TITLE: Non-genetic inheritance of induced defense morphologies across multiple unexposed generations of *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 in one generation can persist across several unexposed 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. While genetic adaptation creates persistent change within a population via allele frequency shifts, reversible plastic phenotypes can be inherited through non-genetic mechanisms. However, most transgenerational plasticity studies examine generations where direct embryonic or germline exposure to environmental cues cannot be excluded. Empirical evidence for persistence definitively beyond the critical threshold for distinguishing true transgenerational plasticity from in utero exposure remains scarce, particularly for vertebrate predator-induced defenses.

We measured phenotypic effects of vertebrate predator exposure in the clonally reproducing water flea, *Daphnia lumholtzi*, isolating environmental effects from genetic variation. We exposed the F0 generation to fish conditioned media, then measured morphological defenses in definitively unexposed F3, F4, and F5 generations characterizing the temporal dynamics of non-genetic inheritance. Predator-induced morphologies persisted through F4 before receding in F5, demonstrating that non-genetic effects extend well beyond the embryonically exposed F1 and germ cell exposed F2 generations. This provides rare empirical evidence for transgenerational plasticity lasting through F4, without confounding genetic variation. We also examined ontogenetic patterns of somatic and defensive trait development, revealing trait-specific temporal dynamics in transgenerational effect expression and decay.

These results highlight how current and ancestral environments interact to determine phenotypic variation across generations and underscores the ecological significance of non-genetic inheritance in natural populations, particularly for understanding population responses to environmental change and predator reintroduction. Characterizing molecular mechanisms

93 underlying transgenerational phenotypic plasticity remains critical for predicting persistence and

94 ecological consequences of non-genetic inheritance.

95

Introduction

Organisms respond to environmental variation via 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 within individuals (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. 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 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 physiological changes in response to short-term fluctuations, illustrating a range of temporal scales over which environmentally induced phenotypic changes can occur.

119 Plasticity within a generation has been well documented across numerous taxa and
120 environmental conditions (Agrawal et al., 1999; Corl et al., 2018; Forsman, 2015). Although
121 plasticity itself is often an adaptive phenotype that has been crafted by natural selection, the trait
122 distributions produced by plasticity within one generation have historically been thought to be
123 ephemeral with little consequence for the phenotypes of future generations (West-Eberhard,
124 2020). However, modern analyses have documented that variation in plastic traits is mediated by
125 changes in gene expression (Hales et al., 2017) and is hypothesized to involve epigenetic
126 regulation (St-Cyr & McGowan, 2015), raising the possibility that induced plastic phenotypes
127 may be inherited across generations (Harris et al., 2012a; Romero-Mujalli et al., 2024). This
128 potential for non-genetic inheritance exemplifies the need to integrate genetic and non-genetic
129 mechanisms of information transmission across generations (inclusive heritability, Danchin &
130 Wagner, 2010) in evolutionary biology.
131 Transgenerational inheritance of induced plastic traits from parent to offspring is
132 expected to evolve when the parental environment covaries with the offspring environment
133 (Agrawal et al., 1999). Although induced plastic phenotypes should return to the baseline state in
134 the absence of the inducing cues over generations, the rate at which this occurs is hypothesized
135 to be determined by the frequency of which the environment shifts (Furrow & Feldman, 2014;
136 Proulx & Teotónio, 2017). The evolution of inherited phenotypic plasticity therefore provides a
137 mechanism for organisms and their offspring to respond adaptively to changing environments
138 without significant genetic load (Ghalambor et al., 2007).
139 Studies have documented that induced phenotypes can be inherited (Bell & Hellmann,
140 2019; MacLeod et al., 2022; Moore et al., 2019; Neylan et al., 2025; Tariel et al., 2020; Yin et
141 al., 2019), but the prevalence and duration of this phenomenon remain unclear, especially under

142 fluctuating environmental conditions (Norouzitallab et al., 2014). A particular challenge is
143 distinguishing true transgenerational inheritance from extended maternal or embryonic effects.
144 Because embryos often exist within the maternal body or egg, they may be directly exposed to
145 the environmental cue (Agrelius & Dudycha, 2025; Skinner, 2008). Furthermore, these embryos
146 may have developing germ cells that can be similarly exposed, necessitating the examination of
147 generations sufficiently removed from the initial exposure to rule out direct effects. The number
148 of generations required to differentiate maternal effects from true transgenerational inheritance
149 varies by organism depending on developmental timing and reproductive mode, but empirical
150 data demonstrating persistence beyond the last generation with possible germline exposure
151 remains limited, partially because embryonic and germ line exposure is often conflated with true
152 transgenerational plasticity (Agrelius & Dudycha, 2025; Skinner, 2008). In fact, a 2020 meta-
153 analysis of transgenerational phenotypic plasticity (TGP) in predator-prey interactions found that
154 only 13% of studies (seven total studies) extended beyond the F0 and F1 generations (Tariel et
155 al., 2020), with studies examining F3+ generations remaining notably rare. Despite these
156 advances, critical questions remain: How long do induced traits persist without ongoing cue
157 exposure? At what generation do transgenerational effects decay? And can we definitively
158 distinguish transgenerational plasticity from extended maternal effects?
159 Convincing examples of non-genetic inheritance that extend beyond temporal limits of
160 potential direct exposure include a handful of examinations in *Caenorhabditis elegans* (Greer et
161 al., 2011; S. W. Kim et al., 2013; Kishimoto et al., 2017; Klosin et al., 2017; Remy, 2010; M.
162 Wang et al., 2019), *Mus musculus* (Anway et al., 2005; Skinner et al., 2013; Van Steenwyk et al.,
163 2018), *Drosophila melanogaster* (Panacek et al., 2011), *Danio rerio* (Alfonso et al., 2019), *Ovis*

aries (Kizilaslan et al., 2025), and the keystone aquatic species, *Daphnia* (Shahmohamadloo et al., 2025).

Often overlooked in such studies is the influence of genetic variation. Asexual reproduction provides a particularly powerful system for studying non-genetic transgenerational effects because it eliminates confounding variation due to recombination, genetic background, and parental sex. Examples of such studies include a striking example in aphids, which examined 27 generations of the inheritance of predation induced phenotypes (Sentis et al., 2018), clonal and hermaphroditic freshwater snail systems (Dejeux et al., 2025; Smithson et al., 2020; Tariel et al., 2020), in which alterations in shells shape and thickness can be inherited across generations, and hermaphroditic *C.elegans* (Baugh & Day, 2020). These examples remain relatively few, leaving it unclear whether true transgenerational inheritance is rare or understudied.

The freshwater microcrustacean *Daphnia* is especially well suited for studying transgenerational plasticity and addressing these questions (Ebert, 2022; Harris et al., 2012; Seda & Petrusek, 2011; Yampolsky et al., 2014). The concept of plasticity was introduced in the early 1900s by Richard Woltereck**,** who demonstrated that head size in *Daphnia* varied with nutrient availability, and proposed that environmentally induced traits could be inherited (Woltereck, 1913). *Daphnia's* parthenogenetic reproduction produces genetically identical offspring, eliminating confounding variation due to recombination, genetic background, and parental sex (Harris et al., 2012). *These ecoresponsive crustaceans* exhibit plastic responses to a range of environmental stressors, including cyanobacteria (Dao et al., 2018; Gustafsson & Hansson, 2004; Schwarzenberger & Martin-Creuzburg, 2021), temperature (Geerts et al., 2015; Landy et al., 2024; Müller et al., 2018), and predation (Hebert & Grewe, 1985; Tollrian & Harvell, 1999), affecting morphology (Horstmann et al., 2021), behavior (Beklioglu et al., 2006), and life history

traits (Adamczuk, 2020; Agrawal et al., 1999). Examples include inducible helmets, spines, and neckteeth in response to predator cues (Horstmann et al., 2021; Laforsch & Tollrian, 2004). Many of these traits persist across clonal generations, such as helmet length in *D. cucullata* (Agrawal et al., 1999) and clutch size in *D. ambigua* (Walsh et al., 2014), suggesting a non-genetic mechanism of inheritance.

The number of generations over which induced traits persist in *Daphnia* in the absence of the original environmental cue is still being resolved. While phenotypic effects extending through the F1-F2 generations (maternal effects) in *Daphnia* are well-documented, evidence for persistence beyond F2 (which eliminates direct embryonic exposure; (Agrelius & Dudycha, 2025) remains limited. Most documented cases track only one or two generations post-exposure, such as helmet length in *D. cucullata* (Agrawal et al., 1999; Laforsch & Tollrian, 2004), and age of maturation in *D. ambigua* (Walsh et al., 2016). Many of these studies have predominantly focused on invertebrate predation, such as *Chaoborus* larvae (Horstmann et al., 2022), *Bythotrephes longimanus* (spiny water flea) (Landy et al., 2024), *Leptodora* (Baludo et al., 2024), *Notonecta* (Horstmann et al., 2023; Weiss et al., 2015), and *Triops* (Pietrzak et al., 2020), or focus on various toxicological stressors (Funke et al., 2024; J. Kim & Choi, 2023; Liao et al., 2023; Liu et al., 2020; Maggio & Jenkins, 2022a, 2022b; Martins & Guilhermino, 2018a, 2018b; Shahmohamadloo et al., 2025; Song et al., 2022; Vandegehuchte, Lemière, et al., 2010; J. Wang et al., 2024). However, studies examining the transgenerational responses to vertebrate predation beyond generations with possible germline exposure remain notably scarce (Tariel et al., 2020).

This gap is particularly important because vertebrate predators impose strong, and often more temporally consistent selective pressure on prey populations (Batabyal, 2023; Salo et al., 2010; Wooster, 1994), making transgenerational responses to fish predation critical for

210 understanding adaptive potential in natural systems, and relevant to predator reintroduction
211 efforts (Bracis & Wirsing, 2021). Molecular studies suggest long term effects are possible, with
212 differential gene expression persisting after predator cue removal (Hales et al., 2017), and
213 methylation differences between naive and unexposed individuals across various stressors
214 (Feiner et al., 2022; Hearn et al., 2019; Jeremias et al., 2018; Trijau et al., 2018). However, the
215 duration of phenotypic persistence and its evolutionary significance remain unclear (Bird, 2024;
216 Feinberg, 2007; Feinberg & Irizarry, 2010; Fitz-James & Cavalli, 2022; Ho & Burggren, 2010;
217 Klibaner-Schiff et al., 2024), requiring empirical evidence beyond generations with possible
218 germline exposure.

219 *Daphnia lumholtzi* develops elongated head and tailspine in response to fish predation
220 (Dzialowski et al., 2003), traits that effectively reduce predation risk and represent a clear
221 example of adaptive phenotypic plasticity (Engel et al., 2014). These distinctive spines are
222 thought to contribute to the species' invasive success in North America (Engel & Tollrian, 2009;
223 Sousa et al., 2017; Work & Gophen, 1999) where *D. lumholtzi* often displaces native North
224 American *Daphnia* populations (Johnson & Havel, 2001). Unlike *D. lumholtzi,* many North
225 American *Daphnia* species typically produce broad, helmet-like adornments rather than
226 elongated spines (Horstmann et al., 2021) exemplifying *D. lumholtzi's* unique defensive strategy.
227 These distinctive morphological defenses, combined with *Daphnia's* parthenogenetic
228 reproduction, make *D. lumholtzi* an ideal system for tracking transgenerational effects across
229 multiple generations without genetic confounds.

230 We measured predator induced morphological traits in *D. lumholtzi* across multiple
231 genetically identical generations following initial exposure, focusing on F3-F5 cohorts where
232 neither the individuals themselves nor their germ cells (as developing gametes in F1-F2) could

have experienced direct exposure to predator cues. Previous work in *D. lumholtzi* shows that continued multigenerational maternal exposure can accelerate defensive trait development (Graeve et al., 2021) and that cyanobacteria-induced life history effects persist for at least one generation (Dao et al., 2018), but no study has examined the long-term persistence of vertebrate predator induced morphology in *Daphnia lumholtzi*, particularly beyond the generations with possible direct exposure. By focusing on F3-F5 generations, our observations document transgenerational inheritance via necessarily non-genetic mechanisms rather than extended effects of direct exposure. Additionally, deriving all experimental lineages from a single founding mother of the Saguaro Lake *D. lumholtzi* clone eliminates confounding effects of standing genetic variation, enabling us to fully isolate non-genetic inheritance.

We found that induced traits persisted through multiple unexposed generations, with measurable effects gradually declining to undetectable levels in F5. We identify distinct ontogenetic trajectories in defensive versus somatic traits across the Daphnia's lifespan. A critical question is whether transgenerational morphological difference reflect inherited plasticity versus size-selective mortality of non-induced phenotypes. To address this alternative hypothesis, we explicitly test for evidence of trait-selective or size-selective mortality and report overlapping survival trajectories between different sized defensive structures, indicating that elongated spines are indeed inherited, rather than a selective artifact. These results demonstrate that true transgenerational plasticity can be inherited across clonal generations, but exhibits limited temporal persistence, with trait-specific patterns of decline that reflect the gradual loss of ancestral environmental influence (Geoghegan & Spencer, 2012; Heard & Martienssen, 2014).

Methods

256 **Experimental Overview (Figure 1).** To explore the dynamics of predation induced phenotypes
257 within and across generations, we reared individual Daphnia with ancestral predation exposure in
258 the F0 generation, alongside unexposed *Daphnia lumholtzi* individuals for five generations
259 following initial predation exposure only in the predator F0 generation (total generations: F0-F5;
260 only F0 exposed). These individuals were the progeny of a single founding mother, whose
261 genetically identical daughters (F0) were randomly assigned to treatment or control to establish
262 replicates, and propagated for an additional four generations without subsequent exposure. This
263 design isolated environmental effects from confounding genetic variation while maintaining
264 independent biological replicates.

265 We focused morphological measurements on F0, F3, F4, and F5 generations. F0
266 established baseline induction effects, while F3-F5 represented generations with no direct
267 embryonic or germline exposure to predator cues - essential for distinguishing true
268 transgenerational inheritance from extended maternal effects (Agrelius & Dudycha, 2025;
269 Skinner, 2008). F1 and F2 generations were propagated but not measured, as phenotypic effects
270 in these cohorts are well-documented and can result from direct embryonic exposure. While this
271 design limits F0 sample size, our primary objective was characterizing persistence of effects in
272 F3-F5 individuals rather than recapitulating characterized F0-F2 responses.

273 *Daphnia* were individually grown in 50 mL beakers containing 30 mL of fish conditioned
274 medium or control medium (COMBO) (Kilham et al., 1998), enabling replication at the
275 individual level. Every other day, until death, during the F0, F3, F4 and F5 generations,
276 individual *D. lumholtzi* were imaged for morphological measurements using `ImageJ` (Schindelin
277 et al., 2012). This experimental framework also allowed us to examine survivorship (as animals
278 were imaged until they died) and life-long ontogenetic trajectories. On this same schedule the

279 media was exchanged, and algae was replenished with 85,000 cells/mL of a 1:1 mixture of
280 *Scenedesmus obliquus* and *Chlorella vulgaris*, hereafter referred to as the standard algae mixture.
281 Variation in sample size between treated and control individuals and across generations
282 reflects differential survival and reproductive success. Predator-exposed individuals showed
283 higher survival and fecundity in some generations, resulting in asymmetric sample sizes.
284 Bayesian hierarchical models can maintain robustness despite imbalance and small sample sizes.
285 See Figure 1 for experimental schematic and sample sizes; See Table 1 for expanded sample
286 sizes, including number of repeated measures observations on individuals.
287 This study focuses on a single genetic background to isolate non-genetic effects. Future
288 work examining multiple clones and populations will be necessary to assess the generalizability
289 of these transgenerational effects.
290
291 **Clonal Genetic Background: *Daphnia Lumholtzi*.** *Daphnia lumholtzi* was collected in the
292 summer of 2019 from Saguaro Lake, AZ, USA. We utilized one Saguaro Lake Daphnia, as our
293 primary objective is to minimize confounding genetic variation, rather than generalize a
294 population's ability to respond to predator cues. This species and genetic background were
295 specifically chosen for its ability to develop unique headspines and tailspines in response to
296 vertebrate predation and for their ecological significance as an invasive North American species,
297 capable of displacing native *Daphnia* species (Engel et al., 2014b; Engel & Tollrian, 2009; Sousa
298 et al., 2017; Work & Gophen, 1999).
299
300 **Common Garden Rearing to Standardize Generational Exposures.** After lake collection, *D.*
301 *lumholtzi* were reared for 6 months in artificial lake medium (COMBO) and fed *Scenedesmus*

302 *obliquus* and *Chlorella vulgaris* ad libitum to dull residual effects of lake predation. Prior to
303 experimental perturbations, *D. lumholtzi* were common garden reared for three generations in
304 COMBO medium (Kilham et al., 1998) and fed a standard algae mixture of *S. obliquus* and *C.*
305 *vulgaris* to minimize the influence of maternal effects on experimental generations.
306
307 **Fish and Control Media Preparation.** To prepare fish (induction) medium, 10L of artificial
308 lake medium (COMBO) was conditioned with five *Gasterosteus aculeatus* for three days prior to
309 filtration (0.45 µm). Fish were fed 10 live *D. lumholtzi* and 10 *D. magna* daily to provide both
310 conspecific and heterospecific prey cues, maximizing predation kairomone concentration. To
311 create control medium, artificial lake medium without fish conditioning was created in parallel
312 with fish medium. Media was frozen and thawed overnight prior to use.
313
314 **Initial F0 Predation Exposure and Experimental Conditions**. From the third generation of
315 common garden reared *Daphnia*, one mother was selected due to observed clutch survivability
316 and clutch size. Nine neonates born within a 12 hour period from the same mother were isolated
317 and randomly assigned to treatment groups, either fish exposure or control. Animals were reared
318 individually in 50 mL plastic beakers with 30mL of the appropriate medium and fed 5 mL
319 standard algae mixture every other day, ensuring replication at the individual level. Individual
320 Daphnia were staged, imaged, and returned to fresh media with algae every other day for the
321 duration of their lives.
322
323 **Propagation across Generations.** Upon reproduction, up to six neonates from each mother's
324 first clutch were randomly collected and reared individually in 50 mL beakers containing 30 mL

control medium. If individuals from the mother's first clutch did not survive to reproduce, neonates from the mother's subsequent clutch were used instead. This ensured propagation of all lineages while maintaining consistency in clutch selection across treatments. See Figure 1 and Table 1 for samples sizes.

Imaging and Data Collection. Every other day, animals in the F0, F3, F4, and F5 generation were staged on a glass slide and imaged using `cellSenseEntry` (Olympus Life Science). Scale bars were added to images during acquisition for calibration during measurements. Morphological measurements of *headspine* (tip of spine to top of compound eye), *body length* (top of eye to base of tail), *tailspine* (base of tail to tip of tailspine), *total body length* (tip of headspine to tip of tailspine) were collected (see Figure 2) using `ImageJ` (Schindelin et al., 2012). All images were calibrated to a consistent scale using the 'set global scale' function in `ImageJ`, which converts pixel distances to micrometers based on the embedded scale bar. See Figure 2 for measurement details.

Ontogenetic Trajectories. To illustrate general patterns of ontogenetic development in somatic and defensive traits, Figure 3 presents scatterplots pooled across generations, using `ggplot2` (Wickham, 2016). Generation- and treatment-specific effects are subsequently analyzed in detail in our primary statistical models (see Primary Hypothesis section below).

Statistical Analysis.

Primary Hypothesis: Phenotype Transmission Across Generations. Because individuals were measured repeatedly within generations, we used hierarchical mixed models to account for non-

independence. We employed complementary Bayesian hierarchical and frequentist mixed-effects approaches to ensure robust inference. Bayesian models provide full posterior distributions enabling probabilistic statements about treatment effects and improved estimation with sparse data through partial pooling (Gelman & Hill, 2006). Frequentist models provide p-value-based hypothesis testing for readers familiar with traditional frameworks. Importantly, both approaches yielded consistent effect size estimates and overlapping confidence/credible intervals across all generations and traits (Table 2, Supplemental Table 2), supporting the reliability of our conclusions regardless of statistical framework.

Morphological variation was analyzed using both frequentist and Bayesian mixed-effects models in `R` (R Core Team, 2021), using the `lme4` (Bates et al., 2015) and `brms` (P. C. Bürkner, 2017) packages respectively. For each generation (*F0, F3, F4, F5*) and each trait (*headspine, tailspine*), we tested for the effects of predation treatment, with *body length* as a covariate, and a random intercept for individual identity to account for repeated measures on individuals. The model formula used was:

```
spine ~ treatment + bodylength + (1|individual)
```

where *individual* represents the individual Daphnia identity, accounting for repeated measures on the same organism over time. The *bodylength* term is a covariate accounting for differences in body length that contribute to spine length, and treatment represents whether the F0 progenitor was exposed to predation (for F3, F4 and F5); F0 treated animals were directly exposed. Spine refers to either the measured head or tailspine length.

We selected this model structure based on established principles in *Daphnia* morphological analysis and our experimental design. *Body length* was included as a covariate because spine length scales allometrically with body size in *Daphnia* and failing to account for this relationship can confound treatment effects with size-related variation (Laforsch & Tollrian,

2004; Petrusek et al., 2009). *Treatment* (predator exposure vs. control) was included as the main fixed effect to test our primary hypothesis of transgenerational inheritance of induced defenses. The random intercept for individual identity *(1|individual)* accounts for repeated measurements on the same individuals, addressing potential pseudo-replication issues common in experimental ecology (Freeberg & Lucas, 2009; Hurlbert, 1984). We included *body length* rather than *age* as a covariate because they are highly correlated (Pearson's r = 0.85; Supplemental Figure 1), and *body length* more directly captures the allometric relationship between body size and spine development (Laforsch & Tollrian, 2004).

Bayesian Hierarchical Models. We implemented Bayesian hierarchical models with the model structure described above. The hierarchical structure allows partial pooling across individuals, improving estimation in the presence of sparse data. This method also improves upon *p*-value based frequentist methods by providing a full posterior distribution for the effect size, allowing probabilistic statements about parameter values rather than relying on a binary significant/non-significant framework. 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. 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. We generated posterior predictions at the mean body length using `posterior_epred()` from the `posterior` package (Bürkner et al., 2025), isolating treatment effects while controlling for individual-level variation. These predictions are visualized in Figure 4. We determined adequate model fit and convergence using $\hat{R}$ values, effective sample sizes (ESS), leave-one-out cross-validation (LOOIC), WAIC (Supplemental Table 1; (Vehtari et al., 2021) , trace plots, and posterior predictive checks

(Supplemental Figure 2 and 3; Gabry et al., 2019). Marginal and conditional R^2 values were computed using the `performance` package (Lüdecke et al., 2021) to assess model fit. Treatment effect estimates, including standard errors, confidence intervals, probability of direction (PD), and R^2 values are presented in Table 2. The predicted effect of treatment on defensive traits across generations is visualized in Figure 4.

Frequentist Mixed-Effects Models. To provide familiar p-value-based hypothesis testing, we also conducted frequentist analysis using the `lme4` (Bates et al., 2015) `R` package with the same model formula. 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 (Supplemental Table 3). Bayesian and frequentist estimates were highly consistent across generations and traits, with overlapping intervals and similar effect sizes (Table 2; Supplemental Table 2). For transparency, raw means comparisons are presented in Supplemental Figure 5.

Primary Analysis: Testing for Size-Dependent Treatment Effects. To specifically assess the effect of treatment on *body length* across generations, we again utilized Bayesian hierarchical modeling using the following formula:

```
bodylength ~ treatment + headspine + tailspine + (1|individual)
```

This model determines whether *treatment* affects body size independent of spine elongation. We used *spine* lengths (and not *age*) as covariates because *age* and *body length* are highly correlated (Pearson's r =0.85, Supplemental Figure 1) and because our spine models used *body length* (not *age*) as a covariate, making this specification more consistent. As with the spine models, we used weakly informative priors and a Student's t distribution and ran 2000 warmup and 4000 iterations using 4 chains and 4 cores utilizing the `brms` `R` package (Bürkner, 2017). Trace plots

and posterior predictive checks confirmed model convergence and adequate model fit and are included in the supplemental materials for reference (Supplemental Figure 6).

Secondary Analysis: 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 (Figure 5). To statistically evaluate the effect of induction on morphological plasticity, we modeled Δ values using linear mixed-effects models, `lme4` (Bates et al., 2015) 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:

```
delta value ~ treatment + age + (1|individual)
```

Because this analysis addressed a secondary question about short-term morphological plasticity rather than our primary hypothesis on spine inheritance, we opted for a parsimonious linear mixed-effects approach. This strategy ensures computational efficiency for secondary analyses while providing the level of inferential detail appropriate for each research question. 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:

```
delta value ~ treatment + ns(age, df = 2) + (1|individual)
```

However, these models suffered from overparameterization, 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. Results were visualized in Table 3, including the estimated effect of treatment, standard error, F-statistic, and *p*-value. For a more granular approach, we also modeled these effects by generation and present the same parameters (Supplemental Table 4).

Secondary Analysis: 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. Because this analysis addressed a secondary question about size-selective mortality rather than our primary hypothesis on spine inheritance, we opted for a parsimonious frequentist mixed-effects approach. Bayesian modeling was unnecessary here because the goal was to test a simple association between body size and spine length, and linear mixed-effects models provided interpretable estimates while accounting for repeated measures. This approach balances computational efficiency with the depth of inference appropriate for each research question. *Survival status* 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 individual identity.

```
spine ~ bodylength + (1|individual)
```

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 (Figure 6).

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 (Figure 7).

Sample Size Considerations. Due to attrition and differences in fecundity and survivorship across 6 generations, the F5 generation suffered from lower sample sizes comparatively, particularly in the control animals. Although we employ Bayesian hierarchical models, which are better equipped to handle unbalanced designs and sparse sample sizes, we conducted a retrospective power analysis using the mean effect size from the F3 and F4 generations (Supplemental Table 6) using the `pwr` R package and the `pwr.t.test` function (Champely et al., 2020).

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, 2019), `gghighcontrast` (Grosenbach, 2021) **,** and `Rmisc` (Hope, 2022) for summary statistics. Estimated marginal means and post hoc comparisons were calculated using `emmeans` (Lenth & Piaskowski, 2017).

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.

490 **Table 1: Sample sizes across experimental generations and treatment showing number of**

491 **individuals and repeated observation details.**

Generation	Treatment	Individuals	Observations	Mean Obs/Ind
F0	induced	5	66	13.2
	control	4	34	8.5
F3	induced	74	480	6.5
	control	58	124	2.1
F4	induced	63	556	8.8
	control	17	131	7.7
F5	induced	41	500	12.2
	control	8	45	5.6

492
493 F0 generation began with 9 founder individuals (4 control and 5 predator induced individuals) produced in the same
494 clutch by the same mother. All subsequent generations (F1-F5) were reared in control medium only. 'Individuals' =
495 unique Daphnia measured within that generation/treatment; 'Observations' = total measurements across repeated
496 sampling (every other day) within generation/treatment; 'Mean Obs/Ind' = average number of repeated measurements
497 per individual. F1 and F2 generations were propagated in control medium but not measured, as their phenotypes can
498 result from direct embryonic exposure (see Methods for detailed rationale). Variation in sample size across treatments
499 and generations reflects biological differences in survival and reproductive success between predator-exposed and
500 control lineages. Note the reduced sample sizes in F5, particularly among control individuals, which is addressed with
501 a retrospective power analysis (Supplemental Table 5).

Results

Defensive and Somatic Traits Display Distinct Ontogenetic Patterns. *Daphnia lumholtzi* exhibited distinct developmental trajectories across their lifespan for body length, headspine, and tailspine structures, regardless of treatment (Figure 3). Defensive spine traits displayed age-dependent plasticity, with early-life elongation followed by gradual reduction in spine length across all individuals, independent of treatment (Fig. 3B-C). Headspine length peaked (~850 μm) within the first 10 days and declined steadily to ~400 μm in older individuals (Figure 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) (Figure 3C). These general developmental patterns provide the ontogenetic context within which treatment-specific differences (Fig. 4) should be interpreted.

Predator cue exposure results in an increase in headspine and tailspine length in the exposed generation. Because most morphological changes occur within the first 20 days of life, before spine growth plateaus (Figure 3), we modeled treatment effects on individuals less than 20 days of age. Consistent with previously documented within-generation plastic responses in other species, F0 *Daphnia lumholtzi* reared in predator cue environments exhibited robust and statistically significant increases in both headspine and tailspine length relative to controls, indicating postembryonic predator cue exposure was sufficient to produce a substantial increase in both headspine and tailspine length. In the F0 generation, with predation cues provided from the first post-embryonic day, Bayesian models estimate significant increases in induced defense trait lengths (Figure 4; Table 2). Specifically, we report an estimated increase in headspine length of nearly 70 μm (95% CI: [1.67,132.90]). These differences correspond to a roughly 10% increase in headspine length in exposed Daphnia over control Daphnia. Tailspine length also

increased dramatically in the exposed F0 individuals, by nearly 100 µm in directly exposed Daphnia (95% CI: [15.35,181.54]), also representing a roughly 10% increase.

Table 2. Posterior mean estimates of treatment-induced changes in spine length across generations

Spine	Generation	Estimate	SE	95% CrI	PD	R^2 (marg.)
Head Spine	F0	68.54	32.90	[1.67, 132.9]	**97.7%**	60.4%
	F3	38.19	18.32	[3.1, 74.71]	**98.5%**	64.9%
	F4	44.36	20.45	[3.99, 84.58]	**98.4%**	65.8%
	F5	-7.07	22.32	[-51.23, 36.78]	62.6%	56.5%
Tail Spine	F0	99.71	41.28	[15.35, 181.54]	**98.6%**	94.5%
	F3	5.81	18.35	[-30.24, 41.81]	62.5%	98%
	F4	85.57	34.76	[16.95, 154.28]	**99.3%**	98.3%
	F5	-19.21	29.07	[-77.17, 37.5]	74.7%	94.8%

Models were fit separately by generation for individuals <20 days old. Each row represents a unique combination of generation and spine type. 'Estimate' is the posterior mean difference in spine length (µm) between predator-induced and control treatments, with positive values indicating elongation in induced individuals. 'SE' is the posterior standard deviation. '95% CrI' denotes the 95% credible interval for the posterior distribution. 'PD' (Probability of Direction) represents the posterior probability that the effect is in the estimated direction (positive indicating higher trait values in treated, and negative indicating lower trait values in treated, compared to control). 'R^2 (marg.)' is the marginal Bayesian R^2, indicating variance explained by fixed effects only. See Methods for full model specification and prior structure.

Defensive Traits Are Inherited Through F4. Phenotypic differences persisted for multiple generations after removal from the inducing environment, in generations sufficiently removed from embryonic and germline exposure. When F0 mothers are exposed, their developing F1 embryos are directly exposed in the brood chamber, and the germline cells within F1 embryos that will produce F2 are also exposed. Thus, F2 represents the last generation with potential germline exposure. Only by the F3 generation are any observed effects transmitted through

547 purely non-genetic mechanisms, as F3 individuals and their germ cells had no direct contact with
548 predator cues.

549 Despite two intervening generations without exposure, F3 individuals with ancestral
550 predation exposure in F0 exhibited a significant increase in headspine length. Bayesian inference
551 yielded an effect size of almost 40 μm (95% CI: [3.10,74.71]). This represents ~56% retention of
552 the F0 headspine response magnitude (38.19 μm vs. 68.54 μm). In contrast, tailspine length in F3
553 showed no significant difference between treated and control individuals (β = 5.81 μm, 95% CI:
554 [-30.24, 41.81]), indicating trait-specific attenuation of the tailspine below a statistically
555 detectable threshold.

556 In the F4 generation, also completely unexposed to predator cues, both headspine and
557 tailspine lengths were again significantly elevated in the induced animals as compared to the
558 control individuals. Ancestral predator exposure increased the headspine length by 44.36 μm
559 (95% CI: [3.99,84.58]) and the tailspine length by 85.57 μm (95% CI: [16.95,154.28]).
560 Therefore, F4 headspine retained ~65% of the F0 response magnitude (44.36 vs. 68.54 μm),
561 while F4 tailspine retained ~86% (85.57 vs. 99.71 μm), demonstrating trait-specific patterns of
562 transgenerational persistence (Table 2). Tailspine showed a distinct pattern from headspine: non-
563 significant in F3 but strongly significant in F4, suggesting potential generational fluctuations in
564 trait expression or differential transgenerational mechanisms between the two defensive
565 structures.

566 By the F5 generation all measurable differences between induced and control individuals
567 had disappeared. We report an estimate of -7.07 (indicating treated animals have a shorter
568 headspine; 95% CI: [-51.23,36.78]) for the headspine and -19.21 μm (95% CI: [-77.17, 37.50]

569 for tailspine length (Table 2, Figure 4). Estimated differences were small with 95% credible
570 intervals widely overlapping zero for both headspine (95% CrI: [-51.23, 36.78]) and tailspine
571 (95% CrI: [-77.17, 37.50]), indicating no detectable treatment effect. These findings indicate that
572 the induced phenotypes ultimately reverted to a statistically undetectable baseline in the absence
573 of continued environmental cues over 5 unexposed generations. Attrition across six generations
574 resulted in a small sample size for this generation, making it difficult to discern if we lack the
575 statistical power to detect a subtle effect.

576 Model fit was strong across all analyses, with marginal R^2 values ranging from 56-66%
577 for headspine models and 94-98% for tailspine models, indicating that treatment and body length
578 together explain substantial variance in spine morphology. Frequentist linear mixed-effects
579 models yielded highly consistent results (Supplemental Table 2), with overlapping confidence
580 intervals and similar effect size estimates, confirming the robustness of our inferences across
581 statistical frameworks.

582 These results provide rare empirical evidence of vertebrate predator-induced
583 transgenerational plasticity persisting through the F4 generation. The temporal trajectory of
584 treatment effects across generations (Figure 4) reveals both the persistence and ultimate decay of
585 transgenerational plasticity. Headspine effects declined from F0 through F5, while tailspine
586 showed an unexpected resurgence in F4 after non-significant F3 effects, highlighting trait-
587 specific dynamics. The persistence of these effects through four generations, particularly in a
588 species with rapid generation times (~2 weeks), demonstrates substantial temporal stability of
589 non-genetic inheritance.

590 **Treatment Effects on Body Length are Minimal.** In contrast to defensive traits, body
591 length increased rapidly as the animals aged in both control and induced individuals, reaching an
592 early asymptote (~2,500 μm) around 25 days. This growth pattern was strongly correlated with
593 age (Pearson's r = 0.85; Supplemental Figure S1). Visual inspection of ontogenetic patterns
594 (Figure 3) showed overlap between body length trajectories between control and induced
595 animals, likely demonstrating trait-specific inheritance of defensive morphologies independent of
596 general body size effects. Indeed, confidence intervals for the Bayesian models illustrating the
597 effect of treatment on body length across generations largely overlap zero, with probability of
598 direction (PD) estimates hovering around 50% (PD > 97.5% typically indicates strong directional
599 evidence), and estimated body size differences between -2.63 and 0.46 μm (treated - control;
600 Table 3), except for the F4 generation. Notably, F4 confidence intervals narrowly overlapped
601 zero (-122.54, 2.22) with an estimated body length difference of -61.45 μm, and a PD of 97.1%,
602 just shy of the conventional 97.5%, providing some support for a smaller body size due to
603 predation exposure in this generation only. Effect sizes were negligible across most generations
604 (Cohen's d = -0.05 to 0.03), with only F4 showing a small to medium effect (d = -1.16), further
605 supporting that ancestral predation exposure primarily affects defensive structures rather than
606 general body size.
607 Together, these ontogenetic analyses demonstrate that transgenerational inheritance of
608 predator-induced traits is largely specific to defensive structures and occurs independently of
609 changes in body size. There is, however, some evidence of smaller body sizes due to ancestral
610 predation exposure in the F4 generation alone.
611
612 **Table 3. Treatment effects on body length controlling for spine morphology**.

Generation	Est. Difference (µm)	95% CrI	Cohen's d	PD
F0	-0.53	[-99.45, 95.21]	< 0.01	50.3%
F3	-2.53	[-39.42, 34.84]	-0.05	55.1%
F4	-61.45	[-122.54, 2.22]	-1.16	97.1%
F5	0.46	[-93.78, 89.51]	0.03	51.7%

Treatment effects on body length across generations. Bayesian hierarchical models tested whether ancestral predator exposure affected body size independent of spine elongation, controlling for headspine and tailspine length, and with a random intercept for individual identity. Negative estimates indicate smaller body size in predator-exposed individuals. See Methods for full model specification. 'Generation' refers to the generation examined, 'Est. Difference (µm)' is the estimated effect of treated – control, therefore negative values represent a smaller estimated body size in treated animals. '95% CrI' depicts the 95% credible intervals. For interpretability, we include the estimated effect sizes in the 'Cohen's d' column. 'PD' (Probability of Direction) represents the posterior probability that the treatment effect is in the estimated direction. Values >97.5% indicate strong evidence for the effect direction; values near 50% indicate high uncertainty.

These results demonstrate that predator-induced morphological plasticity can be inherited through at least four generations and is largely concentrated to defensive spines rather than body length. This inheritance is finite and trait-dependent, with detectable headspine elongation through the F4 generations. The decrease of effect sizes and 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 (Figure 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 individuals could be attributed to variation in growth dynamics (rather than final trait size) or trait distributions, we assessed whether treatment effects could be explained by differences in developmental timing, size-selective mortality, or trait-based survival.

636 The most pronounced morphological changes occurred early in life, prior to 20 days of
637 age (Figure 3). This period includes the onset of reproduction (typically 7–12 days), during
638 which *D. lumholtzi* mothers are not yet fully grown. Morphological change peaked around this
639 time for all traits, particularly for body length and total body size (Figure 5).

640 Importantly, no significant differences in growth rate were detected between treatment
641 groups for any of the four traits examined (body length, headspine, tailspine, and total body
642 size), indicating that predator exposure did not alter the rate of somatic or defensive trait
643 development (Table 4). Specifically, treatment effects were non-significant for body length (Est.
644 = 5.70 ± 7.94 μm, F = 0.52, p = 0.47), total body size (Est. = 9.87 ± 19.20 μm, F = 0.27, p =
645 0.61), headspine (Est. = 6.51 ± 4.57 μm, F = 2.06, p = 0.15), and tailspine (Est. = 1.88 ± 7.57
646 μm, F = 0.06, p = 0.80). Generation-specific analyses similarly revealed no treatment effects on
647 growth rates in any individual generation (all p > 0.05; Supplemental Table 4), confirming that
648 differences in defensive trait expression persist consistently across F0-F5 independent of
649 developmental timing. These results suggest that the observed morphological differences are not
650 due to accelerated or delayed growth in induced individuals but instead reflect differences in
651 final trait expression independent of growth rate.

652 **Table 4: Growth rate is not influenced by treatment**

Trait	Est. Difference (μm)	Std. Error	F statistic	P value
Body Length	5.70	7.94	0.52	0.469
Total Body Size	9.87	19.20	0.27	0.605
Headspine	6.51	4.57	2.06	0.151
Tailspine	1.88	7.57	0.06	0.802

Results of a linear model estimating the effect of treatment on trait change between timepoints. Analysis includes all individuals measured during the first 20 days of life across all generations (n = 183 induced, n = 87 control) 'Trait' represents the morphology examined, the 'Est Difference (μm)' represents the model estimated difference (treated – control; positive values indicate larger estimates in treated animals compared to control). 'Std. Error' describes the standard error of the estimate, and the F-statistic and *p*-value for the corresponding morphology are represented in the 'F statistic' and 'P value' columns.

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 (residuals control for body size) by survival status (Figure 6) showed that while survivors tended to have a larger body length, **residual spine lengths showed overlapping distributions and trajectories** between survivors and non-survivors. Overlapping distributions of spine residuals between survivors and non-survivors (Figure 6) 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: $\beta = -0.0007$, $p = 0.110$). Individuals from predator-exposed lineages showed significantly higher survival than controls ($\beta = 0.678$, $p < 0.001$), suggesting that transgenerational defensive trait expression may confer fitness benefits even in the absence of direct predator exposure. Importantly, this survival advantage was independent of spine length, as neither headspine nor tailspine significantly predicted survival status. 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).

Power Analysis. F5 sample sizes were reduced due to natural attrition across six generations (n=8 control individuals, 41 induced; Table 1). Retrospective power analysis using mean effect sizes from F3-F4 generations yielded estimated power of 0.30 for headspine and 0.66 for tailspine (Supplemental Table 5). These estimates are conservative because they do not account for repeated measures (average 12.2 observations per individual for induced, 5.6 for control; Table 1), which increase effective sample size. While we cannot definitively distinguish between trait decay and insufficient power in F5, the pattern across F0-F5 suggests gradual attenuation consistent with temporal decay of transgenerational effects.

These findings demonstrate that transgenerational plasticity in *D. lumholtzi* operates primarily on defensive structures, persists for at least four generations following ancestral exposure, and is not due to size-selective or trait-selective mortality.

Discussion

Our findings demonstrate that predator-induced morphological defenses in *Daphnia lumholtzi* persist through at least four unexposed generations via non-genetic mechanisms. Critically, measurable effects in F3, F4, and (to a lesser extent) F5 occurred in cohorts with no direct, embryonic, or germline exposure to predator cues, a necessary criterion for distinguishing transgenerational plasticity from extended maternal effects (Agrelius & Dudycha, 2025; Skinner, 2008). This temporal persistence is notable given that F3 represents the first generation where developing germ cells had no contact with the inducing signal, and F4-F5 are even further removed. These data contribute to a limited but growing body of evidence demonstrating that

700 environmentally induced phenotypes can be inherited across multiple generations through
701 epigenetic or other non-genetic mechanisms.
702 Our experimental design intentionally employed a single clonal lineage to isolate non-
703 genetic effects from standing genetic variation, enabling us to definitively demonstrate
704 transgenerational inheritance independent of allelic differences. However, this design limits
705 inference to one genetic background. The existence of 'uninducible' *D. lumholtzi* clones with
706 constitutive defenses (Engel & Tollrian, 2009), even from nearby populations in Arizona,
707 demonstrates substantial genetic variation in plasticity and defense strategies within this species.
708 Future work must examine whether the 4-generation persistence we observed is representative
709 across clones or population-specific, and whether genotype-by-environment interactions
710 influence the temporal dynamics of transgenerational effects (Auld et al., 2010; Scoville &
711 Pfrender, 2010).
712
713 **Induced Phenotypes Persist Across Generations but Fade Without Cues**
714 Headspine and tailspine elongation persisted through four unexposed genetically identical
715 generations, with measurable effects in F3 and F4 despite the absence of continued induction.
716 This transgenerational persistence supports the hypothesis that environmental information can be
717 retained and expressed across generations via non-genetic mechanisms (Gapp et al., 2014; Heard
718 & Martienssen, 2014; Rechavi et al., 2014).
719 By the F5 generation, trait differences between induced and control individuals were not
720 detectable. This regression to baseline aligns with evolutionary expectations that plastic traits
721 fade when environmental cues vanish, minimizing fitness costs associated with unnecessary
722 defenses (DeWitt et al., 1998; Van Buskirk & Steiner, 2009).

723 Headspine and tailspine displayed divergent transgenerational trajectories, suggesting
724 trait-specific non-genetic regulation. Headspine effects declined rather monotonically across
725 generations (68 μm → 38 μm → 44 μm → -7 μm), consistent with gradual erosion of epigenetic
726 marks. In contrast, tailspine showed non-significant effects in F3 (6 μm) but pronounced effects
727 in F4 (86 μm), approaching F0 magnitude (100 μm). This "resurgence" pattern could reflect: (1)
728 differential stability of epigenetic marks regulating the two traits, (2) compensatory
729 developmental mechanisms favoring tailspine expression when headspine inheritance weakens,
730 or (3) stochastic fluctuations in small sample sizes. These trait-specific dynamics highlight that
731 transgenerational plasticity is not uniformly expressed across correlated traits and may involve
732 independent regulatory mechanisms (Bonduriansky & Day, 2009; Herman & Sultan, 2011; Uller
733 et al., 2013). Future work should examine whether these two defensive structures are regulated
734 by distinct epigenetic pathways.
735 Retrospective power analysis for F5 yielded estimated power of 0.30 for headspine and
736 0.66 for tailspine, based on mean effect sizes from F3-F4 generations. While we cannot
737 definitively distinguish between true decay and insufficient power in F5, several lines of
738 evidence support the former: (1) the decline in effect sizes across F0-F4, (2) credible intervals for
739 F5 estimates centered near zero rather than showing directional trends, and (3) the biological
740 expectation that transgenerational effects should eventually attenuate without environmental
741 reinforcement. Achieving high statistical power in ecological studies, especially with
742 multigenerational designs is inherently challenging (Kimmel et al., 2023) but our use of repeated
743 measures and hierarchical modeling maximized inferential strength given practical constraints.
744 The 5-generation persistence of predator-induced defenses has implications for
745 population dynamics in variable predation regimes. In systems with fluctuating predator densities

746 (seasonal fish migrations, predator reintroductions, reproductive timing), prey populations may
747 remain partially defended for 4-5 generations (several months in this *Daphnia* species) after
748 predator removal. This "ecological memory" could buffer populations during temporary predator
749 absences but may incur costs if defenses persist longer than threats. Fitness consequences,
750 including fecundity and survival across generations will be the focus of future work in our
751 predator-prey system.
752
753 ***Daphnia lumholtzi* Induced Defenses Are Robust, Age-Specific, and Not Driven by Selection**
754 Exposure to predator cues in the F0 generation triggered strong morphological defenses
755 in *Daphnia lumholtzi*, with significant elongation of both headspine and tailspine structures.
756 Post-embryonic exposure alone was sufficient to induce these traits in our Arizona clone,
757 demonstrating that embryonic priming is not universally required for defense induction in this
758 genus (Laforsch & Tollrian, 2004). This finding underscores the differences in inducibility
759 between clones and species and highlights the need to assess transgenerational inheritance in a
760 variety of systems (Auld et al., 2010; Scoville & Pfrender, 2010; Shahmohamadloo et al., 2025).
761 This is further illustrated by the characterization of "uninducible" *D. lumholtzi* clones which
762 constitutively express defense phenotypes even in the absence of predation cues (Engel &
763 Tollrian, 2009).
764 Importantly, these trait differences were not attributable to size-selective or trait-selective
765 mortality. Survivors and non-survivors showed overlapping developmental trajectories, and
766 survival probability was primarily associated with body length, not spine morphology. This
767 confirms that the observed phenotypic differences are induced responses rather than artifacts of

768 differential survivorship among individuals based upon armor morphology (Dieckmann et al.,
769 2006; Kingsolver et al., 2001).
770 We also found that spine lengths were most prominent in early life and declined with age,
771 a pattern distinct from somatic growth. This suggests stage-specific optimization of defenses,
772 likely targeting periods of highest vulnerability to predation (Nagano & Yoshida, 2020; E. E.
773 Werner & Gilliam, 1984). Smaller, younger Daphnia likely experience higher predation rates
774 from gape-limited fish predators (Nagano & Yoshida, 2020), making early-life defenses
775 particularly adaptive. The age-dependent decline in spine length we observe may represent
776 adaptive reallocation from defense to reproduction as individuals reach body sizes less
777 vulnerable to predation. This ontogenetic trajectory, previously unreported for *D. lumholtzi*
778 headspines, parallels patterns in other inducible defense systems where defenses are most
779 pronounced during vulnerable life stages (Nagano & Yoshida, 2020) and documented tailspine
780 shortening with age in *Daphnia* (Gu et al., 2021).
781
782 **Mechanisms of Non-genetic Inheritance and Evolutionary Implications**
783 The persistence and gradual decline of induced traits across generations strongly suggests
784 epigenetic inheritance as a likely mechanism. Having established the phenotypic baseline and
785 generational persistence, future mechanistic work can now uncover the molecular mechanisms
786 underlying these patterns. Candidate mechanisms include, DNA methylation, histone
787 modification, and chromatin-level changes that facilitate the transmission of plasticity without
788 altering the genetic code (Banerjee & Roychoudhury, 2017; Casadesús, 2016; Davidowitz, 2009;
789 Ho & Burggren, 2010; Li & Zhang, 2014; Niederhuth et al., 2016; Robichaud et al., 2012; M. S.
790 Werner et al., 2023; Yaniv, 2014). The temporal decay pattern we observed is consistent with

791 incomplete erasure of epigenetic marks (Dias & Ressler, 2014; Gapp et al., 2014; Grishkevich &
792 Yanai, 2013), as documented in other invertebrate and vertebrate systems including
793 *Caenorhabditis elegans* (Kaneshiro et al., 2019), the asexual freshwater snail, *Physa acuta*
794 (Dejeux et al., 2025), *Daphnia magna* (Asselman et al., 2016; Vandegehuchte, De Coninck, et
795 al., 2010), as well as vertebrate systems including mice (Carone et al., 2010) and humans
796 (Mulligan et al., 2025). By identifying these generational phenotypic dynamics, we establish a
797 foundation for uncovering the molecular mechanisms underlying generation-specific adaptive
798 phenotypes, a key priority for future research.

799 Our focus on vertebrate predation addresses an important gap in transgenerational
800 plasticity research. While numerous studies examine *Daphnia* responses to invertebrate predators
801 such as *Chaoborus* larvae (Agrawal et al., 1999; Horstmann et al., 2022), studies of vertebrate
802 predator-induced transgenerational effects remain scarce (Tariel et al., 2020). This gap is
803 consequential because vertebrate predators typically exert more consistent selective pressure than
804 invertebrate predators (Batabyal, 2023; Wooster, 1994) and are central to current conservation
805 efforts. Our findings have direct relevance for predator reintroduction and rewilding efforts,
806 where prey populations may exhibit defenses for 4-5 generations (~2-3 months in *D. lumholtzi*)
807 following ancestral exposure. Understanding the temporal dynamics of these transgenerational
808 responses, especially in a keystone aquatic species, could improve predictions of predator-prey
809 dynamics and ecosystem restoration outcomes (Bracis & Wirsing, 2021; Delibes-Mateos et al.,
810 2019).

811 Our findings align with theoretical predictions that transgenerational plasticity should
812 evolve when parental and offspring environments are positively correlated (Agrawal et al., 1999;
813 Herman & Sultan, 2011). The 4-generation persistence we observe (~2-3 months in this system)

matches timescales over which predator densities fluctuate in natural lakes due to seasonal migrations, overwinter mortality, or breeding events. However, the eventual decay by F5 is also adaptive as prolonged expression of costly defenses in predator-free environments would reduce fitness through allocation trade-offs (DeWitt et al., 1998; Van Buskirk & Steiner, 2009). The temporal dynamics we document therefore suggest fine-tuning of inheritance duration to match ecological predictability, though testing this hypothesis would require comparative studies across populations experiencing different predation regimes.

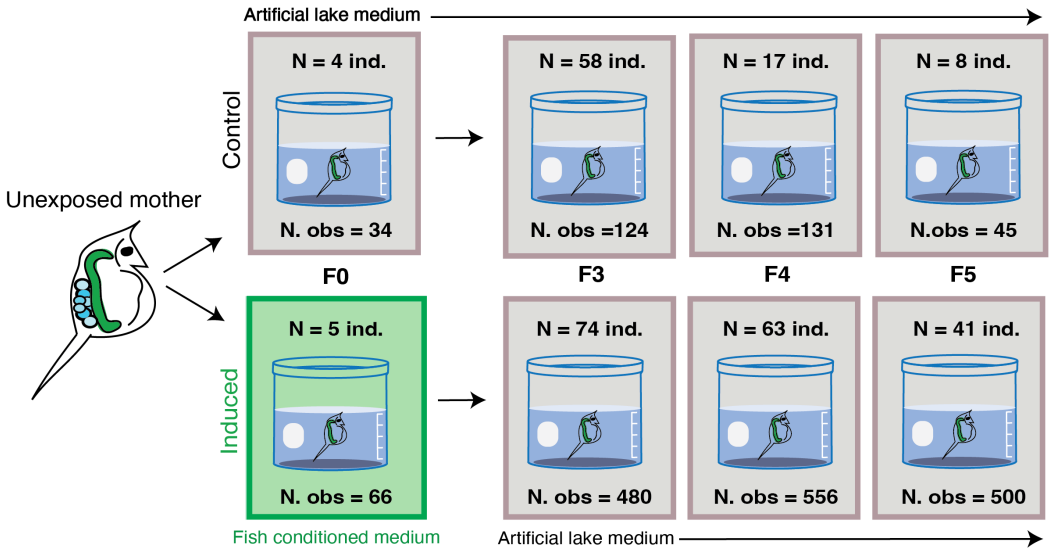
Our findings underscore the evolutionary relevance of environmental history in the absence of genetic variation (Jablonka & Lamb, 2006; Laland et al., 2015; Pigliucci & Müller, 2010). Transgenerational plasticity allows organisms to respond to ancestral conditions, potentially buffering populations against short-term environmental fluctuations (Donelson et al., 2018; Uller et al., 2018). Because our study intentionally used a single *Daphnia* population, future work should 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

This work advances understanding of transgenerational plasticity in a keystone aquatic genus by documenting vertebrate predator-induced morphological effects in key generations sufficiently removed from embryonic and germline exposure, therefore revealing trait-specific temporal persistence in defensive structures. Our findings align with previous work documenting clutch size persistence in *D. ambigua* (Walsh et al., 2014), helmet length inheritance in *D. cucullata* (Agrawal et al., 1999), and multi-generational erosion of plastic traits in *D. pulicaria*

837 (Landy et al., 2024), while crucially extending the temporal window of observation for predator-
838 induced effects to key, previously undocumented generations.
839 This transgenerational plasticity may buffer populations against short-term ecological
840 fluctuations, while its eventual attenuation minimizes the costs of unnecessary trait expression
841 (Herman & Sultan, 2011; Uller et al., 2013). Future research should integrate molecular profiling
842 (DNA methylation, chromatin modifications, differential transcription) across F0-F5 generations
843 with multi-clone comparative studies to determine both the mechanistic basis and generality of
844 these transgenerational effects. Such integrative approaches will advance understanding of how
845 organisms respond to environmental variation across generational timescales and the role of non-
846 genetic inheritance in evolutionary adaptation to changing environments.
847

848 **FIGURES**



849
850 **Figure 1. Schematic illustrating the experimental setup allowing for direct testing of**
851 **transgenerational epigenetic inheritance by induced defenses by comparing individuals with**
852 **and without grandparental exposure to predator cues**. Offspring (F0) from a single unexposed
853 mother were split into two genetically identical groups: control (top), where individuals where
854 continuously reared in artificial lake medium, and induced (bottom), where F0 individuals were
855 exposed to fish-conditioned medium indicated in green. Only the F0 induced generation is exposed
856 to fish conditioned media. Sample size for each generation and treatment is indicated (N ind.), and
857 the number of observations (repeated measures, N.obs) is indicated. Subsequent generations (F3-
858 F5) from both treatment group and control are maintained in artificial lake media. Generations
859 were propagated for six generations using the first viable (successful reproduction) and measured
860 every other day during the F0, F3, F4, and F5 generations.
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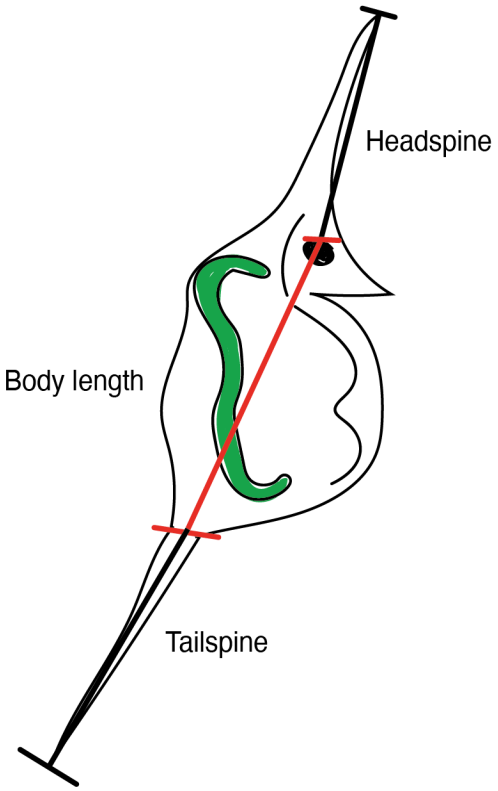


Figure 2. Measurement of inducible morphological defenses in Daphnia in response to predator cues. Daphnia reared in fish-conditioned media develop significantly elongated headspines and tailspines compared to control animals. Representative lateral images of morphological measurements are indicated, 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.

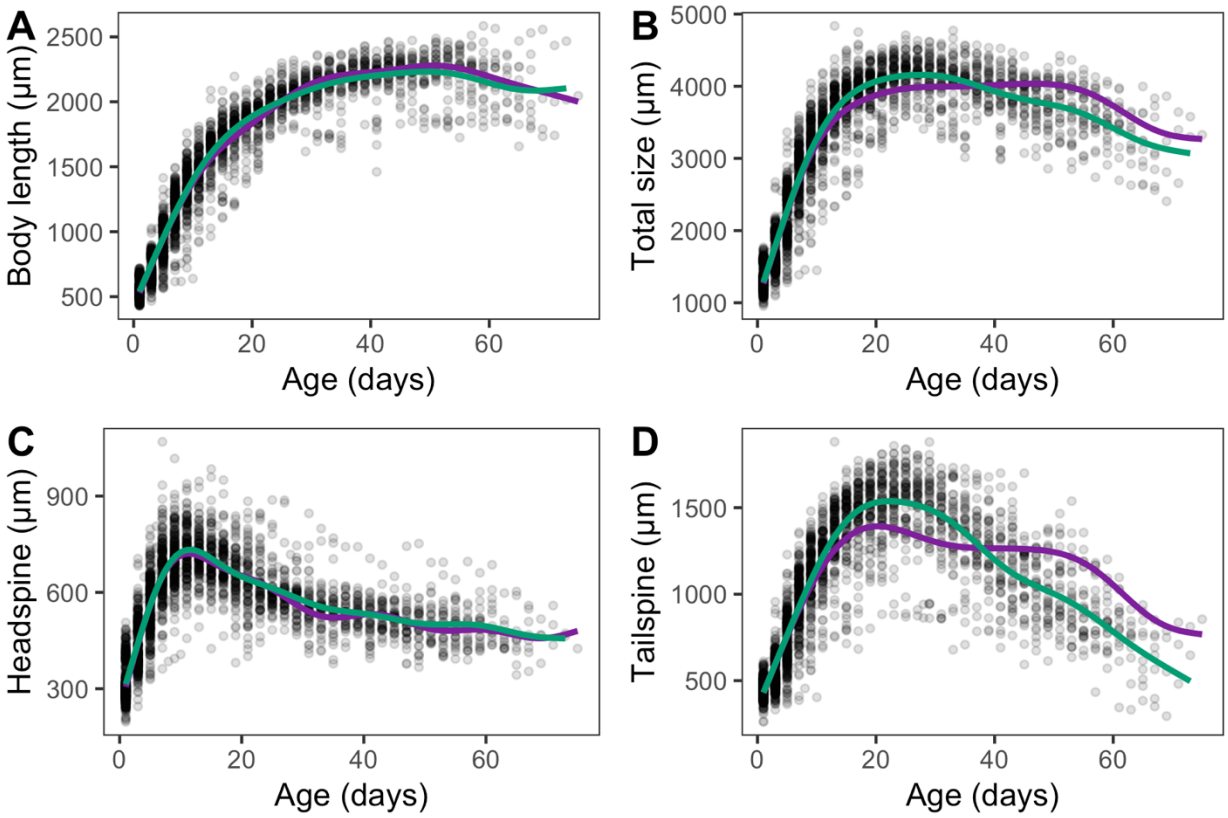


Figure 3. Trajectories of somatic and defensive traits reveal divergence in late-life dynamics. Scatterplots display individual measurements of (A) body length, (B) total body size, (C) headspine length, and (D) tailspine length (in µm) as a function of age (in days). Green line represents locally averaged (LOESS) trends for induced individuals (F0 exposed to fish-conditioned media); purple line represents locally averages (LOESS) trends for control individuals (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. Morphological trajectories demonstrate distinct patterns in defense trait expression, but not body growth, between control and treated individuals, consistent with the inheritance of plastic response initiated by ancestral exposure.

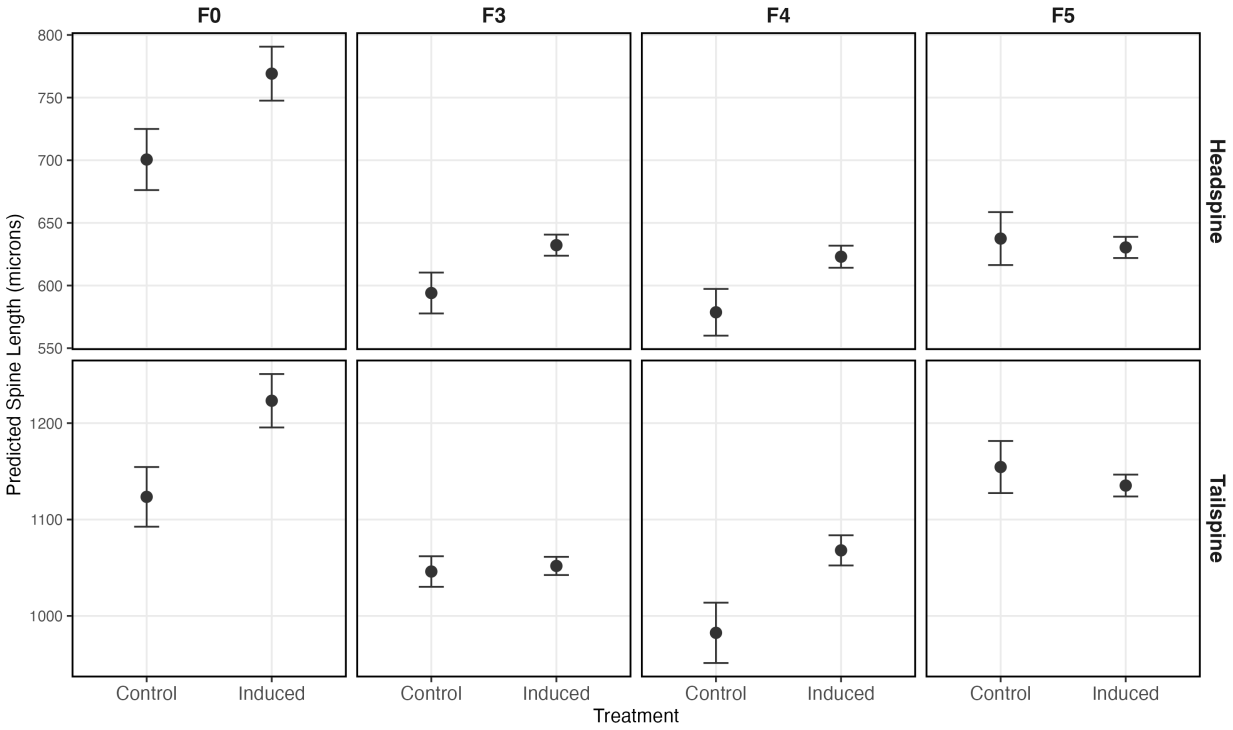


Figure 4. Predicted spine lengths by generation and treatment. Predicted mean headspine length (top row) and tailspine length (bottom row) for *Daphnia* in control and induced individuals across generations (F0, F3 ,F4, F5). Points represent model-predicted means ± standard error. Estimates are based on data from individuals ≤ 20 days old when defensive traits are most pronounced (Figure 3). See Table 2 for underlying estimates and parameters, and Supplemental Table 1 for model diagnostics.

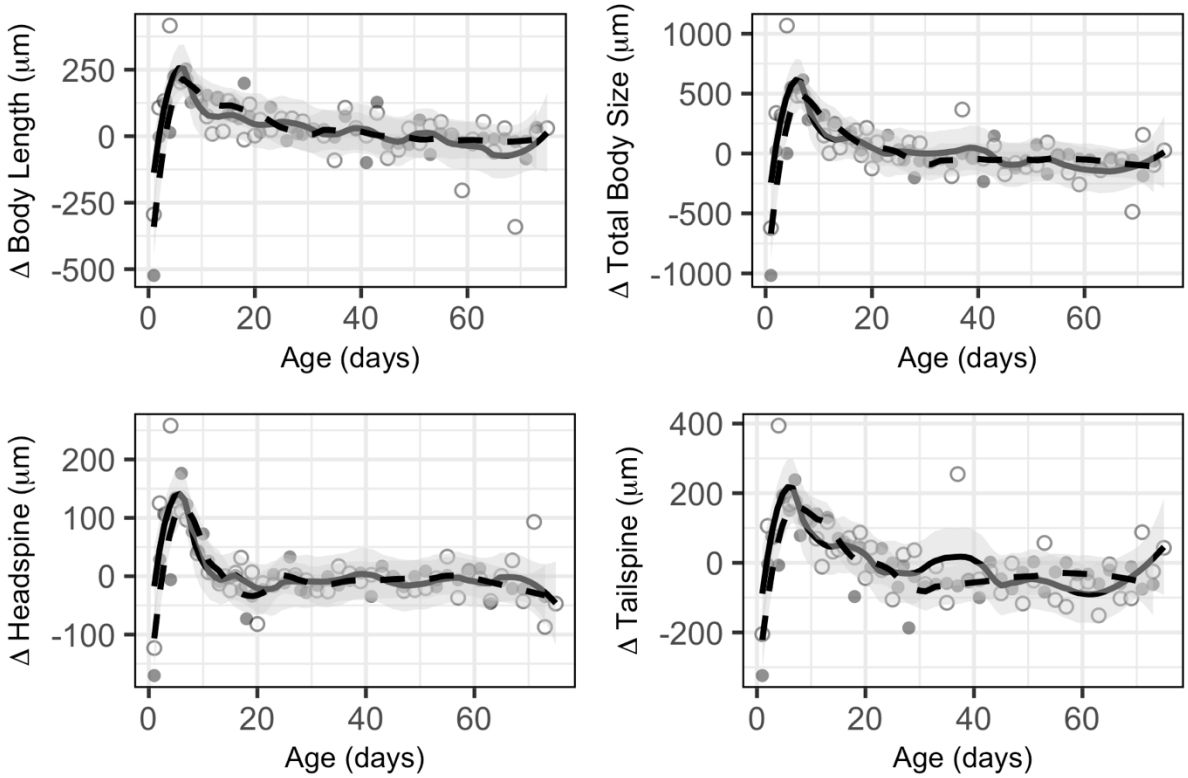


Figure 5. Growth of defensive and somatic traits are most pronounced early in life, followed by a stabilization with age, and were unaffected by treatment. Changes in morphological measurements (body length, total body size, headspine, and tailspine) across the lifespan (days). Each panel shows the mean 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). Induced/treated animals are represented by the solid line, and control animals are indicated by the dashed line.

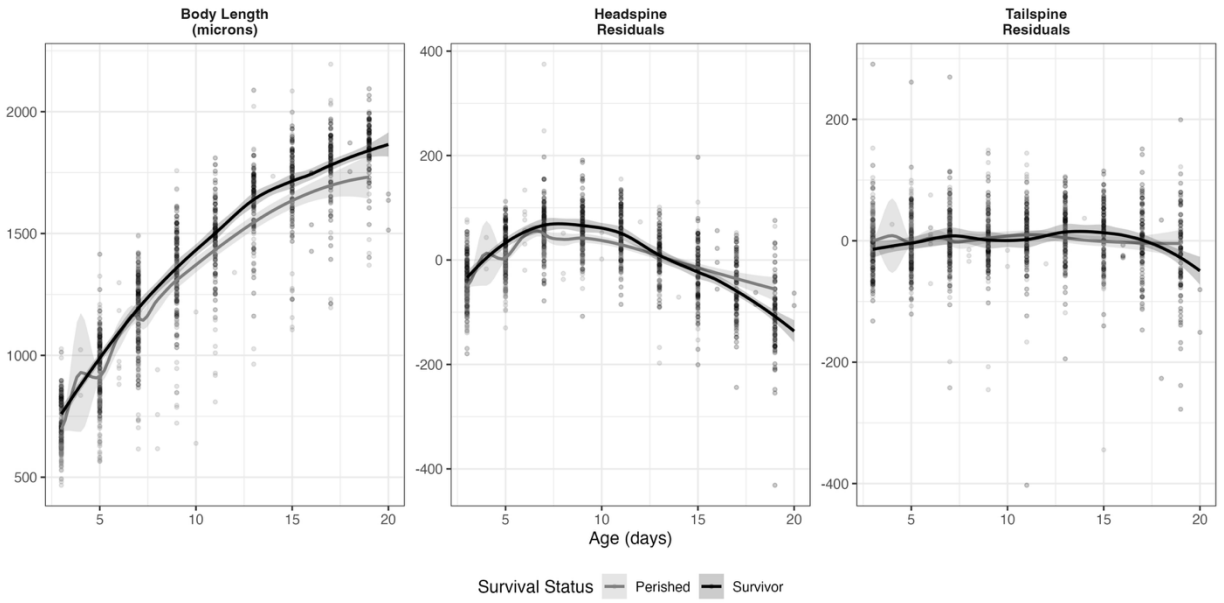


Figure 6. Developmental trajectories during periods of concentrated growth do not indicate treatment or size selective survivorship. Body length, head spine (residual), and tail spine (residual) measurements over first 20 days of age for individuals grouped by survival status (dark grey for those who survived to the next timepoint - survivors, light grey 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.

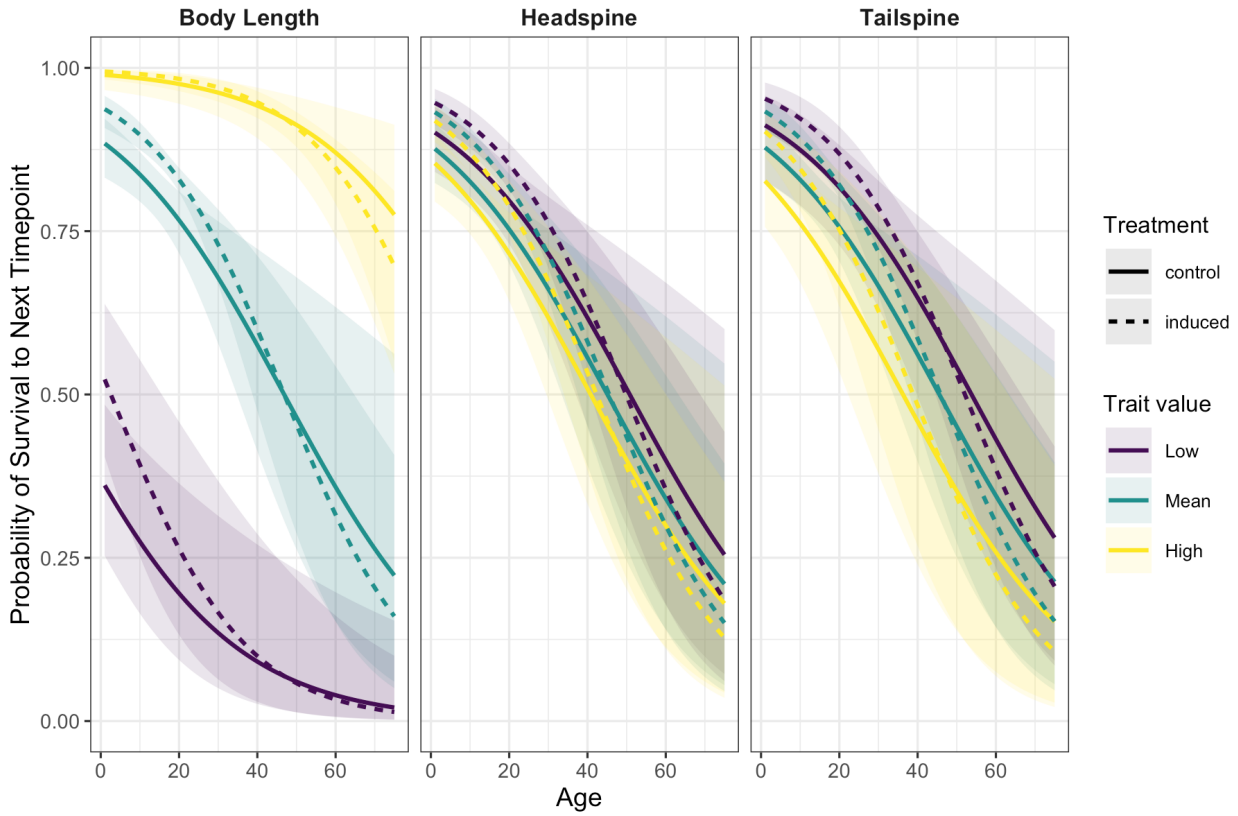


Figure 7. Survivorship and morphological trait distributions do not support size-selective mortality as a driver of morphological differences. Modeled probability of survival to the next timepoint as a function of age, body length, spine length, and treatment, based on a generalized linear model. Survival probability was predicted at 10th (low - purple), 50th (mean – blue), and 90th (high- yellow) percentiles of trait values, with dashed lines representing induced/treated animals and solid lines representing control. Body length values are associated with a greater likelihood of survival at most ages, whereas different spine lengths show minimal effect on predicted survival, regardless of treatment. 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.

SUPPLEMENTAL

Supplemental Table 1: Bayesian Model Diagnostics and Fit Summary.

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

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.

Supplemental Table 2: Linear estimates for headspine and tailspine length

Spine	Generation	Est. Difference (μm)	Std. Error	R^2 marginal	R^2 conditional	P value
Head Spine	F0	68.98	27.19	58.4%	59.7%	**< 0.05**
	F3	37.02	17.61	51.4%	65%	**< 0.05**
	F4	43.81	20.19	47.7%	65.9%	**< 0.05**
	F5	-5.79	21.41	50.7%	52.6%	0.789
Tail Spine	F0	114.73	31.93	93.4%	94.2%	**< 0.01**
	F3	8.19	18.28	95%	98.1%	0.655
	F4	85.45	34.14	90.3%	98.4%	**< 0.05**
	F5	-24.21	39.07	93.1%	96.6%	0.539

Linear mixed-effects model results for headspine and tailspine length across generations. Models were fit identically to Bayesian models for comparison. Treatment effects reach significance ($p < 0.05$) in F0, F3, and F4 but not F5 for headspine, and in F0 and F4 for tailspine, consistent with Bayesian results. See main text for Bayesian results.

Supplemental Table 3: Goodness-of-fit and diagnostic statistics for linear mixed-effects models.

Spine	Generation	Est. (µm)	Std. Error	t-value	Marginal R^2	Conditional R^2	AIC	BIC	Log-Likelihood
Headspine	F0	68.98	27.19	2.54	58.4%	59.7%	881.0	892.5	-435.5
	F3	37.02	17.61	2.10	51.4%	65%	3651.0	3669.8	-1820.5
	F4	43.81	20.19	2.17	47.7%	65.9%	3316.0	3334.3	-1653.0
	F5	-5.79	21.41	-0.27	50.7%	52.6%	2454.2	2470.9	-1222.1
Tailspine	F0	114.73	31.93	3.59	93.4%	94.2%	870.5	882.0	-430.3
	F3	8.19	18.28	0.45	95%	98.1%	3485.1	3503.9	-1737.6
	F4	85.45	34.14	2.50	90.3%	98.4%	3225.7	3244.0	-1607.9
	F5	-24.21	39.07	-0.62	93.1%	96.6%	2427.1	2443.8	-1208.5

Models were fit independently for each combination of experimental generation (F0, F3, F4, F5) and morphological trait (headspine, tailspine). The estimate represents the effect of treatment in µm. Std. error indicates the standard error, and the t-values measures the difference between the sample and population means. 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.

991 **Supplemental Table 4: Generation-specific growth rate analysis.**

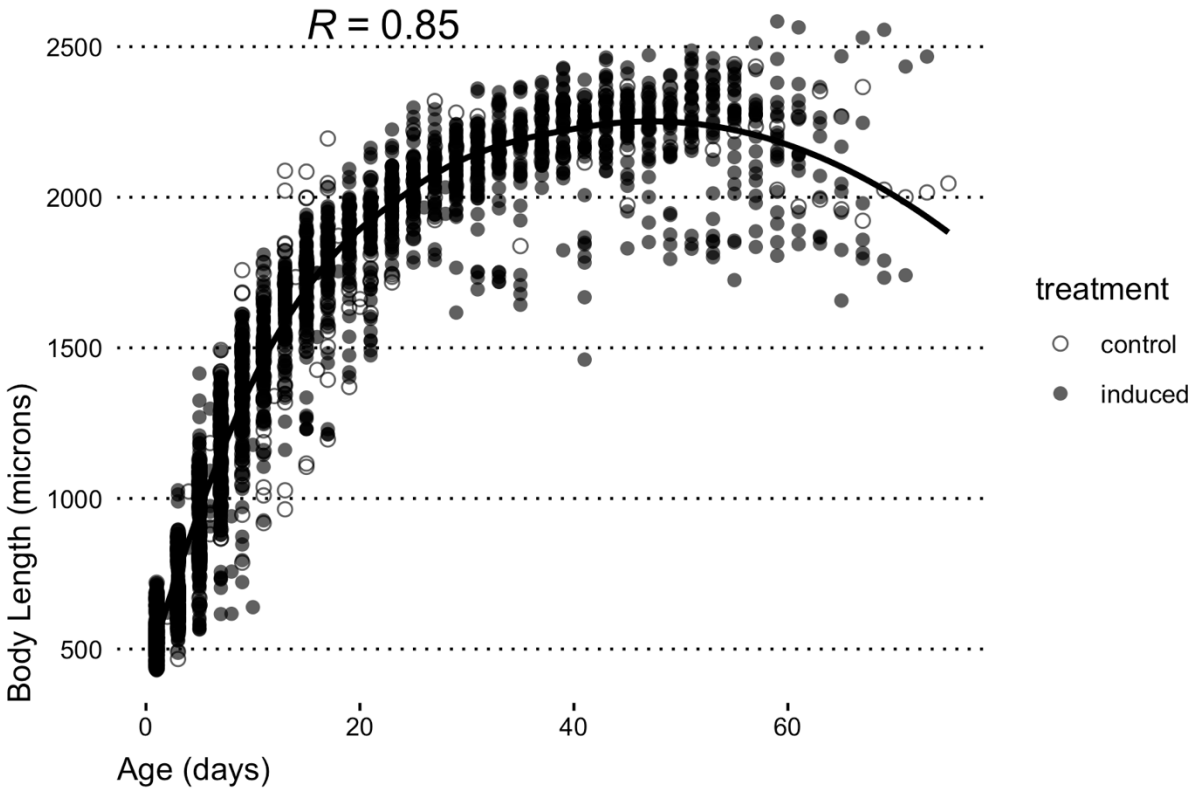
Generation	Trait	Estimate	SE	t value	P value
F0	Body Length	-25.592741	31.10285	-0.82284239	0.413
	Total Body Size	-7.091392	79.88130	-0.08877412	0.929
	Headspine	22.142802	22.23214	0.99598168	0.322
	Tailspine	3.423961	32.21077	0.10629862	0.916
F3	Body Length	16.060122	20.28191	0.79184464	0.429
	Total Body Size	56.924441	61.25339	0.92932721	0.361
	Headspine	-1.379195	16.10992	-0.08561156	0.932
	Tailspine	31.772759	18.21731	1.74409681	0.082
F4	Body Length	-16.610240	14.82382	-1.12051036	0.263
	Total Body Size	-39.774020	33.47196	-1.18827879	0.235
	Headspine	-9.445478	7.87350	-1.19965430	0.231
	Tailspine	-18.663554	14.63455	-1.27530766	0.203
F5	Body Length	-14.346394	27.29111	-0.52568011	0.599
	Total Body Size	-13.165803	66.40782	-0.19825680	0.843
	Headspine	-14.225783	13.31968	-1.06802748	0.286
	Tailspine	2.735027	25.45705	0.10743693	0.914

992 Mixed-effects models tested treatment effects on morphological growth rates (Δ from previous timepoint) separately
993 for each generation. Model: delta ~ treatment + age + (1|individual). Each row represents a unique combination of
994 generation and trait. Significant treatment effects ($p < 0.05$) in specific generations would indicate generation-
995 dependent differences in developmental strategy, however no significant differences were detected.
996
997

998 **Supplemental Table 5: Retrospective power analysis for F5 generation.**

	Effect Sizes (µm)			F5 Sample Sizes		Power Analysis			
Spine	**F3 Effect**	**F4 Effect**	**Avg Effect (µm)**	**F5 n Control**	**F5 n Induced**	**Cohen's d**	**Power**	**Needed n (80%)**	**Est. Power**
Head Spine	37.0	43.8	40.4	8	41	0.57	0.30	49	**29.8%**
Tail Spine	8.2	85.4	46.8	8	41	0.96	0.66	19	**66.4%**

999 Effect sizes for head and tail spine traits were averaged across F3 and F4 generations and used as benchmarks to
1000 estimate statistical power for detecting similar effects in F5 given actual sample sizes (n = 8 control, n = 41 induced).
1001 Cohen's *d* represents standardized effect size (treatment effect divided by pooled SD). Columns report observed
1002 effect sizes (µm), sample sizes, calculated power, and the estimated sample size required to achieve 80% power.



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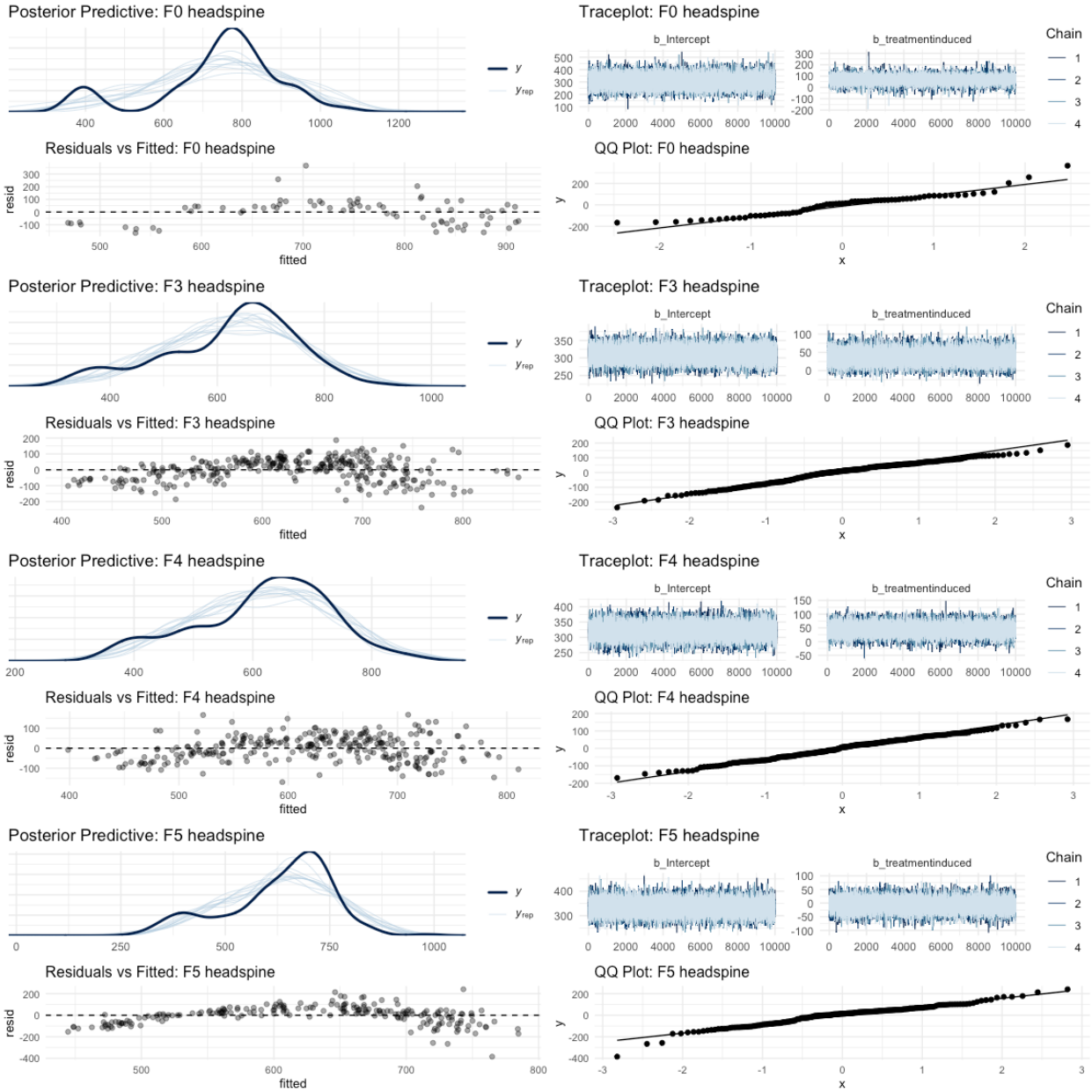
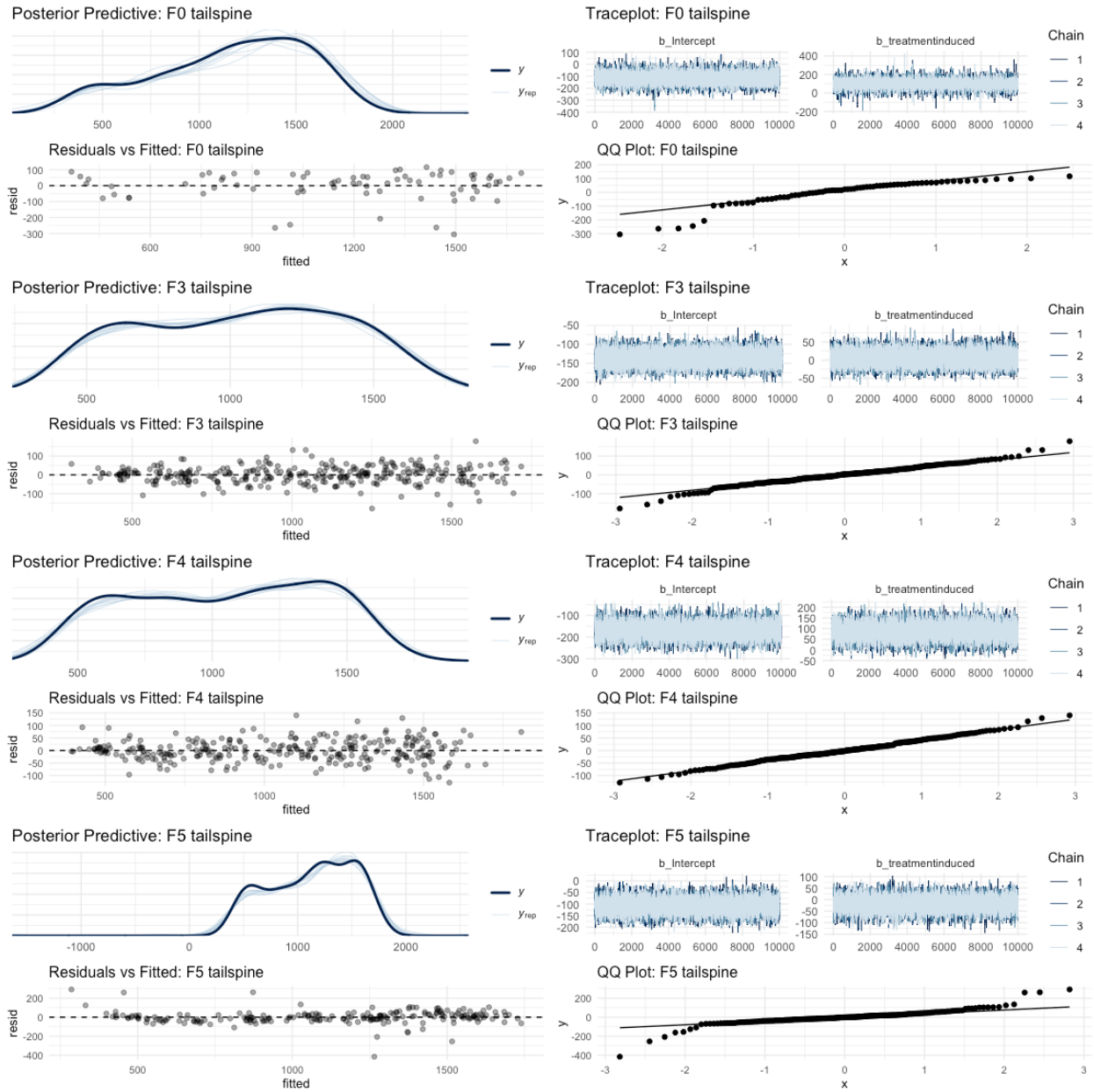
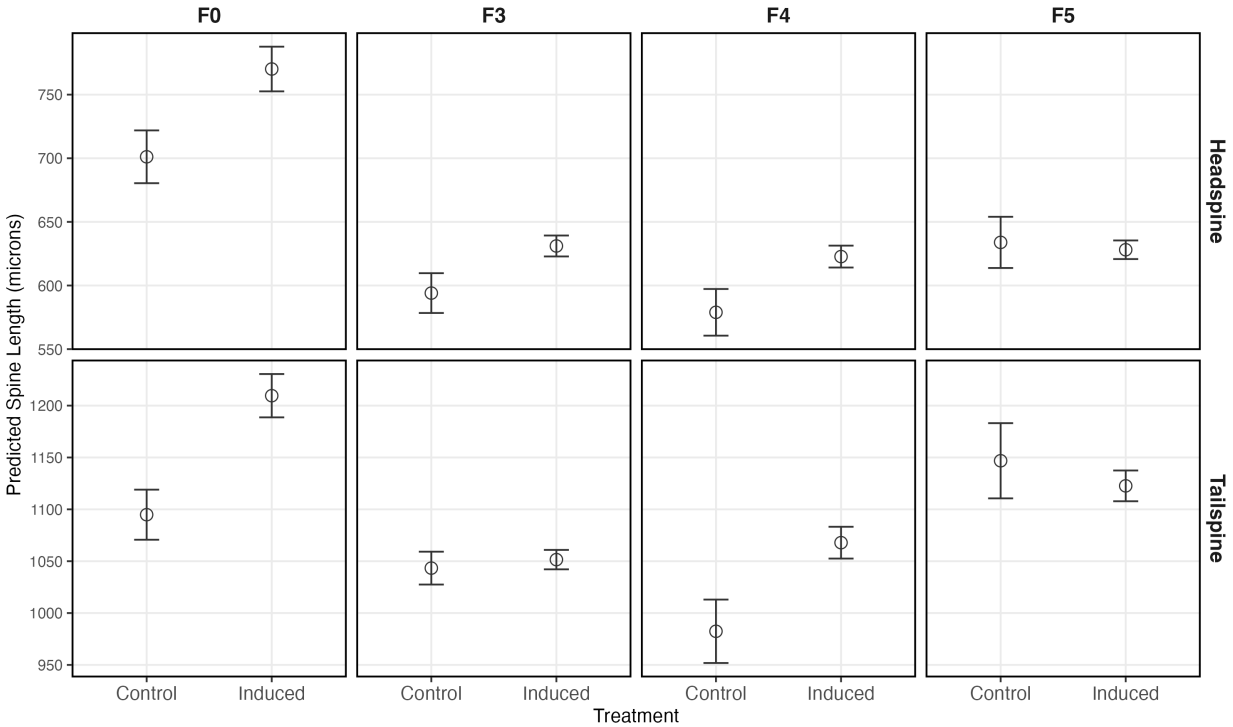
1005 **Supplemental Figure 1. Ontogenetic growth trajectories for Daphnia body length across**
1006 **treatments show high correlation between age and body length.** Scatterplot of individual
1007 body length measurements (µm) as a function of age (days) for control (open circles) and
1008 induced (filled circles) treatment across all generations. The solid line shows the overall fitted
1009 growth curve (Pearson's r =0.85) across all data. Growth patterns are similar between treatment,
1010 indicating that environmental induction principally affects defensive traits rather than somatic
1011 traits in this clone.
1012



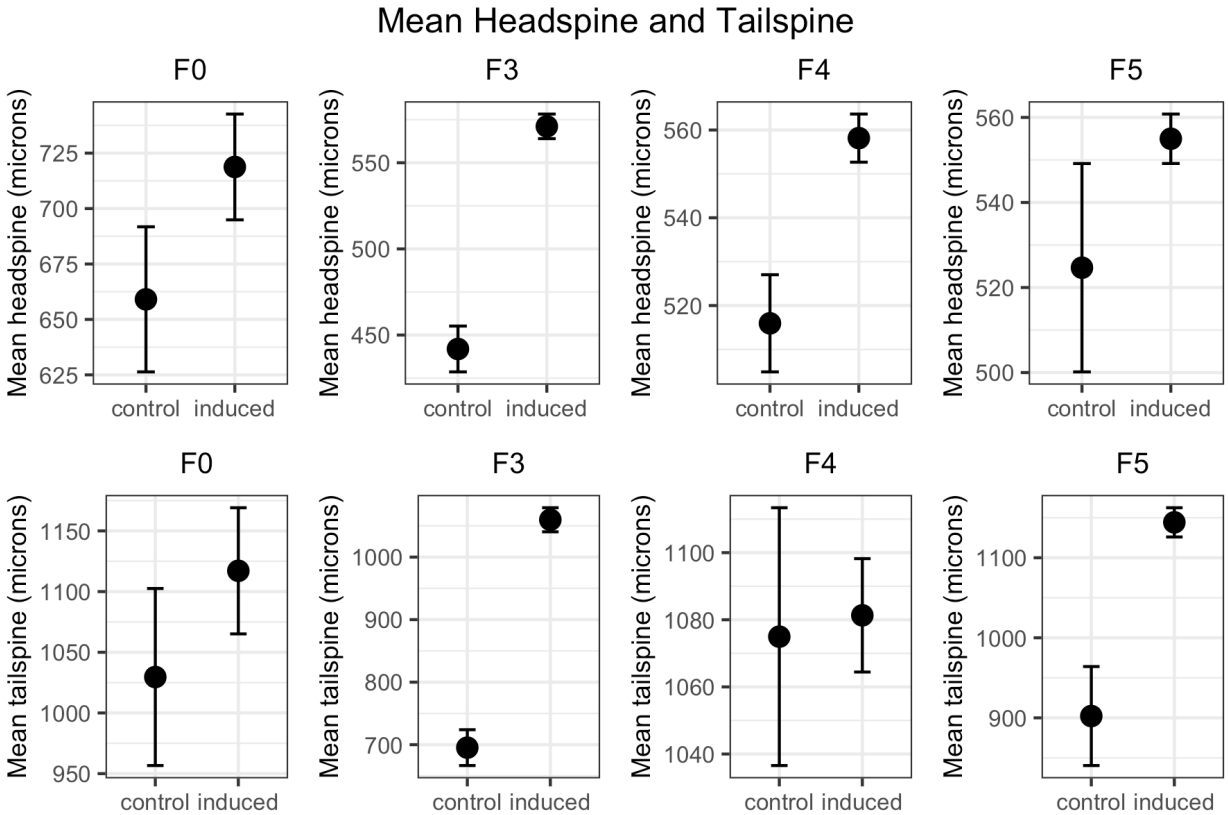
Supplemental Figure 2: **Bayesian model diagnostic plots for the headspine trait across generations.** Each row represents an independent model. The panels display posterior predictive checks (model fit), residuals vs. fitted values (homoscedasticity), MCMC traceplots (convergence), and Quantile-Quantile (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.



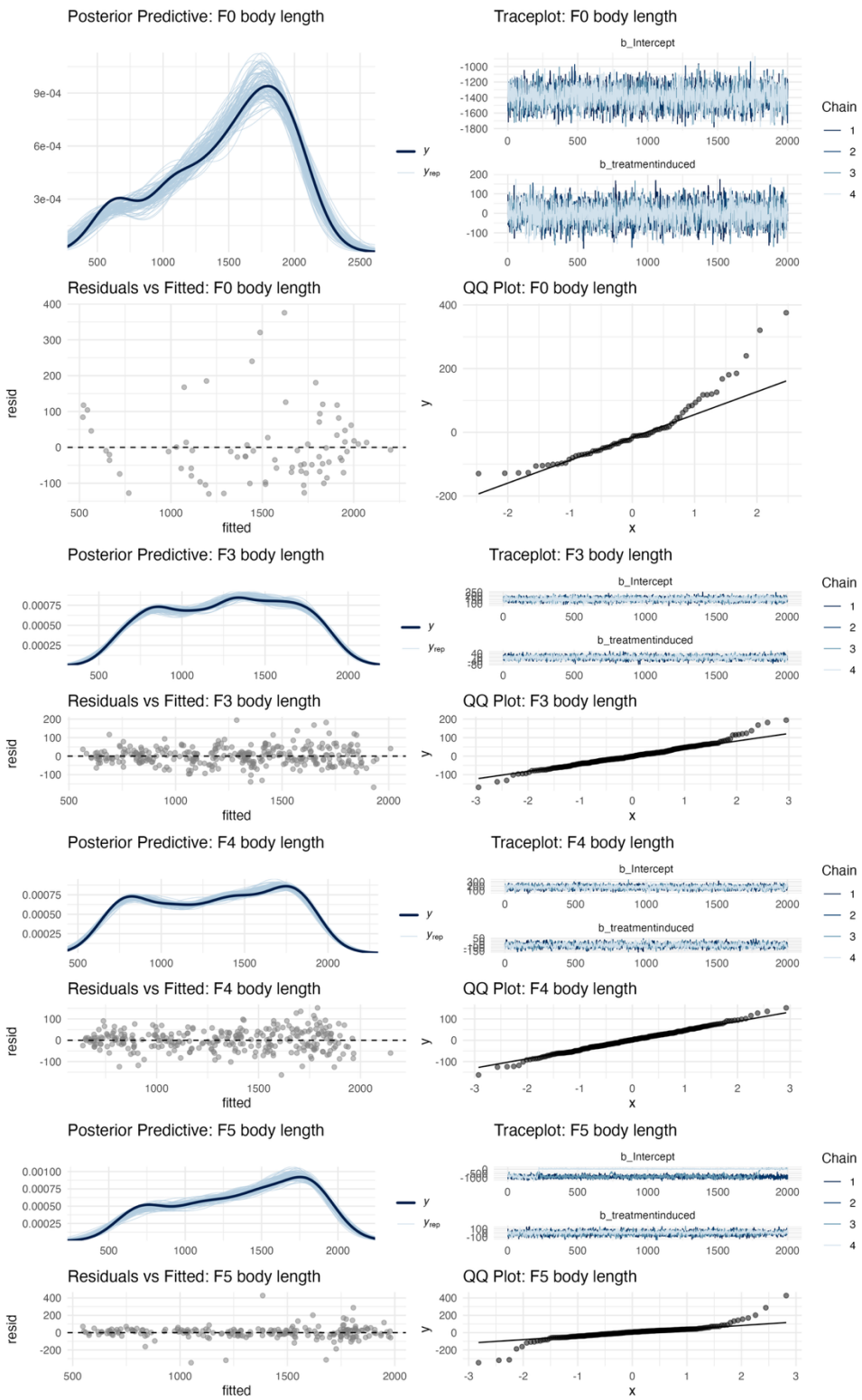
Supplemental Figure 3: Bayesian model diagnostic plots for the tailspine trait across generations. Each row represents an independent model. Plots include posterior predictive checks (model fit), residuals vs. fitted values (homoscedasticity), MCMC traceplots (convergence), and Quantile-Quantile (QQ) 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.



Supplemental Figure 4: Predicted spine lengths by generation and treatment from linear mixed-effects models. Predicted mean headspine length (top row) and tailspine length (bottom row) for Daphnia in control and induced individuals across generations (F0, F3, F4, F5). Points represent model-predicted means ± standard error from linear mixed-effects models.



Supplemental Figure 5. 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 individuals across four non-exposed generations (F3–F5) and the originally exposed generation (F0). Each panel displays pairwise comparisons between control and induced 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. These data illustrate the finite and dynamic pattern of transgenerational plasticity in response to environmental cues. Note: this data is included for transparency, but does not include correction for body sizes, and violates independence assumptions as there are repeated measures.



Supplemental Figure 6: Model diagnostics for body length Bayesian models across generations. For each generation: posterior predictive check (top left), MCMC traceplots (top right), residuals vs fitted (bottom left), and QQ plot (bottom right).

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