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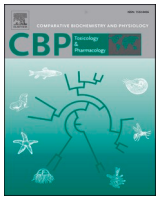

Investigation regarding the physiological effects of cobalt on physiological functions in *Drosophila*, crayfish, and crab: Behavioral, cardiac, neural, and synaptic properties

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ABSTRACT

Cobalt, a metallic element found naturally in the earth's crust, is essential to survival. It is the active center of cobalamins such as vitamin B12 and is also a micronutrient for bacteria, algae, and fungi. The effects of cobalt (II) chloride ($CoCl_2$), the inorganic form of cobalt, are dependent on the dosage. High dosage or chronic exposure to $CoCl_2$ can have negative effects, such as carcinogenic properties, intoxication, and "beer drinker's cardiomyopathy." This investigation was designed to test the effects of acute, high-concentration in cobalt exposure on physiological functions in *Drosophila*, crayfish, and crab, particularly in terms of behavioral, cardiac, neural, and synaptic properties. When exposed to 1 mM of $CoCl_2$, decreased neural transmission was observed at the neuromuscular junction (NMJ) of both crayfish and *Drosophila* larvae. Within the crayfish proprioceptive organ, no conclusive changes in activity were observed due to the high variability among individuals, but activity was observed to increase in the crab proprioceptive organ after 10 min immersion the $CoCl_2$. In larval *Drosophila*, heart rate decreased to near-cessation, though the *in-situ* preparations were able to recover regular heart rates after sufficient saline rinsing. Systemic injections of $CoCl_2$ into crayfish hemolymph produced no significant effects on heart rate or tail flip response. In larval *Drosophila* that consumed food tainted with $CoCl_2$, no effects were observed on behavior, mouth hook movements, or body wall movements; however, this led to adults bearing a slightly decreased lifespan, which indicates that 1 mM $CoCl_2$ has differing effects by tissue and organism.

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1. Introduction

The element cobalt (Co) is an essential trace metal that is an important and necessary nutrient for organisms ranging from bacteria to humans. Bacteria are known to make use of cobamides (family of vitamin B_{12}); in fact, some bacteria species—such as the *Akkermansia muciniphila*, which are present in the human gut microbiome—can even remodel certain unusable forms of B_{12} into structures that can better be processed (Mok et al., 2020). Algae, fungi, and plants also require cobalt; in particular, plants require vitamin B_{12} for proper enzyme function involved in nitrogen fixation (Hu et al., 2021). In humans, cobalt is a cofactor for both the essential vitamin cobalamin (vitamin B_{12}) and for enzymes (i.e., methionine synthase and methylmalonyl-CoA-mutase; Herrmann and Obeid, 2012). However, despite cobalt's significance across many species, high doses are damaging to plants and animals alike (Hu et al., 2021; Catalani et al., 2011).

Exposure to cobalt can occur through various methods, such as inhalation, mining, glassmaking, inks, cosmetics, and consumption. Namely, dietary supplements are currently unregulated by governmental agencies; thus, they pose issues for hazardous substances like cobalt (Leyssens et al., 2017). Athletes who take supplements to increase red blood cell levels are especially at risk of overconsuming cobalt. Cobalt was also once added to beers for stabilization of the foam, which led to cardiomyopathy in consumers (Alexander, 1972; Packer, 2016). Another way exposure to cobalt may occur is through bioaccumulation in an ecosystem; for instance, fertilizers, insecticides, and industrialized antimicrobial or antifungal agents can contaminate water, soil, plants, macroinvertebrates, animals, and humans (Abdallah Alnuwaiser, 2019; Norton et al., 2001; Ouellet et al., 2013; Nasirian and Irvine, 2017). Cobalt added to plants as a fertilizer directly affects both the plant and the organism that consumes it and provides indirect contamination to water sources via environmental runoff (Khan et al., 2023; Stubblefield et al., 2020; Khan et al., 2016). Skaldina et al. (2018) showed that wood ants can accumulate cobalt through exposure to industrial metal processing plants. This pattern is apparent in other environmental sources as well; interestingly, honey and wax combs taken from bee hives can be used as an index of environmental cobalt levels for monitoring the environment (Ćirić et al., 2021; Hassona and El-Wahed, 2023). Cobalt is also used to help combat foulbrood disease in bees by decreasing the spore forming bacterium *Paenibacillus*, though this treatment does risk contaminating the honey (Grigorian, 1971). Finally, cobalt can be transported into cells via nanoparticles (Vales et al., 2013) or a metal transporter (Agranoff et al., 2005), whereupon its ions can enter the animal's systemic circulation and, depending on the exposure concentration and tissue type, have varying effects. In weakened organisms, cobalt has been linked to increased susceptibility to viral and bacterial infections, as well as higher frequencies of additional side effects (Dey et al., 1981; Shapiro, 2001). Such detriments are interestingly paradoxical, as cobalt is also used as a bactericidal agent on surfaces.

Previous studies have noted the effects of cobalt on different animal species. First, while natural cobalt alone is dangerous in excess, the isotopic cobalt found in radiation and radioactive pollutants has even harsher effects on the behaviors of many animal species (Gagnaire et al., 2011). In addition to behavioral effects, cobalt can induce various pathologies in both vertebrates and invertebrates (Karpov, 1964; Leyssens et al., 2017). In bees, cobalt has been noted to promote cell division and encourage cells to remain in the metaphase state, which has led to the use of cobalt in cell cultures intended for induction and analysis of metaphase states (Ueira-Vieira et al., 2013). It would be interesting to determine whether these actions also occur in mammalian tissues. Furthermore, cobalt is known to block ion channels, particularly Ca^{2+} channels in nerve terminals. In fact, cobalt is more effective at blocking Ca^{2+} channels than other divalent ions (e.g., Mg^{2+}), as previously described in various invertebrates and cell types (Washio, 1982; Yamamoto and Washio, 1979; Fukuda and Kawa, 1977; Sattelle and Piddington, 1975; Lee and O'Dowd, 1999; Hsieh et al., 2001; Pannabecker and Orchard, 1989; Jenson and Bloomquist, 2015). In insects, cobalt did not block all forms of synaptic transmission (Yarom and Spira, 1982; Miyan, 1991), though its Ca^{2+} blockage, it affected chemical synaptic transmission and decreased the release rate of hormonal factors and secretion properties in insects (Mitchell et al., 1980; Kindle et al., 1990; Wildemann and Bicker, 1999). The use of various metals, such as Co^{2+} from salt cobalt (II) chloride ($CoCl_2$), has helped with the identification of ion channel subtypes by exploring varied sensitivity to blockage by different divalent ions (Pelzer et al., 1989). Crustaceans and mammals share similar physiological processes to those observed in insects; for example, synaptic transmission in each is moderated by Ca^{2+} ion dependence through the action of voltage-gated Ca^{2+} channels. However, the storage of Ca^{2+} in organelles (e.g., SER, mitochondria) and its actions in second-messenger systems occur throughout the animal kingdom as well. Co^{2+} likely has some impacts on these processes, but more research is needed to fully understand its effects.

The skeletal muscles of many insects (e.g., *Drosophila*) and crustaceans (e.g., crayfish) allow Ca^{2+} influx through the plasma membrane and induction of muscle contractions like that of mammalian cardiac muscle. Cobalt can block a Ca^{2+} channel subtype in *Drosophila* skeletal muscle (Suzuki and Kano, 1977); however, this action has yet to be investigated in crustaceans. Nonetheless, it could also account for mammalian cardiac pathology. In addition, both sensory and muscle cells use stretch-activated ion channels for physiological measures; some channels are Ca^{2+}-permeable and can be blocked by cobalt (Travis and Spencer, 2013; Rae et al., 1992). Cobalt exposure may result in cell toxicity through blockage of ion channel function or through disruption of intracellular processes that may normally link to Ca^{2+} actions (Dagan and Sarne, 1979; Chopikashvili et al., 1989). For years, researchers have used $CoCl_2$ to trace the morphology of neurons and other cells by allowing the compound to be transported along the living cells' axons or cytoplasm, where the cobalt can be precipitated with ammonium sulfide and the tissue processed with alcohol dehydration for visualization. This process indicates that Co^{2+} can diffuse within a cell after entering it.

In this investigation, several physiological models were used to examine the acute effects of Co^{2+} — namely, the direct effects on sensory neuronal function in *Procambarus clarkii* (red swamp crayfish) and *Callinectes sapidus* (crab) proprioceptive neurons and on the neural circuitry, synaptic transmission, and cardiac functions in crayfish and larval *Drosophila melanogaster* (fruit fly). Proprioceptive neurons use stretch-activated channels to detect joint movements, while crayfish and crab sensory nerve models allow for direct assessment of cobalt's neuronal effects without synaptic input. Glutamatergic synapses at isolated crayfish and larval *Drosophila* neuromuscular junctions (NMJs) comprise fitting models for investigating synaptic transmission, as the muscles do not produce action potentials and instead feature graded excitatory junction potentials (Jan and Jan, 1976; Kurdyak et al., 1994). Much like in mammals, evoked transmission in these organisms is dependent on presynaptic voltage-gated Ca^{2+} channels. Larval *Drosophila* also offers *in-situ* access to a myogenic pacemaker heart absent any neural innervation. This preparation can be maintained in physiological saline devoid of hormones and peptides (de Castro et al., 2014; Johnstone and Cooper, 2006). Although invertebrate models are not entirely suitable for investigating some mammalian reactions to cobalt exposure, such as angiogenesis (Hong et al., 2023), the basic cellular process may be similar.

Crayfish and *Drosophila* models can be used to examine the diverse effects of cobalt on cardiac function, neural activity, development, and survival. Crayfish have a neurogenic heart, which is useful for investigating a multitude of physiological functions. A sensory stimulus — such as a physical touch or a vibration of the water around them — causes a measurable cardiac response indicative of central nervous system (CNS) function (Listerman et al., 2000; Schapker et al., 2002; Shuranova et al., 2006). Because the heart rate is dependent upon neural input, the effects of CNS sensory input on the crayfish autonomic nervous system can be assessed with the administration of a forceful telson tap; this results in

the heartbeat pausing for a few seconds, then beating faster (Shuranova et al., 2006). Crayfish survival can also be observed following the systemic injection of various compounds. *Drosophila*, meanwhile, represent an essential research model for biological systems and clinical research (Yamaguchi and Yoshida, 2018; Ugur et al., 2016; Mackay and Mackay and Anholt, 2006; Bier and Bodmer, 2004; Wolf et al., 2006; Souidi and Jagla, 2021), as they provide a reliable model for examining how dietary consumption of a given substance affects an organism. Use of *Drosophila* as a model allows for the assessment of how organisms are affected at different points in the life cycle, such as when exposure occurs during development (including survival rate) and in adulthood (Elliott et al., 2023a; Pankau et al., 2022; Wagers et al., 2024). *Drosophila* can also undergo relatively easy gene alterations, allowing for intentional, controlled abnormalities in gene expression and, thus, enabling the investigation of ionic transport systems and cellular processes.

The use of *Drosophila*, crayfish, and crab models will help develop a core understanding of the processes underlying absorption, transportation, and storage of essential metals like cobalt, which can provide valuable insight into the physiology of various animals (So et al., 2023). This study examines cobalt's various effects on *Drosophila*, crayfish, and crab models to provide a basis for future studies on how it affects other species and to develop a more comprehensive knowledge of cobalt's implications in health and physiology.

2. Methods

Most of the procedures used in this study have been used in previous studies and are detailed in previous publications (Pankau et al., 2022; Wagers et al., 2023; Elliott et al., 2023a, 2023b); some procedures are published in open-source video format and are cited below. These earlier studies also focused on the physiological and developmental effects of acute exposure to high concentrations of the essential metals: manganese (($MnCl_2$ and $MnSO_4$ at 5, 15 and 30 mM), ferric iron ($FeCl_3$ and $(NH_4)_5[Fe(C_6H_4O_7)_2]$ at 10 and 20 mM), and zinc ($ZnCl_2$ at 1 and 10 mM) in the same manner as reported herein for comparative analysis. The procedures are restated in brief for this study and copied for the companion study on acute exposure to high concentrations to sodium selenite (Brock et al., 2025). Invertebrate animal care was approved by the Institutional Animal Care and Use Committee. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cobalt(II) chloride hexahydrate (product number 255599, ACS reagent, 98 % purity) was used throughout this study.

2.1. Animals

Canton S (CS) *Drosophila melanogaster* flies were used in all behavioral and physiological assays. This strain has been isogenic in the lab for several years 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. Blue crabs (*C. sapidus*) were obtained from a local supermarket in Lexington, KY, USA, which were initially delivered from a distribution center in Atlanta, GA, USA. The crabs were bought and maintained in a seawater aquarium for several days prior to use in order to assess their health. Adult crabs in the range of 10–15 cm in carapace width (from point to point) were used and were alive and very active upon autotomizing a leg for experimentation. Red Swamp Crayfish (*Procambarus clarkii*) were obtained from a distribution center in Atlanta, GA, USA and delivered to a local supermarket in Lexington, KY, USA, where they were purchased. Throughout the study, midsized crayfish measuring 6–10 cm in body length and 12.5–25 g in body weight were used. Each animal was housed in individual standardized plastic containers with aerated water (20–21 °C) and were fed dry fish food weekly (Pankau et al., 2022).

2.2. Adult and larval Drosophila melanogaster: survival and development

In order to study the effects of cobalt chloride on *Drosophila* life cycles, food was tainted to a concentration of 1 mM and placed in 11 vials. Ten first instar larvae were also placed in each vial. The time from 1st instar larva to pupation was measured. Another set of ten vials (also each containing ten first instars) was run without exposure to cobalt chloride as experimental controls. Each 1st instar larva was placed into a vial at most 12 h post-hatching. Each control vial contained 1 g of corn meal food mixed with 1 mL of deionized water; however, the eleven experimental vials contained the food (1 g) mixed with 1 mL of 2 mM cobalt chloride instead. Vials were closed with cotton lids to allow for an aerated and moist environment (21 °C). The larvae were then left to develop into pupa and the number of pupae for each condition counted daily. Vials that did not develop pupa after 10 days were isolated, the food removed, and the contents examined for dead larvae.

Assessing adult survival involved the collection of adults within 2 to 3 days of eclosion. Ten adults were randomly placed in plastic vials containing standard *Drosophila* food (1 g) mixed with 1 mL of 2 mM cobalt chloride. Survival was monitored daily, and ten control trials conducted with exposure to *Drosophila* food (1 g) mixed with 1 mL of deionized water.)

2.3. Larval Drosophila melanogaster: body wall movements and mouth hook movements

Larval locomotion was assessed by using a dissecting microscope to visually count the number of body wall movements (BWM) in contractions per minute. Ten late 2nd-instar larvae were placed in food (1 g) mixed with 1 mL of 0.2 mM of cobalt chloride, while another ten were placed in food tainted with 1 mL of 2 mM cobalt chloride. Another ten late 2nd-instar larvae placed in *Drosophila* food (1 g) and 1 mL of deionized water served as control comparisons. In each case, the 1 g of food was assumed to be equivalent to 1 mL for dilution of the stock solutions. Exposure began 24 h before the body wall contractions were assessed. For recording purposes, plastic Petri dishes (about 8.5–9 cm in diameter) were used. Filter paper (Whatman #3) was saturated with pure apple juice to promote locomotion and placed within the dishes, at which point the larvae were observed individually and their BWM counted.

Immediately after measurement of the larval body wall movements, each individual larva was transferred to another dish for measurement of its mouth hook movements (MHM). Small, smooth-bottomed Petri dishes (5.5 cm diameter) were filled with a small volume of yeast solution (a few dried yeast granules mixed with water) to promote feeding without body wall locomotion. The solution level was kept minimal to allow the larval spiracles to protrude safely. The MHM were also counted visually with the use of a dissecting microscope. Both the BWM and MHM were counted in a lightly illuminated environment at room temperature (21–22 °C).

2.4. Larval Drosophila melanogaster: beating heart rate

A detailed description of the process by which the larval heart of early 3rd instars may be exposed is provided in in video format (Cooper et al., 2009). In brief, however, the larvae were dissected via a cut along the ventral surface and pinned open on three 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). Cobalt chloride was added to the saline to produce a concentration of 1 mM, and the pH of the saline was maintained at 7.1.

Heart rate was counted visually with the aid of a dissecting microscope and is presented in beats per minute for graphical analysis.

Measures were taken in the caudal region of the heart tube (Fig. 1): first during initial saline exposure, again after replacing the bathing solution with one containing cobalt chloride, and again after two wash-outs with fresh saline. Each rate was counted as soon as the new solutions were added, and all measures were taken in a lightly illuminated environment at room temperature (21–22 °C).

2.5. *Procambarus clarkii: cardiac responses and survival*

The crayfish heart rate was obtained via an impedance measure. The recording procedure has been described in textual and video format (Bierbower and Cooper, 2009), and involves the same experimental methods as have been described for injection of iron into crayfish hemolymph (Wagers et al., 2023) and re-stated as follows. The heart rate of an intact crayfish was obtained by threading recording wires under the carapace protecting the heart. Insulated, stainless steel (diameter 0.005 in./0.008 in. with coating; A-M Systems, Carlsburg, WA) wires were used, and the insulation was burned off the ends with a flame to provide a good connection with the recording devices. Small holes were poked through the carapace at approximately the thickness of the wires to ensure minimal hemolymph loss and to maximize the likelihood that the wires remain in place during fixation. The wires were placed into the carapace such that they spanned the heart, thus facilitating an accurate impedance measure (UFI, model 2991; Listerman et al., 2000). To avoid damaging the heart, special attention was paid to insert only a short portion of wire (1–2 mm). After the wire occupied an optimal position, fixation was ensured with 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, Australia) with an acquisition rate set at 1 kHz. Heart rate calculation was conducted through direct counting over 1-min intervals and reported as beats per minute (BPM) (Wagers et al., 2023).

Each crayfish was injected in the abdomen with either saline (for control comparisons) or a cobalt chloride solution (for experimental values) by a needle passed through the articulating membrane on the ventral side, close to the lateral aspect; this prevents damage to underlying muscles while allowing direct and rapid mixing with the hemolymph. The control group was used to ensure that neither handling the crayfish nor the injection itself would confound the data. Systemic levels of cobalt chloride were calculated to account for dilution in the hemolymph from a stock concentration of 10 mM. The total amount of hemolymph in each crayfish was estimated on the basis of its weight, using the assumption that 30 % of an animal's weight is hemolymph (Gleeson and Zubkoff, 1977; Guirguis and Wilkens, 1995), which ensured that an appropriate volume of stock would be injected. The physiological saline was comprised of the following: 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). Since the animals have an open circulatory system, injecting a given compound results in it being carried toward the heart, where it bathes the cardiac ganglion and muscle. These experiments were carried out in six trials (i.e., six crayfish were injected with saline controls, and six more were injected with cobalt chloride).

To assess whether the neurogenic heart remained responsive to sensory stimuli (which would typically activate the central nervous system and, in turn, lead to altered neural input at the cardiac ganglion), the crayfish telson was tapped forcefully with a glass rod to elicit a tail flip response and the recording maintained for 20 min afterwards. This was followed by injections of either saline or cobalt chloride and, after a further 20 min of recording, the telson was forcibly tapped again and the effects on heart rate monitored. This tapping process continued periodically over 24 to 48 h post-injection (Fig. 2), which meant that it also provided an assessment on the survival of the injected crayfish over a period of days.

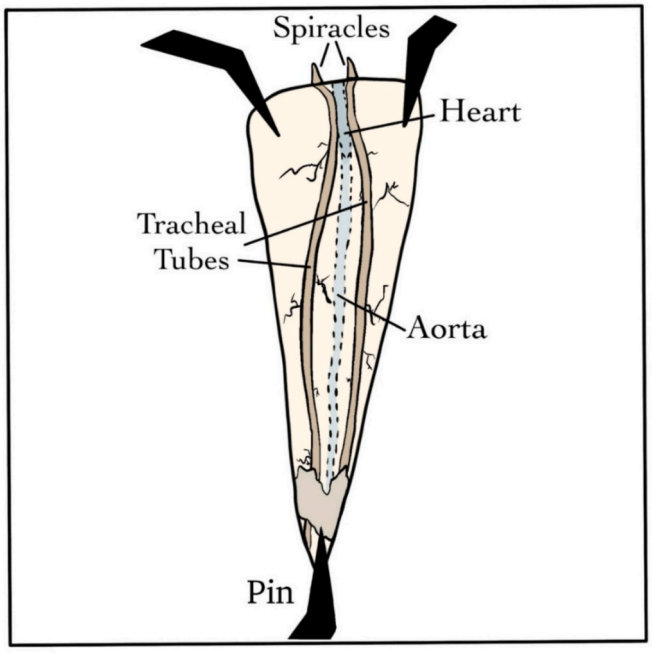

Fig. 1. The filleted larva preparation used for measurement of the heart rate when exposed to cobalt chloride. Heart rates were counted by manual inspection through a dissecting microscope, both before and after switching to the compound of interest. Counts were obtained from the caudal end of the preparation, near the point where the bifurcation point of the two tracheal tubes.

2.6. *Procambarus clarkii: muscle receptor organ*

The dissection procedure is described in video format (Leksrisawat et al., 2010), and comprises the same experimental procedure previously described for assessing the effects of iron exposure at the muscle receptor organ (MRO) (Wagers et al., 2023). In brief, however: the muscle receptor neurons of the crayfish abdominal proprioceptor are fully exposed to the bathing saline and produce spikes — measured with extracellular recording — upon manipulation of the abdominal joint. The segmental nerve only measures two neurons: one sensitive to dynamic movement, and the other sensitive to static movement; as such, relatively few variations in spike amplitudes are recorded. The two neurons each have their sensory endings embedded within a single associated muscle fiber, which each span the joint they monitor and reside directly underneath the dorsal cuticle. By moving the joint within the span of a single second and then holding it steady for at least the next nine, both types of neurons may be monitored. Abdominal joints not involved in the recordings were pinned in a Sylgard-lined dish and covered with crayfish saline, whereupon the MRO nerve was exposed and pulled into a suction electrode (made from glass pipettes fitted with plastic tips; details of this process are available in Baierlein et al., 2011) for recording of extracellular signals from the cut nerves. A P-15 amplifier (Grass Instruments) was used in conjunction with a PowerLab/4s A/D converter and Lab Chart 7 software (ADI Instruments, Colorado Springs, CO, USA) to record signals at a 20 kHz sampling rate. Neural activity is readily distinguished from the baseline noise present with a relaxed and motionless MRO.

During the experiment, an insect dissecting pin was used as a reference point for the maximum displacement range to ensure that each displacement was identical, and each displacement was marked on the computer recording file. Movements were repeated thrice with identical rates and degrees of movement to ensure that each preparation underwent three consistent trials in each condition. These displacements were performed in the presence of saline only, after the bathing media was switched to one with 5 mM cobalt chloride, after 10 min of incubation

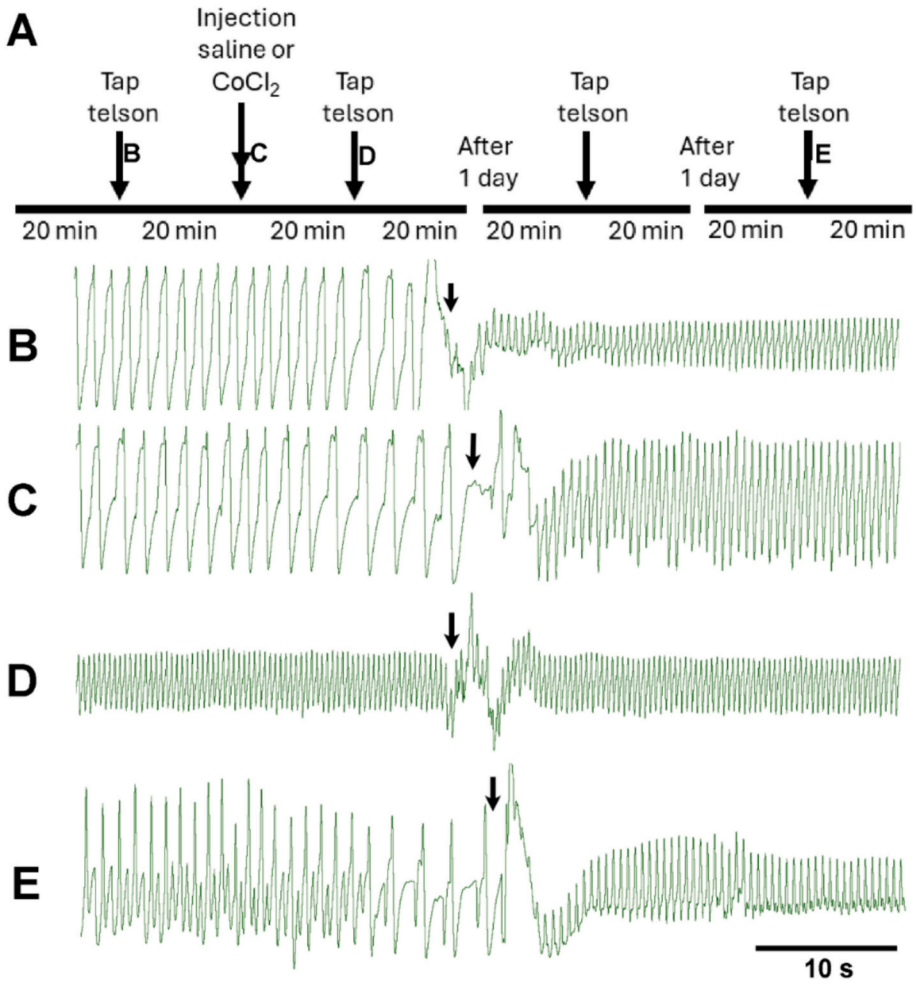

Fig. 2. The experimental paradigm for monitoring the cardiac function of the crayfish. Heart rate was determined both before and after a telson tap, as well as both before and after injection of saline or cobalt chloride. The telson tap (and subsequent monitoring) was repeated 20 min, 24 h, and 48 h after the injection. Responsiveness to the telson tap was determined by two observations: the first, the crayfish tail flip (or other rapid movement), and the second, a pause in the electrical recording. As shown in B through E, the illustrated trace showed a pause due to the telson tap. Part A illustrates when traces B through E took place in context with one another. Note that the beating rate sped up after a telson tap, except after the injection of cobalt chloride (D) for which it is likely that the already-high rate prior to the tap interfered with a possible acceleratory response. However, a noticeable pause in the beating still occurred in response to the tap.

time, and then a final time after two rinses with fresh saline to remove the cobalt chloride. After each displacement, the joint was returned to its starting position and allowed to rest for 10 s before the next displacement occurred.

Analysis was conducted by determining the number of spikes present within 10 s of the initial movement, which was performed for each of the three trials within each condition. In addition, the average number of spikes across the three displacements in each condition was calculated for graphical purposes, and percent changes determined for normalization of the differences among preparations. A paired *t*-test (if a Shapiro-Wilks test passed) or a Wilcoxon Signed Rank test was used to compare response difference before and during exposure to cobalt chloride.

2.7. *Callinectes sapidus: propodite-dactylopodite chordotonal organ*

The dissection procedures and electrophysiological measures used herein are the same as those described thoroughly in text and video format by Majeed et al. (2013) and have been used previously for assessing the effects of zinc exposure on the PD organ (Pankau et al., 2022). In brief, autotomy of the first or second walking leg was induced by lightly pinching at the base of the leg with pliers. The propodite-dactylopodite (PD) chordotonal organ spans the last segment of the leg and a window was cut into the cuticle in the propodite segment on both sides of the leg to expose the organ. The leg was then pinned in place in a Sylgard-lined dish and covered with crab saline. Once exposed, the PD nerve was pulled into a suction electrode for recording (Fig. 3). During the experiment, the dactyl was moved from a position of flexion to one of extension during a 1 s time frame, held extended for at least 9 more seconds, and then returned to the starting position. An insect dissecting pin was used as a reference point to indicate the maximum displacement range, and each displacement was marked on the computer recording file.

The crab saline used during recordings of the sensory nerves consisted of (in mM): 470 NaCl, 7.9 KCl, 15.0 $CaCl_2{\cdot}2H_2O$, 6.98 $MgCl_2{\cdot}6H_2O$, 11.0 dextrose, 5 HEPES acid and 5 HEPES base adjusted to pH 7.4. Cobalt chloride (5 mM) was added to this standard saline for examining how the compound affects neural responses. The number of spikes recorded in the first 10 s of joint displacement provided an index of the neural activity.

The experimental paradigm applied to the crab PD organ was the same as was used at the crayfish MRO (see above). Movements were repeated for three trials in each condition, with identical rates and degrees of movement. This was performed while the prep was exposed to saline only, to cobalt chloride (5 mM), after 10 min of incubation, and after two wash-out rinses with fresh saline. The joint was returned to its

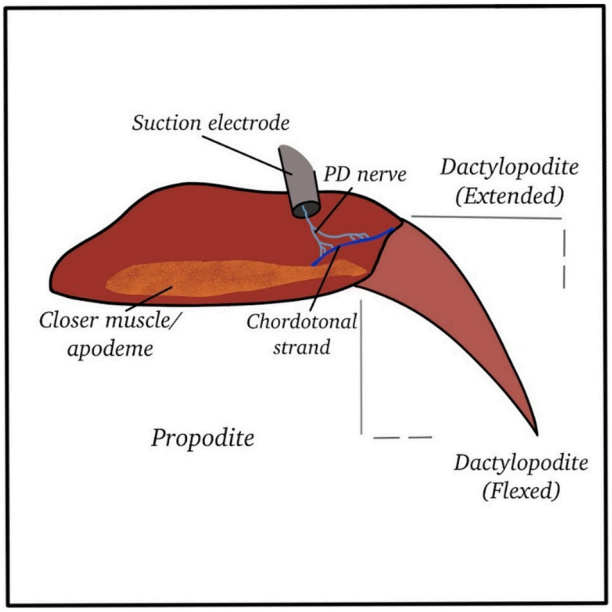

Fig. 3. The first or second walking leg of the crab was used, and the PD organ/associated nerve was exposed. The joint was initially kept at a bend of 90 degrees, whence it was extended out to 180 degrees within 1 s and held for at least another nine. The total activity observed across the full 10 s was used for analysis across the solutions.

starting position and allowed to rest for 10 s after each displacement.

2.8. *Drosophila melanogaster and Procambarus clarkii: synaptic transmission at neuromuscular junctions*

The dissection procedures and electrophysiological measures used here are similar to those thoroughly described in text and video by Cooper and Cooper (2009) and involve the same experimental paradigm as previously described while assessing the effects of zinc exposure at the crayfish and larval *Drosophila* NMJs (Pankau et al., 2022). In brief, the excitatory neuron was isolated from the inhibitor neuron and subsequently stimulated in the meropodite segment via pulse trains at 60 Hz separated by 10 s intervals. 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 using a 20 kHz sampling rate. Dissected preparations were maintained in the crayfish saline describe above (for use with the crayfish MRO preparation). Cobalt chloride (1 mM) was added to the saline for examination of its effects on synaptic transmission.

The effects of cobalt chloride (1 mM) on evoked excitatory junction potential (EJP) amplitude were assessed using the last EJP in the train as an index, with amplitude being measured from base to peak. Recordings were taken over an interval of at least 250 s in saline; 500 s in cobalt chloride (1 mM); and another 250 s in fresh saline (i.e., without cobalt chloride). Stimulus trains were delivered every 10 s, providing enough time (~ 8.3 min) for the acute effects of cobalt chloride exposure to be appraised, as has previously been performed with Zn^{2+}, Mn^{2+} and Fe^{3+} (Elliott et al., 2023a; Pankau et al., 2022; Wagers et al., 2023).

Third-instar larval *Drosophila melanogaster* were dissected in physiological saline (described above for larval *Drosophila* cardiac measures) after which the segmental nerves were cut and pulled into a suction electrode filled with saline. The nerves were then intermittently stimulated at 0.5 Hz (S88 Stimulator, Astro-Med, Inc., Grass Co., West-Warwick, RI, USA) while a sharp intracellular electrode (30 to 40 M resistance) filled with 3 M KCl was inserted into the fiber to monitor transmembrane potentials of body wall muscle m6. An Axoclamp 2B (Molecular Devices, Sunnyvale, CA, USA) amplifier and 1 X LU head stage were used.

2.9. *Statistical methods*

In general, when normality was assumed, Shapiro-Wilks tests were used to validate assumptions and determine whether use of a *t*-test, paired t-test or Wilcoxon Signed Rank Test would be best. An ANOVA was used, when necessary, along with Bonferroni post-hoc *t*-tests.

For survival analysis in *Drosophila melanogaster*, Weibull regression was used to model survival curves (with solution type — control vs. cobalt chloride — as the factor). For the behavioral studies, a t-test was used to determine the difference between the treatments (control, selenium selenite).

Analysis of results obtained regarding heart rate, crayfish MRO, crab PD, synaptic transmission at the crayfish/*Drosophila* NMJs was conducted in the same way; data involved both the initial saline treatment and application of the cobalt chloride. Data from before exposure and during exposure were compared via a paired *t*-test, though a Wilcoxon Signed Rank test was used in cases where the data were not normally distributed. A significance level of 0.05 was used in all studies.

3. Results

3.1. *Adult and larval Drosophila melanogaster: survival and development*

Larvae given food tainted with 1 mM $CoCl_2$ during the 1st-instar stage underwent pupation later than larvae fed a normal, untainted cornmeal diet, as well as exhibiting a higher death rate (Fig. 4). Of the 110 larvae (10 larvae in each of 11 total vials) to receive this tainted diet, only a few formed pupae; those that successfully did so died prior to adulthood, as determined by the fact that they did not hatch for 10 days. Upon examination, 1st-, 2nd-, and early 3rd-instars were found decaying in the food, which indicates that the larvae died during each stage of development. The developmental curves for both control- and cobalt-fed larvae are illustrated in Fig. 4B. The differences in development and survival were significant between the two conditions (ANOVA, $p < 0.0001$).

Adults (2 to 3 days post-eclosion) fed tainted food died more promptly than the control larvae (Fig. 5); the projected survival of controls is significantly longer than those fed $CoCl_2$ (1 mM) (ANOVA, $p < 0.0001$).

3.2. *Larval Drosophila melanogaster: body wall movements and mouth hook movements*

Larvae given food tainted with $CoCl_2$ (1 mM) were assayed for behavioral differences compared to a control diet (standard corn meal), without any significant differences (Fig. 6). The experimental diets were instituted when the larvae were late 2nd instars, and the assays were conducted when the larvae had matured into early 3rd instars. Similar locomotion (measured in body wall movements per minutes) and eating behaviors (mouth hook movements per minute) were observed in both the control and experimental groups, suggesting that consuming food tainted with cobalt did not have an acute effect on intact, developing larvae.

3.3. *Larval Drosophila melanogaster: beating heart rate*

Acute exposure of in situ early 3rd-instar larval heart tubes to $CoCl_2$ led to a rapid decrease in heart rate, or, in some cases, a total halt to the beating (Fig. 7A; $p < 0.05$; paired t-test, $n = 11$). One of the 11 preparations saw an increase in the rate; however, overall, a 66 % decrease in heart rate was observed (Fig. 7B).

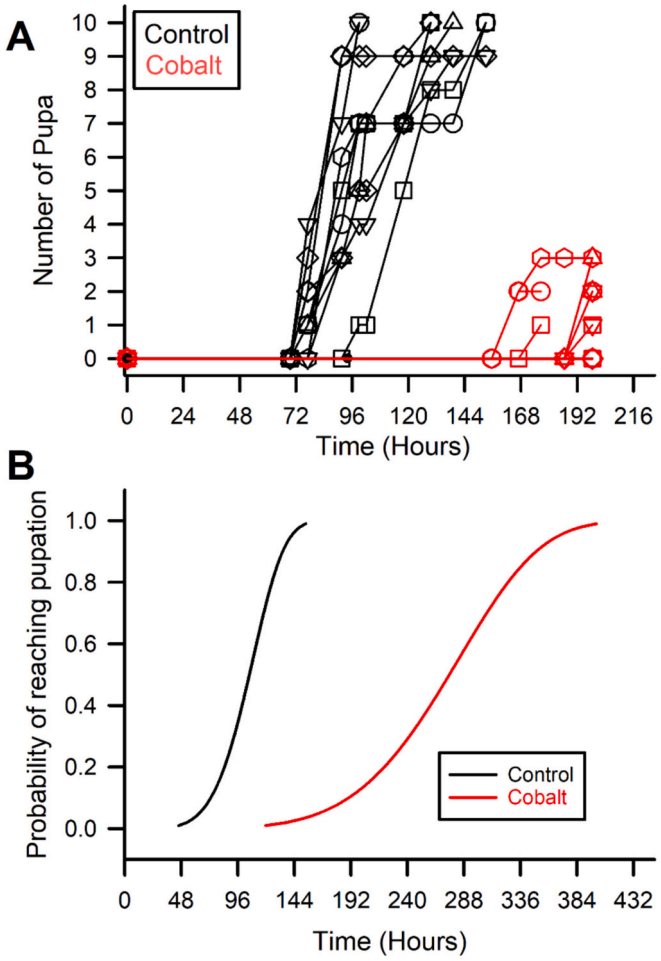

Fig. 4. Results from the larval development and survival study for exposure to 1 mM $CoCl_2$. Early 1st-instar larvae were placed either in standard food or a batch tainted with 1 mM $CoCl_2$. The number of pupae formed was recorded and the food examined for larvae (whether dead or alive). (A) Pupation rates observed for the control and cobalt vials. Each $CoCl_2$ vial contained at most 10 pupae. (B) The projected probability of reaching pupation for groups treated with control or cobalt. The two developmental curves bore significant differences (ANOVA, $p < 0.0001$), with the cobalt group taking longer to reach pupation than the controls and bearing fewer surviving larvae.

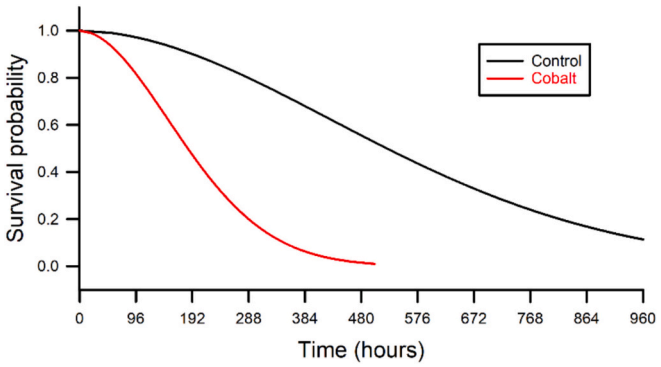

Fig. 5. The survival of adult Drosophila when exposed to dietary $CoCl_2$ two to three days after eclosion from pupa cases. Ten vials of ten adults each served as controls (containing normal food), while six more vials (also containing ten adults each) underwent exposure to 1 mM $CoCl_2$. A significant difference was observed between the survival curves for the two conditions (ANOVA, $p < 0.0001$), with the cobalt group dying out at a quicker rate than the controls.

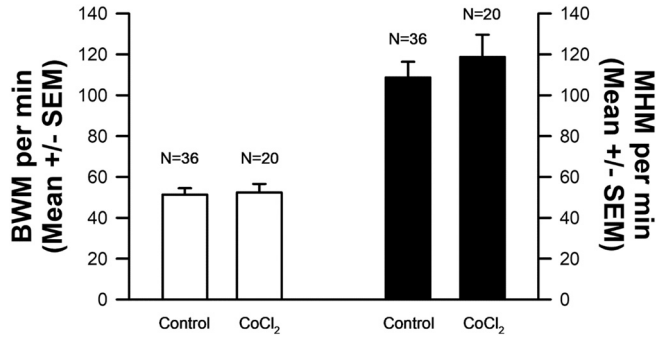

Fig. 6. Larval behaviors after 24 h of exposure to food tainted with 1 mM $CoCl_2$, as compared to controls (fed a standard corn meal diet). Eating behavior (mouth hook movements per minute, MHM) and crawling behavior (body wall contractions per minute, BWM) were highly similar, indicating that exposure to food with $CoCl_2$ (1 mM) did not have a significant effect on either of these behaviors. Late 2nd-instar larvae were placed in the food and then assayed as early 3rd instars (i.e., non-wandering larvae found on the vials' sides or otherwise out of the food). The rates are expressed as a mean +/− SEM; significance was examined as a *t*-test $p > 0.05$, $N > 20$ larvae for each condition.

3.4. *Procambarus clarkii: cardiac responses and survival*

Responsiveness of the crayfish heart to sensory stimulus (i.e., tap on the telson) did not exhibit an acute change upon injection with 1 mM $CoCl_2$ (Fig. 8); however, on the third day post-injection, tapping the telson led to an increased heart rate as compared to the saline-injected controls (Fig. 8A, Mann-Whitney Rank Sum Test, $p = 0.015$). Fig. 8B depicts a graph of the observed percent change and displays this behavioral difference. All six of the saline-injected control crayfish survived for at least two weeks (at which point they were no longer under watch; of the six crayfish injected with cobalt, five also survived the 2 weeks of observation, but one died in the second week.

3.5. *Procambarus clarkii: muscle receptor organ*

Function of the primary sensory neurons associated with the crayfish MRO was not altered by acute exposure to $CoCl_2$ (5 mM); they responded to joint movements in an equivalent manner before, during, and after exposure to a relatively high concentration of $CoCl_2$ (Fig. 9).

3.6. *Callinectes sapidus: propodite-dactylopodite chordotonal organ*

The primary sensory neurons associated with the PD organ of a marine crab walking leg manage the animal's proprioception. Acute exposure to a low concentration of $CoCl_2$ (0.1 mM) had no significant effect on neural responses to joint movement (Fig. 10A); however, at higher concentrations (1 mM), neural activity underwent a significant increase that only strengthened following the ten-minute incubation time (Fig. 10B; $p < 0.05$; paired *t*-test; n = 6). The percent changes from activity in saline to that in experimental conditions allow for normalization of the differences between initial activity and the effects of $CoCl_2$ (Fig. 10C).

3.7. *Drosophila melanogaster and Procambarus clarkii: synaptic transmission at neuromuscular junctions*

Synaptic transmission efficacy at the crayfish NMJ was depressed by acute exposure to $CoCl_2$ (1 mM). Depression in EJP amplitude was observed in the stimulus trains within only a few minutes of exposure to saline containing cobalt, as is illustrated in a representative recording (Fig. 11A) and as a graph (Fig. 11B). This amplitude reduction is observed in all eight preparations and significant (Fig. 11C; paired t-test from initial saline to $CoCl_2$ Shapiro-Wilk; $p < 0.05$; $n = 8$). Since EJP

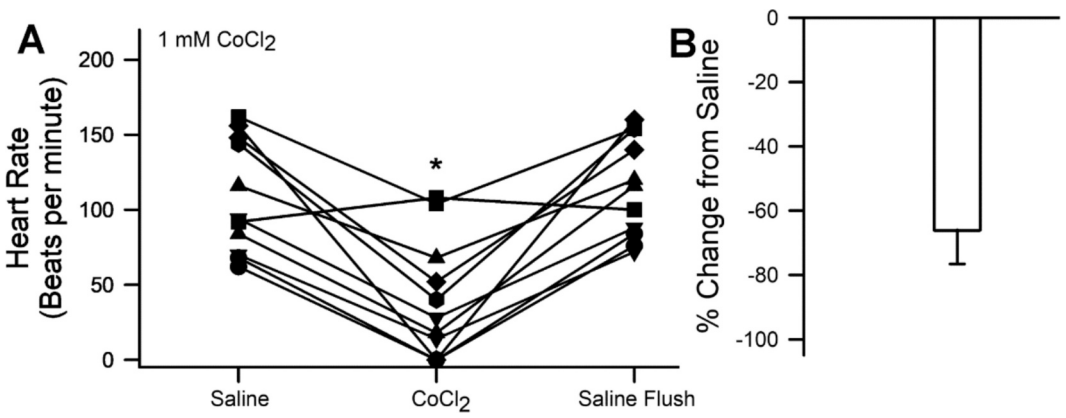

Fig. 7. The effect of $CoCl_2$ exposure on heart rate of in situ 3rd-instar larval hearts. The larvae were dissected and filleted, which exposed the heart tube to the bathing saline and flushed away any endogenous compounds. (A) The heart rate was counted in the initial saline, after exchange of the media to CoCl2 solutions, and after rinsing the preparation with fresh saline to wash out the cobalt. The heart rate dropped significantly upon exposure ($p < 0.05$; paired *t*-test, n = 11). Each line represents an individual larva and illustrates the change in its heart rate throughout medium exchange. (B) On average, heart rate decreased by 66 % when the initial saline was replaced with a $CoCl_2$ solution.

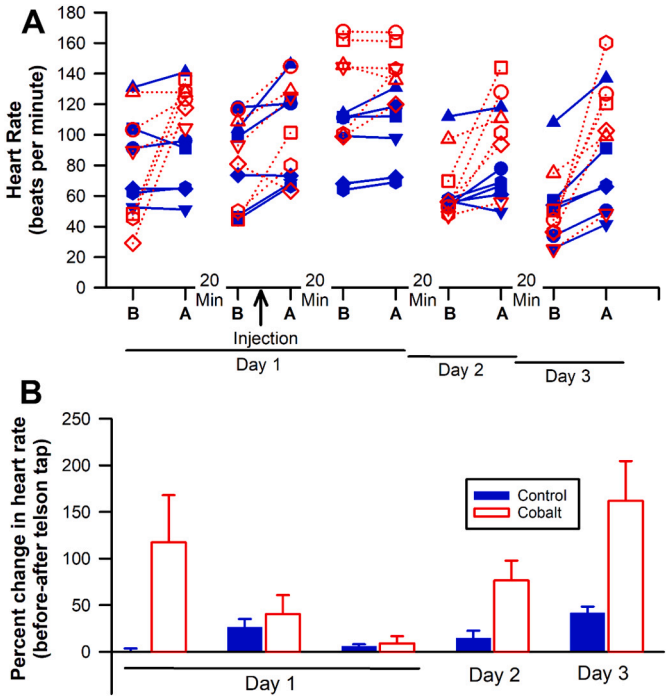

Fig. 8. The effects of $CoCl_2$ injections into the crayfish hemolymph. Six crayfish were injected with saline as controls, while six more were injected with $CoCl_2$. (A) All 12 responded to a telson tap both before and after injections, and this response was also recorded on days 2 and 3. All 6 saline-injected control crayfish survived the two weeks of observation, while 1 of the 6 crayfish injected with $CoCl_2$ died before observation ended. (B) The difference in response to a telson tap (as determined by a quantification of the resultant heart rate increase as a percent change) was significant for the cobalt-exposed crayfish as of the third day (Mann-Whitney Rank Sum Test, p = 0.015).

amplitude varied in saline, a percent change graph for each preparation was utilized to normalize the effects of cobalt exposure as opposed to the starting conditions (Fig. 11D). Upon removing the $CoCl_2$ with fresh saline washout, the EJP amplitudes began returning to baseline conditions (Fig. 11C), which illustrates that these preparations were not irreversibly affected by exposure to $CoCl_2$.

The efficacy of synaptic transmission at the crayfish NMJs was compared to that at the NMJs of larval *Drosophila*, which was similarly depressed by acute exposure to $CoCl_2$ (1 mM); however, the magnitude of the effect was greater in larval *Drosophila* preparations than in crayfish. A representative recording is shown in (Fig. 12A) and graphically expressed (Fig. 12B). A reduction in EJP amplitude was observed in all six preparations (Fig. 12C; paired t-test from initial saline to $CoCl_2$ Shapiro-Wilk; $p < 0.05$; $N = 6$). Since each preparation bore varied EJP amplitudes in the initial saline, a percent change was determined for each preparation for normalization purposes (Fig. 12D). Upon removing the cobalt solution, EJP amplitudes returned to baseline conditions (Fig. 12C), suggesting that the effects of these acute exposures were reversible.

4. Discussion

The experiments contained within this overview surveyed acute responses to $CoCl_2$ in several model preparations in order to provide a better understanding of how the compound altered physiological processes. It appears that larval and adult *Drosophila* fed a diet tainted with $CoCl_2$ may experience slow development and, with continuous exposure to high concentrations (1 mM) over time, lethality. However, developed larvae consuming tainted food for 24 h bore no acute alterations in crawling or eating behaviors. The long-term effects of late-stage larvae eating food laced with cobalt have yet to be examined; but, considering that survival rates in adult *Drosophila* were significantly reduced, late-stage larvae would also likely have reduced survival. Once the isolated NMJs or heart tubes of larval *Drosophila* were exposed to $CoCl_2$, cobalt's influence is rapid and discernable within minutes, as both synaptic transmission and myogenic cardiac function are swiftly compromised. In crayfish, synaptic transmission at the NMJ was likewise depressed, though not as extensively as in larval *Drosophila*. Surprisingly, injections of $CoCl_2$ into crayfish hemolymph did not immediately alter survival or cardiac function, though the preparations appeared hypersensitive to sensory stimuli two days after injections. Action of the primary sensory nerves involved with proprioception in the crayfish and crab underwent no depression, and the crab model even exhibited an enhancement of activity. Initially, it was assumed that stretch activated channels (SACs) in neuronal sensory endings might be blocked by cobalt exposure such that overall neural activity would be reduced, but this was not the case.

When an organism consumes a substance tainted with cobalt, the concentration present in the systemic circulation (i.e., hemolymph or blood) depends on the contaminant concentration and form, the organism's supplemental diet and hydration, and secretions from the body into the lumen. The intestine microbiome also has a large role in processing cobalt, while dietary fat and intestinal health affect absorbance (Wang et al., 2024). On a cellular level, free metal ions compete for

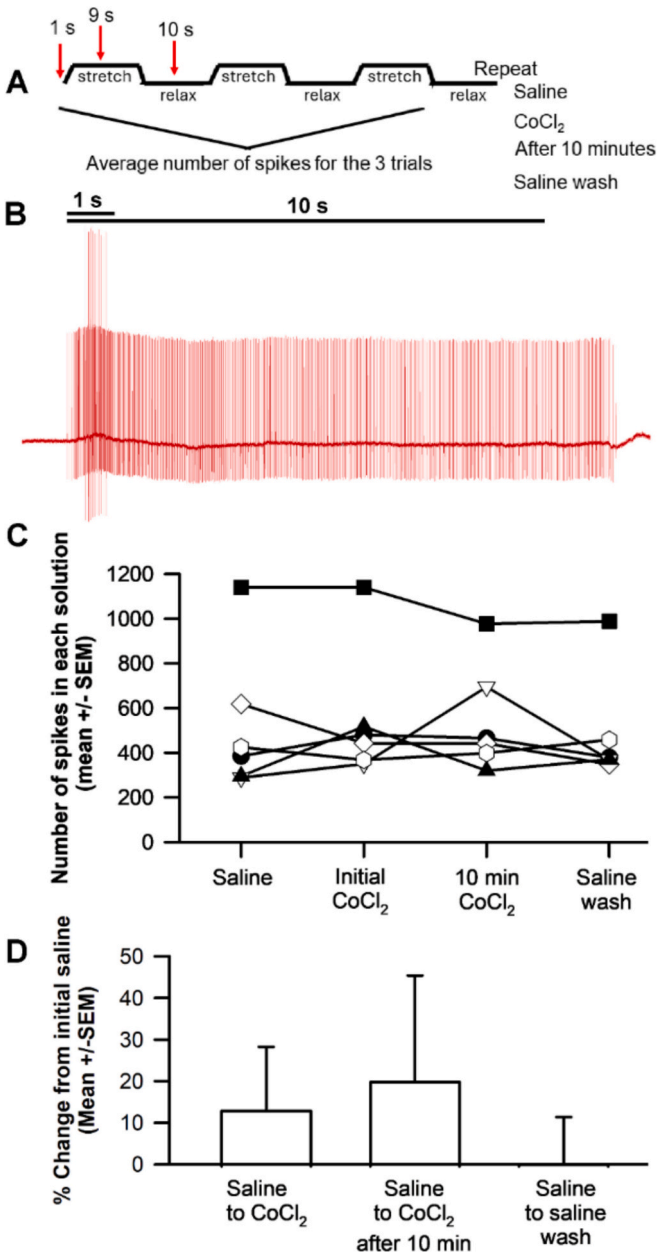

Fig. 9. The effect of $CoCl_2$ on activity at the crayfish muscle receptor organ. (A) A schematic of the procedure for manipulating the abdominal segment in conjunction with cobalt exposure at the MRO. Three trials were conducted within each condition — saline, $CoCl_2$ (5 mM), after ten minutes' incubation time, and during washout — and the neural activity within the first 10 s of each trial examined; a count was taken of the number of spikes present in each ten-second interval, and then the values within each condition were averaged. The joint was kept in its initial, relaxed position during the 10 s of rest between each trial, as well as during the 10 min of incubation time. Each joint movement occurred within 1 s, after which the stretched position was maintained for at least nine more prior to relaxation. (B) A representative trace of one such trial (C) The number of spikes in each of the three trials (+/− SEM). No significant change was observed following acute exposure to $CoCl_2$ (5 mM) (whether assessed immediately or after 10 min of incubation) as compared to baseline activity levels ($p > 0.05$; paired *t*-test; $n = 6$). (D) Alterations in neural activity, as expressed in percent change from initial saline to each experimental condition and calculated from the averaged activity levels. No significant effect from exposure to $CoCl_2$ (5 mM) was observed (Rank sum Sign test; $n = 6$).

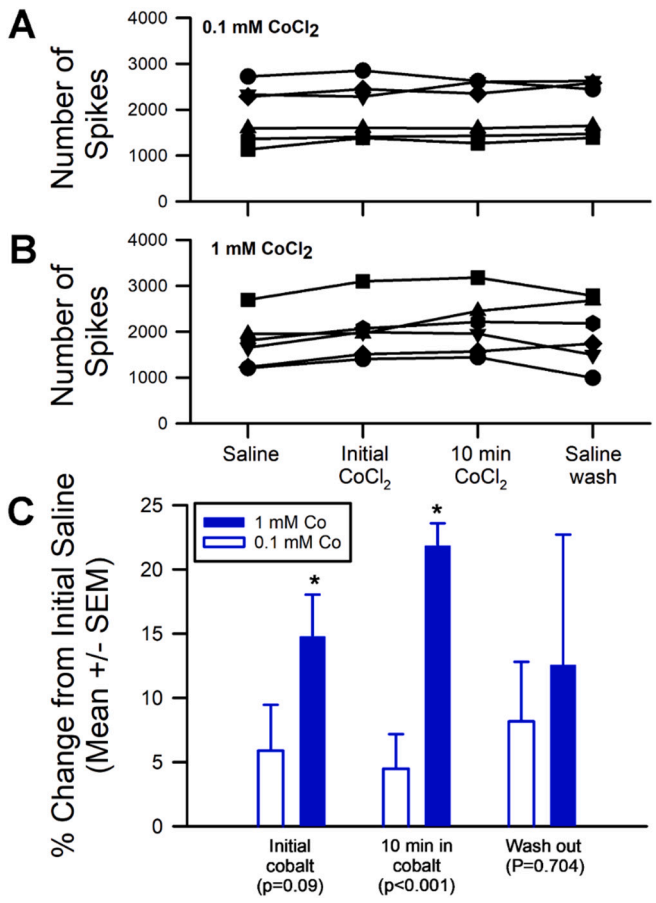

Fig. 10. $CoCl_2$ stimulated sensory nerve activity of the propodite–dactylopodite (PD) chordotonal organ in the crab walking leg. Three trials were conducted in each condition — saline, $CoCl_2$ at (A) 0.1 mM or (B) 1 mM, ten minutes' incubation time, and washout — wherein the joint was extended for 10 s and then flexed for ten more. Neural activity was measured for each of the three trials and averaged (+/− SEM) for each bathing condition. Individual preparations were examined under each condition without repetitive exposure. (C) No significant effects on activity were observed at exposure of 0.1 mM, but a significant increase in activity was observed for both the acute and incubated exposure to 1 mM $CoCl_2$ ($p < 0.05$; paired t-test; $n = 6$).

transporters that may have preferred ions.

In these studies, neither the precise cobalt concentration absorbed nor the potential that it accumulated with continuous feeding could entirely be addressed; it would thus be of interest to try pulsed exposures for comparison in future studies. Even injections of cobalt into the crayfish hemolymph left questions regarding the free ionic concentrations, as some might have been bound by proteins, lipids, or tissues such that the free ionic levels did not reach the estimated 1 mM. Indeed, this is likely the case, as 1 mM exposure would result in a depression of activity at crayfish NMJs and an absent tail flip response following a telson tap; it would also likely prompt a decrease in cardiac rate, rather than the expected increase, due to the chemical synaptic steps of the sensory-CNS-motor circuit. The most logical explanation for synaptic efficacy decreasing at both crayfish and *Drosophila* NMJs is the blockage of presynaptic voltage-gated channels.

Larval *Drosophila* NMJs were affected more by the $CoCl_2$ than the crayfish were at the same concentration. While both are sensitive to changes in the surrounding Co^{2+} concentration, the fact that crayfish physiological saline contains 13.5 mM $CaCl_2(H_20)_2$ while *Drosophila* saline contains only 1 mM may indicate that the addition of 1 mM of $CoCl_2$ would not displace Ca^{2+} as thoroughly in crayfish. In both preparations, cobalt seems to be readily displaced upon sufficient flushing of

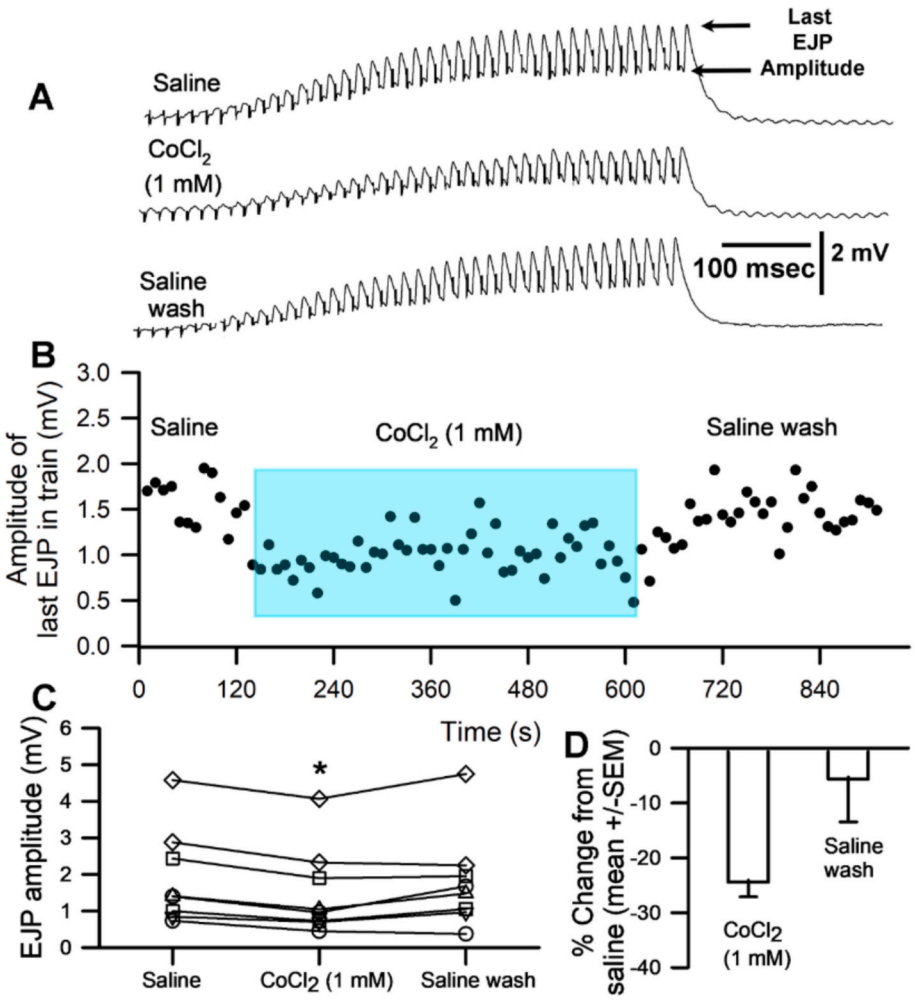

Fig. 11. The effects of $CoCl_2$ (1 mM) on evoked excitatory junction potentials (EJPs) at the neuromuscular junction of the crayfish walking leg opener muscle. (A) The motor nerve was stimulated every ten seconds with 60 Hz pulse trains under each condition: in saline, while bathed in $CoCl_2$ (1 mM), and during washout. (B) The final EJP amplitude of each train was measured throughout the paradigm and graphed, as is illustrated for a representative preparation. (C) For comparative measures, the average amplitude of the final EJP across the 25 trials in the initial saline, the final 25 (of 50) trials in $CoCl_2$, and the 25 trials in washout were used. EJP amplitudes were significantly decreased during exposure to $CoCl_2$ (paired t-test from initial saline to $CoCl_2$ with normality test by Shapiro-Wilk; $p < 0.05$; $n = 8$). (D) To normalize the variation, a mean percent change from saline to $CoCl_2$ was determined for each preparation; specifically, the average change in EJP amplitude with $CoCl_2$ exposure was a decrease of 24 %, and the amplitudes generally returned to their initial height after the preparation was rinsed with fresh saline.

the NMJs with fresh saline, suggesting that competition at the channel level is a feasible explanation for the observed differences in response. Some divalent ions can block voltage-gated Ca^{2+} channels, including Cd^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} (Langner, 2000). It is unlikely that Co^{2+} behaves like Ca^{2+} and blocks NALCN channels (i.e., sodium-ion leak channels; Monteil et al., 2024), as the membrane potentials did not hyperpolarize upon exposure to 1 mM $CoCl_2$. At higher concentrations, divalent ions like Co^{2+} may block these leak channels. Although it is unknown so far whether NALCN channels are present or not in the body wall muscles of crayfish and larval *Drosophila*, it is highly likely that some form of Na^+ leak channel exists in larval *Drosophila* muscles; after all, E_K is about −90 mV (Ikeda et al., 1976; Salkoff and Wyman, 1983) and *Drosophila* muscle's resting membrane potential is around −60 mV.

Organisms exposed to cobalt — whether from industrial settings or other means — should be considered bio-accumulators of the metal. This, as well as the knowledge that cobalt is essential for organisms throughout their life span and supplements as edible materials (whether intentional or otherwise), reinforces the importance of understanding what consequences overconsumption of cobalt may have.

Direct exposure of the larval *Drosophila* heart to cobalt resulted in a rapid decrease in heart rate, which could be due to the element acting upon various targets. It was not established whether the pacemaker potential was depressed or whether electrical signals were present, but it is known that the muscle was unable to contract. The larval heart rate, just like in mammals, is dependent on extracellular Ca^{2+}, the plasmalemmal Na+/Ca2+ exchanger (NCX), the Ca^{2+}-ATPase (PMCA), and the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Desai-Shah et al., 2010; Elliott et al., 2023a; Hove-Madsen et al., 2004; Morgan, 1991). If Ca^{2+} were unable to flux into the myocyte, the cells would thus be unable to contract, so contractions were used as the visual index of cardiac function; however, future studies are required to determine whether the ionic currents for generating pacing were compromised or if only the heart's contractibility was affected.

It is interesting to note that the activity of the primary sensory neurons at the crayfish MRO and crab PD organ were not depressed by $CoCl_2$. The SACs in the sensory endings of these proprioceptive neurons likely allow for Ca^{2+}-ion flux when open (i.e., during joint movements), but a past genomic search for PIEZO1 homologs within crustacean genomes did not locate PIEZO1 genes (McCubbin et al., 2020); so it is likely that a different subtype of SAC exists in these structures. Gd^{3+} is known to rapidly block SACs and, with fresh saline exchange, is quickly removed (Dayaram et al., 2017a, 2017b); additionally, Gd^{3+} is known to

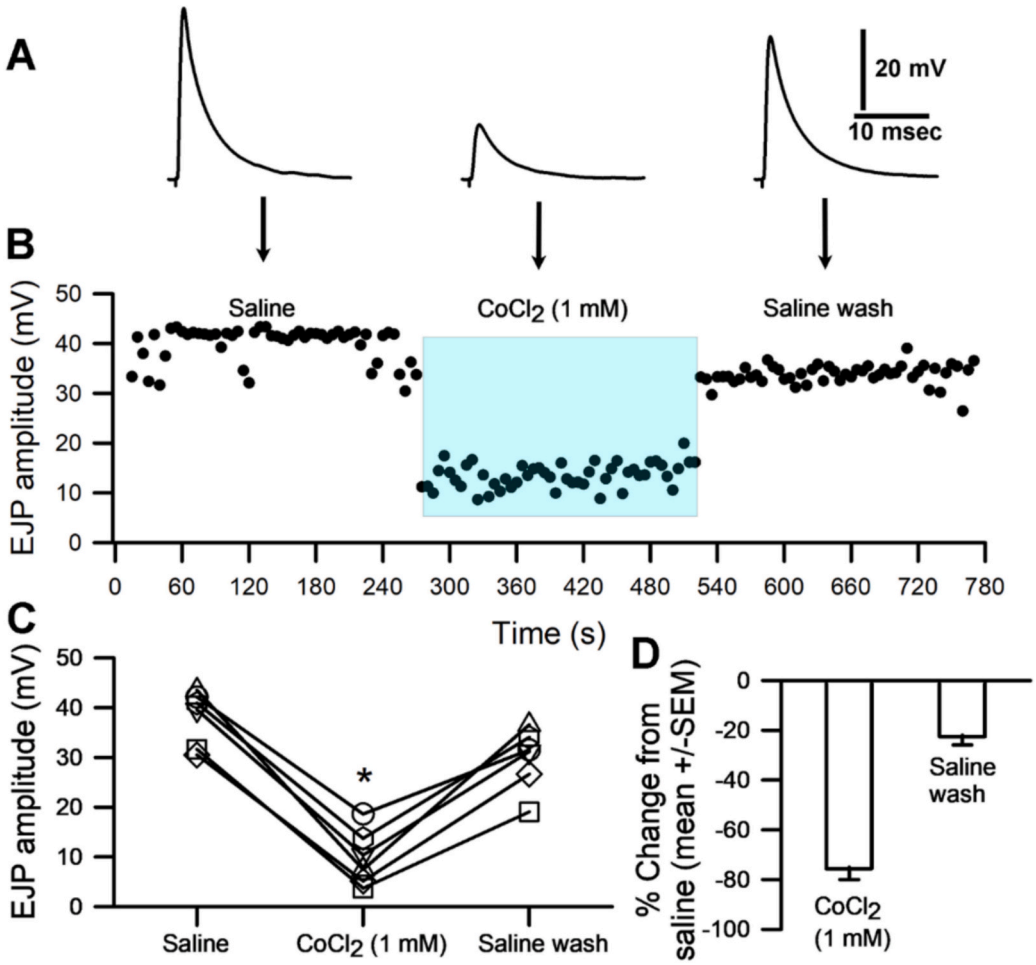

Fig. 12. The effects of $CoCl_2$ (1 mM) on evoked excitatory junction potentials (EJPs) at the larval Drosophila neuromuscular junction. (A) Responses in the EJPs before, during, and after $CoCl_2$ exposure. (B) A representative response with the segmental nerve stimulated (0.5 Hz) in each of the experimental conditions: in saline, during exposure to $CoCl_2$, and during washout. (C) The average EJP amplitudes for each condition are shown for all six preparations. The amplitudes significantly decreased during exposure to $CoCl_2$ (paired t-test from initial saline to $CoCl_2$ with normality test by Shapiro-Wilk; $p < 0.05$; $n = 6$). (D) In normalizing initial variation, a mean percent change from saline to $CoCl_2$ was determined for each preparation, indicating an average decrease of 75 % during exposure to $CoCl_2$, though the amplitudes generally returned to their initial values after washout.

block voltage-gated Ca^{2+} channels and Ca^{2+}-specific SACs. The precise SAC subtype present in these crustacean proprioceptive sensory neurons is thus still unknown, as they do not fit into a traditional pharmacologic profile (McCubbin et al., 2020).

Many unanswered questions remain regarding how, exactly, Co^{2+} affects different tissues/organisms; but a comparative approach in physiological investigations allows for analysis of similarities and differences in the effects across the model preparations used, as well as for a greater overall understanding in the mechanisms of action. Further research must be conducted into how ionic cobalt affects animal and human health, as well as how that might differ between acute and chronic exposure. Currently, no standard treatment exists for excessive cobalt exposure beyond stopping exposure and supportive physiological maintenance to treat the symptoms alone. It is imperative to develop potential new therapeutic treatments, as well as gain an awareness of how cobalt causes physiological disruption in order to help reduce risks of future exposure (Barceloux, 1999; Catalani et al., 2011; Chen and Lee, 2023).

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CRediT authorship contribution statement

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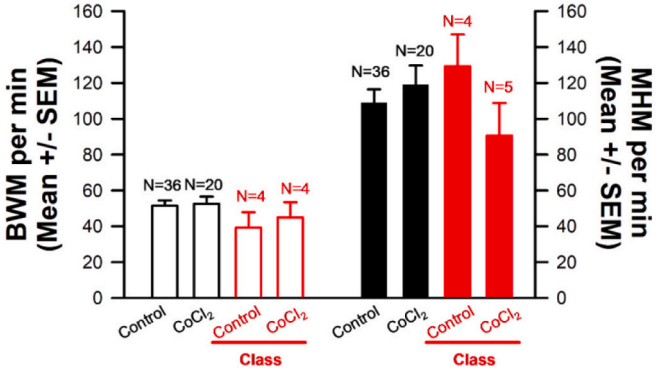
Declaration of competing interest

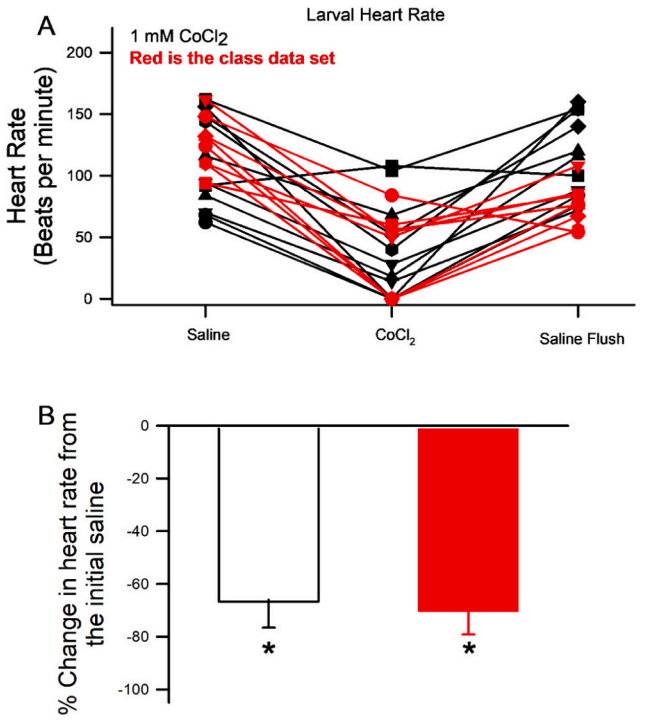
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A

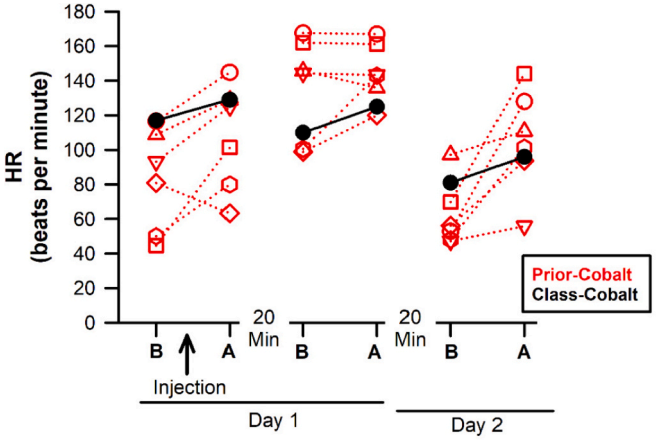
The examination of reproducibility in experimentation and analysis for some of the paradigms presented were addressed by participants in an undergraduate course. All repeated paradigms and analysis revealed similar trends without significant differences.



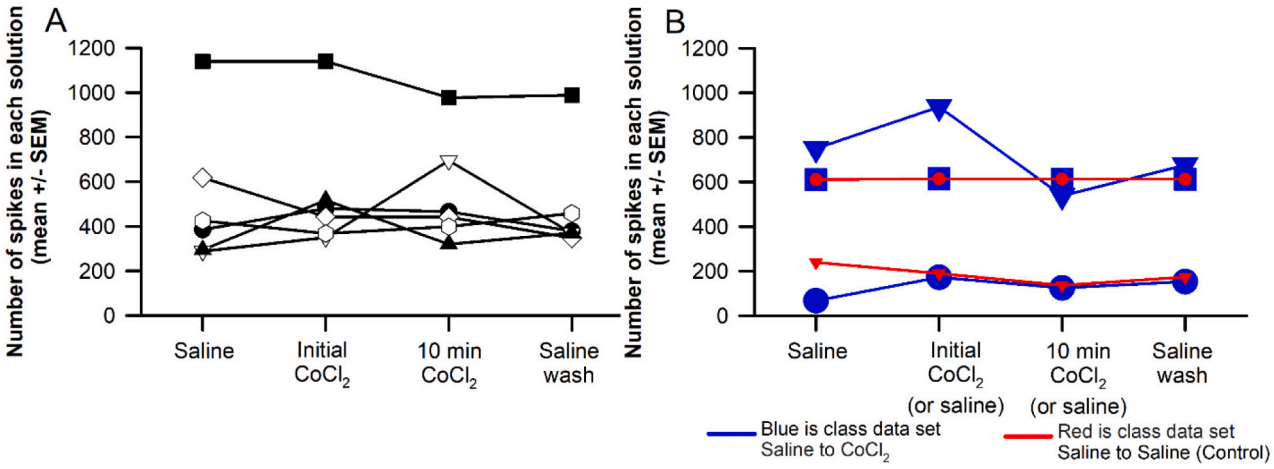
Appendix A1. Repeating the behavioral analysis in body wall movements and mouth hook movements after 24 h of consuming food tainted with 1 mM $CoCl_2$ with independent investigators (i.e. class) revealed the same responses as in the primary investigation 3 months later. Mouth hook movements per minute (MHM) and crawling behavior as body wall contractions per minute (BWM) were determined by direct visual observations.



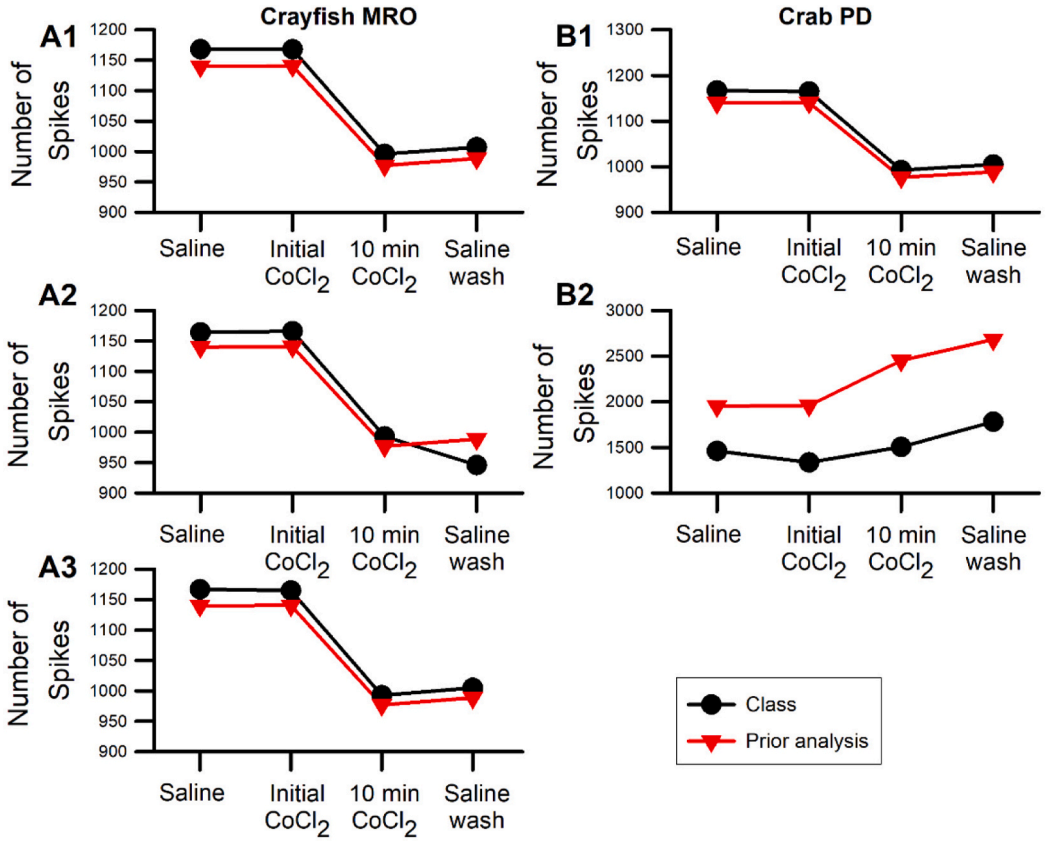
Appendix A2. Repeating the investigation of the acute effects in exposure to the in situ larval heart with 1 mM $CoCl_2$ by independent investigators (i.e. class) revealed the same percent decrease in the heart rate as previous experimentation. (A) Individual preparations are shown as individual lines for experiments conducted in a research laboratory environment with well-trained individuals and a set of students in a course. (B) The percent changes in the heart rate with exposure to $CoCl_2$ by the two groups was not significantly different from each other but both show a significant effect to exposure of $CoCl_2$ (paired *t*-test $p < 0.05$).



Appendix A3. Repeating the investigation of injecting $CoCl_2$ into the hemolymph of crayfish and stimulating sensory input by a tap on the telson to alter the heart rate revealed similar responses as previous investigation three months earlier.



Appendix A4. Repeating the investigation by participants in a class by acutely exposing the MRO of crayfish to $CoCl_2$ produced similar responses as to a saline control as well as to the previous responses to exposure of $CoCl_2$ (1 mM).



Appendix A5. Three different data sets of electrical activity from three different preparations of the crayfish MRO preparations (A1-A3) and two of the crab PD organs (B1and B2) were reanalyzed to compare for reproducibility in analysis. The repeated analysis revealed similar trends to the effects of acute exposure to $CoCl_2$ (1 mM). The variation in the absolute values is due to choosing different thresholds from the baseline in counting the smaller amplitude spikes for the crab PD preparation shown in B2.

Data availability

Data will be made available on request.

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