Effects of ethylenediaminetetraacetic acid (EDTA) on growth and survival of Long-spined Sea Urchin *Diadema antillarum* larvae

Running title: Effects of ethylenediaminetetraacetic acid on Long-spined Sea Urchin larvae

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/naaq.10305

Abstract

Presence of heavy metals in seawater is a major challenge for hatchery culture of sea urchin larvae due to their sensitivity to metal pollution. The impact of heavy metals in seawater for sensitive marine larviculture could be minimized via chelation of metals using chemical compounds such as ethylenediaminetetraacetic acid (EDTA). In this study, the effects of EDTA were tested for the Long-spined Sea Urchin *Diadema antillarum* during larviculture at two concentrations (10 µM and 50 µM) and two exposure durations (6 and 24 days post-fertilization). Growth and survival of larvae were evaluated as response variables. Significant differences in growth and survival of larvae were found among the treatments at different EDTA concentrations and exposure durations. The 10 µM EDTA treatment enhanced growth and survival of larvae relative to control for both exposure durations. The 50 µM EDTA treatment was unsuitable for *D. antillarum* larviculture due to reductions in growth and survival. The use of 10 µM EDTA during embryo incubation and larviculture is expected to improve *D. antillarum* larval fitness leading to improved hatchery production.

Keywords: Sea urchin, *Diadema antillarum*, larvae, heavy metal, EDTA

Introduction

Nearshore ocean waters are often polluted with heavy metals due to anthropogenic input via urban runoff, industrial discharge, and mining activities (Häder et al. 2020). Heavy metal pollution in seawater is a major problem for marine hatchery operations due to sensitivity of embryos and larvae to trace-metal concentrations. Heavy metals trigger oxidative stress andimpair protein functions, leading to abnormal development and mortality in larvae (Gale et al. 2016). Brass and/or stainless steel in water pumps used in a hatchery, as well as metal accumulation in microalgae food sources, can expose marine larvae to heavy metals. Chelating heavy metals in seawater using a chemical compound is a potential approach to minimize the bioavailability of heavy metals to sensitive organisms. Ethylenediaminetetraacetic acid (EDTA) is known to be an effective chelator thereby reducing the bioavailability of toxic heavy metals (Resgalla et al. 2012). EDTA also has inhibitory effects on biofilm formation (Percival and Salisbury 2017) and proliferation of the foodborne pathogen *Escherichia coli* (Castellano et al. 2011; Mohammadi et al. 2022). Several studies have indicated the potential use of EDTA for rearing marine larvae to improve production output (Gale et al. 2016; McDougall et al. 2020; French et al. 2021).

The Long-spined Sea Urchin *Diadema antillarum* is a keystone macroalgae grazer throughout Caribbean coral reefs. This urchin's populations experienced unprecedented 93-98% mortality due to an unknown disease in 1983-1984, which contributed to an ecological phase shift from coral dominated to macroalgae dominated communities on Caribbean reefs (Lessios et al 1984). Restoring *D. antillarum* populations, along with coral outplanting, is a potential approach to maintain ecosystem function while necessary environmental improvements occur (Edmunds and Carpenter 2001; Francis et al. 2019; Williams 2022; Pilnick et al. 2023). The natural recovery of *D. antillarum* populations has been slow and spatially variable, thereby restocking of hatchery propagated *D. antillarum* should be considered. Development of successful hatchery techniques would be a significant step towards implementing *D. antillarum* population recovery via restocking of hatchery propagated urchins.

Developing a reliable large-scale larviculture technique for *D. antillarum* has

been a long-standing challenge due to a lengthy larval duration, negative buoyancy in the culture vessel, and unique larval biology (Leber et al. 2009). Novel systems for *D. antillarum* culture have recently been developed and the nutritional requirements have been explored by our lab and collaborators (Pilnick et al. 2021; Hassan et al. 2022; Pilnick et al. 2022; Wijers et al. 2022). Several challenges remain, however, to improve larval survival to competency and metamorphosis. Larvae of *D. antillarum* are very sensitive to heavy metal exposure, which leads to stunted development during early larval stages at low concentrations (~10 µg/L) of Cu, Ni, Ag or Se (Bielmyer et al. 2005). The seawater used in our lab was previously analyzed to have heavy metal concentrations of 2.6 µg/L Zn, 1.5 µg/L Cu and 0.78 µg/L Ni, whereas levels of Se and Pb were below detection (Pilnick et al. 2021). However, no information is currently available on the efficacy using a metal chelating agent for *D. antillarum* larviculture. This study aims to assess the effects of EDTA on *D. antillarum* larviculture by evaluating the growth and survival of larvae.

Materials and Methods

A total of 17 broodstock Long-spined Sea Urchins were collected from patch reefs at ≤ 8 m depth of off Marathon, Florida Keys, USA. Broodstock were transported in aerated, seawater-filled coolers, and housed in a greenhouse system at The Florida Aquarium's Center for Conservation in Apollo Beach, FL, USA. Broodstock were distributed to three 450 L fiberglass tanks within a 2,380-liter recirculating system under natural photoperiod. Male and female broods were kept separate tanks for easier identification during spawning. The system was maintained with life support components including biological, chemical, and mechanical filtration via live rock, activated carbon, foam fractionators, and 150 µm filter socks. Temperature and salinity of the broodstock system were maintained at ~25 °C and ~35 ppt, respectively. Titanium immersion heaters were used to maintain temperature, and freshwater was added periodically to encounter evaporation and maintain salinity. Spawning was induced by placing broodstock in a spawning tub at ~30 °C water temperature. Details of *D. antillarum* broodstock system maintenance and spawning procedures are described in Pilnick et al. (2021).

Eggs were transported in 20 L buckets to a climate-controlled room and maintained aeration for two hours after fertilization. Over 90% fertilization was achieved, and subsamples of seawater from the bucket containing 190 embryos were transferred to each of fifteen 1 L borosilicate glass bottles. Each bottle was filled to 600 mL of 35 ppt, 1 μm filtered, natural seawater (collected from Gulf of Mexico; initially sand filtered and ozonated before transporting to the lab). The concentrations of heavy metals in the same seawater source were found to be relatively low for separate batches of water samples used for larviculture in our lab (Pilnick et al. 2021). Since seawater source and treatments (such as filtration and ozonation) were exactly same, this study assumed that the heavy metal concentration in seawater was similar to the prior study. Larvae were cultured in a climate-controlled room at 25 °C and seawater pH ranged from 8.1-8.3.

Bottles were placed on orbital shakers (Heathrow Scientific 120460 Digital Orbital Shaker) at 130 rpm to suspend embryos and larvae in the water column. This culture system is described in detail by Wijers et al. (2023). An experimental design with five treatments (three replicates in each; each glass bottle was considered an experimental unit) was employed to test the effects of EDTA on larvae (Table 1). Seawater in the bottles was fully replaced semi-weekly by sieving larvae through 100 μm or 200 μm mesh based on larval size. Initially, 10 mM EDTA stock solution was prepared (0.73 g of EDTA in 250 mL distilled water), and 0.6 mL and 3.0 mL of the stock solution were added to 600 mL seawater to achieve 10 μM and 50 μM EDTA concentrations, respectively. Larvae were fed microalgae *Rhodomonas lens* and *Chaetoceros calcitrans* diet once daily at equal proportions of total carbon throughout the culture period. Cell counts of each microalgae species were standardized based on carbon content considering *R. lens* containing 40.7 pg C/cell (Ohs et al. 2010) and *C. calcitrans* containing 22.2 pg C/cell (Pérez-Morales et al. 2015). Microalgae were initially fed at a total carbon equivalent of 4,000 cells/mL *R. lens* at 4 days post-fertilization (dpf), which was incrementally adjusted to 16,000 cells/mL by 24 dpf. Body length of 10 randomly-selected larvae from each replicate was measured at 3, 10, 17 and 24 dpf, and larvae were returned to the culture bottles after measurements. Larval body length was measured from the base of the body to the edge of the oral hood according to Wheeler et al. (2016). The total number of larvae present in each bottle at the end of experiment was counted and survival was calculated as the proportion of initial embryo stocking. Individual larval measurements within a treatment were treated as subsamples with their mean considered the

replicate value used in statistical analysis. Treatment effect on larval growth of larvae was analyzed using a generalized linear model. Data on final survival were tested for assumptions of normality and homogeneity of variance using Shapiro-wilk and Levene's test. Proportional survival data were arcsine transformed and analyzed with one way ANOVA followed by Tukey's post-hoc test. All data were analyzed using IBM SPSS (v. 27).

Results

Larval growth was significantly affected by EDTA exposure (Wald statistic = 21.9, df = 4, $P < 0.05$). Larvae treated with 10 µM EDTA for 24 days attained the largest size of 476.2 ± 27.2 µm (mean ± SE) and were significantly different to those treated with 50 µM EDTA which attained the smallest size of 373.9 ± 17.5 µm at 24 dpf. Larvae in the control group attained a size of 376.4 ± 21.8 µm and were significantly smaller than T1 at 24 dpf. Growth of larvae with different EDTA concentrations and exposure durations is presented in Figure 1.

Larval survival was significantly affected by EDTA exposure (F = 2.9, df = 4, P < 0.05). Larvae treated with 10 µM EDTA for 6 days attained the highest survival of 22.2 ± 3.8% (mean ± SE) and were significantly different compared to those treated with 50 µM EDTA for 24 days, which attained the lowest survival of 10.0 ± 0.9%. However, none of the treatments were significantly different from the control. Survival of larvae in response to EDTA treatment is presented in Figure 2.

Discussion

In this study, the efficacy of EDTA addition was evaluated to improve best management practices for *D. antillarum* larviculture. Results indicated that a lower concentration (10 µM) of EDTA improved growth and survival, while a higher concentration (50 µM) was detrimental. Significantly higher growth and numerically higher survival was attained by EDTA treatment at the lower concentration (10 µM) relative to the control. At a 10 µm EDTA concentration, larvae treated for a longer exposure duration grew faster than those treated for a shorter duration, but the opposite response was found for survival. This contrasting response to EDTA exposure durations was likely due to a difference in larval density and resource allocation, i.e., at lower survival

more space and food resources were available per larvae, which might have contributed higher growth. Contrasting growth of *D. antillarum* larvae has also been observed due to differences in larval survival in other studies (Pilnick et al. 2022; Wijers et al. 2023).

Heavy metals induce oxidative stress during embryogenesis via production of reactive oxygen species (ROS) that lead to DNA damage and abnormal development (Rainbow 2002). Some metals such as zinc, iron, and copper are essential biological components, therefore, metabolically available forms of these metals are important for biological functions. Once accumulation of these essential metals exceeds a certain threshold, they become toxic or lethal to marine invertebrates (Wang et al. 2018). Other metals such as mercury, lead, and cadmium have no, or limited, biological functionality, thereby any exposure to these metals may have toxic effects (Wang et al. 2009).

Marine animals have an inherent capacity to excrete excess metals, but when the rate of accumulation supersedes excretion and/or detoxification, the metals become toxic and disrupt biological functionality (Rainbow 2002). Bielmyer et al. (2005) found that *D. antillarum* larvae developed with stunted arms at the pluteus stage when exposed to ~10 µg/L concentrations of Cu, Ni, Ag or Se. Since *D. antillarum* is particularly susceptible to trace heavy metals, it is possible that addition of EDTA provided some benefit by chelating heavy metals that existed at sublethal concentrations. The latent impact of low metal concentrations on *D. antillarum* larvae is unknown and warrants future investigation, especially given that our data suggest a benefit from EDTA. In addition, dietary heavy metal contributions could also be a potential source of exposure, but their status was unknown in this study.

It is known that EDTA prevents the production of reactive oxygen species (ROS) by chelating dissolved heavy metals (Guerin et al. 2001). The efficiency of chelation depends on factors such as pH, the affinity of EDTA with dissolved metals, and the presence of other ligands in the seawater. In studies with the Green-lipped Mussel *Perna canaliculus*, incubation of embryos with EDTA resulted in two-fold lower oxidative damage (Gale et al. 2016). Toyota and Nakashima (1998) found a dual function of EDTA in the chelation of toxic metals and an increased bioavailability of essential metals while studying the growth of phytoplankton.

The usage of foam fractionation and UV sterilization in marine hatchery water treatment may cause unintended alterations of organic ligands that change the speciation and concentration of bioavailable metals. The dual function of EDTA could minimize unintended harmful impacts on water treatment, which has direct applicability in hatchery operations.

EDTA is the most widely used chelating agent for heavy metals in marine hatcheries. However, the dosage and length of EDTA exposure for embryo incubation and larviculture needs careful consideration based on species, the stage of embryo-larval development, and dissolved metal concentrations. EDTA treatment with 3 µM to 50 µM have been used during egg fertilization, embryo incubation, and larviculture of the Green-lipped Mussel *Perna canaliculus* (McDougall et al. 2019; McDougall et al. 2020; French et al. 2021). In the current study, a lower concentration of EDTA (10 µM) resulted in higher growth and survival of *D. antillarum* larvae compared to higher concentration (50 µM). Similarly, addition of 12 µM EDTA in seawater for up to 48 hours significantly enhanced growth, survival, food ingestion, and swimming activity of *P. canaliculus* larvae. However, the continuation of EDTA addition beyond 48 hours produced a minimal impact on larval fitness (McDougall et al. 2020; French et al. 2021). These authors argued that embryos appeared to be vulnerable to the presence of trace heavy metals during very early stages of development, but once shell formation is completed, continuation of EDTA treatment was not necessary. Our study suggests that a similar pattern may exist in *D. antillarum* larvae. Since only one low concentration of EDTA (10 µM) was tested in this study, exploring the effects of other lower concentrations of EDTA at different levels of heavy metal concentrations warrants further investigation.

This study concludes that 10 µM EDTA for up to six days can be used to improve growth and survival of *D. antillarum* larvae. Although this study provided information on the beneficial usage of EDTA, the concentration of heavy metals in the body tissue of larvae with or without EDTA treatment was unknown. An evaluation of metal accumulation in the larval body tissue in response to EDTA treatment and larval health status would a step forward to better understand the role of EDTA. Developing strategies to minimize the impact of bioavailable heavy metals would be a critical step for success in scaling up *D. antillarum* larval production.

Acknowledgements

We thank our partners at the Florida Fish and Wildlife Conservation Commission (B. Sharp and G. Delgado) and The Florida Aquarium (A. Petrosino) for helping with broodstock collection and husbandry, respectively. This research was supported by grants from the National Oceanic and Atmospheric Administration Restoration Center (award NA20NMF4630304), the National Fish and Wildlife Foundation (Grant ID 0302.20.068850), Florida Keys National Marine Sanctuary Foundation (award #22-02-J-439 and the Florida Fish and Wildlife Conservation Commission State Wildlife Grants Program (Agreement # 21009).

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Table 1: Concentrations and durations of ethylenediaminetetraacetic acid (EDTA) exposure to Long-spined Sea Urchin, *Diadema antillarum* larvae.

Treatment	Treatment type	Concentrations of EDTA	Duration of exposure
T1	Lower concentration and shorter exposure	10 µM	6 days
T2	Lower concentration and longer exposure	10 µM	24 days
T3	Higher concentration and shorter exposure	50 µM	6 days
T4	Higher concentration and longer exposure	50 µM	24 days
T5	Control	None	None

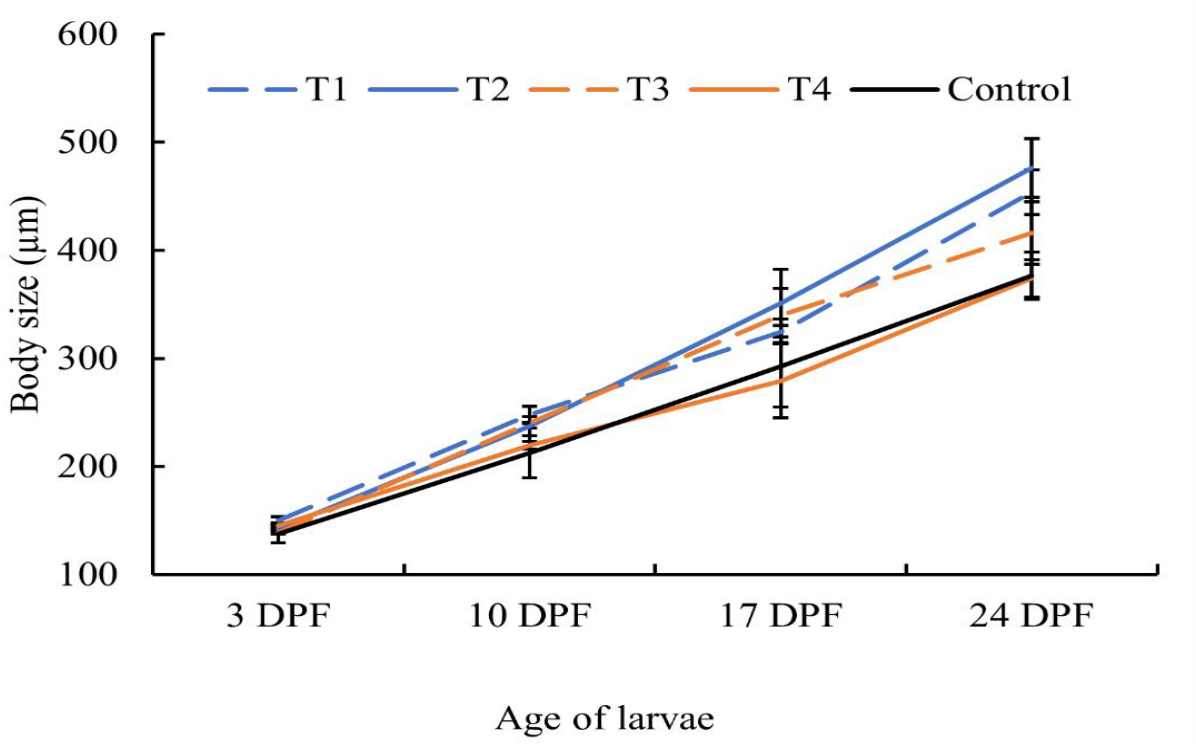

Figure 1: Growth of Long-spined Sea Urchin, *Diadema antillarum* larvae treated with different concentrations of ethylenediaminetetraacetic acid (EDTA). T1 = 10 µM for 6 days; T2 = 10 µM for 24 days; T3 = 50 µM for 6 days; T4 = 50 µM for 24 days; T5 = no EDTA control. Each data point represents mean ± SE (n = 3, 10 larvae per replicate). DPF: Days Post Fertilization.

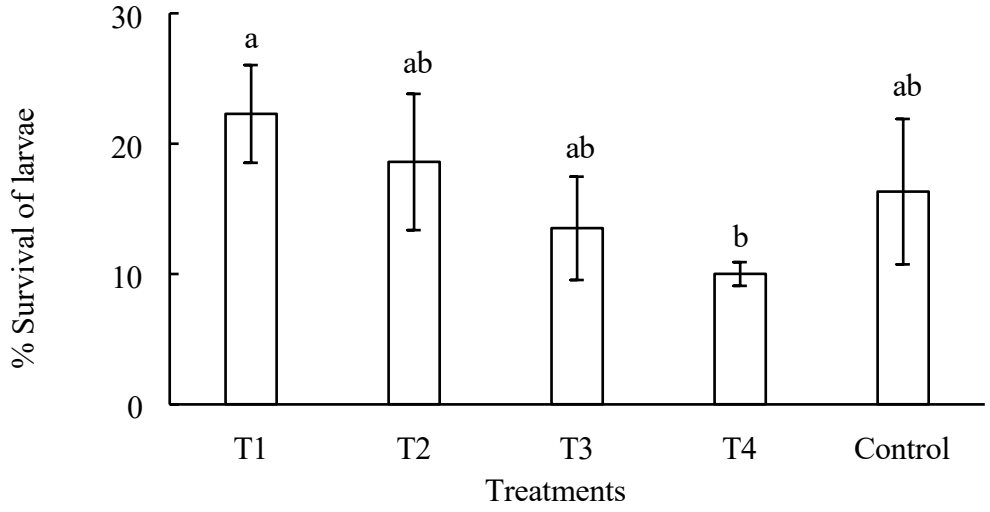

Figure 2: Survival up to 24 days post-fertilization of Long-spined Sea Urchin, *Diadema antillarum* larvae treated with different concentrations of ethylenediaminetetraacetic acid (EDTA). T1 = 10 µM for 6 days; T2 = 10 µM for 24 days; T3 = 50 µM for 6 days; T4 = 50 µM for 24 days; T5 = no EDTA control. Different letters in each column indicate significant differences among the treatments (one-way ANOVA, $P < 0.05$). Each bar represents mean ± SE of three replicates.

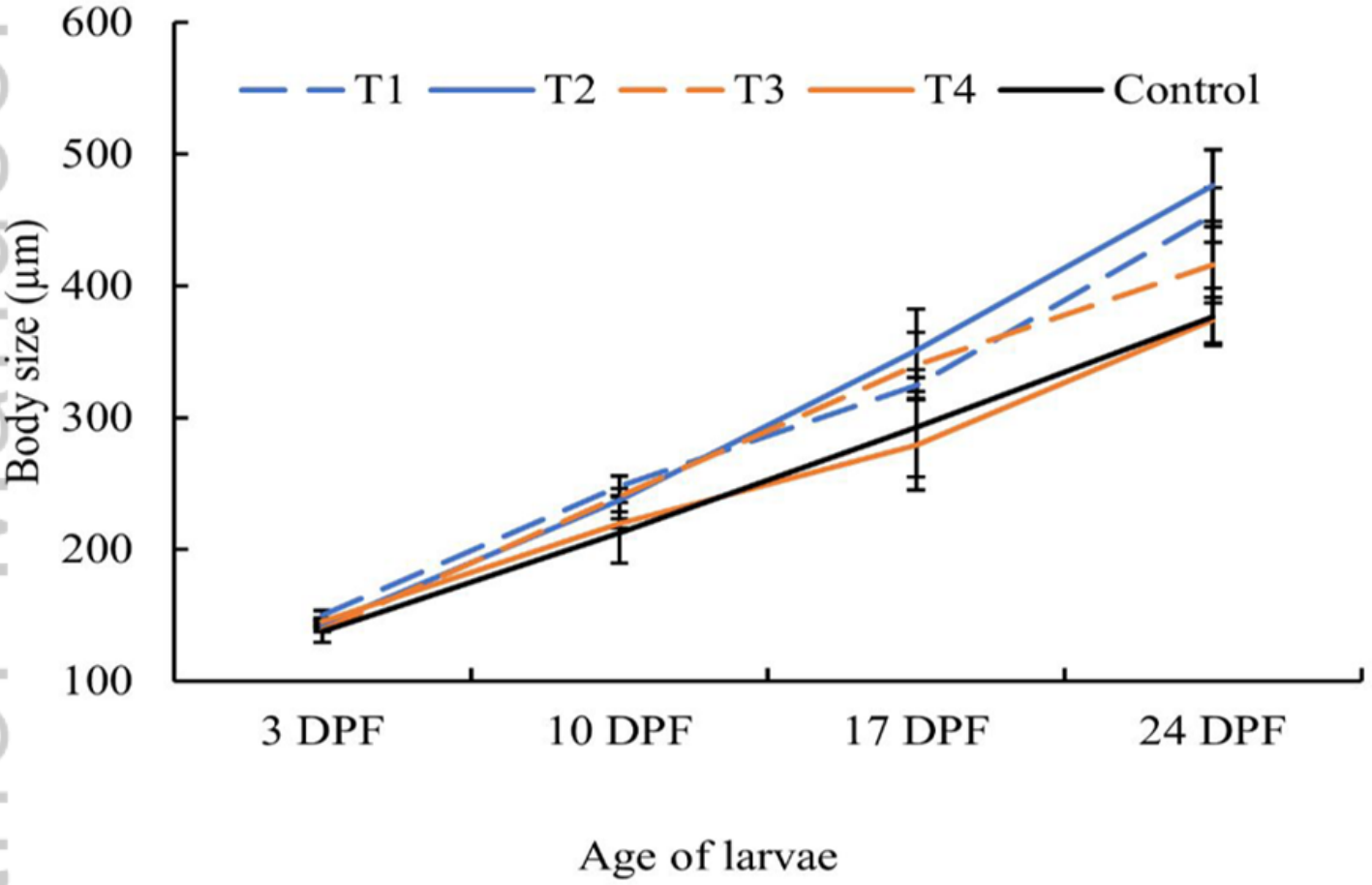

Figure 1.png

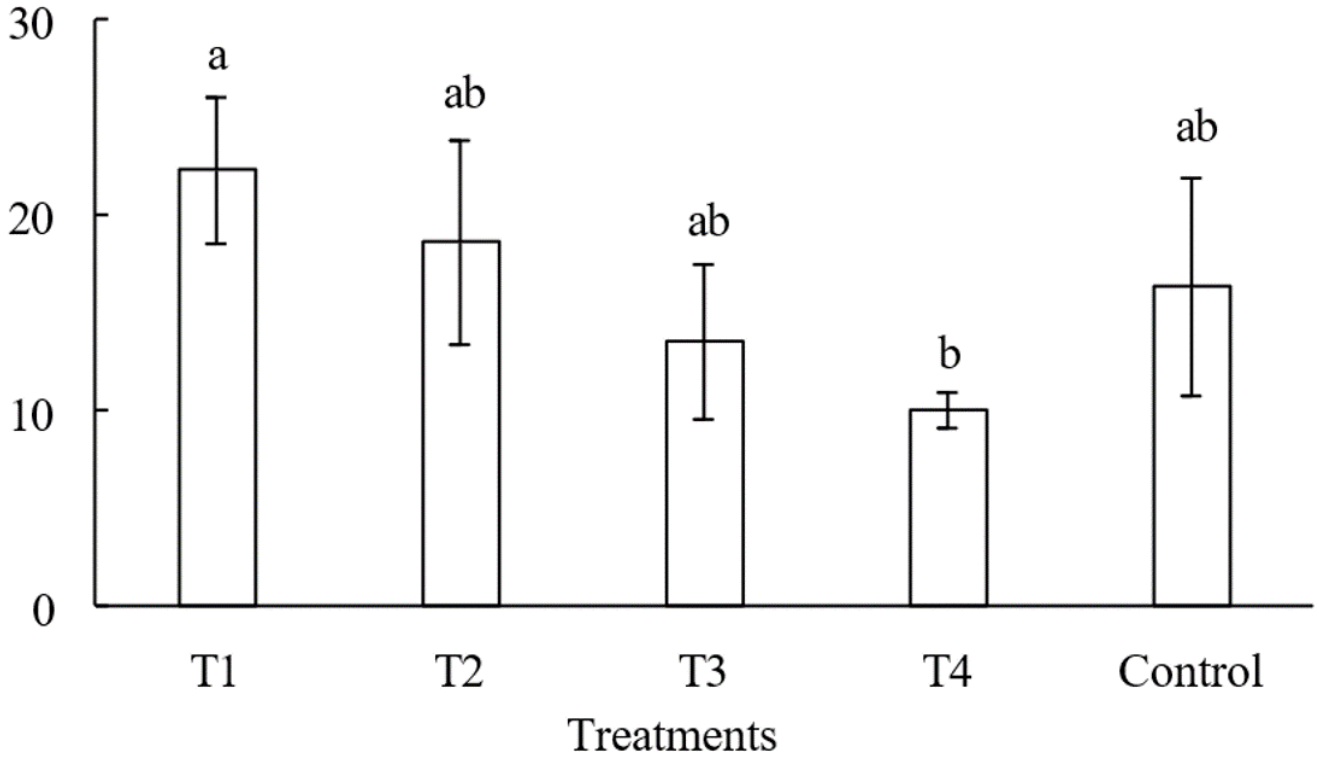

Figure 2.png