Changes in Larval and Imago Behavior in Lead-exposed *Drosophila melanogaster*

Iin Hindun[1], *Yusfiah Amami Dwi* Erwintha[1], *Diani* Fatmawati[1,2], *Siti* Zubaidah[3], *Hendra* Susanto[3], and *Ahmad* Fauzi[1*]

[1]Department of Biology Education, Faculty of Teacher Training and Education, Universitas Muhammadiyah Malang, Jl. Raya Tlogomas 246 Malang 65144, Indonesia
[2]Graduate School of Biotechnology, College of Life Sciences, Kyung Hee University, 1732 Deogyeong-daero, Giheung-gu, Yongin-si, Gyeonggi-Do 17104, Republic of Korea
[3]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Malang, Jl. Semarang No. 5, Malang 65145, Indonesia

Abstract. Lead is a pollutant that is often found in various locations that may affect the behavior of living things. The purpose of this study was to analyze the effect of lead exposure on behavior changes in *Drosophila melanogaster*. Flies were grouped into four levels of lead exposure: 0, 100, 150 and 200 ppm for two generations. Behavioral observations were made before and after lead exposure. The observed behavior was the ability of the pupae to climb through the pupa position assay and the success and speed of the imago climbing through the adult climbing assay. As a result, higher exposure to lead tends to increase the percentage of pupae in the low zone. Pupae with tilted orientation always have the highest percentage in all groups, both in generation 0 and generation 2. Furthermore, the results of the analysis of covariance inform that lead exposure have no significant effect on climbing duration and climbing success. Overall, lead exposure has the potential to affect the behavior of *D. melanogaster*. Further studies involving other behavioral assays need to be conducted to observe the effect of lead on all behavioral variables.

1 Introduction

Lead contamination or pollution has become a serious issue in the environment [1] and human health [2]. Lead contamination can be found in various locations, including soil [3–4], water [5], and air [6–7]. In humans, lead can disrupt the nervous system [8], cognition [9], and the development of children [10]. In particular, children are highly vulnerable to the adverse effects of lead because their nervous systems are still in the developmental stage [11–12]. Exposure to lead can also cause cardiovascular problems [13], kidney disorders [14–15], and other impacts on body systems [16].

In ecosystems, lead contamination can damage water [17] and soil quality [18] and disrupt the lives of organisms within them [19]. Lead may accumulate in aquatic organisms, affecting fish populations and other aquatic creatures [20]. In the food chain, lead can accumulate and

---

* Corresponding author: ahmad_fauzi@umm.ac.id

reach dangerous levels for higher animals in the food pyramid, including humans who consume that food [21–22]. Due to the potential dangers posed by lead contamination, it is important to continue monitoring and reducing lead exposure in the environment and society.

Exposure to heavy metals such as lead not only has short-term impacts, but also the potential for long-term effects that may not be apparent in the first generation [23–24]. Through cross-generational research approaches, changes in behavior and organism responses to prolonged environmental exposure can be studied [25]. Such research provides a more holistic view of long-term adaptation and potential impacts that may emerge in future generations. Thus, research examining the effects of lead exposure for more than one generation provides important insights into identifying potential risks to human health and ecosystems and developing more effective mitigation strategies to protect both aspects from the negative impacts of lead contamination.

While cross-generational research on humans or other mammals would bring deep understanding, ethical challenges, long time frames, and practical limitations pose significant barriers [26–27]. To overcome these obstacles, the use of model organisms such as *Drosophila melanogaster* has proven to be an effective solution [28]. With a short reproductive cycle and the ability to observe cross-generational effects in a relatively short period of time [29], Drosophila provides an ideal platform for understanding the long-term implications of lead exposure [25].

In this context, the importance of selecting the right variables becomes a crucial factor in evaluating the effects of lead exposure on this model organism. Pupa height, which also serves as an indicator of larval climbing ability [30], reflects how well larvae are able to move against gravity. Similarly, pupa orientation, imago climbing time, and imago climbing success can provide insights into organism responses to lead exposure during critical developmental stages [31–32]. Through a combination of these variables, it is hoped that this study will open the door to a deeper understanding of behavioral adaptation or changes that may occur in Drosophila under lead-contaminated environmental conditions. In this context, careful and comprehensive exploration of the complex interactions between lead exposure and these variables will help paint a more complete picture of lead’s impact on Drosophila behavior.

Despite a number of studies attempting to examine the impact of lead exposure, there are still shortcomings in understanding the long-term cross-generational effects of this heavy metal. Most research tends to focus on short-term impacts [33–34], while long-term impacts have not been fully revealed. In particular, research on the influence of lead on the behavior of Drosophila larvae and imago in a cross-generational perspective is still limited [25]. Therefore, this study aims to fill this gap by examining the impact of lead exposure on Drosophila behavior over a broader period, namely through two generations. By embracing the concept of pretest-posttest control group design and utilizing various behavioral variables, this study proposes a new approach to understanding the influence of lead on Drosophila behavior.

2 Method

2.1 Research design

This study uses a pretest-posttest control group design to investigate the impact of lead exposure on the behavior of *D. melanogaster* larvae and adults. The model organism used in this study is the wild-type strain of *D. melanogaster*. This design allows for observation of treatment effects before and after exposure, as well as comparison with a control group not exposed to lead. The study consists of four treatment groups, each receiving lead exposure at different concentrations: 0 ppm (control group), 100 ppm, 150 ppm, and 200 ppm. Drosophila larvae and adults were bred in culture bottles containing standard nutrient media. Lead was added to the culture media at predetermined concentrations to create different lead exposure conditions between treatment groups. Observations were made before and after lead exposure for two generations of Drosophila, with the aim of understanding the long-term effects of lead exposure.

2.2 Pupa position assay

The method employed to observe the pupa's position in this investigation drew inspiration from the methodology outlined by Fauzi *et al.* (2020) [31]. Within each experimental replication, three pairs of flies were introduced into culture tubes that were 30 cm in height. The observation took place on the 10th day of the experiment, where the location and orientation of each pupa were meticulously recorded. The vertical space within the tube was divided into four distinct zones, demarcating different elevations. Subsequently, the pupae's orientations were categorized into three groups—upright, tilted, and flat—in correspondence with their positions within each zone.

2.3 Adult climbing assay

The methodology employed for the Adult Climbing Assay in this study followed a procedure that outlined in a previous work [31]. The assay involved the measurement of climbing performance in adult Drosophila. For the climbing duration assessment, the experimental setup involved preparing culture tubes designed for the climbing behavior of *D. melanogaster*. Each tube was designated with a height marker of 8 cm above its base. Subsequently, five mature *D. melanogaster* flies from each treatment group were gently introduced into these tubes. A brief period of acclimation was allowed to mitigate potential stress effects. Once the acclimation was deemed stable, the tubes were lightly tapped and shaken to ensure that all flies congregated at the lower portion of the vial. Observations were made as the adult flies were given the opportunity to climb and reach the predetermined climbing boundary. The duration taken by each fly to reach the 8 cm mark was recorded, with a recording time limit of 60 seconds imposed. In instances where flies did not attempt to climb or failed to reach the designated height within the recording time, a uniform recording of 60 seconds was assigned to that individual.

Subsequently, for the assessment of climbing success, a similar experimental setup was employed. Culture tubes were again prepared, with each tube clearly demarcated at a height of 8 cm from the base. This time, ten mature *D. melanogaster* flies from each treatment group were gently introduced into the designated tubes. Similar to the previous procedure, a short acclimation period was provided to minimize stress. Once the flies were acclimated, the tubes were tapped and shaken to gather the flies at the base. Observations were made as the adult flies were given a 10-second interval to climb and reach the predefined boundary of 8 cm. The number of flies that successfully reached this height within the stipulated time was recorded, providing essential data on the climbing success of the flies under different treatment conditions.

2.4 Data analysis techniques

The number of pupae in each zone was then analyzed using a two-way Analysis of Covariance (ANCOVA), where treatment is the first factor while the zone is the second factor. Next, to observe pupa orientation, data was analyzed by calculating the percentage of upright, tilted, and flat oriented pupae in each zone, also in both generations (before and after treatment).

Meanwhile, data on climbing duration and climbing success were analyzed using one-way ANCOVA with treatment (lead concentration) as the main factor. Previously, data was tested for normality using the Kolmogorov-Smirnov test and homogeneity of variance using the Levene test. If the normal distribution and homogeneity requirements are met, one-way ANCOVA analysis is continued. The significance results of this analysis will provide information on significant differences between treatments. To determine significant differences among treatment groups, a post hoc test was performed using the Dunn method. All these analyses were performed using specific statistical software and were considered significant at a significance level of $\alpha = 0.05$.

3 Results and Discussion

This paper will reveal the cross-generational impact of lead exposure on *D. melanogaster*. Through the data analysis described earlier, this study aims to gain deeper insights into the long-term effects of lead contamination on the behavior of larvae and imago of this model organism. The results of the analysis will be carefully depicted, and their implications will be discussed to understand behavioral changes in the context of environmental impacts.

The interpretation of the two-way ANCOVA results reveals important insights into the effects of treatment levels and height zones on the number of pupae. The analysis aimed to assess the interaction between treatment levels (0, 100, 150, 200 ppm lead) and height zones (Zone 1, Zone 2, Zone 3, Zone 4) on the dependent variable, which is the number of pupae. The ANCOVA findings, as summarized in Table 1, indicate that the interaction between treatment levels and height zones is not statistically significant ($F = 0.728$, $p = 0.682$). Furthermore, the results demonstrate that treatment levels do not exert a significant influence on the number of pupae ($F = 1.456$, $p = 0.233$).

Table 1. Two-Way ANCOVA test results: The effect of treatment and pupation zone on the number of pupae.

Cases	Sum of Squares	df	Mean Square	F	p
Treatment	7,308.972	3	2,436.324	1.456	0.233
Zone	19,863.555	3	6,621.185	3.957	0.011
Before exposure	392.588	1	392.588	0.235	0.629
Treatment ✻ Zone	10,965.680	9	1,218.409	0.728	0.682
Residuals	132,190.412	79	1,673.296		

On the other hand, the analysis shows a significant effect of height zones on the number of pupae ($F = 3.957$, $p = 0.011$). Further investigation, as presented in Table 2, provides a comprehensive overview of the post hoc results. The mean number of pupae varies across the different zones: Zone 1 has the highest mean pupae count (72.083), followed by Zone 2 (46.958), Zone 3 (32.292), and Zone 4 (0.833). The post hoc analysis conducted using the Dunn Test reveals distinct patterns. Zone 1 displays no significant difference in pupae count compared to Zone 2, yet it significantly surpasses Zone 3 and Zone 4. Zone 2 also exhibits a significant increase in pupae count compared to Zone 3 and Zone 4. Additionally, Zone 3 shows a significant elevation in pupae count compared to Zone 4.

These findings underscore the relationship between treatment levels, height zones, and the resulting number of pupae. The non-significant effect of treatment levels on pupae counts further contributes to our understanding of the phenomenon. This suggests that, despite the introduction of varying lead concentrations over multiple generations, the resulting pupal distribution remains relatively consistent. On the other hand, the significant influence of height zones themselves highlights the importance of considering the vertical dimension in understanding pupal positioning. The post hoc results provide further clarity by revealing specific zones that exhibit substantial differences in pupae counts, underscoring the complex interplay between environmental factors and pupal distribution.

Table 2. Two Summary of the Dunn's Post Hoc Comparison analysis.

Comparison	z	W_i	W_j	p
Zone 1 - Zone 2	0.521	69.479	65.333	0.602
Zone 1 - Zone 3	3.737	69.479	39.729	< .001
Zone 1 - Zone 4	6.284	69.479	19.458	< .001
Zone 2 - Zone 3	3.217	65.333	39.729	0.001
Zone 2 - Zone 4	5.763	65.333	19.458	< .001
Zone 3 - Zone 4	2.547	39.729	19.458	0.011

The post hoc analysis adds another layer of depth to the discussion, revealing specific patterns in pupal distribution among the different height zones. The higher mean pupae count in Zone 1, followed by a decreasing trend across Zones 2 and 3, aligns with the notion that pupae are more likely to accumulate in areas of greater accessibility and favorability for pupation. It is evident that factors related to height and accessibility within the culture tubes play a crucial role in shaping the distribution of pupae.

Upon examining the data presented in Table 3, a consistent trend emerges: pupae with a tilted orientation consistently exhibit the highest proportion across all experimental groups, spanning both generation 0 and generation 2. The prevalence of pupae exhibiting a tilted orientation suggests that this orientation may confer certain advantages or adaptations for pupae within the context of their environment. It is plausible that the tilted orientation provides pupae with enhanced stability, potentially aiding in their attachment to the tube's surface. This could be particularly advantageous during the pupation process, as pupae need to securely anchor themselves to the chosen substrate.

Table 3. The percentage of pupal orientation in each treatment.

Lead concentration	Before Exposure			After Exposure		
	Upright	Tilted	Flat	Upright	Tilted	Flat
0 ppm	13.15%	29.89%	12.02%	8.22%	23.16%	7.36%
100 ppm	8.83%	30.42%	11.77%	7.30%	32.74%	7.41%
150 ppm	10.80%	35.60%	10.99%	8.78%	45.14%	14.42%
200 ppm	9.57%	39.27%	12.99%	10.97%	46.99%	12.52%

The dominance of the tilted orientation warrants further investigation into its potential adaptive significance. This orientation may facilitate optimal resource utilization, as pupae might strategically position themselves to access nutrients or environmental cues critical for their development. Moreover, the prevalence of the tilted orientation across both generations suggests that this trait is not merely a transient response but rather a consistent and potentially adaptive behavior exhibited by Drosophila pupae.

The positioning of Drosophila pupae with respect to the gravitational axis is governed by a multifaceted interplay of variables. One research study discovered that active larvae frequently construct extensive tunnels within agar substrates and encase their pupae within these tunnels [35]. These embedded larvae exhibit a more extended period of development from egg to pupariation compared to larvae that pupate on the surface [35]. This implies that the construction behaviors of larvae, which likely encompass their alignment with the gravitational axis, may be influenced by developmental timing. Contrarily, another study found no substantial correlation between larval development duration and pupation elevation [36], indicating the involvement of additional factors. The underlying reasons for such wandering behavior remain ambiguous but could be attributed to fluctuations in temperature, variations in food quality, or ecological factors such as interactions with conspecifics or exposure to potential threats like parasitoids [37]. Consequently, it appears that the orientation of *D. melanogaster* pupae in relation to the gravitational axis is subject to regulation by a combination of physiological and environmental factors.

The outcomes of the ANCOVA analysis for the climbing duration data are presented in Table 4. The findings indicated that the treatment did not have a statistically significant effect on climbing duration (*F* = 0.446, *p* = 0.723). This outcome highlights that the exposure to different lead contamination levels did not yield notable alterations in the climbing duration behavior of Drosophila. Importantly, it is noteworthy that the mean climbing duration in the 0-ppm treatment group was the shortest (with an average of 5.467 seconds), although this speed did not exhibit significant differences from the climbing durations observed in the three treatment groups (ranging from 8 to 10 seconds). This observation emphasizes that even though the mean duration for climbing was numerically lower in the 0-ppm group, it did not demonstrate substantial statistical separation from the climbing behaviors in the groups exposed to lead at concentrations of 100, 150, and 200 ppm. The consistent climbing duration across treatments prompts us to consider additional factors that may contribute to the robustness of this behavior. It is plausible that the climbing response in *D. melanogaster* is regulated by a complex interplay of physiological and behavioral adaptations, which might include mechanisms that mitigate the immediate effects of lead exposure [38–39].

Table 4. One-Way ANCOVA test results: The effect of treatment on imago climbing duration.

Cases	Sum of Squares	df	Mean Square	F	p
Treatment	82.098	3	27.366	0.446	0.723
G0 Climbing Duration	1.175	1	1.175	0.019	0.891
Residuals	1165.338	19	61.334		

Turning our attention to the analysis of climbing success, the results of the ANCOVA provide insights into the influence of lead exposure on the ability of *D. melanogaster* to successfully climb (Table 5). The non-significant effect of treatment on climbing success (F = 0.748, p = 0.537) indicates that the different levels of lead contamination did not have a significant impact on the flies' ability to successfully climb. Despite the fact that the mean climbing success in the 0-ppm group was the highest (9.33), the lack of statistical significance suggests that exposure to lead did not result in a discernible alteration of the flies' capability to accomplish climbing tasks.

Table 5. One-Way ANCOVA test results: The effect of treatment on imago climbing success.

Cases	Sum of Squares	df	Mean Square	F	p
Treatment	2.042	3	0.681	0.748	0.537
G0 Climbing Success	0.053	1	0.053	0.059	0.811
Residuals	17.28	19	0.909		

The potential impact of lead on climbing speed and climbing success in *D. melanogaster* is an intriguing avenue to explore. Lead, as a heavy metal, has well-documented neurotoxic effects [8–40] that can disrupt various physiological processes [41–42], including neural signaling [43] and muscle function [44]. As reported in other studies, lead exposure can affect neurological signals which are also followed by muscle-mass weakness [45]. When considering the impact of physical exertion on climbing achievement, it becomes evident that the ability to resist muscle fatigue plays a crucial role [46]. In human, climbing activities need a combination of sporadic and sustained contractions of the arm muscles [46]. In instances where climbing poses significant challenges or extends over an extended period, the muscles resort to anaerobic pathways, resulting in fatigue and, consequently, a decline in climbing performance [47]. Given the sensitivity of climbing behavior to alterations in neurological and muscular systems, it is plausible that lead exposure could influence the speed and success of climbing in these flies.

The non-significant effects observed in our study may stem from several factors. Firstly, the concentrations of lead used in the experiment might not have been sufficient to induce noticeable changes in climbing behavior. It is conceivable that higher concentrations of lead or prolonged exposure periods could yield more pronounced effects on climbing ability. This statement is in line with the accumulating evidence that long-term and multi-generational exposure to toxins can result in subtle but significant alterations in behavior, as well as physiological, morphology, and genetic adaptations [25–48–49]. Lead can also damage metabolism in cells by changing several important ions which can cause behavioral problems [23]. As lead exposure persists and accumulates over time, subtle changes in behavior and physiology could accumulate as well [25], leading to more noticeable alterations in climbing ability.

Future studies could delve deeper into the transgenerational effects of lead exposure on *D. melanogaster*. Investigating the potential changes in climbing behavior across multiple generations could provide a more comprehensive understanding of lead's impact on this complex behavior. Moreover, exploring the molecular and cellular mechanisms underlying these changes could shed light on the adaptive responses of Drosophila to environmental stressors. Ultimately, this research not only deepens our understanding of lead's effects on behavior but also highlights the importance of multi-generational studies to uncover the full scope of these impacts.

4 Conclusion

This study investigated the transgenerational effects of lead exposure on climbing behavior in *D. melanogaster*. Through careful examination of pupa positioning, pupa orientation, climbing duration, and climbing success, we gained valuable insights into the potential impacts of lead on this complex behavior. Our findings did not reveal any significant effects of lead exposure on pupa positioning, orientation, climbing duration, or climbing success. While the lack of significant effects may suggest that the concentrations of lead used were not sufficient to induce observable changes in climbing behavior, our study highlights the need for further investigation into the transgenerational effects of lead exposure. The potential for cumulative impacts to emerge over successive generations highlights the importance of considering long-term and multi-generational studies to comprehensively assess the impacts of environmental toxins on behavior. In the broader context, our research contributes to the growing body of knowledge concerning the complex interactions between environmental factors and behavior in model organisms, with implications for understanding similar phenomena in more complex systems.

References

1. I. Manisalidis, E. Stavropoulou, A. Stavropoulos, & E. Bezirtzoglou, Environmental and health impacts of air pollution: A review. *Frontiers in Public Health*, **8** (2020). https://doi.org/10.3389/fpubh.2020.00014.
2. A. L. Wani, A. Ara, & J. A. Usmani, Lead toxicity: A review. *Interdisciplinary toxicology*, **8** (2015) 55–64. https://doi.org/10.1515/intox-2015-0009.
3. M. M. Onakpa, A. A. Njan, & O. C. Kalu, A review of heavy metal contamination of food crops in Nigeria. *Annals of global health*, **84** (2018) 488–494. https://doi.org/10.29024/aogh.2314.
4. J. J. Clark & A. C. Knudsen, Extent, characterization, and sources of soil lead contamination in small-urban residential neighborhoods. *Journal of Environmental Quality*, **42** (2013) 1498–1506. https://doi.org/10.2134/jeq2013.03.0100.
5. K. J. Pieper, R. Martin, M. Tang, L. Walters, J. Parks, S. Roy, C. Devine, & M. A. Edwards, Evaluating water lead levels during the Flint water crisis. *Environmental science & technology*, **52** (2018) 8124–8132. https://doi.org/10.1021/acs.est.8b00791.
6. B. Pandey, M. Agrawal, & S. Singh, Assessment of air pollution around coal mining area: Emphasizing on spatial distributions, seasonal variations and heavy metals, using cluster and principal component analysis. *Atmospheric Pollution Research*, **5** (2014) 79–86. https://doi.org/10.5094/APR.2014.010.
7. M. Megasari, P. Wahyono, R. Latifa, L. Waluyo, A. Fauzi, & D. Setyawan, Lead (Pb) level of fresh and smoked mackerel tuna (Euthynnus affinis) in Tuban, Indonesia. *IOP Conference Series: Earth and Environmental Science* (2019), p. 012032. https://doi.org/10.1088/1755-1315/276/1/012032.
8. L. H. Mason, J. P. Harp, & D. Y. Han, Pb neurotoxicity: Neuropsychological effects of lead toxicity. *BioMed Research International*, (2014) 1–8. https://doi.org/10.1155/2014/840547.
9. V. Karri, M. Schuhmacher, & V. Kumar, Heavy metals (Pb, Cd, As and MeHg) as risk factors for cognitive dysfunction: A general review of metal mixture mechanism in brain. *Environmental Toxicology and Pharmacology*, **48** (2016) 203–213. https://doi.org/10.1016/j.etap.2016.09.016.
10. X. Zeng, X. Xu, Q. Qin, K. Ye, W. Wu, & X. Huo, Heavy metal exposure has adverse effects on the growth and development of preschool children. *Environmental Geochemistry and Health*, **41** (2019) 309–321. https://doi.org/10.1007/s10653-018-0114-z.
11. K. E. Smith & S. D. Pollak, Early life stress and development: potential mechanisms for adverse outcomes. *Journal of Neurodevelopmental Disorders*, **12** (2020) 34. https://doi.org/10.1186/s11689-020-09337-y.
12. S. Hou, L. Yuan, P. Jin, B. Ding, N. Qin, L. Li, X. Liu, Z. Wu, G. Zhao, & Y. Deng, A clinical study of the effects of lead poisoning on the intelligence and neurobehavioral abilities of children. *Theoretical Biology and Medical Modelling*, **10** (2013) 13. https://doi.org/10.1186/1742-4682-10-13.
13. X. Lu, X. Xu, Y. Zhang, Y. Zhang, C. Wang, & X. Huo, Elevated inflammatory Lp-PLA2 and IL-6 link e-waste Pb toxicity to cardiovascular risk factors in preschool children. *Environmental Pollution*, **234** (2018) 601–609. https://doi.org/10.1016/j.envpol.2017.11.094.
14. S. Dai, Z. Yin, G. Yuan, H. Lu, R. Jia, J. Xu, X. Song, L. Li, Y. Shu, X. Liang, C. He, C. Lv, & W. Zhang, Quantification of metallothionein on the liver and kidney of rats by

subchronic lead and cadmium in combination. *Environmental Toxicology and Pharmacology*, **36** (2013) 1207–1216. https://doi.org/10.1016/j.etap.2013.10.003.

15. J. Vizuete, M. Pérez-López, M. P. Míguez-Santiyán, & D. Hernández-Moreno, Mercury (Hg), lead (Pb), cadmium (Cd), selenium (Se), and arsenic (As) in liver, kidney, and feathers of gulls: A review. In P. de Voogt,ed., *Reviews of Environmental Contamination and Toxicology* (Cham: Springer International Publishing, 2019), pp. 85–146. https://doi.org/10.1007/398_2018_16.
16. M. A. Assi, M. N. M. Hezmee, A. W. Haron, M. Y. M. Sabri, & M. A. Rajion, The detrimental effects of lead on human and animal health. *Veterinary world*, **9** (2016) 660–71. https://doi.org/10.14202/vetworld.2016.660-671.
17. R. Bhateria & D. Jain, Water quality assessment of lake water: A review. *Sustainable Water Resources Management*, **2** (2016) 161–173. https://doi.org/10.1007/s40899-015-0014-7.
18. R. G. Morgado, S. Loureiro, & M. N. González-Alcaraz, Changes in soil ecosystem structure and functions due to soil contamination. In A.C. Duarte, A. Cachada, & T. Rocha-Santos,eds., *Soil Pollution* (Elsevier, 2018), pp. 59–87. https://doi.org/10.1016/B978-0-12-849873-6.00003-0.
19. S. Mishra, R. N. Bharagava, N. More, A. Yadav, S. Zainith, S. Mani, & P. Chowdhary, Heavy metal contamination: An alarming threat to environment and human health. In R. Sobti, N. Arora, & R. Kothari,eds., *Environmental Biotechnology: For Sustainable Future* (Singapore: Springer Singapore, 2019), pp. 103–125. https://doi.org/10.1007/978-981-10-7284-0_5.
20. O. Jitar, C. Teodosiu, A. Oros, G. Plavan, & M. Nicoara, Bioaccumulation of heavy metals in marine organisms from the Romanian sector of the Black Sea. *New Biotechnology*, **32** (2015) 369–378. https://doi.org/10.1016/j.nbt.2014.11.004.
21. M. Aslam, A. Aslam, M. Sheraz, B. Ali, Z. Ulhassan, U. Najeeb, W. Zhou, & R. A. Gill, Lead toxicity in cereals: Mechanistic insight into toxicity, mode of action, and management. *Frontiers in Plant Science*, **11** (2021). https://doi.org/10.3389/fpls.2020.587785.
22. A. Kumar, A. Kumar, C.-P. M.M.S., A. K. Chaturvedi, A. A. Shabnam, G. Subrahmanyam, R. Mondal, D. K. Gupta, S. K. Malyan, S. S. Kumar, S. A. Khan, & K. K. Yadav, Lead toxicity: Health hazards, Influence on food chain, and sustainable remediation approaches. *International Journal of Environmental Research and Public Health*, **17** (2020) 2179. https://doi.org/10.3390/ijerph17072179.
23. A. C. Olufemi, A. Mji, & M. S. Mukhola, Potential health risks of lead exposure from early life through later life: Implications for public health education. *International Journal of Environmental Research and Public Health*, **19** (2022) 16006. https://doi.org/10.3390/ijerph192316006.
24. P. Mitra, S. Sharma, P. Purohit, & P. Sharma, Clinical and molecular aspects of lead toxicity: An update. *Critical Reviews in Clinical Laboratory Sciences*, **54** (2017) 506–528. https://doi.org/10.1080/10408363.2017.1408562.
25. D. Fatmawati, D. Khoiroh, S. Zubaidah, H. Susanto, M. Agustin, & A. Fauzi, Wing morphological changes of Drosophila melanogaster exposed with Lead in nine generations. *AIP Conference Proceedings* (AIP Publishing, 2022).
26. R. A. Powell & G. Proulx, Trapping and marking terrestrial mammals for research: Integrating ethics, performance criteria, techniques, and common sense. *ILAR Journal*, **44** (2003) 259–276. https://doi.org/10.1093/ilar.44.4.259.

27. O. Van Cauwenbergh, A. Di Serafino, J. Tytgat, & A. Soubry, Transgenerational epigenetic effects from male exposure to endocrine-disrupting compounds: a systematic review on research in mammals. *Clinical Epigenetics*, **12** (2020) 65. https://doi.org/10.1186/s13148-020-00845-1.
28. F. P. Fischer, R. A. Karge, Y. G. Weber, H. Koch, S. Wolking, & A. Voigt, Drosophila melanogaster as a versatile model organism to study genetic epilepsies: An overview. *Frontiers in Molecular Neuroscience*, **16** (2023). https://doi.org/10.3389/fnmol.2023.1116000.
29. M. Yamaguchi & H. Yoshida, Drosophila as a model organism. *Drosophila Models for Human Diseases, Advances in Experimental Medicine and Biology* (Singapore: Springer Nature Singapore Pte Ltd, 2018), pp. 1–10. https://doi.org/10.1007/978-981-13-0529-0_1.
30. X. Han, B. Geller, K. Moniz, P. Das, A. K. Chippindale, & V. K. Walker, Monitoring the developmental impact of copper and silver nanoparticle exposure in Drosophila and their microbiomes. *Science of The Total Environment*, **487** (2014) 822–829. https://doi.org/10.1016/j.scitotenv.2013.12.129.
31. A. Fauzi, S. Zubaidah, & H. Susanto, The study of larva and adult behavior of Drosophila melanogaster: Do strains affect behavior? In A. Taufiq, H. Susanto, H. Nur, M. Aziz, C.-R. Chang, H. Lee, M. Diantoro, N. Mufti, N.A.N.N. Malek, I.C. Wang, D.T. Iskandar, G. Elbers, S. Sunaryono, S. Zubaidah, S. Sumari, A. Aulanni'am, A.B. Nandiyanto, I. Wibowo, & A.Y. Handaya,eds., *AIP Conference Proceedings* (Malang: AIP Publishing, 2020), pp. 0400141–0400147. https://doi.org/10.1063/5.0002429.
32. D. Khoiroh, L. Hindun, D. Fatmawati, S. Zubaidah, H. Susanto, & A. Fauzi, Drosophila melanogaster behavior study: Does plumbum affect pupation and climbing ability of imago? *AIP Conference Proceedings* (AIP Publishing, 2023), p. 020099. https://doi.org/10.1063/5.0111891.
33. S. Zhou, S. E. Luoma, G. E. St. Armour, E. Thakkar, T. F. C. Mackay, & R. R. H. Anholt, A Drosophila model for toxicogenomics: Genetic variation in susceptibility to heavy metal exposure. *PLOS Genetics*, **13** (2017) e1006907. https://doi.org/10.1371/journal.pgen.1006907.
34. Z.-H. Liu, J. Shang, L. Yan, T. Wei, L. Xiang, H.-L. Wang, J. Cheng, & G. Xiao, Oxidative stress caused by lead (Pb) induces iron deficiency in Drosophila melanogaster. *Chemosphere*, **243** (2020) 125428. https://doi.org/10.1016/j.chemosphere.2019.125428.
35. S. Narasimha, S. Kolly, M. B. Sokolowski, T. J. Kawecki, & R. K. Vijendravarma, Prepupal building behavior in Drosophila melanogaster and its evolution under resource and time constraints. *PLOS ONE*, **10** (2015) e0117280. https://doi.org/10.1371/journal.pone.0117280.
36. P. Welbergen & M. B. Sokolowski, Development time and pupation behavior in the Drosophila melanogaster subgroup (Diptera: Drosophilidae). *Journal of Insect Behavior*, **7** (1994) 263–277. https://doi.org/10.1007/BF01989734.
37. C. J. Reaume & M. B. Sokolowski, The nature of Drosophila melanogaster. *Current Biology*, **16** (2006) 623–628. https://doi.org/10.1016/j.cub.2006.07.042.
38. H. V. B. Hirsch, G. Lnenicka, D. Possidente, B. Possidente, M. D. Garfinkel, L. Wang, X. Lu, & D. M. Ruden, Drosophila melanogaster as a model for lead neurotoxicology and toxicogenomics research. *Frontiers in genetics*, **3** (2012) 68. https://doi.org/10.3389/fgene.2012.00068.

39. S. Perveen, S. Kumari, H. Raj, & S. Yasmin, Effects of sodium fluoride and Ocimum sanctum extract on the lifespan and climbing ability of Drosophila melanogaster. *The Journal of Basic and Applied Zoology*, **82** (2021) 32. https://doi.org/10.1186/s41936-021-00229-8.
40. O. Shilpa, K. P. Anupama, A. Antony, & H. P. Gurushankara, Lead (Pb) induced oxidative stress as a aechanism to cause neurotoxicity in Drosophila melanogaster. *Toxicology*, **462** (2021) 152959. https://doi.org/10.1016/j.tox.2021.152959.
41. M. Balali-Mood, K. Naseri, Z. Tahergorabi, M. R. Khazdair, & M. Sadeghi, Toxic mechanisms of five heavy metals: Mercury, lead, chromium, cadmium, and arsenic. *Frontiers in Pharmacology*, **12** (2021). https://doi.org/10.3389/fphar.2021.643972.
42. Z. Chen, X. Huo, G. Chen, X. Luo, & X. Xu, Lead (Pb) exposure and heart failure risk. *Environmental Science and Pollution Research*, **28** (2021) 28833–28847. https://doi.org/10.1007/s11356-021-13725-9.
43. J. G. Paithankar, S. Saini, S. Dwivedi, A. Sharma, & D. K. Chowdhuri, Heavy metal associated health hazards: An interplay of oxidative stress and signal transduction. *Chemosphere*, **262** (2021) 128350. https://doi.org/10.1016/j.chemosphere.2020.128350.
44. T. Sanders, Y. Liu, V. Buchner, & P. B. Tchounwou, Neurotoxic effects and biomarkers of lead exposure: A review. *Reviews on Environmental Health*, **24** (2009). https://doi.org/10.1515/REVEH.2009.24.1.15.
45. H. N. Mustafa & A. M. Hussein, Does allicin combined with vitamin B-complex have superior potentials than alpha-tocopherol alone in ameliorating lead acetate-induced Purkinje cell alterations in rats? An immunohistochemical and ultrastructural study. *Folia Morphologica*, **75** (2016) 76–86. https://doi.org/10.5603/FM.a2015.0076.
46. Climbro Team, Performance factors in sport climbing. (2020). https://climbro.com/2020/04/performance-factors-in-sport-climbing/.
47. A. W. Sheel, Physiology of sport rock climbing. *British Journal of Sports Medicine*, **38** (2004) 355–359. https://doi.org/10.1136/bjsm.2003.008169.
48. M. Saaristo, T. Brodin, S. Balshine, M. G. Bertram, B. W. Brooks, S. M. Ehlman, E. S. McCallum, A. Sih, J. Sundin, B. B. M. Wong, & K. E. Arnold, Direct and indirect effects of chemical contaminants on the behaviour, ecology and evolution of wildlife. *Proceedings of the Royal Society B: Biological Sciences*, **285** (2018) 20181297. https://doi.org/10.1098/rspb.2018.1297.
49. K. Świacka, A. Michnowska, J. Maculewicz, M. Caban, & K. Smolarz, Toxic effects of NSAIDs in non-target species: A review from the perspective of the aquatic environment. *Environmental Pollution*, **273** (2021) 115891. https://doi.org/10.1016/j.envpol.2020.115891.