SEXUAL ALTERATION IN LITTORARIA ANGULIFERA (GASTROPODA: LITTORINIDAE) FED WITH AN ORGANOTIN-CONTAMINATED ARTIFICIAL DIET

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ABSTRACT

Purpose: This study verified the presence of sexual morphological changes in Littoraria angulifera fed with an artificial diet with tributyltin (TBT).

Theoretical framework: Littoraria angulifera (Lamarck, 1822) is a conspicuous estuarine gastropod from tropical regions. It shows generalist feeding habits and fulfills several selection criteria for estuaries monitoring.

Method/design/approach: For 30 days female L. angulifera (n=360) were placed in culture plates and fed with paper fragments soaked with a control (sea water and Dimethyl sulfoxide (DMSO)) and TBTCl (0.1 and 0.5 μg g−1) solutions. After this period, the specimens that survived were anesthetized (10% MgCl2) and euthanized for pallial oviduct length (POL) and distance between the anus opening to vulva (DAV) analysis.

Results and conclusion: The paper consumption decreased after the first week of treatment in all groups, and the specimens fed with diet contaminated with TBT stopped eating during the second week. The specimens exposed to TBT exhibited elongated POL, while DAV was reduced, changes that might compromise reproductive function. Penial development was not detected in females fed with the diet contaminated with TBT.

Research implications: L. angulifera can be an estuarine species for assessing organotin contaminants, which also elevates its importance in biomonitoring programs.

Originality/Value: This study highlights the use of contaminated artificial diet with paper is significant in proposing a material with low costs, widespread access, and ease of handling and measurement as a low cost alternative for invasive ecotoxicological experiments with L. angulifera.

Keywords: Estuaries, Gastropods, Morphometrical Alteration, Monitoring, Tributyltin.

ALTERAÇÃO SEXUAL EM LITTORARIA ANGULIFERA (GASTROPODA: LITTORINIDAE) ALIMENTADA COM DIETA ARTIFICIAL CONTAMINADA COM TRIBUTILESTANHO

RESUMO

Objetivo: Este estudo verificou a presença de alterações sexuais morfológicas em Littoraria angulifera alimentadas com dieta artificial com tributilestanho (TBT).

Referencial teórico: Littoraria angulifera (Lamarck, 1822) é um gastrópode conspicuo estuarino de regiões tropicais. Apresenta hábitos alimentares generalistas e cumpre vários critérios de seleção para monitorização de estuários.

Método/desenho/abordagem: Durante 30 dias fêmeas de L. angulifera (n=360) foram colocadas em placas de cultura e alimentadas com fragmentos de papel embebidos em um soluções controle (água do mar e Dimetil...
sulfóxido (DMSO)) e com TBTCl (0,1 e 0,5 μg g⁻¹). Após esse período, os espécimes que sobreviveram foram anestesiados (10% MgCl²) e eutanasiados para análise do comprimento palial do oviduto (POL) e distância entre a abertura do ânus à vulva (DAV).

Resultados e conclusão: O consumo de papel diminuiu após a primeira semana de tratamento em todos os grupos, e os espécimes alimentados com dieta contaminada com TBT pararam de comer durante a segunda semana. Os espécimes expostos ao TBT exibiram POL alongado, enquanto DAV foi reduzido, alterações que podem comprometer a função reprodutiva. Não foi detectado desenvolvimento peniano em fêmeas alimentadas com dieta contaminada com TBT.

Implicações para pesquisa: *L. angulifera* pode ser uma espécie estuarina para avaliação de contaminantes organoestânicos, o que também eleva sua importância em programas de biomonitoramento.

Originalidade/Valor: Este estudo destaca que o uso de dieta artificial contaminada com papel é significativo ao propor um material de baixo custo, de amplo acesso e facilidade de manuseio e medição como uma alternativa de baixo custo para experimentos ecotoxicológicos invasivos com *L. angulifera*.

Palavras-chave: Estuários, Gastrópodes, Alteração Morfométrica, Monitoramento, Tributilestanho.

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1 INTRODUCTION

The genus *Littoraria* Griffith and Pidgeon, 1834 (Gastropoda: Littorinidae) is found exclusively in mangroves (Vermeij, 1973). The strong association with this environment is evidenced by its several adaptations to the habitat include shell architecture (Reid, 1986), polymorphism (McKillup & McKillup, 2008), ovoviviparous development, plasticity of radular teeth (Reid, 1986) and dietary innovations (Lee & Williams, 2002; Silliman & Newell, 2003). *Littoraria angulifera* (L.) are a generalist grazer (Lee & Williams, 2002) and depends on trees and substratum for food, shade, and protection from predators (Lozouet & Plaziat, 2008; Reid, Dyal, Lozouet, Glaubrecht, & Williams, 2008).

Due to the ecological characteristics, ease of collection and manipulation (Merkt & Ellison, 1998; Boehs & Freitas, 2021), *L. angulifera* fulfills several criteria for monitoring estuaries and mangroves (McCarty & Munkittrick, 1996). Some studies reported the use of *L. angulifera* and congeneric species as an indicator of organisms in mangrove areas after changes in morphology caused by chemical pollution, such as the organotin compounds like tributyltin (TBT) (Melo, Maia, & Rocha-Barreira, 2012; Costa, Zamprogno, Pedruzzi, Dalbem, & Tognella, 2013).

Due to the worldwide ban in 2003 due to its toxic effects on several aquatic organisms, the TBT continue been used in antifouling paints on ships and fishing nets in marine and estuarine areas in South America (Martínez, Piol, Nudelman, & Guerrero *et al*. 2017; Van Gessellen, Bouwman, & Averbuj, 2018), where the Littorinidae species can be used to evaluate and biomonitoring TBT in mangrove systems (Syahrial & Ezraneti, 2021).

Despite the criteria for biomonitoring, few studies have used *L. angulifera* as an environmental quality sentinel species in mangroves/estuaries (Tanaka & Maia, 2006; Sánchez-Arias, Rodíguez, Caballer, Asmussen, & Medina, 2011; Melo, Maia & Rocha-Barreira, 2012). In order investigate this gap, the aim of this study was to verify possible morphological changes in sexual parameters in *L. angulifera* submitted to a non-invasive experiment using artificial diet with TBT. Our hypothesis was that females exposed to TBT by an artificial diet contaminated will increase POL and decrease DAV as a morphological
Sexual Alteration in *Littoraria Angulifera* (Gastropoda: Littorinidae) Fed with an Organotin-Contaminated Artificial Diet

response to TBT contamination in the lowest concentration.

2 THEORETICAL FRAMEWORK

Estuaries and mangroves feature a variety of ecological niches that provide a diverse fauna with representatives of various phyla (Macnae, 1969). Among these, a small group of organisms has a mandatory association with the estuarine environments (Plaziat, 1984). This group includes mesogastropods of like species of the genus *Littoraria* Gray, 1833, that adapted to live in these environments (Reid, 1986; Merkt and Ellison, 1998). Due the response in their morphological to the specific environment they occupy, as well as to human impacts, these mollusks are becoming attractive for use in environmental monitoring (Zeidan, Freitas, Santos, Silva-Neto & Boehs, 2018; Tanaka and Maia, 2006; Melo et al., 2012; Costa et al., 2013; Martínez et al., 2013).

In relation to the use of the morphometric parameters (prostate length and distance between the opening of the prostate to the anus in males; oviduct length and distance between the vulva to the anus in females), Zeidan et al. (2018) confirmed the positive correlation between oviduct length and shell height. Thus, this parameter can be used in reproductive evaluation of this species. However, to reinforce the adequacy of *L. angulifera* use in estuarine monitoring, the effect of pollutants also needs to be investigated.

In general, environmental damage on the coast of Bahia is mainly associated with the disorderly rural and urban occupation in the surroundings of rivers and estuaries, which also often receive untreated domestic sewage, cellulose industry effluents, as well as pesticide residues from upstream crops (Schiavetti *et al*., 2002; De Matos & De Moura Pires, 2020). Also, because it is an area with intense naval activity, as well as nautical tourism and artisanal fishing, several impacts are also due to maritime activities (de Oliveira Aguiar & Cancado, 2020). One of these impacts is the organotin contamination that can cause sexual alterations and reproductive unfeasibility in marine gastropods, as verified in the region by Zeidan and Boehs (2017) in the neogastropod *Stramonita rustica* (Lamarck, 1822). Although mesogastropods are considered less sensitive than neogastropods to some types of chemical contamination (Bauer *et al*., 1995), these are interesting alternatives to places where there are no neogastropods, as is the case of mangroves.

Sexual alterations in the genus *Littoraria* were first reported by Bauer, Fioroni, Ide, Liebe, Oehlmann, Stroben, and Watermann (1995). They found that female *Littorina littorea* (L.) exposed to antifouling paints containing TBT showed a gradual replacement of the oviduct by prostate resulting in sterilization, a disfunction called *intersex*. Rank (2009) observed the high sensitivity to TBT pollution in *L. littoria* in a highly contaminated harbor in Denmark. Costa, Zamprogno, Pedruzzi, Dalbem, and Tognella (2013) verified the incidence of *intersex* development in *L. angulifera* in harbor areas along the Brazilian Southeast coast contaminated by TBT. Our group (Zeidan, Barroso, & Boehs, 2018) observed signs of pallial oviduct length (POL) elongation and reduction in the distance between the anus and the vulva (DAV) in *L. angulifera* from regions of the Brazilian Northeast with high levels of chemical contamination indicating masculinization.

Within the assumptions for bioindication referred by Mccarty and Munkittrick (1996), *L. angulifera* meets several selection criteria for estuaries and mangroves monitoring, among them, easy collection and manipulation and its conspicuous presence in these systems. Nevertheless, few studies have used it as a sentinel species of environmental quality in mangroves/estuaries (Martínez *et al*., 2013). Recent studies in Southeast (Costa *et al*., 2013) and Northeast Brazilian coast (Zeidan *et al*., 2018), suggest the feasibility of using *L. angulifera* as bioindicator for organotin contamination that still been used in antifouling paints of boats.
3 METHOD

3.1 Samples

Female of *Littoraria angulifera* (n = 920) identified macroscopically *in situ* by the absence of the penis were obtained by active search/manual capture during low tide, in September and November of 2016, on stems and aerial roots of *R. mangle* in a well-preserved mangrove (Zeidan, Freitas, Santos, Silva Neto, & Boehs, 2019) in South Atlantic (14°48'34.48"S; 39°2'21.76"W) in the municipality of Ilhéus, Bahia State, in the northeast of Brazil. The specimens were placed in polypropylene bags labeled and transported to the State University of Santa Cruz - UESC (Ilhéus) for laboratory procedures. The samplings were authorized by the Chico Mendes Institute for Biodiversity Conservation - ICMBio, Brazil (License Number 20912-3/2014).

3.2 Biometric Parameters

The criteria for grouping by shell height were defined according to Lenderking (1953), who described the sexual maturity of *L. angulifera*. According to this methodology females were divided in two shell height groups, Group A: measuring between 15 and 16 mm and Group B: between 19 and 20 mm.

The following sexual parameters were evaluated: pallial oviduct length (POL) and distance between the anus opening to vulva (DAV), as criteria of sexual morphometry of *L. angulifera* (*Figure 1*) (Zeidan et al., 2019). The measurements were performed under a stereoscopic microscope with the aid of a digital caliper and then transcribed to data sheets and finally to the statistical software.

![Figure 1](image.png)

**Figure 1.** Microscopic view of a female of *Littoraria angulifera* with indication of the sexual parameters measured in the study, being: DAV = Distance between Anus (AA) and Vulva (V) and POL = Pallial Oviduct (OP) Length.

**Source:** Prepared by authors.

3.3 Artificial Diet Trial

In a previous study we were surprised to observe that *L. angulifera* had a preference for white office paper from a marking tags aquarium. The papers were totally eaten even in the presence of natural food sources (boughs with fungi and lichens). After this *in loco* observation, we performed a trial to verify if an exclusively white office paper diet for two months has any impact on the survival of *L. angulifera*. For this, we initially used 360 females, divided into two groups with 180 specimens according to the shell height category (Group A:
15.53±0.53 mm; Group B: 20.15±0.37 mm). The specimens were labeled in numerical sequence with ink pen and then, placed in a 5 L capacity aquarium for acclimatization at a temperature of 25±1ºC, constant controlled aeration and with a small volume of seawater (100-150 ml) in the bottom to maintain moisture in the aquarium (dilution of 30±1). The animals were fed with wet white office paper and observed every day to survival, foraging, and aquarium maintenance.

At the end of two months, 100% of the specimens had survived and avidly fed with the diet that had been offered. After this trial, we released the specimens into their natural habitat. We decided did not use them in the next set of experiments because we believed that the stress generated by this first trial could interfere with their survival in the second experiment. For this reason, a second cohort of 360 different females were sampled in the same station and separated into two groups according to size, following Lenderking’s (1953) criterion, to perform the contaminated diet trial. The specimens were measured, weighed, and maintained for five days in a 5 L capacity aquarium for acclimatization according to the same abiotic criteria used in the first essay. The specimens were fed with white office paper moistened in sea water at dilution of 30. After acclimatization, the individuals were measured again, weighed, and then divided into four treatments in triplicate (n=15/triplicate), placed in different culture plates with 24 well (n = 12) each one and fed with the artificial diet over 30 days. The choice of white paper in this trial was due to the palatability, low cost, easy handling and measurements, and easy reproducibility.

The paper used to feed the animals was cut into fragments of 1cm², soaked with different solutions: control treatment with sea water (T0), control treatment with Dimetil sulfoxid (DMSO) (T1), TBTC1 (0.1 μg g⁻¹) (T2), and TBTC2 (0.5 μg g⁻¹) (T3). Then, the papers were dried to ambient temperature in the absence of light and before being placed in the bottom of each pit they were moistened in sea water. The concentrations were designed according to Oehlmann, Bauer, Minchin, Schulte-Oehlmann, Fioroni, & Markert (1998) and adapted from Bauer et al. (1995) to ensure a good correspondence between nominal and real aqueous TBT exposure in polluted areas with recorded sexual alteration in congeneric species L. littoria. The tributyltin chloride (TBT (CAS number 1461-22-9) was purchased from Sigma Aldrich. The stock solution (4.8 μg L⁻¹ of TBT) was prepared in DMSO and stored at 4 °C in the dark before use. The TBT solutions in different concentrations were prepared by diluting the stock solution in DMSO according to each treatment. Every week the paper fragments were changed in each treatment and the area of the remaining papers was measured in millimeters. Then, we calculated the amount eaten subtracting the total area minus the remaining area in each paper fragment. The food supply was constant (ad libitum) according to the foraging of the specimens. The contaminated diet trial was conducted in an open box with no light to avoid TBT degradation. Laboratory conditions were maintained at 20 °C and the aeration was constant and available through the perforated cover culture plate to allow air circulation.

After the experiment, all the specimens that had survived were anesthetized with 10% MgCl₂ saline for 1 hour (Zeidan et al., 2018) and euthanized. Stereoscopic microscopy analysis was performed to observe the sexual parameters and a possible alteration of pallial oviduct length (POL) and distance between the anus opening to vulva (DAV) in specimens treated with the contaminated diet, as a criterion of sexual morphometry of L. angulifera, as previously observed by Zeidan et al. (2019).

3.4 Data Analysis

The paper consumption was measured weekly in all treatments. The paper plotted in the bottom of plats was removed and placed on a millimetric rule. The total mortality until the
end of the trial was calculated using descriptive statistic. To compare the survival between the groups a t Test was used. The correlation of height with the parameters POL and DAV was verified by the Pearson correlation coefficient (r). The biometric parameters analyzed were POL and DAV, which were compared among treatments using Kruskal-Wallis analysis followed by the Dunn post hoc test. The confidence level was set at 95% and the statistical package was the Bioestat 5.3.

4 RESULTS AND DISCUSSION

This experiment was the first to show sexual alteration in *L. angulifera* submitted to an artificial diet as the food source. It is usual to conduct long-term ecotoxicological experiments with contaminant injections to induce sexual changes in gastropods (Bauer et al., 1995; Horiguchi, 2017). Before the beginning of this study, we previously injected the same concentrations of TBT and DMSO used along this experiment and verified mortality in the majority the organisms in both treatments. However, high survival at the end of this experiment allowed us to conduct the study and verify the sensitivity of *L. angulifera* to invasive experiments. A total of 179 individuals (49.72%) survived, 21.25±4.64% in Group A and 23.5±9.18% in Group B, with no significant difference (p=0.03) between the groups. Despite the fact that the treatment T3 contains TBT, Group B showed the highest percentage survival among treatments and groups (Table 1).

Table 1. Survival results of diet experiment with *Littoraria angulifera* females of two height classes (groups A and B) in different treatments (T0, T1, T2 and T3).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>% of survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP A</td>
<td></td>
</tr>
<tr>
<td>T0</td>
<td>60</td>
</tr>
<tr>
<td>T1</td>
<td>51.11</td>
</tr>
<tr>
<td>T2</td>
<td>37.77</td>
</tr>
<tr>
<td>T3</td>
<td>40</td>
</tr>
<tr>
<td>GROUP B</td>
<td></td>
</tr>
<tr>
<td>T0</td>
<td>51.11</td>
</tr>
<tr>
<td>T1</td>
<td>46.66</td>
</tr>
<tr>
<td>T2</td>
<td>31.11</td>
</tr>
<tr>
<td>T3</td>
<td>80</td>
</tr>
</tbody>
</table>

Source: Prepared by authors.

In a previous experiment, the preference of *L. angulifera* for cellulose fiber derived from paper compared to the natural source (fungi and algae) was confirmed. Due to this observation, it was possible to conduct the present trial. Representatives of genus *Littoraria* are opportunistic feeders. This was confirmed for *L. angulifera* by Kohlmeyer and Bebout (1986) and Lee and Williams (2002), who reported the existence of four food items in the intestine of this species: cork cells, cellulose fibers, fungal propagules, and algae. According to these authors, the food proportions vary depending on to the type of the substratum that each snail had fed upon. This might be explained by the abundance source of cellulose from mangrove trees in comparison to other available items (Lee & Williams, 2002) and the easier ingestion of celluloses cells that are in part corroded enzymatically, while fungi and microalgae are excreted unchanged in their feces (Kohlmeyer & Bebout, 1986).

During the experiment, a gradual decrease in paper consumption was observed after the first week in all groups and treatments. In the second week all animals receiving the contaminated diet with the TBT solution stopped eating in both classes, evidencing the non-palatability and toxicity of the diet, modifying the food intake (Figure 2). On the other hand, the consumption in the treated groups without the TBT also decreased. While it was not possible to explain the reason for this reduction, we hypothesized the decrease in food...
consumption in the control groups might be related to the experiment conditions, including confinement in the culture plates, the aphotic environment, the proximity of treatments with TBT, the volatility of this compounds. In animals submitted to the contaminated diet, the TBT may influence the palatability and toxicity of the diet, modifying the food intake, because the animals treated at T2 and T3 doses stopped eating from the second week.

Figure 2. Evolution of paper consumption by *Littoraria angulifera* throughout the experiment. First graphic= Group A; Second graphic= Group B.

Source: Prepared by authors.

The biometric results are shown in the Table 2. In both groups we observed statistical differences in the sexual parameters in the specimens fed with TBT (T2 and T3). Elongation of POL was found in females exposed to TBT, inducing a consequent decrease in DAV. In groups A and B, we observed a weak correlation ($r>0.28$) between shell height and DAV. In the group B, a high correlation was found ($r<0.74$) between shell height and POL in the specimens that received the control treatments (T0 and T1). This observation means that a TBT-contaminated diet might compromise the natural development of the reproductive organ responsible for storage and release of the eggs. This hypothesis was confirmed by the high negative correlation ($r=-0.74$) between POL and DAV in adult females exposed to the higher concentration of TBT (T2).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
<th>Shell height</th>
<th>POL</th>
<th>DAV</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>T0</td>
<td>15.90±0.75</td>
<td>3.51±0.28</td>
<td>2.12±0.24</td>
</tr>
<tr>
<td></td>
<td>T1</td>
<td>15.55±0.81</td>
<td>3.46±0.29</td>
<td>2.12±0.23</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>15.99±0.56</td>
<td>4.25±0.48</td>
<td>1.16±0.50</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>15.74±0.50</td>
<td>4.49±0.28</td>
<td>0.96±0.23</td>
</tr>
<tr>
<td></td>
<td>T0</td>
<td>19.86±0.60</td>
<td>4.47±0.73</td>
<td>2.45±0.26</td>
</tr>
<tr>
<td>B</td>
<td>T1</td>
<td>20.02±0.62</td>
<td>4.72±0.74</td>
<td>2.55±0.26</td>
</tr>
<tr>
<td></td>
<td>T2</td>
<td>19.90±0.58</td>
<td>5.73±0.59</td>
<td>1.04±0.50</td>
</tr>
<tr>
<td></td>
<td>T3</td>
<td>19.99±0.61</td>
<td>5.67±0.72</td>
<td>1.35±0.56</td>
</tr>
</tbody>
</table>

Source: Prepared by authors.

The consistent alterations in POL and in the DAV observed showed a plasticity of sexual parameters due to toxicity of TBT, corroborating previous findings of Zeidan et al. (2018). In our experiment, in both height classes the pallial oviduct was elongated, while the DAV
Sexual Alteration in *Littoraria Angulifera* (Gastropoda: Littorinidae) Fed with an Organotin-Contaminated Artificial Diet

The pallial oviduct is related to the transport and storage of sperm and the production of planktonic eggs before spawning in water (Lenderking, 1953). The oviduct of the females lies along the rectum cavity and terminates in the final projecting extremity just outside the anus. It opens to the mantle cavity at a small and distal aperture (vulve), where the females firstly spawn their eggs in the mantle cavity and after veliger larvae in water (Reid, 1986). This anatomical position and size of pallial oviduct represents a reproduction strategy. The development of eggs occurs in the mantle cavity before releasing them to environmental since this species does not have capsule organs (Merkt & Ellison, 1998). Larger females may have larger litters, as they have more space to store and to hatch the eggs in the mantle cavity (Zeidan et al., 2019), assuming that the fertility increases in larger animals, converging with the interpretation of (Merkt & Ellison, 1998).

In both size classes and concentration of TBT (T2 and T3), POL and DAV showed significant differences (p<0.001) among females from control treatments (sea water and DMSO) (Table 3). In these first, the pallial oviduct became more elongated and closer to the anal opening like the prostate in males. According to Reid (1986), in male *L. angulifera* the prostate is the organ responsible for sperm nutrition and transport. It is located in the right lateral portion of pallial cavity, parallel to the anus and above the columellar muscle, being in the distal portion of prostate. It opens to the spermatic duct located in the right lateral region of the head, which carries sperm forward the over lateral surface of head-foot to the penis, located on the right side of the head behind the eye. The decrease in DAV and its proximity to the anal opening in females fed with TBT indicates masculinization and compromising the release of larvae to the environment.

The post-hoc test showed that the TBT did not cause (p=ns) a dose-dependent effect on the parameters analyzed in both size classes. The POL became more elongated, and the DAV reduced in females that were exposed to TBT, as shown in Table 1. No penis development was observed in the females as previously observed by Zeidan et al. (2018).

**Table 3.** Results obtained for two sexual parameters of *Littoraria angulifera* from control treatments (sea water and DMSO) and exposed to contaminated diet of 0.5 μg.g⁻¹ and of 0.1 μg.g⁻¹ TBTCl. POL: Pallial Oviduct Length; DAV: Distance between Anus and Vulva; H*: test value; p values denote statistically significant differences between control and TBT fed females.

<table>
<thead>
<tr>
<th>Group</th>
<th>Variables</th>
<th>Degrees of Freedom</th>
<th>H*</th>
<th>p values</th>
<th>Post-hoc Dunn</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>POL</td>
<td>3</td>
<td>55.974</td>
<td>&lt;0.0001</td>
<td>T0 and T1 (p= ns)</td>
</tr>
<tr>
<td>A (n=85)</td>
<td></td>
<td></td>
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<td>T0 and T2 (p&gt;0.05)</td>
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<td>T0 and T3 (p&gt;0.05)</td>
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<td>T1 and T2 (p&gt;0.05)</td>
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<td>T1 and T3 (p&gt;0.05)</td>
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<td>T2 and T3 (p=ns)</td>
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<tr>
<td></td>
<td>DAV</td>
<td>3</td>
<td>55.974</td>
<td>&lt;0.0001</td>
<td>T1 and T2 (p&gt;0.05)</td>
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<td>T2 and T3 (p=ns)</td>
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<td>T0 and T2 (p&gt;0.05)</td>
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<td></td>
<td>T0 and T3 (p&gt;0.05)</td>
</tr>
<tr>
<td></td>
<td>POL</td>
<td>3</td>
<td>50.009</td>
<td>&lt;0.0001</td>
<td>T1 and T2 (p&gt;0.05)</td>
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<tr>
<td>B (n=94)</td>
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<td></td>
<td></td>
<td>T1 and T3 (p&gt;0.05)</td>
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<td>T0 and T1 (p= ns)</td>
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<td>T0 and T2 (p&gt;0.05)</td>
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<td>T0 and T3 (p&gt;0.05)</td>
</tr>
</tbody>
</table>
The penis development is the last stage (stage 4) of masculinization induced by TBT in *L. littoria* (Bauer et al., 1995) and it is by far the least frequent stage in field populations (Oehlmann et al., 1998). Since the sexual alteration in littorinids occurs gradually in mature females the absence of a penis may be related to the duration of this experiment. To answer this question properly, it would be necessary to carry out a long-term experiment to verify if the masculinization stages in *L. angulifera* follow similar patterns to *L. littoria*, as they are species with different reproductive strategies.

Sexual alterations in the congeneric species *L. littorea* were firstly reported by Bauer et al. (1995). The females were experimentally exposed to Sn at a concentration of approximately 15 ng. This resulted in a transformation of capsule gland in a prostate, causing sterility and confirming the endocrinal disruption called *intersex*. Since then, more than 20 studies were published investigating *intersex* in periwinkle *L. littorea*, as species for TBT biomonitoring in estuarine environments on the north Atlantic Coast and in the Mediterranean Sea (Syahrial & Ezraneti, 2021). So far, only one study was conducted on *L. angulifera* to verify its potential for biomonitoring of TBT. Costa et al. (2013) used the same index proposed by Bauer et al. (1995) and found high levels of *intersex* in *L. angulifera* collected on the Southeastern coast of Brazil. Nevertheless, it is not possible to attribute *intersex* stages proposed by Bauer et al. (1995) to female *L. angulifera* because it has distinct reproductive strategies compared to *L. littorea*. While *L. littorea* is an oviparous species that show capsule glands, *L. angulifera* does not have any capsule gland due to its ovoviviparity (Reid, 1986). However, phenotypic sexual disturbance occurs in the genital tract in the specimens submitted to TBT treatment and this organotin can affect population reproduction in the natural environmental.

In addition to the sexual changes observed in the field by Zeidan et al. (2018) and confirmed in this study, changes in morphometry and sensitivities of *L. angulifera* in coastal areas, especially mangrove ecosystems, enable the use of this species as a bioindicator, as already suggested by Syahrial and Ezraneti (2021). In this context, some studies have been carried out in recent years using *L. angulifera* on the Brazilian coast. Melo et al. (2012) reported variations in shell morphology in response to mangrove deforestation. Additionally, Boehs and Freitas (2021) found an influence of mangrove tree cover on the size and weight of the species in the coast of Bahia. Thus, *L. angulifera* seems to fill the requirements to be used in biomonitoring programs (McCarty & Munkittrick, 1996), especially in estuaries, as *L. angulifera* is a species found only in of mangroves.

5 CONCLUSION

This study highlights the use of contaminated artificial diet as an alternative for invasive ecotoxicological experiments with *L. angulifera* instead of a natural diet. The use of white paper in this experiment is significant in proposing a material with low costs, widespread access, and ease of handling and measurement. Although paper consumption decreased during the study, due to the unpalatable nature of TBT and the confining condition of the experiment, the changes observed in females exposed to a contaminated diet, evidenced a trophic source of contamination. Additionally, the results obtained confirmed the consistency of sexual alterations in both size classes and concentration attesting sexual alterations caused by TBT in *L. angulifera* females from 15 mm. The extension of the pallial oviduct making it closer to the anal opening, can compromise larval spawning and, consequently, population reproduction.

Source: Prepared by authors.

<table>
<thead>
<tr>
<th>DAV</th>
<th>3</th>
<th>57.079</th>
<th>&lt;0.0001</th>
<th>T1 and T2 (p&gt;0.05)</th>
<th>T1 and T3 (p&gt;0.05)</th>
<th>T2 and T3 (p=ns)</th>
</tr>
</thead>
</table>
Due to several ecological attributes, the exclusive occurrence in mangroves and morphological responses to low TBT concentrations already observed in the field and confirmed in this study, it is possible to conclude that . Despite the prohibition on use of TBT compounds in antifouling paints, they continue to be used in South America, as evidenced by several investigations (Peraza, Russel, Castro, & Fillmann, 2022). This indicates that the prohibition has not been effective in those countries. Continuous monitoring to assess the evolution of this scenario in marine and estuarine environments is essential to propose legally effective measures for the non-use of TBT-based antifouling paints.

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