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ORIGINAL ARTICLE

Sublethal effects of malathion insecticide on growth of the freshwater crab *Poppiana dentata* (Randall, 1840) (Decapoda: Trichodactylidae)

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ABSTRACT

Pesticides can interfere with various aspects of growth and the normal molt cycle of a crustacean. *Poppiana dentata* (Randall, 1840), an indigenous crab species, spends most of its life cycle in, and proximal to, benthic sediments in which pesticide residues can reside. This study sought to assess the sublethal effects of a locally-used, commercial malathion insecticide on growth aspects of *P. dentata*. Juvenile crabs were obtained from berried females collected in northwest Trinidad. Young crabs were placed in a control (insecticide-free) treatment and an exposure treatment involving continuous exposure to the malathion insecticide, at 10 µg/L concentration over five months (n = 4 crabs/treatment). Carapace width (CW), length (CL) and intermolt period were recorded and used to derive size increment, specific growth rate (SGR), growth curves and logistic equations. Malathion-exposed crabs exhibited irregular patterns in SGR and size increment. Exposed crabs also exhibited a delay in molting and longer intermolt periods, compared to the control crabs ($p < 0.05$). Breakpoint (17.5 mm CW) and maximum size ($CW = 25.77\ (1+\exp\ (1.500–0.056t))^{-1}$) for exposed crabs were relatively smaller than those of the control (22.11 mm CW; $CW = 34.30\ (1+\exp\ (1.774–0.035t))^{-1}$). Findings indicate that sublethal exposure to malathion insecticide altered growth patterns in *P. dentata,* some of which can influence maturity and later cascade into secondary consequences for local populations.

KEYWORDS

Endpoint, organophosphate pesticide, specific growth rate, Trichodactylidae

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Introduction

Growth is a key endpoint for evaluating the effects of both endogenous and exogenous factors. Growth is characterized as a discontinuous process for crustaceans, whereby increases in size take place when the rigid, calcified cuticle is shed during ecdysis or molting. The release of ecdysteroid (*e.g.,* ecdysone) into circulation by the paired Y-organs is usually associated with the onset of molting, but neurohormones produced in the X-organ-sinus gland complex (*e.g.* molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH)), regulate ecdysteroid release and synthesis by the Y-organs (Dell *et al.*, 1999; Nakatsuji *et al.*, 2000; Chung, 2010). This interplay of hormones along intricate pathways, coupled with limb regeneration, control the molting cycle in crustaceans.

Sublethal effects of toxicants can compromise an organism's fitness through impairing essential traits, including growth (Connell *et al.*, 1999). Growth can also be indirectly impacted through modification of energy allocation and sudden metabolic demands, brought on by the stress responses of an organism exposed to a toxicant. Xenobiotics, in particular, can interfere with the normal intermolt period (number of days between two successive molts) of the molt cycle of a crustacean, along with negatively influencing size increments and growth rates. Xenobiotic compounds are considered 'foreign compounds' which are not normally present in the environment, but rather introduced and/or produced by human activities (Connell *et al.*, 1999). Even at sublethal levels, toxicants like pesticides, can increase respiratory energy demands for estuarine crustaceans while consequently lowering the energy reserved for new tissues, as well as growth efficiency rates for juvenile development (Negro *et al.*, 2011). These toxicants can even impair the foraging ability of organisms, compromising energy acquisition, and by extension, growth.

Alteration of growth in life stages sensitive to toxicants can be useful as early warning indicators for detrimental effects in aquatic populations (Negro *et al.*, 2011). Disruption in growth and development at an earlier, vulnerable stage of life in an organism (*e.g.,* juvenile stage) can eventually lead to a succession of deleterious consequences, extending from the individual to population and community levels. This is because growth is essentially linked to other crucial aspects like reproduction and fitness. Juvenile development is a fundamental phase in the lifespan of a species, such that this stage, along with reproduction, represents common endpoints used to evaluate interspecific vulnerability, in relation to chronic toxicity (Amiard-Triquet and Amiard, 2013). Examining how a toxicant modifies the typical growth pattern in the juvenile phase can provide in-depth understanding of the long term, ecological risks faced in adulthood. Various studies have looked at the impacts of organophosphate (OP) insecticides on embryo and juvenile stages of freshwater decapods. In all cases OPs were shown to disrupt the normal growth of the life stage. These studies record the effects of sublethal and lethal concentrations of chlorpyrifos-based insecticide on growth and survival of the freshwater prawn, *Palaemonetes argentinus* Nobili, 1901 (Montagna and Collins, 2007), chronic exposure of a commercial chlorpyrifos formulation on growth of the trichodactylid crab, *Trichodactylus borellianus* Nobili, 1896 (Montagna, 2010), Diazinon 60EC exposure on young-of-the-year and juveniles of two crayfish species, *Pacifastacus leniusculus* (Dana, 1852) and *Orconectes limosus* (Rafinesque, 1817) (Buřič *et al.*, 2013) and sublethal concentrations of chlorpyrifos on embryonic hatching success, hatching time and post-hatching survival of the burrowing crab, *Zilchiopsis collastinensis* (Pretzmann, 1968) (Negro *et al.*, 2014).

Assessing the effects of non-discriminate biocides, like OP insecticides, on growth can be beneficial in terms of understanding the long-term consequences faced by non-target populations. Freshwater, non-target species are particularly vulnerable to impacts of insecticides and require high research priority since contamination of their aquatic habitats can easily occur due to extensive spraying from nearby agricultural lands or via community vector control. OP pesticides represent popular biocides for combating insects, due to their cost-effectiveness, high toxicity toward pests and relatively quick environmental degradation. OPs, such as malathion, encompass the top insecticides for governmental and commercial use (Fulton and Key, 2001). It is commonly applied as part of vector control measures for combating

mosquito-borne diseases in many tropical countries, including in Trinidad and Tobago. A total of 15 pesticide products are registered for use in Trinidad and Tobago and contain malathion as the active ingredient (AI) (Ministry of Health, 2020). An active ingredient refers to the chemical constituent contained in a pesticide product that controls pests and is usually listed by its name and percentage by weight on the product label (USEPA, 2019). Malathion is an organophosphorus compound that represents an anticholinesterase insecticide, such that the mode of action is inhibition of the neurotransmission enzyme, acetylcholinesterase (Bookhout and Costlow, 1976). The latter has been designated as a feasible biomarker for indicating exposure to an OP insecticide. Despite its higher environmental biodegradability in relation to organochlorine pesticides, it still remains a highly toxic chemical for both target and non-target biota. A consequence of its biodegradable nature would be the need for repeated applications of the chemical and the development of insect pest resistance; both of which would incite higher malathion concentrations during application and introduce greater risk to non-target biota. Early research attributes malathion to delayed, late offspring development of brachyurans, such as for the mud crab, *Rhithropanopeus harrisii* (Gould, 1841) and blue crab, *Callinectes sapidus* Rathbun, 1896 (Bookhout and Costlow, 1976). The authors noted a delayed zoeal development and lengthening of the time period between hatching and the first crab stage, with increasing concentrations of malathion.

To date, there is no information on the effects of OP pesticides on the freshwater crab species, native to Trinidad. *Poppiana dentata* (Randall, 1840) is one of only three freshwater, indigenous crab species reported for Trinidad (Singh *et al.*, 2020a) and its lifecycle is spent mainly in the freshwater environment, living and feeding in close proximity to the water and benthic sediments where pesticides can reside. Organophosphates, like malathion, are toxic to freshwater species (Pham, 2017), so there is a need for investigating the exposure risk faced by non-target biota, residing in contaminated aquatic habitats. Therefore, the goal of this study was to conduct such an assessment, by examining the chronic effects of a commercial malathion insecticide, at sublethal concentration, on the growth of juvenile *P. dentata* crabs under laboratory conditions. Growth patterns of crabs in this bioassay were evaluated by: (1) specific growth rate (SGR) and (2) breakpoint sizes, growth curves and logistic equations derived from growth components of intermolt period and size, as measured by carapace width (CW) and carapace length (CL).

Material and Methods

Acquisition of crabs and experimental design

This study comprised juvenile crabs obtained from wild stock females. Berried females of *P. dentata* (Family Trichodactylidae) were collected from Bamboo, in the northwest of Trinidad (10°37'49.2"N 61°25'51.2"W). This site is a freshwater waterway that drains into the Caroni River, located in the northwestern region of Trinidad. Mesh traps, with mesh size of 3.18 mm, were deployed along three accessible points along the waterways, approximately 50 m apart. Each site in which the captured *P. dentata* resided was shown to have low or minimal biocide presence, as affirmed by negative test results derived from prior qualitative (presence/absence) testing of water from the collection points (*in situ*), using rapid pesticide detection Agri-Screen® Tickets (Neogen® 2019). Berried females (n = 2) were removed from the traps and their species identification confirmed using the species key of Magalhães and Türkay (2008).

The growth period for this bioassay was selected as five months, since growth beyond this point would have been possibly influenced by natural reproductive maturation processes. This period was selected using a prior baseline study on *P. dentata*, which yielded breakpoint sizes at structural reproductive maturity of 16.84 mm CW (female) and 28.40 mm CW (male). The 5-month period was selected based on the length of time for male crabs to reach a size of 28.75 ± 2.98 mm CW (Singh *et al.*, 2020b).

The commercial malathion insecticide was chosen for this study as it is extensively used in local vector control and agricultural applications and was assumed to be prevalent in indigenous aquatic environments. Malathion is one of the pesticides that has been involved in dengue prevention and vector control strategies for the *Aedes aegypti* (Linnaeus, 1762) mosquito throughout Caribbean countries,

for approximately 20 to 30 years (Rawlins, 1998). In addition, previous work assessed sublethal concentrations (0.1, 1 and 10 µg/L) of the same malathion insecticide on acetylcholinesterase (AChE) activity in *P. dentata*, revealing significantly high inhibitions (> 70 %; $p < 0.05$) across tissues for 10 µg/L (Singh, unpublished data). Sublethal effects of a toxicant can still adversely impair organism fitness and imply a change in crucial physiological processes, such as growth (Newman and Unger, 2003). It was, therefore, reasonable to hypothesize that growth in *P. dentata* would be affected by this OP and at the normal working concentration.

Chemical description and preparation

The organophosphate insecticide used in this bioassay is commercially available as an emulsifiable concentrate (EC) formulation, Fyanon® (CHEMINOVA), containing 57 % malathion as the active ingredient (AI). This insecticide product is state registered for use (locally) and is available for purchase through many local commercial retailers by agricultural and domestic users. The Material Safety Data Sheet (MSDS) for the insecticide was obtained from the distributing commercial company from which the pesticide was purchased. The original concentration of malathion contained within the total formulation volume (listed by the manufacturer's MSDS) was then computed. From this, the quantity of commercial formulation that had to be added to the test solution, to maintain a 10 µg/L concentration of malathion in the test water, was determined. Dilution of the original formulation was then performed using the relevant volumes and according to application directions on the product label. Dilution was carried out with distilled water, in order to prepare the exposure test solutions at 10 µg/L concentration. Diluting the pesticide with water (*versus* an organic solvent like acetone) according to the preparation guidelines resulted in a more practical test solution that was comparable to solutions generally prepared by pesticide users, and by extension, what would typically be introduced into the habitats of *P. dentata*. The same concentration was analyzed prior to the experiment, using Gas Chromatography-Electron Capture Detector (Shimadzu GC 2010). This affirmed that the concentration of malathion contained within the insecticide formulation was maintained and was close to the nominal concentration in the test water (retention time: 7.473 minutes; concentration: 9.36 µg/L). It should also be noted that any reference to malathion in this study results, refers explicitly to effects caused by the commercial formulation of the malathion insecticide and not the analytical grade form of malathion.

Experimental conditions and growth measurements

Newly hatched crabs were selected and assigned to two treatment groups; a control group ($n = 4$ crabs) and an exposure group ($n = 4$ crabs). The latter group is referred to as the malathion-exposed cohort or exposure cohort throughout this work. It should be noted that any newly hatched crabs with missing or lost appendages were not used in this study. Therefore, the number of hatched crabs that met these selection criteria, as well as the material resources (lab infrastructure and resources) that were available prior to initiation of the experiment, both limited the replicate number and insecticide concentrations that could be evaluated. In addition, the number of replicates within the designated laboratory space was limited, to ensure ethical conditions for the animals were met.

The growth patterns associated with the control cohort were taken to represent growth under normal conditions and were monitored synchronously with the exposure cohort, under the same physiochemical and photoperiod conditions. All replicate crabs were of the same age. The malathion-exposed cohort were of initial size 2.96 ± 0.29 mm CW, 2.29 ± 0.10 mm CL (mean ± SD) and those of the control group were 3.04 ± 0.36 mm CW, 2.60 ± 0.22 mm CL. Each crab was reared in a glass aquarium (length 8 inches × width 6 inches × height 6 inches) containing 1600 mL of laboratory prepared, de-chlorinated water. Crabs were observed daily over a 5-month period, under laboratory conditions of dissolved oxygen (DO) at 6.6 ± 0.1 mg/L, pH at 7.60 ± 0.36, temperature at 26.1 ± 0.1 °C and a photoperiod of 12 h light:12 h dark. Previous monitoring at the specimen collection site revealed similar temperatures (mean ± SD: 26.1 ± 0.3 °C; range: 25.8–26.4 °C), pH (7.59 ± 0.31;

6.79–7.93) and DO concentration (6.1 ± 0.9 mg/L; 6.02–7.84 mg/L). The photoperiod was chosen since it is analogous to the diurnal cycle that occurs throughout most of the year in Trinidad.

The newly hatched crabs were allowed to acclimate to laboratory conditions for a period of six to seven days, during which they were allowed to undergo their first three molts under the same pesticide-free growing conditions as those of the control. After their third molt and hardening of their exoskeletons, crabs of the exposure cohort were then subject to continual exposure of 10 µg/L malathion insecticide for the remainder of the 5-month period. Any variations in growth during the pesticide exposure phase, therefore, would have been due to the toxicant, and not from acclimation to the laboratory conditions. In addition, this initial period in which growth is typically highest, allowed the crabs to grow unhindered in pesticide-free conditions, and by extension, ensured that the crabs were healthy and well acclimated.

A renewal of freshly prepared (10 µg/L malathion insecticide) test solution was performed every two days for the exposure cohort. The quantity of insecticide formulation was added to the test solution, such that malathion was maintained at a 10 µg/L concentration. Therefore, the cohort was exposed to a fairly continuous concentration of the insecticide malathion (at 10 µg/L) in the test water, at this renewal rate. Montagna and Collins (2007) applied a similar renewal regime when assessing the effects of another OP insecticide (Terminator Ciagro 48 % chlorpyrifos) on juvenile *Pa. argentinus* freshwater prawns. The authors applied a renewal rate of 70 % of the test solution in each container, every two days. In our experiment water renewal was done for the control cohort with laboratory-prepared (pesticide-free) water only. The water in each test aquarium was also monitored weekly, for temperature, DO and pH to ensure that water quality remained constant, so as to avoid varying the malathion concentration.

The feeding regime entailed crabs being fed daily, using a varied diet of Hikari crab cuisine pellets (Kyorin Food Industries Co. Ltd.) and segments of the aquatic waterweed, *Egeria densa*. The latter represents a common weed species generally found throughout habitats of *P. dentata* (personal observation). The regime involved crabs of CW < 5 mm being fed 2 g of pellets and 2 g of *E. densa* segments, CW ≥ 5–15 mm were fed 5 g of the pellets and 4 g of *E. densa* segments, CW > 15–25 mm were fed 10 g of pellets and 6 g of *E. densa* plant segments, and CW > 25 mm were fed 24 g of pellets and 6 g of *E. densa* segments. This diet and regime were considered suitable for proper growth since it was similarly applied in the prior, baseline growth study (Singh *et al.*, 2020b) in which crabs were observed to exhibit normal growth and behavior. Removal of uneaten food and feces was performed along with renewal of test water. It should be noted that there were no occurrences of mortality throughout the monitoring period.

Carapace width (CW) and carapace length (CL) were measured from the molted exoskeletons or exuviae, in order to avoid stress by handling molted crabs. A digital caliper (VINCA DCLA) was used to measure to the nearest hundredth of a millimeter. CW was measured as the maximum width of the carapace and CL as the distance between the median notch of the frontal margin and the posterior margin of the carapace. General observations were also made on feeding when they consumed their exuviae molt and the appearance of their exoskeletons following molting. These observations were taken during their early growth and served as qualitative indications of how fast crabs of the exposure and control cohorts hardened and advanced through their molt cycles.

Any size increase was computed as the difference between the CW or CL measurements (mm) between two consecutive molts. Size increments for each molt were then expressed as percentages of the corresponding premolt CW or CL. This represented the percent size increment, or the extent to which the CW or CL postmolt exceeded that of the respective premolt (Hartnoll, 1982). The intermolt period was recorded as the number of days between two successive molts, with the actual day of ecdysis not included in this period. Breakpoint analysis of CW differences was used to estimate structural maturity for each cohort, using scatter plots created in R (R Development Core Team, 2016) and the package 'strucchange' (Zeileis *et al.*, 2002). Specific growth rate (SGR) was calculated for each molt from the relevant CW or CL size increment and intermolt period, using the method from Romano and Zeng (2006). Monthly SGR was also calculated using the same procedure.

The mean size class (in terms of CW) and cumulative intermolt period were also derived for each molt and used to plot separate growth curves for the exposure and control groups. Variation in CW size (mm) over time (cumulative intermolt period) was also fitted to a standard logistic equation, similar to that used by Jin *et al.* (2001). Moreover, variation in CW served as the dimensional change used to estimate structural maturity for this study.

Data analysis

All statistical analyses and graphical depictions were done using R version 3.3.1 (R Development Core Team, 2016). An alpha level of 0.05 was used for all statistical tests. A number of statistical procedures require datasets to come from normal populations and have homogeneity of variances (Zar, 1999). The current datasets were found to be non-normal (Shapiro-Wilk test; $p < 0.05$), with unequal variances or heteroscedasticity (Levene's test; $p < 0.05$). Accordingly, non-parametric tests were used. Specifically, the Spearman rank correlation coefficient was used to assess the relationship between carapace dimension and intermolt period. The Mann-Whitney U test was used to detect differences between the control and exposure cohorts, in terms of CL, CW, intermolt period, size increment and SGR. The Kruskal-Wallis and post hoc Dunn's test were also used for pairwise comparisons of SGR of different molts as well as for SGR of different months. The package 'dunn.test' (Dinno, 2014) was used for conducting both of these tests.

Results

Initial sizes of crabs at the start of monitoring were similar. Juvenile *P. dentata* crabs of the malathion-exposed group were initially 2.96 ± 0.29 mm CW, 2.29 ± 0.10 mm CL at the start of the monitoring period. This was similar to the control group, which had an initial size of 3.04 ± 0.36 mm CW, 2.60 ± 0.22 mm CL. A general observation was made on the extent of crabs feeding, such that those of the control cohort consistently ate all food provided with little to no items remaining in their aquaria. However, the malathion-exposed crabs were noted to eat only a portion of their total pellets and plant segments administered, while the uneaten remainders were removed in each water renewal. Juvenile crabs of the control treatment were observed to consume their molted exuviae within a few hours of their initial molts. This growth behavior was similarly noted for the exposure cohort but only for their first three molts during the pesticide-free period, prior to their contact with 10 µg/L malathion insecticide. The exoskeletons of both control and exposure cohorts changed from translucent to opaque within 24 to 48 hours of molting. The postmolt consumption behavior and fast transitioning of the cuticle appearance indicated a typical and rapid hardening of the exoskeleton. This behavior changed for the control crabs, as they grew older (> 15 mm CW), whereby consumption of exuviae and opacity of the exoskeletons took place within 1 to 3 days after molting. However, juveniles of the exposure treatment were observed to take relatively longer to consume their exuviae (4 ± 2 days), during their exposure to the malathion insecticide.

Within the 5-month study period, all *P. dentata* crabs that were exposed to 10 µg/L malathion insecticide ($n = 4$) only reached their eighth molt, by the fourth month of the monitoring period. A total of 32 molts were recorded for this group over the five months period and molt events halted thereafter; with this cohort failing to attain their ninth and tenth molts (Tab. 1). In contrast, those of the control cohort ($n = 4$) experienced a total of 40 molts, each crab having undergone 10 molt events by the fifth month.

Generally, CW increased with intermolt period for both cohorts (Fig. 1) and this was congruent with CW being significantly correlated with intermolt period for the malathion-exposed ($rho = 0.89$, $p < 0.05$) and control ($rho = 0.99$, $p < 0.05$) groups. This was also reflected in the size class distributions for control and malathion-exposed crabs, where mean intermolt period increased with mean CW size class (Fig. 1A, 1B). However, patterns in size increment for exposed crabs were different from the control, following exposure to the malathion insecticide. Initially (pre-exposure), CW and CL size increases and respective percent increments of the exposure cohort were similar to those of the control cohort (Tab. 1), however, this was only observed for the initial three molts, prior to insecticide exposure (pesticide-free period).

Table 1. Mean size increase for carapace width (CW) and carapace length (CL) with standard deviations (SD), along with percentage size increments for control and malathion-exposed cohorts of *Poppiana dentata* crabs.

Moult number	1	2	3	4	5	6	7	8	9	10
	Control									
CW size increase (mm ± SD)	1.18 ± 0.38	1.26 ± 0.64	1.50 ± 0.46	2.05 ± 0.30	2.46 ± 0.16	3.14 ± 0.42	3.30 ± 0.70	4.12 ± 0.06	4.60 ± 1.76	5.17 ± 1.83
CW percent increment (%)	44	31	29	31	29	27	23	23	20	19
CL size increase (mm ± SD)	0.79 ± 0.22	1.07 ± 0.62	1.28 ± 0.50	1.57 ± 0.37	1.93 ± 0.08	2.48 ± 0.53	2.75 ± 0.76	3.40 ± 0.42	3.31 ± 0.49	4.32 ± 0.28
CL percent increment (%)	30	32	29	28	27	25	22	22	17	20
	Pre-exposure			Exposure – 10 µg/L concentration of malathion insecticide						
CW size increase (mm ± SD)	1.08 ± 0.47	1.36 ± 0.37	1.58 ± 0.74	1.73 ± 0.36	4.64 ± 2.41	3.34 ± 2.09	4.16 ± 3.37	4.25 ± 2.26	No moult	No moult
CW percent increment (%)	38	34	29	23	50	23	28	21	No moult	No moult
CL size increase (mm ± SD)	0.74 ± 0.13	1.09 ± 0.46	1.39 ± 0.66	2.82 ± 0.89	4.14 ± 1.79	3.00 ± 1.36	2.91 ± 0.82	3.59 ± 0.93	No moult	No moult
CL percent increment (%)	26	38	33	56	50	24	20	21	No moult	No moult

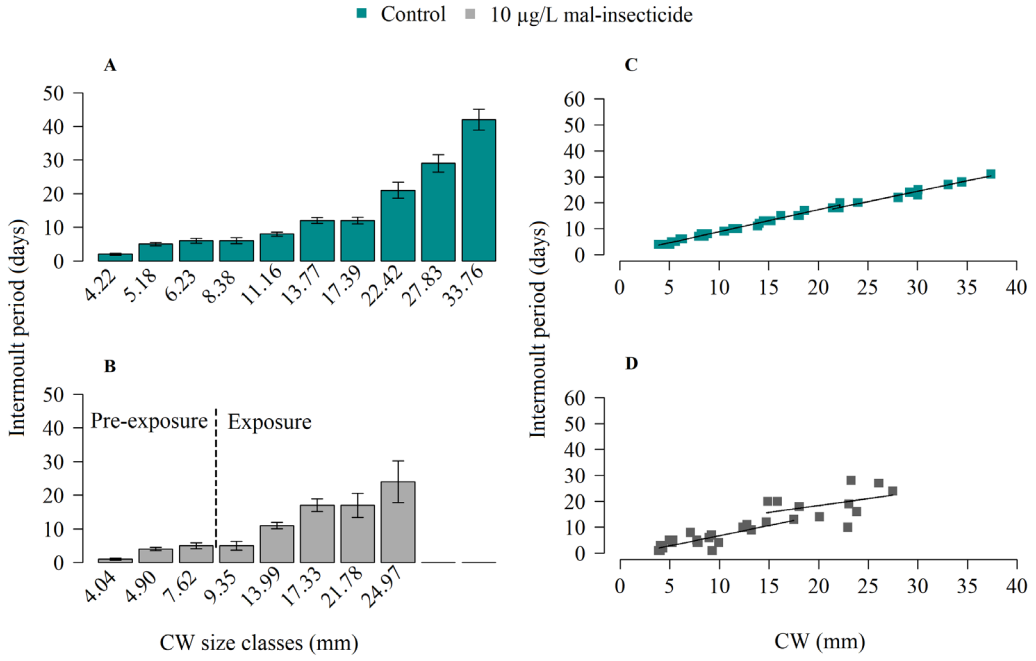

Figure 1. Growth aspects for *Poppiana dentata* in relation to: mean intermolt period and CW size classes of control (**A**) and malathion-exposed (**B**) crabs over the 5-month period; intermolt period and carapace width (CW) for control (**C**) and malathion-exposed (**D**) crabs. Error bars in A and B plots represent the standard errors for the intermolt periods. The dashed line in B separates pre-exposure (no pesticide) conditions for the malathion-exposed cohort and exposure to the malathion insecticide at 10 µg/L concentration. Breakpoints in C and D are denoted by segmented lines for the respective cohort.

Size increases of juveniles within this pre-exposure period followed a corresponding trend to that of control crabs, in which high percent increments were associated with initial molts, namely 38 % for CW for the first molt and 38 % CL for the second molt (Tab. 1). Likewise, CW and CL size increments of 44 % and 32 %, for control crabs were the highest for the first molt and second molts, respectively (Tab. 1).

Following exposure to the insecticide (after the third molt), inconsistent patterns in CW and CL size increase were noted for the subsequent five molts of malathion-exposed crabs. Moreover, maximum

percent CL (56 %) and CW (50 %) size increments occurred around the fourth and fifth molts for these crabs (Tab. 1). This contrasted with the pattern observed for the control cohort whereas the crabs grew older, their dimensional growth slowed down and the intermolt period became longer toward the end of five months (42 ± 6 days). Consequently, their minimum CW (19 %) and CL (17–19 %) percent increments were also associated with the later molts, at around the ninth or tenth molt, toward the end of the five months (Tab. 1).

Despite the irregularity in size increase for malathion-exposed crabs, differences in CW, CL and associated size increments were not significant between the exposure group and the control group (CW: $W = 29, p > 0.05$; CL: $W = 30, p > 0.05$; CW size increment: $W = 24$, $p > 0.05$; CL size increment: $W = 604$, $p > 0.05$). However, intermolt periods for the malathion-exposed cohort were substantially different from those of the control cohort ($\chi^2 = 6.821, p < 0.05$). The exposed cohort also showed signs of a delay in molting and an extension in intermolt period upon exposure to the malathion insecticide; as evidenced by the lack of molt events after the eighth molt (Fig. 1B).

With regards to breakpoint determination and scatter plots, a segmented relationship was noted between CW and intermolt period for both cohorts (Fig. 1C, D). Breakpoint for the exposure cohort was 17.5 mm CW, at 13 days intermolt period, with the overlapping region of the slopes coinciding with a range of 14.74 to 17.5 mm CW and 12 to 13 days intermolt period. This size range was below the breakpoint for the control group (22.11 mm CW; 18 days intermolt period). This disparity was also noted in the growth equations and growth curves. The fitted logistic equation for the control cohort is expressed as $CW = 34.30\ (1+\exp\ (1.774–0.035t))^{-1}$, however, dissimilar components were noted for the malathion-exposed cohort, expressed as $CW = 25.77\ (1+\exp\ (1.500–0.056t))^{-1}$. Growth curves also reflected the variation in dimensional growth between cohorts (Fig. 2A, B). Generally, fast growth

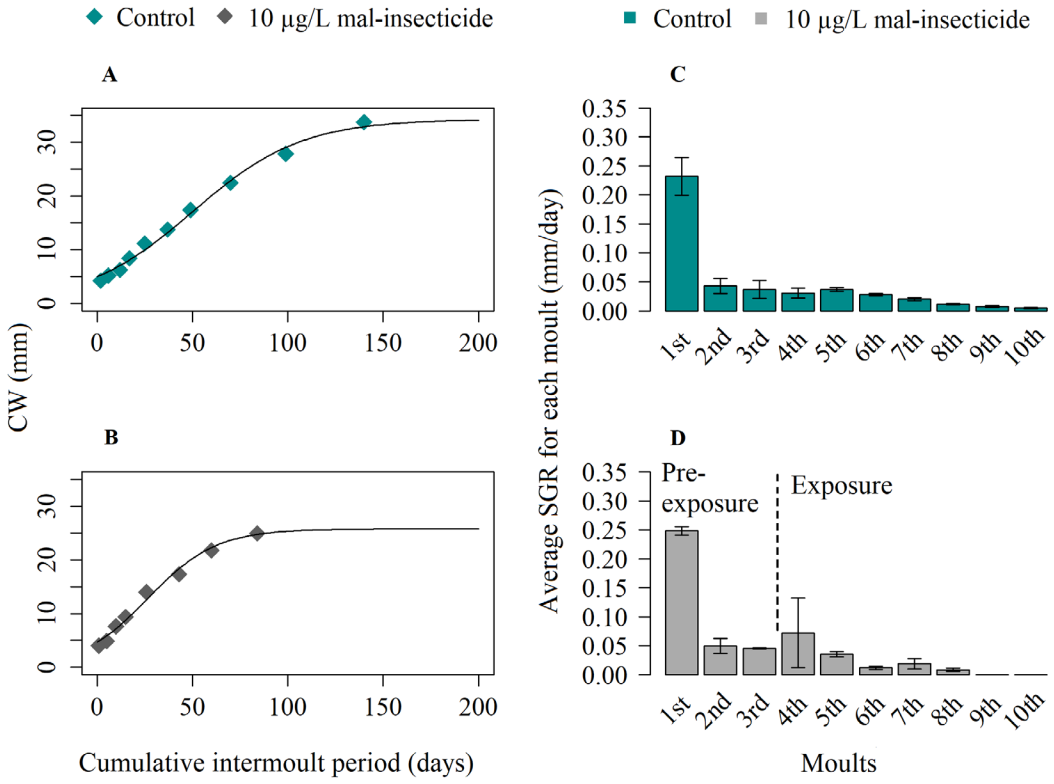

Figure 2. Growth curves for *Poppiana dentata* in relation to: variation in CW size (mm) over time (cumulative intermolt period) for control (**A**) and malathion-exposed (**B**) crabs over the 5-month period; mean specific growth rate (SGR) for control (**C**) and malathion-exposed (**D**) crabs for the same 5-month period. Error bars in C and D represent the standard errors for SGR. The dashed line in D separates the initial three molts under pre-exposure (no pesticide) conditions for the malathion-exposed cohort and those molts experienced during exposure to the malathion insecticide at 10 µg/L concentration.

of CW was seen early in the monitoring period but eventually slowed down toward the end. While this trend was noted across the two cohorts, CW growth for the exposure group reached asymptote before 100 days at around 25 mm CW (Fig. 2B); a much earlier time and smaller size compared to that of the control at around 150 days and a CW of 35 mm (Fig. 2A).

The patterns in SGR for the malathion-exposed group were only like the control group during the pre-exposure or pesticide-free period. This was evident from a high and similar SGR for the first molt of the control (CW = 0.23 ± 0.07 mm/day; CL = 0.24 ± 0.09 mm/day) and the exposure group, prior to malathion exposure (CW = 0.25 ± 0.01 mm/day; CL = 0.22 ± 0.09 mm/day). The initial CW SGR was also significantly higher than that of the second molt for both cohorts (control: $Z = 2.451$, $p < 0.05$; malathion-exposed: $Z = 2.861$, $p < 0.05$) (Fig. 2C, D).

Following exposure to the malathion insecticide, differences in CW SGR between control and malathion-exposed groups were not significant ($W = 29$, $p > 0.05$). Likewise, variations in CL SGR were not significantly different between control and malathion-exposed groups ($W = 31$, $p > 0.05$). Despite these minimal differences, the trend in CW SGR for the malathion-exposed group was noted to deviate from the control group. A sudden increase in SGR was noted for the exposed group at their fourth molt and this coincided with the time at which these crabs began their exposure to the malathion insecticide (Fig. 2D).

CW SGR also differed across the months for malathion-exposed crabs ($\chi^2 = 18.396$, $p < 0.05$) and was the highest in the first month, and significantly greater than the second ($Z = 2.703$, $p < 0.05$) and subsequent months. This corresponded with the highest CW percent increment (50 %) of the exposure group (Tab. 1) occurring within the initial month, as well as this group experiencing up to five molts events within the first month. This was similar to crabs of the control cohort, where their CW SGR for the first month was also considerably higher than that of the second (control: $Z = 2.333$, $p < 0.05$) and subsequent months. Additionally, the control crabs experienced three to six molt events within their first month. These molts of the control cohort were also associated with the highest CW percent increment (44 %) (Tab. 1). CL SGR also differed significantly across molts and months for the malathion-exposed group (across molts: $\chi^2 = 5.355$, $p < 0.05$; monthly: $\chi^2 = 21.451$, $p < 0.05$). This was dissimilar to the pattern exhibited by the control crabs, where CL SGR differences across molts and months were not significant (across molts: $\chi^2 = 5.355$, $p > 0.05$; monthly: $\chi^2 = 6.132$, $p > 0.05$).

Discussion

Exposure to the malathion insecticide appears to alter the molting behavior and growth patterns of *P. dentata* crabs. While the new exoskeletons of molted malathion-exposed crabs transitioned in appearance, similar to the control group, juveniles of the former treatment were observed to take relatively longer to consume their exuviae (4 ± 2 days), following exposure to the malathion insecticide. This could have been due to the bioconcentration of malathion within the exoskeleton, thereby decreasing palatability. Though this was only qualitatively assessed, it still indicates alteration in molting behavior for juveniles in terms of their normal prompt feeding on their molted exoskeleton. This can delay assimilation of calcium from the molted shell, prolong the completion of recalcification and exoskeleton hardening, and by extension, increase juvenile vulnerability. Rapid hardening of the brachyuran exoskeleton after ecdysis can be facilitated by prompt ingestion of the molted, calcified shell. This exuvia contains a considerably amount of calcium (30–70 %) and this can be re-ingested during postmolt and used for restoring calcium deficits (Wheatly, 1999). Therefore, rapid hardening conveys the advantage of efficient growth, through rapid turnover of molt cycles, while reducing juvenile susceptibility to predators and intraspecific cannibalism.

Patterns in CW and CL size increment also appear to be altered from exposure to the malathion insecticide. In addition, exposed crabs show signs of a delay in molting and an extension in intermolt period upon exposure; as evidenced by the lack of molting events after the eighth molt. The delay in the molting cycle is expected since pesticides, among other pollutants, have been known to inhibit molting (Rodríguez *et al.*, 2007). Insecticides, in particular, have been reported to delay molting in decapods as Buchanan *et al.* (1970)

observed a delay in molting for young (zoeae stage) *Cancer magister* Dana, 1852, resulting from exposure to a carbamate insecticide (Sevin) at concentrations as low as 0.1 µg/L. Disruption to the normal molt cycle from toxicant exposure can be attributed to disturbances to the paired Y-organs, and neurosecretory structures, such as the X-organ and sinus gland, thereby resulting in altered levels of MIH and other neurohormones (Montagna and Collins, 2005; 2007; Negro *et al.*, 2011). This was demonstrated in the effects of 24 h exposure to heptachlor (organochlorine) of larvae of the marine lobster, *Homarus americanus* H. Milne Edwards, 1837, whereby the suspension in the onset of molting was correlated with significant reductions in ecdysteroid levels present in circulation (Snyder and Mulder, 2001). Increased levels of cytochrome P450 dependent enzymes were also observed in *H. americanus* and considered to be upregulated by ecdysteroid hormones, alluding to the connection between these detoxifying enzymes and molting; as well as the possibility that their induction was induced by the heptachlor toxicant (Snyder and Mulder, 2001). Aside from growth hormonal disturbances, the malathion insecticide could also disrupt energy homeostasis of *P. dentata*, upon exposure, slowing growth and possibly affecting reproduction. Biotransformation of pollutants, such as pesticides, typically involves ATP consumption, leading to higher energy consumption and deviation of energy from normal growth.

The lack of the ninth and tenth molts in the exposure cohort was especially suggestive of longer intermolt periods, for these molts, and subsequent ones beyond the end of the 5-month monitoring period. These later intermolt periods would have been longer than the corresponding molt periods of the control group (ninth molt: 29 ± 3 days; tenth molt: 42 ± 3 days) and that reported for a baseline group (ninth molt: 33 ± 11 days; tenth molt: 41 ± 8 days) from a previous study (Singh *et al.*, 2020b). The intermolt period of freshwater decapods can be quite sensitive to pesticides; as highlighted by the longer intermolt periods for juveniles of another trichodactylid, *T. borellianus*, following a 4-month exposure to sublethal concentrations (0.62, 1.25 and 2.50 µg/L) of a chlorpyrifos insecticide, Terminator Ciagro® (Montagna, 2010), and the irregular intermolt periods of the freshwater prawn, *Pa. argentinus*, from exposure to low concentrations of chlorpyrifos (0.005–0.022 µg/L) and endosulfan (0.122–0.488 µg/L) insecticides (Montagna and Collins, 2007). Juveniles of the same prawn species have been reported to lengthen their intermolt period after two molts, in response to exposure to the Roundup® herbicide (Montagna and Collins, 2005), as well as from exposure to sublethal concentrations of a cypermethrin commercial insecticide (Collins and Cappello, 2006). Similarly, the intermolt period lengthened across successive molts of the *P. dentata* exposure cohort.

The breakpoint for the *P. dentata* exposure cohort occurs at relatively smaller values (17.5 mm CW, at 13 days intermolt period) than those of the control cohort (22.11 mm CW; 18 days intermolt period), along with a smaller size at which structural maturity (14.74 to 17.5 mm) was reached. This is also reflected in CW growth approaching asymptote at a much smaller size and a smaller maximum size (25.77 mm CW). Relative to the control group, these reduced maximum and structural maturity sizes of the exposure group may indicate a constraint on the lifespan of these crabs, caused by continuous exposure to the OP insecticide. Xenobiotics, in particular, have been known to affect growth of decapods through their molting events, causing adults to maintain smaller sizes than the average conspecific (Negro *et al.*, 2011). This indirectly has consequences for reproduction success, in terms of mate selection and capacity for bearing young, as well as for longevity. However, further examination of *P. dentata* under similar toxicant conditions, over a longer test period, is needed to confirm this.

Following exposure to the malathion insecticide, patterns of CW and CL SGR for *P. dentata* became irregular, with a noticeable elevation in rates at the fourth molt, in relation to CW (Fig. 2D). Insecticides can modify dimensional growth rates, as evident by the overall negative or null growth rate for CL of *Pa. argentinus* exposed to a cypermethrin insecticide, at correspondingly low concentrations of 0.0001, 0.001 and 0.01 µg/L (Collins and Cappello, 2006). Alterations in dimensional growth and intermolt period can be attributed to toxicant disturbances of the neurohormonal system of the X-organ-sinus gland complex (Negro *et al.*, 2011) and/or by disruption of

energy homeostasis and increased energy expenditure for survival (versus using the energy for growth).

Growth of an organism involves feeding, assimilation and energy expenditure, but exposure to toxicants can compromise all these processes, through modification of energy allocation and sudden metabolic demands associated with detoxification and stress responses. For instance, the decline in size increased per molt and extended intermolt period for *T. borellianus* exposed to chlorpyrifos insecticide were attributed to reduced levels of proteins generally used for tissue growth and repair (Montagna, 2010). Stress responses from OP exposure, like neurotoxicity, can ultimately induce a shift in the normal energy allocations, to facilitate possible coping mechanisms of the affected organism. OPs are neurotoxic and act by inhibiting the acetylcholinesterase (AChE) enzyme, thereby resulting in accumulation of acetylcholine (enzyme substrate), disruption in regular neurotransmission across neuromuscular junctions, and followed by continuous muscular contractions (Bookhout and Costlow, 1976; Fulton and Key, 2001; Mulchandani *et al.*, 2001; Rickwood and Galloway, 2004; Bolton-Warberg *et al.*, 2007; Lionetto *et al.*, 2011). This neurotoxicity was previously demonstrated for *P. dentata*, where significant AChE inhibition resulted from exposures to sublethal concentrations of the same malathion insecticide (Singh, unpublished data).

A reduction in overall energy availability for both somatic and gonadal growth can also result from impaired feeding in an organism experiencing toxic stress. Based on delayed consumption of exuviae and observations on the unconsumed food that was removed during water renewal, it seems that feeding was reduced in *P. dentata* under malathion insecticide exposure (as opposed to the control cohort in which little to no food remained after feeding events). This reduced feeding would ultimately reduce the available energy for these crabs to undergo normal growth. A portion of the total pellets and plant segments were consumed by the exposure group, each time these were provided, and so it cannot be fully verified that feeding was impaired.

The responses of *P. dentata* in this study highlight that their growth is sensitive to malathion insecticide and even at sublethal levels this OP biocide can significantly modify the molting cycle. Disruption in energy homeostasis and the hormonal endocrine system involved in molting may have occurred in exposed *P. dentata*, delaying the intermolt period and cycle for later molts. However, this remains quantitatively unconfirmed and requires further investigation into metabolism energetics, and levels of MIH, ecdysteroids and other related growth hormones in crabs exposed to malathion insecticide. Malathion can be broken down to its toxic intermediate, malaoxon, through hydrolysis, and the latter is known to be more toxic than the parent compound, despite its fast ability to degrade (Wendel and Smee, 2009). It is reasonable to infer that commercial malathion insecticide, at sublethal levels, can alter growth patterns of *P. dentata* juveniles and subsequently influence their maturity. Consequently, the presence of malathion insecticide in the aquatic habitats of *P. dentata* could indirectly impact reproduction in indigenous populations, through altered energy investment for growth and reproductive processes (*e.g.*, oocyte/sperm development), reduced success for smaller males in mate competition and limited egg carrying capacity of smaller females.

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Compliance with ethical standards

Ethical sanction was granted by the Campus Ethics Committee of The University of the West Indies St. Augustine (Trinidad and Tobago) for conducting research on *Poppiana dentata*.

Conflict of interest

The authors declare they have no conflict of interest, both financial and non-financial.

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