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**Acute and multigenerational toxicity of polylactic acid microplastics on a copepod
bioindicator**

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30 **Abstract**

31

32 The increasing production and improper management of petrochemical plastic wastes have led
33 to widespread environmental issues. Bioplastics, particularly polylactic acid, are promoted as
34 eco-friendly alternatives, but their environmental impacts remain poorly understood. We
35 investigated the acute and multigenerational effects of polylactic acid microplastics on the
36 copepod *Eurytemora affinis*, a remarkable bioindicator species of zooplankton. Our research
37 revealed distinct 96-hour median lethal concentration values, significantly higher ($p < 0.05$) for
38 adult males (134.6 mg/L) than for adult females (106.9 mg/L). In multigeneration exposure, we
39 found that a lower concentration of 400 $\mu\text{g/L}$ polylactic acid microplastics significantly ($p <$
40 0.05) induced higher mortality, reduced reproduction through the production of smaller-sized
41 eggs, elongation of the naupliar phase, and offspring with lower fitness. The lower fitness in
42 offspring led to a significant ($p < 0.05$) reduction in female body size, including prosome length,
43 width, and volume. Interestingly, a recovery trend in copepod survival and reproductive
44 parameters was observed in the generation of decontamination, fifth filial generation. Our
45 findings highlight the potential ecological risks that could be posed by polylactic acid
46 microplastics in marine environments and emphasize that their environmental fate should not
47 be underestimated.

48

49 **Keywords:** Bioplastics; Polylactic acid; Microplastic; Copepod; Multigeneration; Toxicity.

50

51 **1.Introduction**

52

53 Global plastic production increased to over 400 million metric tons in 2022, and if current
54 management trends continue, 12,000 million metric tons of plastic waste will be disposed of by
55 2050 (John et al. 2022; Ali et al. 2024). Microplastics originating from petrochemical plastics
56 have been reported to exert negative effects on biota, accumulate in the food chain, and have
57 adverse effects on ecosystems (Osman et al. 2023; Eze et al. 2024). Bioplastics have emerged
58 and been promoted as a safe alternative, and their production has reached 2.1 million tons in
59 2023 (EuropeanBioplastics 2023). Among the bioplastics, polylactic acid, a biobased polymer,
60 constituting approximately 31% of all bioplastics produced in 2023 which has found extensive
61 applications across various sectors, including medical applications, packaging, and now also
62 emerging in textile applications (Ali et al. 2023b; Ferreira et al. 2024).

63 Polylactic acid has been reported to produce microplastics under aquatic environmental
64 conditions (Le Gall et al. 2022), and these microplastics have been detected in the aquatic
65 environment (Kazour et al. 2019). Recently, we reviewed the negative impacts of these
66 biobased microplastics on the exposed aquatic biota and concluded that these microplastics
67 could be as toxic as petrochemical ones (Ali et al. 2023a). We emphasized the long-term toxicity
68 evaluation of these microplastics, including their multigenerational effects, which have never
69 been studied.

70 Considering this knowledge gap, this study is the first to investigate polylactic acid
71 microplastics' acute toxicity and assess its intergenerational chronic toxicity on *Eurytemora*
72 *affinis*, a bioindicator species, for five generations using environmentally relevant
73 concentrations. We focused on exploring rarely addressed biomarkers associated with the
74 morphological and reproductive characteristics of *E. affinis*. Our primary objective was to gain
75 a comprehensive understanding of the organism's responses and the inheritance of developed
76 traits when exposed to next-generation microplastics in successive generations through a
77 multigenerational assay. Additionally, we aimed to examine the reversible vulnerability of the
78 studied biomarkers in a recovery generation to gain an insight to polylactic acid induced
79 toxicity.

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81 **2.Experimental**

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83 2.1 Microplastics and model organism

84

85 In this study, we used Ingeo™ Biopolymer 4032 D polylactic acid (Nature Works LLC,
86 United States) as starting material to synthesis microplastics of the size range 2 to 10 µm (Fig.
87 S1). A detailed on the synthesis and characterization are presented in Supplementary Material
88 (text S1).

89 The model organism used in this study was *E. affinis*, an estuarine zooplankton that
90 transports energy from primary producers to higher trophic levels, supports commercially
91 important fishes, and contributes to the biological carbon pump. Thus, it has been widely used
92 as a model organism for intergenerational toxicity studies of natural and anthropogenic stresses
93 (Souissi et al. 2016a; Souissi et al. 2021; Das et al. 2023; They et al. 2023). The selected model
94 organism originated from the wild which were collected at oligohaline zone of the Seine
95 estuary, France, in September 2014. Since then, this culture is maintained at the Laboratory of

96 Oceanology and Geosciences, Wimereux, using copepods culturing and rearing protocol
97 developed by Souissi et al. (2016a).

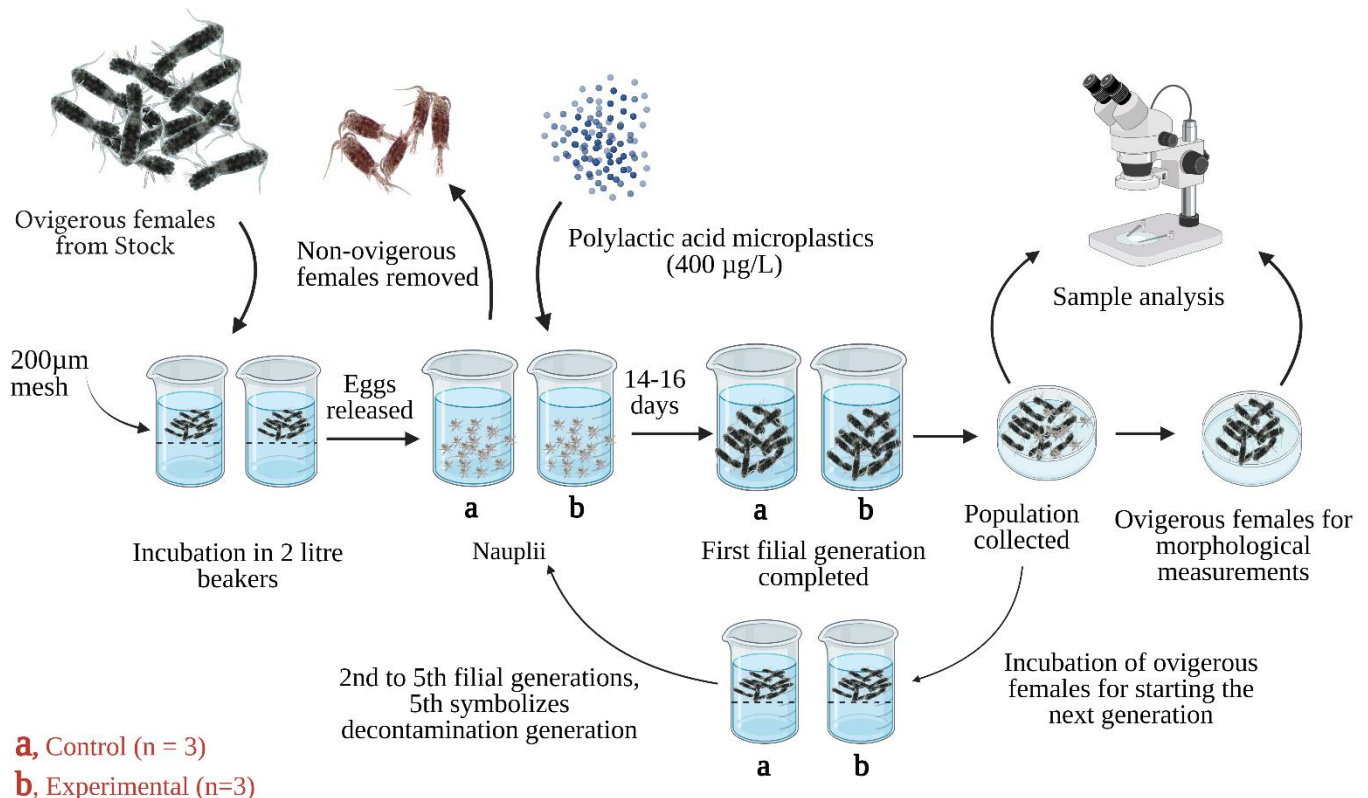
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99 2.2 Acute toxicity and multigeneration exposure

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101 The 96-hour lethal concentration (LC50) of polylactic acid microplastics in *E. affinis* adults
102 were determined based on our previous work (Kadiene et al. 2017), with details in the
103 Supplementary Material (text S2). For multigeneration experiment, we exposed copepods to
104 400 µg/L polylactic acid microplastics for five generations following the protocol of They et
105 al. (2023). We chose this concentration considering the highest reported environmental
106 petrochemical microplastic concentration of 2.5 mg/L (Cózar et al. 2014) and the limited data
107 on biomicroplastics in aquatic environments. Although our selected concentration may exceed
108 from those found in the environment but remaining lower than concentrations used in other
109 studies (An et al. 2024; Savva et al. 2023), bringing our experiment closer to realistic
110 contamination conditions.

111 The experiment comprised a control group and a polylactic acid microplastics group, each
112 with three replicates. 30 ovigerous females were initially selected for each replicate, and after
113 egg release, non-ovigerous females were removed. Microplastics were added to the treatment
114 group, and after 14 to 16 days, the population was filtered to complete the first filial generation.
115 Subsequently, 30 ovigerous females from each replicate were introduced into new beakers for
116 subsequent generations from the second to fifth filial generation. The fifth filial generation
117 served as a recovery group without microplastic exposure. Selected endpoints, including total
118 population, mortality, sex ratio, morphological, and reproductive parameters, were analyzed
119 following established methods (Souissi et al. 2016b; Das et al. 2023), with details provided in
120 the Supplementary Material (text S3). Results were analyzed according to in text
121 Supplementary Material (text S4).



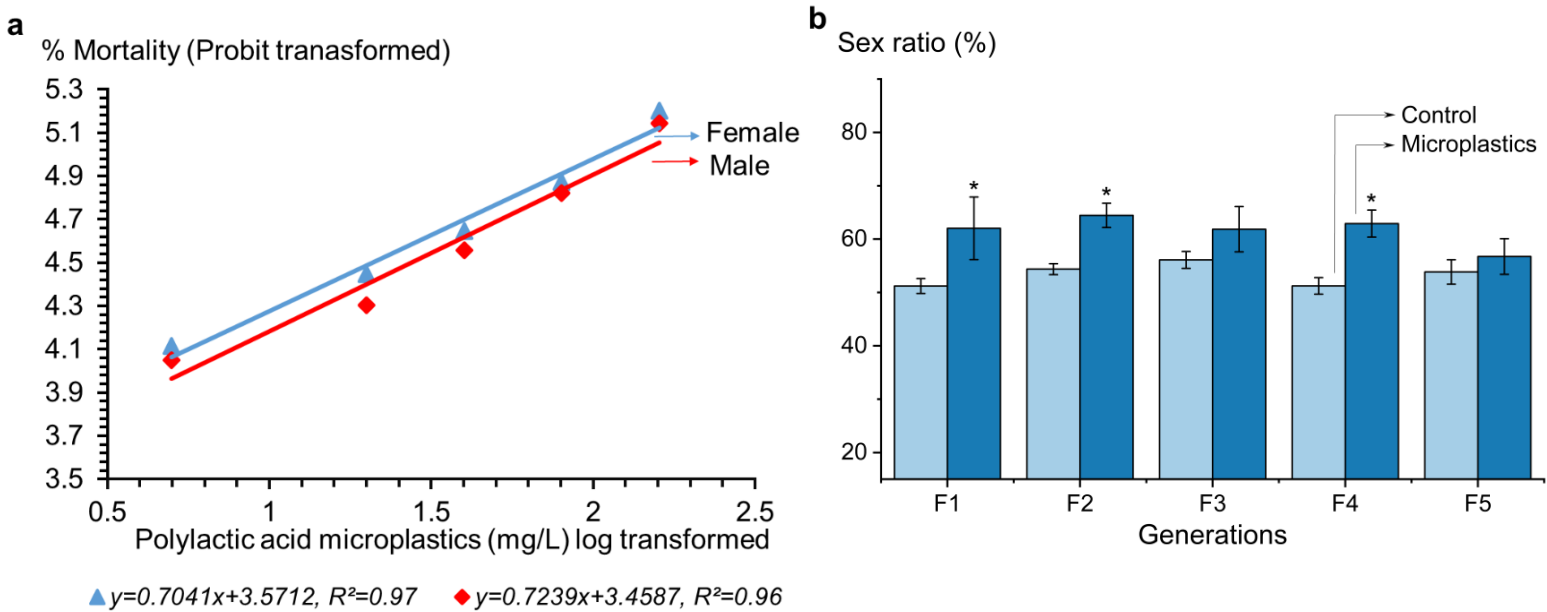
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Figure 1. Protocol for multigenerational exposure of *Eurytemora affinis* to polylactic acid microplastics, analyzing morphological and reproductive parameters at the end of each filial generation. Four generations assess transgenerational toxicity, with a fifth generation for recovery potential.

3. Results and discussion

3.1 Acute toxicity and multigenerational effects on sex ratio

We calculated the average 96-hour LC50 value for *E. affinis* as 120.8 mg/L. Gender-specific responses were observed, with adult males exhibiting a significantly higher ($p < 0.05$) tolerance, with LC50 of 134.6 mg/L (95% CI= 60–239 mg/L), compared to adult females who showed more susceptibility, with LC50 of 106.9 mg/L (95% CI = 64–203 mg/L) (Fig. 2a). Additionally, multigeneration exposure altered the sex ratio, with males comprising 62 percent of the population compared to 52 percent in the control group (Fig. 2b).



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Figure 2. 96-hour concentration to mortality curves for male and female *Eurytemora affinis* exposed to polylactic acid microplastics (a). Sex ratio (% of male) at the end of generations from the first filial (F1) to the fifth filial (F5) with mean \pm SE (b). Asterisks (*) represent statistical significance ($p < 0.05$).

Standard error (SE).

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Gender-specific responses to contaminants have been reported in crustacean populations (Kadiene et al. 2017, 2019). While other studies showed no lethal effects of polylactic acid microplastics on jellyfish and water flea with an effective concentration 50% (EC50) values of 77.4 and 16.4 mg/L (Di Giannantonio et al. 2022; An et al. 2024), our study demonstrates distinct LC50 values for male and female *E. affinis*. Similar to our findings, Kadiene et al. (2017) reported that *E. affinis* females are more sensitive to cadmium (LC50: 90.04 μ g/L) than

152 male (LC50: 127.75 µg/L). Sroda and Cossu-Leguille (2011) observed higher sensitivity to
153 copper in females of *Gammarus roeseli* and *Dikerogammarus villosus* compared to males.
154 Furthermore, we propose that the toxicity is caused by microplastics rather than the associated
155 chemicals, aligning with Zimmermann et al. (2020) and Jemec et al. (2016).

156 Exposure of *Daphnia magna* to polylactic acid and polyethylene terephthalate microplastics
157 increased the male offspring ratio (An et al. 2024). In our study, the increase in the male ratio
158 may be attributed to the higher sensitivity of females, indicated by a lower LC50 value. The
159 observed effects on the sex ratio in the exposed group could be linked to the larger size of *E.*
160 *affinis* females, prompting increased energy demand for foraging and a higher likelihood of
161 encountering microplastics. Higher grazing rates in female copepods have been associated with
162 size differences (Gréve et al. 2017). Moreover, our findings contribute to the understanding of
163 microplastic-induced alterations in sex ratios and their potential ecological consequences. If
164 female copepods are more susceptible to microplastic toxicity than males, it could lead to
165 increased female mortality and decreased recruitment production, potentially disrupting the
166 aquatic food chain due to lower food supply at higher trophic levels.

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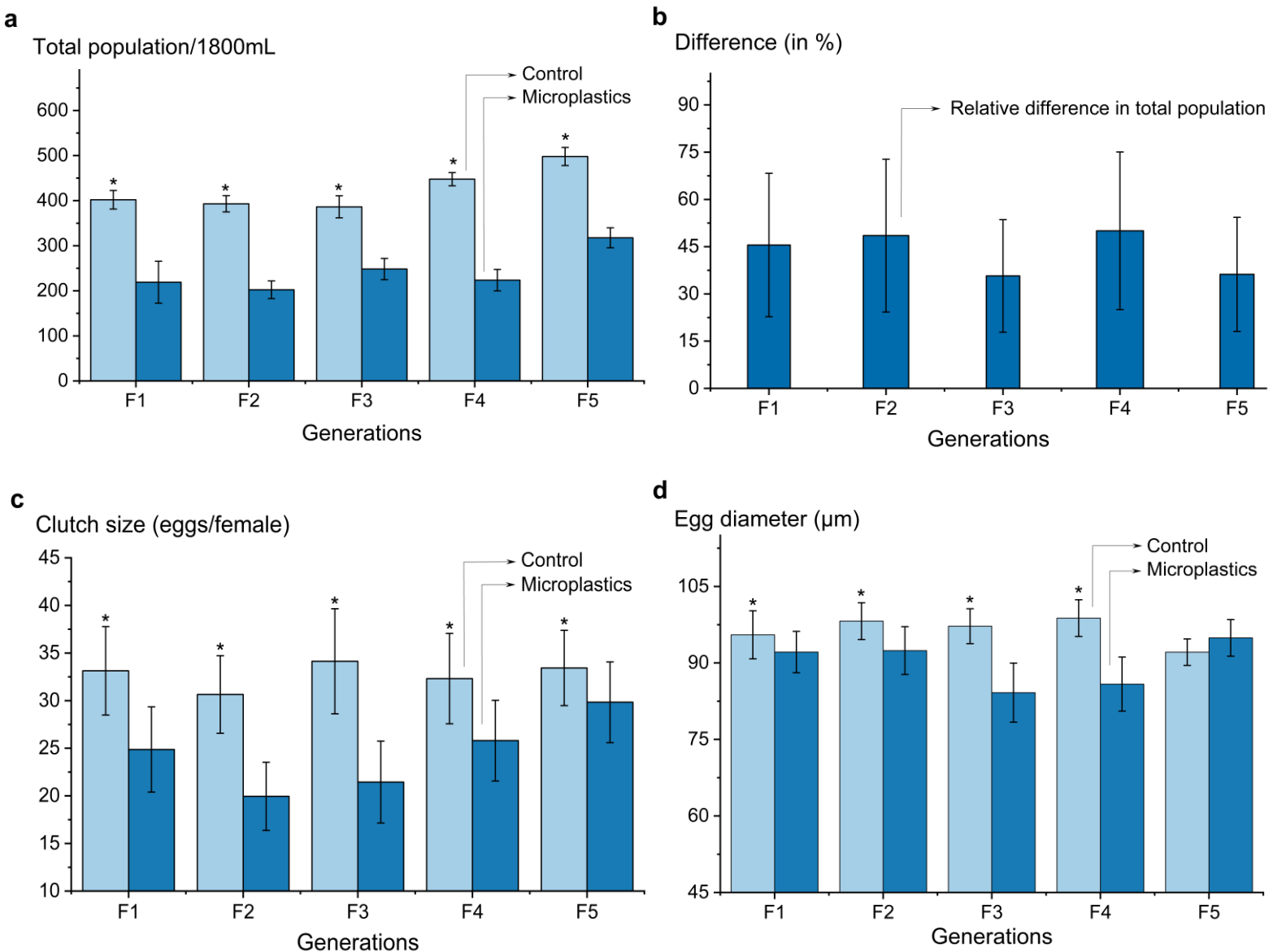
168 3.2 Effects on total population and mortality

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170 In the multigeneration exposure, we found that the exposed group had a significantly lower
171 population density ($p < 0.05$) than the control group, with 202 to 317 individuals per 1.8L versus
172 393 to 498 individuals in the control group (Fig. 3a). Notably, the decontamination generation,
173 the fifth filial generation, had lower mortality (47.68%) and a greater total population than the
174 exposed groups, suggesting a reversible vulnerability of reproductive parameters. The relative
175 difference in total population between the control and exposed groups varied across the
176 generations, ranging from 35.72 to 50.04% (Fig. 3b). Mortality data consistently showed higher
177 rates in the exposed group across all five generations (Fig. S4). Similarly, a significant
178 relationship was observed between the total population and successive generations in the
179 control group ($r^2 = 0.59$, $p = 0.016$), but not in the exposed groups ($r^2 = 0.41$, $p = 0.16$) (Fig.
180 S5a). Thus, the multigenerational exposure resulted in lower population density and
181 consistently higher mortality rates, indicating disrupted reproductive patterns with potential
182 significant ecological impact.

183 The ingestion of microplastics by zooplankton has been linked to increase bioconcentration
184 and oxidative stress, resulting in gastrointestinal tract obstruction and mortality (Na et al. 2023;
185 Jemec et al. 2016; Eltemsah and Bøhn 2019). In our study, the higher mortality in the exposed
186 could be attributed to the accumulation of microplastics in the gut, followed by the associated
187 implications. The increasing trend for mortality observed in the first and second generations
188 aligns with previous research on microplastics exposure (Schür et al. 2020), indicating a
189 substantial adverse impact during the initial exposure. Furthermore, fluctuations in resources
190 availability can significantly impact fitness (Hämäläinen et al. 2017). The decline in the total
191 population in subsequent generations could be attributed to the production of offspring with
192 lower fitness. In the third generation, a decrease in mortality and an increase in the total
193 population could be linked to the increased egg diameter in the second generation. These results
194 support the findings of Jamieson et al. (2003) who reported that large eggs in the copepod
195 *Cyclops kolensis* produce offspring with high fitness. The decontamination generation, the fifth
196 generation, displayed lower mortality and the highest total population, which could be attributed

197 to their proteomic plasticity, leading to an increase in energy metabolism and stress defense. In
 198 conclusion, the exposure of *E. affinis* to polylactic acid microplastics has led to increased
 199 mortality with transgenerational effects, affecting the population of subsequent generations and
 200 highlighting the potential implications of these microplastics for the aquatic food web.
 201



202

203 Figure 3. Total population (a), relative difference in total population (b), clutch size (c), mean
 204 egg diameter (d) of *Eurytemora affinis* in the control and polylactic acid microplastics-exposed
 205 groups at the end of generations from the first filial (F1) to the fifth filial (F5) with Mean ± SE.
 206 Asterisks (*) represent statistical significance ($p < 0.05$).

207 Standard error (SE).

208

209 3.3 Clutch size and egg diameter

210

211 In the multigeneration exposure, we found that clutch size significantly ($p < 0.05$) decreased
 212 compared to the control group, with the lowest value (19.5) in the second generation (Fig. 3c).

213 However, from the second to the fifth generations, the clutch size showed an increasing trend,
214 suggesting a tradeoff between fecundity and egg diameter. Moreover, mean egg diameter in the
215 exposed groups also significantly ($p < 0.05$) decreased, with a notable decline from the second
216 to third generation (92.41 to 84.17 μm), followed by an increase from the third to the fifth
217 generations (Fig. 3d). The control group exhibited an average of 32.7 eggs per female with an
218 average egg diameter of 96.35 μm , while the exposed groups had an average of 24 eggs per
219 female with an average egg diameter of 89.89 μm . No significant correlation was observed
220 between clutch size and subsequent generations in the control ($r^2 = 0.02$, $p = 0.99$) or exposed
221 ($r^2 = 0.37$, $p = 0.61$) groups (Fig. S5e). In the control group, egg diameter showed a moderately
222 positive correlation with subsequent generations ($r^2 = 0.27$, $p = 0.02$), while the exposed group
223 showed a negative correlation ($r^2 = 0.51$, $p = 0.00$) (Fig. S5f).

224 Microplastics, despite reports of fragmentation in some species' digestive tracts, such as
225 rotifers (Zhao et al. 2023), cannot be digested or absorbed by aquatic filter feeders. This may
226 result in energy depletion and consequently a decline in reproductive outputs. Exposure to
227 polystyrene microplastics in copepods resulted in reduced fecundity and smaller egg size,
228 attributed to an energy deficit and oxidative stress (Cole et al. 2013, 2015). These findings are
229 consistent with our results, where clutch size consistently decreased from the first to the second
230 generation. Our results also showed an increasing trend in clutch size and a decreasing egg
231 diameter from the third to the fourth generation, attributed to the tradeoff between clutch size
232 and egg size, as previously reported in *E. affinis* under environmental stress (Souissi and Souissi
233 2021). Microplastic exposure in marine medaka led to reduced fecundity, with offspring from
234 exposed parents exhibiting compromised antioxidant enzyme activities, resulting in decreased
235 fertility and hatching rates (Wang et al. 2019). Thus, the ingestion of polylactic acid
236 microplastics by *E. affinis* presumably leads to similar consequences as those observed for
237 petroleum-based microplastics, resulting in a reduction in food intake, which, in turn, prioritized
238 energy allocation for growth and survival, ultimately leading to reduced reproduction.

239

240 3.4 Effects on the life stages *Eurytemora affinis*

241

242 The copepod life stages of *E. affinis* in control and microplastics-exposed groups at the end
243 of each generation are shown in Table S1. The number of nauplii in the exposed groups was
244 significantly higher ($p < 0.05$) than in the control group in most generations, with the control
245 group averaging 15.6 nauplii, while the exposed group averaged 33 nauplii. In contrast, the
246 number of all other copepod life stages, including copepodite females and males, and adult
247 females and males, was significantly higher ($p < 0.05$), except for the adult males in the fifth
248 generation and copepodite males in the third to fourth generations, where the difference was
249 not significant ($p < 0.05$). Polylactic acid microplastics have resulted in premature hatching,
250 leading to the production of substandard nauplii, followed by an increase in the naupliar phase
251 and subsequent high mortality.

252 Microplastics ingestion in copepods could decrease food uptake, potentially causing an
253 energy deficit and impacting growth and development to adulthood (Botterell et al. 2019).
254 *Daphnia pulex* exposed to polystyrene microplastics significantly reduced the total number of
255 offspring (Liu et al. 2019). The development of *Tigriopus japonicus* from naupliar to copepodite
256 was delayed when exposed to polyamides and polyethylene microplastics (Yu et al. 2020).
257 Exposure to polystyrene microbeads resulted in slower growth in the larvae of the marine

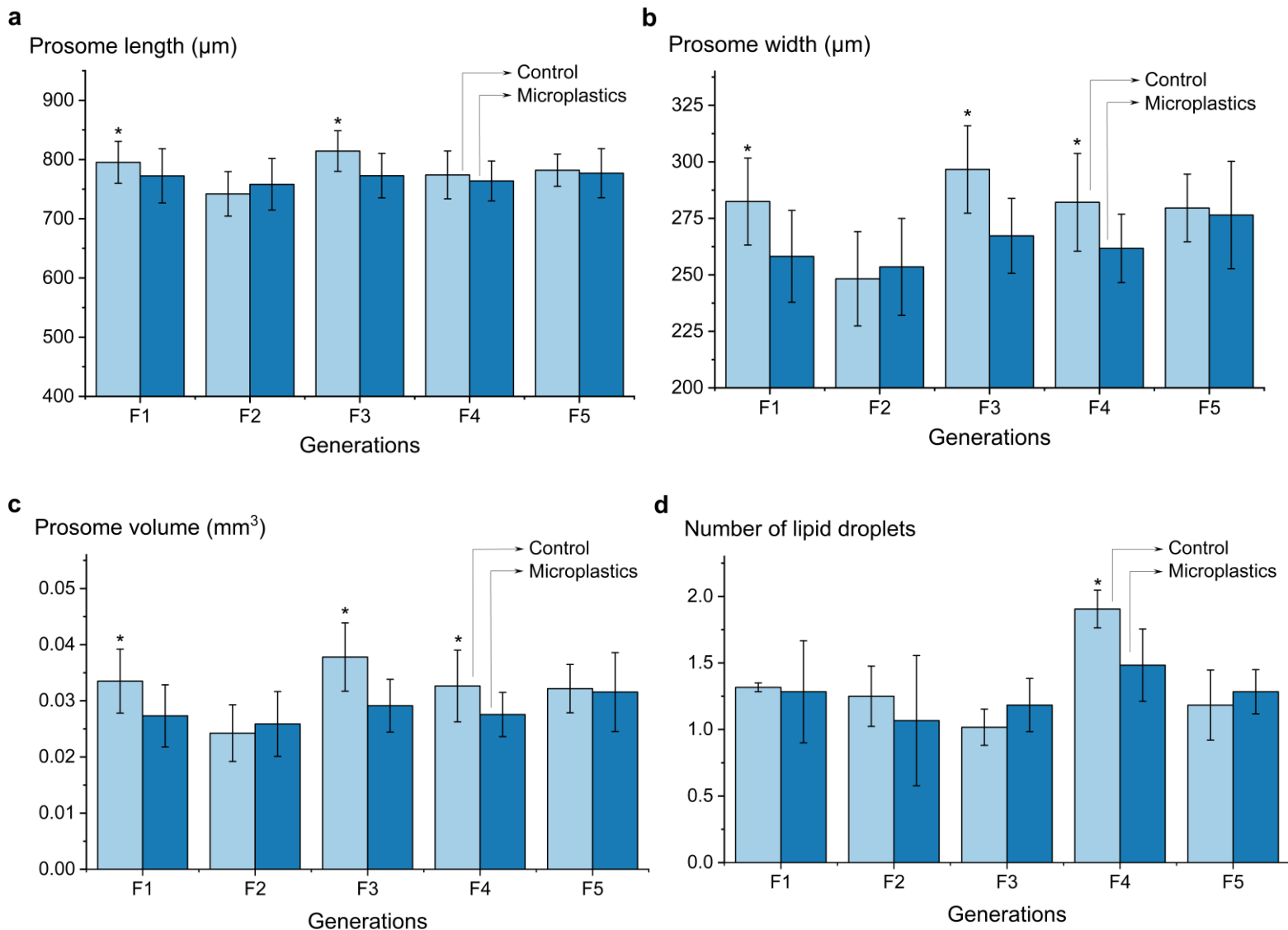
258 gastropod *Crepidula onyx* (Lo and Chan 2018). Exposure of *Acartia tonsa* copepods to
259 polystyrene microbeads resulted in reduced nauplii survival (Shore et al. 2021). Microplastics
260 exposure in copepods increases the naupliar phase and leads to reduced egg hatching and
261 premature hatching (Lee et al. 2013; Beiras et al. 2018). Consistent with these results, we found
262 that exposure to microplastics from polylactic acid affects reproduction by reducing egg
263 hatching, increasing the nauplii phase, and consequently reducing the transition to copepod
264 stages.

265

266 3.5 Prosome dimensions and number of lipid droplets

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268 Over generations, the exposed group exhibited significant changes in prosome length,
269 width, and volume compared to the control group, indicating considerable impact on copepod
270 morphology (Fig. 4 a–c). We also found that the prosome dimensions of the control group
271 showed fluctuations, likely due to higher density. The control group demonstrated an average
272 prosome length and width of 781.5 and 277.7 μm , respectively. In contrast, the exposed groups
273 exhibited a reduced prosome size, with average lengths and widths of 768.8 and 263.4 μm . The
274 regression results for the prosome dimensions show a stable trend in successive generations for
275 both the control and exposed groups, with all p -values for the tests being greater than 0.05 (Fig.
276 S5 b–d). Furthermore, the number of lipid droplets in the exposed group varied across
277 generations, with reductions in the early generations (first to second) and subsequent increases
278 in the third to fifth generations, suggesting potential stress responses (Fig. 4d). The observed
279 changes in copepod morphology and lipid droplet dynamics suggest adaptive responses to
280 microplastic exposure over successive generations. The fluctuations in prosome dimensions in
281 the control group highlight the influence of population density on zooplankton morphology.



283

284 Figure 4. Prosome length (a), prosome width (b), prosome volume (c), and number of lipid
 285 droplets (d) of *Eurytemora affinis* in the control and polylactic acid microplastics-exposed
 286 groups at the end of generations from the first filial (F1) to the fifth filial (F5) with Mean \pm SE.
 287 Asterisks (*) represent statistical significance ($p < 0.05$).

288 Standard error (SE).

289

290 The body size of zooplankton is a useful ecological indicator however it could also be
 291 influenced by the density of the population (Corona et al. 2021), which explain the variation in
 292 the prosome dimensions we found in the control group. *Daphnia magna* exposure to
 293 microplastics resulted in significantly reduced body length and produced offspring with small
 294 body size (Martins and Guilhermino 2018). The body size of offspring produced by
 295 microplastic-exposed mothers was significantly smaller compared to the control (Eltemsah and
 296 Bøhn 2019), which is consistent with our findings. An increase in the prosome dimension of
 297 the exposed group during the second generation could be attributed to a tradeoff between
 298 population size and individual body size, which is in line with the findings of Souissi and
 299 Souissi (2021).

300 Lipid droplets maintain energy reserves when food availability is low however, copepods
301 are unable to utilize lipid droplets under stressful conditions (Das et al. 2023). In our study, an
302 increase in the number of lipid droplets in the third and preceding generations could be
303 attributed to the stress response. The decrease in the number of lipid droplets observed in the
304 decontaminated generation is in line with the findings of Souissi et al. (2016b), where survival
305 exhibited a negative correlation with the number of lipid droplets. Overall, we found that
306 polylactic acid microplastics induced effects on the morphological parameters of *E. affinis* that
307 are consistent with those observed with other contaminants and, notably, with other types of
308 microplastics.

309

310 **4. Conclusion**

311

312 Together, using *Eurytemora affinis* as an experimental model, we investigated the acute and
313 multigenerational effects of effect of polylactic acid microplastics. Key findings of this research
314 indicate that polylactic acid microplastics induced gender-specific acute toxicity, while
315 multigenerational exposure, at lower environmentally relevant concentration, resulted in
316 reduced survival, altered reproductive parameters, and morphological changes. This study
317 highlights the potential ecological risks posed by microplastics, generated from bioplastics, in
318 estuarine and marine environments and emphasizes the importance of understanding the long-
319 term consequences on marine organisms. As the use of bioplastics like polylactic acid continues
320 to rise, it becomes crucial to assess their environmental impacts comprehensively and identify
321 strategies to address the challenges associated with their persistence and accumulation in marine
322 ecosystems.

323

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325

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328 fragments on zooplankton ecology via video tracking and behavioral analysis*' (TOPAZ).

329

330 Declarations

331

332 Competing interest

333

334 The authors declare that we have no known competing interests.

335

336 Code availability

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338 Not applicable.

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340 Ethics approval

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342 Not applicable.

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344 Consent to participate

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Not applicable

Consent to publication

Not applicable

Availability of data and materials

Data will be made available on request.

Code availability

Not applicable

Authors' contributions

WA contributed to the experiments, data collection, analysis, and writing of the original draft. SD and JT contributed to the experimental design and editing. HJ contributed to the experiment, data analysis, and revision. J-.SL was involved in supervising and editing. PZ and SS contributed to supervising, editing, and funding acquisition.

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