THE CANADA SCIENCE FAIR JOURNAL

ARTICLE

Biology

Non-Genetic Inheritance of Developmental and Immune Disorder from Heavy Metal Exposure and Low-Temperature Stress Using Fruit Flies

Written By: Kristin Cho
Edited by: Langola Andrianarison
Layout By: Ahmed Nadeem

Awards: Toronto Science Fair Gold, Sanofi Biogenius Award, Canada Wide Science Fair Bronze, Pacific Asia Science Olympiad Silver

Heavy metal exposure is known to cause adverse health effects because of its ability to damage membrane and DNA, perturb protein function, and disturb enzyme activity (Witkowska et al., 2021). By interfering with immune homeostasis, heavy metal exposure results in a disease state, in which the body fails to make appropriate immune activation and suppression in tissues and organs (Duarte et al., 2018). Heavy metals as environmental pollutants can become even more toxic by mixing with different environmental elements, such as water, soil, and air (Mitra et al., 2022). Due to their characteristics of toxicity, longevity in the atmosphere, and ability to accumulate in the human body via bioaccumulation, humans and other living organisms can be exposed to heavy metals through ingestion or inhalation of contaminated food, drinking water, and polluted air or through occupational exposure in the workplace (Mitra et al., 2022).

Heavy metals have been identified as neurodevelopmental toxins since they can be responsible for fetal damage which leads to neurological defects, developmental delays, learning disabilities, and behavioural abnormalities (Gorini et al., 2014). For instance, in *Drosophila melanogaster*, heavy metal exposure causes irreversible neurobehavioural damage, reducing lifespan and locomotor activity(Bonilla-Ramirez et al., 2011). Accumulation of heavy metal in the fruit fly's head is associated with the neurodegeneration of several dopaminergic neuronal clusters, while also destroying DAergic neurons in the fly's brain, thereby impairing their movement capabilities (Bonilla-Ramirez et al., 2011). Several studies have demonstrated that elevated levels of heavy metal exposure can affect the behaviour of organisms. However, one study highlighted a concerning finding; it showed that songbirds with heavy metal exposure displayed an increase in aggressive behaviour (Janssens et al., 2003).

Exposure to temperature fluctuations is a common source of naturally occurring stress for organisms. For many insect species, exposure to low temperatures induces a reversible comatose state known as chill coma (Findsen et al., 2014). This condition is caused by the loss of neuromuscular function and leads to progressive loss of ionic and metabolic homeostasis, as well as hemolymph ions (Williams et al., 2018; Davis et al., 2021). One area of focus in this field is rapid cold hardening (RCH), which is a protective response against cold shock. The RCH response protects against cold shock, a form of non-freezing injury, which represents a major obstacle to the successful cryopreservation of many types of cells and tissues (Lee Jr et al., 2010). Research shows that RCH improved both survival and reproductive output after a subsequent cold shock, but the RCH treatment alone was associated with costs in terms of reduced survival and reproductive output (Overgaard et al., 2007). In addition, cold activation of potential immunity in Drosophila may be a compensatory mechanism to maintain stable immune function during or after a low-temperature cold shock (Salehipour-shirazi et al., 2016). A study identified the most pronounced changes following the RCH treatment to be elevated levels of glucose and trehalose (Overgaard et al., 2007). While some studies did not find changes in gene expression levels, a gene expression analysis study revealed that stages of cold response elicited gene expression changes involved in heat shock response, circadian rhythm, and metabolism (Vesala et al., 2012).

The purpose of this study is to investigate the effects of environmental stress on adult flies, specifically heavy metal exposure and cold temperature, and how this relates to developmental and immunological problems in their offspring. This study explores whether the first generation's response to environmental stressors closely resembles that of the second generation. Moreover, whether the adverse effects are passed on to the second generation without direct exposure to the stress. Lastly, the study aims to determine how exposure to multiple stressors affects the organisms and whether these effects are aggravated or cancelled out. If heavy metal exposure indeed decreases development and immunity in fruit flies, then it follows that the second generation would also likely be affected by the diminished immunological functions inherited from their parents. Similarly, if cold shock does enhance survival and reproduction in adult flies, it is reasonable to hypothesize that adult flies exposed to both heavy metal and cold shock would exhibit improved immunological functions compared to those exposed solely to heavy metal.

MATERIALS AND METHODS

1) Extraction of Heavy Metal

5g of coiled copper, lead, brass, and aluminum were each added into a 50mL conical tube, which was filled up to 25mL with distilled water. The heavy metals were then extracted into the water of each test tube by using a rocker for 48 hours as shown in Figure 1. The water metal level was measured by dipping one strip of the water metal kit (SenSafe Water Metals Check Test, USA) into the heavy metal extracts for 30 seconds and moving it back and forth. Then excess water was removed on a paper towel for 2 minutes before comparing it to the table indicating the level of heavy metal contents. The heavy metal solutions were sterilized with a syringe (KOVAX-syringe, Korea) and 0.20 µm (GVS Single Use Filter, USA). Then, each heavy metal extract was mixed at a ratio as shown in Table 1 to create a heavy metal mixture at 100 ppb for the heavy metal level.

2) Extraction of heavy metal from daily items and labware

A 2.19g floating foam tube rack was put into a 50mL conical tube, which was filled up with distilled water. A tumbler mug with a logo and painting on the outside was submerged in water in a plastic container. The test tube and plastic container were placed on a rocking shaker for 48 hours. The water metal level of the extract was measured by using one strip of the water metal kit. The paint extract was sterilized with a syringe (KOVAX-syringe, Korea) and 0.20 µm (GVS Single Use Filter, USA).

Extract	Volume (mL)
copper extract	0.5
lead extract	1.0
brass extract	0.5
aluminium extract	1.0
distilled water	1.0

Table 1. Heavy metal mixture ratio used for fruit fly exposure condition.

3) Fruit fly culture media preparation

A 250mL beaker was heated on a hot plate and 50mL distilled water, 8.4g of corn meal, 3.75g of sugar, and 2.4g of dry yeast were added. Then 40mL distilled water, 0.75g of agar, 1mL of molase, and 0.57mL of propionic acid was added. 10mL distilled water was added and while creating the mixture, the mixture was constantly stirred with a spatula to prevent the sinking of precipitate / solid. When the mixture was fully mixed, the 5mL of mixture was poured into 20 plastic tubes for fruit fly culture and covered with cotton.

4) Preparation and anesthesia of fruit fly

Fruit flies, *Drosophila melanogaster*, were purchased from Biozoa Biological Supply Company, Korea, in which there were 10 wild-type fruit flies in each tube,

Figure 1. Heavy metal mixture and pain extract extraction process

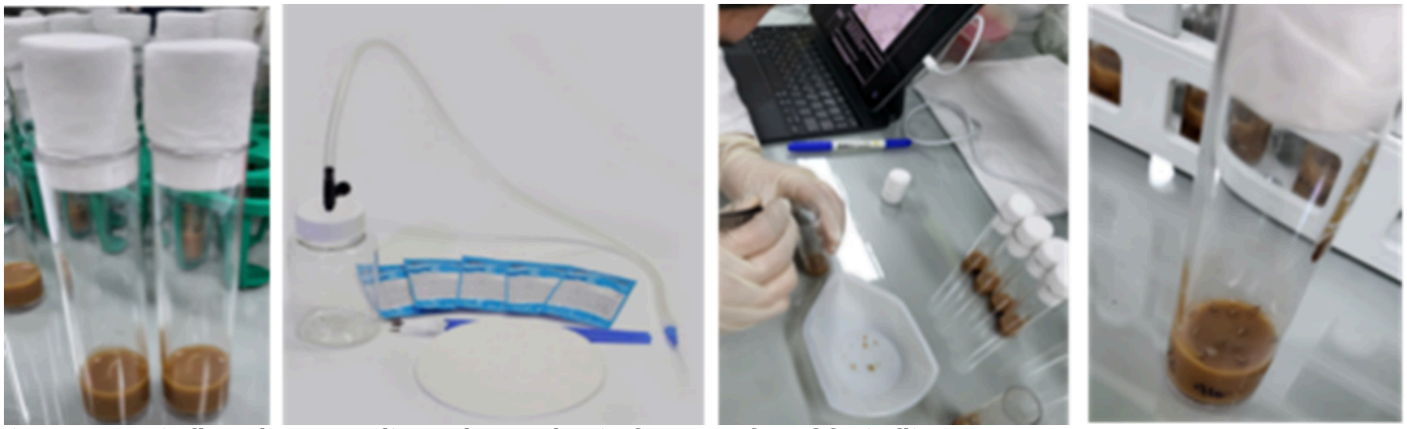

Figure 2. Fruit fly culture media and anesthesia for transfer of fruit flies

male and female mixed. Adult fruit flies were anesthetized, as shown in Figure 2, to help with the process of transfer and collection of fruit flies. The given anesthesia kit (Biozoa Biological Supply Company, Korea) consists of an anesthesia bottle, CO2 effervescent (Alka-Seltzer, Mexico), a syringe needle, and filter paper. Distilled water was poured at least halfway into the anesthesia bottle and the lid was closed immediately after the CO2 effervescent was put in. The fruit fly test tube was flipped and the needle from the anesthesia kit was put in between the gap between the tube and the cotton stopper. The valve of the aesthesia bottle was opened and after 1-2 minutes, the fruit flies were anesthetized. The fruit flies were put on filter paper and transferred into each test tube with culture media.

5) Fruit fly cultivation with paint extract and heavy metal exposure

The 20 plastic tubes with fruit fly culture were fully dried and then 100µl of paint extract 1/10, paint extract 1/100, heavy metal extract mixture 1/10, and heavy metal extract mixture 1/100 were pipetted onto the surface of the fruit fly culture media. After heavy metal and paint extract was absorbed into the culture media and dried, adult flies were transferred into the test tubes. 4 of each extract and control, totaling 20 plastic tubes with media culture were prepared.

To test the direct effect of the stressors, adult fruit flies were exposed to heavy metal, paint extract or treated with a cold exposure at 3°C for 4 hours as indicated in Table 2. To test the long-lasting and hereditary effects, offspring (second generation) born from stress-exposed adults moved to normal conditions were tested (Figure 3).

Table 2. Treatment conditions for adult fruit fly and pupae.

Developmental stage	Test conditions	
Adult (Single stress exposure)	Control	
	Paint 1/100, 1/10	
	Metal 1/100, 1/10	
	3°C for 4 hrs	
Pupa (Single stress exposure)	Control	
	Paint 1/100, 1/10	
	Metal 1/100, 1/10	
Adult (Double stress exposure)	Control	
	Paint 1/100, 1/10	3°C for 4 hr
	Metal 1/100, 1/10	

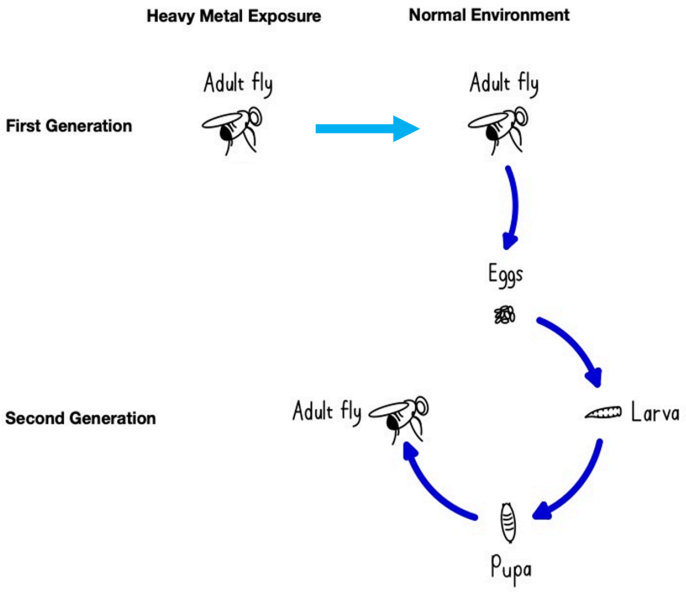

Figure 3. A representative diagram of a fly exposure condition to test the effect on its offspring (second generation). Adult flies were exposed to heavy metal and then moved to normal conditions to lay eggs. Adult flies hatched from these eggs were tested to make observation of the second generation.

6) Adult fly activity measurements

To observe the activity of the differently treated adult flies, once the flies reached the top of the test tubes, test tubes were hit 10 times to drop all the flies to the

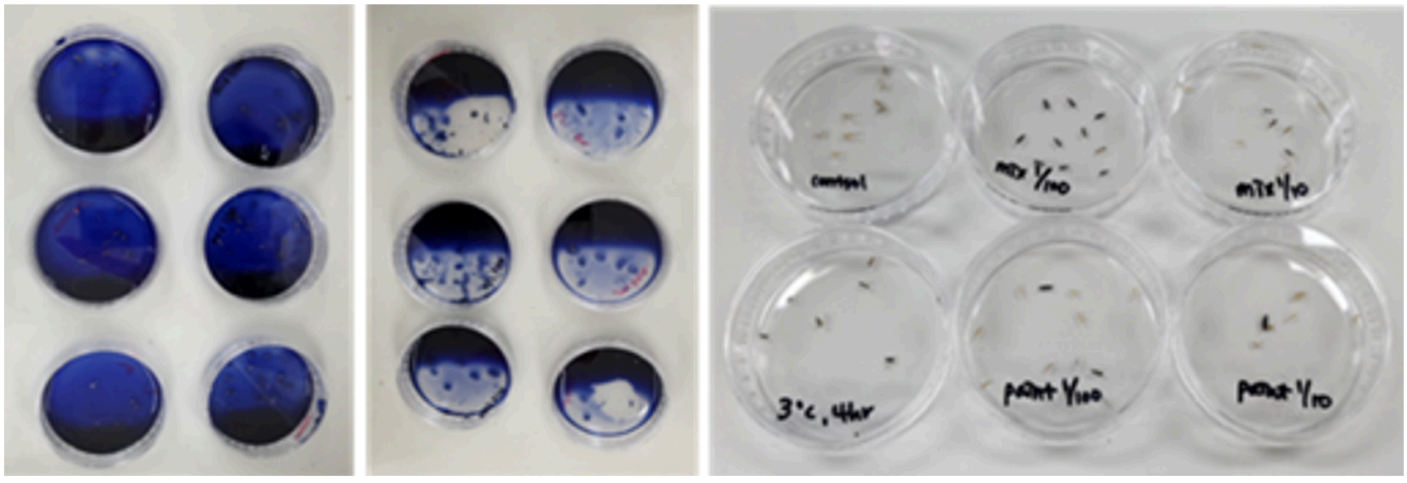

Figure 4. Images of staining process of larvae and stained larvae from differently exposure environments.

bottom. The time it took the flies to reach the top of the test tube again was recorded.

7) Larvae staining

To observe the damage of the environmental stressors (heavy metal, paint extract, low temperature) on human intestinal epithelial homeostasis, the fruit flies' organ system, specifically the midgut cells, were examined by staining larvae of each test condition sample (Capo et al., 2019). After larvae (and pupa if necessary) were collected, they were washed with Phosphate Buffered Saline (PBS) and then stained with 0.02% Trypan Blue (Figure 4). The stained larvae were rinsed with PBS until the larvae surface was destained and then were observed under a stereo microscope.

8) Measurement of relative mRNA abundance with PCR

A. RNA extraction

To collect RNA of the different adult flies after different exposures, RNA was isolated using the RNeasy Mini Kit (QIAGEN, Germany). 10 adult flies of each sample were collected and fly tissues were disrupted and homogenized by centrifuging the tissues with 400uL of Lysis buffer and five 2.3mm diameter ZIRCONIA/SILICA beads (BioSpec Products, USA). Then, following the instructions of the RNeasy Mini Kit, 40ul of RNase-free water was added directly to the spin column membrane, and then it was centrifuged to elute the RNA.

B. cDNA synthesis

5ul of total RNA, 0.5ul of Oligo dT primer, 2ul of 5X reaction buffer, 0.2ul of RTase, and 2.3ul of RTase-Free Water were each added into 200ul tubes and put into a PCR machine at 42 °C for 1 hour.

C. PCR

5ul of cDNA sample, 4ul of F, R primer, 2ul of dNTP, 2ul of 10x reaction buffer, and 6.5ul of distilled water were added into each microtube. The microtubes were put into a PCR machine and went through initial denaturation at 95 °C for 2 min, 35 cycles of denaturation (95 °C, 15s), annealing (60 °C, 35s), and extension (75 °C, 45s), and a final extension at 75 °C

Table 3. Sequences and reaction temperature of the primers used in this research.

Primer	Characteristics	Forward and Reverse Primers
diptericin-A	host-pathogen interactions	5'-AGGTGTGGACCAGCGACAA-3', 63 °C 5'-TGCTGTCCATATCCTCCATTCA-3', 63 °C
drosomycin-B	reporter gene for Toll pathway activation is drosomycin, which encodes an antimicrobial peptide	5'-CTCCGTGAGAACCTTTTCCA-3', 60 °C 5'-GTATOTTCCGGACAGGCAGT-3', 59 °C
TotA-1	expressed in response to stress in the fat body by the JAK-STAT pathway	5'-TGAGGAACGGGAGAGTATCG-3', 60 °C 5'-GCCOTTCACACCTGGAGATA-3', 60 °C
metchnikowin	antibacterial and antifungal properties	5'-CTACATCAGTGCTGGCAGAG-3', 60 °C 5'-CGGTOTTGGTTGGTTAGGATTG-3', 58 °C
beta-actin	housekeeping gene	5'-GCATAGTTTCATCCGCCAGTTG-3, 62 °C 5'-CAAGGTGCTACGAAATCCGTTGT-3, 62.9 °C

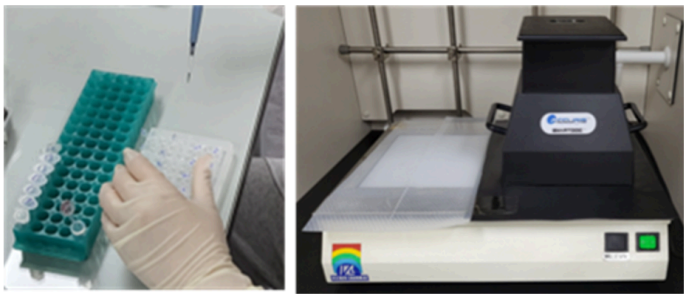

Figure 5. PCR, Gel electrophoresis and UV illumination.

for 10 minutes. The primers were designed with the sequences as follows in Table 3, then were produced at Macrogen, Korea.

D. Gel electrophoresis and DNA band intensity measurements

50mL of 1x TAE buffer and 1.00g of (2.0%) of Agarose LE Master were mixed and microwaved to dissolve the solute. 5uL of StaySafe Nucleic Acid Gel (Dongin Biotech, Korea) was added to the gel solution and poured into the mold. The gel was put into the gel mini electrophoresis system. PCR product was 20uL, so 4uL of 6x DNA loading dye was added for a total of 24uL (Figure 5). 10uL of each sample was loaded into the gel well and then 100bp DNA size marker (Biorad, USA) was loaded after every 6 samples. After loading, the gel mini electrophoresis system ran for 18 minutes at 100V.

The gel was viewed under the UV illuminator (Figure 5) and the band images were taken. With the images, the band intensity was measured and calculated using a program called ImageJ.

RESULTS

1)Extraction of heavy metal for exposure testing

From metals, copper and brass had the highest heavy metal level of 400 ppb and aluminum had a heavy metal level of 100 ppb (Table 4). The copper, lead, brass, and aluminum extract each had a water metal level of 400, 10, 400, and 100 ppb. From environmental exposures, the foam tube had a heavy metal level of 50 ppb and tumbler had level lower than 10 ppb. Each metal extract was mixed to create a heavy metal mixture at 50-100 ppb to match with the heavy metal level of the floating rack.

As shown in Table 5, there was no noticeable difference in the number of survivor adult flies between the heavy metal exposure samples, all with 6 or 7 survivors flies out of the original 10 flies. Compared to the control, heavy metal exposure had a considerable negative impact on the viability of fruit flies. On the other hand, cold shock did not impact the number of survivor adult flies compared to the control.

2) Percentage of eclosion

By day 2, the percentage of eclosion was the highest with 30% for paint extract 1/10, then 20% for both the control and heavy metal mixture 1/10, 10% for heavy metal mixture 1/100, and lastly 0% for paint extract 1/100. By day 4, the heavy metal exposure group had a percentage of eclosion lower than that of the no exposure group. In addition, for heavy metal mixture 1/100, paint extract 1/000, and paint extract 1/10, one adult fly was dead due to the environment by day 4. Heavy metal conditions seem to have a greater negative impact in the long term than in the short term as on day 2, there was no noticeable difference, but by day 4 there was a significant difference in development and viability.

Table 5. Number of survivor adult flies in different environmental stressor conditions.

Conditions		Number of survivors
No exposure	Control	10
Heavy metal mixture	1/100	6
	1/10	6
Paint extract	1/100	7
	1/10	6
Low temperature	3°C, 4hrs	10

3) Activity of adult fruit flies exposed to heavy metal or cold temperature

On day 2, for the control flies, it took 10 times to drop the flies to the bottom while for the heavy metal exposed flies, it only took 4 times of tapping the test tube.

In addition, compared to the control, the heavy metal exposed flies took a significantly increased climbing-up time. Taking longer for their climbing behaviour, two days after moving to normal condition, for heavy metal mixture 1/10, 1 out of 6 flies didn't reach the top while for heavy metal mixture 1/100 and 2 out of 6 flies didn't make it to the top of the test tube. After 4 days of being moved to normal condition, the trend of adult flies not reaching the top of the test tube became more prevalent. Apart from the control and heavy metal mixture 1/10, 2 from paint extract 1/10, 3 from paint extract 1/100, 3 from heavy metal mixture 1/100 were not able to reach the top of the test tubes. Locomotive defects and a decline in the natural upward climbing behaviour can be a result from possible neurodegenerative disorders, caused by heavy metal exposure that exceeds reduced activity from aging.

Temperature also effected fly activity. Three flies from 3°C 4 hrs were not able to reach the top of the

Table 6. Percentage of eclosion in different ratios of heavy metal conditions

Conditions		% eclosion		Comments
		Day 2	Day 4	
No exposure	Control	20	70	-
Heavy metal mixture	1/100	10	50	1 fly dead
	1/10	20	60	-
Paint extract	1/100	0	60	1 fly dead
	1/10	30	40	1 fly dead

test tubes. As ectotherms, fruit flies seem to be impacted by the surrounding temperature. In temperatures under the optimal temperature, decrease in metabolic reaction and the inactivation of metabolism-related enzymes could cause the flies to slow down. However, when the temperature increases again to normal range, the fruit flies return back to normal behaviour and thus this doesn't seem to be a permanent damage for the fly but a temporary status.

4) Activity of unexposed second generation fruit flies of parent flies exposed to heavy metal or cold temperature

Wildtype flies all flew back to the top of the tube within 7 seconds. However, flies from heavy metal exposed parent flies dropped towards the bottom within 4 times of tapping the test tube. Compared to the control, the heavy metal exposed flies had a significantly increased climbing-up time, taking longer for their climbing behaviour. This was observed in both heavy metal mixture and paint extract exposure, but more predominately in heavy metal exposed flies as all flies in this case crawled up. Interestingly, deterioration in activity of cold temperature exposed parent flies was also passed on to the second generation of flies. Flies took 12 seconds to climb up and mostly crawled up without flying.

In all, these results demonstrated that even though these adult flies were never directly exposed to the environmental stressors, such as heavy metal or cold temperature, they were born with defects in their activity.

Table 8. Climbing time of second generation adult flies of stress exposed parent flies that were moved to normal environment after stressor conditions.

Conditions		Time (sec)	Comments
No exposure	Control	7	-
Paint extract	1/100	14	only crawled up without flying
	1/10	11	some only crawled up
Heavy metal mixture	1/100	15	mostly crawled up
	1/10	10	mostly crawled up
Low temperature	3°C, 4hrs	12	mostly crawled up

5) Activity of fruit flies exposed to heavy metal and low temperature

After observation of flies exposed to either heavy metal or cold temperature, activity tendency of the flies exposed to both heavy metal and cold temperature was observed to see if the concurrence of both stressors has a different impact. All double stress exposed flies dropped towards the bottom within 4~6 times of tapping the test tube. For heavy metal exposure and cold shock adult flies, there is no difference in the climbing time with the control fruit flies.

6) Cell damage in Drosophila larvae under the heavy metal or cold temperature exposure

Compared to the control group, which had 2 stained larvae, ratios 1/10 and 1/100 of both paint extract and

Table 7. Climbing time of adult flies in different environmental stressor conditions.

Conditions		Time (sec)		Comments	
		Day 2	Day 6	Day 2	Day 6
No exposure	Control	5	16	-	-
Paint extract	1/100	17	29	-	3 flies stopped at middle
	1/10	10	20	-	2 flies stopped at middle
Heavy metal mixture	1/100	15	29	2 flies stopped at middle	3 flies stopped at middle
	1/10	15	21	1 fly never came up	-
Low temperature	3°C, 4 hrs	10	20	-	3 flies stopped at middle

Table 9. Climbing time of adult flies exposed to both stressors, exposure to heavy metal and low temperature.

Conditions		Time (sec)
No exposure	Control	6
Paint extract	1/100	6
	1/10	6
Heavy metal mixture	1/100	6
	1/10	6

heavy metal mixture had a higher number of stained larvae. ,paint extract 1/10 had 2 more stained larvae than paint extract 1/100; heavy metal mixture 1/10 had 3 more stained larvae than heavy metal mixture 1/10. The cold exposed larvae had 7 stained larvae. These results demonstrate the damages ratio of over 1/100 for both paint extract and heavy metal mixture as well as a cold exposure to the cells of larvae.

Larvae from few generation down, probably 2nd to 3rd generation of stressor exposed flies were also tested for their developmental damage. The number of stained larvae in sample control, paint 1/100, paint 1/10, and mix 1/10 were all similar. However, for mix 1/100 and cold temperature, there were not 10 larvae at the time of selection for both samples and majority of those had cell damage.

8) Cell damage in Drosophila larvae under the heavy metal and cold temperature exposure

Cell damage that may be caused by both heavy metal and cold exposure was also tested. Compared to the control group, which had no stained larvae, ratios 1/100 of paint extract and 1/10 of heavy metal mixture had a higher number of stained larvae. These results confirm that a ratio of 1/100 for paint extract and 1/10

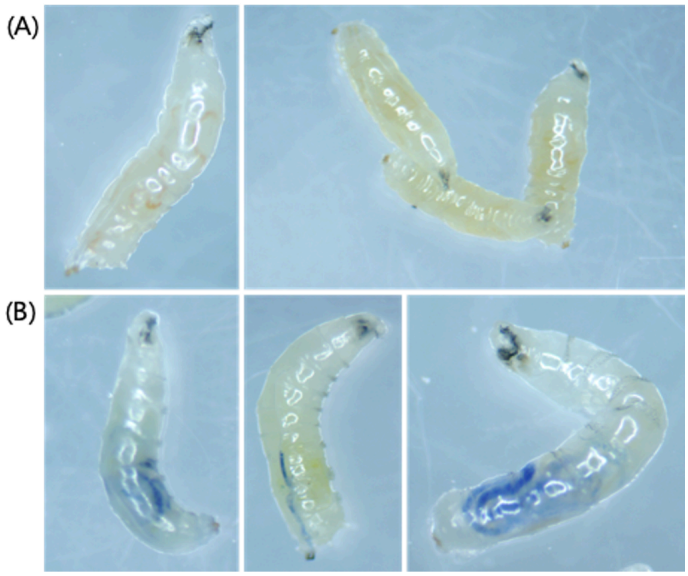

Figure 6. Microscope images of stained and unstained larvae (A) larvae with no stain (B) larvae with stain

Table 10. Larvae born from flies directly exposed to heavy metal or low temperature without transfer to normal condition.

Conditions		Stained number of larvae
No exposure	Control	2
Paint extract	1/100	3
	1/10	5
Heavy metal mixture	1/100	4
	1/10	7
Low temperature	3°C, 4hrs	7

heavy metal mixture damages the cells of larvae. A ratio of over 1/100 for paint extract and heavy metal mixture as well as a cold exposure damages the cells of larvae.

9) Effects of heavy metal and low temperature on immune related gene expression

To discover the negative impacts of heavy metal exposure and cold shock on the immunity of fruit flies, the four immune-related gene expressions of Diptericin-A, Drosomycin-B, Metchnikowin, and Turandot-A were observed.

For the first generation of directly exposed adult flies, there was an overall slight decrease in expression of Diptericin-A. With paint 1/100 having the least expression, it can be seen that the capability of making antimicrobial peptides (AMPs), which are essential components of insect innate immune system, decreases with exposure to either heavy metal or a cold shock (Bolouri et al., 2017). There is a more significant decrease in the expression of Drosomycin-B, a reporter gene for Toll pathway activation is drosomycin, which encodes an antimicrobial peptide. In particular, for heavy metal mixture 1/10, there is approximately a 23% drop in comparison to the control. There is also a significant decrease in the expression of Metchnikowin, in particular for paint extract 1/100. For Metchnikowin, both paint extract 1/10 and 1/100 have a greater decrease than heavy metal mixture 1/10 and 1/100. For both Drosomycin-B

Table 11. Offspring larvae born in normal condition from first generation of flies exposed to heavy metal or cold shock

Conditions		Stained number of larvae	Comment
No exposure	Control	4	
Paint extract	1/100	3	
	1/10	3	
Heavy metal mixture	1/100	0, 9	0 larvae and 10 pupae were collected
	1/10	3	
Cold temperature	3°C, 4hrs	2, 6	3 larvae and 7 pupae collected

Table 12. Larvae from flies exposed to heavy metal and cold temperature

Conditions		Stained number of larvae
No exposure	Control	0
Paint extract	1/100	4
	1/10	0
Heavy metal mixture	1/100	0
	1/10	1

and Metchnikowin, heavy metal mixture 1/10 has a greater decrease than heavy metal mixture 1/100, while paint extract 1/100 has a greater decrease than paint extract 1/10. For Turandot-A, which is a gene that response to stress in the fat body by the JAK-STAT pathway, there was no significant difference among control, mix 1/10, mix 1/100, paint 1/10, and paint 1/100. However, there was about 10% rise in the expression of Turandot-A in the low temperature sample.

For the double stressor exposed adult flies, there wasn't a significant difference between the control and the double stressor exposed flies. Overall, heavy metal mixture 1/10 had the least expression for Drosomycin-B and Metchnikowin,.Drosomycin-B had the most general decrease in all double stressor exposed adult flies. For Metchnikowin, as the heavy metal concentrations rose, the expression decreased despite exposure to low temperature. While both heavy metal mixture 1/100 and paint extract 1/100, the low temperature seems to compensate as shown in the expression of Metchnikowin, this effect does not continue when the heavy metal concentration rises to 1/10. The overexpression of Turandot A in paint extract 1/100 and paint extract 1/100 in double stressor condition suggests prolonged survival and retain normal activity. For Turandot A, exposure from paint extract of daily use material seems to have a greater significance than the exposure from artificial heavy metal mixture as the gene expression increase was most prominent in paint 1/10 exposed flies.

DISCUSSION

In Drosophila melanogaster, heavy metal exposure causes irreversible neurobehavioral damage and significantly reduces locomotor activity (i.e. climbing abilities). The general locomotion of flies was tested through climbing and flight activity, by dropping all the flies to the bottom and then recording the time it took the flies to reach the top of the test tubes. Compared to the control, the heavy metal and cold exposed flies had a significantly increased climbing-up time. This suggests that heavy metal exposure and surrounding temperature defects locomotive behaviour. To see if the tendency of the first generation passes onto the second generation and similarly impacts the second generation, the 1st generation was exposed to heavy metal or cold temperature while the 2nd generation was born in normal conditions. Compared to the control, the heavy metal exposed flies had a significantly increased climbing-up time. This result

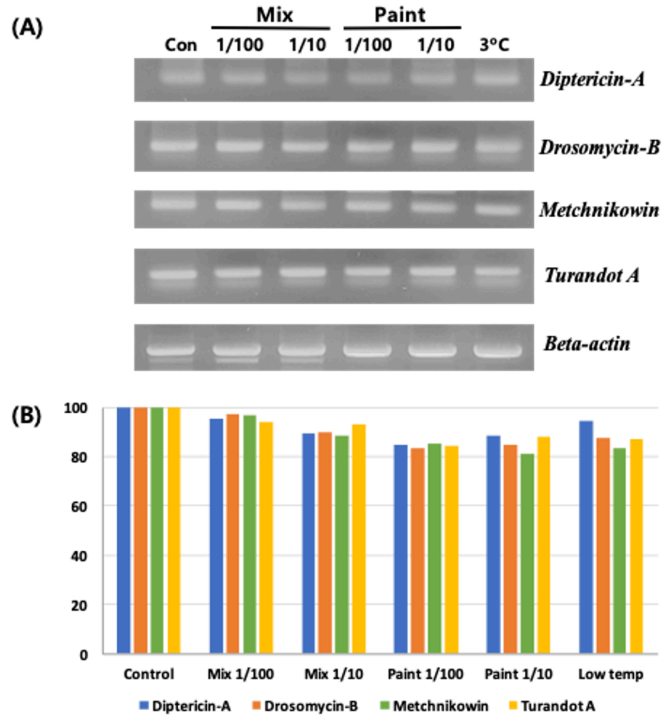

Figure 8. Immune related mRNA expression levels of second generation flies from parents that were exposed to stress conditions.(A) gel electrophoresis (B) mRNA expression levels

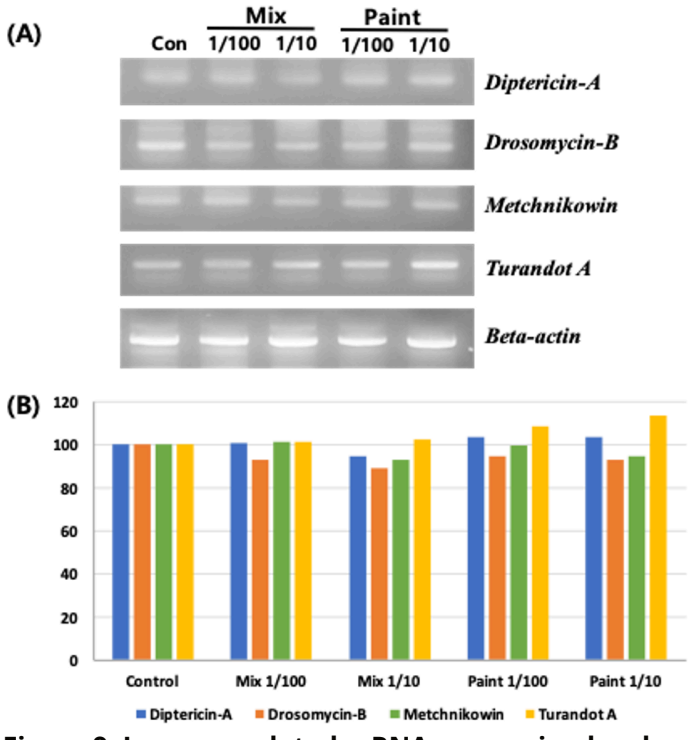

Figure 9. Immune related mRNA expression levels of adult flies exposed to heavy metal and low temperature (3°C for 4hrs) conditions before being moved to normal condition.(A) gel electrophoresis (B) mRNA expression levels

suggests that parents exposed to heavy metal cause their offspring to have defects in activity. For double stressor exposed adult flies, there was no difference in the climbing time with the control fruit flies. In this experiment, the cold shock restored adult flies' activity after both stressors were applied.

The percentage of eclosion, which shows the developmental stage from pupa to adult fly, allows identifying the damage heavy metal exposure has on the developmental stages of adult fruit flies. By day 2, the percentage of eclosion was the highest with 30% for paint extract 1/10, then 20% for both the control and heavy metal mixture 1/10, 10% for heavy metal mixture 1/100, and lastly 0% for paint extract 1/100. By day 4, the heavy metal exposure group had a percentage of eclosion lower than that of the no exposure group. In addition, for heavy metal mixture 1/100, paint extract 1/000, and paint extract 1/10, one adult fly was dead due to the environment by day 4. Heavy metal conditions seem to have a greater negative impact in the long term than in the short term as on day 2, there was no noticeable difference, but by day 4 there was a significant difference in development and viability.

Observing cell damage via larvae staining, both heavy metal and cold exposure appear to inflict significant damage to the cells of larvae, indicating that even during developmental stages, notable cell damage occurs within the intestinal tract, a crucial organ within immunometabolic networks (Witkowska et al., 2021). Larvae from subsequent generations, likely the 2nd to 3rd generation of flies exposed to stressors, were also evaluated for developmental damage. For the 1/100 mixture and cold temperature conditions, fewer than 10 larvae were available for selection in both samples, with the majority exhibiting cell damage. This suggests a slower development compared to the control group. Both the 1/100 ratio of paint extract and heavy metal mixture, along with cold exposure, resulted in larval cell damage. Heavy metal exposure has been associated with alterations in the composition of the intestinal microbiome and an increase in pathogenic bacteria, while cold exposure and freezing of body water can lead to mechanical damage to cells.

Change in immunity was measured by checking the expression level of genes related to antimicrobial, antibacterial, and antifungal activation. To see if directly exposed flies and 2nd generation of those flies has a decrease in immunological response, expressions from primers Diptericin-A, Drosomycin-B, Metchnikowin, and Turandot-A were observed. There was an overall decrease in the expression of all the primers in the 2nd generation. For Turandot-A, which is a gene that response to stress in the fat body by the JAK-STAT pathway, there was no significant difference among the control and heavy metal exposed samples; however, there was about 10% rise in the expression of Turandot-A in the low temperature sample, signifying that flies in low temperature overexpress to survive and preserve normal condition.

Surprisingly, even the short 4 hours of cold temperature stress from the parents, seems to impact the 2nd generation, particularly for Drosomycin-B, Metchnikowin, and Turandot A. Unlike the parent, which had over expressions of Turandot A, the second generation does not show over expressions in Turandot A, suggesting that the cold compensating trait of the parent flies were not passed on.Decrease in immunity was observed in both stress exposed parent and their offspring flies. A difference was seen in Turandot A where unlike the parent, which had over expressions, 2nd generation flies did not show over expressions in Turandot A.

CONCLUSION

Through this study, it was found that adult parent flies that are impacted by both environmental stressors, heavy metal exposure and/or cold shock, impact their offspring to have developmental and immunological problems. From this study, it can be inferred that humans can have the same reactions to environmental stressors, and that can have negative impacts on offspring to have developmental and immunological issues. Furthermore, in future research, the mechanism behind why cold shock can restore the activity of adult flies that are negatively impacted by heavy metal exposure can be studied. Moreover, why environmental stressors have a greater and longer-lasting negative impact than artificial stressors can be explored.

REFERENCES

Bolouri Moghaddam, M. R., Vilcinskas, A., & Rahnamaeian, M. (2017). The insect-derived antimicrobial peptide metchnikowin targets Fusarium graminearum β(1,3)glucanosyltransferase Gel1, which is required for the maintenance of cell wall integrity. *Biological chemistry*, 398(4), 491–498. https://doi.org/10.1515/hsz-2016-

Bonilla-Ramirez, L., Jimenez-Del-Rio, M., & Velez-Pardo, C. (2011). Acute and chronic metal exposure impairs locomotion activity in *Drosophila melanogaster*: a model to study Parkinsonism. *Biometals*: an international journal on the role of metal ions in biology, biochemistry, and medicine, 24(6), 1045–1057. https://doi.org/10.1007/s10534-011-9463-0

Capo, F., Wilson, A., & Di Cara, F. (2019). The Intestine of *Drosophila melanogaster*: An Emerging Versatile Model System to Study Intestinal Epithelial Homeostasis and Host-Microbial Interactions in Humans. *Microorganisms*, 7(9), 336. https://doi.org/10.3390/microorganisms7090336

Davis, H.E., Cheslock, A. & MacMillan, H.A. Chill coma onset and recovery fail to reveal true variation in thermal performance among populations of *Drosophila melanogaster*. *Sci Rep*, 11, 10876 (2021). https://doi.org/10.1038/s41598-021-90401-5

Duarte, J., Woods, K., Andrews, M. C., & Behren, A. (2018, February 2). The good, the (not so) bad and the ugly of immune homeostasis in melanoma. *Wiley Online Library*. https://onlinelibrary.wiley.com/doi/full/10.1111/imcb.12001

Findsen, A., Andersen, J. L., Petersen, A. B., Nielsen, N. C., Rasmussen, L. J., & Overgaard, J. (2014). Why do insects enter and recover from Chill Coma? Low temperature and high extracellular potassium compromise muscle function. *Journal of Experimental Biology*, 217(8), 1297-1306. https://doi.org/10.1242/jeb.098442

Gorini, F., Muratori, F. & Morales, M.A. The Role of Heavy Metal Pollution in Neurobehavioral Disorders: a Focus on Autism. *Rev J Autism Dev Disord*, 1, 354–372 (2014). https://doi.org/10.1007/s40489-014-0028-3

Janssens, E., Dauwe, T., Van Duyse, E., Beernaert, J., Pinxten, R., & Eens, M. (2003). Effects of heavy metal exposure on aggressive behavior in a small territorial songbird. *Archives of environmental contamination and toxicology*, 45(1), 121–127. https://doi.org/10.1007/s00244-002-0133-7

Lee Jr, R. E., & Denlinger, D. L. (2010). Rapid cold-hardening: Ecological significance and underpinning mechanisms. In Low Temperature Biology of Insects (pp. 35-58). *Cambridge University Press*. https://doi.org/10.1017/CBO9780511675997.003

Mitra, S., Chakraborty, A. J., Tareq, A. M., Emran, T. B., Nainu, F., Khusro, A., Idris, A. M., Khandaker, M. U., Osman, H., Alhumaydhi, F. A., & Simal-Gandara, J. (2022). Impact of heavy metals on the environment and human health: Novel therapeutic insights to counter the toxicity. *Journal of King Saud University* - Science, 34(3), 101865. https://doi.org/10.1016/j.jksus.2022.101865

Overgaard, J., Malmendal, A., Sørensen, J. G., Bundy, J. G., Loeschcke, V., Nielsen, N. C., & Holmstrup, M. (2007). Metabolomic profiling of rapid cold hardening and cold shock in *Drosophila melanogaster*. *Journal of insect physiology*, 53(12), 1218–1232. https://doi.org/10.1016/j.jinsphys.2007.06.012

Salehipour-shirazi, G., Ferguson, L. V., & Sinclair, B. J. (2016). Does cold activate the *Drosophila melanogaster* immune system? Journal of Insect Physiology, 96, 29-34. https://doi.org/10.1016/j.jinsphys.2016.10.009

Vesala, L., Salminen, T. S., Laiho, A., Hoikkala, A., & Kankare, M. (2012). Cold tolerance and cold-induced modulation of gene expression in two Drosophila virilis group species with different distributions. Insect molecular biology, 21(1), 107–118. https://doi.org/10.1111/j.1365-2583.2011.01119

Williams, C. M., Rocca, J. R., Edison, A. S., Allison, D. B., Morgan, T. J., & Hahn, D. A. (2018). Cold adaptation does not alter ATP homeostasis during cold exposure in Drosophila melanogaster. Integrative zoology, 13(4), 471–481. https://doi.org/10.1111/1749-4877.12326

Witkowska, D., Slowick, J., Chilicka & C. (2021). Heavy metals and human health: Possible exposure pathways and the competition for protein binding sites. *Molecules*, 26(19), 6060. https://doi.org/10.3390/molecules26196060

ABOUT THE AUTHOR KRISTIN CHO

Kristin Cho is a graduate of Havergal College, class of 2023, and a passionate youth advocate for environmental science and public health. Her dedication to making a difference has led her to organize campaigns involving 300 local pharmacies, aimed at addressing improper medicine disposal at the community level, fundraisers supporting indigenous water treatment operators, and donations for Oxfam, facilitating access to clean water in underdeveloped nations through eco-runs. In addition to her activism work, Kristin has received recognition for her research, including a Gold Medal and the Sanofi Biogenius Award at the Toronto Science Fair, a Bronze Medal at the Canada Wide Science Fair, and another Bronze Medal at the Korea Science and Engineering Fair.