2+} is present (Oudit et al., 2006). It is also known that Fe^{2+} can block Ca^{2+} currents in the presence of 2 mM extracellular Ca^{2+} for Ca(V)3.1 channels in HEK293 cells (Lopin et al., 2012). Such blockages would additionally affect synaptic transmission. As far as we know, the effects of acute Fe^{3+} exposure on reducing synaptic transmission at the NMJ have not been addressed.

As for why larval *Drosophila* skeletal muscle depolarized with exposure to Fe^{3+} in a dose-dependent manner, but not crayfish skeletal muscle, remains unresolved. It is possible that Fe^{3+} blocks a K2P channel subtype in *Drosophila* muscle that is not present in crayfish skeletal muscle. Evidence for the differential expression of K2P channels responsible for maintaining a resting membrane potential is present. The use of doxapram in a recent study, a blocker of the pH-sensitive K2P channel subtype, depolarizes larval *Drosophila* skeletal muscle but not crayfish skeletal muscle (Elliott et al., 2024).

4.6. Future goals and implications for other organisms

In conclusion, two invertebrate models, *Drosophila melanogaster* and *Procambarus clarkii*, were utilized to investigate the physiological effects of acute Fe^{3+} overload. The rich history of experimentation linked to these model organisms provides researchers with well-documented knowledge of the anatomical structures, along with simple procedures by which to reliably accesses the organ systems of interest (Sattelle and Sattelle and Buckingham, 2006). In the future, additional studies are vital to further document the cellular mechanisms by which organisms respond to Fe^{3+} overload. Identifying specific membrane channels that

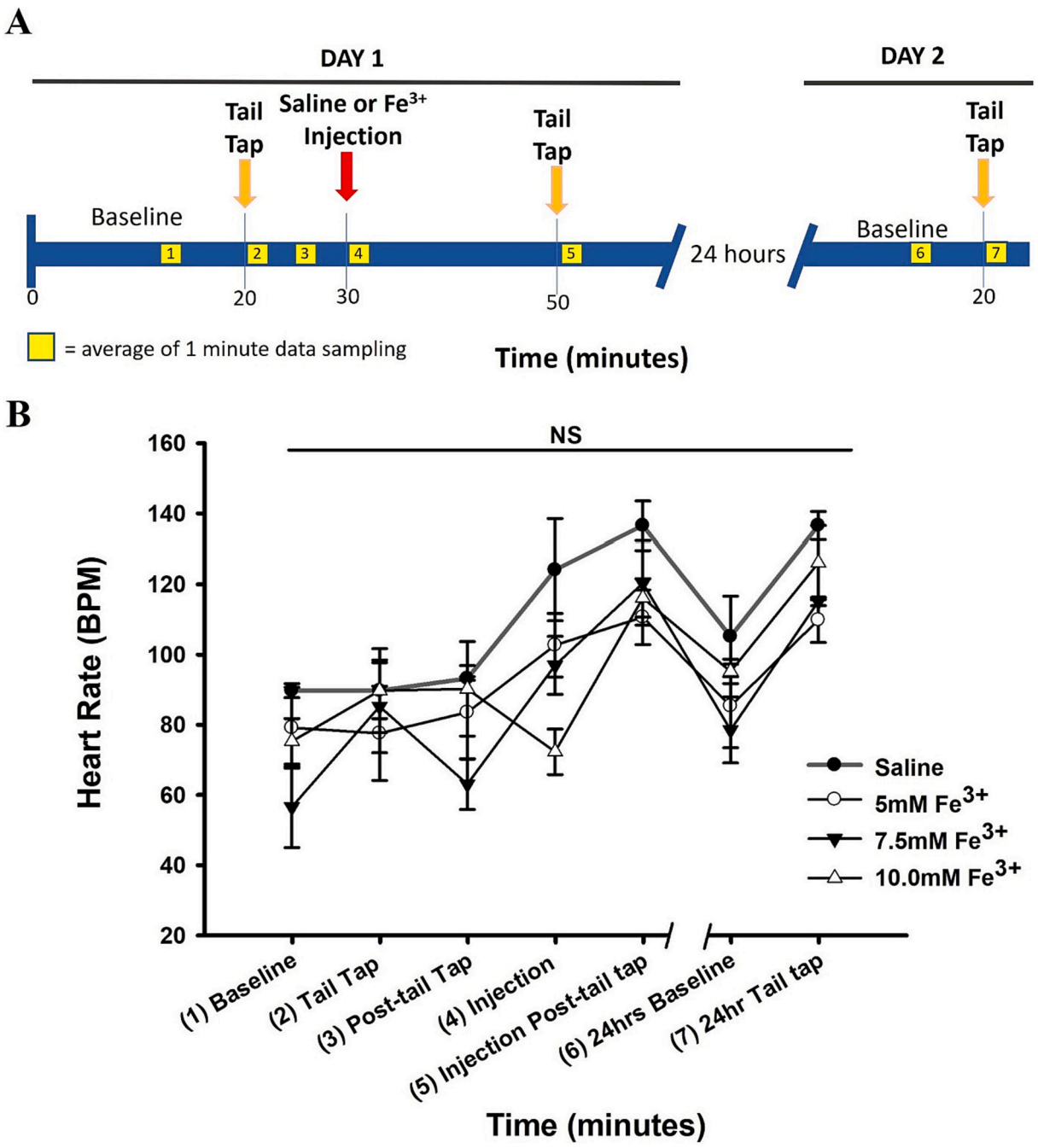

Fig. 12. The effect of acute exposure of iron on the heart of a freely moving crayfish. The crayfish heart was exposed to ferric ammonium citrate by injection into the hemolymph within the abdominal cavity at concentrations of 5 mM, 7.5 mM and 10 mM. The rate was determined by direct counts of each beat over 1-minute intervals (bpm) at multiple time points. There was no significant difference of increasing iron concentrations on heart rate when compared to the saline control groups across all time points (ANOVA; $P > 0.05$; N = 6).

are affected in the nervous and cardiac system during acute exposure is of particular interest, along with pinpointing the window of time in which tissue recovery is maximized. Recording electrical events, function for various ionic channels, and identifying effects on ionic pumps or exchangers are also welcomed topics for future exploration. The present animal models provide a means by which to preliminarily uncover such information with more ease and less expense prior to progressing towards the use of mammalian models. However, caution must be taken to address how translational the outcomes are. With mammalian models, information from such studies can be applied towards the development of pharmacological agents utilized for acute Fe^{3+} exposure in various tissues, such as in the cases of hemorrhagic strokes or pericardial tamponades.

The effects of ferroptosis in humans and animals are known to cause various pathological conditions, but many of the specific mechanisms on whole organs are a combination of cellular action (Solntseva et al., 2015). Some directed studies on ion channels have revealed Fe^{3+} can block K^{+} channels in neurons (Bukanova et al., 2007); however, many ionic channels, pumps, ion exchangers, endocrine and neurotransmitter receptors have not been fully investigated with respect to the specific effects of acute Fe^{3+} exposure (Jiang et al., 2019; Agarwal, 2001; Winegar et al., 1991). The damage from long-term iron exposure, from minutes to hours, is complex due to production of free radicals through the Fenton reaction and other cellular processes (Chen et al., 2011;

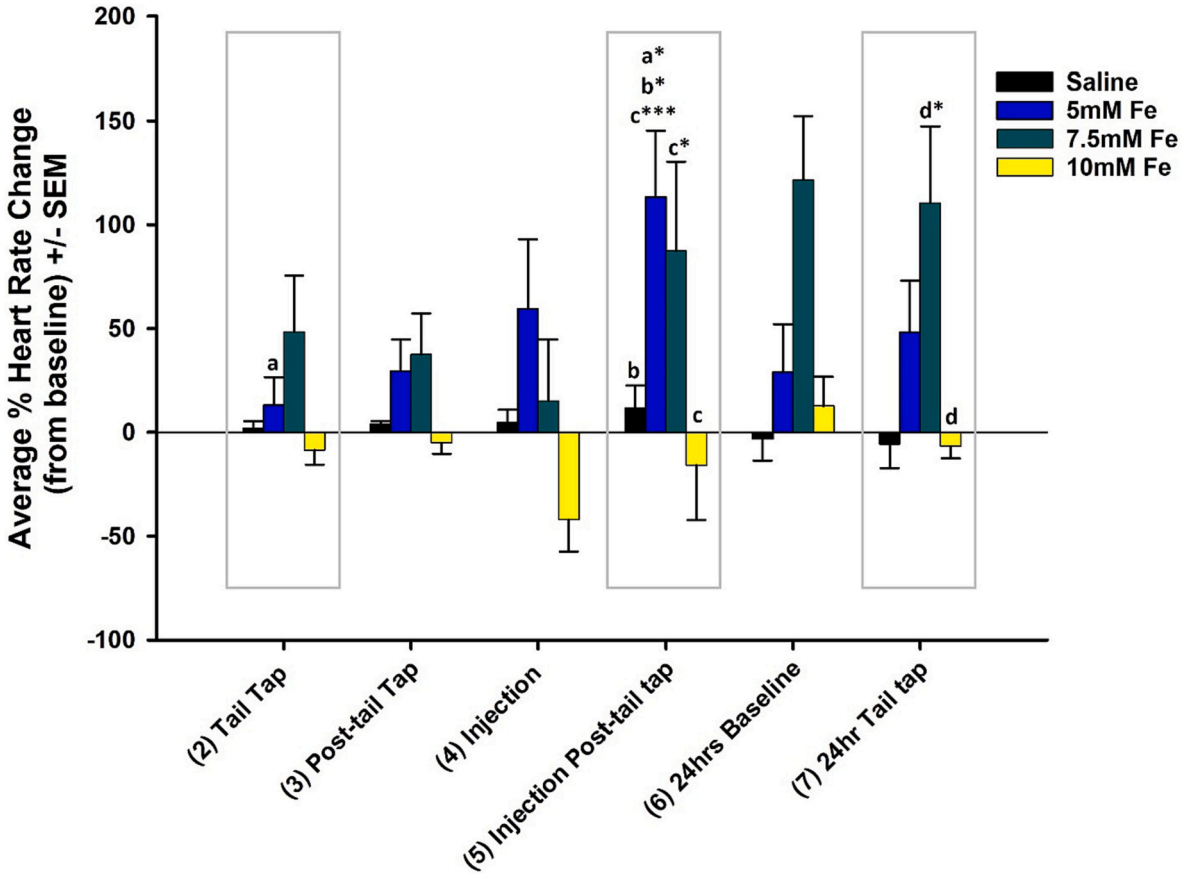

Fig. 13. The average effect of ferric ammonium citrate on heart rate. Percent changes from the initial values designated as baseline recordings for each individual prior to and during exposure to ferric ammonium citrate were used due to the variation within each group. There was a significant difference between pre-injection tail tap and the 5 mM Fe post-injection tail tap (ANOVA; Holm-Sidak post hoc analysis; $P < 0.05$; $N = 6$) and within the post-injection post-tail tap time point for both 5 mM ($P < 0.05$, $N = 6$; $P < 0.001$, $N = 6$) and 7.5 mM ($P < 0.05$, $N = 6$).

Nakamura et al., 2005). It is not fully understood why iron accumulates in the substantia nigra of the mammalian brain (Snyder and Connor, 2009), but this accumulation and the production of free radicals is thought to relate to the development of Parkinson's disease (Snyder and Connor, 2009) and is associated with Alzheimer's and Friedreich ataxia (Delatycki et al., 2000). The cellular localization is assumed to be related to iron transport (Anderson and Vulpe, 2009; Kress et al., 2002; Liu et al., 2021; Wessling-Resnick, 2000). Likely, similar damage to additional tissues such as the heart results from free radical production. Current therapeutic treatments for ferroptosis reduce iron levels with chelating agents or flushing lysed red blood cells away from exposed tissue before iron accumulation occurs (Almutairi et al., 2019; Farr and Xiong, 2021; Fang et al., 2019).

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Institutional review board statement

Not applicable for invertebrates.

CRediT authorship contribution statement

Mikaela L. Wagers: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ashley Starks:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jeremy Nadolski:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sonya M. Bierbower:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sean Altenburg:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Blake Schryer:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Robin L. Cooper:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

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