ENVIRONMENTAL SENSITIVITY AND PLASTIC MORPHOLOGICAL DEFENSES IN *DAPHNIA LUMHOLTZI*

by

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Primary Thesis Advisor

Understanding the plasticity of morphological defenses in *Daphnia lumholtzi* in response to predator chemical cues is crucial for interpreting the adaptive strategies of this organism and its role as a model for studying transgenerational epigenetic inheritance. This study focuses on the sensitivity of embryos reared in the mother's brood chamber and examines how morphological defense plasticity is controlled by inheritance through epigenetic mechanisms or induced by environmental cues during embryonic development.

Our research demonstrates novel findings in which predation responses occur due to environmental signaling across variable times in embryonic development indicating a robust and flexible response mechanism to environmental stressors. Interestingly, our research also found no innate ability of developing embryos to respond to the same environmental signals during in-vitro rearing.

By exploring these mechanisms, our study contributes to a deeper understanding of how predator cues influence morphological defenses across generations in Daphnia lumholtzi. Further investigation is needed to unravel the molecular pathways involved and to confirm these preliminary findings, paving the way for more comprehensive studies on the epigenetic regulation of adaptive traits.

Acknowledgements

I would like to sincerely thank my graduate student mentor within the Cresko Lab, Shannon Snyder. Without her expertise, mentorship, and care for my personal and academic development, none of this research would be possible. My growth as a student and scientist has relied on her efforts for which I am extremely grateful. Working closely with Shannon Snyder has fostered my curiosity and passion for science in a way that no other mentor has.

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Chapter 1: Introduction

Daphnia lumholtzi as a Model Organism for Transgenerational Epigenetic Inheritance

Daphnia lumholtzi, a species of small planktonic crustacean, has emerged as a significant model organism for studying epigenetic inheritance and phenotypic plasticity. Its unique reproductive strategy, which includes cyclical parthenogenesis and the ability to produce clonal offspring, allows researchers to control genetic variability and focus on environmental and epigenetic factors influencing trait development. The ability of *Daphnia lumholtzi* to rapidly adapt to environmental changes makes it an ideal subject for investigating how organisms respond to external stressors and transmit adaptive traits across generations.

Daphnia lumholtzi exhibits a life cycle characterized by both asexual and sexual reproduction. During favorable conditions, females produce clonal offspring through amictic parthenogenesis, leading to rapid population growth. In response to environmental stressors, such as overcrowding or unfavorable temperatures, *Daphnia* switch to sexual reproduction, producing haploid eggs that can survive harsh conditions. This reproductive flexibility, combined with their ability to develop distinct morphological defenses like elongated head and tail spines, makes *Daphnia lumholtzi* an excellent model for studying phenotypic plasticity and adaptive responses.

One of the most notable adaptive responses in *Daphnia lumholtzi* is their sensitivity to predator kairomones. Chemical cues released by predators, such as stickleback fish, can induce significant morphological changes in Daphnia. These changes, primarily the elongation of head and tail spines, enhance their survival by making them less susceptible to predation (Kolar et al., 1998). This ability to detect and respond to environmental signals in the absense of genetic variation highlights the sophisticated mechanisms underlying Daphnia's phenotypic plasticity.

The plasticity of spine lengths in *Daphnia lumholtzi* has been extensively documented, particularly through the work conducted at the Cresko lab and by doctoral student Shannon Snyder. Their research has demonstrated that exposure to predator kairomones can induce spine elongation in both maternal and offspring generations, suggesting a complex interaction between direct environmental conditioning and inherited epigenetic modifications. These findings underscore the importance of understanding the mechanisms driving morphological responses and their potential implications for adaptive evolution.

Within the anatomy of *D. lumholtzi*, a few characteristics are important as well for the following experimental methods. As seen in *Figure 1,* inside the carapace a large portion of a female Daphnia's body contains the brood chamber where embryos are reared. Eggs are initially formed from germline cells in the ovaries and soon moved to the brood chamber. Eggs are first deposited with an egg casing, but as embryos develop, they shed the casing and become close to fully formed inside the brood chamber. Once fully developed, the Daphnia neonates will be released from the brood chamber fully formed and swimming.

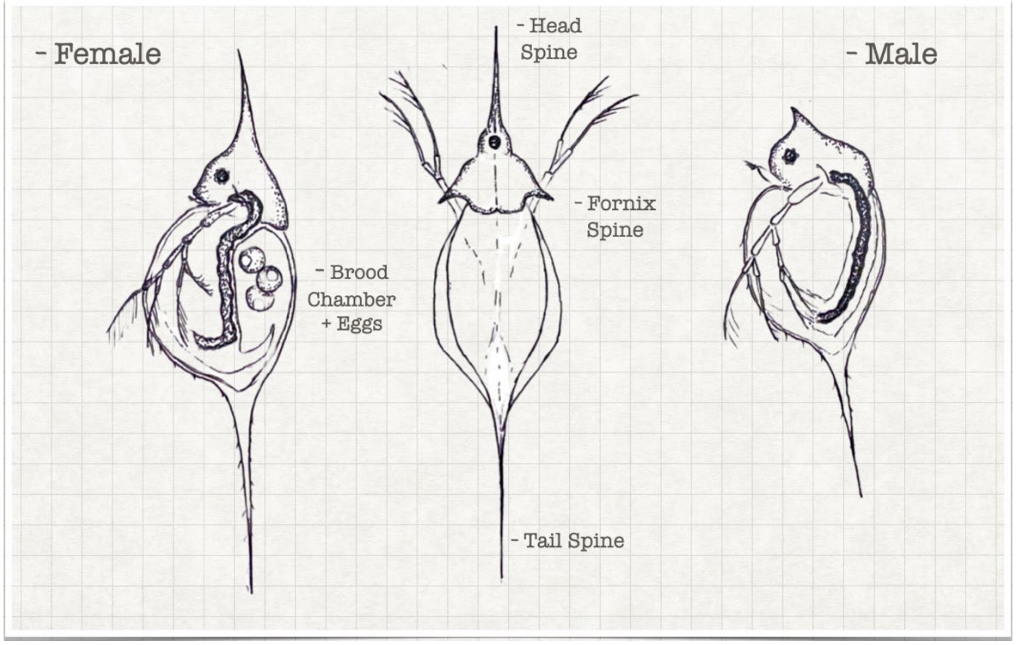

Figure 1.1) *Daphnia lumholtzi* Anatomy

Female and male D. lumholtzi shown from dorsal and side profile. In the side profile of female Daphnia, the brood chamber housing a typical 3 round embryos inside is a key feature. From a dorsal profile, head, tail, and fornix spines can all be seen and are key plastic phenotypes involved in predation response. Male daphnia are seen with considerable smaller spines and the lack of a brood chamber.

Transgenerational Epigenetic Inheritance in *Daphnia lumholtzi*

Epigenetics encompasses the study of heritable changes in gene expression that do not involve alterations in the DNA sequence. This includes both mitotic and meiotic epigenetic inheritance, wherein changes in gene expression patterns are passed onto daughter cells or offspring, respectively, without changes to the underlying DNA sequence. Transgenerational eipgenetic inhertiance (TGEI) considers the possibility that epigenetic adaptations in gametes could confer non-genetic information through meiosis across multiple generations.

Evidence from diverse organisms, ranging from lower eukaryotes such as yeast (Tang et al., 2012) and nematodes (Werner et al., 2023) to higher-order eukaryotes such as mammals

(Feinberg, 2007), highlights the prevalence and importance of epigenetics and increasingly builds a case for TGEI. For instance, studies in the genus *Daphnia* have demonstrated phenotypic plasticity inherited across generations upon environmental triggers . In nematodes, heritable changes in gene expression mediated by small RNAs have been documented as possible drivers of traits exhibiting TGEI (CITE). Current evidence suggests these processes occur even in mammals, including humans, where environmental factors can lead to heritable epigenetic changes with implications for health and disease (Feinberg, 2007).

Debate Over TGEI Versus Maternal Rearing Effects

The concept of TGEI has sparked considerable debate within the scientific community. While some studies provide compelling evidence for the epigenetic inheritance of traits, others question the robustness and reproducibility of these findings. In *Daphnia lumholtzi*, research has shown that both maternal rearing effects and direct environmental exposures can influence the expression of adaptive traits such as spine elongation (Graeve, et al 2021). The challenge lies in distinguishing between these influences and understanding how each contributes to phenotypic outcomes.

For instance, Agrawal et al. (1999) found that Daphnia mothers exposed to fish kairomones produced offspring with enhanced defensive traits, suggesting a significant role for maternal effects. However, other studies, such as those conducted by Walsh et al. (2016), provided evidence for TGEI, where offspring of mothers exposed to predator cues exhibited similar traits even without direct exposure. These conflicting findings underscore the need for more rigorous experimental designs to isolate and understand the contributions of maternal rearing, environmental conditioning, and epigenetic inheritance.

To unravel the complexities of phenotypic plasticity in *Daphnia lumholtzi*, it is crucial to discern the relative influences of embryonic environmental sensitivity, maternal rearing effects, brood chamber conditioning, and TGEI. By employing controlled experimental designs that isolate specific variables, researchers can better understand how these factors interact and contribute to the development and inheritance of adaptive traits.

Objectives of Thesis Experiments

The objectives of this research are to unravel the complexities of phenotypic plasticity and epigenetic inheritance in *Daphnia lumholtzi*, focusing on the influences of embryonic environmental sensitivity, maternal rearing effects, brood chamber conditioning, and true trans-generational epigenetic inheritance. By leveraging *Daphnia lumholtzi* as a model organism, these experiments aim to elucidate the genetic and epigenetic mechanisms that facilitate the development and inheritance of adaptive traits in response to environmental cues.

Experiment 1

The primary objective of Experiment 1 is to compare the relative contributions of maternal rearing effects and direct embryonic conditioning by analyzing the phenotypic outcomes of early and late-stage kairomone inductions. *Daphnia lumholtzi*'s ability to exhibit inducible defenses in response to predator cues suggests a complex interaction between direct environmental conditioning and maternal influences. By separating the timing of kairomone exposure into early-stage (pre-embryonic) and late-stage (post-embryonic) inductions, this experiment aims to clarify how maternal rearing and brood chamber conditions contribute to the expression of adaptive traits.

To achieve this, three experimental groups were established: early-stage kairomone induction, late-stage kairomone induction, and continuous kairomone exposure. In the early-stage induction group, maternal *Daphnia* were exposed to kairomones for the first seven days of life, before embryogenesis, to assess potential epigenetic inheritance or maternal rearing effects without direct embryonic exposure. In the late-stage induction group, maternal Daphnia were exposed to kairomones from days 8-14 of life, coinciding with embryonic development within the brood chamber, to isolate the effects of direct kairomone exposure on embryos while in the brood chamber. The continuous exposure group will expose both F0 (parental) and F1 (offspring) generations to kairomones throughout the entire experimental period to assess the cumulative effect of sustained environmental stressors.

Morphological changes, specifically head and tail spine lengths, were measured and compared in the offspring of each experimental group at multiple developmental stages (e.g., 72 and 168 hours post-dissection). The data were analyzed to determine the relative importance of maternal rearing, direct embryonic conditioning, and sustained environmental exposure in shaping adaptive traits.

Experiment 2

The objective of Experiment 2 is to isolate and examine the direct effects of predator kairomones on the embryonic development of *Daphnia lumholtzi*, focusing on the embryos' susceptibility to environmental cues without maternal influence. Preliminary findings have demonstrated that *Daphnia lumholtzi* embryos can be successfully dissected from the maternal brood chamber and reared in a controlled environment. This novel method allows for precise assessment of the embryos' response to predator-induced chemical cues, independent of maternal rearing effects. By exposing isolated embryos to kairomones, this experiment seeks to determine the extent to which these environmental signals induce morphological defenses, such as the elongation of head and tail spines.

In this approach, early-stage embryos will be dissected from the maternal brood chamber and reared in controlled environments, with and without exposure to predator kairomones. Morphological changes, particularly head and tail spine lengths, will be measured and analyzed to assess the direct impact of kairomone exposure on embryonic development. Additionally, differential gene expression in response to kairomone exposure will be documented to identify the genetic factors involved in the induction of defensive phenotypes.

Understanding the interplay between embryonic environmental sensitivity, maternal rearing effects, brood chamber conditioning, and TGEI is crucial for unraveling the complexities of phenotypic plasticity and inheritance in *Daphnia lumholtzi*. These experiments will provide valuable insights into the genetic and epigenetic mechanisms underlying adaptive trait expression, solidifying *Daphnia lumholtzi*'s status as an emerging model organism in the field of epigenetics. By documenting differential gene expression and morphological changes in response to predator cues, this research aims to advance our understanding of how environmental factors influence the evolution and inheritance of adaptive traits across generations.

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Chapter 2: Transgenerational Sensitivity to Variation in Kairomone Induction Timing

Transgenerational epigenetic inheritance and maternal rearing effects are both mechanisms through which traits can be passed down from one generation to the next, but they operate through different mechanisms and have different scopes of influence.

Transgenerational epigenetic inheritance involves the transmission of epigenetic modifications from parents to offspring, which can influence gene expression and phenotype without altering the DNA sequence. Epigenetic modifications, such as DNA methylation and histone modifications, can be influenced by environmental factors experienced by the parents and can persist across generations. This means that traits influenced by transgenerational epigenetic inheritance can be inherited even if the offspring themselves were not directly exposed to the environmental factors that induced the epigenetic changes in their parents. For example, studies have suggested that exposure to certain environmental stressors or dietary factors in one generation can lead to changes in DNA methylation patterns that are passed down to subsequent generations, influencing traits such as behavior, metabolism, and disease susceptibility.

Maternal rearing effects, on the other hand, refer to the influence of maternal care and nurturing behaviors on offspring development and phenotype. Maternal behaviors, such as grooming, nursing, and licking, can have profound effects on offspring physiology, behavior, and stress responses. These effects are often mediated through alterations in the offspring's neuroendocrine system, including changes in hormone levels and neurotransmitter activity. Maternal rearing effects are typically observed within the same generation and are dependent on the quality of maternal care received by the offspring. Offspring that receive high levels of maternal care tend to exhibit lower stress reactivity, better cognitive function, and enhanced social behavior compared to offspring that receive low levels of maternal care.

While transgenerational epigenetic inheritance and maternal rearing effects are distinct mechanisms, they can also interact and overlap in their effects on trait expression. For example, maternal behaviors and environmental exposures experienced by the mother can influence epigenetic modifications in her offspring, thereby affecting trait expression across generations. Additionally, maternal care can modulate the effects of transgenerational epigenetic inheritance by influencing the offspring's response to environmental stressors and their ability to cope with epigenetically inherited traits.

In summary, transgenerational epigenetic inheritance involves the transmission of epigenetic modifications across generations, while maternal rearing effects refer to the influence of maternal care on offspring development within the same generation. While these mechanisms operate through different pathways, they can interact and overlap to influence trait expression in offspring.

Experimental Design

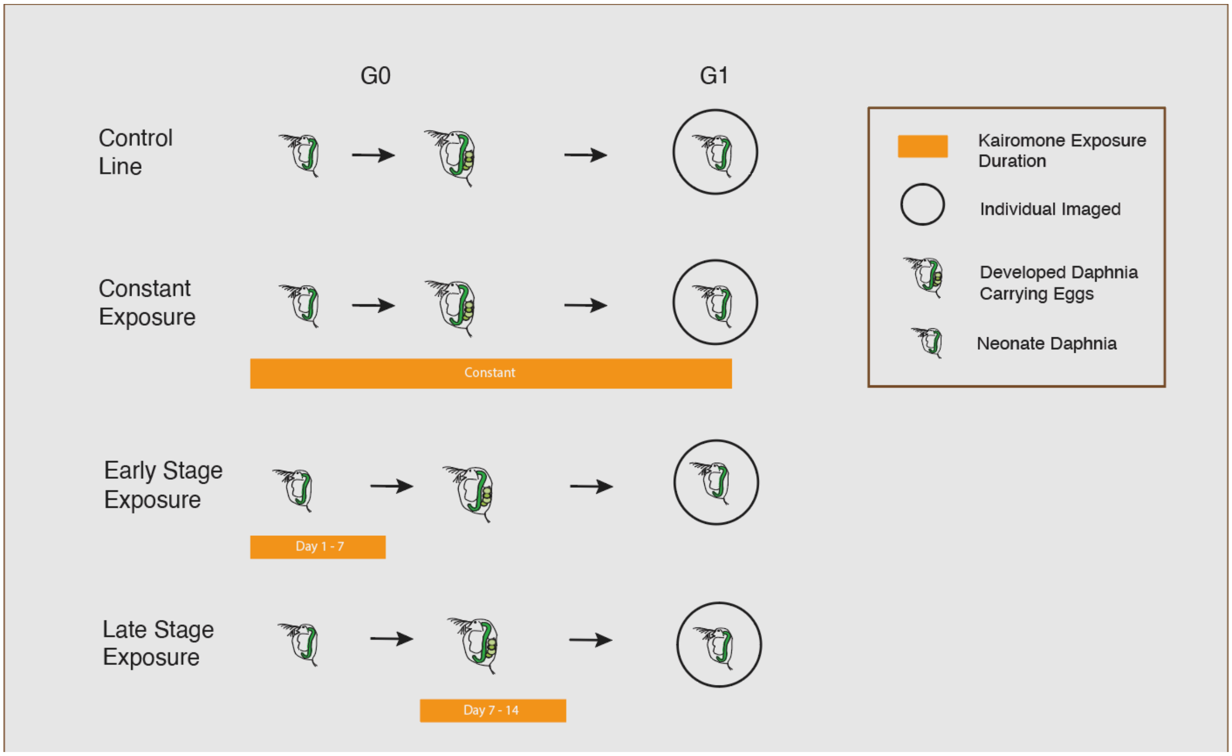


Figure 2.1) Experimental Design

Experimental schematic showing kairomone exposure timelines throughout development and reproduction of two generations of Daphnia. Orange bars indicate duration of kairomone exposure at days since birth of G0 (maternal) Daphnia. G1 daphnia in circles indicate the second-generation daphnia in which phenotypic data was taken. Treatments contained the following samples sizes respectively: n = 16, n = 18, n = 12, n =23.

When analyzing trans-generational trait expression as *Daphnia lumholtzi*, it is important to have a comprehensive understanding of how and when maternal rearing effects can muddy phenotypic analysis of epigenetically controlled traits. To achieve this, the following experiment was designed to tease apart the most environmentally sensitive developmental time frames in D. lumholtzi embryos.

During embryonic development, embryos are reared from the single cell stage to neonatal form inside the mother's brood chamber (refer to life cycle in introduction). During this embryonic development, either epigenetic markers inherited from the mother or environmental and maternal conditioning have the potential to affect adult trait expression. Here, we tested for susceptibility of the defensive spine growth phenotype to various treatments of kairomone induced media representative of chemical cues associated with predation pressure. As previously documented in experiments from Shannon Snyder's work in the Cresko lab, D. lumholtzi individuals experiencing two generations of kairomone exposure will exhibit longer head and tail spines than control. Along with repeating these exposure lines, this experiment added three new test groups: an early stage induction, late stage induction, and constant induction.

Early Stage Induction: Early stage induction established a line induced with kairomone for the first seven days of a maternal daphnia's life, "priming" the mother with environmentally induced adaptations with the potential for non-genetic inheritance. The seven-day timeline for maternal kairomone induction was established as a stage of adulthood without the opportunity for fully formed embryos to exist within the brood chamber. By ending kairomone induction before this seven-day mark, not all embryos in the "early stage induction" line could be directly exposed to kairomone chemicals inside the brood chamber or in neonatal form. In this test line, effects of kairomone treatment on trait expression can be hypothesized to come from epigenetic inheritance or kairomone-dependent maternal rearing effects. Without kairomone present through brood chamber development, no direct signaling from kairomone to embryos through post-deposition development could occur.

Late Stage induction: Late stage induction established a line induced with kairomone from days 8-14 of a maternal daphnia's life. This timeframe coincides with embryonic development within the brood chamber. During these seven days after embryos are deposited into the brood chamber, embryos begin as single-cell embryos surrounded by a chorion casing and progress through independently and morphologically developed daphnia before being released from the maternal brood chamber into the outside environment. This timeframe was chosen to eliminate inheritance of induced trait expression during embryogenesis and isolate possible maternal rearing effects and direct kairomone exposure to embryos within the brood chamber environment.

Constant Induction: In addition to separating kairomone induction between pre and post brood chamber rearing, a third experimental line was added to receive maximum kairomone exposure and consequently maximum expected trait expression across two generations. In this line, both G0 and G1 were reared in kairomone media throughout the full length of experimentation.

After isolating early stage induction, late stage induction, constant kairomone, and control lines through G0 daphnia, treatments were followed through accordingly to G1. Once G1 neonates were isolated, all lines were raised through adulthood in respective environments. At Day 4 of adult life, G1 individuals were imaged to capture three data points from each Daphnia: head spine, tail spine, and body length. After imaging at day 4, individuals were reared for an additional four days in their respective environments and imaged again at day 8 capturing a parallel dataset at a later stage of adulthood. Both day 4 and day 8 datasets were created as trait expression differences have been documented to exist across adult life in *Daphnia lumholtzi* (Shannon snyder).

This experiment was first run as a pilot experiment with 2 first generation daphnia in each group. After initial phenotype analysis, power estimates and sample size approximation for the larger experiment. Figure 2.2 shows that as the sample size increases, the test power also increases, reaching a plateau at around 20 samples per group. The blue dashed line indicates the optimal sample size needed to achieve a power of approximately 80%, which is commonly considered adequate for detecting a statistically significant effect. Based on the graph, a sample size of 15 Daphnia in each second-generation test group is recommended for the final larger experiment to ensure sufficient power. With this in mind, we chose to move forward with the full experiment with the goal of 15 to 20 second generation daphnia in each test group.

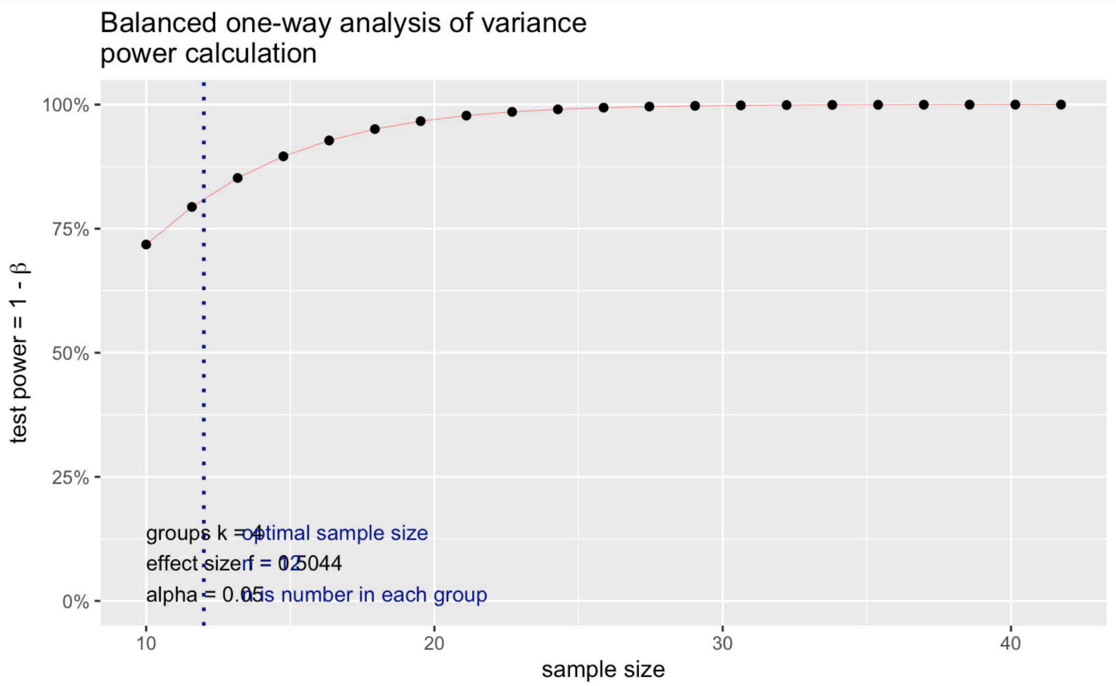


Figure 2.2) Balanced One-Way Analysis of Variance Power Analysis

The graph displays a power analysis conducted to determine the appropriate sample size for a balanced one-way ANOVA in a larger experiment. The y-axis represents the test power (1 - β), while the x-axis shows the sample size per group. The analysis was based on data from a small pilot experiment with the following parameters: four groups (k = 4), an effect size (f) of 0.5044, and an alpha level (α) of 0.05.

Results

The results of Thesis Experiment 1 provide insights into the phenotypic plasticity of *Daphnia lumholtzi* in response to predator kairomones, focusing on head and tail spine lengths across different treatment groups: control, early stage induction, late stage induction, and constant kairomone exposure.

To interpret phenotypic data, a linear model was fit to individual data sets with body length measurements and treatment groups as explanatory variables for linear trends in both head and tail spines. This model's explanatory power gives confidence in this approach with an R^2 of

0.92. With these linear models, ANCOVA interpreted the effects of treatment groups taking into account the interactions of the predictive covariate body length.

At Day 4, the head spine response in the control group was the lowest among all groups, indicating minimal morphological changes without kairomone exposure. In contrast, the early stage induction group showed a slight increase in head spine length compared to the control group, suggesting that initial maternal exposure to kairomones had a modest effect. The late stage induction group exhibited head spine lengths similar to those of the early stage group, indicating that the timing of kairomone exposure, whether during maternal or embryonic stages, did not significantly differ in their effects on head spine elongation. The constant kairomone exposure group had the highest head spine length, suggesting that continuous exposure to kairomones had a cumulative effect on head spine elongation.

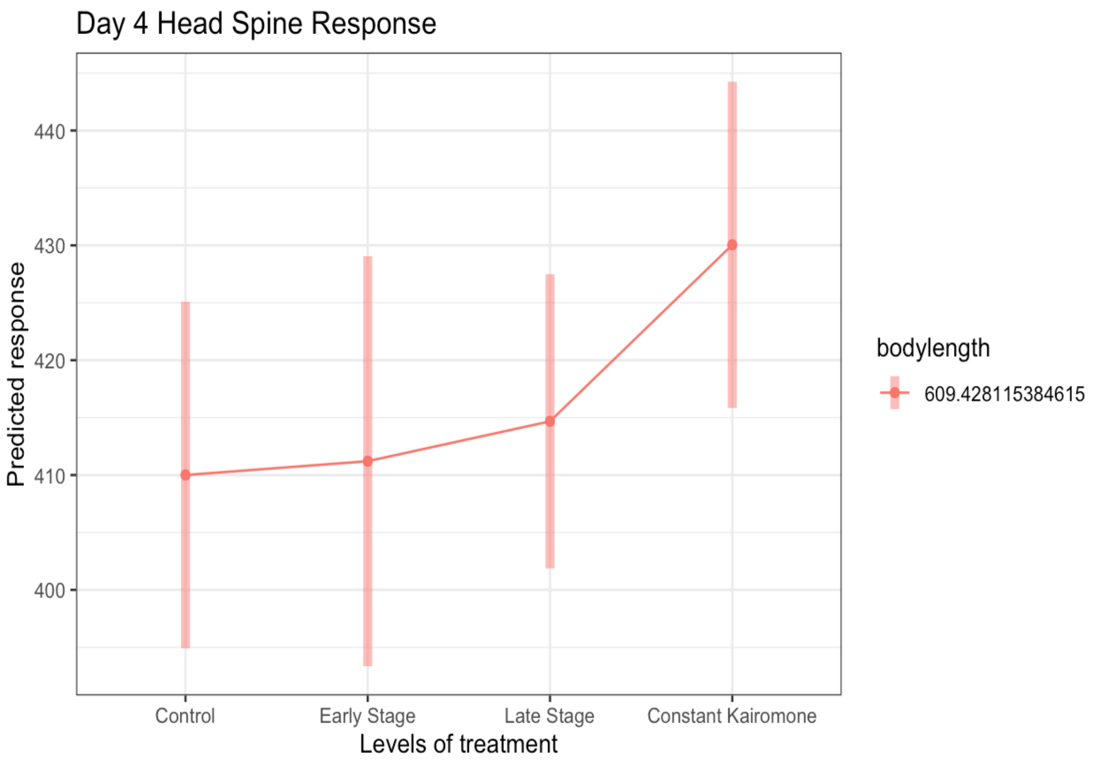


Figure 2.3) Head spine Response at Day 4

The graph illustrates the predicted head spine lengths of *Daphnia lumholtzi* at Day 4 across four treatment groups: Control, Early Stage, Late Stage, and Constant Kairomone exposure. The predicated head spine response shows the estimated mean for the dataset based on a fitted linear model. ANCOVA results show significant effects of treatment ($F(3, 73) = 10.562$, $p < 0.001$) and body length ($F(1, 73) = 107.897$, $p < 0.001$) on head spine length. Constant kairomone exposure resulted in the highest head spine length, while early and late-stage inductions had similar lengths, both slightly higher than the control group, which exhibited the lowest head spine length. Pairwise comparisons revealed no statistically significant differences between specific treatment groups ($p > 0.05$ for all comparisons). These results suggest that constant kairomone exposure tends to increase head spine length the most, while early and late-stage inductions have comparable effects.

The figure and statistical analysis suggest that while there is a trend for increased head spine length with constant kairomone exposure, the differences between the treatment groups are not statistically significant after adjusting for multiple comparisons using Tukey's method. The significant effects of body length and treatment in the ANCOVA indicate that both factors contribute to the observed variation in head spine lengths, though the pairwise comparisons reveal that the specific differences between treatment levels are not robust.

After conducting a ANOVA analysis on the significance of the effect of an interaction term between treatment and body length on head spine length, this interaction was proven to be significant and thus included in the ANCOVA results show in figure 2.3.

By Day 8, the trends observed at Day 4 persisted. The control group continued to have the lowest head spine length, showing minimal changes in the absence of kairomone exposure. Both early and late-stage inductions resulted in head spine lengths that were slightly above the control group, indicating that maternal priming and embryonic conditioning contributed similarly to head spine trait expression. The constant kairomone exposure group again exhibited the longest head spines, reinforcing the notion that continuous exposure to predator cues led to the strongest morphological response.

After conducting a ANOVA analysis on the significance of the effect of an interaction term between treatment and body length on head spine length, this interaction was proven to be insignificant and thus excluded from the ANCOVA results show in figure 2.4.

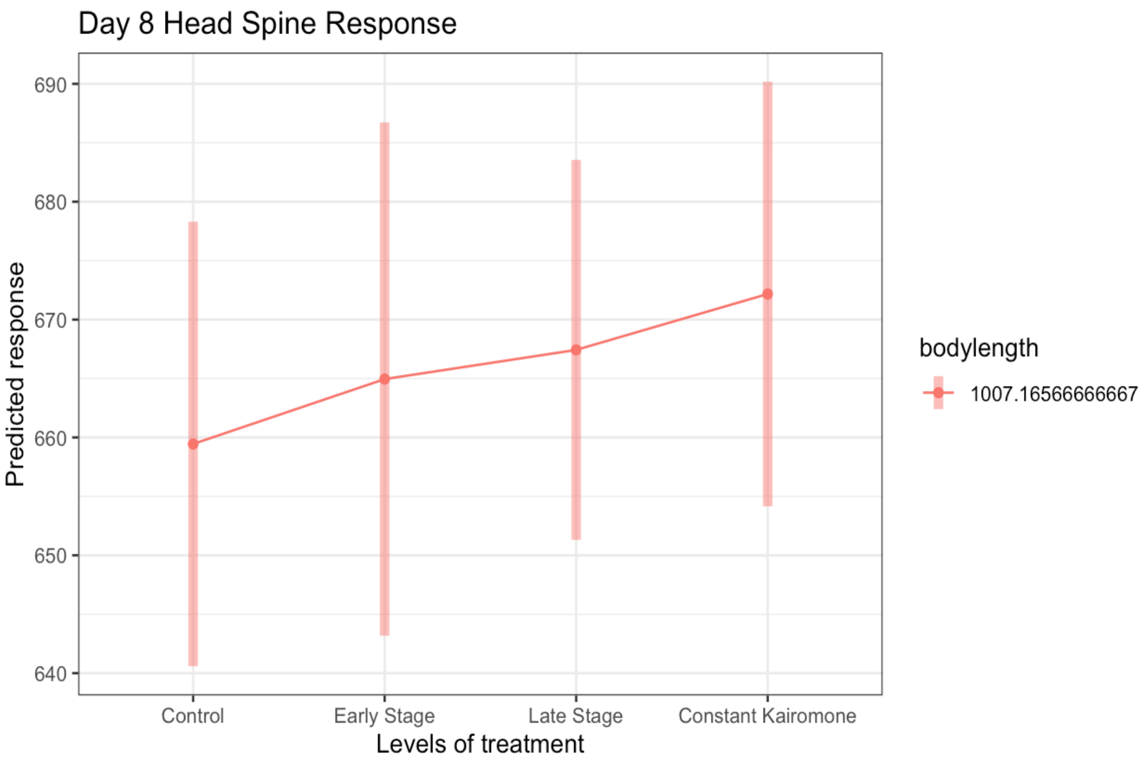


Figure 2.4) Head spine Response at Day 8

The graph illustrates the predicted head spine lengths of *Daphnia lumholtzi* at Day 8 across four treatment groups: Control, Early Stage, Late Stage, and Constant Kairomone exposure. The predicated head spine response shows the estimated mean for the dataset based on a fitted linear model. ANOVA results show significant effects of treatment (F(3, 133) = 3.356, p = 0.021) and body length (F(1, 133) = 1416.882, p < 0.001) on head spine length. Constant kairomone exposure resulted in the highest head spine length, while early and late-stage inductions had similar lengths, both slightly higher than the control group, which exhibited the lowest head spine length. Pairwise comparisons revealed no statistically significant differences between specific treatment groups (p > 0.05 for all comparisons). These results suggest that constant kairomone exposure tends to increase head spine length the most, while early and late-stage inductions have comparable effects.

The tail spine lengths at Day 4 showed that the control group had the shortest tail spines, indicating no significant morphological changes without kairomone exposure. The early stage induction group had slightly increased tail spine lengths compared to the control group, suggesting a modest effect of initial maternal exposure to kairomones. Similarly, the late stage induction group exhibited tail spine lengths comparable to the early stage group, indicating that both maternal and embryonic exposures had similar effects. The constant kairomone exposure group had the longest tail spines, demonstrating a strong morphological response to continuous kairomone exposure.

After conducting a ANOVA analysis on the significance of the effect of an interaction term between treatment and body length on head spine length, this interaction was proven to be significant and thus included in the ANCOVA results show in figure 2.5.

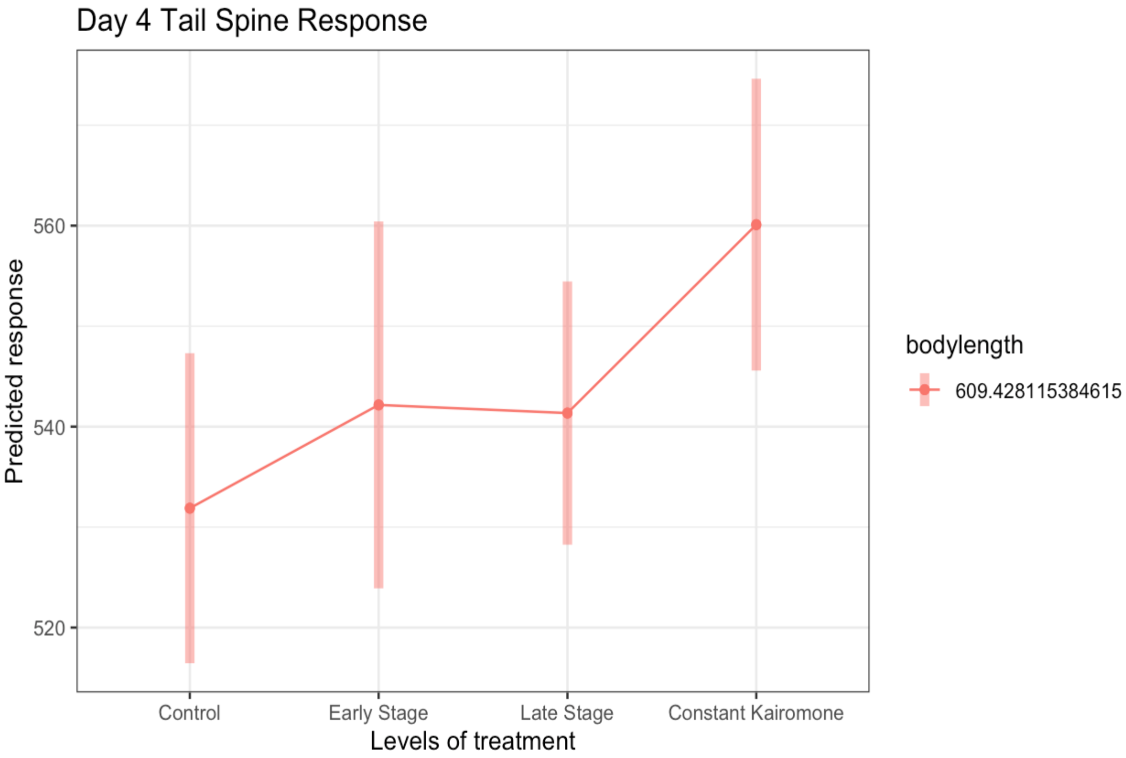


Figure 2.5) Tail spine Response at Day 4

The graph illustrates the predicted tail spine lengths of *Daphnia lumholtzi* at Day 4 across four treatment groups: Control, Early Stage, Late Stage, and Constant Kairomone exposure. The predicated head spine response shows the estimated mean for the dataset based on a fitted linear model. ANOVA results show significant effects of treatment and body length on tail spine length. Constant kairomone exposure resulted in the highest tail spine length, while early and late-stage inductions had similar lengths, both slightly higher than the control group, which exhibited the lowest tail spine length. Pairwise comparisons indicated that constant kairomone exposure showed a trend towards significantly longer tail spines compared to the control and early-stage groups, though the differences were not statistically significant after adjustment ($p > 0.05$ for all comparisons). These results suggest that continuous kairomone exposure leads to the most pronounced tail spine elongation, while early and late-stage inductions have similar effects.

At Day 8, the tail spine length trends remained consistent with those observed at Day 4. The control group continued to have the shortest tail spine lengths, while the early and late-stage inductions resulted in comparable lengths, indicating similar effects of maternal and embryonic exposure. The constant kairomone exposure group again exhibited the longest tail spines, highlighting the cumulative effect of sustained kairomone exposure on morphological changes.

After conducting a ANOVA analysis on the significance of the effect of an interaction term between treatment and body length on head spine length, this interaction was proven to be insignificant and thus excluded from the ANCOVA results show in figure 2.6.

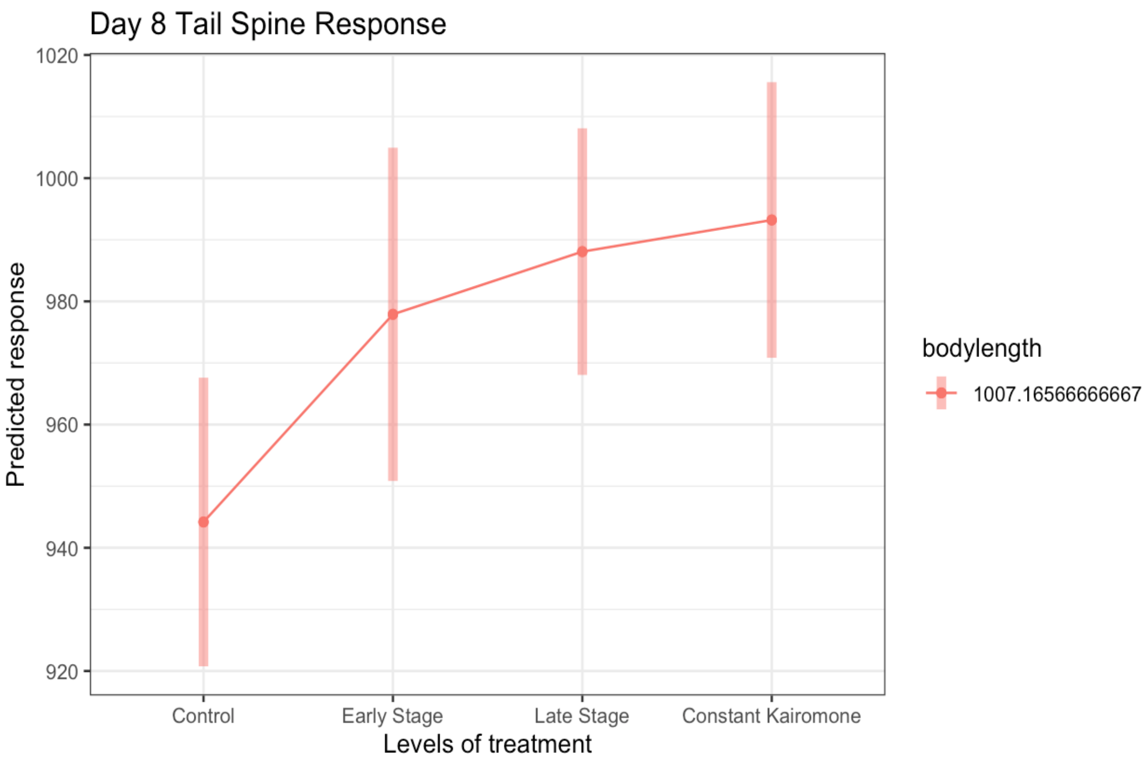


Figure 2.6) Tail spine Response at Day 8

The graph illustrates the predicted tail spine lengths of *Daphnia lumholtzi* at Day 8 across four treatment groups: Control, Early Stage, Late Stage, and Constant Kairomone exposure. The predicated head spine response shows the estimated mean for the dataset based on a fitted linear model. ANOVA results show significant effects of treatment and body length on tail spine length. Constant kairomone exposure resulted in the highest tail spine length, significantly higher than the control group ($p = 0.005$). The early-stage and late-stage inductions resulted in similar lengths, both higher than the control group but not significantly different from each other or from the constant kairomone group ($p > 0.05$ for all comparisons). These results indicate that constant kairomone exposure has the strongest effect on tail spine length, while early and late-stage inductions provide moderate elongation.

The results indicate that late and early stage inductions resulted in similar trait expressions, suggesting that both maternal priming and embryonic conditioning contribute equally to spine elongation. The emphasis on maternal priming versus embryonic conditioning does not seem to lend more strongly to trait expression in adult *Daphnia lumholtzi*. Continuous kairomone conditioning across all developmental stages yielded the most pronounced adult life trait expression, highlighting the importance of sustained environmental pressure. Additionally, the induction of traits post-deposition into the brood chamber suggests that both maternal rearing and neonatal sensing play roles in trait expression. However, the study's small sample size limits the statistical power, and while trends are evident, they should be interpreted with caution and verified with larger sample sizes.

These findings contribute to the understanding of phenotypic plasticity and epigenetic inheritance in *Daphnia lumholtzi*, emphasizing the roles of maternal and environmental factors in shaping adaptive traits.

Discussion

Our experiments on *Daphnia lumholtzi* revealed that both late and early stage inductions of predator presence result in comparable trait expressions in terms of head spine and tail spine development. Measurements taken at different developmental stages, specifically during early embryonic phases and later larval phases, showed no significant differences in the degree of spine elongation. This indicates that Daphnia are capable of mounting similar inducible defenses regardless of whether the predator cues are detected early or later in their developmental timeline, suggesting a robust and flexible response mechanism to environmental stressors.

Maternal Priming versus Embryonic Conditioning

The comparison between maternal priming and embryonic conditioning did not show a significant difference in the expression of inducible traits in adult *Daphnia lumholtzi*. Offspring of mothers exposed to predator cues did not exhibit stronger or more pronounced trait expressions than those directly exposed to predator cues during embryonic development. This suggests that the maternal environment alone, without direct embryonic exposure, is insufficient to enhance the inducible defenses in the adult stage. Therefore, the role of direct embryonic conditioning appears to be equally important in shaping the defensive phenotypes.

Constant Kairomone Conditioning Across All Timepoints

Daphnia lumholtzi exposed to constant kairomone conditioning throughout their developmental stages exhibited the most pronounced inducible traits in adulthood. Consistent exposure to predator cues from the embryonic stage through to adulthood resulted in the longest head and tail spines, indicating a cumulative effect of continuous environmental stressors. This finding underscores the importance of sustained predator presence in the environment for the maximal expression of defensive traits, highlighting a dose-response relationship between kairomone exposure duration and trait development.

Induction Post-Deposition into Brood Chamber

The inducible traits of head and tail spine elongation can also be triggered after deposition into the brood chamber. This suggests that *Daphnia lumholtzi* have the capability to continue sensing and responding to environmental cues during the neonatal stage. Maternal rearing conditions and neonatal sensing could both contribute to the induction of defensive traits, allowing for a window of phenotypic plasticity that extends beyond the immediate embryonic phase. This finding expands our understanding of the temporal flexibility in inducible defense mechanisms.

It is important to note that our study was limited by a small sample size, which affects the statistical power and the generalizability of our findings. While trends indicate potential patterns in trait expression across different induction timings and conditions, the results should be interpreted with caution. Larger sample sizes and further replication studies are necessary to confirm these preliminary trends and to draw more robust conclusions about the epigenetic mechanisms underlying inducible defenses in *Daphnia lumholtzi*.

The following power analysis in figure 2.7 was conducted on the final data set providing a more robust analysis than found in figure 2.2. In this post hoc analysis, an optimal sample size proved to be considerably larger than what was used at 49 individuals per test group.

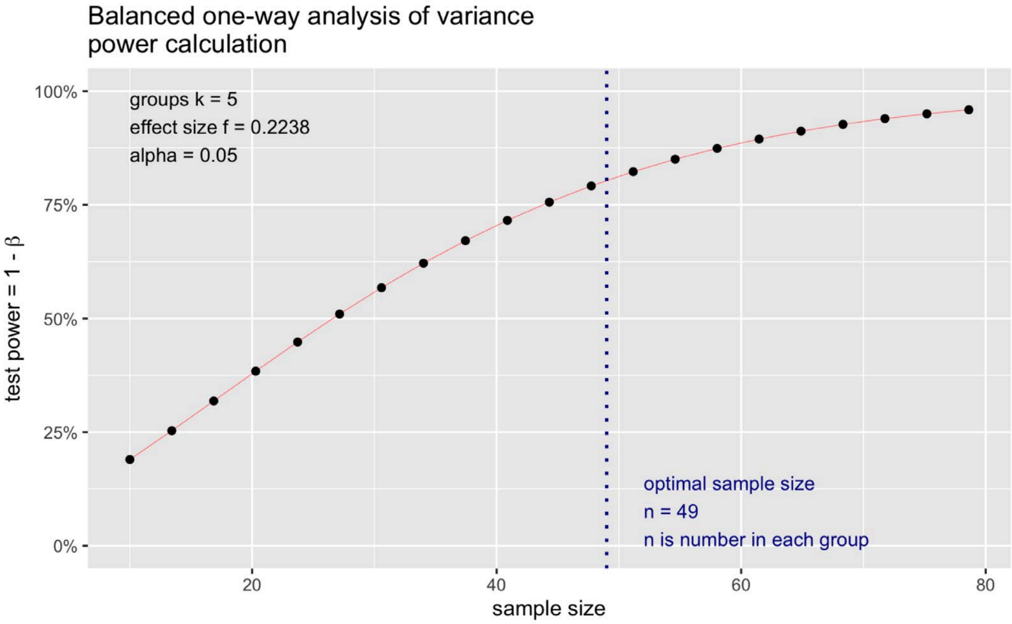


Figure 2.7) Post Hoc ANOVA Power Analysis

The graph displays a power analysis conducted to determine the appropriate sample size for a balanced one-way ANOVA in a larger experiment. The y-axis represents the test power (1 - β), while the x-axis shows the sample size per group. The analysis was based on the following parameters: five groups (k = 5), an effect size (f) of 0.2238, and an alpha level (α) of 0.05.

Chapter 3: Innate Embryo Sensitivity to Kairomone

The study of inducible defenses in aquatic organisms provides critical insights into the adaptive strategies that prey employ to survive in environments with fluctuating predation pressures. Daphnia, a genus of small planktonic crustaceans, exhibit phenotypic plasticity, which enables them to develop morphological defenses in response to chemical cues (kairomones) released by predators. These inducible defenses, such as elongation of head and tail spines, enhance survival by making them less susceptible to predation by fish. The present experiment investigates the epigenetically controlled trait expression of head and tail spine elongation in Daphnia in response to kairomone exposure.

In natural settings, maternal Daphnia carry embryos in their brood chambers, where the embryonic environment may be influenced by various unknown maternal factors. With findings indicating inducible trait expression of daphnia experiencing kairomone during brood chamber rearing, unanswered questions of how that induction can occur remained. To precisely control the developmental environment and isolate the effects of kairomones on trait induction, embryos were dissected from maternal Daphnia and reared in vitro. This method allows for a controlled assessment of how direct exposure to kairomones impacts the morphological development of the Daphnia spines.

Experimental Design

Two lines of Daphnia embryos were prepared for the experiment: a control group and a kairomone-induced experimental group. The embryos were dissected and immediately exposed to kairomones, 8 hours post-formation, continuing the exposure until key neonatal traits had developed by the 48-hour mark. This specific timeframe corresponds to the developmental stage of a Daphnia embryo at approximately 12 days of age in vivo. Subsequently, the Daphnia were reared to adulthood, and the spine phenotypes were measured at 72 and 168 hours post-dissection, which correspond to roughly 3-day-old and 7-day-old Daphnia reared naturally within the maternal brood chamber, respectively.

This experimental design allows for the investigation of the direct effects of predator-released kairomones on the epigenetic regulation of defensive trait expression in Daphnia. By comparing the control and experimental groups, the study aims to elucidate the mechanisms through which environmental cues can induce morphological changes and the developmental timeline required for these changes to manifest. The findings will contribute to a deeper understanding of phenotypic plasticity and its evolutionary significance in predator-prey interactions.

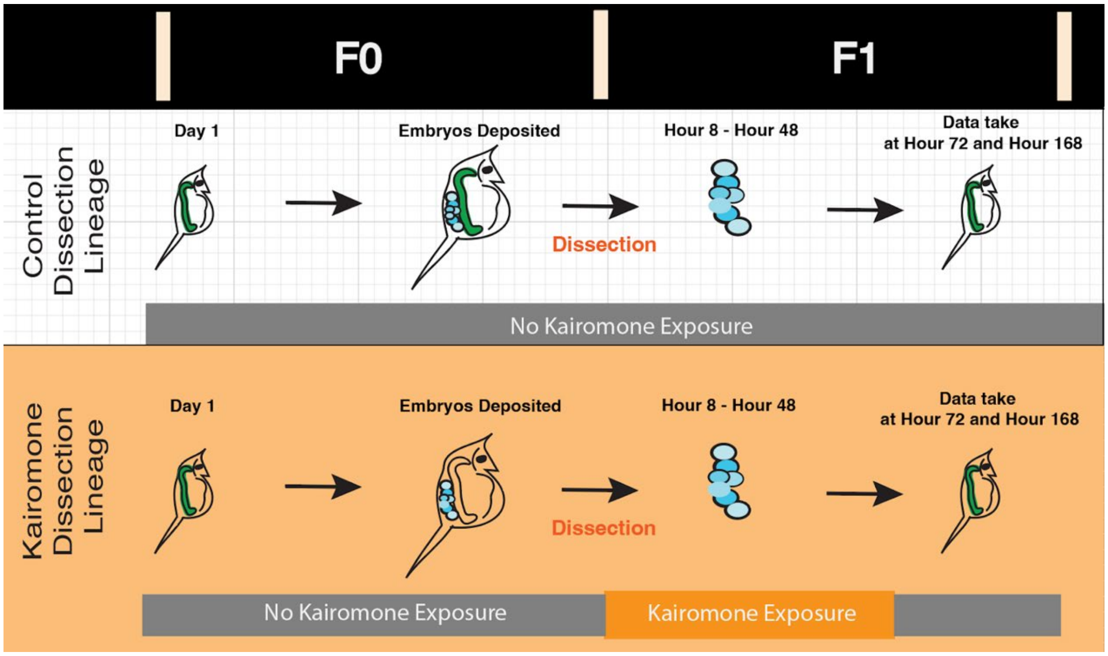


Figure. 3.1) Experimental Design

Experimental schematic showing a control without kairomone signals and a test line with kairomone signals. The gray bars indicate time without kairomone exposure while the orange bar indicates time with kairomone exposure. The position of exposure bars aligns with experimental steps such as embryo dissection. In the Kairomone Dissection Lineage, exposure occurred from dissection at hour 8 through hour 48 of embryonic development.

Results

Data analysis focused on comparing the lengths of head and tail spines between the control and kairomone-induced experimental lines of Daphnia. The measurements were taken at two developmental stages, 72 hours (day 3 equivalent) and 168 hours (day 7 equivalent) post-dissection, to assess the impact of kairomone exposure on spine elongation.

The one-way ANOVA analysis was performed to evaluate the impact of kairomone exposure on head and tail spine lengths in Daphnia at different developmental stages. Specifically, the focus was on the effect of the treatment (kairomone exposure) compared to the control group. The following results were obtained:

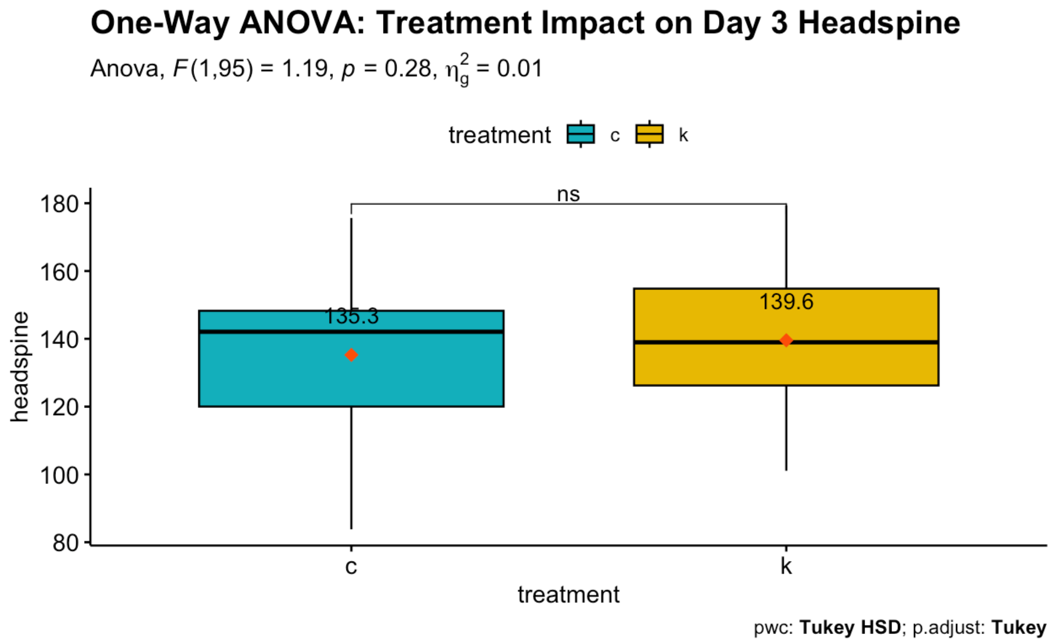


Figure 3.2) Treatment Impact on Day 3 Head spine Length

The graph displays the head spine lengths of *Daphnia lumholtzi* at Day 3 across control (c) and kairomone exposure (k) groups. The ANOVA results show no significant effect of treatment on head spine length ($F(1, 95) = 1.19$, $p = 0.28$, $\eta^2 = 0.01$). The mean head spine lengths were 135.3 µm for the control group and 139.6 µm for the kairomone group. While there was a slight increase in the kairomone group, the difference was not statistically significant (ns).

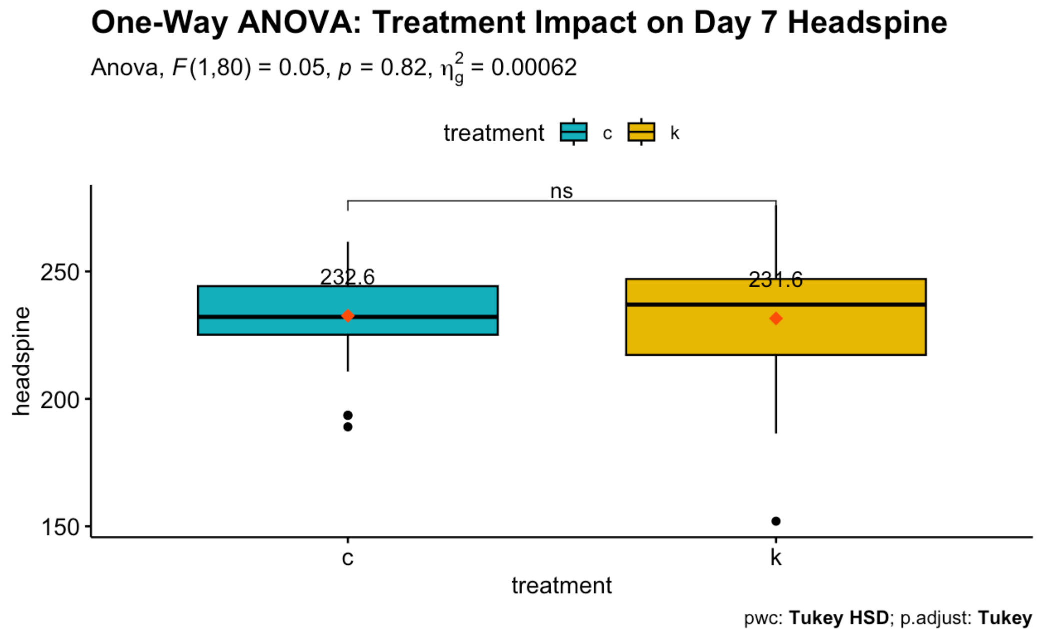


Figure 3.3) Treatment Impact on Day 7 Head spine Length

The graph illustrates the head spine lengths of *Daphnia lumholtzi* at Day 7 for the control (c) and kairomone exposure (k) groups. The ANOVA results indicate no significant effect of treatment on head spine length (F(1, 80) = 0.05, p = 0.82, $\eta^2 = 0.00062$). The mean head spine lengths were 232.6 µm for the control group and 231.6 µm for the kairomone group. The difference between the groups was minimal and not statistically significant (ns).

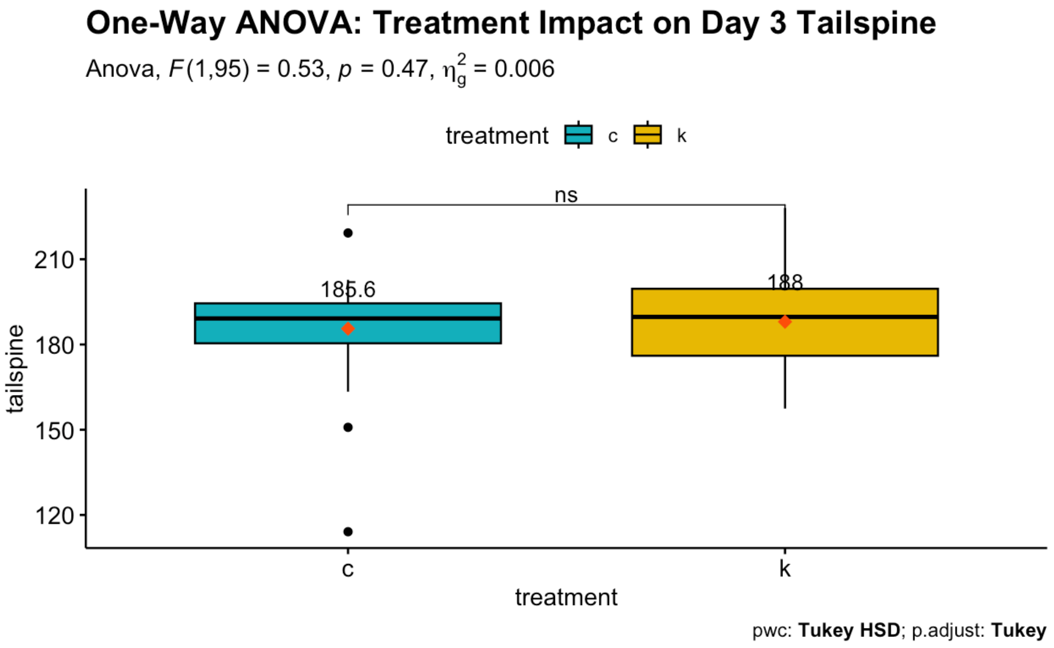
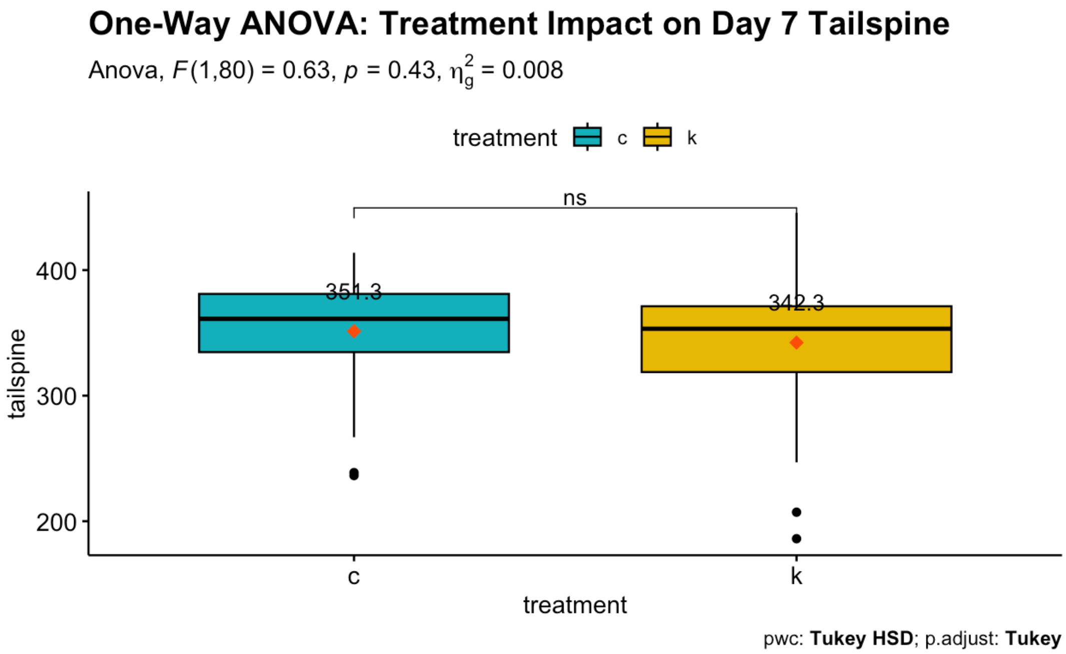


Figure 3.4) Treatment Impact on Day 3 Tail spine Length

The graph presents the tail spine lengths of *Daphnia lumholtzi* at Day 3 across two treatment groups: control (c) and kairomone exposure (k). The ANOVA results indicate no significant effect of treatment on tail spine length ($F(1, 95) = 0.53$, $p = 0.47$, $\eta^2 = 0.006$). The mean tail spine lengths for the control and kairomone groups were 185.6 µm and 188 µm, respectively. Despite the slightly higher mean in the kairomone group, the difference was not statistically significant (ns).



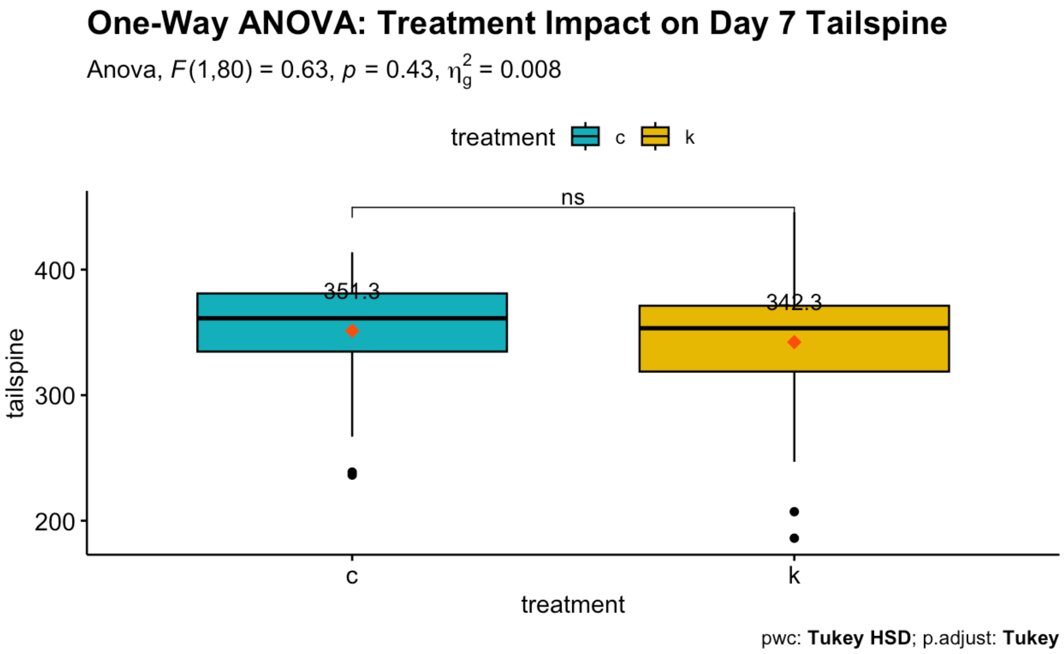


Figure 3.5) Treatment Impact on Day 7 Tail spine Length

The graph shows the tail spine lengths of *Daphnia lumholtzi* at Day 7 for control (c) and kairomone exposure (k) groups. The ANOVA results reveal no significant treatment effect on tail spine length (F(1, 80) = 0.63, p = 0.43, η² = 0.008). The mean tail spine lengths were 351.3 µm for the control group and 342.3 µm for the kairomone group. Although there was a slight decrease in the tail spine length for the kairomone group, the difference was not statistically significant (ns).

The ANOVA analysis revealed that there were no significant differences in head or tail spine lengths between the control and kairomone-treated groups at both day 3 and day 7 post-dissection. Despite the treatment, the measured spine lengths remained statistically similar across all comparisons. With sample sizes of 49 (control) and 50 (experimental) for head spine, and similar sample sizes for tail spine, more experimentation with larger sample sizes might be needed to detect any subtle trends that could yield significant phenotype differences. However, based on the current data, embryonic in-vitro kairomone exposure did not significantly increase the lengths of head or tail spines.

Discussion

The data from this study suggest that embryonic *Daphnia lumholtzi* do not possess an innate ability to sense or respond to kairomone chemicals. This conclusion is based on the absence of significant morphological changes in head and tail spine lengths when Daphnia embryos were exposed to kairomone media during in-vitro rearing. The results indicate no predation response in the morphological plasticity of defensive spines following embryonic conditioning with predator cues.

Key to this interpretation was the experimental design that involved dissecting embryos from the mother's brood chamber and rearing them to adulthood in a controlled environment, isolated from maternal influence. This methodology allowed for the specific assessment of the embryos' direct response to kairomone exposure, ruling out any maternal priming effects. The statistical analyses showed no significant differences in spine lengths between the control and kairomone-exposed groups at both Day 3 and Day 7.

Thus, the findings highlight that the morphological defenses typically seen in Daphnia in response to predator presence are likely influenced by maternal or post-embryonic environmental factors rather than an inherent embryonic capacity to detect and respond to kairomones. Further research is needed to explore the exact mechanisms through which these defenses are induced, with a focus on post-embryonic stages and maternal effects.

Chapter 4: Conclusion

The collective findings from the experiments on *Daphnia lumholtzi* provide a nuanced understanding of the mechanisms underlying inducible defenses in response to predator kairomones. The comparison between the conclusions from the two sets of experiments underscores the critical role of maternal mediation in the development of defensive traits.

The current study concludes that *Daphnia lumholtzi* embryos do not possess an innate ability to sense or respond to kairomone chemicals. This conclusion is based on the lack of significant morphological changes in head and tail spine lengths when embryos were exposed to kairomone media during in-vitro rearing. The absence of a predation response in the morphological plasticity of defensive spines suggests that direct embryonic exposure to predator cues is insufficient to induce these traits. This finding was facilitated by the isolation of embryos from maternal influence through in-vitro rearing, allowing for a clear assessment of embryonic conditioning without maternal priming effects.

In contrast, the first experiment demonstrated that both early and late-stage inductions of predator presence result in comparable trait expressions. Measurements taken at different developmental stages, including early embryonic and later larval phases, showed no significant differences in the degree of spine elongation. This suggests that Daphnia are capable of mounting similar inducible defenses regardless of whether predator cues are detected early or later in their developmental timeline. These results indicate a robust and flexible response mechanism to environmental stressors.

The critical insight from comparing these experiments is the role of the mother in mediating the inducible defenses. The first experiment indicated that kairomone exposure to both the mother Daphnia and its developing offspring leads to the expression of defensive traits in adult life. However, the second experiment, which exposed developing Daphnia to kairomones without the mother or rearing in the mother's brood chamber, did not result in the development of defensive traits in adulthood. This stark contrast suggests that the expression of defensive traits induced during embryonic development is mediated by the mother and not by neonatal sensing or any innate ability of embryos to sense or respond to environmental kairomone cues.

These findings collectively suggest that maternal influence is crucial for the induction of defensive traits in *Daphnia lumholtzi*. While direct embryonic exposure to kairomones without maternal involvement does not induce these traits, the presence of the mother and her exposure to predator cues plays a pivotal role. This highlights the importance of maternal priming in the development of adaptive defenses and suggests that the environmental conditions experienced by the mother can significantly influence the phenotypic outcomes in her offspring.

This research contributes to our broader understanding of phenotypic plasticity and adaptive strategies in response to environmental challenges. It emphasizes the intricate interplay between maternal effects and environmental cues in shaping the adaptive traits of *Daphnia lumholtzi*, offering valuable insights into the evolutionary mechanisms underlying these responses.

Future Directions

The findings from these experiments offer a foundation for further research to deepen our understanding of the mechanisms behind inducible defenses in *Daphnia lumholtzi*. Several avenues for future investigations are proposed to address the limitations and unanswered questions from the current study.

One of the primary future directions involves repeating these experiments with larger sample sizes. The trends observed in the current data need corroboration through studies with greater statistical power. Increased sample sizes will help validate the findings and ensure the robustness of the conclusions drawn about the role of maternal mediation and the insufficiency of embryonic conditioning alone in inducing defensive traits.

Beyond phenotypic analysis of spine lengths, future studies should include mRNA analysis on embryonic Daphnia exposed to kairomones. This would involve comparing gene expression profiles of embryos reared with and without kairomone exposure to identify differential gene expression patterns. Such an approach could reveal insights into the molecular pathways and specific genes involved in the organism's response to environmental stressors. For example, analysis of genes involved in the stress response, development, and morphological changes could provide a more comprehensive understanding of how kairomone exposure affects Daphnia at the genetic level.

To further explore the mechanism of kairomone sensing, in-situ assays during embryonic development can be conducted. These assays would check for the expression of key proteins responsible for environmental sensing. Proteins and genes such as those related to the stress response, sensory receptors, and signaling pathways identified in related studies (Weiss, 2019) can be targeted. By identifying whether these proteins are expressed in embryos exposed to kairomones, researchers can verify if the embryos have any innate capability to sense environmental cues.

A crucial unanswered question is through what mechanisms mother Daphnia influence offspring trait expression during embryonic development in the brood chamber. Investigating whether there is a form of brood chamber conditioning, alongside epigenetic mechanisms, that primes offspring with defensive traits, is essential. Experiments could involve manipulating the brood chamber environment to see if changes affect offspring trait expression. Additionally, studies on the transfer of signaling molecules or hormones from mother to embryo during development could provide insights into the maternal effects observed.

The precise mechanisms by which mother Daphnia influence the trait expression of their offspring during embryonic development remain unclear. To address this, potential experiments could employ molecular techniques to track the transfer of substances from the mother to the embryos. By observing changes in offspring traits when these transfers are inhibited or altered, researchers can gain insights into how maternal influence operates. Furthermore, the concept of brood chamber conditioning warrants investigation. Experiments could involve altering the brood chamber environment and observing whether offspring still develop inducible defenses. Gene expression analysis can be used to identify potential conditioning signals, providing further understanding of the mechanisms involved.

By addressing these questions and expanding the scope of analysis, future research can build a more detailed and mechanistic understanding of the factors driving phenotypic plasticity and inducible defenses in *Daphnia lumholtzi*. This will enhance our knowledge of ecological interactions and evolutionary processes governing adaptive traits in response to environmental stressors.

Chapter 5: General Methods

1. Algae Production

Scenedesmus obliquus and Chlorella sp. algae were cultivated separately by inoculating each species in 75 ml of Woodshole media. The cultures were maintained under constant temperature and ample light for one week. After the initial growth period, 20-30 cultures of 75 ml each were combined into one gallon glassware. The combined cultures were bubbled for aeration and allowed to grow for an additional week. Subsequently, the cultures were centrifuged to pellet the algae, and the separated algae were combined with combo media to create feeding strength stock algae. The stock algae for each species was kept separate.

2. Feeding

A 50:50 mix of Scenedesmus obliquus and Chlorella sp. algae was prepared using an optical density of 25 cm for each species before combining. For individual Daphnia, 10 ml of this algae combination was fed every other day. For Daphnia reared in communal beakers in a common garden environment, 50 ml of the algae mix was fed every other day.

3. Experimental Environment

For each experiment, adult Daphnia were isolated in 50 ml beakers filled with either control media or kairomone-treated media. The media was changed every other day to maintain experimental conditions.

4. Dissection

To dissect embryos post deposition into the brood chamber, two fine-tipped tweezers were used to gently pull apart the underside of a mother Daphnia, exposing the brood chamber.

The embryos were then nudged out into the surrounding media. This procedure caused little to no trauma to the embryos but resulted in nearly 100% fatality of the mother Daphnia.

5. In-Vitro Embryo Rearing

Dissected embryos were transferred to a 1.5% agar and combo media plate topped with 50 ml of control or kairomone-treated water. During the setting of the agar, approximately 50 glass beads were spread across the surface before complete cooling. Once set, the beads were removed, creating spherical wells in the agar. Embryos were then transferred from the microscope slide where dissection took place using a glass Pasteur pipette and placed into individual agar wells. This setup ensured that embryos were isolated from contact with other embryos, facilitating individual tracking throughout analysis, imaging, and note-keeping.

6. Kairomone Treated Media and Control Media Preparation

Kairomone-treated media and control media were prepared over a three-day process. Two 10-gallon buckets were filled with nanopure water and aerated with bubbling tubes. In the bucket for kairomone-treated media, five three-spine stickleback fish were added and fed 10 *Daphnia lumholtzi* and 10-20 Daphnia magna daily. The control bucket received daily additions of Daphnia but no fish. After three days, the media from both buckets were filtered through Steri-Flip filters to remove debris from fish, algae, and Daphnia, and to sterilize the media from potential contaminants and bacteria. The media was then frozen for preservation and thawed 24 hours before use in the experiments.

7. *Daphnia lumholtzi* clone

Daphnia lumholtzi Saguaro Lake (SAG) clones were generously donated by Michael Lynch. SAG was collected in the summer of 2019 from Saguaro Lake, AZ, USA. This clone was specifically chosen for its ability to develop novel headspines and tailspines in response to predation.

Common Garden Rearing

D.lumholtzi clones were reared for 6 months in artificial lake media (COMBO) and fed *Scenedesmus obliquus* and *Chlorella vulgaris* ab libitum to dull residual effects of lake predation. Prior to experimental perturbations, ancestral *D. lumholtzi* were reared for three generations in COMBO media and fed a standard algae mixture of *S. obliquus* and *C. vulgaris* to minimize the influence of maternal effects on experimental generations.

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