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CONCENTRATION-DEPENDENT EFFECTS OF SUBLETHAL CADMIUM ON DNA INTEGRITY IN DAMSELFLIES

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ABSTRACT

Environmental pollution by heavy metals, particularly cadmium (Cd), poses serious ecological threats due to its persistence and genotoxic potential. This study examined the DNA-damaging effects of Cd exposure on two damselfly species, *Pseudagrion microcephalum* and *Pseudagrion pruinosum* (Odonata: Coenagrionidae), used as bioindicators of aquatic ecosystem health. Larvae were collected from Air Puteh and Kulim Hi-Tech rivers in Kedah, Malaysia, representing different environmental conditions. Final instar larvae were exposed to Cd concentrations of 0.01 mg/L and 1 mg/L for ten days. DNA damage was assessed using the comet assay, quantifying fragmentation through tail moment, olive tail moment, and DNA percentage in the tail. Both species exhibited significant DNA damage upon Cd exposure, with increased tail moments and DNA fragmentation in a dose and time-dependent manner. For *P. microcephalum*, tail moments at 0.01 mg/L ranged from 0.06 to 0.75, and from 0.10 to 0.86 at 1 mg/L, peaking around day 6. Olive tail moments and tail DNA percentages also peaked on days 6 and 7, indicating heightened genotoxic stress. *Pseudagrion pruinosum* showed increased DNA damage, with tail moments ranging from 0.01 to 0.52 (0.01 mg/L) and 0.02 to 0.70 (1 mg/L), peaking on day 7. Notably, *P. microcephalum* exhibited greater DNA damage across all parameters compared to *P. pruinosum*, suggesting species-specific susceptibility linked to genetic or metabolic differences. One-way ANOVA showed significant differences ($P<0.05$) across treatments and durations. Fluctuations after day seven suggest activation of repair mechanisms, though prolonged exposure may compromise these responses. This study highlights the effectiveness of comet assays on Odonata larvae for genotoxicity monitoring and underscores the ecological risks posed by chronic Cd exposure in freshwater systems.

Keywords: Cadmium; Comet assay; DNA damage; heavy metal pollution; *Pseudagrion* sp.

ABSTRAK

Pencemaran alam sekitar oleh logam berat, khususnya kadmium (Cd), menimbulkan ancaman ekologi yang serius disebabkan sifatnya yang kekal dan berpotensi menyebabkan kerosakan genetik. Kajian ini meneliti kesan kerosakan DNA akibat pendedahan Cd terhadap dua spesies pepatung jarum, *Pseudagrion microcephalum* dan *Pseudagrion pruinosum* (Odonata: Coenagrionidae), yang digunakan sebagai bioindikator kesihatan ekosistem akuatik. Larva

dikumpulkan dari Sungai Air Puteh dan Sungai Kulim Hi-Tech di Kedah, Malaysia, yang mewakili keadaan persekitaran yang berbeza. Larva instar akhir didedahkan kepada kepekatan Cd sebanyak 0.01 mg/L dan 1 mg/L selama 10 hari. Kerosakan DNA dinilai menggunakan ujian komet (comet assay) yang mengukur pemecahan DNA melalui momen ekor, momen ekor olive, dan peratusan DNA dalam ekor. Kedua-dua spesies menunjukkan kerosakan DNA yang ketara selepas pendedahan kepada Cd, dengan peningkatan momen ekor dan pemecahan DNA yang bergantung kepada dos dan masa. Bagi *P. microcephalum*, momen ekor pada 0.01 mg/L berada dalam julat 0.06 hingga 0.75 dan dari 0.10 hingga 0.86 pada 1 mg/L, dengan kemuncak sekitar hari ke-6. Nilai momen ekor olive dan peratusan DNA dalam ekor juga mencapai kemuncak pada hari ke-6 dan ke-7, menunjukkan tekanan genotoksik yang tinggi. *Pseudagrion pruinosum* turut menunjukkan peningkatan kerosakan DNA, dengan momen ekor antara 0.01 hingga 0.52 (0.01 mg/L) dan 0.02 hingga 0.70 (1 mg/L) dan kemuncak pada hari ke-7. Menariknya, *P. microcephalum* menunjukkan tahap kerosakan DNA yang lebih tinggi dalam semua parameter berbanding *P. pruinosum*, mencadangkan kerentanan spesies yang berbeza-beza disebabkan perbezaan genetik atau metabolik. Ujian ANOVA sehala menunjukkan perbezaan yang signifikan ($P<0.05$) antara rawatan dan tempoh pendedahan. Fluktuasi selepas hari ke-7 mencadangkan pengaktifan mekanisme pembaikan, walaupun pendedahan yang berpanjangan boleh menjejaskan keupayaan tindak balas ini. Kajian ini menyerlahkan keberkesanan ujian komet pada larva Odonata untuk pemantauan genotoksisiti dan menekankan risiko ekologi akibat pendedahan kronik Cd dalam sistem air tawar.

Kata kunci: Kadmium; Ujian komet; Kerosakan DNA; Pencemaran logam berat; *Pseudagrion* sp.

INTRODUCTION

Freshwater ecosystems in Malaysia are under growing threat from anthropogenic contamination, as rapid urbanisation, industrial discharges, agricultural runoff, and domestic wastewater contribute to the accumulation of heavy metals in aquatic habitats. Such pollutants may persist in the water column or bind to sediments, thereby posing a chronic risk to benthic and nektonic organisms alike. Among these organisms, aquatic insects play a critical role in structuring freshwater communities and function as valuable bioindicators of ecological health. In particular, molecular biomarkers of genotoxic stress especially DNA strand breaks, serve as early-warning signals of sublethal toxicity that can precede adverse population-level effects. The alkaline single-cell gel electrophoresis or comet assay, has emerged as a powerful and sensitive tool for detecting such DNA damage in aquatic invertebrates.

One of the most concerning metals in Malaysian freshwaters is cadmium (Cd), which is non-essential, highly toxic, and capable of inflicting oxidative and genotoxic damage at relatively low exposure levels. Industrial operations such as metal smelting, battery manufacturing, electroplating, and pigment production release Cd into air, water, and soil (Abd Elnabi et al. 2023; Barik et al. 2025). Mining activities, especially those involving zinc and lead ores, also contribute to Cd contamination (Barik et al. 2025). Furthermore, the use of phosphate fertilizers in agriculture introduces Cd into soils, which can leach into water bodies. These pathways facilitate the entry of Cd into aquatic ecosystems, where it can persist and accumulate, posing serious ecological threats (Abd Elnabi et al. 2023). Once introduced into aquatic environments, Cd exhibits high toxicity even at low concentrations. It can bioaccumulate in aquatic organisms, moving up the food chain and affecting higher trophic levels, including fish, birds, and ultimately humans (Orata & Sifuna 2023). Cadmium interferes with essential biochemical and physiological processes by displacing vital metals such as zinc

and calcium in enzymes and proteins, disrupting cellular metabolism. One of the primary mechanisms of Cd toxicity is the induction of oxidative stress through the generation of reactive oxygen species (ROS), which damage cellular components including lipids, proteins, and DNA (Lushchak 2011; Unsal et al. 2020). The persistence and mobility of Cd in the environment underscore the urgency of assessing its biological impacts on aquatic taxa.

Studies on the toxicity of pesticides on insect survival have often been conducted by researchers (Masdah et al. 2023; Siti Asma' et al. 2024; Paramita et al. 2025). Genotoxicity, or the ability of a substance to damage genetic material, is a critical endpoint for assessing the impact of environmental contaminants (Alnasser 2025). DNA damage can lead to mutations, impaired cell function, and even cell death, which in turn affect organismal health, reproduction, and population dynamics (Wojtczyk-Miaskowska & Schlichtholz 2018). Understanding the genotoxic effects of Cd is essential for evaluating its ecological risks and for developing effective environmental management strategies. Molecular biomarkers such as DNA strand breaks provide sensitive indicators of sub-lethal stress before more overt toxic effects become apparent.

Thus far, most of the molecular-level genotoxicity research in Malaysia has concentrated on non-biting midges (family Chironomidae), especially *Chironomus kiiensis*. In a seminal laboratory study, Al-Shami et al. (2012) exposed *C. kiiensis* larvae to sublethal concentrations of Cd (0.1, 1, and 10 mg/L), as well as copper (Cu) and zinc (Zn) for 24 hours, and detected significant increases in DNA strand breaks using the comet assay. Notably, Cd induced more severe genotoxic effects than Cu or Zn, as measured by both tail moment and olive tail moment parameters (Al-Shami et al. 2012). Such findings provide critical mechanistic insight, linking Cd exposure to DNA damage in a model tropical aquatic insect species. Furthermore, field-relevant evidence complements these laboratory results. In a study of northern Peninsular Malaysia, Al-Shami et al. (2012) exposed *C. kiiensis* larvae to sediments collected from rivers exhibiting various degrees of contamination. Their results revealed that larval DNA damage, measured via the comet assay, increased in a time-dependent manner and correlated with sediment pollution levels, including Cd and other heavy metals (Al-Shami et al. 2012). This work underscores the ecological relevance of sediment-borne Cd and demonstrates that chironomid larvae can reliably reflect genotoxic stress in situ.

Besides, acute toxicity data also support the rationale for investigating sublethal genotoxic effects. Ebau et al. (2012) conducted acute exposure tests on different larval instars (first to fourth) of *Chironomus kiiensis* and *Chironomus javanus* using Cd and lead (Pb). They found that Cd was significantly more toxic than Pb, with younger instars exhibiting greater sensitivity, and that *C. javanus* was particularly susceptible (Ebau et al. 2012). Such mortality data help to calibrate environmentally relevant exposure concentrations for subsequent sublethal genotoxicity assays.

Despite this substantial body of work on chironomids, there is a conspicuous lack of molecular-genotoxicity research focused on Odonata (dragonflies and damselflies) in Malaysia. This is notable because odonate nymphs are well-suited as bioindicators: they often occupy a higher trophic level, they persist in freshwater habitats for months to years, and they can accumulate contaminants both from the water column and sediments. However, Malaysian studies on odonates to date have mainly focused on heavy-metal accumulation patterns rather than direct measures of genomic integrity. For instance, a recent study by Ahmad Hadri and Suhaila (2023) quantified the bioaccumulation of Cd, Cu, Mn, and Zn in damselfly (Zygoptera) larvae collected from Malaysian rivers. They found that the concentrations of metals in insect

tissue were consistently higher than in sediments, and calculated biota–sediment accumulation factors (BSAF) that exceeded 1 for several metals, including Cd, indicating strong bioaccumulation potential (Ahmad Hadri & Suhaila 2023). Similarly, Nurul Huda et al. (2017) and Suhaila et al. (2016) reported on the community composition of odonate larvae in Malaysian streams and the influence of water quality parameters on their distribution. Yet none of these studies applied cytogenetic or molecular biomarkers, leaving a critical gap in understanding the genotoxic risk of Cd to odonate species.

Within the Odonata, the genus *Pseudagrion* (damselflies) is particularly promising for genotoxicity studies in Malaysia. *Pseudagrion* species are widely distributed across a variety of freshwater systems including rivers, swampy areas, peatlands, and anthropogenic waterways and their nymphs frequently inhabit littoral zones where sediments can concentrate heavy metals. Their trophic position, life span, and habitat preferences make them effective integrators of exposure to both dissolved and sediment-bound contaminants. Yet, despite their ecological significance, there is currently no published study examining cadmium-induced DNA damage (or other genotoxic endpoints) in *Pseudagrion* species in Malaysia. This gap in the literature has important implications. DNA damage in odonate nymphs could impair development, reduce emergence success, and lower reproductive fitness, thus negatively affecting population viability. Given their role as predators, any genotoxic harm in *Pseudagrion* may also propagate through the food web, potentially influencing ecosystem structure. Furthermore, molecular genotoxic biomarkers such as the comet assay provide mechanistic insight that complements traditional accumulation and survival metrics, thereby enhancing risk-assessment frameworks.

To address this critical knowledge gap, the present study aims to elucidate the molecular effects of Cd exposure on *P. microcephalum* and *P. pruinosum* by quantifying DNA damage induced by oxidative stress. By exposing larvae to environmentally relevant Cd concentrations over a controlled duration, the research seeks to establish the relationship between Cd dose, exposure time, and genotoxic outcomes. The comparative analysis between two damselfly species will enhance understanding of species-specific vulnerabilities and resilience. The findings will contribute valuable data for ecological risk assessments, informing policymakers and conservationists about the potential hazards of Cd contamination in freshwater systems. Moreover, the study underscores the importance of integrating molecular biomarkers into environmental monitoring programs to detect early signs of pollution-induced damage. Ultimately, this research supports efforts to develop mitigation strategies that safeguard freshwater biodiversity and maintain ecosystem services.

MATERIALS AND METHODS

Sampling Locations and Sample Collection

Final instar larvae of *P. microcephalum* (Figure 1) and *P. pruinosum* (Figure 2) were collected from two rivers in Kedah, Malaysia: Air Puteh River (N 05º 11’ 16.0”, E 100º 36’ 11.0”) and Kulim Hi-Tech River (N 05º 25’ 47.4” E 100º 34’ 46.8”). These locations were chosen based on preliminary data indicating a high abundance of the target Coenagrionidae species. Air Puteh River, characterized by a mixed substrate of gravel and sand with partial shading, is bordered by oil palm plantations and vegetation such as *Colocasia esculenta* (taro) and *Nephrolepsis* sp. (fern). In contrast, Kulim Hi-Tech River flows through an industrial park with no canopy cover, featuring banks lined with grass, *Brachiaria* sp. and taro, *Colocasia esculenta* and a similar gravel-sand substrate.

Figure 1. Larvae of *Pseudagrion microcephalum* used in the present study to assess the cadmium induced DNA damage

Figure 2. Larvae of *Pseudagrion pruinosum* used in the present study to assess the cadmium induced DNA damage

Larvae Identification

Final instar larvae, measuring 15–20 mm in body length, were identified using established taxonomic keys; Burmeister (1839), Rambur (1842), Morse et al. (1994) and Yule & Yong (2004). In the laboratory, the larvae were sorted and individually housed in plastic containers with dechlorinated tap water and vegetation, providing both substrate and space. The containers were maintained at a controlled room temperature of 27±2°C under a 12:12-hour light-dark photoperiod, with larvae allowed 12 hours to recover from transportation stress before further experimentation.

Cadmium (Cd) Exposure

After a 12-hour acclimation period, final instar larvae of *P. microcephalum* and *P. pruinosum* were individually placed in plastic containers. These containers, measuring 12 cm in height, 8 cm in top diameter, and 4 cm in bottom diameter, were filled with 100 ml of Cd solutions at concentrations of 0.01 mg/L and 1 mg/L. The exposure durations ranged from Day 1 to Day 10. Each *Pseudagrion* sp. was placed individually in a container and was fed three times during the ten-day exposure period with mosquito, *Culex quinquefasciatus* (Okude et al. 2017).

Isolating each larva in separate containers prevented cannibalism and maintained experimental integrity. There were 90 containers for each 0.01 mg/L and 1 mg/L of Cd, with a total of 360 containers with individual tested odonates for both *Pseudagrion* species. The plastic containers ensured inertness, preventing any chemical interactions with the Cd and maintaining controlled laboratory conditions essential for toxicity testing. At the end of the exposure periods (Days 1 through 10), larvae were collected and stored at -20°C until further analysis using the comet assay, as outlined by Jackson et al. (2013). Distilled water was used as a negative control, establishing a baseline for comparison and ensuring that any DNA damage observed was a result of Cd exposure rather than external environmental factors. This control also distinguished between natural background DNA damage and damage specifically induced by Cd.

Comet Assay (Single Cell Gel Electrophoresis)

Collected samples of *Pseudagrion* sp. that were exposed to 0.01 and 1 mg/L of Cd from each day (day 1 until day 10) were used to observe the occurrence of DNA damage. For this study, 360 larvae of *P. microcephalum* and *P. pruinosum* were used to assess DNA damage using the OxiSelect™ Comet Assay Kit (Cell Biolabs, Inc). To prepare the slides for comet assay analysis, three larvae were needed per slide. Reagents such as lysis buffer and alkaline electrophoresis solution were prepared according to the manufacturer's instructions. A base layer of 75 µl of Comet Agarose was applied to each well of the OxiSelect™ Comet Slide, ensuring complete coverage. The slides were then kept horizontally and transferred to 4°C for 15 minutes. Three larvae were pooled and ground in 1 ml of chilled 1X phosphate-buffered saline (PBS) containing 20 mM EDTA and 10% DMSO, as described by Lee & Choi (2009). After centrifuging at 13,000 rpm for 3 minutes, 10 µl of the supernatant was combined with 100 µl of low-melting-point agarose (OxiSelect™ Comet Agarose). The resulting mixture (75 µl) was carefully placed on top of the base layer of Comet Agarose on the slide, which was then incubated for 15 minutes at 4°C in the dark. Following this, the slide was immersed in pre-chilled lysis buffer (~25 ml per slide) for 40 minutes at 4°C in the dark. Afterward, the lysis buffer was aspirated and replaced with pre-chilled alkaline solution (~25 ml per slide), where the slides were left for 30 minutes at 4°C in the dark.

Following the incubation in the alkaline solution, the slide was carefully moved to a horizontal electrophoresis chamber, which was filled with cold Alkaline Electrophoresis Solution until the buffer level covered the slide. A voltage of 30 volts (1 volt/cm) was applied for 15-30 minutes. The volume of the solution was adjusted to achieve a current setting of 300 mA. Once the electrophoresis run was completed, the slide was transferred horizontally into a clean container with approximately 25 ml of pre-chilled distilled water. The slide was immersed for 2 minutes, aspirated, and this rinsing process was repeated twice. The final rinse was performed with 70% ethanol for 5 minutes. After this step, the slide was carefully removed from the ethanol and allowed to air dry. Once the agarose was completely dry, 100 µl of diluted Vista Green DNA Dye was added to each well, and the slide was incubated at room temperature for 15 minutes. The slide was then ready for image analysis.

Image and Data Analyses

DNA migration on the prepared slides was visualized and photographed using a fluorescence microscope (Olympus BH-2, Japan) at 400X magnification, equipped with a ProgRes Cloplus camera (Laser Optic Systeme, Germany) and filters for 495 nm excitation and 515 nm barrier. ImageJ software (version 1.52e) was utilized to measure tail moment length, tail DNA intensity, and cell DNA intensity. One way ANOVA was used to evaluate differences in tail

moment length, DNA percentage in the tail, and olive tail moment between the two *Pseudagrion* sp.

RESULTS

Genotoxic Effects of Cadmium (Cd) on *Pseudagrion microcephalum* and *Pseudagrion pruinosum*

Figure 3 illustrates the progression of DNA comet damage in *P. microcephalum* and *P. pruinosum* over a ten-day exposure to Cd, from day 1 to day 10 (a-j). Each subfigure represents a specific day, highlighting the increasing DNA damage caused by Cd exposure. On days 1 and 2, the nuclei of both species appeared mostly rounded (Figures 3a, 3b). By day 3, the nuclei began to display a blurry tail, and by day 7, a distinct tail was visible, indicating significant DNA damage and strand breaks in the insect cells.

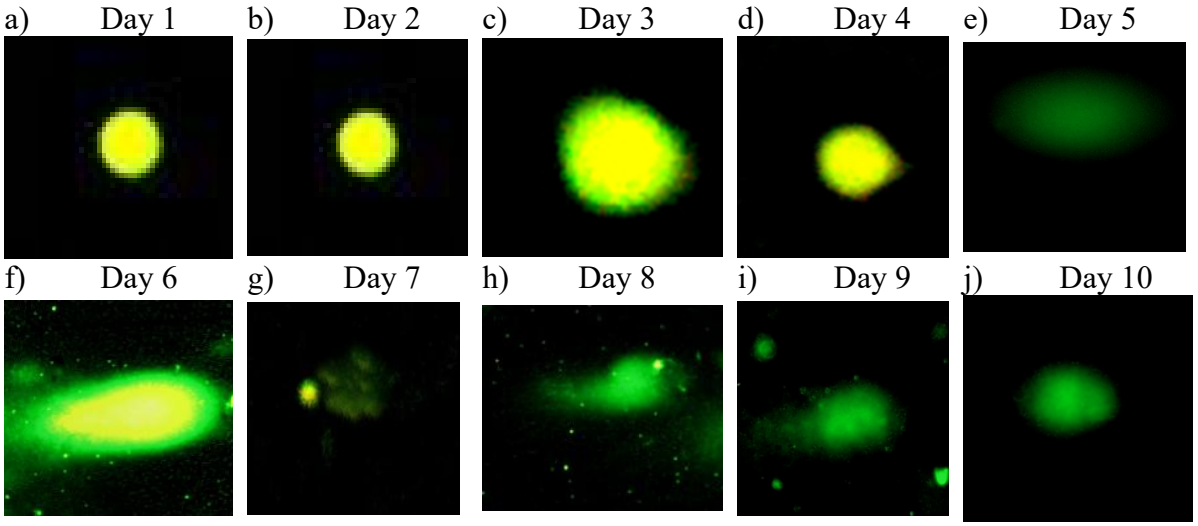

Figure 3. Representative DNA comets of *P. microcephalum* and *P. pruinosum* following Cd exposure at 0.01 mg/L and 1 mg/L

The study revealed that *P. microcephalum* exhibited increasing DNA damage with higher Cd concentrations over time. At 0.01 mg/L of Cd, tail moment lengths ranged from 0.06 to 0.75, peaking on day 6, while at 1 mg/L, they ranged from 0.1 to 0.86, also peaking on day 6, indicating a more significant effect at higher concentrations. Similarly, the DNA percentage in the tail increased with Cd concentration, ranging from 36.9% to 90.7% at 0.01 mg/L and 38% to 108.6% at 1 mg/L, with maximum values recorded on days 6 and 7, respectively. Olive tail moment, another indicator of DNA damage, followed a similar trend, with values ranging from 2 to 67.8 at 0.01 mg/L and 3.6 to 88.7 at 1 mg/L, peaking on day 6 for both concentrations. These findings highlight a dose-dependent increase in DNA damage metrics, as shown in Table 1. These findings highlight the escalating DNA damage in *P. microcephalum* with increasing Cd concentration and exposure duration.

The study found that the tail moment length of *P. pruinosum* increased with higher concentrations of Cd, ranging from 0.01 to 0.52 pixels/mm at 0.01 mg/l and 0.02 to 0.70 pixels/mm at 1 mg/l, with the highest values for both concentrations observed on day 7 (Table 2). The olive tail moment, a more sensitive measure of DNA damage that considers DNA distribution in both the head and tail, showed significant differences compared to tail moment

length. Statistical analysis using one-way ANOVA ($P<0.05$) confirmed significant variations in tail moment length, olive tail moment, and DNA percentage in the tail for both *Pseudagrion* species exposed to 0.01 and 1 mg/L of Cd.

Table 1. Mean tail moment length, olive tail moment, and DNA percentage in the tails±SE of *P. microcephalum* larvae following exposure to various concentrations of Cd for different durations

	Cd Concentration (Mg/L)	Mean of Tail Moment Length±SE	Mean of Olive Tail Moment±SE	Mean of DNA Percentage in Tail±SE
Day 1	0.01	0.06±0.001	2.0±1	36.9±0.1
	1	0.10±0.03	3.6±0.8	38.0±0.3
Day 2	0.01	0.04±0.004	3.0±0.8	68.8±0.2
	1	0.10±0.03	7.4±0.4	71.7±0.6
Day 3	0.01	0.19±0.03	13.1±1.2	68.5±0.2
	1	0.20±0.02	14.6±1.2	73.0±0.4
Day 4	0.01	0.25±0.02	21.4±1.2	87.4±0.2
	1	0.25±0.08	23.6±1.0	96.3±0.7
Day 5	0.01	0.46±0.06	40.3±1.2	87.9±0.2
	1	0.50±0.05	49.4±1.6	98.1±0.5
Day 6	0.01	0.75±0.03	67.8±0.9	90.7±0.3
	1	0.86±0.07	88.7±1.8	103.4±0.5
Day 7	0.01	0.55±0.05	49.7±0.8	90.6±0.2
	1	0.60±0.03	65.5±1.4	108.6±0.3
Day 8	0.01	0.35±0.03	26.4±1.8	76.0±0.3
	1	0.46±0.04	45.7±1.8	99.4±0.5
Day 9	0.01	0.50±0.03	40.0±1.6	79.2±0.4
	1	0.58±0.04	49.7±0.7	85.4±0.6
Day 10	0.01	0.20±0.02	16.5±1.2	82.3±0.2
	1	0.42±0.05	34.9 ± 1.8	82.8±0.6

Table 2. Mean of the tail moment length, olive tail moment and DNA percentage in tail±SE of *P. pruinosum* larvae after exposure to several concentrations of Cd at several durations

	Cd Concentration (mg/L)	Mean of Tail Moment Length±SE	Mean of Olive Tail Moment±SE	Mean of DNA Percentage in Tail±SE
Day 1	0.01	0.010±0.005	0.3±0.05	34.9±0.6
	1	0.024±0.003	0.9±0.07	35.2±0.4
Day 2	0.01	0.027±0.001	1.3±0.2	48.52±0.6
	1	0.029±0.002	2.0±0.8	68.7±0.2
Day 3	0.01	0.133±0.05	6.7±0.2	50.2±0.3
	1	0.158±0.04	11.1±0.4	70.0±0.4
Day 4	0.01	0.185±0.04	12.3±0.3	66.7±0.8
	1	0.211±0.01	17.9±0.3	85.0±0.2
Day 5	0.01	0.251±0.03	19.3±0.6	77.1±0.2
	1	0.410±0.06	39.0±0.4	95.1±0.5
Day 6	0.01	0.500±0.01	44.5±0.4	89.0±0.1
	1	0.689±0.03	61.8±0.7	98.4±0.1
Day 7	0.01	0.524±0.04	47.1±0.6	89.8±0.2
	1	0.702±0.02	63.2±0.1	99.2±0.1
Day 8	0.01	0.233±0.02	15.9±0.3	68.2±0.5
	1	0.322±0.07	38.9±0.2	90.2±0.6
Day 9	0.01	0.485±0.07	34.1±0.4	70.2±0.4
	1	0.550±0.08	40.0±0.6	80.4±0.3
Day 10	0.01	0.188±0.02	15.1±0.5	80.3±0.5
	1	0.381±0.01	25.9±0.3	82.0±0.1

DISCUSSION

The Genotoxic Impact of Cadmium (Cd) on *Pseudagrion microcephalum* and *Pseudagrion pruinosum* From Day 1 To Day 10

The present study demonstrates that exposure of *P. microcephalum* and *P. pruinosum* to Cd at environmentally relevant concentrations (0.01 and 1 mg/L) induces significant genotoxic effects, as evidenced by increased tail moment length, DNA percentage in the tail, and olive tail moment in comet assays. These findings are consistent with previous reports highlighting the genotoxic potential of Cd in aquatic insects and other biota (Ahmad Hadri & Suhaila 2021; Michailova et al. 2018). Besides, another study done by Al-Shami et al. (2012) on DNA damage in *Chironomus kiiensis* (Diptera: Chironomidae) showed that Cd exposure caused significantly higher DNA fragmentation compared to copper and zinc, as measured by increased tail moment and olive tail moment in comet assays after 24 hours of exposure. Furthermore, the rapid onset of DNA damage observed in this study from day 1, peaking around days 6 to 7, and subsequent fluctuations suggest a dynamic interplay between Cd-induced DNA damage and cellular repair mechanisms.

The comet assay parameters employed in this study, particularly tail moment, are well established indicators of DNA strand breaks and fragmentation, integrating both the extent of DNA migration and the proportion of damaged DNA (Jiang et al. 2023). The temporal pattern of DNA damage observed in this study aligns with the concept that initial exposure to Cd generates reactive oxygen species (ROS), leading to oxidative DNA lesions and strand breaks (Tang et al. 2015). The subsequent decline and fluctuation in DNA damage markers likely reflect activation of DNA repair pathways, including nucleotide excision repair (NER) and base excision repair (BER), which are critical for removing bulky adducts and oxidative base damage, respectively (Ma et al. 2024). Similarly, the observed fluctuations in DNA damage levels over time in the study done by Al-Shami et al. (2012) imply that *Chironomus kiiensis* larvae may activate DNA repair mechanisms in response to genotoxic stress. Despite the activation of these repair mechanisms, chronic Cd exposure is known to impair DNA repair efficiency by disrupting the expression and function of repair enzymes (Augustyniak et al. 2020). This impairment may result from Cd induced oxidative stress overwhelming cellular antioxidant defenses or direct interference with repair proteins (Prorok et al. 2021). Consequently, persistent DNA damage accumulates, which can lead to genomic instability, mutations, and compromised cellular function. Findings from this current study corroborate with the previous studies reporting that prolonged Cd exposure leads to increased chromosomal aberrations, micronucleus formation, and sister chromatid exchange in aquatic organisms (Michailova et al. 2018), underscoring the genotoxic threat posed by Cd contamination.

Species specific differences in susceptibility to Cd induced DNA damage were evident with *P. microcephalum* showing higher tail moment values and greater DNA fragmentation than *P. pruinosum* under identical exposure conditions. This observation aligns with earlier research suggesting that genetic makeup and metabolic capacity influence the detoxification and repair responses to heavy metals (Ahmad Hadri & Suhaila, 2023; Kusakabe et al. 2019). For instance, differential expression of metallothioneins and antioxidant enzymes may confer varying degrees of protection against Cd toxicity (Carter & Parsons 2016). Such interspecies variability has important ecological implications, as it may affect species resilience, population dynamics, and community structure in contaminated habitats (Barraclough 2015). Besides, the ecological consequences of Cd induced genotoxicity in *Pseudagrion* species extend beyond individual-level effects. DNA damage can impair critical biological processes such as development, metamorphosis, and reproduction, ultimately reducing fitness and survival (Kim

et al. 2023; Badisa et al. 2007). Given the role of dragonflies as both predators and prey within freshwater ecosystems, their decline can disrupt trophic interactions and ecosystem functioning (Prorok et al. 2021). Furthermore, as bioindicators, changes in the genetic health of *Pseudagrion* populations may serve as early warning signs of broader environmental degradation (Ahmad Hadri & Suhaila 2023; Kietzka 2019).

Furthermore, the dose-dependent increase in DNA damage observed in this study highlights the environmental risks associated with Cd contamination in aquatic ecosystems. While laboratory exposures often involve controlled, constant Cd concentrations, natural environments typically experience fluctuating and lower Cd levels, which may require longer exposure durations to elicit detectable genotoxic effects (Nagaraju et al. 2022; Pinot et al. 2000; Tang et al. 2015). This variability underscores the importance of considering both concentration and exposure time in ecological risk assessments. Moreover, the cumulative and synergistic effects of Cd with other pollutants, such as zinc, have been shown to exacerbate DNA damage in aquatic organisms (Michailova et al. 2018), suggesting that complex pollutant mixtures pose heightened threats to aquatic biodiversity (Reid et al. 2019; Fleeger et al. 2003). Understanding the mechanisms and dynamics of DNA damage and repair in *Pseudagrion* species is crucial for developing effective conservation strategies. Monitoring genotoxic biomarkers like comet assay parameters can provide sensitive indicators of sublethal pollutant effects, enabling early intervention before population declines occur (de Lapuente et al. 2015; Kumaravel & Jha 2006). Conservation efforts should focus on reducing heavy metal inputs through improved wastewater treatment, stricter industrial regulations, and habitat restoration to enhance water quality (Augustyniak et al. 2020). Protecting genetic diversity within *Pseudagrion* populations may also improve their adaptive capacity to withstand environmental stressors. Future research should aim to elucidate the molecular pathways governing Cd detoxification and DNA repair in these species, employing genomic and proteomic approaches to identify key genes and proteins involved (Ma et al. 2024). Investigations into potential transgenerational effects and adaptive responses to chronic Cd exposure will further inform risk assessments and conservation planning. Additionally, field studies assessing genotoxicity in natural populations exposed to variable pollutant regimes will provide ecologically relevant data to complement laboratory findings (Prorok et al. 2021).

CONCLUSION

In conclusion, this study confirms that Cd exposure induces significant DNA damage in *P. microcephalum* and *P. pruinosum*, with temporal patterns reflecting both damage induction and repair. The species-specific differences in susceptibility underscore the need for tailored conservation approaches. Given the ecological importance of these dragonflies and their sensitivity to heavy metal pollution, protecting their genetic integrity is essential for maintaining freshwater ecosystem health in the face of increasing anthropogenic pressures.

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AUTHORS DECLARATIONS

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No conflict of interest

Ethics Declarations
No ethical violations

Data Availability Statements
The data from this study were obtained from a PhD thesis research project entitled 'The Potential of Coenagrionidae Larvae (Odonata) as a Biological Indicator for Freshwater Ecosystem.'

Author's Contributions
Siti Hamidah Ismail (SHI) collected samples, prepared the slides, analyzed the data and drafted the initial version of the manuscript. Suhaila Ab. Hamid (SAH) designed the research and rewrote and revised the manuscript.

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