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ASSESSMENT OF MALATHION INDUCED TOXICITY IN *DATTAPHRYNUS MELANOSTICTUS* TADPOLES: A BIOCHEMICAL INVESTIGATION

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ABSTRACT: Environmental pollutants apart from other factors like habitat loss and interventions of invasive species, is regarded as a likely cause towards a worldwide decline in amphibian population. The present study attempts to elucidate the toxicity of commercially formulated malathion (MAT) by investigating biochemical aspects in *Dattaphrynus melanostictus* tadpoles. Three sublethal concentrations of MAT (1.0, 1.8, and 2.5 mg/l) were considered to which the tadpoles (Gosner stage 27) were exposed for five days. The outcome of the present investigation revealed a significant decline ($P<0.05$) in activities of catalase, superoxide dismutase, glutathione peroxidase while suggesting a significant elevation in lipid peroxidation. The inhibition acetylcholinesterase activity confirmed MAT as anticholinesterase product. The overall outcome of the present investigation suggests the toxic potentials of MAT; which could have possibly resulted in compromised antioxidant status and neurobiochemical makeup of the exposed tadpoles. Thus, based on the results obtained, it could be ascertained that the commercial grade MAT may pose a potential threat to the tadpoles of *D. melanostictus* under the selected sublethal concentrations. The study further validates the feasibility to measure the intensity of aquatic pollution in the course of regulatory surveillance and monitoring the waters with suspected organophosphate contamination.

INTRODUCTION: Large scale anthropogenic activities have been associated with the drastic decline of amphibian populations globally [1, 2, 3]. Evaluation by the International Union for Conservation of Nature, category for vulnerable or critically endangered species suggested that 32.5% of total amphibian species have declined in terms of their number, which is far critical than for birds and mammals [4, 5].

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Amphibians, unlike other animals, constitute a unique group among many ecosystems due to their active and multiple roles as, prey, predators and herbivores [6, 7]. Their contribution to trophic dynamics makes them one of the crucial features in determining the survival ability of other organisms through food chain [8, 9]. Hence, their existence at a certain population ratio could be considered as accountable for the continuity of other species as well. Even though the loss of habitat is considered to be the primary reason behind amphibian decline [10], the role of pesticide contamination in freshwater habitats often questions its contribution in survival rate and reproduction of anurans [11].

In addition to this, tadpoles are known to complete a part of their life cycle (larval development) in an

aquatic medium like ponds and lakes to which the pesticidal effluents from agricultural runoffs often find their way [12, 13, 14]. The rich permeability of skin and egg, which often results in getting absorbed, persist, and bioaccumulate further explains the vulnerability of tadpoles to environmental xenobiotics [15].

A number of factors like morphological deformities, compromised reproducing ability, immune-suppression, and reduction in growth and development has indicated the potential risk of pesticide contamination against anurans [16]. The use of integrated biomarker approaches for studying the inter-cascading changes in biochemistry has become an advanced strategy for reporting the overall health of tadpoles under the toxicological point of view [17, 18]. Amphibian susceptibility to insecticides has been very well acknowledged in the past [15] and proven to be critical enough for compromising growth and development [19].

These insecticides are further known to indirectly increase susceptibility to parasite infection by decreasing the activity patterns in tadpoles [20]. Since tadpoles are capable of avoiding free-swimming parasites by either moving away or swimming in erratic patterns [21], the response through means of locomotory perception upon suffering pesticide-induced physiological trauma is highly critical in determining their survival tendencies [22] and hence, cannot be overlooked.

Previous reports have suggested the involvement of toxicants in imparting oxidative stress by random generation of reactive oxygen species [23, 24, 25] and also known to pose catastrophic potentials against neurotransmission in tadpoles that may result in impaired swimming abilities [26]. However, literature support on evaluations of MAT toxicity in tadpoles of *Dattaphrynus melanostictus* is found to be limited. Even though *D. Melanostictus* is found being fairly resistant as compared to other species of tadpoles [27], susceptibility in terms of antioxidant enzyme status and neurobiochemical activity cannot be ignored under the declining trend of its population. In addition to this, the effect of MAT on activities of free oxyradical scavenging enzymes and neurotransmission capabilities lacks in literature. Therefore, an attempt has been made in the present study to investigate the toxic potentials of sublethal concentrations of commercially formulated MAT on *D. melanostictus* tadpoles.

MATERIALS AND METHODS:

Toxicant Selected and Test Solutions: Commercial grade malathion of 50% EC (MAT) was selected as the toxicant for the present study and was procured from the local market (Dharwad, Karnataka, India). The stock solution was prepared by dissolving 1 gram of MAT in 100 ml of double distilled water. The requisite test concentrations were freshly prepared by diluting the stock solution before the initiation of toxicity studies.

Procurement and Maintenance of Tadpoles: Five hundred and fifty tadpoles of *Dattaphrynus melanostictus* (Stage 20) were collected from uncontaminated ponds located in Karnatak University campus, Dharwad city (Karnataka, India) and were transported to the laboratory with care. The tadpoles were acclimatized to laboratory conditions, during which they were fed with plant origin feed and boiled spinach *ad libitum*. The water in aquaria was renewed every two days, and excess of food and feces were removed. For exposure studies, tadpoles were divided into four groups, namely, control (C) and Exposure 1 (E1), Exposure 2 (E2), Exposure 3 (E3). The groups namely C, E1, E2, and E3 received MAT concentrations of 0.0, 1.0, 1.8 and 2.5 mg/l respectively. Each group consisted of 10 tadpoles (n=10) and were maintained in triplicates. Tadpoles were transferred to glass aquaria only after thorough inspection and identification of its stage (Stage 27) according to Gosner [28]. Each glass aquaria consisted of 10 liters of water, which was dechlorinated through aeration. All the experiments were carried out at 25 ± 2 ºC with a light-dark cycle of 14:10 hours, which was being maintained throughout the completion of the investigation. Besides, temperature, pH, and dissolved oxygen levels were monitored daily **Table 1**. The mean values of individual group were taken into account for the present study.

Antioxidant Assay: The antioxidant status was determined by analyzing variation in the activity of antioxidant enzymes. The antioxidant enzymes, catalase, superoxide dismutase, glutathione peroxidase, and lipid peroxidation level was determined by the methods of Luck [29], Kakkar *et*

al.,[30], Paglia and Valentine [31] and Buege and Aust [32] respectively.

TABLE 1: SHOWING VALUES FOR QUALITY ASSESSMENT OF WATER USED IN THE PRESENT INVESTIGATION

Parameter	Values obtained
Temperature	24 ± 1 ºC
pH	7.1 ± 0.3
Dissolved oxygen	6.1 ± 0.4 mg/L
Total Hardness	37.3 ± 3.1 mg as $CaCO_3$/L
Salinity	Nil
Specific gravity	1.003
Calcium	21.31± 0.27 mg/L
Phosphate	0.9 ± 0.04 mg/L
Magnesium	0.85 ± 0.3 mg/L

Determination of AChE Activity: The activity of acetylcholinesterase was determined by the methodology as described by Ellman *et al.* [33]

Statistical Analysis: The enzymatic activities of neurobiochemical and antioxidant status are reported as the mean ± standard error of the mean (SEM) obtained from triplicates. The data were subjected to one-way analysis of variance and further subjected to Tukey's test for post hoc analysis by defining the significance level at P<0.005.

Ethical Committee: All procedures implemented in the present study were in accordance with the guidelines of the Institutional Animal Ethics Committee (IAEC). The animals subjected to experimentation were handled as per the guidelines issued by the Committee for the Purpose of Control and Supervision of Experiments for Animals (CPCSEA), New Delhi, India.

RESULTS: The results from the present investigation suggested the toxic insult due to MAT intoxication which was by inducing oxidative stress due to variations in all the considered antioxidant enzyme activities and curtailing acetylcholinesterase activity in all groups affecting the biochemistry exposed tadpoles. The variation of catalase activity in'E1' was noticed which showed a variation of +18.43%, followed by the change of -28.49% and -60.41% in 'E2' and 'E3' respectively when compared with control 'C' **Fig. 1**.

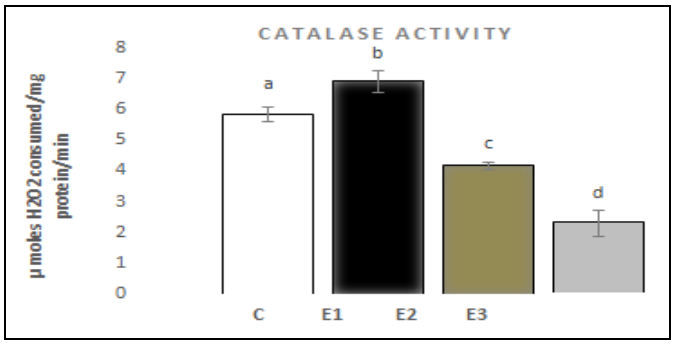

FIG. 1: CHANGES IN CATALASE ACTIVITY OF *DUTTAPHHRYNUS MELANOSTICTUS* FOLLOWING EXPOSURE TO MAT FOR DURATION OF 5 DAYS. Values are mean ± SE with level of significance defined at P≤0.05. Similar alphabets indicate insignificant difference

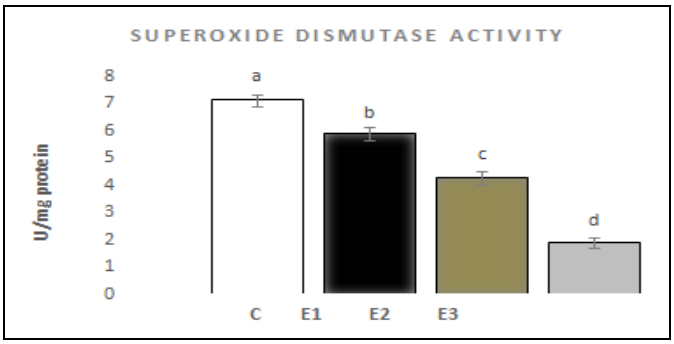

FIG. 2: CHANGES IN ACTIVITY OF SUPEROXIDE DISMUTASE IN *DUTTAPHHRYNUS MELANOSTICTUS* FOLLOWING EXPOSURE TO MAT FOR DURATION OF 5 DAYS. Values are mean ± SE with level of significance defined at P≤0.05. Similar alphabets indicate insignificant difference

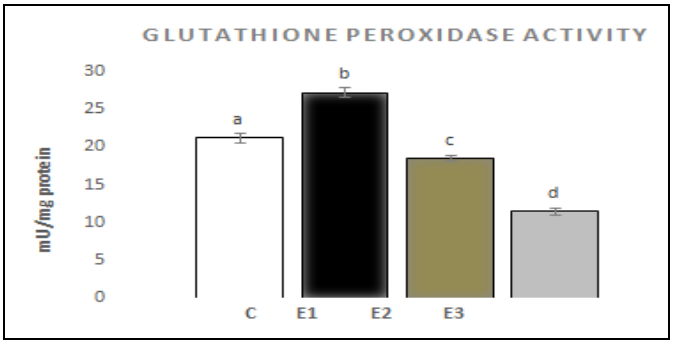

FIG. 3: CHANGES IN ACTIVITY OF GLUTATHIONE PEROXIDASE IN *DUTTAPHHRYNUS MELANOSTICTUS* FOLLOWING EXPOSURE TO MAT FOR DURATION OF 5 DAYS. Values are mean ± SE with significant difference at P≤0.05. Same alphabets indicate insignificant difference

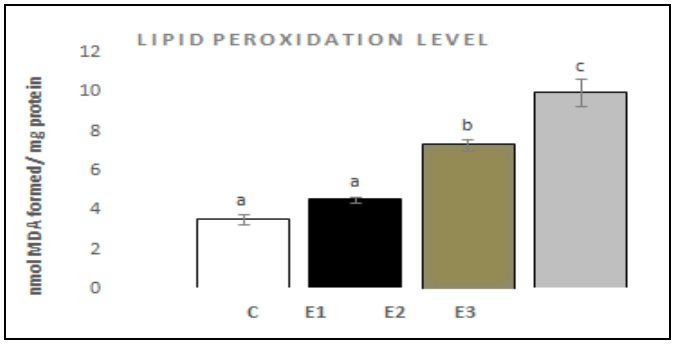

FIG. 4: CHANGES IN ACTIVITY OF LIPID PEROXIDATION IN *DUTTAPHHRYNUS MELANOSTICTUS* FOLLOWING EXPOSURE TO MAT FOR DURATION OF 5 DAYS. Values are mean ± SE with significant difference at P≤0.05. Same alphabets indicate insignificant difference

The decline in the activity of SOD was observed in a uniform trend which demonstrated the continuous destruction of its enzymatic activity. The percent decline in SOD activity was -17.13%, -39.60% and -73.73% in'E1', 'E2' and 'E3' respectively as compared to 'C' **Fig. 2**. A significant difference was also noticed in the activity of GPx in the tadpoles of different groups, **Fig. 3**. The percent change in expression patterns of GPx was found to be; +28.27%, -12.48% and -45.85% in 'E1', 'E2' and 'E3' respectively when compared with 'C.'

The lipid peroxidation was found to elevate under the influence of MAT, and the variations of the same have been presented in **Fig. 4**. The increase in lipid peroxidation of exposed tadpoles witnessed in the present study was continuous, and the increase in MDA formation has been presented in **Fig. 4**. The significant difference in 'E2' and 'E3' was noticed in exposed tadpoles unlike 'E1'. Upon correlation, the results indicated the frequency of damage to be highest in tadpoles belonging to 'E3' which endured the highest amount of toxicity caused by the maximum concentration of MAT after 5 days. AChE activity in tadpoles was also found to be affected under the influence of the MAT. Changes in the activity of AChE were noticed in all the exposed tadpoles as compared with control **Table 2**.

TABLE 2: CHANGES IN ACETYLCHOLINESTERASE ACTIVITY OF CONTROL AND EXPOSED TADPOLES OF *DUTTAPHRYNUS MELANOSTICTUS* FOLLOWING MAT EXPOSURE FOR 5 DAYS, VALUES ARE EXPRESSED AS nmole OF PRODUCT FORMED min/ mg/ PROTEIN

Parameter/group	C	E1	E2	E3
Acetylcholinesterase	7.81 ± 0.24^{a}	6.64 ± 0.2^{b}	5.02 ± 0.25^{c}	2.15 ± 0.16^{d}
Percent change	-----	-14.98	-35.72	-72.471

Values are means ± SEM. Means with different superscripts are significantly different ($p<0.01$), while means with the same superscripts indicate non-significant changes according to one-way ANOVA with Tukey's post hoc test.

DISCUSSION: Aquatic pollution due to indiscriminate use of pesticides has been acknowledged to be one of an important anthropogenic source against amphibian survival and is known to affect a large fraction of the aquatic ecosystems around the globe. Amphibian species are vulnerable to contaminants because of the greater permeability of the skin and the presence of gills during the larval stage [34, 35]. Oxidative metabolism is a regular phenomenon in the cell environment [36, 37,] and its controlled limits are governed by biochemical makeup in different aquatic organisms including fish [38, 39], and amphibians [40]. The biochemical makeup, constitutes several sections, of which, antioxidant enzymes form an imperative part [36]. The antioxidant pool further comprises a cluster of free oxyradical scavenging enzymes, *viz.* catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) which are known to be the first line of defense against oxidative stress triggered by arbitrary metabolic process *in-vivo* [41]. Thus, their over and under expressions are known to play a crucial role in the maintenance of the health status of an organism [42].

The present investigation reveals the interference of MAT in oxidative metabolism and thus initiating the cascading changes by the inducement of oxidative stress in *Dattaphrynus melanostictus* tadpoles. Metabolic breakdown of intoxicated xenobiotics can lead to ROS production at a considerable amount, which is sufficient to compromise the health status of an organism [43]. The significant variation in the activity of catalase enzyme witnessed in the present study is in agreement with the studies of David *et al.*, [44] who reported the decline in catalase enzyme activity under the influence of a synthetic pyrethroid, cypermethrin.

In the present study, the elevation of CAT at 'E1'and depletion at 'E2' and 'E3' are in agreement with the previously reported studies of Costa *et al.*, [45] who suggested the discrepancies in CAT activity of bullfrog under the influence of Round up®. Variation in catalase activity in the present study did not follow a uniform pattern and was inconsistent with MAT concentration. This may be due to some other extraneous factors which perhaps may not be captured within the boundary of the present study. However, it could be emphasized that the biochemical demolition and repair are the two simultaneous yet individual processes, and this could have accounted for uneven patterns of catalase expression.

Further, the present report on the significant decline in CAT activity in the *D. melanostictus* tadpoles begs the question of whether the exposed tadpoles could be more tolerant to MAT induced oxidative stress and whether high levels of MAT deactivates CAT or overwhelms the tadpoles to synthesize CAT under mild concentrations. The SOD activity in tadpoles was found to decline under the presence of MAT. Ojha *et al.*, [42] suggested the active role of organophosphorus pesticides in modulating the activity of SOD in a significant fashion. The intracellular environment could be the space for the generation of superoxide anion, which may be the consequence of foreign chemical component participation [46]. This generally initiates the active role of SOD upon which it swiftly terminates the harmful radical under the action of body defense mechanism. The constant interaction between elevated levels of superoxide radicals and inadequate magnitude of its counterpart SOD might have resulted in an incessant enzymatic decline in the present investigations.

The glutathione peroxidase activity is known to be a crucial indicator in determining the levels of oxidative stress [47]. The elevated activity of GPx at 'E1' and its subsequent reduction at 'E2' and 'E3' may be due to its active role during countering of the free oxyradicals generated during MAT detoxification. The other reason may be perhaps due to the GPx sharing of its substrate H_2O_2 with CAT. Since the affinity of CAT towards H_2O_2 is much lesser than GPx [48], and its induction under mild MAT stress could have been a biochemical response of exposed tadpoles. The uniform and irregular trend in decline of SOD and CAT respectively, in the tadpoles exposed to MAT, begs the need for an alternative mechanism to reimburse enzymatic status which was seen as a response under the compensatory act of GPx [49].

This mechanism further convinces the marked increase in GPx activity at E1, during which there was an insignificant change of CAT and significant change in SOD activity. These statements are in support of studies reported by Santos *et al.,* [50] who witnessed the changes in GPx activity in *Phyllomedus aiheringii* exposed to polluted water. This phenomenon may perhaps be adequate to convince the metabolic synthesis of GPx activity under varying concentrations of MAT.

MDA, being one of the major end products of lipid peroxidation [51], is also thought to be a cause in fabricating the arrest of cell function through elevating the condition of oxidative stress [52]. Increased LPO was observed in the present investigation which can be implicated to MAT interaction with cellular metabolism [53], stated that the increased LPO may be due to intervention of a toxic substance such as pesticides, which matches the outline of present condition, wherein, increase in LPO was clearly found to be based on concentration of investigated toxicant MAT. The present findings of increased LPO and decreased antioxidant enzyme activity can reaffirm strong support to suggested hypothesis stating induced LPO could be the consequence of decreased antioxidant enzyme status or *vice versa* [54].

The acetylcholinesterase activity in tadpoles exposed to different concentrations of MAT is presented in **Table 2**. From the outcome of the present investigation, it could be noted that, *in-vivo* exposures to sublethal concentrations of MAT led to a concentration-dependent inhibition of AChE activity. The significant decline in activity of acetylcholinesterase of exposed tadpoles which was observed in the present investigation can be compared with the previously the findings of Lopez-Lopez *et al.*, [55] who reported the significant decline in AChE activity of *Girardinichthys viviparous* (Bustamante) to untreated domestic wastewater, agricultural runoff and sewage treatment plant effluent. AChE is recognized as a crucial neurotransmitter is known to play a vital act in regulation of cholinergic nervous transmission.

In a report given by Scaps and Borot [56] suggested the inhibition of AChE in the polychaete *Nereis diversicolor* upon exposure to a carbamate insecticide- carbamyl. Detrimental effects of pesticide mediated inhibition of AChE activity are evident from the current investigation; this is in the background of their sensitivity and urgent need towards their declining population worldwide [57]. Reports provided by few authors have suggested digressed locomotory behavior in *Neomysis integer* [58] and *L. vannamei* [59] that could be due to inhibition in cholinesterase activity of exposed animals. Several reports have indicated locomotive distress which in turn has been linked to the impaired status of AChE activity in the freshwater oligochaete

Lumbriculus variegatus two days after of exposure to azinphos-methyl [60]. Therefore, links established in our present study could reaffirm the outcome of AChE inhibition as a biomarker which may further contribute in evaluating the detrimental speculations that could be caused by organophosphorus insecticide MAT on health status and survival ability of *D. melanostictus* tadpoles. The current study further clearly implicates the possibilities of MAT in causing impaired swimming abilities in tadpoles, following exposure to sublethal concentrations for 5 days.

CONCLUSION: The present investigation suggests that the commercial formulations of MAT can induce oxidative stress through a decline in antioxidant enzyme activity, which may further lead to oxidative damage in *D. melanostictus* during metamorphosis. MAT was also known to demonstrate antagonistic property against acetylcholinesterase activity in exposed tadpoles. Thus, based on the outcome of the present investigation, it is suggested that necessary precautions should be taken before its use and disposal under the proximity of active amphibian habitats, to conserve the vanishing amphibian species. Further, the assays included in present study validate the feasibility to measure the toxicity of MAT in terms of antioxidant stability, anticholinesterase potentials in the course of regulatory surveillance and monitoring the waters with suspected MAT contamination.

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CONFLICT OF INTEREST: The authors hereby declare no conflict of interest.

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