

1 **Effect of short and long-term cadmium exposure on**
2 **behaviour and cardiac function in *Drosophila*.**

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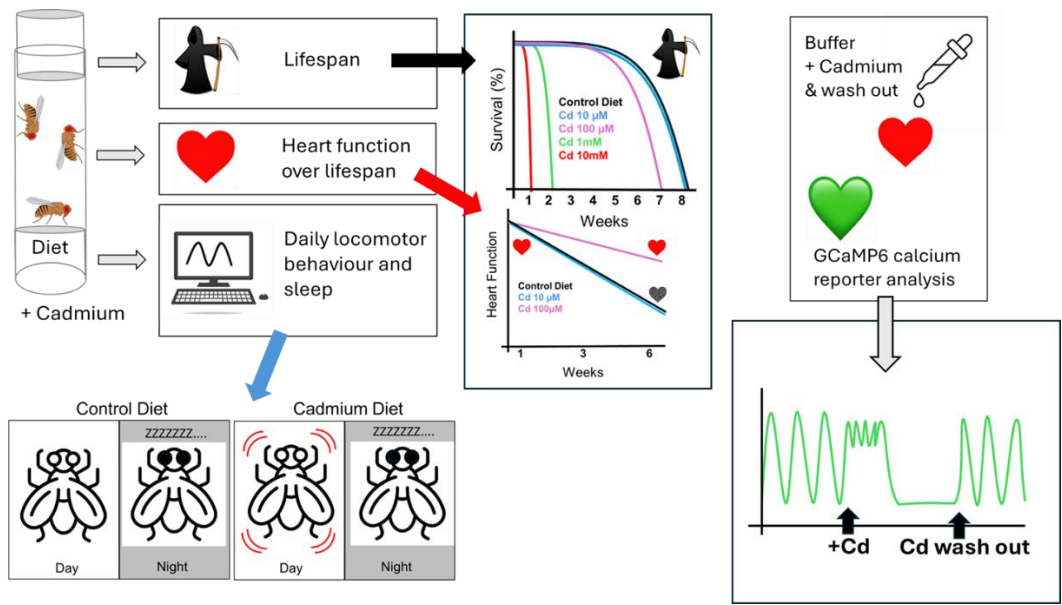
19 **Highlights:**

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- 21 1. Dietary cadmium causes daytime hyperactivity in *Drosophila*.
- 22 2. At low doses, lifespan and cardiac ageing are unaffected by dietary cadmium
- 23 3. At doses that shorten lifespan, dietary cadmium can reduce cardiac ageing.
- 24 4. Direct exposure of cardiomyocytes to cadmium disrupts calcium signalling.
- 25 5. Direct exposure to cadmium causes reversible cardiac arrest.

26

Graphic Abstract.



Abstract.

Cadmium is a non-essential heavy metal and environmental pollutant that causes a range of pathologies across different species. In humans, cadmium exposure has recently been directly linked to heart disease. Understanding how long-term cadmium exposure affects cardiac physiology is therefore important. In this work we employed a tractable *Drosophila melanogaster* model to study the effects of cadmium exposure on behaviour, lifespan and cardiac physiology. Dietary experiments established that cadmium at 10 μ M and 100 μ M was tolerated for several weeks, whereas doses in the mM range caused lethality within days. It was estimated that 10 μ M dietary exposure represented an approximately 60-fold excess of the maximum exposure recommended for humans. Although 10 μ M cadmium had no impact on lifespan compared to the control diet, it did cause significant daytime hyperactivity. Direct exposure of the heart to cadmium caused reversible cardiac arrest and disrupted calcium signalling. Compared to controls, 10 μ M dietary cadmium had no impact on the rate of cardiac ageing over a six-week period. The higher dose of 100 μ M shortened the flies' lifespan but it slowed the rate of cardiac ageing. The findings indicate that *Drosophila* can be used to model the direct effects of cadmium on cardiomyocyte function and also demonstrate the existence of cardioprotective pathways triggered by dietary cadmium exposure. The data also indicate that cadmium at doses that do not affect lifespan or heart function, do cause daytime hyperactivity. Identifying and the cardioprotective mechanisms and understanding the hyperactivity phenotype in *Drosophila* may yield important findings of applied relevance to insects in general, as well as humans exposed to cadmium.

Introduction.

Cadmium is a non-essential heavy metal found in the Earth's crust and atmosphere, as well as commercial products, including cigarettes, batteries, plastics and paints ("Compendium of Chemical Hazards: Cadmium," 2016). Cadmium is also a pollutant of foods, water supplies, the atmosphere and workplaces (WHO, 2019). In the mid twentieth century Itai-itai disease, a severe condition characterised by intense pain, kidney dysfunction and bone disease was linked with cadmium poisoning. There is now extensive evidence that humans chronically exposed to cadmium develop cardiovascular pathologies including kidney disease (Ferraro et al., 2010), an increased risk of cardiovascular disease (Lamas et al., 2023; Tellez-Plaza et al., 2013), reduced heart rate and electrocardiographic abnormalities (Feng et al., 2015; Thapa et al., 2018) and increased risk of heart failure in non-smokers (Sears et al., 2022). Whilst it is well-established that cadmium can affect cardiovascular physiology, it remains unclear which mechanisms are involved and whether these differ according to the route of exposure, the length of exposure time and the life history stage that exposure occurs. Understanding this is important because it guides measures that may mitigate or treat cadmium toxicity.

Evidence from experimental studies using mammalian systems indicates that cadmium is an effective blocker of L-type voltage-gated calcium channels which are crucial for calcium entry into cardiomyocytes (reviewed by DeCoursey, (DeCoursey and Cherny, 2007)). Direct exposure of primary rat cardiomyocytes to cadmium negatively affects contraction rates (Limaye and Shaikh, 1999) and can reduce ATP levels, drive ER stress and promote cell death (Chen et al., 2015). In other rodent models, cadmium can cause oxidative damage to cardiomyocytes, as well as mitochondrial dysfunction, lipotoxicity and inflammation (Reviewed by Renu *et al* (Renu et al., 2022)). The effect of cadmium exposure in rodents via their diet or drinking water results in a plethora of pathologies involving multiple organ systems, with cadmium notably accumulating in, and damaging the kidneys. Studies where

heart physiology was quantified have shown that dietary cadmium can interact with high cholesterol in ApoE^{-/-} mice to significantly increase the risk of heart failure via a mechanism involving fibrosis (Türkcan et al., 2015). Additionally, mice given cadmium-laced water can develop left ventricular dysfunction, an effect linked to disrupted cardiomyocyte calcium signalling (Fitch et al., 2023). Collectively, these findings indicate that cadmium can affect cardiac physiology via evolutionarily conserved, non-cell autonomous (e.g. fibrosis) and cell-autonomous mechanisms (e.g. calcium signalling). However, experimentally modelling the impact of chronic cadmium exposure is not trivial because it requires mammalian systems that are time consuming and resource intensive. As an alternative, it may be possible to model the impact of cadmium (and other heavy metals) on heart physiology using a simpler, more tractable system such as that offered by the fruit / vinegar fly *Drosophila melanogaster*.

Drosophila have a contractile heart (also known as the dorsal vessel) which runs almost the entire length of the dorsal midline from the head to the final abdominal segment. The heart consists of two regions, a non-contractile aorta (typically in the thorax and head) and a contractile 'ventricle-like' region in the abdomen. As well as contractile cardiomyocytes, the abdominal dorsal vessel comprises specialised valve cells that direct the flow of haemolymph and ostia which allow haemolymph to flow into and out of the vessel's interior (reviewed in Blice-Baum et al (Blice-Baum et al., 2019)). This simple system, in combination with complex genetic manipulations, has been used to study cardiac physiology in a range of biological contexts, including development (Vogler et al., 2014; Meyer et al., 2022), potassium channel abnormalities (Ocorr et al., 2007), tropomyosin physiology (Madan et al., 2020), metabolism (Birse et al., 2010), mechanotransduction (Zechini et al., 2022) and ageing (Vaughan et al., 2018). Despite the strengths of *Drosophila* as a model for heart physiology, there appears to be only one study of cadmium's cardiac effects which found that injection of 10 mM CdCl₂ into larvae increased heart rate, as well as the frequency of arrhythmias (Zabihihesari et al., 2022).

112 In this work, the impact of cadmium on cardiac function and cardiac ageing were evaluated
113 using adult *Drosophila melanogaster*. The studies explored how cardiomyocytes respond to
114 direct cadmium exposure using semi-intact heart preparations but also how the heart would
115 react to long term exposure in ageing flies. The findings demonstrate that whilst cadmium
116 can directly affect cardiomyocyte function, the impact of indirect exposure via the diet is
117 more complex, with some cadmium doses slowing the heart's age-related function decline,
118 despite increasing mortality. These findings help establish the *Drosophila* heart model as a
119 means of exploring the impact of heavy metal pollutants on cardiac function and highlight the
120 existence of cardioprotective mechanisms of potential relevance to human heart physiology.
121

Methods:

***Drosophila* Stocks and husbandry.**

The wild type Oregon-R-C strain from the Bloomington Drosophila Stock Centre was used throughout these studies. For calcium imaging of cardiomyocytes *TinCΔ4*; *GCaMP6* flies were generated and described in (Zechini et al., 2022). Flies were maintained on a standard cornmeal-sucrose-agar diet (1.4 L distilled water, 100 g sucrose, 102 g cornmeal, 12 g agar, 35 g dried yeast, 3.8 g Nipagin and 7 mL propionic acid). Food was decanted into polypropylene vials which were capped with cotton stoppers. Flies were housed under 12 hr:12 hr light-dark cycles at 25°C.

Justification of Cadmium doses used in the study.

Circulating Cadmium levels in humans are typically below 5 ng/mL (<44.6 nM), whereas acute toxicity manifests >50 ng/ mL (>446 nM) ("Cadmium, Blood - Laboratory Test Catalog," n.d.). Primary neonatal rat cardiomyocytes are recorded to lose contractile function within 30 minutes of exposure to doses from 100 nM to 500 nM (Limaye and Shaikh, 1999). Cadmium at doses from 100 μM to 1.5 mM in a standard cornmeal-agar diet affected measures of *Drosophila* development and adult lifespan (Nanda and Firdaus, 2022). Finally, the larval heart is known to respond to injections of 10 mM cadmium (Zabihhesari et al., 2022). Accordingly, the dose range used in the current work was from 100 nM to 10 mM. We know how much a female fly eats per day (~1mg of food) and how much they weigh (wet weight ~12mg). We can extrapolate this to a 'mg ingested per kg body weight per day' exposure used to describe exposure in larger animals including humans. We calculated that flies on the 10 μM diet have an exposure of 153 μg cadmium / kg body weight / day. Extrapolating this dose to humans, it is ~60 fold greater than the recommended maximum for humans which is 2.5 μg/kg/day (according to guidelines from the European Food Safety Authority (Authority (EFSA), 2009).

Ageing of flies.

As heart analysis (see below) uses females flies, ageing studies also used female flies. Females were collected within one week of eclosion and it was assumed that all were mated. Twenty females were added to vials containing the standard diet described above, or the standard diet supplemented with CdCl₂ (10 µM, 100 µM, 1 mM and 10 mM) and tipped to fresh food every 3 days for the first two weeks (to avoid flies getting stuck in wet food containing large numbers of feeding larvae) and then every 7 days thereafter. Mortality was recorded until all flies were dead. The flies' heart function was analysed at one, three and six weeks of aged. The percentage of flies surviving as they aged was presented as a Kaplan Meier plot.

Measurement of behaviour using Trikinetics Locomotor Activity Monitor (LAM).

For LAM analysis, groups of 20 female *Drosophila* were added to vials containing equal volumes of the different diets. Six vials were used per treatment. Vials were capped with cotton stoppers and placed in the LAM device housed in an incubator with a 12 hr:12 hr light-dark cycle at 25°C. The LAM device uses infrared beams which are crosses as active flies move about in the tube. Every time a fly crosses a beam, it counts as a single unit of activity. These counts are tallied over the subjective day and night periods. Beam crosses were counted continuously every 5 minutes and analysis of each population's 24 hr behavioural activity was conducted using the average of two consecutive days' data, these being the 3rd and 4th day after females were placed in the vials. Periods of five minutes where no beam crosses are recorded are regarded as sleep (Cirelli et al., 2005), accordingly the average total length of sleep periods was presented as means (+/-SEM).

Recording heart function.

Heart function was recorded as previously described (Ivy et al., 2015), in semi-intact abdominal preparations where flies are dissected in the presence of calcium-replete HBSS (with CaCl₂ at 2 mM). Hearts were recorded using a Zeiss Axiolab A1 widefield microscope

fitted with a water-dipping 10x objective. This was coupled to a Ximea xiD camera controlled by CamTool software (Lambda Photometrics Ltd, Hertfordshire, UK). Approximately 11 seconds of footage was recorded at a frame rate of 50 frames per second (fps), allowing analysis of at least 10s or 500 frames of footage. Footage was saved as a sequence of Tiff images and analysed using FIJI, which was used to generate m-modes (contraction kymograms). The m-modes were then used to measure the heart rate, the diastolic and systolic intervals (DI and SI, respectively) for each heart recording, the length of the cardiac cycle (calculated as DI+SI), as well as the fractional shortening (FS, the distance moved by the heart during a contraction, expressed as a percentage of the heart's width at diastole). All data were presented as the mean +/- the SEM.

Calcium imaging.

Flies were dissected for heart analysis and above and calcium signal images at the 1st pair of valves where the signal is relatively strong compared to other regions of the heart. Videomicroscopy sequences (500 frames corresponding to 10s of data collection) were opened in ImageJ and a region of interest was drawn around a single valve so that fluorescence data were collected throughout the cardiac cycle. The mean grey scale value was collected for each frame and entered into an Microsoft Excel spreadsheet. Baseline values (five frames at diastole with the lowest fluorescence) were averaged to form a single value (F_0). This value was then compared to subsequent values (F_1) to generate the F_1/F_0 ratio. Single representative traces from flies treated with different cadmium doses were then depicted as line graphs.

Statistical Analysis.

A Shapiro Wilk test was used to establish if data were parametric or non-parametric. For comparing two means, either a *t*-test (parametric data) or Mann Whitney U-test (non-parametric data) was performed. Where there were more than two means, either a 1-Way ANOVA (parametric) or a Kruskal-Wallis test (non-parametric) was conducted with post-hoc

206 analysis (Tukey's HSD test (after ANOVA) or Dunn's test (after Kruskal Wallis)). Graphs
207 present mean data for experimental groups +/- Standard Error of the Mean (SEM).

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Results.

Establishment of a tolerated dose of dietary cadmium.

For dietary experiments looking at the effect of cadmium on heart, the initial aim was to identify a dose that caused minimal mortality before three weeks of age. Flies on 1 mM and 10 mM CdCl₂ all died within 9 days (Figure 1A), whereas flies on diets containing 100 μM and 10 μM showed significantly greater tolerance with >90% survival at three weeks (Figure 1B). Flies on the 10 μM CdCl₂ diet reached 50% mortality at 57.3 ±1.2 days, which was similar to flies on the control diet (59.3 ±0.7 days; P=0.83, Figure 1B). In contrast, flies on the 100 μM CdCl₂ diet reached 50% survival at 35.3 ±5.6 days (P<0.01, Figure 1B).

By three weeks of age there was no significant mortality in any experimental group, including controls (Figure 1C). However, all groups showed reduced survivability by 6 weeks (Figure 1D), with the flies on the 100 μM CdCl₂ diet showing the greatest level (survival was 90.0 ±2.0% (control); 95.0 ±0.0% (10 μM CdCl₂) and 31.7 ±6.6% for the 100 μM CdCl₂ diet). These data ruled out the use of the two highest doses (1 mM and 10 mM) for use as chronic dietary cadmium exposure regimens; whereas 10 μM and 100 μM CdCl₂ were well tolerated, with 10 μM having no impact on lifespan and 100 μM CdCl₂ shortening lifespan but only after the 'early' period of their lifespan. Hence, dietary experiments focused on diets containing 10 μM or 100 μM CdCl₂.

Effect on locomotor behaviour.

The lower doses of dietary cadmium appeared to have little impact on mortality during the flies' first three weeks of life, however this does not mean the flies were 'healthy'. It was therefore reasoned that dietary cadmium may affect overall locomotor function. To assess this, the locomotor activity (measured by infrared beam crossing) of female flies was monitored every five minutes using an Locomotor Activity Monitor (LAM). Groups of one-week old flies had *ad libitum* access to the respective control and cadmium-containing diets

and data were collected for two consecutive 24hr periods, starting one day after flies had been placed into the monitoring tubes.

The beam crossing activity of flies on all diets exhibited a similar diurnal pattern of activity, with onset of activity coinciding with lights on, followed by a period of daytime quiescence and an increase in activity in anticipation of lights out. After lights off, the flies' activity significantly reduces until just before lights on (Figure 2A). However, flies on both cadmium diets displayed increased activity just after lights on relative to controls, as well as increased activity just before lights off (arrows, Figure 2A). Quantitatively, this amounted to an increase to total beam crossing activity of 24% (10 μ M cadmium diet) and 31% (100 μ M cadmium diet) compared to controls (Figure 2B; $P < 0.05$ for both cadmium diets compared to the control). This increase was limited to daytime activity, as there was no difference to beam crossing activity during the night (Figure 2C; no significant difference between experimental groups), nor was there any impact on total nocturnal sleep (Figure 2D; no significant difference between experimental groups). These data indicate that dietary cadmium caused modest, yet statistically significant, daytime *hyperactivity* but no impact on overall nocturnal activity and sleep.

Cadmium's direct effect on the insect heart.

Based on preceding research findings from numerous groups using rodent models, it was predicted that cadmium would directly and negatively affect *Drosophila* heart function. As well as exposing flies to cadmium via their diet, the heart's response to direct exposure of CdCl₂ (in dissected heart preparation) was also examined. At the highest dose used (10 μ M), hearts stopped beating within milliseconds (see an example m-mode in Figure 3A). Similarly, when 1 μ M CdCl₂ was applied, the heart stopped but sometimes restarted, suggesting the heart could recover from arrest (Figure 3B). Hearts exposed to the lowest dose (100 nM CdCl₂) continued to beat, albeit with periods of arrhythmia and a longer cardiac cycle (example in Figure 3C).

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270 In order to test whether heart function could resume after arrest, a series of 'wash-out and
271 recovery' experiments using 1 μM CdCl_2 were performed. As expected, a 10-minute direct
272 exposure to 1 μM CdCl_2 caused cardiac arrest ($P < 0.001$, Figure 3D). After two hours the
273 heart had started and exhibited a rate that was not statistically different to that of controls
274 (Figure 3D).

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276 **Calcium:**

277 To firmly establish a cell-autonomous role for cadmium's effect on the heart, the impact on
278 cardiomyocyte calcium signalling was recorded. To achieve this, flies expressing a calcium
279 reporter (*UAS-GCaMP6*) under the control of a heart-restricted driver (*TinC44-Gal4*) were
280 exposed directly to 1 μM cadmium chloride (Figure 4). The heart reacted as described above
281 by initially stopping rhythmic beating and then restarting. In the absence of cadmium the
282 heart had rhythmic calcium spikes corresponding to diastolic-systolic cycle. Upon the
283 addition of cadmium, the calcium signal remained high and failed to return to baseline.
284 Within a few milliseconds the signal showed weak spikes and then returned to baseline,
285 whereupon rhythmic calcium signals re-developed but at a reduced frequency (Figure 4A
286 and B). These data confirmed that cadmium could cell-autonomously influence
287 cardiomyocyte function by causing a reversible form of cardiac arrest.

288

289 **Dietary cadmium's effect on cardiac ageing.**

290 Given that the data had indicated cadmium could directly and negatively affect cardiac
291 function, it was reasoned that flies on the two cadmium diets (10 μM and 100 μM) would
292 develop severe cardiomyopathy that worsened with age and that this might help explain the
293 shortening of lifespan seen in the higher dose (100 μM).

294 Therefore, one week old, female flies were aged for six weeks on the respective diets and
295 heart function was assessed at one, three and six weeks (Figure 5). Representative m-
296 modes of flies show that by three weeks there was a lengthening (i.e. slowing) of the cardiac

cycle in flies on the control diet and lower cadmium dose. In contrast to those results and contrary to our hypothesis, flies on the higher cadmium dose retained a 'younger' heart function phenotype (Fig 5A). By week six, the ageing phenotype had continued to develop in flies on the control diet and lower cadmium dose, whereas there was no evidence of a similar age-dependent decline of cardiac function in flies on the higher cadmium dose (even though this dose was causing increased mortality by this time).

Heart function was quantified and this confirmed that heart rate decreased from 2.1 ± 0.1 bps (week 1) to 1.4 ± 0.1 beats per seconds (bps, week 3) and 1.0 ± 0.1 bps (week 6) for controls (Figure 5B; $P < 0.01$; for the effect of ageing). Flies on the lower dose cadmium ($10 \mu\text{M}$) diet showed a near identical age-dependent change to heart rate as controls. In contrast, flies on $100 \mu\text{M}$ cadmium had a heart rate at both three and six weeks which was significantly greater than that seen in controls and the lower dose cadmium diet ($P < 0.01$ compared to the other experimental groups).

Accordingly, the cardiac cycle of flies lengthened in flies on the control and $10 \mu\text{M}$ cadmium diets but this age-dependent change was not evident in flies on the higher ($100 \mu\text{M}$) cadmium dose at either three or six weeks (Figure 5C; $P < 0.01$ for the effect of ageing; $P < 0.01$ for flies on the $100 \mu\text{M}$ dose being different to the other groups at both three and six weeks). Notably, the length of the cardiac cycle for six-week-old flies on the higher cadmium dose was not different to one-week-old flies.

As flies age their heart rate declines due to lengthening of the cardiac cycle and this is widely reported to be a consequence of changes to both the diastolic and systolic intervals. In agreement with this, the systolic and diastolic intervals lengthened in both controls and flies on the lower cadmium dose (Figures 5D and 4E; $P < 0.01$ for the effect of time in both experimental groups). In contrast, flies on the higher cadmium dose had systolic and diastolic intervals that were significantly different to the other experimental groups ($P < 0.01$ at

both three and six weeks). Again, by six weeks there was no significant difference between the systolic and diastolic intervals recorded at one and six weeks for the flies on the higher cadmium dose.

Finally, the ageing heart shows a reduction in the distance that the heart walls move during contraction, known as fractional shortening (FS, which is expressed as a percentage; the lower the percentage, the shallower the contraction). In agreement with other findings, we found that FS declined by three weeks of age in controls (from 44.5% to 38.4%) and also flies on the lower cadmium dose (from 44.5% to 36.9%; figure 6, $P < 0.01$). In contrast, FS in flies on the higher cadmium dose showed barely any decline by three weeks (from 44.5% to 43.1%; not significantly different from week 1 and significantly different ($P < 0.05$) compared to the other experimental groups at three weeks). By six weeks of age there was further decline in FS within flies on the control and lower dose cadmium diets (to 28.5% and 29.9%, respectively), whereas in flies surviving on the higher cadmium dose, FS had declined to only 34.6%, a figure comparable to FS in the other groups at 3-weeks (Figure 6, $P < 0.001$, for 100 μM cadmium group compared to the other experimental groups).

These data indicate that the expected age-dependent changes to heart function occurred for the controls, as well as flies on the lower (10 μM) dose cadmium. In contrast, flies on the higher (100 μM) cadmium dose showed significantly reduced signs of cardiac ageing, which was evident even at three weeks of age (please refer to supplementary videos for representative examples of heart function in flies at 1 week of age and after 6 weeks on the respective diets).

Discussion.

This work aimed to examine the long-term effects of low dose cadmium on cardiac ageing using a *Drosophila* model of heart physiology. It was demonstrated that *Drosophila* could tolerate low-dose cadmium in their diet with no impact on lifespan at the lowest dose (10 μ M) and a significant effect on lifespan after three weeks of life (100 μ M). It was also shown that direct exposure of the adult heart to cadmium caused arrest and that this was reversed after wash-out of the cadmium. Chronic dietary cadmium exposure caused mild hyperactivity and either had no effect on cardiac ageing (10 μ M CdCl₂), or slowed cardiac ageing (100 μ M), despite the latter dose shortening lifespan.

Diurnal hyperactivity.

Although millimolar levels of cadmium in the diet caused rapid mortality, doses in the micromolar range (10 μ M and 100 μ M) were well tolerated for several weeks. Both of the lower doses caused significant hyperactivity that was restricted to the daytime period of the light-dark cycle. This result contrasts that from another study where flies fed a diet containing 500 μ M CdCl₂, showed reduced locomotor activity (Hu et al., 2023). The dose used in that study was 5 to 10-fold greater than the ones we used in our study, prompting the possibility that cadmium exerts qualitatively distinct behavioural responses according to its dose. Whether the effect on behaviour of low dose cadmium exposure translates to other species is unclear because behavioural studies are relatively uncommon and involve a wide range of animals (and therefore different measures of behaviour), at different life-cycle stages. For example, sub-lethal cadmium reduced swimming activity of the freshwater shrimp *Gammarus aequicauda* (Morillo-Velarde et al., 2011); whereas green shore crabs *Carcinus maenas* given 63 μ M in their water showed no overall change to locomotion activity, or shelter-seeking behaviour in open-field tests (Blewett et al., 2017). A recent study highlighted that sub-lethal doses of cadmium disrupt cardiac development and the behaviour of adult zebrafish (Ma et al., 2024). It is also worth noting that prenatal cadmium exposure is associated with Attention Deficit Hyperactivity Disorder (ADHD) in six year old girls (Kim et

al., 2020). These data, and our own data, suggest more research is required to understand the different outcomes that developmental and adult exposure to chronic, low-dose cadmium has on different species. Given the persistence of cadmium in the environment, studies on its behavioural effects using *Drosophila* as a model, may yield important insights of relevance to other species.

Effect on the Heart.

The data confirmed that cadmium, when directly added to cardiomyocytes, could arrest the heart. This result was partly expected given that cadmium is used experimentally to block L-type calcium channels critical for calcium entry into cardiomyocytes (refer to review by DeCoursey, (DeCoursey and Cherny, 2007)). **In these acute, direct exposure experiments it is probable that cadmium disrupted calcium influx but also efflux across the plasma membrane, as well as its return to intracellular calcium stores; the culmination being a stochastic, yet negative impact on heart function.** In addition, the wash-out experiment indicated that cardiac arrest after direct exposure to cadmium is a reversible state in *Drosophila*. However, free cadmium is not likely to directly affect cells when ingested because it forms a complex with circulating proteins (see below), so studying its toxicology in a whole animal model is important and the genetic tractability of *Drosophila* heart model offers advantages over rodent models.

The findings that showed cardiac ageing on the lower (10 μ M) cadmium diet were unexpected given that it neither affected lifespan nor the rate that the heart's function declined over a six-week period, while the same dose increased daytime activity. It was also unexpected to find that the higher dose (100 μ M) of cadmium slowed cardiac ageing *in vivo*. This result contrasts the association of cadmium with cardiovascular disease in humans and cadmium-induced cardiac dysfunctions in mice (Fitch et al., 2023), as well as findings indicating that direct exposure to cadmium disrupts cardiomyocyte contractions (this study and also (Zabihihesari et al., 2022)). One possible explanation for our result in the ageing

studies is the induction of metallothioneins (MTs), an evolutionarily conserved family of cysteine-rich metal-interacting proteins with anti-oxidative effects that mitigate cadmium toxicity in a range of model systems (reviewed in (Nordberg and Nordberg, 2022)). In mammalian models, whilst only a fraction of ingested cadmium is transported into circulation (by mechanisms that are not well understood), the bulk of it initially binds high molecular weight proteins (such as albumin) and then MTs. The Cd-MT complexes predominantly accumulate in the kidney proximal tubule cells (and causes renal toxicity after liberation in the lysosomes) but are also distributed to other tissues. The expression of MTs is up-regulated in response to the activity of Metal-responsive Transcription Factor 1 (MTF-1), a transcription factor whose expression is sensitive to the presence of zinc and cadmium (Giedroc et al., 2001). In terms of cardiac ageing in rodent models the experimental over-expression of MTs in murine cardiomyocytes can protect the heart from ageing by mitigating oxidative stress (Cong et al., 2016). Data on the cardiac effects of cadmium in *Drosophila* are limited but one group recently infused high doses of cadmium into *Drosophila* larvae and reported that the over-expression of *MTF-1* prevented cadmium-induced arrhythmias and changes to heart rate (Zabihhesari et al., 2022). Interestingly, in that study, the heart rate increased, a result that contrasts with our findings where direct exposure to cadmium stopped the adult heart. Quite why there should be such a difference is not clear but it may reflect the difference between the presence of cadmium-interacting molecules in the haemolymph of larvae that are washed away using the semi-intact methodology to study adults in our own study. How systemic cadmium affects the endogenous biology of cardiomyocytes in mice and *Drosophila* models is unclear but clearly the results can be different and dependent on differing mechanisms and developmental timepoints. Consequently, more work is required to establish the relationship between cadmium exposure and cardiomyocyte function at a range of life-history stages and this would benefit from directly comparing responses in a range of species.

Conclusion

In summary, the *Drosophila* heart can tolerate long term cadmium exposure at doses that increase daytime behaviour and shorten lifespan. These results appear at odds with those from rodent models, as well as the wider epidemiological data linking cadmium exposure to cardiovascular disease in humans. These differences need to be understood as they highlight mechanisms that robustly protect the insect heart from cadmium toxicity.

References.

- Authority (EFSA), E.F.S., 2009. Cadmium in food - Scientific opinion of the Panel on Contaminants in the Food Chain. EFSA Journal 7, 980.
<https://doi.org/10.2903/j.efsa.2009.980>
- Birse, R.T., Choi, J., Reardon, K., Rodriguez, J., Graham, S., Diop, S., Ocorr, K., Bodmer, R., Oldham, S., 2010. High-fat-diet-induced obesity and heart dysfunction are regulated by the TOR pathway in *Drosophila*. Cell Metab 12, 533–544.
<https://doi.org/10.1016/j.cmet.2010.09.014>
- Blewett, T.A., Newton, D., Flynn, S.L., Alessi, D.S., Goss, G.G., Hamilton, T.J., 2017. Cadmium bioaccumulates after acute exposure but has no effect on locomotion or shelter-seeking behaviour in the invasive green shore crab (*Carcinus maenas*). Conserv Physiol 5, cox057. <https://doi.org/10.1093/conphys/cox057>
- Blice-Baum, A.C., Guida, M.C., Hartley, P.S., Adams, P.D., Bodmer, R., Cammarato, A., 2019. As time flies by: Investigating cardiac aging in the short-lived *Drosophila* model. Biochim Biophys Acta Mol Basis Dis 1865, 1831–1844.
<https://doi.org/10.1016/j.bbadis.2018.11.010>
- Cadmium, Blood - Laboratory Test Catalog [WWW Document], n.d. URL <https://spectrumhealth.testcatalog.org/show/LAB736> (accessed 5.2.23).
- Chen, C., Zhang, S., Liu, Z., Tian, Y., Sun, Q., 2015. Cadmium toxicity induces ER stress and apoptosis via impairing energy homeostasis in cardiomyocytes. Biosci Rep 35, e00214. <https://doi.org/10.1042/BSR20140170>
- Cirelli, C., Bushey, D., Hill, S., Huber, R., Kreber, R., Ganetzky, B., Tononi, G., 2005. Reduced sleep in *Drosophila* Shaker mutants. Nature 434, 1087–1092.
<https://doi.org/10.1038/nature03486>
- Compendium of Chemical Hazards: Cadmium, 2016.
- Cong, W., Niu, C., Lv, L., Ni, M., Ruan, D., Chi, L., Wang, Yang, Yu, Q., Zhan, K., Xuan, Y., Wang, Yuehui, Tan, Y., Wei, T., Cai, L., Jin, L., 2016. Metallothionein Prevents Age-Associated Cardiomyopathy via Inhibiting NF-κB Pathway Activation and Associated Nitritative Damage to 2-OGD. Antioxid Redox Signal 25, 936–952.
<https://doi.org/10.1089/ars.2016.6648>
- DeCoursey, T.E., Cherny, V.V., 2007. Pharmacology of voltage-gated proton channels. Curr Pharm Des 13, 2400–2420. <https://doi.org/10.2174/138161207781368675>
- Feng, W., He, X., Chen, M., Deng, S., Qiu, G., Li, X., Liu, C., Li, J., Deng, Q., Huang, S., Wang, T., Dai, X., Yang, B., Yuan, J., He, M., Zhang, X., Chen, W., Kan, H., Wu, T., 2015. Urinary Metals and Heart Rate Variability: A Cross-Sectional Study of Urban Adults in Wuhan, China. Environ Health Perspect 123, 217–222.
<https://doi.org/10.1289/ehp.1307563>

- Ferraro, P.M., Costanzi, S., Naticchia, A., Sturniolo, A., Gambaro, G., 2010. Low level exposure to cadmium increases the risk of chronic kidney disease: analysis of the NHANES 1999-2006. *BMC Public Health* 10, 304. <https://doi.org/10.1186/1471-2458-10-304>
- Fitch, M.L., Kabir, R., Ebenebe, O.V., Taube, N., Garbus, H., Sinha, P., Wang, N., Mishra, S., Lin, B.L., Muller, G.K., Kohr, M.J., 2023. Cadmium exposure induces a sex-dependent decline in left ventricular cardiac function. *Life Sci* 324, 121712. <https://doi.org/10.1016/j.lfs.2023.121712>
- Giedroc, D.P., Chen, X., Apuy, J.L., 2001. Metal response element (MRE)-binding transcription factor-1 (MTF-1): structure, function, and regulation. *Antioxid Redox Signal* 3, 577–596. <https://doi.org/10.1089/15230860152542943>
- Hu, Y., Wu, H., Lu, C., Xu, H., Li, B., Guan, W., Wu, M., Gao, Y., Tong, H., 2023. Cadmium chloride exposure impairs the growth and behavior of *Drosophila* via ferroptosis. *Science of The Total Environment* 865, 161183. <https://doi.org/10.1016/j.scitotenv.2022.161183>
- Ivy, J.R., Drechsler, M., Catterson, J.H., Bodmer, R., Ocorr, K., Paululat, A., Hartley, P.S., 2015. Klf15 Is Critical for the Development and Differentiation of *Drosophila* Nephrocytes. *PLoS One* 10, e0134620. <https://doi.org/10.1371/journal.pone.0134620>
- Kim, W., Jang, Y., Lim, Y.-H., Kim, B.-N., Shin, C.H., Lee, Y.A., Kim, J.I., Hong, Y.-C., 2020. The Effect of Prenatal Cadmium Exposure on Attention-deficit/Hyperactivity Disorder in 6-Year-old Children in Korea. *J Prev Med Public Health* 53, 29–36. <https://doi.org/10.3961/jpmph.19.175>
- Lamas, G.A., Bhatnagar, A., Jones, M.R., Mann, K.K., Nasir, K., Tellez-Plaza, M., Ujueta, F., Navas-Acien, A., American Heart Association Council on Epidemiology and Prevention; Council on Cardiovascular and Stroke Nursing; Council on Lifestyle and Cardiometabolic Health; Council on Peripheral Vascular Disease; and Council on the Kidney in Cardiovascular Disease, 2023. Contaminant Metals as Cardiovascular Risk Factors: A Scientific Statement From the American Heart Association. *J Am Heart Assoc* 12, e029852. <https://doi.org/10.1161/JAHA.123.029852>
- Limaye, D.A., Shaikh, Z.A., 1999. Cytotoxicity of cadmium and characteristics of its transport in cardiomyocytes. *Toxicol Appl Pharmacol* 154, 59–66. <https://doi.org/10.1006/taap.1998.8575>
- Ma, L., Yang, H., Xiao, X., Chen, Q., Lv, W., Xu, T., Jin, Y., Wang, W., Xiao, Y., 2024. Co-exposure to sodium hypochlorite and cadmium induced locomotor behavior disorder by influencing neurotransmitter secretion and cardiac function in larval zebrafish. *Environ Pollut* 342, 123070. <https://doi.org/10.1016/j.envpol.2023.123070>
- Madan, A., Viswanathan, M.C., Woulfe, K.C., Schmidt, W., Sidor, A., Liu, T., Nguyen, T.H., Trinh, B., Wilson, C., Madathil, S., Vogler, G., O'Rourke, B., Biesiadecki, B.J., Tobacman, L.S., Cammarato, A., 2020. TNNT2 mutations in the tropomyosin binding region of TNT1 disrupt its role in contractile inhibition and stimulate cardiac dysfunction. *Proc Natl Acad Sci U S A* 117, 18822–18831. <https://doi.org/10.1073/pnas.2001692117>
- Meyer, C., Breitsprecher, L., Bataille, L., Vincent, A.J.M., Drechsler, M., Meyer, H., Paululat, A., 2022. Formation and function of a highly specialised type of organelle in cardiac valve cells. *Development* 149, dev200701. <https://doi.org/10.1242/dev.200701>
- Morillo-Velarde, P.S., Lloret, J., Marín, A., Sánchez-Vázquez, F.J., 2011. Effects of cadmium on locomotor activity rhythms of the amphipod *Gammarus aequicauda*. *Arch Environ Contam Toxicol* 60, 444–451. <https://doi.org/10.1007/s00244-010-9563-9>
- Nanda, K.P., Firdaus, H., 2022. Dietary cadmium induced declined locomotory and reproductive fitness with altered homeostasis of essential elements in *Drosophila melanogaster*. *Comp Biochem Physiol C Toxicol Pharmacol* 255, 109289. <https://doi.org/10.1016/j.cbpc.2022.109289>
- Nordberg, M., Nordberg, G.F., 2022. Metallothionein and Cadmium Toxicology—Historical Review and Commentary. *Biomolecules* 12, 360. <https://doi.org/10.3390/biom12030360>

- Ocorr, K., Reeves, N.L., Wessells, R.J., Fink, M., Chen, H.-S.V., Akasaka, T., Yasuda, S., Metzger, J.M., Giles, W., Posakony, J.W., Bodmer, R., 2007. KCNQ potassium channel mutations cause cardiac arrhythmias in *Drosophila* that mimic the effects of aging. *Proc Natl Acad Sci U S A* 104, 3943–3948. <https://doi.org/10.1073/pnas.0609278104>
- Renu, K., Mukherjee, A.G., Wanjari, U.R., Vinayagam, S., Veeraraghavan, V.P., Vellingiri, B., George, A., Lagoa, R., Sattu, K., Dey, A., Gopalakrishnan, A.V., 2022. Misuse of Cardiac Lipid upon Exposure to Toxic Trace Elements-A Focused Review. *Molecules* 27, 5657. <https://doi.org/10.3390/molecules27175657>
- Sears, C.G., Eliot, M., Raaschou-Nielsen, O., Poulsen, A.H., Harrington, J.M., Howe, C.J., James, K.A., Roswall, N., Overvad, K., Tjønneland, A., Meliker, J., Wellenius, G.A., 2022. Urinary Cadmium and Incident Heart Failure: A Case-Cohort Analysis Among Never-Smokers in Denmark. *Epidemiology* 33, 185–192. <https://doi.org/10.1097/EDE.0000000000001446>
- Tellez-Plaza, M., Jones, M.R., Dominguez-Lucas, A., Guallar, E., Navas-Acien, A., 2013. Cadmium Exposure and Clinical Cardiovascular Disease: a Systematic Review. *Curr Atheroscler Rep* 15, 10.1007/s11883-013-0356–2. <https://doi.org/10.1007/s11883-013-0356-2>
- Thapa, S., Delhey, L., Jin, J., Abouelenein, S., Morad, W., Delongchamp, R., Faramawi, M.F., 2018. Association Between Urinary Cadmium and QRS/T Angle Among Adults in the United States. *J Occup Environ Med* 60, e412–e415. <https://doi.org/10.1097/JOM.0000000000001382>
- Türkcan, A., Scharinger, B., Grabmann, G., Keppler, B.K., Laufer, G., Bernhard, D., Messner, B., 2015. Combination of cadmium and high cholesterol levels as a risk factor for heart fibrosis. *Toxicol Sci* 145, 360–371. <https://doi.org/10.1093/toxsci/kfv057>
- Vaughan, L., Marley, R., Miellet, S., Hartley, P.S., 2018. The impact of SPARC on age-related cardiac dysfunction and fibrosis in *Drosophila*. *Exp. Gerontol.* 109, 59–66. <https://doi.org/10.1016/j.exger.2017.10.011>
- Vogler, G., Liu, J., Iafe, T.W., Migh, E., Mihály, J., Bodmer, R., 2014. Cdc42 and formin activity control non-muscle myosin dynamics during *Drosophila* heart morphogenesis. *J Cell Biol* 206, 909–922. <https://doi.org/10.1083/jcb.201405075>
- WHO, 2019. Exposure To Cadmium: A Major Public Health Concern.
- Zabihhesari, A., Parand, S., Coulthard, A.B., Molnar, A., Hilliker, A.J., Rezai, P., 2022. An in-vivo microfluidic assay reveals cardiac toxicity of heavy metals and the protective effect of metal responsive transcription factor (MTF-1) in *Drosophila* model. *3 Biotech* 12, 279. <https://doi.org/10.1007/s13205-022-03336-7>
- Zechini, L., Camilleri-Brennan, J., Walsh, J., Beaven, R., Moran, O., Hartley, P.S., Diaz, M., Denholm, B., 2022. Piezo buffers mechanical stress via modulation of intracellular Ca²⁺ handling in the *Drosophila* heart. <https://doi.org/10.1101/2022.05.06.490878>

FIGURES.

Figure 1. Effect of low Cadmium on lifespan.

Vials of twenty female flies were maintained on standard Bloomington diet either with or without Cadmium at four different doses (10 μ M, 100 μ M, 1 mM and 10 mM). Flies were transferred to fresh food every 5-7 days and mortality monitored for the duration of their lifespan. (A) The Kaplan-Meier plot shows percentage survival of three to six vials $\pm$ SEM. Flies provided with 100 μ M, 1 mM and 10 mM cadmium had a shorter lifespan than flies in the other experimental groups, $**P < 0.01$ using Kruskal-Wallis. (B) The time at which 50% survival was reached is expressed as the mean ($\pm$ SEM) of three to six vials for each experimental group. $**P < 0.01$ by one-way ANOVA and Tukey's HSD. (C and D) percentage survival at three and six weeks respectively for flies on the lower cadmium doses 10 μ M and 100 μ M; $**P < 0.01$, *ns* = not statistically different from control.

Figure 2. Low dose cadmium causes mild daytime hyperactivity in female *Drosophila* but has no impact on sleep. Flies were housed in 12 hr:12 hr light:dark cycles at 25°C with ad libitum access to the respective diets throughout the recording period. Data from two days recording is averaged and presented. (A) Beam crossing activity across the 24hr light-dark cycle (ZT0 = lights on, ZT12 = lights off; black is control, magenta is 10 μ M and orange is 100 μ M). Arrows indicate periods where flies on either of the cadmium diets were more active than flies on the control diet. (B) Average total beam crossings per day. Cadmium caused a statistically significant, yet small increase to daytime locomotor activity. (C) Average total beam crossings per night and (D) the total nocturnal sleep were both unaffected by cadmium ($*P < 0.05$ by 1-way ANOVA; *ns* = not significant; *n* = 20 flies per LAM vial, six vials per treatment).

Figure 3. The heart's response to direct Cadmium exposure.

Hearts in semi-intact preparations were incubated in **Hanks Basic Saline Buffer (HBSS)** and then provided with a HBSS containing different concentrations of CdCl₂ (white triangle). Movement modes (M-mode) kymogram of *Drosophila* heart contractions were then obtained from video-microscopy footage. (A) The heart is beating regularly prior to the addition of 10 µM CdCl₂ (triangle) and then abruptly stops. (B) The heart stops and then starts again when 1 µM CdCl₂ was added (triangle). (C) The heart contractions become highly erratic after 5 minutes in 100 nM CdCl₂, with periods of arrhythmia as well as periods of contractions displaying signs of a long cardiac cycle. (D) **Quantified heart rate data showing that Cadmium stops the heart (treatment) but it can recover after replacing the buffer with cadmium-free HBSS (recovery).** **P<0.001 for the effect of the cadmium treatment; *ns* = not significantly different at the recovery time; n = 9-10 flies per treatment group. A Kruskal-Wallis test followed by a Dunn's post hoc test were used to compare groups.

Figure 4. Cadmium's effect on cardiomyocyte calcium signal.

Response of cardiomyocytes expressing the calcium reporter GCaMP6 to direct administration of CdCl₂ (1 µM). (A) The trace shows the fluorescence signal of the GCaMP6 reporter before and immediately after administration of cadmium. The immediate effect is a brief period of shallow yet bright calcium waves followed by lengthening of the cardiac cycle due to long periods of diastole where the calcium signal is lowest. (B) Shows a kymogram of the corresponding fluorescence signal from the heart that the quantified data were derived from.

Figure 5. The impact that dietary Cadmium had on heart function in ageing *Drosophila*.

Female flies were maintained for up to 7 weeks on a standard Bloomington diet supplemented with Cadmium Chloride (10 µM or 100 µM). (A) M-modes show representations of an individual fly's heart contractions at weeks 1, 3 and 6 on the respective

diets. (B-E) Heart function was analysed in flies on the control (blue line), 10 μM CdCl_2 diet (orange line) or 100 μM CdCl_2 diet (grey line). (B) Mean beats per second (bps) declined over time in all groups ($P < 0.01$), however the 100 μM diet slowed the decline in heart dysfunction. (C) The cardiac cycle declined in controls and flies on the 10 μM diet but not in flies on the 100 μM diet. (D) Similarly, flies on the 100 μM diet were protected from age-dependent lengthening of the systolic interval and (E) diastolic interval. (** $P < 0.01$; 2-Way ANOVA); $n = 17-32$ flies per data point.

Figure 6. Effect of dietary cadmium on heart contraction (fractional shortening).

Female flies were maintained for up to 7 weeks on a standard Bloomington diet supplemented with Cadmium Chloride (10 μM or 100 μM). Fractional shortening was calculated from videomicroscopy footage of the beating heart (calculated as the distance the heart wall moves during a contraction and presented as a percentage of the heart's diameter at diastole); flies on the 100 μM Cadmium diet aged differently compared to the controls; * $P < 0.05$; ** $P < 0.01$. Aging had a highly statistically significant effect on fractional shortening, *** $P < 0.001$ by 2-Way ANOVA); $n = 15-28$ flies per data point.

FIGURE 1:

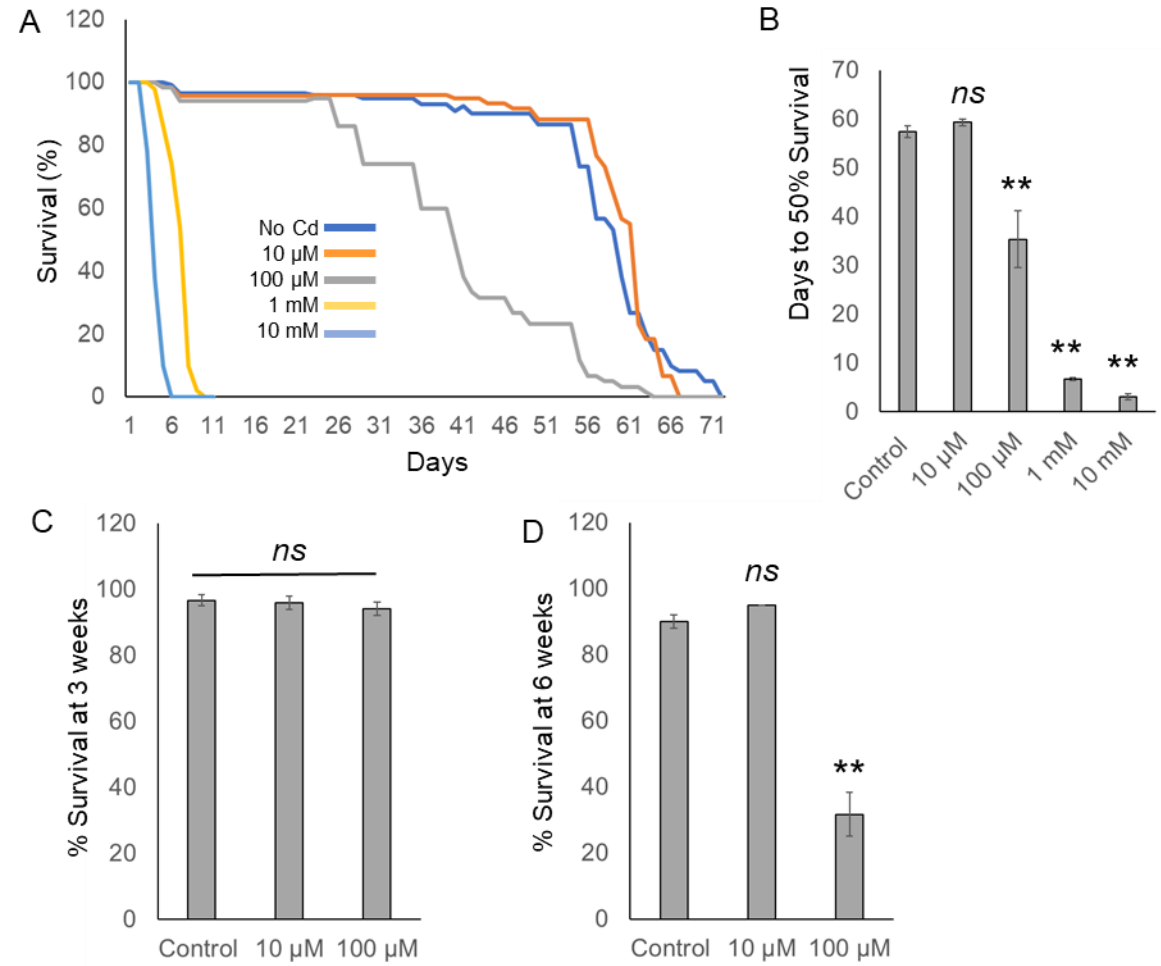
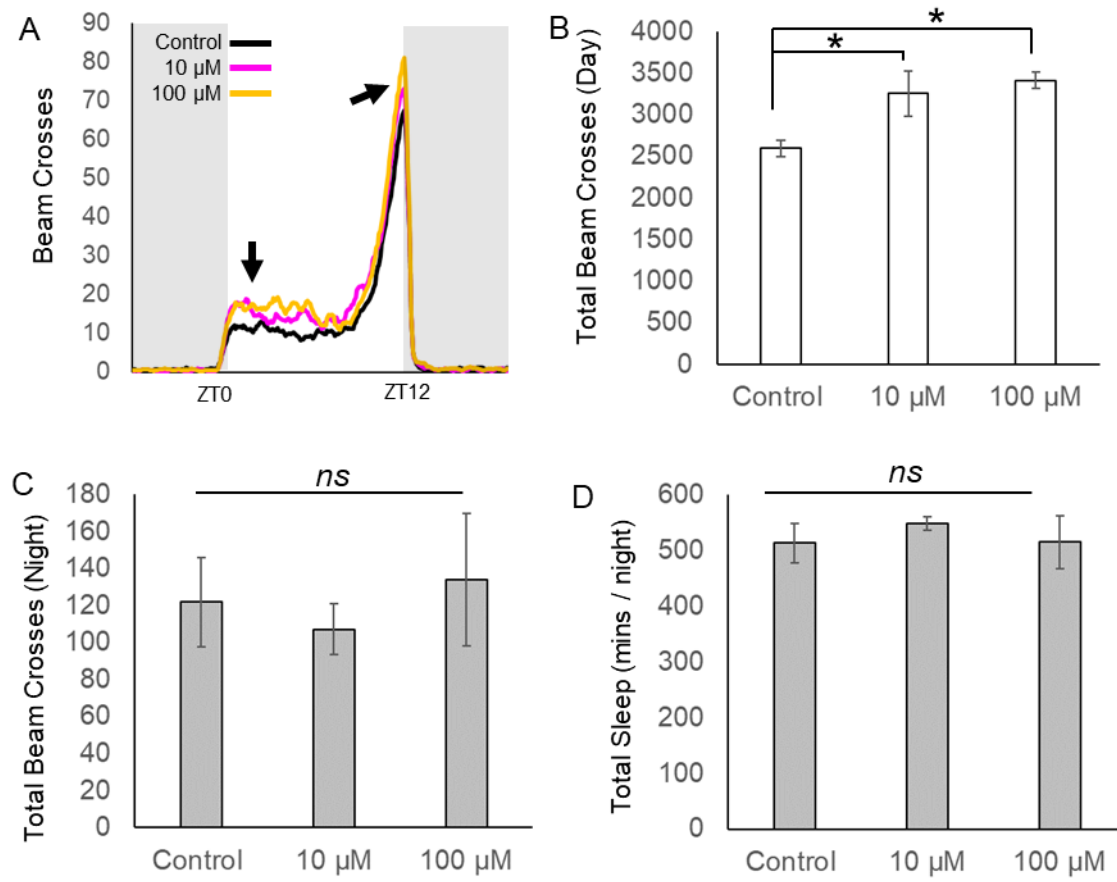
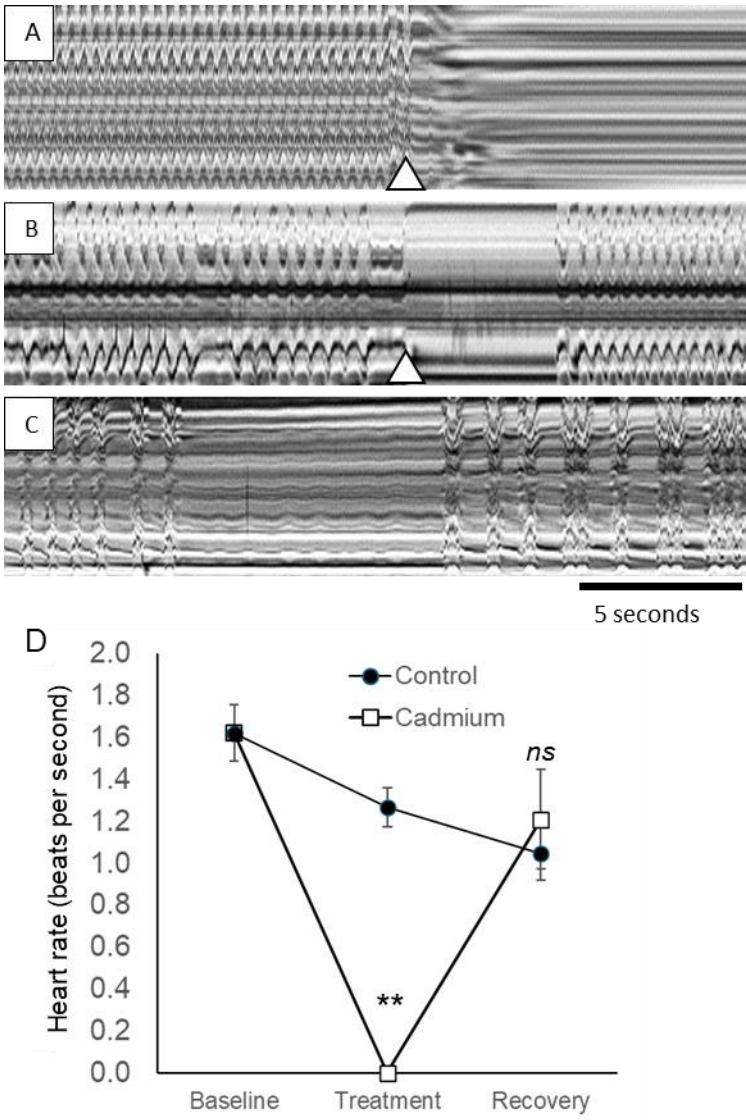


FIGURE 2:



660 FIGURE 3.

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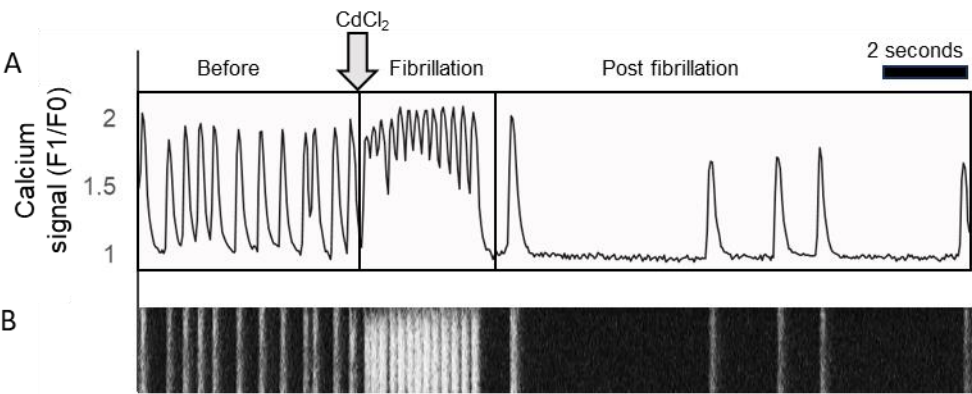


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664 Figure 4.

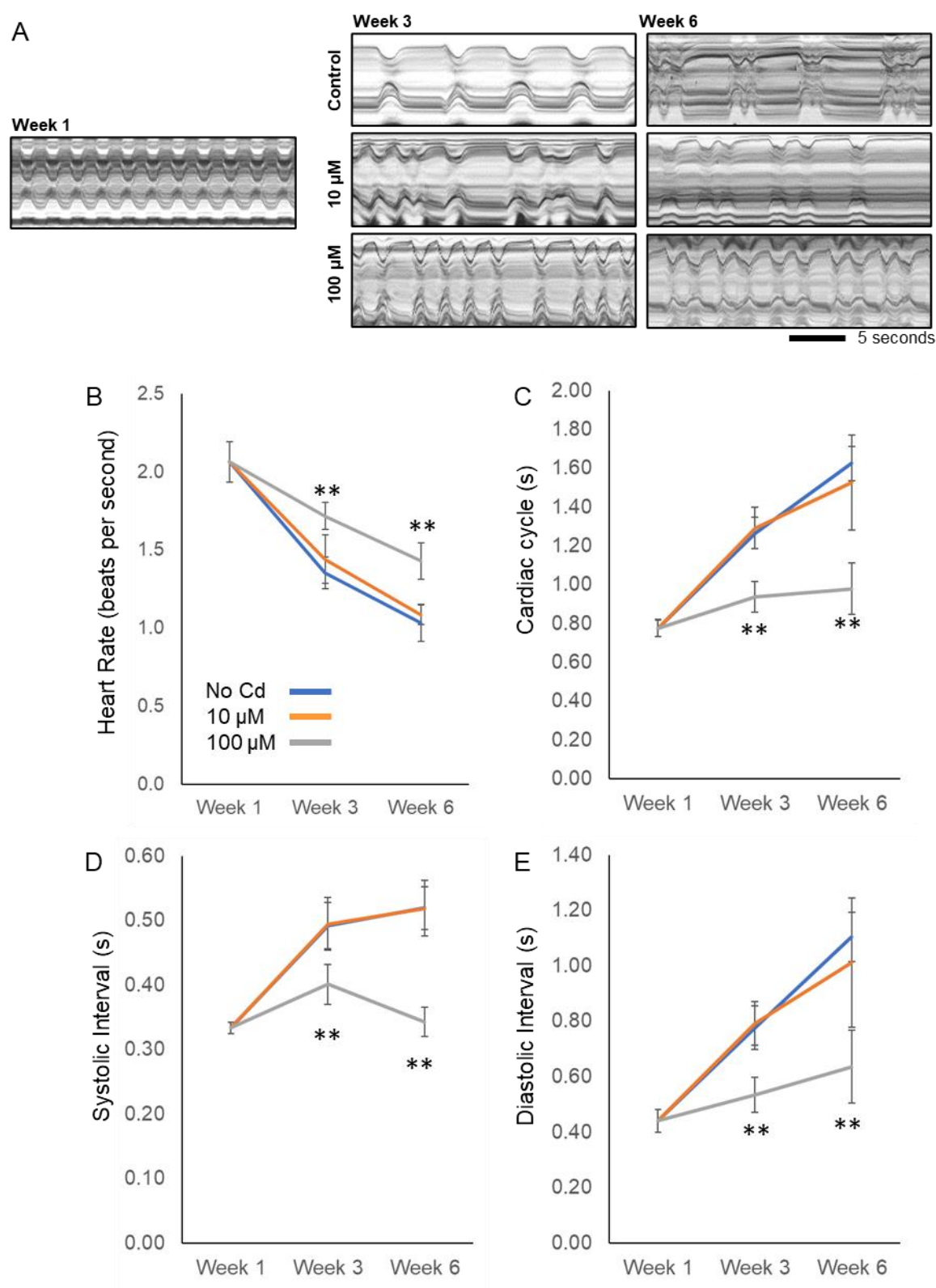
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667 Figure 5.

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FIGURE 6.

