TITLE: Predator induced phenotypes are inherited over four generations in genetically identical *Daphnia lumholtzi*

RUNNING TITLE: Transgenerational Inheritance in *Daphnia lumholtzi*

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Data availability statement: The raw data and full analysis scripts that support the findings of this study are openly available in Markdown on GitHub (https://github.com/shannonsnyder/daphnia-transgenerational-plasticity).

Author contributions: **Conceptualization and Methodology:** SNS and WAC conceived and designed the study. **Investigation:** SNS, WCM, and SLJ reared the animals and collected the imaging data. **Data Collection and Curation:** SNS, EBC and TB curated the morphological measurements. **Formal Analysis:** SNS and WAC analyzed the data. **Visualization:** SNS created the data visualizations. **Writing – Original Draft:** SNS wrote the manuscript. **Writing – Review & Editing:** WAC provided revisions. All authors approved the final manuscript.

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Teaser Text

Can the ghosts of environments past shape organismal phenotypes of future generations? In an experiment with clonal *Daphnia lumholtzi*, we reveal that predator-induced changes in body shape can persist across several generations, even when the predator signal is long gone. Exposing only the first generation to predator cues, we tracked morphological shifts through five generations of genetically identical individuals. We found that these induced plastic phenotypes persisted to the F4 generation before fading in F5. This discovery supports the importance of non-genetic inheritance and underscores the powerful interplay between ancestral and current environments in shaping organismal form and function.

Abstract

Environmental variation can induce phenotypic changes through adaptation of populations and acclimation of individuals. Whereas adaptation can lead to persistent changes within a population via allele frequency shifts, induced plastic phenotypes have been thought to be ephemeral and lasting only through the affected generation. A growing number of studies have shown that induced phenotypes may be inherited, but it is unclear how persistent the inheritance of induced plastic phenotypes is across organisms. To directly address these questions, we measured the phenotypic effects of exposure to a predator cue in the water flea, *Daphnia lumholtzi*, which reproduces clonally allowing the isolation of the effects of direct environmental exposure from allelic variation. We measured the phenotypic effects of exposure to fish conditioned media in the F0, F3, F4, and F5 generations of genetically identical generations following cue exposure only in the F0 generation. We found evidence of predation induced morphologies into the F4 generation, with phenotypes receding in the F5 generation, four generations after cue removal. These results highlight the importance of the interaction between current and ancestral environments in determining phenotypic variation and highlights the importance in future studies of characterizing the molecular mechanisms of within and transgenerational phenotypic plasticity.

Introduction

Organisms respond to environmental variation through changes in morphology, behavior, and life history traits (Donelson et al., 2018; Szűcs et al., 2019; West-Eberhard, 2005) . These phenotypic responses may arise from shifts in allele frequencies across generations or from non-genetic mechanisms acting directly on individuals, such as developmental noise (Levis & Pfennig, 2019; Uller et al., 2018; West-Eberhard, 2020). Increased phenotypic variation can enhance the probability that some offspring will express trait values suited to novel environmental conditions (Botero et al., 2015; Simons, 2009; Tufto, 2015). However, this variation also increases the likelihood of mismatched phenotypes, reducing individual fitness for those with the maladaptive traits. While genetic adaptation can improve population-level fitness over time, subsequent environmental shifts may render previously adaptive traits maladaptive (Lande, 2009; Radchuk et al., 2019).

Regularly experienced environmental variation presents a particular challenge for evolving populations of organisms because it can lead to persistent mismatch of organisms and the environment, and thus ongoing genetic load (Ghalambor et al., 2007; Via et al., 1995). An alternative biological strategy for dealing with such variable conditions is the evolution of plastic traits (Hendry, 2016) in which phenotypic expression covaries with environmental variation (Bradshaw, 1965; Schlichting & Levin, 1986). Phenotypic plasticity allows a single genotype to consistently produce different phenotypes depending on the environment, without requiring genetic change (Pfennig et al., 2010; Pigliucci et al., 2006). Plasticity includes developmental plasticity (Nettle & Bateson, 2015; Uller et al., 2018), where early-life cues shape long-term traits, and acclimatory plasticity (Angilletta, 2009; Beaman et al., 2016), involving reversible

107 physiological changes in response to short-term fluctuations, illustrating a range of temporal
108 scales over which environmentally induced phenotypic changes can occur.

109 Plasticity within a generation has been well documented over numerous organisms and
110 environmental conditions (Agrawal et al., 1999a; Corl et al., 2018; Forsman, 2015). Although
111 plasticity itself is often an adaptive phenotype that has been crafted by natural selection, the trait
112 distributions produced by plasticity within one generation have historically been thought to be
113 ephemeral with little consequence for the phenotypes of future generations (West-Eberhard,
114 2020). However, modern molecular genetic and genomic analyses have documented that
115 variation in plastic traits is mediated by changes in gene expression (Hales et al., 2017) and are
116 hypothesized to involve epigenetic regulation (St-Cyr & McGowan, 2015). These molecular
117 mechanisms lead to the possibility that induced plastic phenotypes may be inherited across
118 generations (Harris et al., 2012a; Romero-Mujalli et al., 2024).

119 Transgenerational inheritance of induced plastic trait values is expected to evolve when
120 the parental environment has some covariance with the offspring environment (Agrawal et al.,
121 1999a). Although the inherited phenotypes should return to the parental state in the absence of
122 the inducing cues over generations, the rate at which this occurs is hypothesized to be determined
123 by the number of generations of which the environment shifts (Furrow & Feldman, 2014; Proulx
124 & Teotónio, 2017). In this way the evolution of inherited phenotypic plasticity provides a
125 mechanism for organisms and their offspring to respond adaptively to changing environments
126 without significant genetic load (Ghalambor et al., 2007). A small number of studies have
127 documented that induced phenotypes may be inherited (Hellmann et al., 2020; Tariel et al.,
128 2020), but it is unclear how common this is across organisms. Even less is known about how

129 long environmentally induced phenotypes persist across generations once the environmental cue
130 is removed (Norouzitallab et al., 2014).

131 The freshwater microcrustacean *Daphnia* is well suited for studying transgenerational
132 plasticity and addressing these questions (Harris et al., 2012b; Seda & Petrusek, 2011;
133 Yampolsky et al., 2014). The concept of plasticity was introduced in the early 1900s by Richard
134 Woltereck**,** who demonstrated that head size in *Daphnia* varied with nutrient availability, and
135 proposed that environmentally induced traits could be inherited (Woltereck, 1913). *Daphnia's*
136 parthenogenetic reproduction produces genetically identical offspring, eliminating variation due
137 to recombination, genetic background, and parental sex (Harris et al., 2012b). These
138 ecoresponsive crustaceans exhibit plastic responses to a range of environmental stressors,
139 including cyanobacteria (Dao et al., 2018; Gustafsson & Hansson, 2004; Schwarzenberger &
140 Martin-Creuzburg, 2021), temperature (Geerts et al., 2015; Müller et al., 2018), and predation,
141 affecting morphology (Horstmann et al., 2021), behavior (Beklioglu et al., 2006), and life history
142 traits (Adamczuk, 2020; Agrawal et al., 1999b). Classic examples include inducible helmets,
143 spines, and neckteeth in response to predator cues (Horstmann et al., 2021; Laforsch & Tollrian,
144 2004). Several of these traits persist across clonal generations, such as helmet length in *D.*
145 *cucullata* (Agrawal et al., 1999a) and clutch size in *D. ambigua* (Walsh, Cooley, et al., 2014),
146 suggesting a non-genetic mechanism of inheritance.

147 The number of generations over which induced traits persist in *Daphnia* in the absence of
148 the original environmental cue remains poorly resolved. Most studies track only one or two
149 generations post-exposure, such as helmet length in *D. cucullata* which disappears by the second
150 generation (F2) (Agrawal et al., 1999a; Laforsch & Tollrian, 2004), and clutch size in *D.*
151 *ambigua* which persists to the third generation (F3) (Walsh, Cooley, et al., 2014). Molecular

152 studies show differential gene expression persisting into F2 after predator cue removal (Hales et
153 al., 2017), and methylation differences between exposed F0 and naïve F1 individuals (Feiner et
154 al., 2022; Hearn et al., 2019; Jeremias et al., 2018; Trijau et al., 2018). These findings suggest
155 that induced traits can persist beyond the directly exposed generation and decline gradually, but
156 the duration of this persistence and its evolutionary significance remain unclear (Feinberg &
157 Irizarry, 2010; Ho & Burggren, 2010).

158 A challenge with such studies is that embryos exist within the mother's brood chamber,
159 making it possible that they too are directly exposed to the environmental cue (Agrelius &
160 Dudycha, 2025). Furthermore, those embryos may have developing germ cells that can similarly
161 be exposed, making it necessary to examine beyond the third (F2) generation (Shahmohamadloo
162 et al., 2025). Here we directly address the question of how long induced phenotypes can be
163 inherited in *Daphnia lumholtzi* with a specific focus on periods after which direct exposure must
164 not have occurred. We measured predator-induced morphological traits in *D. lumholtzi* across the
165 fourth (F3), fifth (F4), and sixth (F5) generations following initial exposure in F0. *D. lumholtzi*
166 develops elongated head and tailspines in response to fish predation (Dzialowski et al., 2003),
167 traits thought to contribute to its invasive success in North America (Engel & Tollrian, 2009;
168 Sousa et al., 2017; Work & Gophen, 1999) as other *Daphnia* species typically produce helmets
169 (Horstmann et al., 2021). Previous work in *D. lumholtzi*, shows that maternal exposure can
170 accelerate defensive trait development in F1 (Graeve et al., 2021) and that cyanobacteria-induced
171 life history effects persist for at least one generation (Dao et al., 2018), but no study has
172 examined the long-term persistence of predator-induced morphology in *D. lumholtzi*. We found
173 that induced defensive traits persisted through four unexposed generations, with measurable
174 effects disappearing in F5. These results demonstrate that transgenerational plasticity has a

175 limited temporal duration, with trait-specific patterns of decline that reflect the gradual loss of
176 ancestral environmental influence (Geoghegan & Spencer, 2012; Heard & Martienssen, 2014).
177

178 **Methods**

179 **Experimental Overview (Fig. 1).** To explore the dynamics of predation induced phenotypes
180 within and across generations, we reared five lineages of predation exposed, and four lineages of
181 unexposed, *Daphnia lumholtzi* for five generations following initial predation exposure and cue
182 removal. *Daphnia* were individually grown in 50 mL beakers containing 30 mL of fish
183 conditioned medium or standard artificial lake medium (COMBO)(Kilham et al., 1998). Every
184 other day during the F0, F3, F4 and F5 generations, individual *D. lumholtzi* were imaged for
185 morphological measurements using ImageJ (Schindelin et al., 2012). On this same schedule the
186 media was exchanged, and algae was replenished with 85,000 cells/mL of a 1:1 mixture of
187 *Scenedesmus obliquus* and *Chlorella vulgaris*, hereby referred to as the standard algae mixture.
188 See Fig. 1 for experimental schematic and sample sizes.

189 ***Daphnia Lumholtzi* Clone.** *Daphnia lumholtzi* clones were collected in the summer of 2019
190 from Saguaro Lake, AZ, USA. This clone was specifically chosen for its ability to develop
191 headspines and tailspines in response to vertebrate predation. These predator response
192 phenotypes are only found in *D. lumholtzi*, whereas other species develop different armor
193 morphologies.

194 **Common Garden Rearing to Standardize Generational Exposures.** *D. lumholtzi* clones were
195 reared for 6 months in artificial lake medium (COMBO) and fed *Scenedesmus obliquus* and
196 *Chlorella vulgaris* ad libitum to dull residual effects of lake predation. Prior to experimental
197 perturbations, ancestral *D. lumholtzi* were reared for three generations in COMBO medium and

198 fed a standard algae mixture of *S. obliquus* and *C. vulgaris* to minimize the influence of maternal
199 effects on experimental generations.
200 **Fish and Control Media Preparation.** To prepare fish (induction) medium, 10L of artificial
201 lake medium was conditioned with five *Gasterosteus aculeatus* for three days prior to filtration
202 (0.45 µm). Fish were fed 10 *D. lumholtzi* and 10 *Daphnia magna* each day to maximize the
203 inducing effect. To create control medium, artificial lake medium without fish conditioning was
204 created in congruence with fish medium. Media was frozen and thawed overnight prior to use.
205 **Initial F0 Predation Exposure and Experimental Conditions**. From the third generation of
206 common garden reared *Daphnia*, one mother was selected due to observed clutch survivability
207 and size. Nine neonates born within 24 hours from the same mother were isolated and randomly
208 assigned to treatment groups, either fish exposure or control. Animals were reared individually in
209 a 50 mL plastic beaker with 30mL of the appropriate medium and fed 5 mL standard algae
210 mixture every other day. Individual *Daphnia* were staged, imaged, and returned to fresh media
211 with algae every other day.
212 **Propagation across Generations.** Upon reproduction, up to six neonates were randomly
213 collected and reared individually in 50 mL beakers containing 30 mL of control media only. If
214 the first clutch did not produce a viable lineage, the next viable clutch was utilized. See Fig. 1 for
215 samples sizes.
216 **Imaging and Data Collection.** Every other day, animals in the F0, F3, F4, and F4 generation
217 were staged on a glass slide and imaged using `cellSenseEntry` (Olympus Life Science). Scale
218 bars were added to images during acquisition for calibration during measurements.
219 Morphological measurements of *headspine* (tip of spine to top of compound eye), *body length*
220 (top of eye to base of tail), *tailspine* (base of tail to tip of tailspine), *total body length* (tip of

221 headspine to tip of tailspine) were collected (see Fig.2) using `ImageJ` (Schindelin et al., 2012).
222 All images were calibrated to a consistent scale using the 'set global scale' function in `ImageJ`,
223 which converts pixel distances to micrometers based on the embedded scale bar. See Figure 2 for
224 measurement details.

225 **Statistical Analysis.**

226 **Phenotype Transmission Across Generations.** Morphological variation was analyzed using
227 both frequentist and Bayesian mixed-effects models in `R` (R Core Team, 2021), using the `lme4`
228 (Bates et al., 2015a) and `brms` (Bürkner, 2017) packages respectively. For each generation (*F0*,
229 *F3*, *F4*, *F5*) and each trait (*headspine*, *tailspine*), we tested for the effects of predation *treatment*,
230 with *body length* as a covariate, and a random intercept for sample identity to account for nested
231 sampling. The model formula used was the following:

232 `spine ~ treatment + bodylength + (1|sample)`

233 We selected this model structure based on established principles in *Daphnia* morphological
234 analysis and our experimental design. *Body length* was included as a covariate because spine
235 length scales allometrically with body size in *Daphnia* and failing to account for this relationship
236 can confound treatment effects with size-related variation (Laforsch & Tollrian, 2004; Petrusek
237 et al., 2009). *Treatment* (predator exposure vs. control) was included as the main fixed effect to
238 test our primary hypothesis of transgenerational inheritance of induced defenses. The random
239 intercept for sample identity accounts for the hierarchical structure of our data, where multiple
240 individuals were measured from each experimental replicate, addressing potential pseudo-
241 replication issues common in experimental ecology (Freeberg & Lucas, 2009; Hurlbert, 1984).
242 We did not include *age* as a covariate due to its high correlation with *body length* (Pearson's R =
243 0.85; Supp. Fig. 1). Linear mixed models were evaluated using Kenward-Roger F-tests and *post*

hoc Tukey tests to assess treatment effects. Conditional and marginal R^2 values, AIC, and BIC were calculated to assess model fit (See Supplemental Table 1 for model diagnostic summary).

We implemented Bayesian hierarchical models using the `brms` (Bürkner, 2017) package to estimate treatment effects on spine morphology while accounting for individual-level variation. These models included fixed effects for *treatment*, *body length*, and random intercepts for individual identity to capture repeated measures. The hierarchical structure allows partial pooling across individuals, improving estimation in the presence of sparse data. Posterior distributions were used to quantify uncertainty, and model diagnostics (e.g., Rhat, ESS, posterior predictive checks) confirmed convergence and reliability. Marginal and conditional R^2 values were computed using the `performance` (Lüdecke et al., 2021) package to assess model fit. See Supplemental Figures 3 and 4 for full diagnostic plots for headspine and tailspine models across generations.

Models were fit using a Student's t-distribution to account for potential outliers and heavy-tailed data which are common in ecological trait data. This choice was supported by posterior predictive checks and traceplot diagnostics indicating stable convergence and reliable inference. We used weakly informative priors and ran three chains with 5,000 iterations each (2,000 warm-up), ensuring convergence ($\hat{R} < 1.01$ for all parameters). Posterior distributions were summarized to extract treatment effect estimates and 95% credible intervals. Bayesian R^2 values (marginal and conditional) were calculated to assess model fit (See Supplemental Table 2 for model diagnostic summary). Posterior predictions were generated using `posterior_epred()` (Gabry et al., 2025) to estimate treatment effects at the mean body length for each generation, controlling for sample-level variation. Models were evaluated using leave-one-out- cross validation and WAIC (Vehtari et al., 2017). Bayesian and frequentist estimates were highly

consistent across generations and traits, with overlapping intervals and similar effect sizes, supporting the robustness of our findings across inferential frameworks. Diagnostic plots for Bayesian models are shown in Supplemental Figures 3 and 4

Age-Dependent Morphological Plasticity (Delta). To assess how morphological traits change across *age* and *treatment*, we calculated within-individual morphological change (Δ) for *body length*, *total body size*, *headspine*, and *tailspine*. Δ values were computed as the difference between successive measurements for each individual (`Δtrait = trait_current - trait_previous`), capturing short-term plastic responses over time. Age trajectories (*Δ trait* vs. *age*) were visualized using scatterplots with LOESS-smoothed trend lines and 95% confidence intervals. To statistically evaluate the effect of induction on morphological plasticity, we modeled Δ values using linear mixed-effects models `lme4` (Bates et al., 2015b) and `lmerTest` package (Kuznetsova et al., 2017), with *treatment* and *age* as fixed effects and individual identity as a random intercept to account for repeated measures `(formula: delta value ~ treatment + age + (1|sample))`. We initially explored more complex temporal modeling approaches, including natural splines with varying degrees of freedom (df = 2 to 4) to capture potential non-linear age-related changes in growth patterns (`formula: delta value ~ treatment + ns(age, df = 2) + (1|sample))`. However, these models suffered from overparameterization given our sample size, and model significance was sensitive to the choice of degrees of freedom, indicating unstable parameter estimates. Consequently, we adopted the more parsimonious linear approach with *age* as a continuous covariate, which provided robust and interpretable results while adequately capturing the age-related trends in our data. Model significance was assessed using ANOVA to compare treatment effects. Results were visualized in a 2×2 panel plot showing estimated trait changes and associated standard errors across treatments.

Size Selective Survivorship Analysis. To test whether size-selective mortality, rather than treatment, could explain differences in spine lengths, we constructed mixed-effects linear models using binary survival status. Survival was defined as the presence or absence of a subsequent timepoint for each individual. Separate models were fit for *headspine* and *tailspine* lengths as a function of *body length*, with a random intercept for sample identity (`spine ~ bodylength + (1|sample)`). Residuals from these models represent spine length variation independent of body size. To visualize trait dynamics across age and survival status, we plotted body length and spine residuals using LOESS-smoothed trend lines with 95% confidence intervals. Survivors and non-survivors were distinguished by color, and traits were faceted for comparison.

To further evaluate survival probability, we constructed a binomial generalized additive model (GAM) with *survival status* as the response variable and *age*, *body length*, *spine* lengths, treatment, and the interaction between *age* and *treatment* as predictors `(status ~ bodylength + headspine + tailspine + age * treatment)`. Trait values representing the 10th, 50th, and 90th percentiles were used to generate survival predictions across age. Predicted survival probabilities and 95% confidence intervals were plotted to assess how trait variation influences survivorship over time.

Statistical Software and Reproducibility. All statistical analyses and visualizations were conducted in `R` (R Core Team, 2021). Data manipulation and cleaning were performed using the `dplyr` package (Wickham et al., 2022) , and visualizations were generated using `ggplot2` (Wickham, 2016), with additional formatting and layout support from `patchwork` (Pedersen, 2025), `gghighcontrast` (Grosenbach, 2021) , and `Rmisc` (Hope, 2022) for summary statistics. Estimated marginal means and post hoc comparisons were calculated using `emmeans` (Searle et al., 2025).

All analyses were conducted using `Rstudio` (Posit Team, 2025) in `RMarkdown` (Allaire et al., 2019) and `Quarto` (Allaire et al., 2025), ensuring full reproducibility of code, figures, and statistical outputs. Annotated Quarto files and datasets are available on GitHub at https://github.com/shannonsnyder/daphnia-transgenerational-plasticity.

Results

Ontogenetic Trajectories Demonstrate Environmental Effects Targeted to Defensive Traits

Daphnia lumholtzi exhibited distinct developmental trajectories across their full lifespan for body length, headspine, and tailspine structures (Fig. 3). Body length increased rapidly as the animals aged in both control and induced lineages, reaching an early asymptote (~2,500 µm) around 25 days. This growth pattern was strongly correlated with age ($R = 0.85$; Supp. Fig. S1), and no significant differences were observed between treatments, indicating that predator cue exposure did not affect overall somatic growth.

In contrast, defensive spine traits displayed age-dependent plasticity, with early-life elongation followed by a gradual reduction in spine length. Headspine length peaked (~850 µm) within the first 10 days and declined steadily to ~400 µm in older individuals (Fig. 3B). Tailspine length reached a delayed maximum (~1,800 µm) around 20 days before declining to values comparable to those observed in early developmental stages (~700 µm) (Fig. 3C). These patterns suggest that predator-induced plasticity is most pronounced during early developmental stages and diminishes progressively across the lifespan of *Daphnia*, consistent with a life-stage–specific defense strategy.

Together, these ontogenetic analyses demonstrate that transgenerational inheritance of predator-induced traits is specific to defensive structures and occurs independently of changes in body size or growth rate, supporting the hypothesis that environmental induction targets adaptive traits without altering general somatic development.

Predator cue exposure results in an increase in headspine and tailspine length in the exposed generation.

341 Postembryonic predator cue exposure resulted in a substantial increase in both headspine
342 and tailspine length relative to unexposed controls. In the F0 generation, with predation cues
343 provided from the first post-embryonic day, both linear and Bayesian models estimate significant
344 increases in induced defense trait lengths (Fig. 4; Tables 1,2). The linear mixed-effects model
345 provided an estimated increase in headspine length of 68.98 ± 27.19 μm (F-stat = 6.44,
346 $p = 0.035$), closely aligning with the Bayesian estimate of 68.94 ± 32.26 μm. These differences
347 correspond to a roughly 10% increase in headspine length over control *Daphnia* never exposed
348 to fish conditioned medium. Tailspine length also increased dramatically in the exposed F0
349 lineage relative to controls, by 114.73 ± 31.93 μm (F-stat = 12.91 , $p = 0.008$; linear model) and
350 100.65 ± 39.75 μm (Bayesian model), representing a roughly 10% increase. Because we noted
351 that most of the morphological changes occur within the first 20 days of life, before spine growth
352 plateaus (Fig. 4), we modeled these effects on *Daphnia* less than 20 days of age.

353

Gen	Spine	Est. Difference (μm)	Std_Error	R^2 marginal	R^2 conditional	F statistic	P value
F0	Headspine	68.98	27.19	0.58	0.60	6.44	0.035
F0	Tailspine	114.73	31.93	0.93	0.94	12.91	0.008
F3	Headspine	37.02	17.61	0.51	0.65	4.42	0.038
F3	Tailspine	8.19	18.28	0.95	0.98	0.20	0.655
F4	Headspine	43.81	20.19	0.48	0.66	4.71	0.034
F4	Tailspine	85.45	34.14	0.90	0.98	6.26	0.015
F5	Headspine	-5.79	21.41	0.51	0.53	0.07	0.789
F5	Tailspine	-24.21	39.07	0.93	0.97	0.38	0.539

354
355 **Table 1. Linear mixed-effects model estimates of treatment-induced changes in spine length across**
356 **generations in Daphnia**. Models were fit separately by generation for individuals ≤ 20 days old. Each row
357 represents a unique combination of generation and spine type. "Gen" indicates the generation of the experimental
358 cohort (e.g., F0, F3, etc.), and "Spine" refers to the type of spine measured, either headspine or tailspine. "Est.
359 Difference (μm)" is the estimated mean difference in spine length (in micrometers) between predator-induced and
360 control treatments, where positive values indicate longer spines in induced individuals. "Std_Error" is the standard
361 error of the estimated difference. "R^2 marginal" and "R^2 conditional" represent the proportion of variance explained
362 by fixed effects alone and by both fixed and random effects, respectively. The "F statistic" and associated "P value"
363 test the significance of the treatment effect; p-values < 0.05 indicate statistically significant differences. See
364 Methods for full model specification and random effect structure.

Gen	Spine	Est. Difference (μm)	Std_Error	R2_marginal	R2_conditional
F0	Headspine	68.94	32.26	0.60	0.047
F0	Tailspine	100.65	39.75	0.95	0.003
F3	Headspine	37.95	18.19	0.65	0.025
F3	Tailspine	6.21	18.24	0.98	0.001
F4	Headspine	44.58	20.79	0.66	0.025
F4	Tailspine	85.82	34.29	0.98	0.001
F5	Headspine	-6.78	22.28	0.56	0.034
F5	Tailspine	-19.34	29.32	0.95	0.004

Table 2. Posterior mean estimates of treatment-induced changes in spine length across generations in *Daphnia*, based on Bayesian hierarchical models. Models were fit separately by generation for individuals ≤ 20 days old. Each row represents a unique combination of generation and spine type. "Gen" indicates the generation of the experimental cohort, and "Spine" refers to the type of spine measured - either headspine or tailspine. "Est. Difference (μm)" is the posterior mean estimate of the difference in spine length (in micrometers) between predator-induced and control treatments, with positive values indicating elongation in induced individuals. "Std_Error" is the standard error of the posterior estimate. "R^2 marginal" and "R^2 conditional" represent the proportion of variance explained by fixed effects alone and by both fixed and random effects, respectively. See Methods for full model specification and prior structure. Note: Lower R^2 values in some models likely reflect limited variation among clonal individuals, consistent with the use of genetically identical lineages in this study.

Defensive Traits Are Inherited Through F4 but Disappear in F5.

Consistent with well-established within-generation plastic responses, F0 *Daphnia* reared in predator cue environments exhibited robust and statistically significant increases in both headspine and tailspine length relative to controls. Frequentist estimates indicated increases of 68.98 μm ($p = 0.035$) for headspine and 114.73 μm ($p = 0.008$) for tailspine, with Bayesian estimates closely corroborating these results (headspine: 68.94 ± 32.26; tailspine: 100.65 ±39.75; Tables 1 and 2).

Strikingly, phenotypic differences persisted for multiple generations after removal from the inducing environment. F3 represents the first generation that was not exposed to predator cues either directly or indirectly. When F0 mothers are exposed, their developing embryos (F1) and the germline cells that will become the F2 generation are also exposed. Only by the F3

generation are the effects of induction potentially transmitted through purely epigenetic mechanisms.

Despite two intervening generations without exposure, F3 individuals from the induced lineage exhibited a significant increase in headspine length relative to controls. Frequentist analysis estimated a 37.02 µm increase (SE = 17.61, $F = 4.42$, $p = 0.038$), with Bayesian inference yielding a nearly identical effect size (37.95 µm, SE = 18.19). This represents approximately 50% retention of the original F0 headspine response. In contrast, tailspine length in F3 showed no significant difference between lineages (frequentist: 8.19 µm, $p = 0.655$; Bayesian: 6.21 µm), indicating trait-specific attenuation of the induced phenotype.

In the F4 generation, also completely unexposed to predator cues, both headspine and tailspine lengths were again significantly elevated in the induced *Daphnia* as compared to the control *Daphnia*. Frequentist estimates showed a 43.81 µm increase in headspine length ($p = 0.034$) and an 85.45 µm increase in tailspine length ($p = 0.015$), with Bayesian estimates closely matching (headspine: 44.58 µm; tailspine: 85.82 µm). These values reflect retention of about 50% of the original F0 effect sizes, similar to the F3 estimates (Tables 1 and 2).

By the F5 generation all measurable differences between induced and control lineages had disappeared. Frequentist analysis estimated a negligible difference in headspine length (–5.79 µm, $p = 0.789$) and tailspine length (–24.21 µm, $p = 0.539$), with Bayesian estimates similarly non-significant. These findings indicate that the induced phenotypes ultimately reverted to baseline in the absence of continued environmental cues over 5 unexposed generations. See Supplemental Figure 2 for raw trait values.

Taken together, these results demonstrate that predator-induced morphological plasticity can be inherited for at least four generations, but this inheritance is finite and trait-dependent. The disappearance of measurable trait differences in F5 underscores the transient nature of this plasticity and suggests that the mechanism of transgenerational epigenetic inheritance decays over time without environmental cue reinforcement within several generations (see Fig. 4).

Differences in Growth Rate and Trait Distributions Do Not Explain Induced Trait Expression.

To evaluate whether the observed morphological differences between induced and control lineages could be attributed to variation in growth dynamics or trait distributions, rather than transgenerational induction, we assessed whether treatment effects could be explained by differences in developmental timing, size-selective mortality, or trait-based survival.

The most pronounced morphological changes occurred early in life, prior to 20 days of age (Figure 4). This period includes the onset of reproduction (typically 7–12 days), during which *D. lumholtzi* mothers are not yet fully grown. Morphological change peaked around this time for all traits, particularly for body length and total body size.

Importantly no significant differences in growth rate were detected between treatment groups for any of the four traits examined (body length, headspine, tailspine, and total body length), indicating that predator exposure did not alter the rate of somatic or defensive trait development (Figure 5). Specifically, treatment effects were non-significant for body length (Est. = 7.73 ± 16.72 μm, $F = 0.22$, $p = 0.642$), total body size (Est. = 0.78 ± 33.30 μm, $F = 0.00$, $p = 0.981$), headspine (Est. = -2.00 ± 6.33 μm, $F = 0.10$, $p = 0.751$), and tailspine (Est. = -4.96 ±

12.11 μm, F = 0.17, p = 0.681). These results suggest that the observed morphological differences are not due to accelerated or delayed growth in induced individuals but instead reflect differences in trait expression independent of growth rate.

To assess whether trait distributions might reflect selective mortality, such as the preferential survival of individuals with longer spines, we compared growth trajectories of survivors and non-survivors during the first 20 days of life. Plotting developmental trajectories for body length, headspine residuals, and tailspine residuals (controlling for body size) by survival status (Fig. 6) showed that while survivors tended to be slightly larger in body length, residual spine lengths showed overlapping distributions and trajectories between survivors and non-survivors. These patterns indicate that trait-based selection is unlikely to account for the observed morphological differences.

The absence of an effect of spine length on survivorship was further supported by a generalized linear model (GLM) predicting survival status from body length, headspine, tailspine, age, and treatment. The model revealed that body length was positively associated with survival ($\beta = 0.0030$, $p < 0.001$), while headspine and tailspine lengths were not significant predictors (headspine: $\beta = -0.0010$, $p = 0.058$; tailspine: $\beta = -0.0007$, $p = 0.110$). Treatment had a significant positive effect on survival ($\beta = 0.678$, $p < 0.001$), but the interaction between age and treatment was not significant ($\beta = -0.0145$, $p = 0.096$). The model explained a substantial portion of deviance (ΔDeviance = 328.8; AIC = 2554.1), but no evidence emerged for size- or trait-selective mortality based on spine length (Figure 7).

Discussion

Induced Defenses Are Robust, Age-Specific, and Not Driven by Selection

Exposure to predator cues in the F0 generation triggered strong morphological defenses in *Daphnia lumholtzi*, with significant elongation of both headspine and tailspine structures. Notably, post-embryonic exposure alone was sufficient to induce these traits in our clone collected from Arizona, USA, contrasting with previous findings in other *D. lumholtzi* populations suggesting embryonic exposure was necessary for trait development (Graeve et al., 2021; Laforsch & Tollrian, 2004). This finding underscores the differences in inducibility between clones of the same species and highlights the need to assess transgenerational inheritance in a variety of systems (Auld et al., 2010; Scoville & Pfrender, 2010; Shahmohamadloo et al., 2025). This is further illustrated by the characterization of "uninducible" *D. lumholtzi* clones which constitutively express defense phenotypes in the absence of predation cues (Engel & Tollrian, 2009), collected just miles away from our Saguaro Lake, AZ clone at Canyon Lake, AZ, USA.

Importantly, these trait differences were not attributable to size-selective or trait-selective mortality. Survivors and non-survivors showed overlapping developmental trajectories, and survival probability was primarily associated with body length, not spine morphology. This confirms that the observed phenotypic differences are induced responses rather than artifacts of differential survivorship among individuals based upon armor morphology (Dieckmann et al., 2006; Kingsolver et al., 2001).

We also found that induced spine lengths were most prominent in early life and declined with age, a pattern distinct from somatic growth. This suggests stage-specific optimization of defenses, likely targeting periods of highest vulnerability to predation (Nagano & Yoshida, 2020;

474 E. E. Werner & Gilliam, 1984). The age-dependent regression of defensive traits, including
475 previously unreported headspine shortening with age, further highlights the dynamic nature of
476 plastic responses and the influence of genetic background (DeWitt et al., 1998; Scheiner, 1993;
477 Via et al., 1995).
478
479 **Induced Phenotypes Persists Across Generations but Fades Without Cues**
480 Our results demonstrate that predator-induced defenses are not confined to the directly
481 exposed generation, nor the immediate offspring of the exposed generation. Headspine and
482 tailspine elongation persisted through four unexposed generations, with measurable effects in F3
483 and F4 despite the absence of continued induction and lack of indirect exposure through
484 germline conditioning. This multigenerational persistence supports the hypothesis that
485 environmental information can be retained and expressed across generations via non-genetic
486 mechanisms (Gapp et al., 2014; Heard & Martienssen, 2014; Rechavi et al., 2014).
487 By the F5 generation, however, trait differences between induced and control lineages
488 had disappeared. This regression to baseline aligns with evolutionary expectations that plastic
489 traits fade when environmental cues vanish, minimizing fitness costs associated with
490 unnecessary defenses (DeWitt et al., 1998; Van Buskirk & Steiner, 2009). The trait-specific
491 dynamics, headspine persistence in F3, tailspine resurgence in F4, suggest that transgenerational
492 plasticity is not uniform and may fluctuate across lineages (Bonduriansky & Day, 2009; Herman
493 & Sultan, 2011; Uller et al., 2013).
494
495 **Mechanisms of Non-genetic Inheritance and Evolutionary Implications**

496 The persistence and gradual decline of induced traits across generations lead to the strong
497 testable hypothesis of epigenetic inheritance as a likely mechanism. DNA methylation, histone
498 modification, and other chromatin-level changes may facilitate the transmission of plasticity
499 without altering the genetic code (Banerjee & Roychoudhury, 2017; Casadesús, 2016;
500 Davidowitz, 2009; Ho & Burggren, 2010; Li & Zhang, 2014; Niederhuth et al., 2016; Robichaud
501 et al., 2012; M. S. Werner et al., 2023; Yaniv, 2014). This pattern is consistent with incomplete
502 erasure of epigenetic marks and has been observed in other invertebrate and vertebrate systems
503 (Dias & Ressler, 2014; Gapp et al., 2014; Grishkevich & Yanai, 2013). In *Caenorhabditis*
504 *elegans*, the histone mark H3K27me3 was shown to persist across generations, influencing gene
505 expression and development in offspring (Kaneshiro et al., 2019). In the freshwater snail, *Physa*
506 *acuta*, predator-induced gene expression changes were maintained across two generations,
507 supporting epigenetic regulation (Dejeux et al., 2025). In mice, paternal diet altered offspring
508 DNA methylation and metabolic gene expression (Carone et al., 2010). In humans, trauma-
509 related DNA methylation patterns were found to persist across three generations (Mulligan et al.,
510 2025). Similarly, in *Daphnia magna*, differential DNA methylation patterns induced by
511 environmental stressors have been documented to persist for multiple generations before
512 returning to baseline (Asselman et al., 2016; Vandegehuchte et al., 2010)
513 Our findings underscore the evolutionary relevance of environmental history even in the
514 absence of genetic variation (Jablonka & Lamb, 20062; Laland et al., 2015; Pigliucci & Müller,
515 2010). Transgenerational plasticity allows organisms to respond to ancestral conditions,
516 potentially buffering populations against short-term environmental fluctuations (Donelson et al.,
517 2018; Uller et al., 2018). However, because our study used a single clone, future work should
518 explore responses across diverse genotypes (Auld et al., 2010). The existence of "uninducible"

clones with constitutive defenses raises intriguing questions about genetic assimilation and the long-term fate of plastic traits (Lande, 2009; West-Eberhard, 2003).

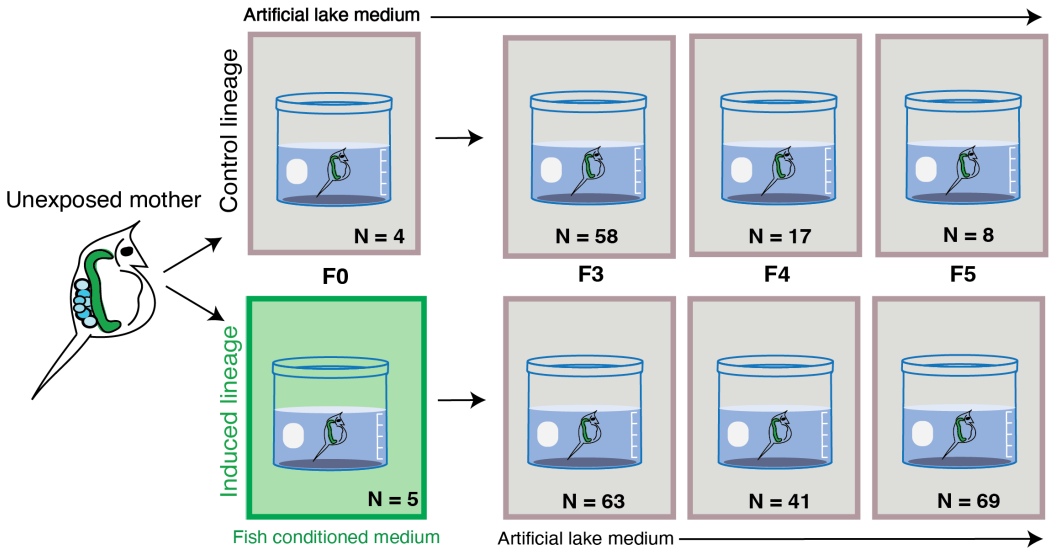
Conclusions

In evolutionary genetics, it is still unclear how general the non-genetic inheritance of induced phenotypes is across organisms, and when it occurs, how long the induced phenotypes will persist in the absence of the signal. Our work contributes to answering both questions by demonstrating that predator-induced morphological defenses in *Daphnia lumholtzi* can persist for up to four generations through non-genetic mechanisms but ultimately regress in the absence of continued environmental cues. This finding aligns with previous work in *Daphnia*, such as clutch size persistence to F3 in *D. ambigua* (Walsh, Whittington, et al., 2014), helmet length inheritance to F2 in *D. cucullata* (Agrawal et al., 1999a) and multi-generational erosion of plastic traits in *D. pulicaria* (Landy et al., 2024), but is novel in demonstrating morphological trait persistence through four unexposed generations. Were this phenomenon to occur in humans, it would be equivalent to the environmental conditions experienced by our great-grandparents in the 19th century shaping our phenotypes today, a striking reminder of how ancestral environments may echo across generations. This transgenerational plasticity may buffer populations against short-term ecological fluctuations, while its eventual attenuation minimizes the costs of unnecessary trait expression (Herman & Sultan, 2011; Uller et al., 2013). Future research should aim to identify the molecular basis of this inheritance (Klosin et al., 2017; Trijau et al., 2018) and explore its evolutionary consequences across diverse genetic backgrounds and dynamic environments.

Acknowledgements

We would like to thank Michael Lynch for his contribution of SAG *Daphnia lumholtzi* clones utilized in this study. We would also like to thank Mark Currey for this assistance in stickleback fish husbandry and handling and Emily Williams for the collection and maintenance of *D. lumholtzi* lineages prior to 2020.

549 **FIGURES**



550
551 **Figure 1. Schematic illustrating the experimental setup allowing for direct testing of**
552 **transgenerational epigenetic inheritance by induced defenses by comparing lineages with**
553 **and without grandparental exposure to predator cues**. Offspring (F0) from a single
554 unexposed mother were split into two lineages: control (top), where individuals where
555 continuously reared in artificial lake media, and induced (bottom), where F0 individuals were
556 exposed to fish-conditioned media indicated in green. Only the F0 induced lineage is exposed to
557 fish conditioned media. Sample size for each generation and treatment is indicated. Subsequent
558 generation (F3-F5) are from both lineages but are maintained in artificial lake media. Lineages
559 were propagated for six generations and measured every other day during the F0, F3, F4, and F5
560 generations. Schematic distinguishes plasticity from persistent heritable effects.
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562
563

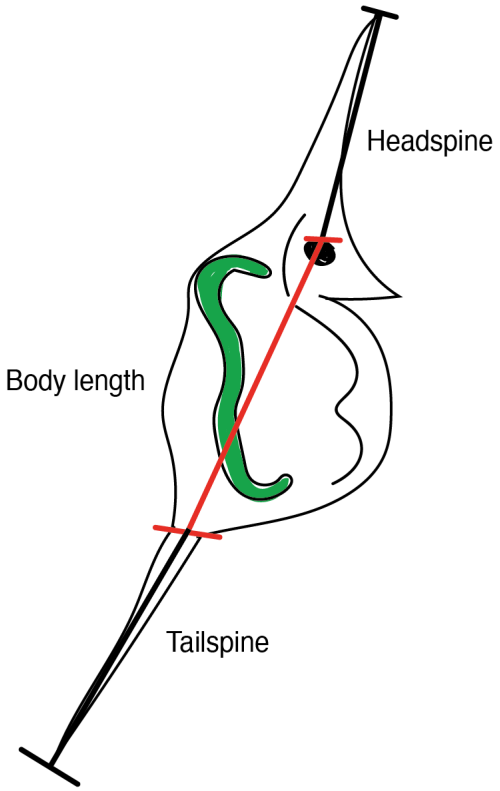


Figure 2. Inducible morphological defense in Daphnia in response to predator cues. Daphnia reared in fish-conditioned media develop significantly elongated headspine and tailspines compared to control. Representative lateral images of uninduced animal reared in artificial lake media (left) and induced animal, reared in fish conditioned media (right). Individuals exposed to fish cues (right) exhibit pronounced elongation of the headspine and tailspine, consistent with a predator-induced defense phenotype. Green lines indicate locations and dimensions for standard measurements of headspine length, body length, and tailspine length, detailed as follows: Headspine was measured from tip of spine to top of compound eye, body length was measured from top of compound eye to base of tailspine, tailspine was measured from base to tip of spine. Total body size was measured from top of headspine to tip of tailspine. The scale bar corresponds to 500 µm.

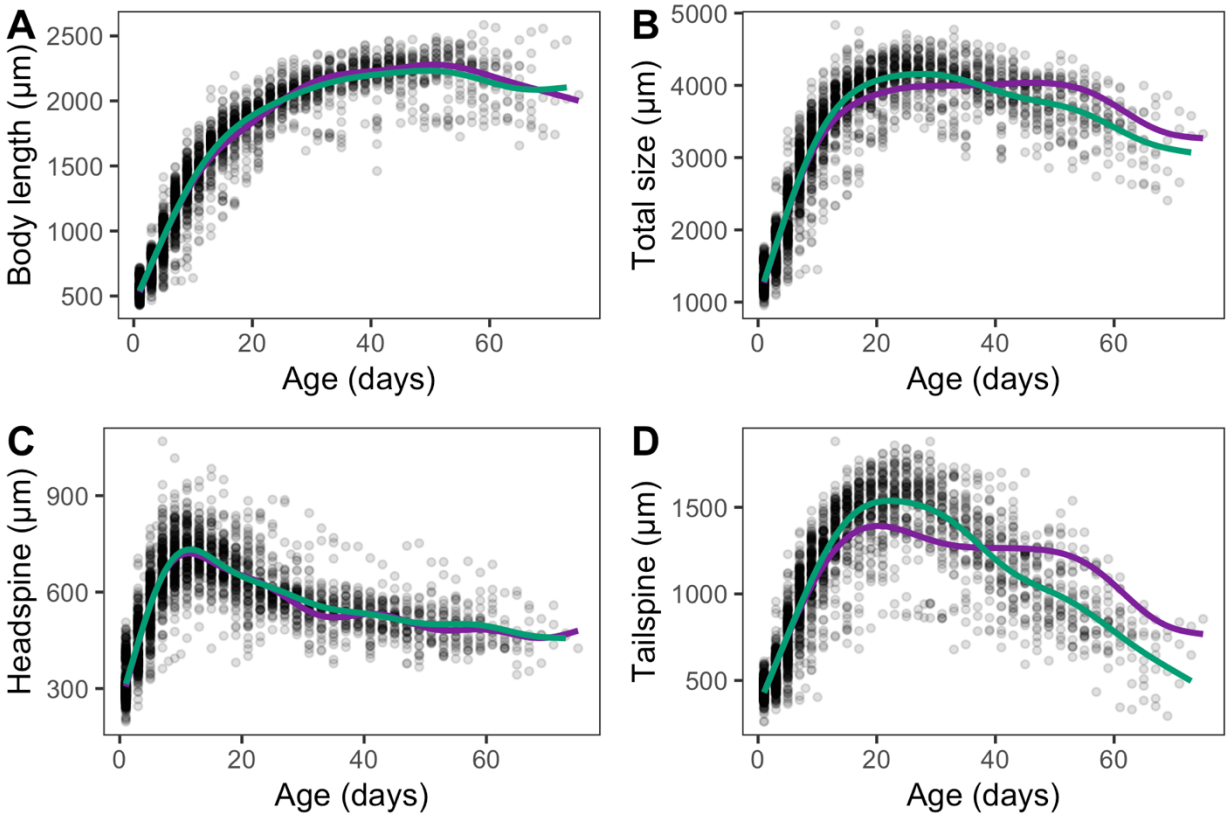


Figure 3. Trajectories of somatic and defensive traits reveal divergence in late-life dynamics. Scatterplots display individual measurements of (A) body length, (B) total body size, (C) headspine length, and (D) tailspine length (in microns) as a function of age (in days). Green line represents locally averaged (GAM) trends for induced lineage (F0 exposed to fish-conditioned media); purple line represents locally averages (GAM) trends for control lineage (no F0 exposure). Somatic traits - body length (A) and total body size (B) - increase rapidly during early juvenile stages, then reach a plateau in mid-life, with little elongation even at advanced ages. In contrast, defensive traits - headspine (C) and tailspine (D) – regress later in life. These results illustrate that the timing and persistence of morphological development vary substantially between defensive and somatic phenotypes. Morphological trajectories demonstrate distinct patterns in defense trait expression, but not body growth, between lineages, consistent with the inheritance of plastic response initiated by ancestral exposure.

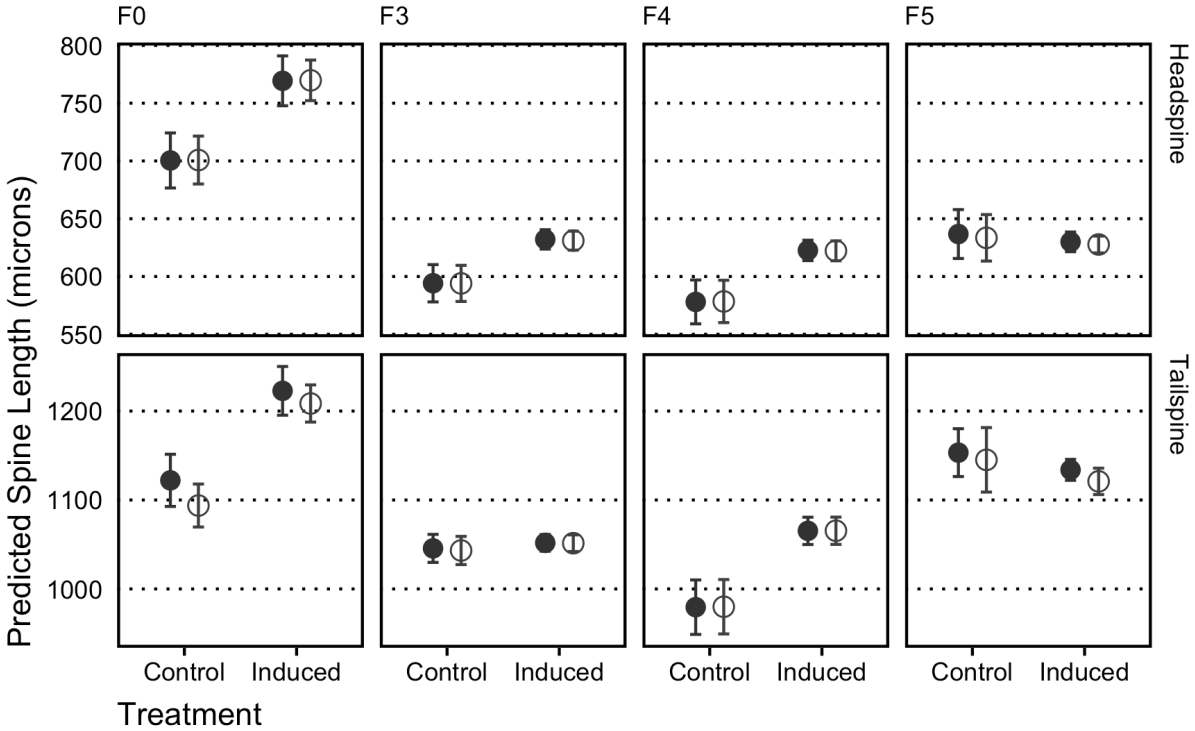


Figure 4. Predicted spine lengths by generation and treatment from linear and Bayesian models. Predicted mean headspine length (top row) and tailspine length (bottom row) for *Daphnia* in control and induced lineages across generations (F0,F3,F4,F5). Points represent model-predicted means ± standard error, with black circles for linear mixed-effect model estimates and open grey circles for Bayesian hierarchical model posterior means. Both model frameworks show increased spine length in induced (fish-conditioned) lineages and yield qualitatively similar inferences, supporting robustness of transgenerational treatment effects. Estimates are based on data from individuals ≤ 20 days old. See tables 1 and 2 for underlying estimates and model parameters.

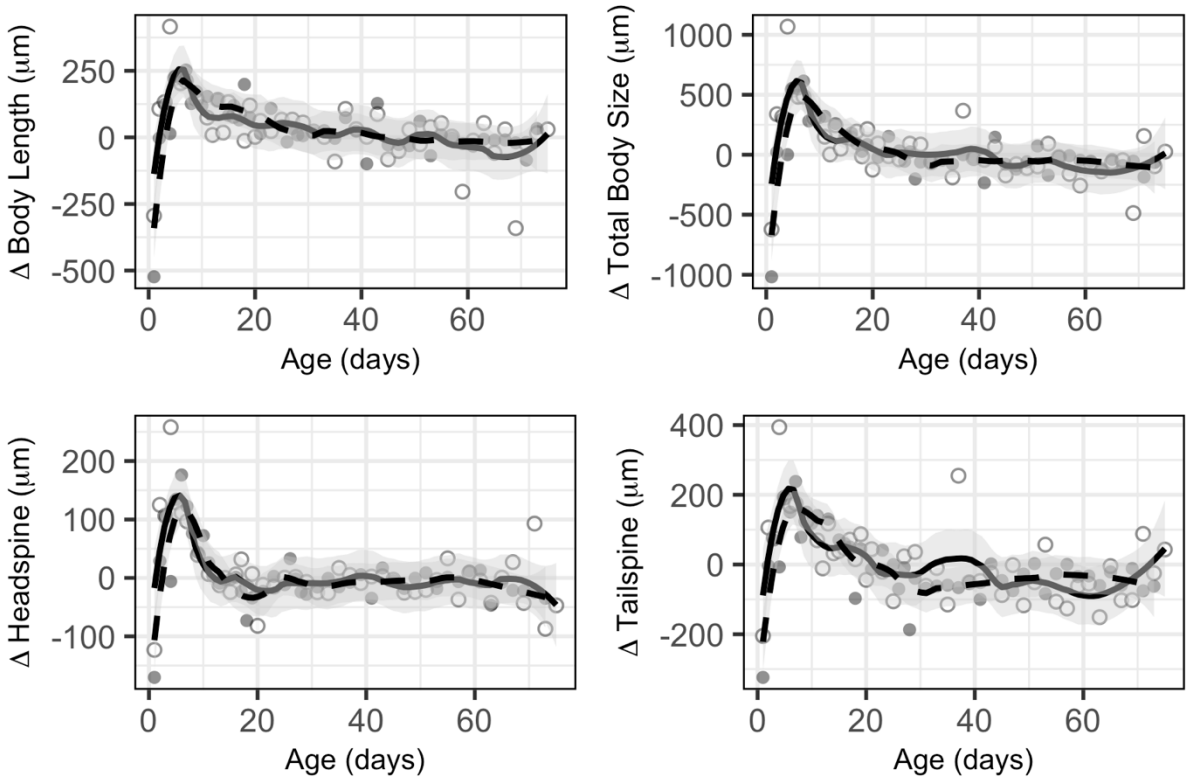


Figure 5. Growth of defensive and somatic traits are most pronounced early in life, followed by a stabilization with age and were unaffected by treatment. Changes in morphological measurements (body length, total body size, headspine, and tailspine) across the lifespan (days). Each panel shows the mean growth (Δ; in μm) from the previous timepoint for the indicated trait. Morphological changes are largest early in life and stabilize as individuals age, as shown by the bold smoothed trend line with 95% confidence intervals (shaded area).

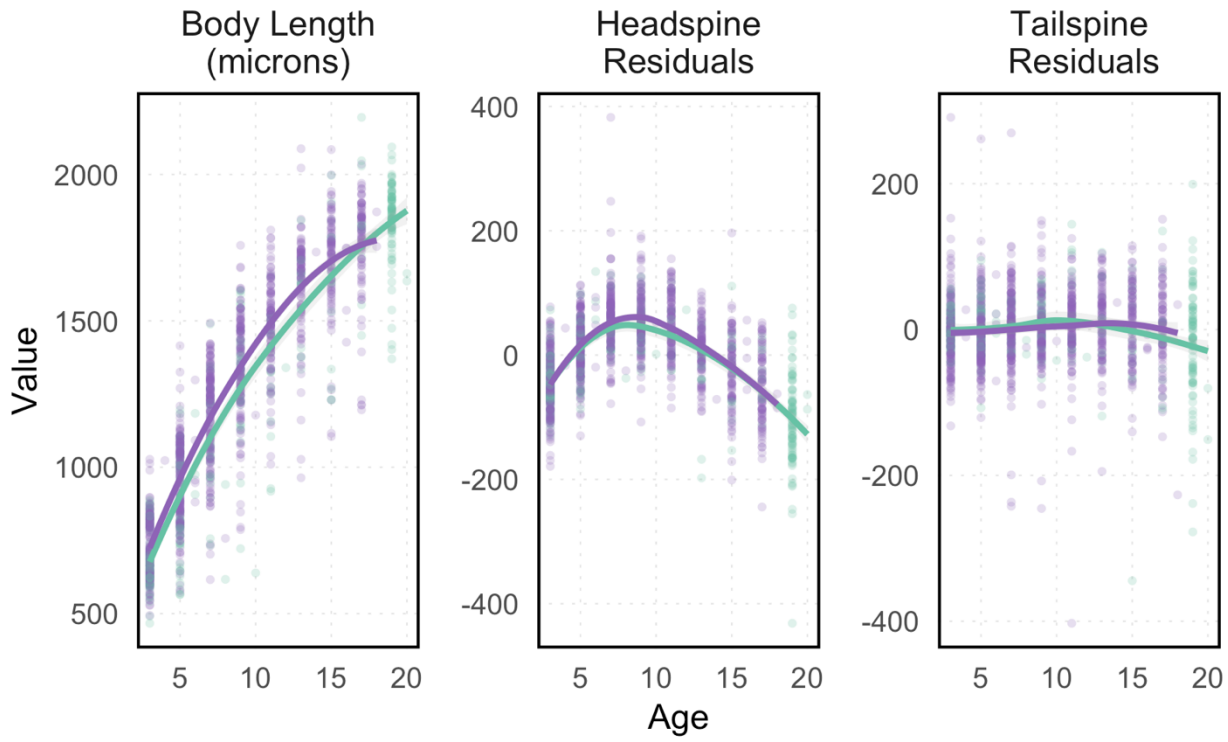


Figure 6. Developmental trajectories during periods of concentrated growth do not indicate treatment or size selective survivorship. Body length, head spine (residual), and tail spine (residual) measurements over first 20 days of age for individuals grouped by survival status (purple for those who survived to the next timepoint - survivors, teal for those that perished). Residual values for head and tail spines are calculated from linear mixed-effects models that account for body length, isolating variation in spine length independent of body size. Both survivors and non-survivors display overlapping developmental trajectories and trait distributions, providing no evidence that size-selective or trait-selective mortality underlies observed group differences.

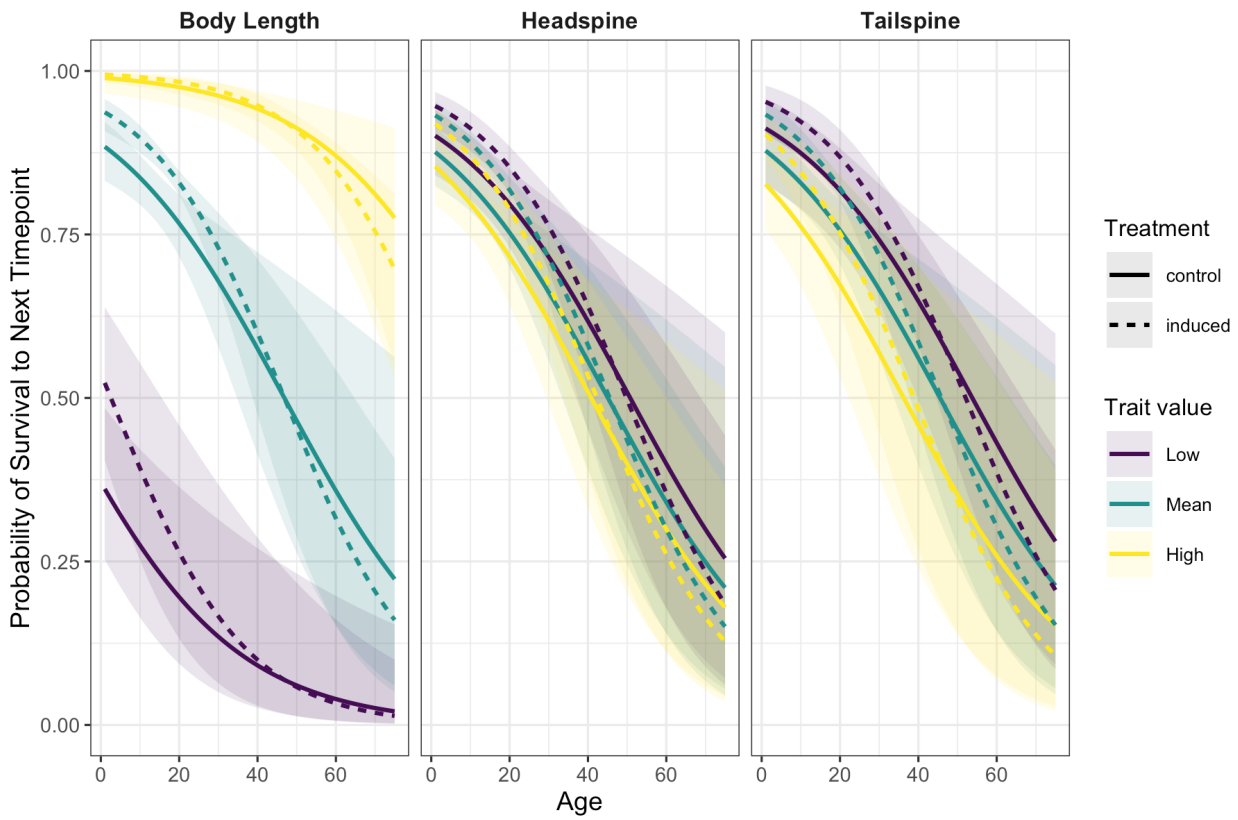


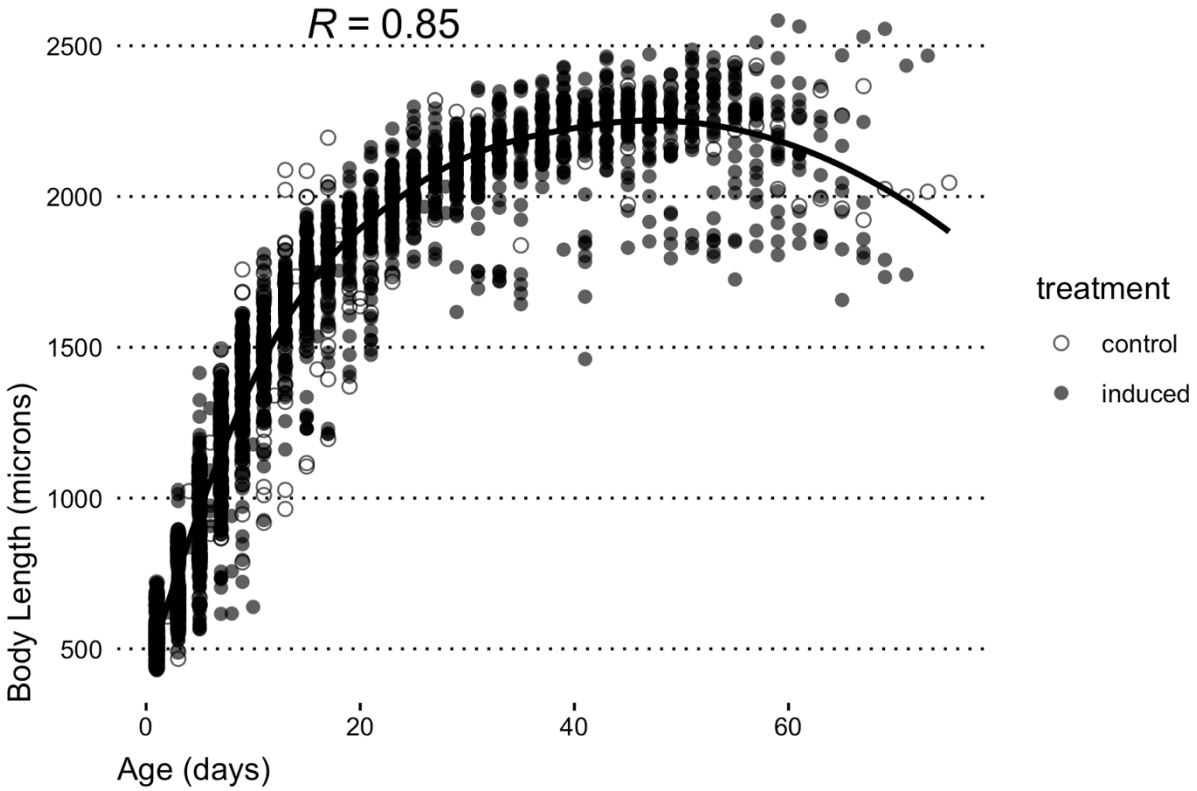
Figure 7. Survivorship and morphological trait distributions do not support size-selective mortality as a driver of morphological differences. Modeled probability of survival to the next timepoint as a function of age, body length, spine length, and treatment, based on a generalized linear model. High body length values are associated with a greater likelihood of survival at most ages, whereas spine lengths show minimal effect. This suggests that while body length does relate to survival probability in the model, size-selective mortality is not strong enough to cause large differences in defensive morpholgies.

Model	Generation	Trait	Marginal R^2	Conditional R^2	AIC	BIC
Linear	F0	headspine	0.584	0.597	881.039	892.492
Linear	F0	tailspine	0.934	0.942	870.549	882.001
Linear	F3	headspine	0.514	0.650	3,651.041	3,669.772
Linear	F3	tailspine	0.950	0.981	3,485.125	3,503.856
Linear	F4	headspine	0.477	0.659	3,315.954	3,334.286
Linear	F4	tailspine	0.903	0.984	3,225.705	3,244.037
Linear	F5	headspine	0.507	0.526	2,454.164	2,470.852
Linear	F5	tailspine	0.931	0.966	2,427.085	2,443.773

Supplemental Table 1: Goodness-of-fit and diagnostic statistics for linear mixed-effects models. Models were fit independently for each combination of experimental generation (F0, F3, F4, F5) and morphological trait (headspine, tailspine). The proportion of variance explained by the models is presented as Marginal R^2 (variance explained by fixed effects alone) and Conditional R^2 (variance explained by both fixed and random effects). Model fit was assessed using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC), where lower values indicate a better trade-off between model fit and complexity. The consistently high Conditional R^2 values, particularly for the tailspine trait, indicate that the random effects structure captured a significant portion of the unexplained variance.

Generation	Trait	Max Rhat	Min Bulk ESS	Min Tail ESS	LOOIC	WAIC	p(Direction)	Marginal R^2
F0	headspine	1.00	10025	14059	889.10	888.85	0.98	0.60
F0	tailspine	1.00	9045	15377	854.56	854.44	0.99	0.95
F3	headspine	1.00	10641	21785	3,634.53	3,632.48	0.98	0.65
F3	tailspine	1.00	11265	18072	3,408.11	3,401.26	0.62	0.98
F4	headspine	1.00	11813	21833	3,296.61	3,294.12	0.98	0.66
F4	tailspine	1.00	5359	10355	3,111.48	3,101.43	0.99	0.98
F5	headspine	1.00	7366	13477	2,451.79	2,451.35	0.63	0.56
F5	tailspine	1.00	7322	9952	2,274.57	2,270.67	0.75	0.95

Supplemental Table 2: Bayesian Model Diagnostics and Fit Summary. Diagnostics are presented for the Bayesian mixed-effects models fit separately for each combination of experimental generation (F0–F5) and trait (headspine, tailspine). Model convergence was assessed using the maximum potential scale reduction factor (Max Rhat), which should be approximately 1.0, and the minimum effective sample sizes for the bulk (Min Bulk ESS) and tails (Min Tail ESS) of the posterior distributions, with higher values indicating more reliable estimates. Model fit and predictive accuracy were compared using the Leave-One-Out Information Criterion (LOOIC) and the Widely Applicable Information Criterion (WAIC); lower values suggest better out-of-sample fit. The probability of direction, p(Direction), represents the posterior probability that the treatment effect is positive. The Marginal R^2 is the proportion of variance explained by the model's fixed effects. All models demonstrated convergence.



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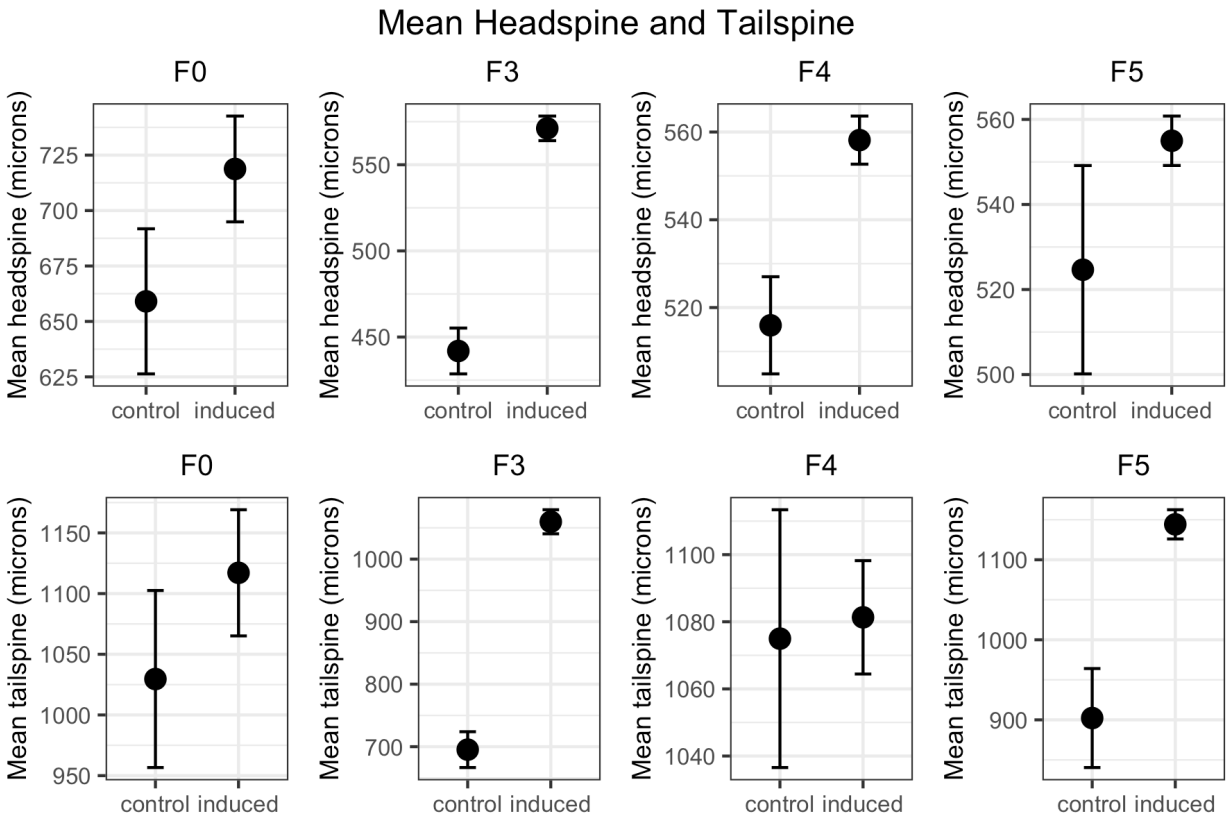
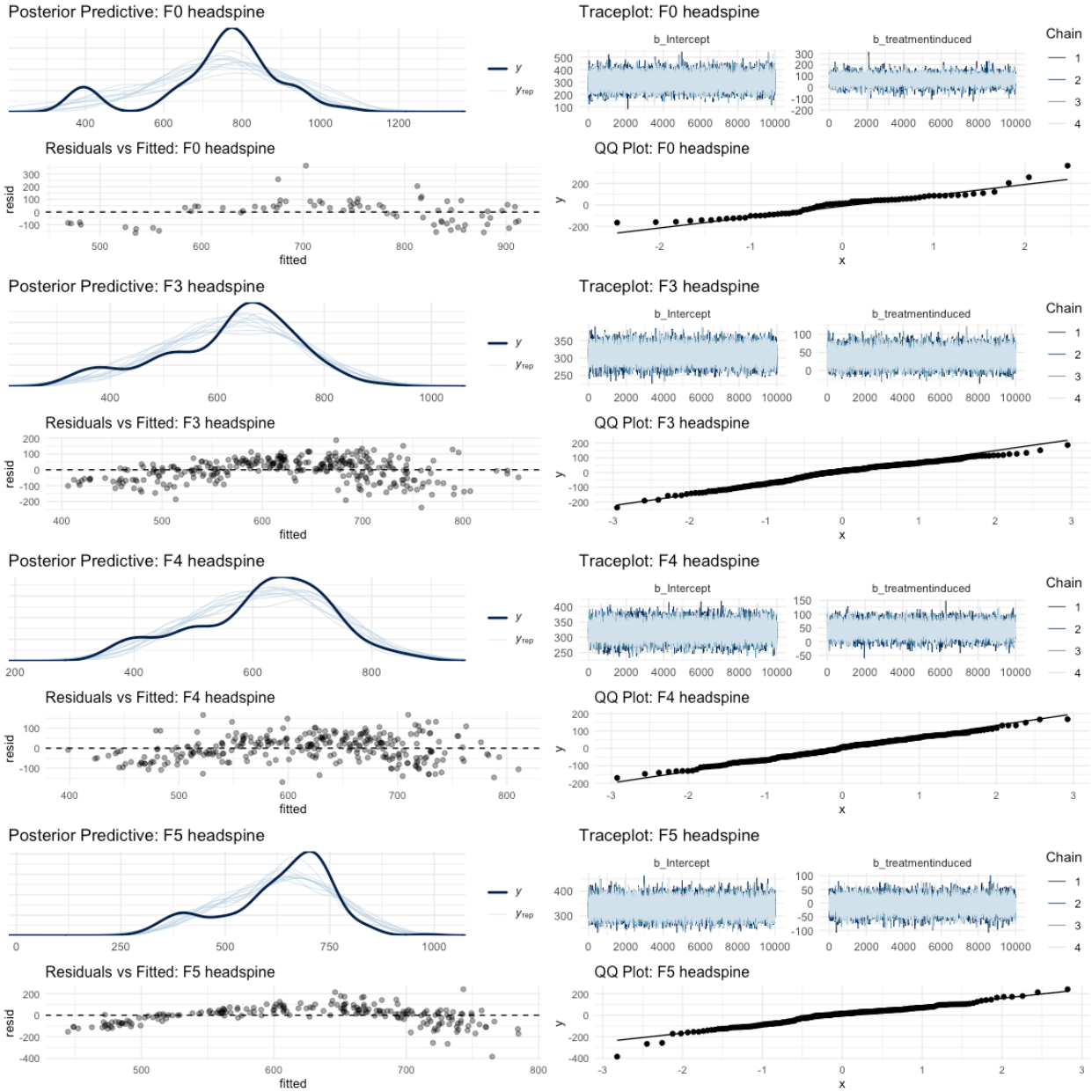
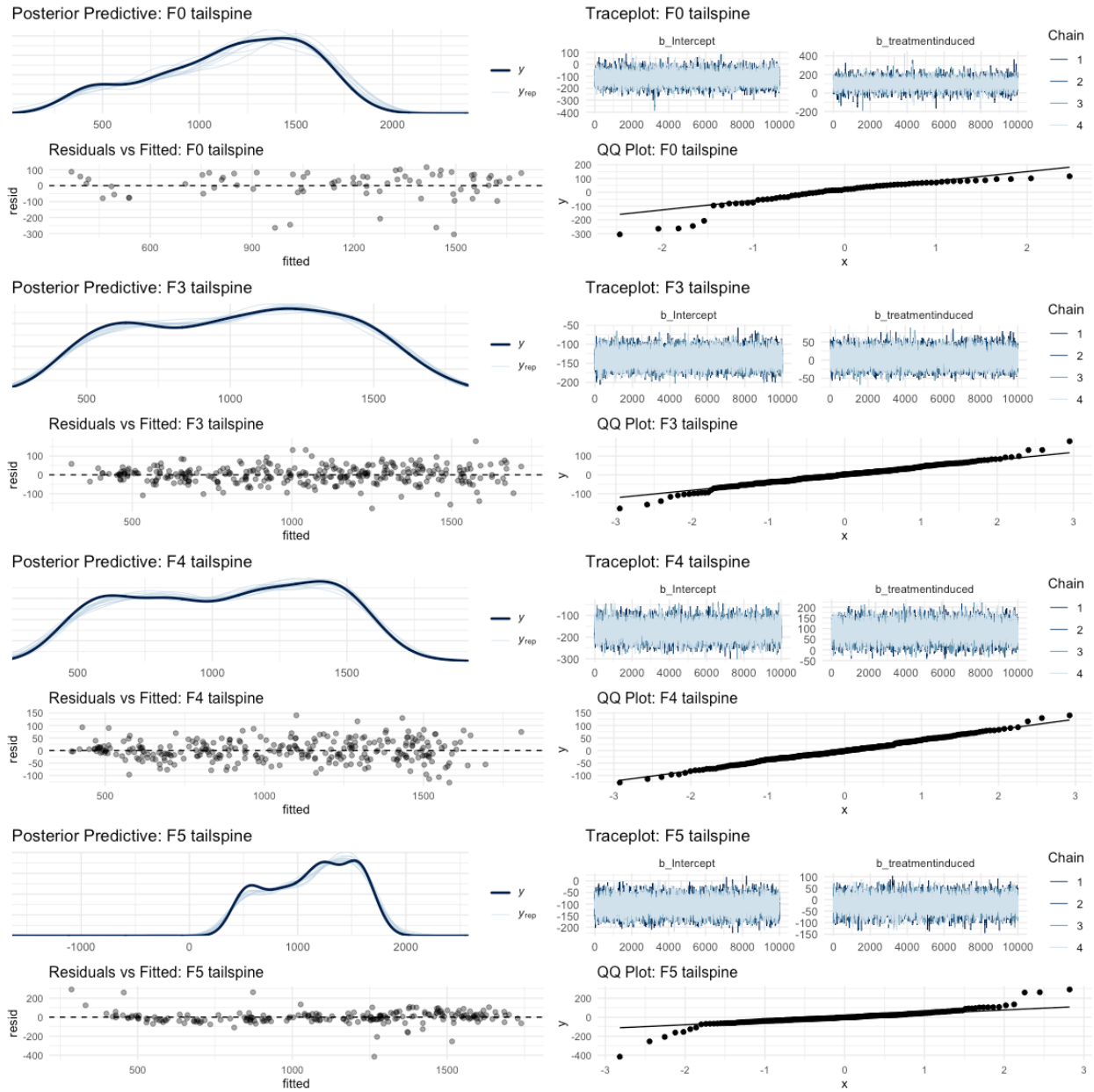
675
676 **Supplemental Figure 1. Ontogenetic growth trajectories for Daphnia body length across**
677 **treatments show high correlation between age and body length.** Scatterplot of individual
678 body length measurements (µm) as a function of age (days) for control (open circles) and
679 induced (filled circles) treatment. The solid line shows the overall fitted growth curve (R=0.85)
680 across all data. Growth patterns are similar between treatment, indicating that environmental
681 induction principally affects defensive traits rather than somatic traits in this clone.
682



Supplemental Figure 2. Transgenerational patterns of headspine and tailspine length in Daphnia following ancestral predator cue exposure. Mean (± standard error) headspine (top panels) and tailspine (bottom panels) values are shown for control and induced lineages across four non-exposed generations (F3–F5) and the originally exposed generation (F0). Each panel displays pairwise comparisons between control (open circle) and induced (closed circle) groups for the indicated generation. Significant induction of both headspine and tailspine length is evident in predator-cue–induced F0 animals, with differential persistence of these defensive traits in subsequent generations. While headspine elongation remains significant through F3 and F4 before declining in F5, tailspine induction fluctuates, with significant retention re-emerging in F4 but lost by F5. These data illustrate the finite and dynamic pattern of transgenerational plasticity in response to environmental cues. See Methods for statistical details and Tables 1,2 for full effect sizes.



Supplemental Figure 3: **Bayesian model diagnostic plots for the headspine trait across generations.** Each row represents an independent model. The panels display posterior predictive checks (model fit), residuals vs. fitted values (homoscedasticity), MCMC traceplots (convergence), and Quantile-Quantile (QQ) plots (normality of residuals). The successful convergence of the MCMC chains and the adequate fit shown by the predictive checks and residual plots support the validity of the models for inference.



Supplemental Figure 4: Bayesian model diagnostic plots for the tailspine trait across generations. Each row represents an independent model. Plots include posterior predictive checks (model fit), residuals vs. fitted values (homoscedasticity), MCMC traceplots (convergence), and Quantile-Quantile (QQ) plots (normality of residuals). The posterior predictive checks show good overlap between observed and simulated data, and the traceplots indicate stable, well-mixed chains. The residuals show no major patterns and are approximately normally distributed, supporting the validity of the models.

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