Impacts of UV radiation on inducible defense traits of *Daphnia pulex*

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Abstract

In aquatic environments predator threats can be present as chemical cues which can induce defensive traits in prey. Such predatory induced cues can be responsible for changes in morphology, life histories and behavior. Predator-induced plasticity has allowed for prey such as *Daphnia pulex* to avert capture by common predators such as *Chaoborus* larvae. Planktonic crustaceans offer a good opportunity to recreate aquatic ecosystem predator-prey dynamics such as *Daphnia-Chaoborus* interactions by use of microcosms in short term experiments. In this study two distinct clones *Daphnia pulex* were exposed to predatory cues, in order to observe the response rate of neckteeth formation in juvenile offspring. To test for the combined effect of kairomones, and an ever-present additional stressor, ultraviolet radiation (UVR) were applied in ecologically relevant levels in a two by two factorial experimental design. Results found that UVR exposure has a significant impact on the offspring's ability to produce the defensive trait and resulted in inter-clonal differences in response rates to body size development. The use of a multifaceted design allowed for the investigation of the allocation of energy to abiotic and biotic stressors found in the natural environment of the key stone species *Daphnia pulex*.

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1 Introduction

1.1 PREDATOR-PREY DYNAMICS

Predator-prey dynamics have provided powerful insight into the ability of prey organisms to respond and adapt to predators by morphological or behavioral responses (Ghalambor et al., 2015). Historically, stressors from predator-prey interaction (Abrams 1986; Marrow et al. 1992) have serve as a major driver in population composition for both prey and predators (Abrams, 1986; Marrow, Law, & Cannings, 1992). The coevolution of predator-prey interactions is fueled by the antagonistic biotic interactions, also referred to as the Red Queen Theory, favoring the multifaced response of defense traits (Clay & Kover, 1996; Marrow et al., 1992). The ability for prey to respond to the dynamic nature of environmental stressors in the presence of predators, however, requires further research.

Predator induced plasticity is prevalent among many taxa as a form of continuous plasticity (Dennis, LeBlanc, & Beckerman, 2014). Inducible defenses allow for prey organisms to respond to a varying risk of predation (Christjani, Fink, & Elert, 2016). Studies have supported the predator-prey plasticity responses to be defined as continuous multivariate, and adaptive, ultimately influencing population dynamics (Dennis et al., 2014; Harvell, 1990). The hypothesis for adaptive plasticity states that phenotypic plasticity will be favored if a phenotype has a higher fitness in the inducing environment than alternative phenotypes, but lower fitness in other environments (Hoverman & Relyea, 2009).

Organisms with inducible defenses can be used as model systems for addressing the ecology and evolution of phenotypic plasticity (Hoverman & Relyea, 2009; McCollum & Van Buskirk, 1996; Nilsson, Brönmark, & Pettersson, 1995). Phenotypic plasticity is the ability of one genotype to express different phenotypes in response to stimuli, either active or passive (Colbourne et al., 2011). Phenotypic plasticity can be classified into three classes of traits: morphology, life history, and behavior (Dennis et al., 2014; Dodson, 1989).

As prey defend against one environmental stressor, this could lead to greater vulnerability to other environmental stressors. The stress produced by UV radiation for example, could lead to the allocation of energy to UV defense, impacting the ability of the organism to defend

against any additional environmental stressors, such as predation or infection (Hoverman & Relyea, 2009). This mechanism can be referred to as mechanistic interference among defenses (Hoverman & Relyea, 2009). Phenotypic plasticity is critical for the success of organisms in dynamic environments. In a study conducted by Hoverman et al. (2009), freshwater snails were used to measure survival costs and benefits of developed plastic traits induced by the presence of predators. Results showed that defense traits that are induced by the presence of specific conditions (i.e. presence of predators), may not be the optimal phenotype for the organism in all environments. Moreover, showing that the costs of adaptive defensive traits are closely linked to the costs of the allocation of available energy.

1.2 DAPHNIA PULEX AS A MODEL ORGANISM FOR PHENOTYPIC PLASTICITY

The ecoresponsive nature of the crustacean *Daphnia pulex* genome provides a great opportunity to observe the impacts of environmental stressors on phenotypically plastic trait which are expressed as a defense morphological trait (Colbourne et al., 2011). Due to their short generation time, parthenogenetic life cycle, large brood sizes and ease of culturing under laboratory conditions, *D. pulex* serve as ideal model organisms for ecological and evolutionary research. Furthermore, with the available information of the *D. pulex* genome, easier observation of environmental influences and gene functions is possible compared to other genomic model species (Colbourne et al., 2011).

By using *D. pulex* as a model organism, we can observe the shifts in physical defensive traits in the presence of predatory info-chemical cues (kairomones) released by feeding *Chaoborous flavicans* larvae. *D. pulex* can be found in soft-water ponds and lakes throughout North America and Europe (Ebert, 2005). *D. pulex* species have been used for a multitude of studies regarding predator-prey dynamics, as *Daphnia* serve as prey to an array of predators. This has ultimately resulted in the dynamic nature of defense traits of *D. pulex*.

D. pulex can possess inter-clonal differences in response rates of neckteeth induction based on kairomone concentrations (Christjani et al., 2016; Hansson, Hylander, & Sommaruga, 2007; Krueger & Dodson, 1981; Sterr & Sommaruga, 2008; Tollrian, 1993). Although inter-clonal differences in response rates between clones has been studied for a few selected clones (Dodson, 1989; Hebert & Crease, 1983; Loaring & Hebert, 1981; Tollrian, 1993), the majority of publications have focused on the induction of defensive response rates of one

particular clone of *D. pulex.* Hence the inter-clonal response rates for the majority of clones is largely unknown, and the generality of these responses may be questioned (Boeing et al., 2006)

1.3 DEFENSE TRAITS OF DAPHNIA PULEX

Defensive traits are costly, and may thus be involved trade-off strategies to ensure energy allocations to e.g. growth and survival. The predator-induced morphological defenses in *D. pulex* are neckteeth (also known as neck spines). Neckteeth are formed by a dose-dependent reaction in the juvenoid hormone signaling pathway, a highly conserved endocrine signalling pathway common in most arthropods (Dennis et al., 2014). The dynamic nature of the induction of neckteeth is the result of a form of optimization between predation risk and protective investments with costs limiting the ability of this trait to be expressed throughout the juvenile stages, as this is commonly seen in predator-prey dynamics (Hoverman & Relyea, 2009). Recorded costs of neckteeth induction include longer development time for offspring, reduced survival, and reduced clutch size (Tollrian, 1995).

Kairomones are the chemical signals responsible for the induction of neckteeth. Info-chemicals produced by *Chaoborus flavicans* have been used in an array of experiments specifically with *Daphnia* species. *Chaoborus flavicans*, also known as phantom midge or glass worm, are a common predator to *Daphnia* species. *Chaoborus* larvae prey on *D. pulex* offspring and other smaller species due to their limited engulfment range of their mouth gape (Sell, 2000; Tollrian, 1995). Induced morphological defenses in the *D. pulex* includes the formation of neckteeth, increased body size, and strengthening of the carapace (Riessen, 2012). Increases in size are linked to greater swimming speed and thus a higher escape and avoidance rate (Tollrian, 1993).

Neckteeth formation in *D. pulex* serves as a defense trait with high costs and limited availability, as the species are not able to express this defensive trait constantly (Dicke & Sabelis, 1988). *D. pulex* have the capability to express neckteeth formation from 2-5 in response to kairomones, depending on the clone observed (Tollrian, 1993). The degree of neckteeth formation in *D. pulex* juveniles has been proven to be directly linked to the concentration of kairomones exposed to and within instars, following a Michaelis-Menten like saturation curve (Christjani et al., 2016; Hansson et al., 2007; Krueger & Dodson, 1981;

Sterr & Sommaruga, 2008; Tollrian, 1993). Neckteeth formation has been proven to increase survival rates up to 45% (Dennis et al., 2014; Hammill et al., 2008; Nilsson et al., 1995) supporting its costly use when needed.

Recent studies have shown that shifts in environmental conditions can negatively impact *Daphnia pulex's* ability to induce neckteeth formation with the presence of predatory cues (Rautio & Tartarotti, 2010; Weiss et al., 2018). Changes in water chemistry such as low calcium concentrations, resulted in the increase vulnerability by decreasing the effectiveness of antipredator defenses. Solar ultraviolet radiation is an environmental stressor that is ever-present and could potentially interact with predatory responses.

1.4 EFFECTS OF ULTRAVIOLET RADIATION ON ZOOPLANKTON

Ultraviolet radiation (UVR) is a common threat to the life histories and population structure of many species in aquatic environments (Vadadi-Fülöp et al., 2012). Although increases in UV radiation to surface bodies of water caused by the reduction of the stratospheric ozone has come to a halt (Williamson et al., 2014). Increases in UV radiation caused by climate change can still potentially limit the inhabitants of the photic zone in freshwater lakes and ponds (Bais et al., 2018; Dokken, 2014; Dugo, Han, & Tchounwou, 2012; Rose et al., 2014). Laboratory and field studies have shown living organism to be negatively affected when exposed to high intensities of UV radiation (Hansson et al., 2007; Hessen, Van Donk, & Andersen, 1995; J. Kim et al., 2009; Rose et al., 2014; Studer, Lamare, & Poulin, 2012; Wolf, Andersen, Hessen, & Hylland, 2017).

UV radiation has been linked to DNA damage, reduced growth rates and decreased fecundity (Rautio & Tartarotti, 2010). UV radiation consists of UV-C (100-280nm), UV-B (280-320mn) and UV-A (400-700nm). The absorption maximum of DNA is estimated to be near 260 nm (de Jager et al., 2017). UV radiation is an environmental threat that acts within the temporal and spatial scale (Hansson et al., 2007). UV-C radiation, the most harmful form, is filtered out through the earth's atmosphere while UV-B radiation is absorbed partially by the stratospheric ozone (Williamson et al., 2014). UV-A radiation is responsible for DNA breaks and reactive oxygen species (ROS) formation in natural systems (Cullen & Neale, 1994; Wolf et al., 2017). As seen in the intertidal copepod *Tigriopus japonicus*, UV radiation

exposure induced the production of oxidative stress agents such as ROS within organisms (Kim et al., 2015).

Zooplankton have evolved to use several protection strategies against UV radiation. Photo-reactivation mechanisms for *Daphnia* species include photo-protective pigments (the accumulation of red carotenoids or synthesis of black melanin), vertical migration and the increased activity of antioxidant enzymes that determine sensitivity to UV radiation (Dieter Ebert, 2005; Hansson et al., 2007; Rautio & Tartarotti, 2010; Wolf et al., 2017). Studies have shown that survival increased for *D. pulex* when UV radiation was filtered out (Rautio et al., 2003). The UV radiation sensitivity of *D. pulex* is dependent on species, season, age and geographical location (Rautio & Tartarotti, 2010).

UV radiation damage creates breaks in DNA caused by either direct radiation or the photo-activation of dissolved organic carbon (DOC), producing free radicals and harmful reactive oxygen species (ROS). ROS formation in organisms is mainly produced by UV-A, as UV-B and UV-C are partially and completely absorbed in the stratosphere (Williamson et al., 2014; Wolf et al., 2017). Studies have shown that while DOC concentrations have been linked to reducing UV radiation exposure by absorption, this reaction may also in turn increase ROS molecules effecting inhabitants of the body of water negatively. *Chaoborus* predatory cues are organic molecules composed of carbon chains. Waters which contain high concentrations of kairomone extracts could possibly induce the adverse reaction of the photoactivation of kairomone molecules as ROS formation with the presence of UV radiation. Moreover, *Daphnia* which inhabit bodies of water with higher concentrations of organic carbon molecules could be affected by the detrimental effects of cellular damage with increases in UV radiation.

1.5 PURPOSE

The focus of this experiment was to observe the effects of UV-A radiation on neckteeth formation of two clones of *Daphnia pulex* from two distinct geographical locations.
For this project I created a multi stressor experimental two by two factorial design (Figure 1).

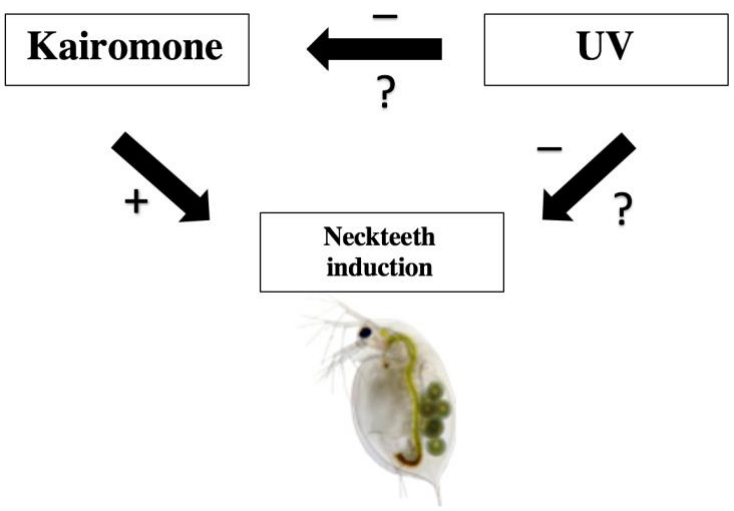

Figure 1. Conceptual visualization of factorial experiment. Potential interaction of kairomone and UV radiation could result in antagonistic influence neckteeth induction development. UV radiation could produce a possible synergistic or antagonistic reaction with kairomone molecules. The interaction of stressors is tested by a two by two factorial design.

The ability to compare studied response rates in new experimental designs provides insight into the sensitivity of defense traits to external relevant stressors in observed natural systems. The observation of the induced morphological defense traits in *Daphnia longicephala* was tested by Trotter et al. (2019), with various types of plastic waste in a multi-faceted factorial design. By creating a two by two factorial design and comparing two clones found in different locations and conditions, response rate defense traits could be measured and compared on the clone and individual level (Tollrian, 1993). The use of clones allows for the opportunity to observe individuals with the same genetic background that express variation in morphological defenses.

Abiotic and biotic environmental stressors have proven to affect inducible defense traits of *Daphnia* species (Sterr & Sommaruga, 2008; Linda C. Weiss, Pötter, et al., 2018; Wolf et al., 2017), but the effect of interactions of the abiotic factor UV radiation and the biotic factor of infochemicals known as kairomones on neckteeth formation has yet to be thoroughly investigated. The probability of whether or not UV radiation has a synergistic or antagonistic effect on the predator-prey dynamics has yet to be tested. Therefore, this project aims to contribute to the understanding of on how organisms allocate limited resources to different functions when under threat (Dennis et al., 2014).

1.6 HYPOTHESES

This study aims to investigate the effects of ecologically relevant levels of UV radiation on the induction of neckteeth in *Daphnia pulex* by testing the following:

1. UV-A will limit neckteeth formation in *D. pulex* juveniles, by either UV radiation denaturation of the kairomone or due to direct stress of UV radiation imposed on *D. pulex*.
2. Clone Group P5 will respond less to UV radiation due to its natural habitat being a very shallow rockpools with higher exposure to sunlight.

2 Materials & Methods

Over the course of several months *Daphnia pulex* were sampled, cultured, and with the help of an experimental design, tested to complete this master's thesis project. In the following pages I will provide a summary of sampling techniques, culturing techniques, the experimental design, and measurements.

2.1 SAMPLING OF DAPHNIA PULEX

Daphnia pulex samples were collected from the UiO AQUA department *Daphnia* lab, which were obtained during sampling excursions in spring of 2017. *D. pulex* samples from the available UiO lab collections were collected from two distinct geographical locations, rock pools and a shaded inland seasonal pond found outside of the Biology building at the University of Oslo (Figure 1). The two clones, named Pond 5-17 (P5) and UNI-17 (UNI), were identified to be of the same species (Appendix 7.1.3) but were identified as different clones. Clone Pond 5-17 was collected from a small rock pool (~1 m) with humic brown water surrounded by vegetation by the coast of Oslo. Clone Uni-17 was collected in a seasonal pond (~1 m) located outside of the Kristine Bonnevie science building at the University of Oslo campus. All *D. pulex* used in this experiment were obtained in January 2018 from previously maintained stock cultures of these two clones at UiO and maintained until February 2019.

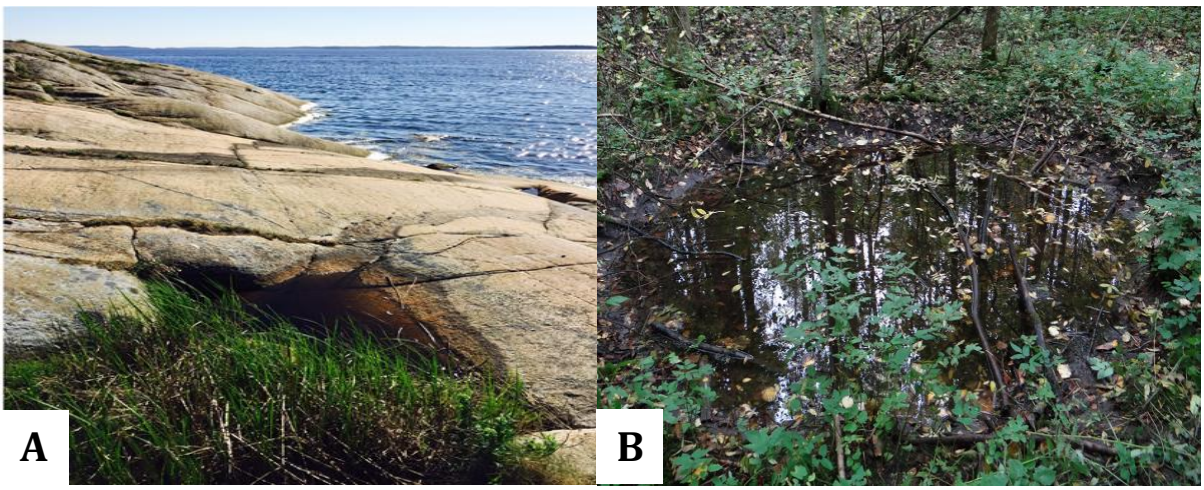

Figure 1. Images of sampling locations for *Daphnia pulex* clones. A) Rock pool location of clone Pond 5-17. B) Inland seasonal pond of clone UNI-17 at the University of Oslo.

2.2 CULTURING OF DAPHNIA PULEX

In the spring of 2017, *D. pulex* were cultured in controlled laboratory conditions using common culturing techniques (Appendix 7.1.3). Cultures were kept in a 21˚C ± 1 ˚C temperature-controlled climate room with a 16:8 light cycle. Both *D. pulex* clones were cultured in an Artificial Daphnia Medium (ADaM) (Ebert, 2013), which is used to mimic the natural water conditions of the *D. pulex* habitat.

Chlamydomonas reinhardtii was selected as an algae food source for all clones. The *Chlamydomonas reinhardtii* species (strain CC-1690) was cultured in a monoclonal chemostat in controlled culture rooms set to 21˚C ± 1˚C with constant LED lighting. Algae was grown in Wright Crypophyte (WC) (a variation of Bold's basal medium), harvested during the log phase of algae growth (Appendix 7.1.3), and replaced every 14 days. All samples of algae were spun down with a centrifuge (Eppendorf Centrifuge 5810 R). WC media was then removed and replaced with ADaM medium before fed to *D. pulex* cultures to reduce bacterial content (Appendix 7.1.3). The optical density (OD) of resuspended algae was then measured at 800 nm using a spectrophotometer (Shimadzu UV 160-A, Japan) to calculate the desired algae concentration. The ratio of algae was then calculated using techniques established by Erik Sperfeld (Unpublished) (Appendix 7.1.3). The formula used for algae concentrations:

$$\frac{1}{Optical\ Density\ x\ 0.3385 - 0.02}\ x\ target\ food\ concentration\ x\ volume\ in\ glass\ (L)$$

D. pulex were fed a strict diet of *Chlamydomonas* once a day (0.5 g C/L of each), to limit the overgrowth of harmful bacteria in the *D. pulex* cultures, as this expedites the degradation of kairomones (Tollrian, 1993).

Frozen kairomone extracts were collected from live *Chaoborus flavicans* samples by Erik Sperfeld at UiO in 2017 (Appendix 7.1.3) and preserved by refrigeration in a freezer at -18˚C in room 412 at UiO in the AQUA department. Studies have shown that neckteeth induction is dose dependent, and with purified concentrations of kairomones ranging from 5 µL to 50 µL a plateau is reached with neckteeth induction (Figure 2). Unpurified kairomone concentrations of 60 µL were used for all treatments of kairomone to ensure the expression of

neckteeth. All *D. pulex* were treated with the same isolated samples, as there can be variability in the neckteeth response rate due to differences in collected kairomone concentrations.

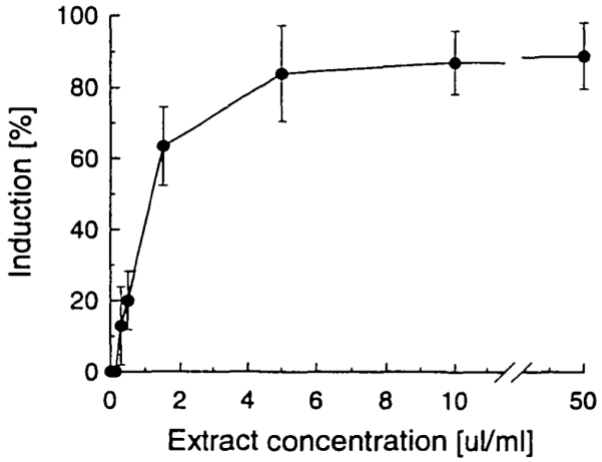

Figure 2. Dose-dependent curve for *Chaoborus* concentrations and neckteeth induction in second juvenile instar. Error bars = ± 1 SD (n=20) (Tollrian, 1993).

2.3 UVR SET UP

Photosynthetically active radiation (PAR) lights were used to mimic natural light conditions (400-700 nm) with fluorescent lamps set to 16/8 light cycles. UV-A lamps (UVA-340, Q-Lab) were selected due to close simulation of sunlight in the critical short-wave UV region (340-400 nm) and to mimic natural ecologically relevant UV exposure (Q-Lab 2019). Spectral distribution of the UV-A lamp is given in Figure 2. The experimental light set up was divided into two sections: UV-A treatment and PAR. The UV-A treatment group used UV-A radiation lamps using the experimental set up used by Wolf & Heuschele (2018). UV-A radiation lamps were measured at 1900 lux using a spectrometer to measure the luminosity of the UV lamp to see ensure the 340-400 nm range was obtained with the experimental set up (SpectraPen LM-500-UVIS). The spectrometer does not cover the full range of UV light sources (only 340-780 nm). For the natural light treatment (PAR) 2 fluorescent 36-W fluorescent lamps were installed 0.15 m above the open glass jars containing *D. pulex* to simulate solar radiation. Each surface area was treated with the same amount of light intensity.

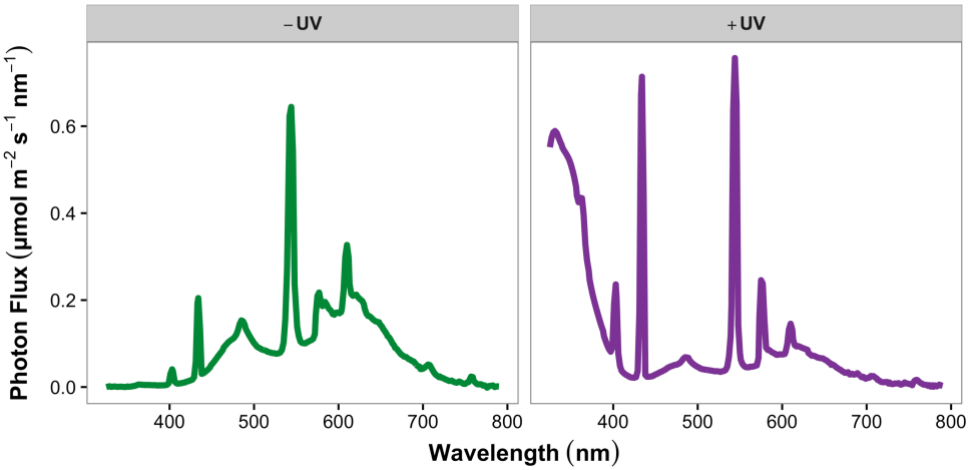

Figure 3. Comparison of the photon flux (μmol m^{-2} s^{-1} nm^{-1}) spectra for the non-UV ("−UV") and the UV treatments ("+UV"). The perceived luminous power was 1500 lux in both irradiance regimes. Note the contribution of UV light (< 400 nm) in the UV treatments (Wolf & Heuschele, 2018).

2.4 PILOT EXPERIMENT

Preliminary experiments were run from July of 2018 to October of 2018 in order to ensure that kairomones were not denatured by UV (Appendix 7.1.2). A study conducted by Sterr & Sommaruga (2008) found UV radiation for 5 to 10 hours to have a significant effect on the efficacy of the predator cue, decreasing neckteeth by 31%. In order to observe the ability of kairomones and neckteeth expression within a shorter time frame (0-4 hours), unfiltered kairomone samples were treated and then exposed to mother *D. pulex*. Additionally, the pilot experiment aimed to test the potency of kairomone extracts collected in 2017.

The integrity of kairomone collections were tested with the following treatments: UV (340-700nm), PAR (400-700nm) and no light alongside a control which contained no kairomones and no light treatment. Kairomone solutions containing 60 µL of unpurified kairomone concentrations in 50 mL glass jars with 40 mL of ADaM medium were sampled every 2 hours for a total of 8 hours. Selected mature mother *D. pulex* of the UNI clone were then placed in target treatment group jars immediately after each 2-hour collection increment. After 2 days of treatment, released offspring were scored to quantify neckteeth induction for instar 2.

2.5 MAIN EXPERIMENT

For the main experiment it was tested whether kairomone induced neckteeth formation would be affected in the presence of UV radiation. Both *D. pulex* clones were exposed to a two by two factorial experiment with the following four treatments: Control, kairomone treatment, UV treatment, and UV + kairomone treatment.

2.5.2 COLLECTION OF MOTHER DAPHNIA

Daphnia pulex mothers treated in identical conditions during the culturing process in order to limit maternal influences (Agrawal et al., 1999), preventing any previous exposure to kairomones from inducing the defensive trait. Mature female *D. pulex* were individually selected from each clone group after the release of their 3rd clutch, as collection of *D. pulex* during the maturation stage after the 3rd clutch release is critical limiting unwanted size variability observed in clutch groups 1-3 (Coakley et al., 2018).

For each treatment, third-generation mothers who had released their third clutch and had eggs in the second embryonic stage of development were selected to ensure established prenatal time for induced morphological change in offspring (Dennis et al., 2014; Naraki et al., 2013) The selection process was done by observing the egg development of mother *D. pulex* under an inverted light microscope (Lecia MZ 8). It was critical to select mother *D. pulex* from the same clutch to ensure synchronization of embryonic development of offspring. A total of 28 mother *D. pulex* were selected from the P5 clone group and 20 from UNI, which were then treated, and offspring scored.

At the beginning of the experiment desired *D. pulex* mothers were treated individually with UV and kairomone treatments in transparent 50 mL open glass jars with 40 mL of ADaM medium. For the kairomone treatment groups, *D. pulex* were placed into *Chaoborus* treated ADaM media before the release of their third clutch (Tollrian, 1993). Mothers (and finally offspring) were transferred daily into new jars with freshly prepared kairomone suspensions for selected groups until the release of the fourth clutch (1-3 days).

Neonates were then collectively isolated from the mother when released as the 4th clutch and observed for the following 2 days. Neonates were observed and assigned a random number

for clutch group during neckteeth and neck-keel scoring. To ensure the development of neckteeth and reinforce the experimental design, selected juveniles in target treatments were exposed to the same experimental treatment as mothers the following day of hatching (day 1) to ensure the complete treatment for instar 1 and 2 of juvenile development and to mimic natural conditions with continuous exposure the environmental stressors.

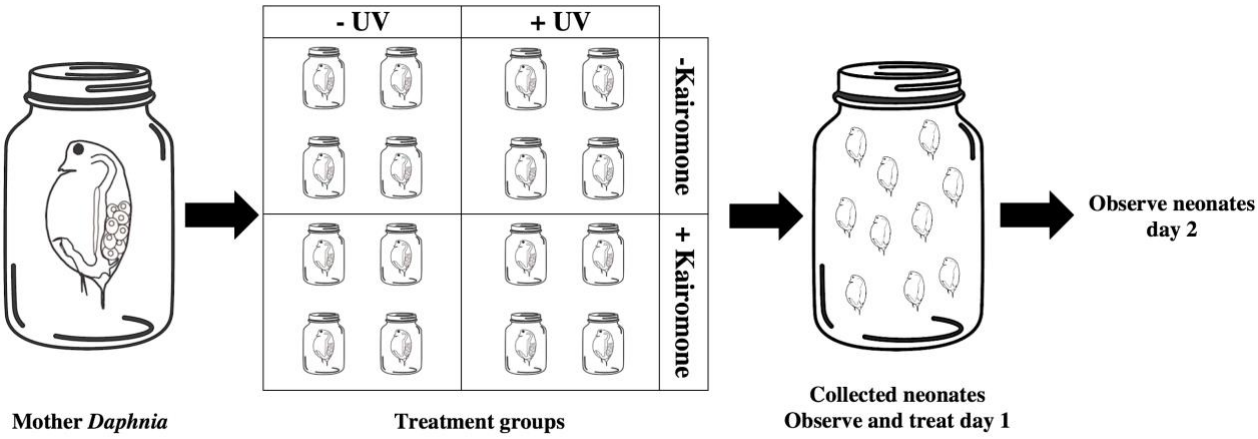

Figure 4. Flow chart of experimental design. Selected mother *D. pulex* were isolated and subject to four different treatment groups (2-3 days). Collected neonates were then observed for defense traits for juvenile stage 1 and treated the first day and observed again the second day for juvenile stage 2 (day 2).

2.6 NECKTEETH SCORING PROTOCOL

Measurements of observed defense traits were scored using Tollrian's (1993) algorithm. The scoring of neckteeth induction for juveniles is computed from the neckteeth count and an ordinal index for neck-keel development. The induction of neckteeth refers to the entire neck region. Induction scores range from 0-100%. A fully developed tooth is given an induction value of 10%, and smaller less visible teeth 5%. Tollrian identified three types for scoring of neckteeth induction. Type A with a normal head shape with no bump or keel is given an induction score of 0% and contains up to three neckteeth. Type B is neck-keel formation which adds an induction value of 30% with 2-3 neckteeth. Type C is a full neck-keel with pedestal increases this to 50% and carries 3-5 teeth. The full expression of neckteeth induction is referred to as the maximum keel which includes 5 neckteeth and a neck-keel with pedestal (Tollrian, 1993).

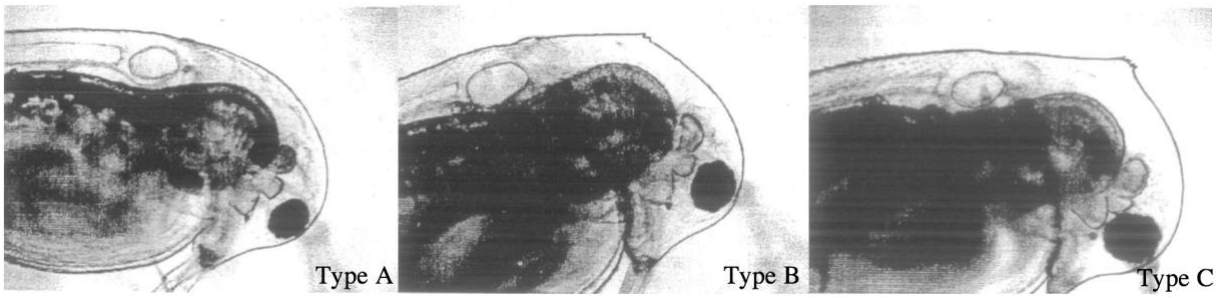

Figure 5. Images of scoring of neckteeth induction types in the second juvenile instar (Tollrian 1993).

2.7 OBSERVATION OF DEFENSE TRAIT AND SIZE

Collected *D. pulex* neonates from treated mothers from the experiment were viewed under a Nikon SMZ-U Stereomicroscope with a zoom of 1:10 and digitally photographed at 100x magnification. *D. pulex* juveniles were observed in the 1st and 2nd instar (day 1 and day 2) according to an established neckteeth scoring system (Tollrian, 1993). The images had to be taken at a specific time each day to ensure the correct juvenile stage and prevent overlaps with shedding time and size variability. *D. pulex* juveniles molt every 24 hours until the 4th instar (Weiss et al., 2015). Neckteeth count was scored from 0-5 and bump-keels produced by *D. pulex* also observed and scored by type using the Tollrian technique (Figure 5).

Figure 6. Scanning electron microscopy (SEM) images prepared by Jannicke Wiik-Nielsen from the Norwegian Veterinary institute. Image of individual P5 clone offspring in instar 2 used in the main experiment treated with kairomones. Image includes full-body, headshot and close-up of induced neckteeth with neck-keel type B.

Size measurements were completed simultaneously using a previously made *D. pulex* protocol (Appendix 7.1.4) when imaging *D. pulex* offspring. A micrometer scale (1 mm) (Zeiss) was used to calibrate the digital microscope photographs. Body length and width were measured using the image analysis program Fiji Image J with preset codes for *D. pulex*

(appendix 7.1.7). Body length and width (mm) was measured from the top of the head to the base of the tail spine.

2.8 STATISTICAL ANALYSES

All statistical analyses were completed using the open-source statistical software R statistics (R Core Team, 2017).

2.8.1 ANALYSIS OF PREDATOR INDUCED MORPHOLOGY

The effects of UV radiation on kairomone in relation to instar and clone ID were analyzed using different statistical models in order to see if (i) UV radiation had an effect on neckteeth formation and body size, (ii) there were significant differences in response rates between clones, and (iii) if mother ID played a significant role (see details below). Graphical representations with ggplot2 (Figures 8-16) and linear models were used initially to explore effects of and interactions between clone, treatment, and instar. Since neckteeth scores have statistical properties that cannot be fully represented with the Gaussian distribution use of standard linear models (constrained between 0% and 100%, with an overabundance of extreme scores (0% and 100%, corresponding to no neckteeth and fully developed neck-keel with the maximal number of teeth (5)), we used Bayesian Multilevel models (MLMs) to develop the final models for the neckteeth induction scores.

2.8.2 BAYESIAN ANALYSIS

BAYESIAN ANALYSIS FOR BODY SIZE

Bayesian analysis was used for the analysis of body size of treated offspring with model development. For the *D. pulex* species, neckteeth formation has been proven to be linked to body length and width (Sell, 2000). Body length and width in juveniles is linked to the defense mechanism of induced morphology by predator cues by reducing capture rate by engulfment of *Chaoborus* species. Based on limited differences in measured values for body length and width, body length was selected as a variable for statistical analysis. To account for the statistical properties of body length with values varying from 0.5 to 1.6 (mm), the brms normal distribution (family = gaussian) was used. A total of 4 chains were run with

4000 iterations and 2000 warm-ups, totaling 8000 post-warmup samples. The mu and sigma estimate parameters were used in this model. The sigma parameter is used to account for residual error variance (Bürkner, 2017). Mother ID was also nested in model development as a group level random effect.

BAYESIAN ANALYSIS FOR NECKTEETH INDUCTION

Bayesian models were developed in close collaboration with Tom Andersen at the University of Oslo and Raoul Wolf at the Norwegian Water Institute. Multilevel models (MLMs) allow for the modeling of data with a grouping structure, such as neonates from the same mother. The brms package (Bürkner, 2017) was used to make Bayesian multilevel models for the neckteeth induction score of instar 2 offspring. Individuals in instar 2 were exclusively selected for model development as neckteeth expression is prevalent in instar 1 for individuals treated with no kairomones however, the induction of neckteeth in instar 2 is exclusively for individuals treated with kairomones (Agrawal et al., 1999; Tollrian, 1993). We also excluded infected individuals from this data set since the number was small (29 to 578) and since exposure to infection was not part of the original design.

To account for the particular statistical properties of the neckteeth induction scores, we chose to model the observations using the so-called 0/1-inflated beta distribution. The beta distribution, which is only defined on the unit interval (from 0 to 1), is a common model for probabilities and other quantities defined over a restricted range such as Tollrian's (1993) neckteeth induction scores. The brms package implements the 0/1-inflated beta distribution (family = zero_one_inflated_beta) as a so-called hurdle model with 3 parameters, mu, zoi, and coi. The zoi parameter is the hurdle probability of an observation being 0/1-inflated or not, while the coi parameter is the conditional probability of being equal to 1 given that the observation is 0/1-inflated. The mu parameter gives the expected observation value given that the 0/1-inflation hurdle is not exceeded (i.e., non-hurdled). All three parameters (mu, zoi, and coi) can be modelled as linear functions of the treatment combinations and clonal or maternal grouping

The brms package serves as a frontend for the STAN system for Bayesian computing (Bürkner, 2017), which allows STAN models to be defined in a syntax very similar to other R functions like lm, glm, lme, lmer, etc. The STAN code generated by brms is then

forwarded to STAN which generates samples from the conditional distribution of the model parameters given the observations, which is also called the posterior distribution. The posterior distribution samples can then be used to compute confidence intervals for model parameters and other model performance indicators like information criteria and leave-out-one cross-validations. A typical brms model would look like this:

```
brm(brmsformula(Neck_teeth_induction ~ Kairomone * UVR + (1 | Mother_ID),
        zoi ~ Kairomone, coi ~ Kairomone), data =.,
        family =zero_one_inflated_beta, iter=4000)
```

This means that the expectation (mu) of the neckteeth induction score (rescaled to the unit interval by dividing by 100) is modeled by the full factorial effect of Kairomone and UVR (i.e., both marginal and interaction effects) with a random effect of maternal grouping. The hurdle parameters (zoi and coi) are both modelled by only the kairomone treatment, since this treatment is expected to have the strongest impact on extreme scores of full or no neckteeth development. The setting shown above means that a STAN sampling chain is run for 4000 iterations, of which the first half is discarded, and the rest retained for analysis. Since STAN can use multiple processor cores the current setting allows for running 4 independent chains in parallel, giving 8000 samples per run. The use of multiple chains also allows for monitoring model convergence by the so-called Rhat statistic, such that a Rhat value close to 1.00 means that the model has converged successfully.

Maternal influences were selected as random effect. Maternal influences are prevalent in the *Daphnia pulex* species, and responsible for the induction of defense traits (Dodson, 1989). Use of mother ID as a random effect in model development help to investigate any possible variation in response due to the mothers' genetic background. Use of mother ID as a random effect would provide insight as to whether the response rates of neonates were a collective mechanism or more specific to the clutch group. Observing maternal influences helps determine if the random effect of mother ID was significant for the response variable of neckteeth formation and body size in this experimental design. *Daphnia pulex* are known to have the ability exhibit strong maternal effects (Kim et al., 2015; Tollrian, 1995) on offspring. The residual standard error (RSE) for maternal influences was measured by comparing the standard deviation intercept of mother ID with the inverse of the family parameter Phi. Results from the measured RSE value for mother ID is meant to serve as the

standard deviation of the observed neckteeth induction values deviate from the values predicted by the model.

3 Results

The development of a two by two factorial design produced significant differences in response rates of defensive morphological traits in *Daphnia pulex.* Over the course of the experiment, collected mothers of two clones of *D. pulex* were exposed to *Chaoborus* kairomones and UV radiation. The development of defensive traits was then observed in the first and second instar of offspring (Fig. 7-11). Statistical analyses were executed to determine the statistical backing of the varying response rates to treatments.

3.1 PILOT EXPERIMENT

Results from the pilot experiment were deemed successful as neckteeth formation was induced in all offspring exposed to UV treated kairomones. UV-A radiation exposure on kairomones of up to 8 hours allowed for the full expression of neckteeth (Figure 7). The main experiment was then able to commence as the integrity of kairomones had been proven sufficient to use in the two by two factorial design.

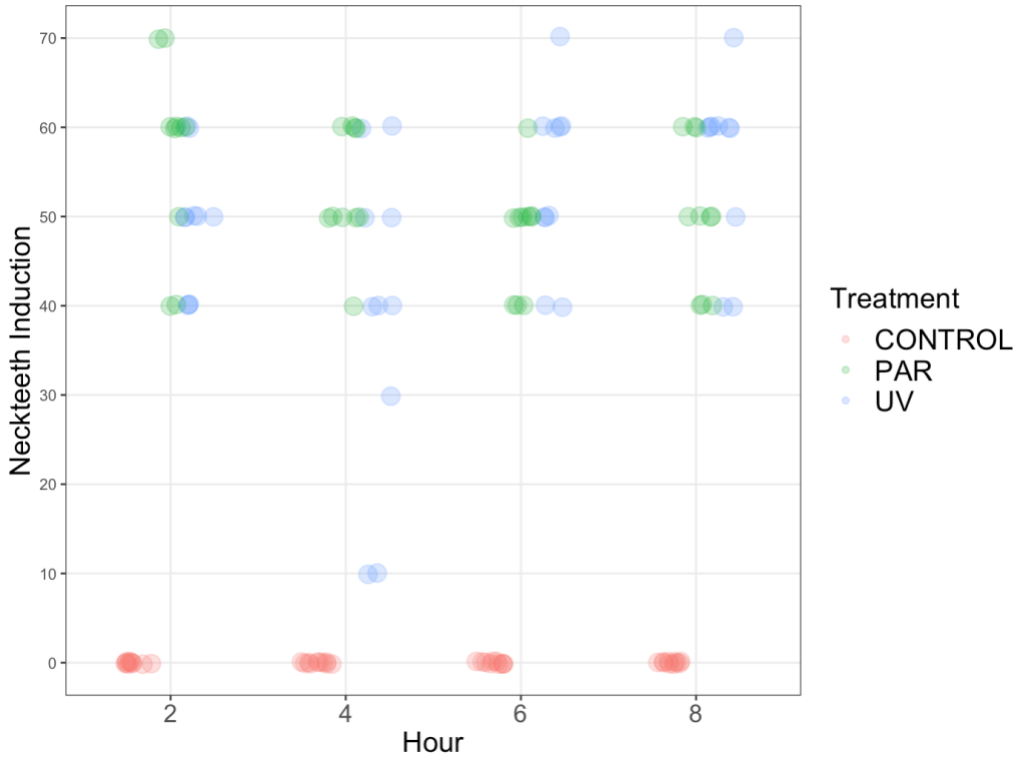

Figure 7. Measured values of neckteeth induction of offspring of UNI clone in instar 2 from pilot experiment. Measured induction values of treated kairomones collected in 2-hour increments.

3.2 EFFECTS OF TREATMENTS ON BODY SIZE

The body length and width of offspring was measured using analyzed microscope images with the ImageJ program (Appendix 7.2.5). Body length, body width and spina length were all measured, but body length was select to represent the measured value for body size. Clone group UNI showed a significant decrease in body size for +UV/+K treated individuals when compared to the P5 +UV/+K treatment group (Figure 8). UV radiation treatment had a significant impact on body length of the UNI clone, while the coastal P5 clone showed greater values in body length than the -UV/+K treatment group.

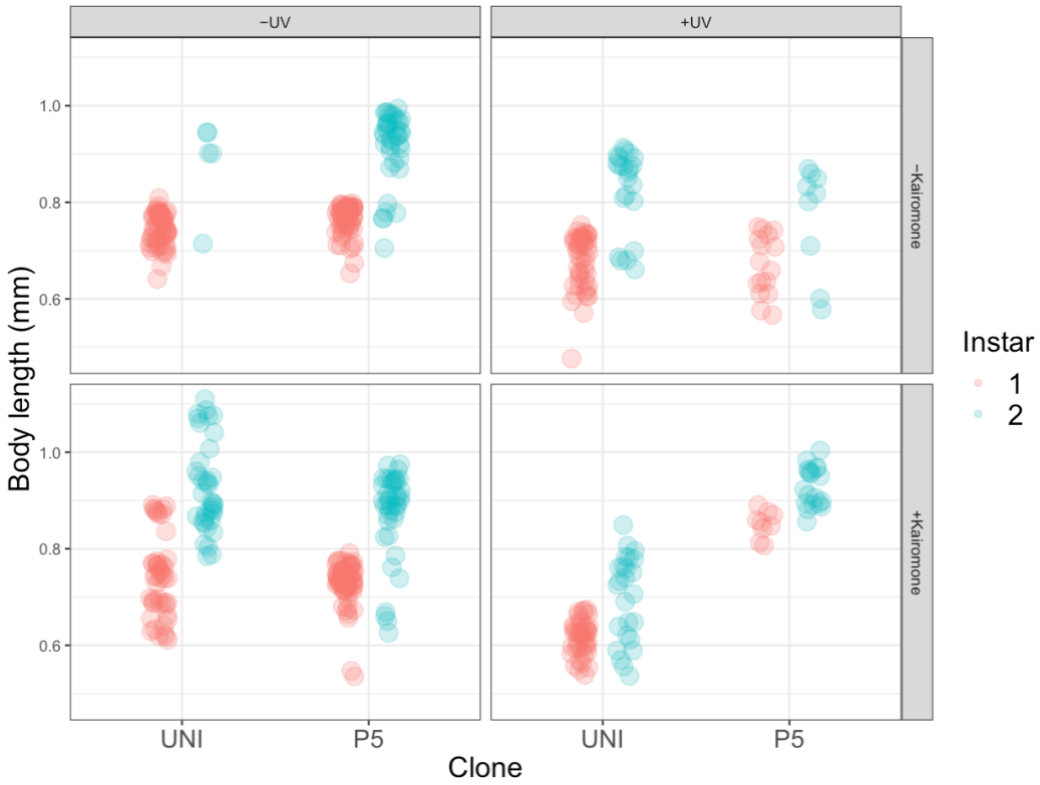

Figure 8. Measured values for body length (mm) of collected offspring for both clones (UNI – P5) in instar 1 and 2 of two by two factorial design.

Clone group UNI expressed the values than P5 in body length for the -UV/+K treatment group of values up to 1.6 mm, consisting of the greatest values measured among all treatments and clones (Figure 8). Observed instar 2 values were greater than instar 1 as expected.

Variability in length for P5 was seen for treatment groups exposed to UVR (figure 8), groups treated with UVR expressed body lengths greater than those treated with kairomones with no UV. This clone P5 treatment group +K +UV responded in an unpredicted manner, as for the UNI clone groups treated with UVR (+ K/ -K) expressed a significant decrease in body size for kairomone treated individuals when compared to all other treatments.

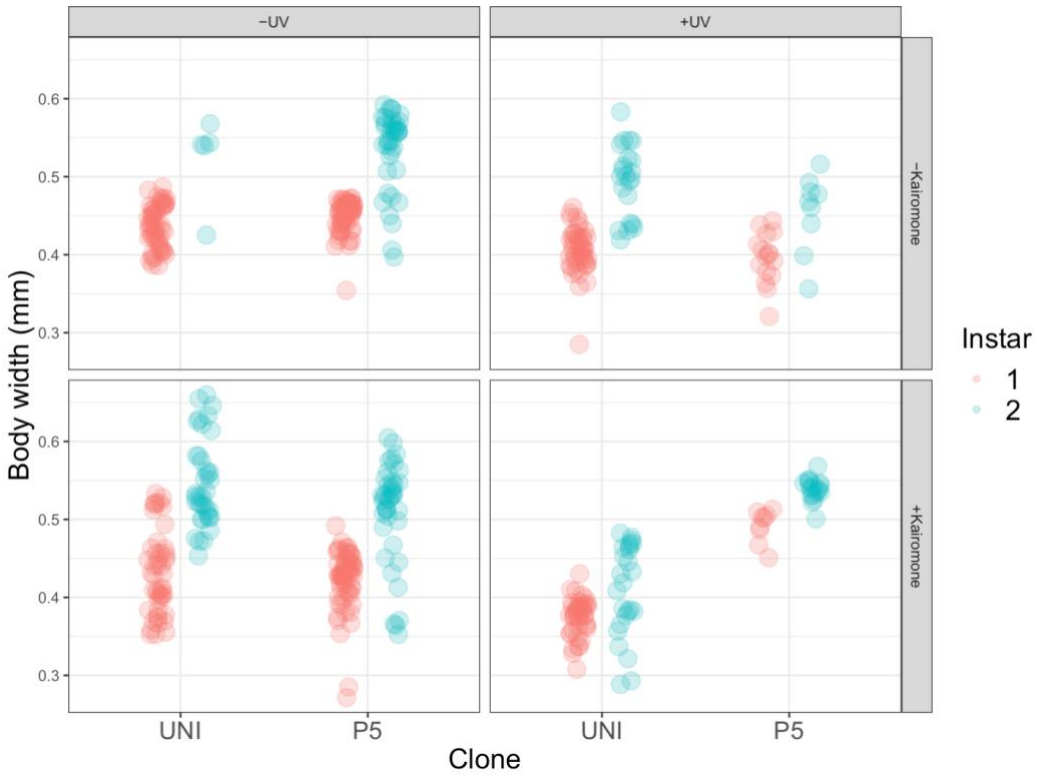

Figure 9. Measured body width values of collected offspring both clones (UNI – P5) in two by two factorial design.

Body width was measured to compare response rates between clones. The UNI clone group was able to express the highest body width value in instar 2 at 0.6596 mm for the -UV/+K treatment group. The P5 clone group however, expressed the lowest value for body width in the same treatment group at 0.2715 mm in instar 1.

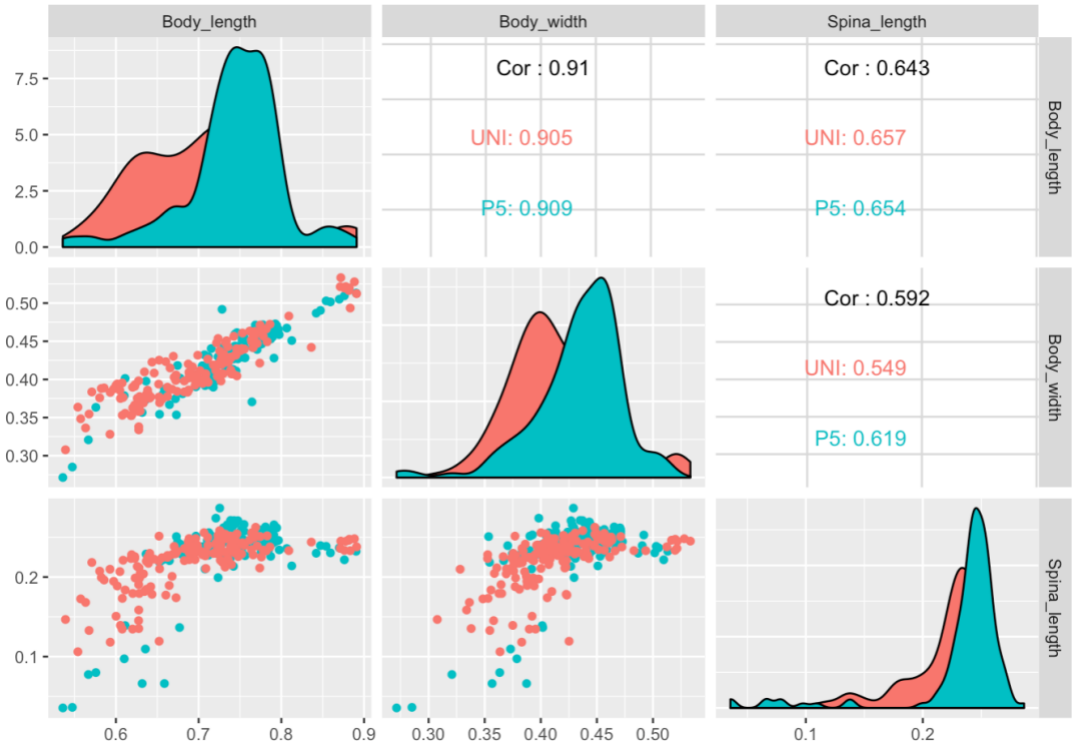

Figure 10. Scatterplot of measured body length, body width and spina length (mm) of collected offspring both clones (UNI – P5) for instar 1 of two by two factorial design.

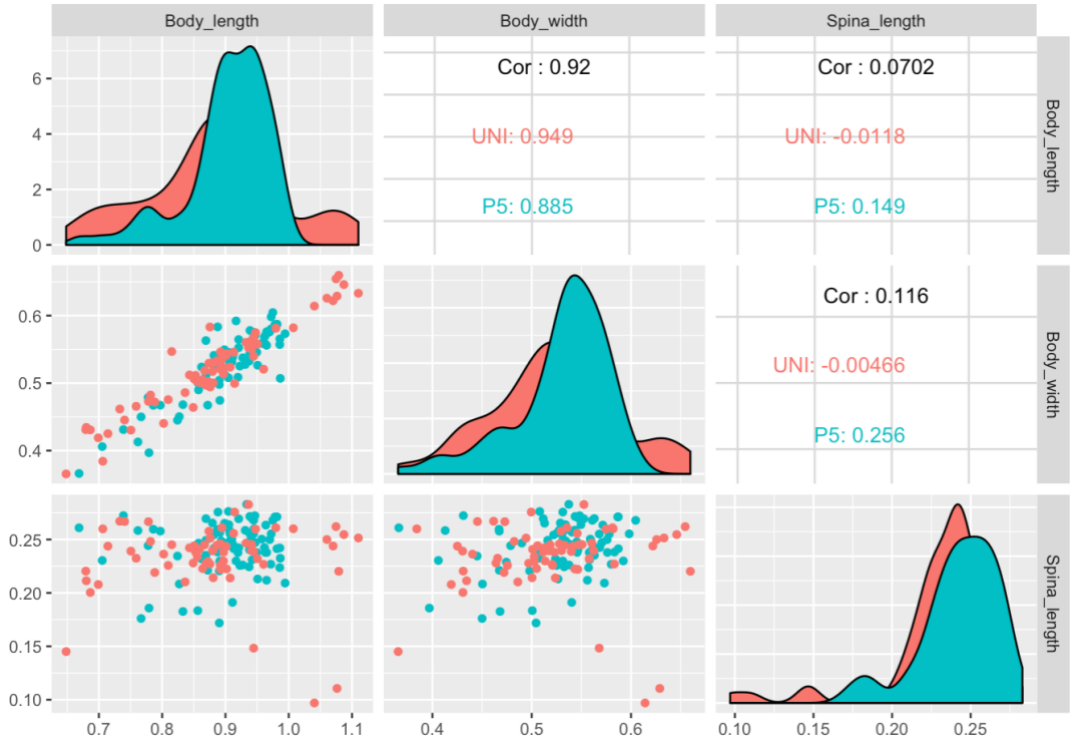

Figure 11. Scatterplot of measured body length, body width and spina length (mm) of collected offspring both clones (UNI – P5) for instar 2 of two by two factorial design.

Body length was selected for statistical analysis due to the limited differences in measured values for body length, body width and spina length (Figures 10 & 11). Clones selected in this experiment had not previously exhibited varying lengths in spina length in response to different kairomone concentrations, so this value was purposefully excluded from analysis (Erik Sperfeld, Unpublished). Body length was selected as a sufficient variable for analysis of differences in body size between clones and treatment groups.

3.3 BAYESIAN ANALYSIS OF BODY SIZE

Bayesian analysis of body length was conducted in order to obtain statistical support for inter-clonal differences in measured body length in instar 2 to the two by two factorial design. A simple gaussian brms model was used for body length values varied from 0.5 to 1.6,

MODEL	BRMS FORMULA
BRM.A	Body_length ~ Kairomone + (1| Mother_ID)
BRM.B	Body_length ~ Kairomone + UVR (1|Mother_ID)
BRM.C	Body_length ~ Kairomone + UVR + Clone + (1|Mother_ID)
BRM.D	Body_length ~ Kairomone + Clone + (1|Mother_ID)
BRM.E	Body_length ~ Kairomone * UVR + (1|Mother_ID)
BRM.F	Body_length ~ Kairomone * UVR * Clone + (1|Mother_ID)
BRM.G	Body_length ~ Kairomone * UVR + Clone + (1|Mother_ID)
BRM.H	Body_length ~ Kairomone * Clone + UVR + (1|Mother_ID)

Table. 1 Formulated Brms formulas for body length used for comparison with WAIC values in R. Model column is for the model ID and brms formula represents the formula composition of each model tested.

Watanabe-Akaike information criterion (WAIC) values with the use of the leave-one-out cross-validation (loo) method was used for model selection (Table 1). Simpler brms formulas were used to prevent over-estimation by over-parameterization of the models. Top selected models based on measured WAIC value were models G, F, and E (Table 2). Models G and E had the lowest WAIC values however, model G which included the clone variable carried the greatest model weight at 0.67 out of 1 (Table 2).

Model	***WAIC diff***	***SE diff***	***Model weight***
BRM.G	0.0	0.0	0.00
BRM.E	0.20	0.87	0.09
BRM.F	3.63	6.85	0.60
BRM.B	11.60	9.58	0.00
BRM.C	11.78	9.90	0.00
BRM.A	14.11	12.73	0.00
BRM.D	14.24	12.80	0.32
BRM.H	14.59	10.15	0.00

A

Model	***WAIC diff***	***SE diff***	***Model weight***
BRM.G	0.00	0.00	0.67
BRM.F	0.77	0.76	0.33
BRM.E	4.09	6.92	0.00

B

Table 2. A) Results of Compared Watanabe-Akaike information criterion differences (WAIC diff), standard error differences (SE diff), and compared model weighted values. B) Final comparison of best fit brms models for body length in instar 2.

The final model that was selected was model G, which contained the interaction of UV and kairomone added with clone ID. Plots were created using the shinystan package in order to observe patterns in plots of group-level and population-level effects of posterior distribution. The mean for the posterior distribution of the population level effect clone P5 and the +UV/+K treatment group were positive (Appendix 7.1.8). This indicates that the model predicts these a greater length in offspring size for the P5 clone and those in the treatment group +UV/+K.

Results from Model G predictions show that kairomone and UV treatment groups had an antagonistic effect on measured body length for both clones. The model estimated the kairomone treatment group to have no effect on body length (-0.02 ± 0.02 mm). It was predicted that the UV treatment group would have a 0.12 ± 0.03 mm decrease in body length. The UV x Kairomone interaction was estimated to have a 0.01 increase (-0.12 – 0.02 + 0.15 = 0.01) in body length compared to the control group as well. Model predictions did not support observed differences between clones and treatment groups and that there (Figure 12). Observed vs. predicted values from model G show that the values for predicted body size were much higher than the observed.

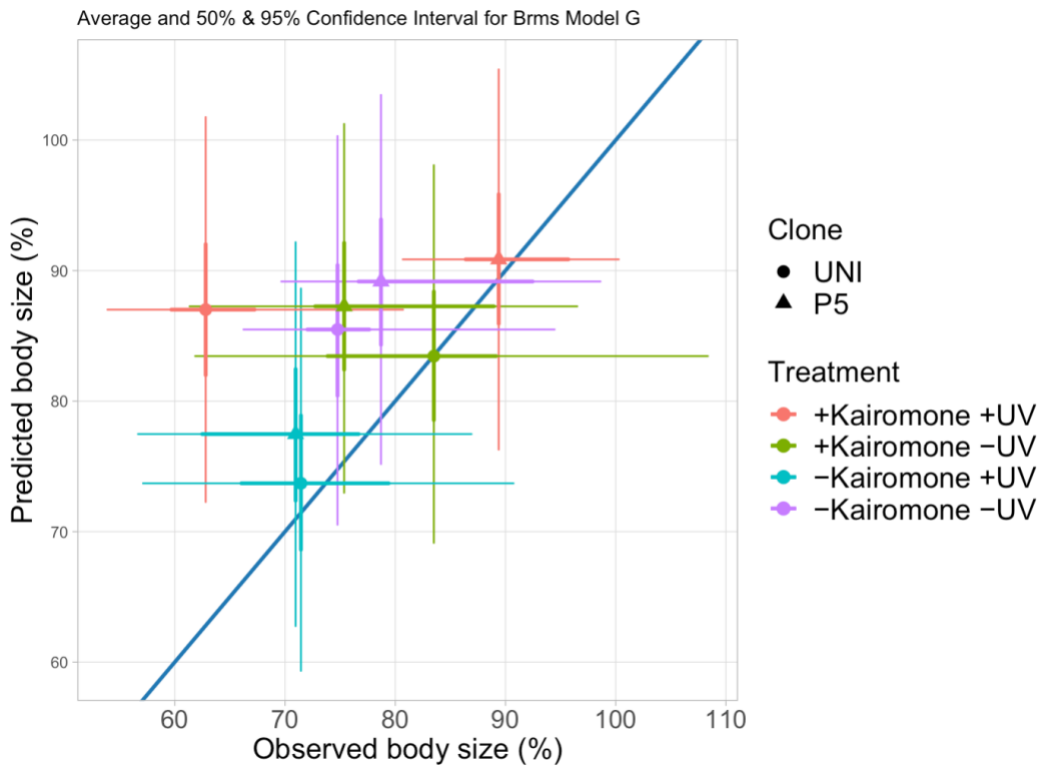

Figure 12. Plot of observed vs predicted values for body length (mm) for offspring of both clones (UNI – P5) in instar 2 using brms model G. Average 50% and 95% confidence interval values for predicted values versus the mean values (%) of observed body size values with linear regression line. Treatment values were placed together in order to visualize differences between response rates of clones in treatments.

Maternal influences were nested in the model as a group level random effect. The variance contribution for mother ID in model G was 2.8 times the residual variance. This allowed us to conclude that the random effect of mother ID played a large role for measured body length in offspring in instar 2 and justified the use of mother ID as a nested random factor in neckteeth induction model development.

3.4 TRENDS IN BUMP-KEEL SCORES

For the first portion of the experiment, morphological traits were observed and scored using Tollrian methods (Tollrian, 1993). Bump-keel scores of all neonates were categorized by type and recorded. Figure 11 shows scoring results of bump-keel development in neonates. The expression of Type A and Type B were prevalent in both clone groups. Bump-keel induction was greatly reduced for groups treated with UV and kairomones, as compared to the

kairomone only treatment group in which a portion expresses the a fully developed Type C neck-keel with pedestal.

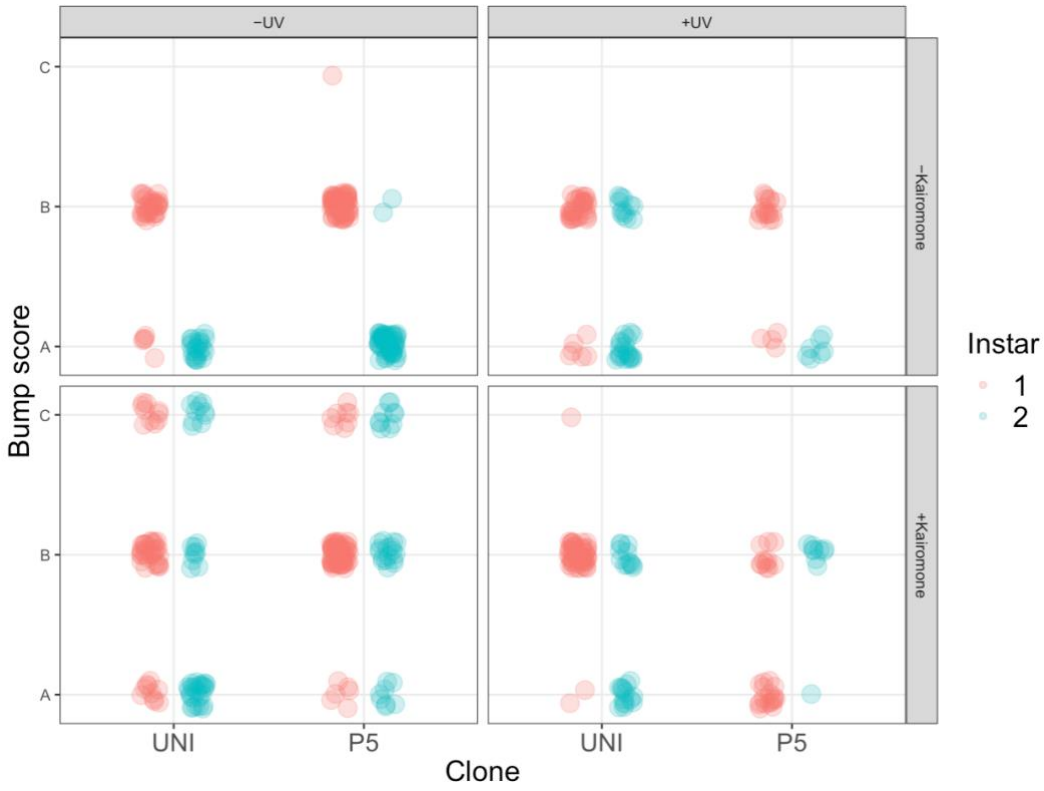

Figure 13. Scatter plot of measured bump score (A-C) for both clones (UNI – P5) values using Tollrian (1993) scoring system in two by two factorial design.

Overall, the observed bump-keel scores were greatly reduced in both clones for UV treatments, with only 1 individual in the +UV column one with a bump score of Type C in the UNI clone group, expressing a fully developed neck-keel with pedestal. This is important to note, as bump-keels play a significant role in the induction score calculation (accounting for up to 50%). As seen in figure 13, majority of bump-keel formation was Type B, this is surprising as the concentration of kairomones used (60µL) should have allowed for the full induction of defense traits for instar 2 in the -UV/+K treatment group.

3.5 EFFECTS ON NECKTEETH FORMATION

Individual neckteeth formation was observed and scored to later be used for calculating the induction values of the morphological defense trait of neckteeth formation. Examined neonates expressed neckteeth count ranged from 0 to 5. A limited number of individuals from

groups exposed kairomone showed the full expression of five neckteeth in both clones. The addition of the UV radiation as a stressor had a consequential effect on neckteeth formation for both clones of *D. pulex*. Overall, UV radiation had a significant impact on the juveniles' ability to express neckteeth. Values for the control groups were as expected for day 2 with no induction, with the exception of two individuals in the P5 clone group. Average values for the kairomone treatment groups were notably low, as only a small portion of offspring were able to produce induction values of 100% (Figure 16).

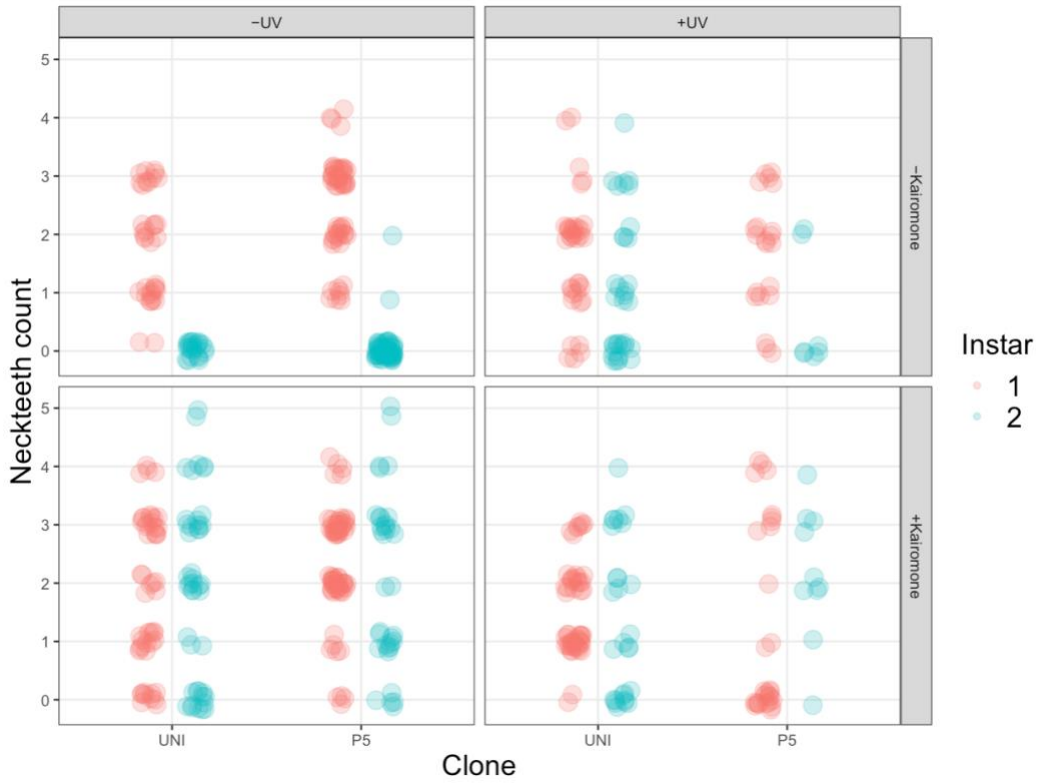

Figure 14. Scatterplot of observed neckteeth count (1-5) for both clones (UNI – P5) in instar 1 and 2 in two by two factorial design.

Although UV radiation had an obvious impact on neckteeth induction values, neonates from both UV groups were able to express up to 4 neckteeth. The expression of neckteeth formation in the second instar is highly conserved for individuals exposed to kairomones as the induction of neckteeth formation is very costly. Notably a few individuals however, appeared to be an exception for reduced induction in instar 2 with no kairomones. A conspicuous portion of individuals treated with UV but no kairomones expressed neckteeth in the second instar of juvenile development. As seen in Figure 14, individuals treated with UV-A and no kairomones expressed neckteeth greater than 1 (2-3) in second instar of juvenile

development. Under these conditions, a portion of individuals treated with +UV/ -K expressed neckteeth induction in the second instar in both clone groups. Two neonates of the P5 clone were observed to expressed neckteeth in the second instar of the control group,

Measured bump-keel values in instar 1 and 2 were compared to neckteeth count to determine if the expression of both traits had expressed a similar trend in development for all treatment groups (Figure 15). Neck-keel development scores showed a great range in neckteeth count per type of bump-keel with values ranging from 1-4. Examined results in variability of neckteeth score for each neck-keel type is in support of the findings in Tollrian's scoring system (Tollrian, 1993).

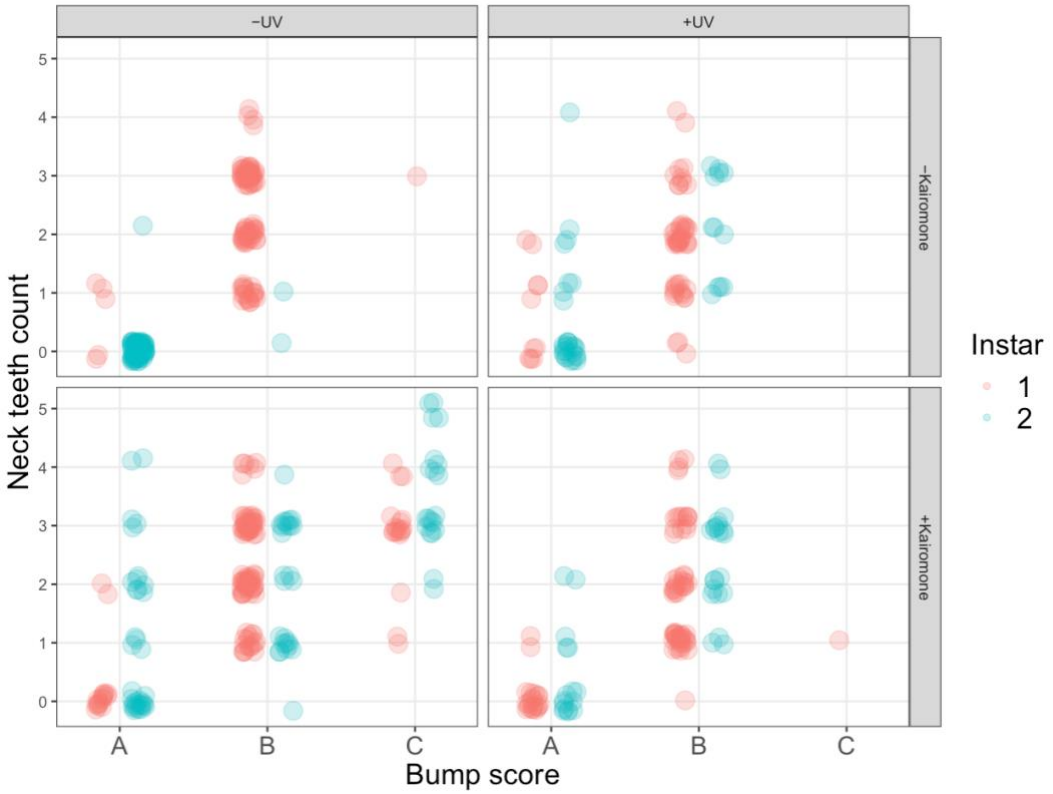

Figure 15. Scatterplot of observed neckteeth count vs bump score for both clones in instar 1 and 2 in two by two factorial design.

The expression of a Type C neck-keel in the second instar is only present in the -UV/+K treatment group. There were few individuals whom were able to express a Type C neck-keel in the first instar. The response rate across all clones show a significant decrease in neckteeth induction. As seen in figure 8, the expression of Type C bump-keel was uncommon for neonates exposed to UV. This could provide insight into the high costs of bump-keel

development when compared to neckteeth count, as neckteeth were expressed from 0-4 for UV treated groups. The most prevalent type of neck-keel was Type B among all treatment groups for instar 1. Among all treatment groups, the expression of Type B neck-keel is the most prevalent, as seen in Figure 15 as well.

The induction of neckteeth was consistently lower in UV treated groups. Measured values were given induction scores using protocol created by Tollrian (1993) with values ranging from 0-100% (Figure 15). Results shows that neckteeth induction was greatly reduced in groups treated with UV (Fig. 7-9). Kairomone treatment had the most impact on neckteeth formation for both clones (95% CI: 40% to 100% increase in number of neckteeth). The kairomone x UVR interaction resulted in a decrease in neckteeth for both clones as well (95% CI: 20% to 50% decrease).

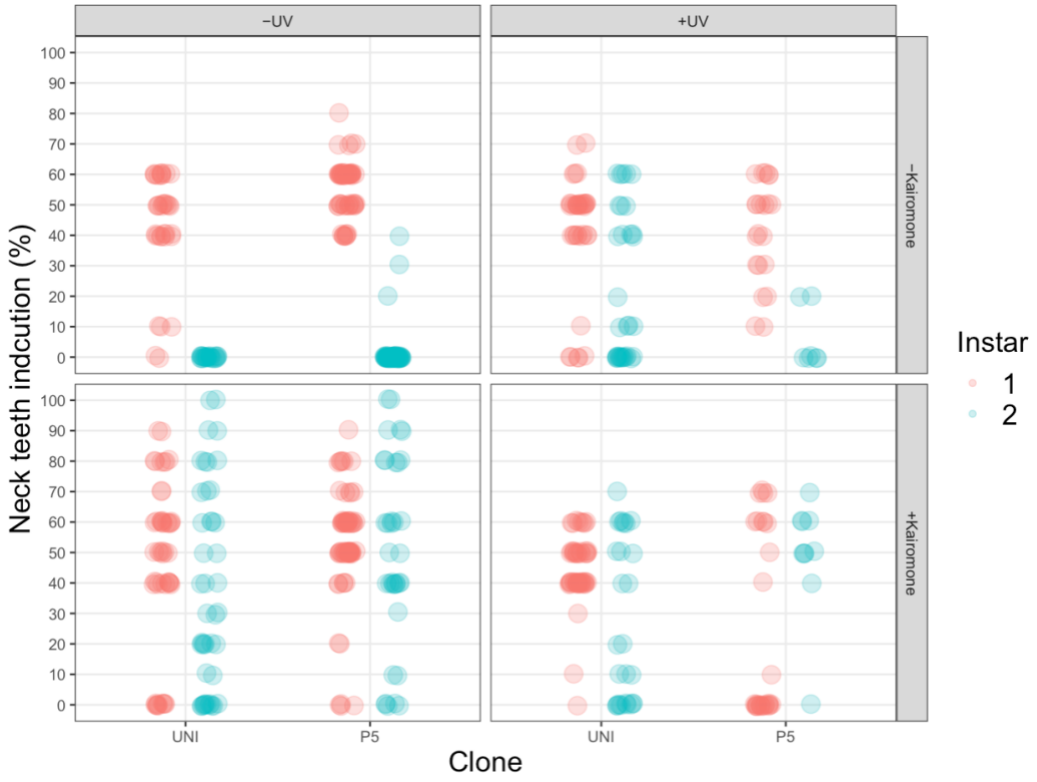

Figure 16. Scatterplot of calculated neckteeth induction (%) of collected offspring of both clones in two by two factorial design using Tollrian scoring system (1993).

The UV and kairomone treatment group showed great differences in response rates between clones. The P5 clone showed an overall reduced induction rate for instar 1 (20%), while the UNI clone group expressed a mean induction values of 50% for instar 1. Trends in major

differences between clones is largely in part due to the varying neck-keel values scored between clones. As seen in Figure 17, the P5 clone expressed a reduced value for neck-keel type, affecting the overall score of neckteeth induction for this clone for the +UV/+K treatment group.

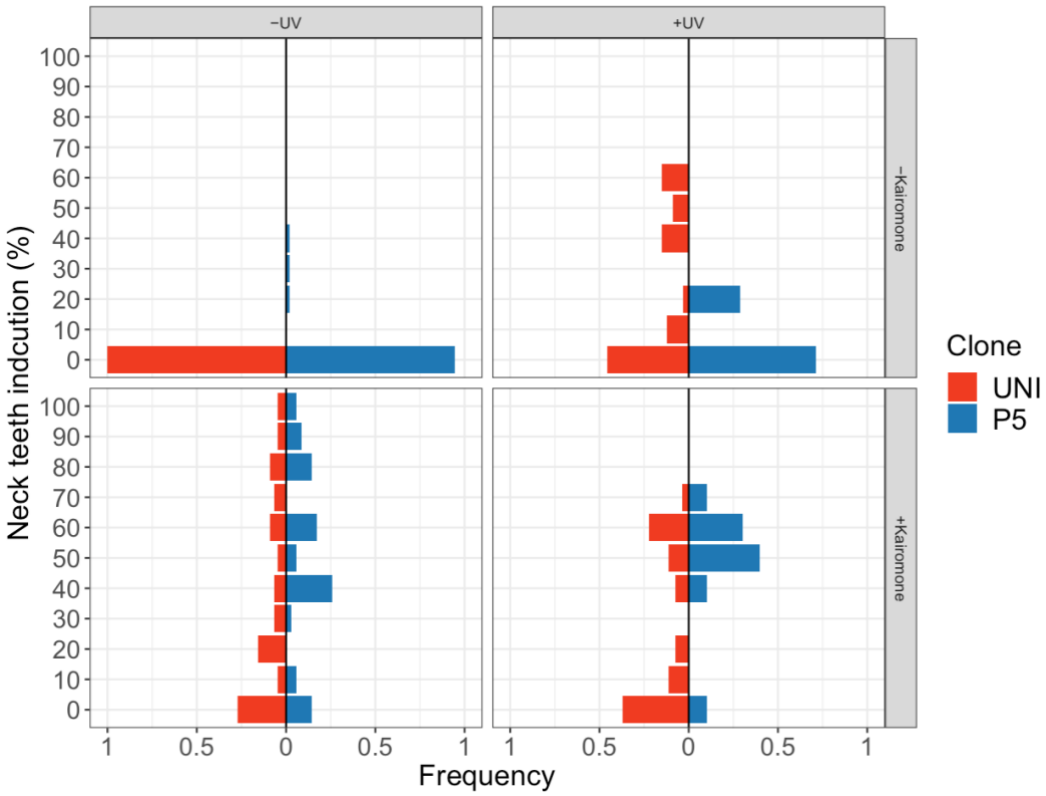

Figure 17. Boxplots of neckteeth induction (%) vs frequency of offspring in instar 2 of all treatment groups for both clones.

Variation in the frequency of response to predator cues was prevalent among the two clones used. As seen in Figure 17, the UNI clone group expressed a greater range of neckteeth induction for UV treated groups in instar 2. While the P5 clone expressed greater neckteeth induction values for the +UV/+K treated groups. The plotting of neckteeth induction versus the frequency of individuals in instar 2 shows that both clones responded to treatments with similar trends for control groups (-UV/-K), as neckteeth formation was greatly reduced for control groups as expected and increased for kairomone treatments (Figure 17). Maximum induction of neckteeth (100%) did not occur in groups treated with UV and was exclusively for groups treated with kairomones. The distribution of neckteeth induction is similar in both clones for the -UV/+K treatment group (Figure 17). Results supports the use of potent kairomone use, as induction was possible in both clones.

Figure 17 shows possible support for inter-clonal differences in neckteeth induction as there were significant differences between response rates of treatment groups. Neckteeth formation in clone group P5 had an overall greater expression of neckteeth in all treatments when compared to UNI (Figure17/Appendix 7.1.8). As the clone P5 neonates had a greater induction value for the frequency of individuals treated with kairomone only (Figure 17). *Daphnia* in the P5 and UNI clone group showed unexpected expression in neckteeth for groups not treated with kairomones for instar 2. Treatment +UV/-K had a small portion of induction of neckteeth for instar 2 for both clones (Figures 14-17). As seen in Figure 11, clone UNI expressed a greater range of neckteeth induction in response to UV with no kairomones (0-65% induction). Versus the coastal P5 clone which exhibited a much lower induction value for the +UV/-K treatment group (0-20% induction).

3.6 BAYESIAN ANALYSIS OF NECKTEETH INDUCTION

Bayesian multilevel models (MLMs) were used in order to observe the statistical relevance of results from experimental treatments. Results show the odds for neckteeth induction for juveniles treated with kairomone to be a 1.18-fold greater than the odds for the control group (Appendix 7.1.7). The developed Bayesian MLM predicts greater indication that induction for kairomone treatment, but also shows that UVR treatment and clone ID were also relevant to the neckteeth induction response in instar 2.

MODEL	BRM FORMULA
BRM.8	Neckteeth_induction ~ Kairomone * UVR + Clone + (1| Mother_ID)
BRM.9	Neckteeth_induction ~ Kairomone + (1|Mother_ID)
BRM.10	Neckteeth_induction ~ Kairomone * UVR + (1|Mother_ID)
BRM.11	Neckteeth_induction ~ Kairomone + Clone + (1|Mother_ID)
BRM.12	Neckteeth_induction ~ Kairomone + UVR + Clone + (1|Mother_ID)
BRM.13	Neckteeth_induction ~ Kairomone * Clone + UVR + (1|Mother_ID)
BRM.14	Neckteeth_induction ~ Kairomone * UVR * Clone + (1|Mother_ID)
BRM.15	Neckteeth_induction ~ Kairomone * Clone + (1|Mother_ID)

Table 3. Formulated simple brms formulas for neckteeth induction in instar 2 for both clones used in model comparison.

Model selection was done by comparing Watanabe-Akaike information criterion (WAIC) values with the use of the leave-one-out cross-validation (loo) method in R (Table 4). Simpler brms formulas were used to prevent over-estimation by over-parameterization of the models. brms formulas for neckteeth induction contained adjusted zero one probability (zoi) and conditional one probability (coi) parameters for the kairomone effect in order to prevent over estimation. Variables were added as interactive or additive when investigating the best fit model. Mother ID was selected as a random fixed variable

Model	*WAIC diff*	*SE diff*	*Model weight*
BRM.14	0.0	0.0	0.50
BRM.10	0.63	2.8	0.44
BRM.12	18.57	11.08	0.00
BRM.13	19.36	11.66	0.00
BRM.8	19.61	16.19	0.03
BRM.9	19.61	16.19	0.03
BRM.11	20.31	16.34	0.00
BRM.15	20.95	16.63	0.00

A

Model	*WAIC diff*	*SE diff*	*Model weight*
BRM.10	0.00	0.00	1
BRM.9	18.97	14.47	0
BRM.8	18.97	14.47	0

B

Table 4. A) Compared Watanabe-Akaike information criterion differences (WAIC diff), standard error differences (SE diff), and compared model weighted values. B) Final comparison of best fit brms models for neckteeth induction in instar 2.

The brms leave-one-out cross-validation (loo) function was used in R to obtain the values found in Table 4. Models were ranked ordered from lowest (BRM.14) to highest (BRM.15) based on the WAIC difference values. The compared WAIC difference values were calculated using the value of lowest valued model BRM.14 minus the comparison model. Through model comparison, there was strong statistical support for simpler models with the kairomone effect than clone and UV effects and their interactions (Table 4).

Model 14 represented the interaction of kairomone, UVR and clone (Table 3). Although this model weighed to be significant when compared to other models (Table 4A), Model 14 alone did not hold enough model weight, as chains did not fully converge during model development. The model with the second lowest WAIC value Model 10 was then used to

compare to the top two models with greatest compared model weight (Table 4B). Model 10 argued for the kairomone and UV interaction (Table 3) and was fully supported with a model weight of 1 when compared to the second highest ranked model based on WAIC values. Model 10 values were used for statistical analysis of neckteeth response rates of offspring. Model 14 was used for visualization of clone response rates to treatments and the unexpected induction of offspring in instar 2 for the +UV/-K treatment group.

As kairomone treatments result in great differences in instar 2 between control and treatment groups, the drastic differenced had to accounted for in order to prevent the over-estimation of values. The zero one probability (zoi) and conditional one probability (coi) were given the logistic links of the kairomone effect to solve this issue. The odds for zero one inflation were estimated to be 0.063 folds less in the kairomone treatment than the control. While the odd of conditional one probability was 134-fold more for the kairomone treatment than the control. These results support the observed results of neckteeth induction as individuals treated with kairomones expressed a greater range of expression of the defense trait (Figures 13-17).

Model 10 estimated that the kairomone treatment group will have 1.18-fold greater odds in neckteeth induction than the control group. UVR treatment odds were predicted to result in 43% less neckteeth induction than the control. While using a zero one inflated beta distribution with brms, zoi and coi values had to be adjusted to fit the parameters of the model. As seen in figure 17, inter-clonal differences were evident for both of the UV treatment groups (+UV/+K and +UV/-K). Predicted values expressed a greater range in neckteeth induction and observed.

Model 14 was selected to show observed vs predicted values due in order to observe the Kairomone x UVR x Clone interaction. Plots were then implemented in order to show range of observed values when compared to predicted values in the 50% and 95% confidence interval (Figure 18). Model predictions were the closest fit to observed data for the -UV/+K treatment group. Predicted values for treatment groups produced much lower values than the observed. For the +UV/+K treatment group, the observed values for the UNI clones responded more closely to the predicted values. As seen in Figure 18, inter-clonal differences were evident for both of the UV treatment groups (+UV/+K and +UV/-K). Predicted values expressed a greater range in neckteeth induction and observed.

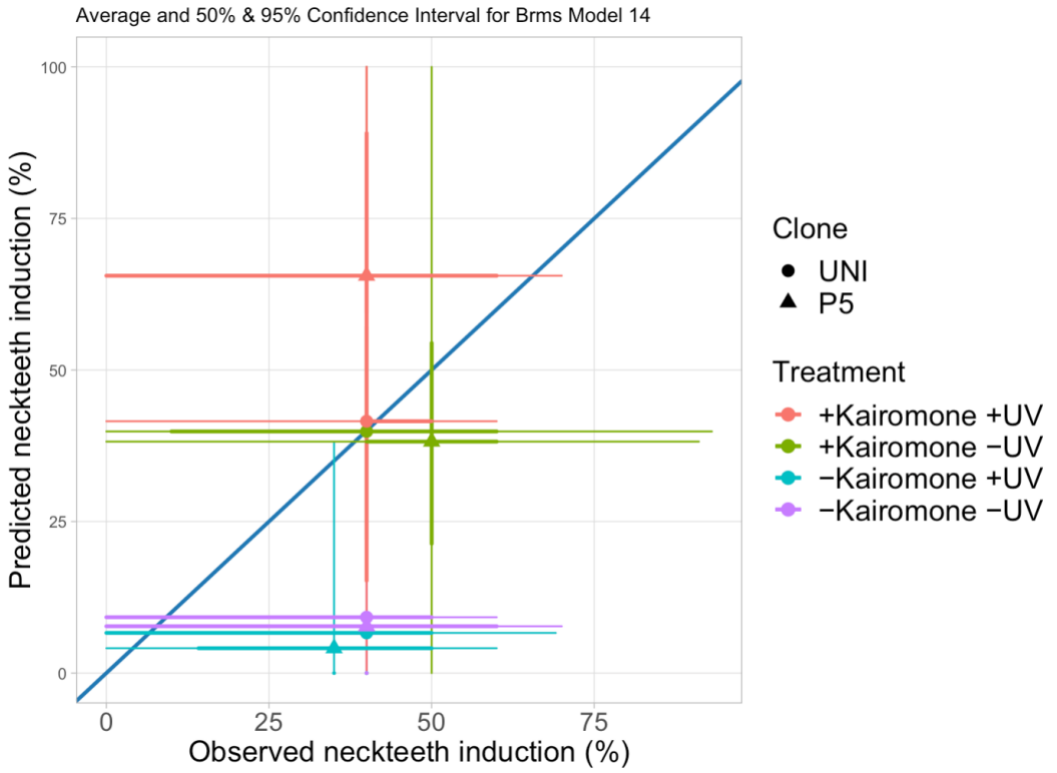

Figure 18. Plot of observed vs predicted values for neckteeth induction (%) for offspring of both clones in instar 2 using brms model 14 with Kairomone x UVR x Clone interaction. Average 50% and 95% confidence interval values for predicted values versus the mean values (%) of observed neckteeth induction values with linear regression line. Treatment values were placed together in order to visualize differences between response rates of clones in treatments.

Mother ID served as a group effect in model development (Table 3). The estimated odds ratio for mother ID was 3.63 indicating a strong influence on the induction of neckteeth. Maternal influences for neckteeth induction were measured using the Residual Standard Error (RSE). The calculated value was estimated to be 0.06. The low value indicates the close fit of the model to observed values.

3.7 INFECTION RATE IN NEONATES

A total of 29 individuals of the 578 neonates scored were identified with an unidentified parasite (see Appendix 7.1.9). These were all in the UV radiation treatment groups (+UV/+K and +UV/-K), represented by both clones suggesting possibly an immunosuppressive response to UV radiation. For the treatment group +UV/+K, the presence the parasite was

only present in the UNI clone (Figure 19). There were no signs of infection for the control groups.

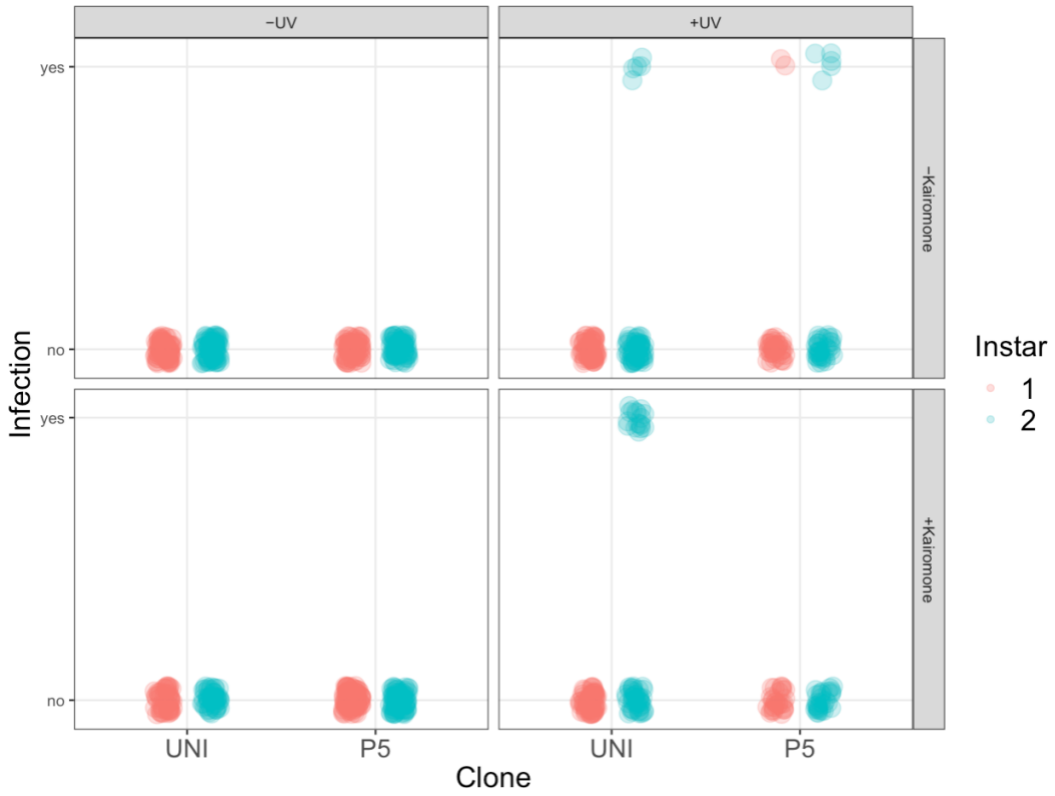

Figure 19. Scatterplot of infected offspring for both clones in instar 1 and 2 in two by two factorial design.

Infection results were small and not by effects of the design but noteworthy in the results. No statistical support was done as a way by factor of the experiment, but a naturalistic design by the presence of infection for UV treated groups.

4 Discussion

4.1 SUMMARY OF EXPERIMENTAL RESULTS

The impacts of UV radiation on inducible defenses in *Daphnia pulex* were observed in the developed two by two factorial design. First, a pilot experiment was conducted in order to test the first portion of hypothesis (1), to see if UV-A radiation would denature kairomones used in the main experiment. Second, neckteeth induction values and size among all treatments for both clones were compared (Figures 8-17). Third, the development of model systems provided sufficient statistical support in response rates of neckteeth induction of treatment variables. The experiment also resulted in a portion of individuals treated with UV-A to become contaminated with an unidentified pathogen. Results and statistical analyses support the hypotheses that (1) UV-A radiation treatment was a significant stressor on neckteeth formation when interacting with the kairomone variable and (2) inter-clonal differences were evident in the development of body size of offspring.

4.1.2 BODY SIZE AND MATERNAL INFLUENCES

Use of a two by two factorial design allowed for the observation in varied responses of offspring between clones and treatment groups. Measured values in body length showed distinct differences in response rates between clones. P5 clones responded to +UV/+K with increases in body length, with values equivalent and greater than those in the-UV/+K treatment group. While clone UNI responded poorly to UV, with the +UV/+K treatment group having the lowest body length values of all treatment groups. Increases in body length are a response to predatory cues. The P5 clones was able to induce an increase in size despite the additional UV stressor even in the +UV/K treatment group, which is an goes against previous studies conducted, where UV radiation as a main stressor was found to cause a decrease in body size (Hansson et al., 2007). Limited values in measured values for control group instar 2 values was due to technical difficulties caused by image file corruption.

Results from Bayesian analysis showed that while there was strong support for maternal influences on body size, body size in relation to the treatment groups was limited. Response rates among individuals of different mother ID provides insight on the convergent phenotypes of genetic differences in response rates among clones on the individual level

(Dennis et al., 2014). By focusing on response rates of clutch groups from different mothers, this provides the opportunity to look at the effects of UV radiation on the individual level versus the population level.

Variability in the response rates for neonates from different clutch groups (different mother ID) could be the result of the mothers treated ability to respond to the infochemicals created by *Chaoborus*, while body size is dependent on the neonates after receiving cues from the mother and kairomones during embryonic development. Although studies have shown that the body size of mothers directly effects the offspring size (source). Varied values in size between different clutch groups shows that offspring reacted differently to treatments in the development of body length and width and statistical analysis proposes that maternal influences are evident for body size. The response of predatory cues on body size within instars and during embryonic and between clones still requires to be further investigated.

Moreover, differences in body size between clones could due to clone P5 is from a shallow rock pool, with UVR-exposed locality, it could have greater ability to respond to both environmental stressors. Increases in body length for clone group P5 for the +UV/+K treatments supports the hypothesis (2) that the coastal P5 clone group would respond better to UV radiation treatment than the inland UNI clone. The UNI clone group is found in a shaded inland seasonal pond with limited exposure to UV radiation. More likely, the addition of UV-A radiation may have been within an exposure range that *D. pulex* UNI clones were not able to acclimate to.

4.1.3 NECKTEETH INDUCTION

Results showed that all neonates responded adversely to UV radiation for neckteeth induction, as exposure to UV-A resulted in an antagonistic effect on the inducible defense trait of *D. pulex*. As shown in Figure 16, UV radiation treatment groups expressed lower rates of neckteeth induction. For the +UV/+K treatment groups however, there was a higher range of neckteeth induction, suggesting that kairomones were not denatured by the experimental design. Clone P5 and UNI did not show significant inter-clonal differences neckteeth induction for treatment groups with no UV. There was greater variability in observed responses for UV-radiation treatment groups for neckteeth induction values between clones P5 and UNI (Figure 17).

The appearance of an unidentified parasite was an unintended response. Induction values for the +UV/+K treatment group were low, with many individuals in this treatment group expressing signs of infection (Appendix 7.2.7), possibly indicating a potential trade-off between neckteeth induction and infection rates in the presence of predators and environmental stressors such as UV. The latter could suppress the immune system and thus make the animals more vulnerable to infection. Notably, high rates of mortality (50% and higher) were prevalent among the UV treated groups, resulting in limited neckteeth scoring of the UV treated groups compared to those without exposure to UV radiation.

As neckteeth induction has been proposed to be exclusively for offspring exposed to kairomones during embryonic development and juvenile stages, neonates in the +UV/-K treatment group expressed an array of neckteeth induction values. The induction of neckteeth in instar is believed to be exclusively for individuals exposed to predator cues, as neckteeth formation is extremely costly resulting in the dynamic expression of the plastic trait. The expression of neckteeth for individuals exposed to UV radiation raises questions as to whether the plastic trait of neckteeth induction can be induced by other abiotic or biotic stressors than kairomones. The induction of neckteeth in juveniles has only been studied with predatory cues (Christjani et al., 2016; Hansson et al., 2007; Krueger & Dodson, 1981; Sterr & Sommaruga, 2008; Tollrian, 1993). The hypothesis (1) that neckteeth formation will decrease in UV radiation treatments is supported by the results of this study, as defense traits were decreased significantly in animals exposed to UV radiation.

4.2 EXPERIMENTAL DESIGN

4.2.1 UVR TREATMENT

The use of ecologically relevant doses of UV radiation supported the experimental setup for testing *D. pulex* with kairomone treated waters. Kairomone and UV radiation could have resulted in the degradation of the info-chemical, but studies have shown that kairomone molecules are carbon based water soluble molecules composed of long chained (>C14) fatty acids and thus the process of denaturation of the compound was limited and allowed for this experiment to produce valid results (Dodson, 1989; Sterr & Sommaruga, 2008; Linda C. Weiss, Pötter, et al., 2018).

The signaling pathway involved in receiving kairomone cues is highly conserved among *Daphnia* (Colbourne et al., 2011). I found general support for the hypothesis that responses would be induced by kairomone presence while reduced when exposed to UV radiation as an additional stressor. The effects of UV radiation on zooplankton has been tested on a number of species, with an emphasis on ROS formation and UV radiation defense mechanisms (B.-M. Kim et al., 2015; J. Kim et al., 2009; Rautio & Tartarotti, 2010; Wolf et al., 2017). The inducible response of neckteeth formation using UV radiation as a co-stressor has not been previously tested, however.

Adverse reactions on kairomones was observed by Sterr & Sommaruga (2008), where kairomone potency was greatly reduced with UV-A radiation treatment after 5 hours. Results from this paper were used to favor the use of UV radiation on neckteeth formation, as the potency of kairomones still remained active within 0-4 hours as seen in the preliminary experiment (Appendix 7.1.2). The potency of kairomones in unfiltered water with no light lasts up to 7 hours (Dodson, 1989). Thus, the limited half-life of kairomones supports the use of UV radiation as a treatment for mother *Daphnia* as the short time period required for the induction of neckteeth limits the deteriorating effects of UV radiation on kairomones (Sterr & Sommaruga, 2008).

4.2.2 CLONES

Clones used in this experiment were obtained from bodies of water containing no signs of *Chaoborus* predators. Although the lack of predators has no effect on the induction ability of *D. pulex*, it is important to note that both clones had no previous exposure to kairomones which could affect the response rate in the experiment. The waterbodies from which the clones were sampled were also devoid of fish predatory fish which could have impacted body and clutch size (Maurone et al. 2018). Clone selection was based purely on geographical collection sites.

The use of different clones for neckteeth induction experiments has been limited to a few clones known to be responsive to kairomones (Sterr & Sommaruga 2008; Tollrian 1993), and most works rely on commonly tested clones such as C9C, R9, and KWK9 (Dufresne et al. 2008; Weiss et al. 2018; Tollrian 1993). By use of two different clones in a two by two

factorial design, neckteeth induction of offspring was able to also be compared between clones. This introduces an additional layer to the measurement of neckteeth induction in response to UV radiation between two clones with different genetic backgrounds (Coulbourne et al. 2012; Dennis et al. 2017).

Inter-clonal differences in response to UV radiation exposure alone have been studied (Rautio & Tartarotti 2011). Examples include pigmented vs. non-pigmented *D. pulex* clones, which exhibit differences in response rates to increases in UV radiation such as decreases in clutch and body size (Hansson et al., 2007). The induction of defense traits with the presence of UV radiation however, had yet to be explored in relation to variation in response rates between clones from distinctive geographical locations.

The question of a possible kairomone and UV interaction resulting in ROS formation in the water creating an additional stressor was not addressed in this experimental design. Some limitations in the experiment were amount of experiments run and mother *Daphnia* available. The time constraint of the master's thesis limited the amount of times the experiment could be replicated. The pilot experiment could be run twice, but the main experiment, however, the main experiment was not replicated. This was due to time restrictions and limitations in *Daphnia* available for the experiment. A total of 28 mother *Daphnia* were used for the P5 clone and 20 for the UNI clone. Available mother *Daphnia* were restricted as clutch size numbers for desired mother *Daphnia* groups were limited (numbers ranging from 2-5 healthy individuals produced) for long periods of time in the fall of 2018, delaying the main experiment by several months. The use of more clones could have aided in the observation of inter-clonal differences.

4.3 USE OF MULTI-STRESSOR EXPERIMENTAL DESIGN

The ability for *Daphnia pulex* to adapt to changing environmental conditions by shifts in expression of genes has allowed for the distribution of *Daphnia* to inhabit an array of bodies of water. Phenotypic plasticity has provided the necessary tools for *Daphnia* to respond to shifts in predator dynamics, environmental stressors, and limits in food availability (Thiel & Wellborn 2018). Observing differences in plastic responses of neckteeth formation is a multivariate issue (Dennis et al. 2017). The notion that metabolic cost incurred during the

acclimation to UV-A stress may explain the organisms reduced capacity to deal with other stressors (J. Kim et al., 2009).

A decreased value in neckteeth formation for UV treated groups could indicate a shift in energy allocation between predatory defenses and cellular defenses against UV damage. As inducible defenses are adaptive under predation pressures (Harvell, 1990), potential fitness costs of neckteeth formation became a limiting factor when exposed to the additional stressor of UV-A. Studies have shown that exposure to UV-A radiation results in DNA damage, in a study conducted by Wolf et al. (2017), *Daphnia magna* exposed to UV radiation resulted in measured DNA breaks with a comet assay. By presenting a common environmental factor such as UV-A, it possible to see the detrimental effects on the ability of neonates to defend against predators.

The multiple stressor approach presents a complexity to the results. The allocation of energy to different functions when under threat of multiple stressors has a significant effect on morphology and life history (Dennis et al., 2014; Sterr & Sommaruga, 2008; Linda C. Weiss, Pötter, et al., 2018). Measuring the impact of the combination of abiotic and biotic environmental stressors poses a challenging task when facing multifaced defense responses.

The addition of the two environmental stressors, has proven to have an antagonistic effect on inducible defense traits (Weiss et al., 2018). In a study conducted by Weiss et al., varying levels of the abiotic factor CO_2 were tested on *D. pulex* in order to see the effects on the expression of neckteeth. Results showed that increases in CO_2 negatively altered chemical communication between predator and prey by reducing the ability of *D. pulex* to sense its prey resulting in a reduction in neckteeth formation. The addition of abiotic stressors to natural biotic predator-prey dynamic systems presents a challenging feat for animals which induce defense traits. Not only is the allocation of energy to defense traits costly, it can also be inhibited by the addition of additional stressors.

Studies on the plastic response of system against UV radiation between two clones has been done before. A study by Hansson et al. (2007) proposed that genetic differences within clones, prohibited one of two different clones from expressing the same amount of photoprotective pigmentations against the same treatment of UV-B radiation. Hansson et al., (2007) compared the behavioral escape mechanisms of two clones of *D. pulex* from distinct

geographical locations (Arctic and temporal region). Increased mortality and reduced offspring were some of the ways in which the clone from the temporal region which had not previously been exposed to high rates of UV radiation and the evolutionary drive for selection for quick responses to UV radiation, responded to the experimental design created by Hansson et al. (2007). Results support the comparison in response rates of two clones. Furthermore, this introduces the idea that *D. pulex* in low lateral regions are at greater risk to increases in UV damage (Rautio & Tartarotti, 2010). Inter-clonal differences in neckteeth formation based on geographical location, however, still requires more research in combination with multi-stressor experiments.

4.4 IMPACTS OF UVR ON DEFENSE

As seen in the study conducted by Hoverman & Relyea (2009), there was general support in their prediction that responses to one predator will reduce the risk of predation for that particular predator but can increase the risk of predation by functionally different predator. As seen in our results, individuals treated with UV-A were more susceptible to infection in those who have not been treated with UV radiation. Interactions of UV radiation not only reduced the response variable of neckteeth formation but could be responsible for the development of infections from foreign bodies, by lowering natural defense mechanisms against pathogens to presumably allow for the allocation of energy towards UV radiation repair. UV radiation can have a significant impact on interactions between pathogens and hosts (Häder et al., 2015). Increases in UV radiation have been proven to reduce immune defense mechanisms causing higher infection rates to those exposed (Studer et al., 2012).

Daphnia pulex are vulnerable to infection from epibionts, parasites and pathogens (Dieter Ebert, 2005) in their natural habitat. The morphological structure of *Daphnia* species, whose bodies are composed of a uncalcified shell which encloses the appendages and abdomen, can serve as an ideal place for epibionts and parasites in aquatic environments (Dieter Ebert, 2005). Although the immune system of *Daphnia* species still requires much needed investigation (Ebert, 2005; McTaggart et al., 2009), studies conducted thus far on *Daphnia* have been on observing the modes of infection and immune system responses (Ebert et al., 1998; Dieter Ebert, 2005).

Differences in rates of infection between clones could be possibly linked to genetic differences, as some genotypes invest more into defense mechanisms at the expense of other bodily functions (Carius et al., 2001; Little & Killick, 2007). The mechanisms involved in co-evolution of host-parasites which affect population genetic structures are intertwined with the costs of immunity on fitness. Genetic variation for resistance to infectivity has been observed by Carius et al. (2001) in *Daphnia magna*, providing insight to frequency-dependent selection system between hosts and parasites fueling coevolution between the two. Differences in response rates for *Daphnia pulex* used in this experiment could be the product of different genetic backgrounds and abilities to respond to additional environmental stressors such as UV radiation, kairomones and pathogens. The susceptibility to infection of *Daphnia pulex* caused by environmental conditions still requires however, further investigation.

4.5 IMPACTS ON ENVIRONMENTAL STRESSORS ON NATURAL SYSTEMS

Increases in UV radiation in aquatic systems may lead to detrimental effects on key stone species such as *D. pulex*. Many studies have presented data that supports increases in UV radiation over the past decade (Williamson et al., 2014; Wolf et al., 2017; Wolf & Heuschele, 2018). *D. pulex* found in clear and shallow lakes and ponds are subject to significant UV stress which could affect both the fitness and survival of this keystone species. Studies have shown that the ability for prey such as *Daphnia pulex* to express defensive traits against predators play a critical role of stabilizing the effects on community structures (Weiss et al., 2018). The inability for *D. pulex* to produce induced morphologies to defend against predators would have serious implications on trophic levels, community species composition, and ultimately the entire ecosystem in which *D. pulex* are involved (Verschoor et al., 2004). The effects of UV radiation on neckteeth formation in juveniles exposed to kairomones has provided insight on potential effects of increases in UV radiation on *D. pulex* populations in shallow transparent lakes and ponds in their ability to defend against predators.

5 Conclusion

5.1 AIMS OF MASTERS THESIS

The results of this study emphasize the need for further investigation of present-day environmental threats on highly specialized evolved defense traits (Colbourne et al., 2011; Weiss et al., 2018). This project aimed to address the probability as to whether or not UV radiation had a synergistic or antagonistic effect on kairomone induction of defense traits. The aims and objectives of this experiment were met. Results showed that UV radiation did have an antagonist effect on neckteeth formation. The addition of UV radiation on *D. pulex* mothers led to limited neckteeth formation in juveniles (1) and the coastal clone group P5 responded notably better to UV radiation treatment by an increased size in instars 1 and 2 compared to the inland clone UNI (2). The experiment also presented new findings with an increased rate of infection and the induction of neckteeth in UV radiation treated groups with no kairomones, anomalies were observed in the +UV/-K treatment groups, where juveniles who had not been previously exposed to kairomones, expressed neckteeth in the second instar.

5.2 FUTURE DIRECTIONS

The status of molecular pathways involving the regulation of neckteeth formation requires more investigation (Christjani et al., 2016). The use of molecular techniques to measure expression of plastic phenotypes would provide future insight to the *Daphnia* and *Chaoborus* predator-prey dynamic. First, a screening of gene expression related to neckteeth formation for all 4 treatments should be performed to see if expression of different morphological traits is up or down regulated. Second, a greater sampling of *Daphnia pulex* clones from different geographical locations (cf Christjani et al. 2016), would provide greater insight on the sensitivity of *Daphnia pulex* species per se, and thus the generality of the observed responses.

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7 Appendixes

1. Pilot Experiment Protocol
2. Pilot Experiment Overview
3. Daphnia Culturing Protocol
4. Neckteeth Counting Technique
5. Daphnia Size Protocol
6. R Code
7. BRMS plots and tables

7.1.2

PILOT EXPERIMENT PROTOCOL
UV Kairomone Protocol for *Daphnia pulex*

Purpose:
Test if timed UVB exposure of 8 hours significantly denatures kairomone proteins enough that defensive neckteeth formation cannot be expressed by *Daphnia pulex* juveniles. For this pilot experiment we will be focusing on the development of neckteeth, so clutch number will not be considered.

Materials:
2 UVB light tubes
2 LED lights
6 mother D. pulex in stage 2 of embryonic development
60 µL kairmones per individual
ADaM media
40 mL glass jars
20 °C climate room

Methods:
Conduct experiment in enclosed 20 °C climate room.
Day 1: Obtain 12 40mL glass jars. Fill with 40 mL ADaM media. Insert 60 µL of kariomones using pipette in each jar. Place 6 of the 12 jars under direct UVB lamp set up. Place the remaining 6 jars away from UV exposure in UV room (preferably on shelf above UV lamp). Begin timed UV exposure. Remove 2 jars from each group (UV and non UV) every 2 hours and relocate out of UVB and LED light exposure in 21 °C climate room. Place mother *Daphnia* in embryonic stage 2 in collected media immediately.

Day 2: Treat kairomones again in 2 hour increments and place mother daphnia in new jars from kairomones treated for day 2.
*repeat steps each day until juveniles are released.

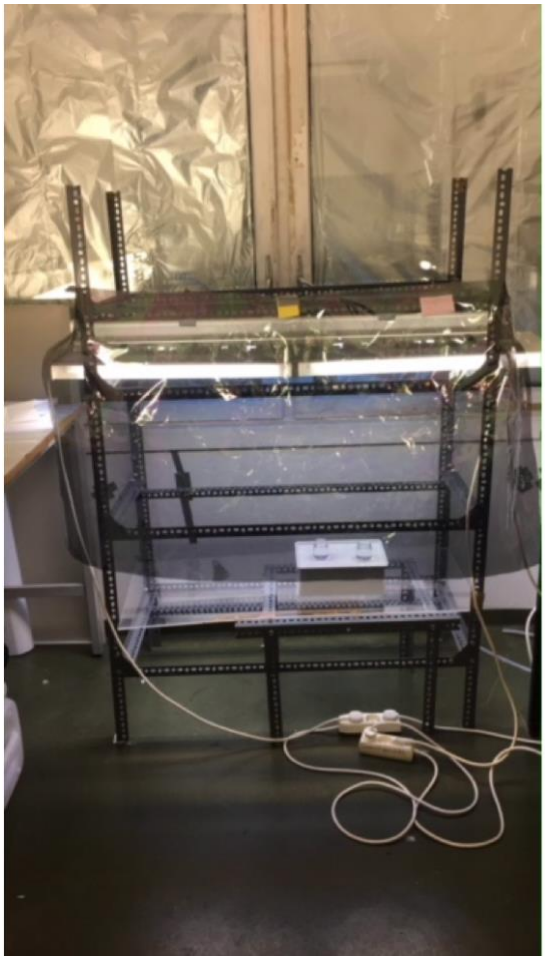
Day 3/ 4: Once juveniles appear, score neckteeth for juvenile stages 1 and 2 to determine if neckteeth formation is possible with UV treated kairomones.

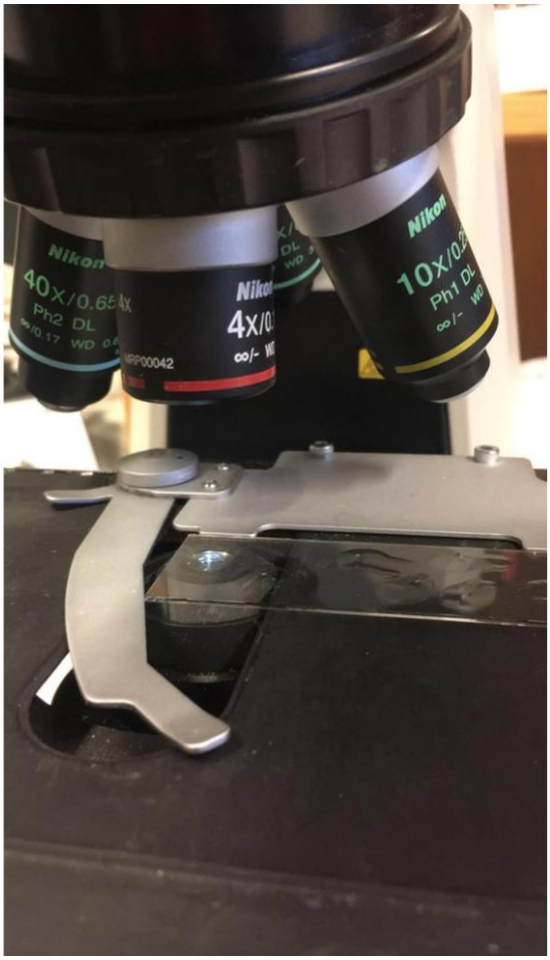
PILOT EXPERIMENT OVERVIEW

Kairomone (0–4 h PAR pre-incubation): 2 mother daphia, 5 neonates each (10 total)
Kairomone (4–8 h PAR pre-incubation): 2 mother daphia, 5 neonates each (10 total)
Kairomone (0–4 h UVB pre-incubation): 2 mother daphia, 5 neonates each (10 total)
Kairomone (4–8 h UVB pre-incubation): 2 mother daphia, 5 neonates each (10 total)

Treatment group	Mean neckteeth no. count
Control (no kairomone):	no neck teeth
Kairomone (0–4 h PAR pre-incubation):	4.5 neck teeth
Kairomone (4–8 h PAR pre-incubation):	3.0 neck teeth
Kairomone (0–4 h UVB pre-incubation):	1.5 neck teeth
Kairomone (4–8 h UVB pre-incubation):	2.5 neck teeth

Daphnia pulex climate room





Pilot experimental light set up and scoring with light microscope technique.

7.1.3

DAPHNIA CULTURING PROTOCOL

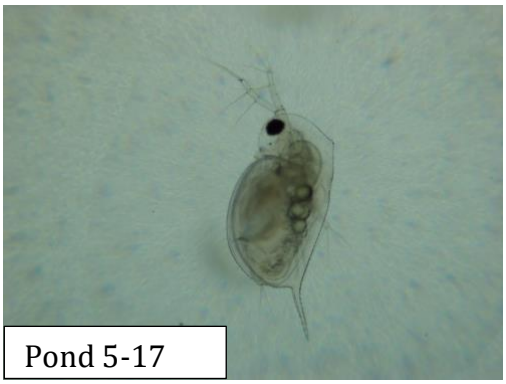
SAMPLING OF CLONES

Clone Pond 5-17

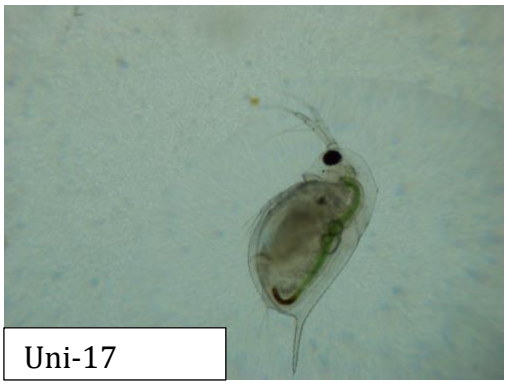
Sampled from Dag O. Hessen 16/17. September 2017, island near Sponvika (~GPS: 59.098405, 11.198153) from a small rock pool (~1-2m) surrounded by vegetation (water was humic/brown colored). No obvious signs of *Chaoborus* at this site, perhaps Corixidae present.

Clone Uni-17

Sampled from Erik/Jessica/Elke on 22. September 2017, in pond behind Kristine Bonnevies Building (~GPS: 59.937767, 10.722368). No obvious signs of *Chaoborus*. *Daphnia* were noted to be very red and many were carrying ephippia.



Pond 5-17



Uni-17

ADaM Media

Artificial Daphnia Medium (ADaM) protocol was provided by Ebert et al. (2013). 10 L glass jars were used for ADAM media. Jars were constantly aerated and held in *Daphnia* climate rooms (21˚C +-1).

Back to Online View

Artificial *Daphnia* medium: ADaM (Aachener Daphnien Medium)

We grow *Daphnia* in artificial medium. The original recipe for the medium was developed by Klüttgen, B., Dülmer U., Engels, M. & Ratte H. T. 1994. ADaM, an artificial freshwater for the culture of zooplankton. Water Research 28:743-746. We use a modified version of this medium (altered Seleniumdioxid concentration). To produce a given amount of medium add the amounts of sea salt and stock solutions as given in the following table.

Water	Sea salt [gram]	Stock solution A (ml)	Stock solution B (ml)	Stock solution C (ml)
10 l	3.33	23	22	1
50 l	16.6	115	110	5
60 l	19.9	138	132	6

60 l medium tank in our lab

We use a special sea salt which is produced by combining all chemical to make up the mixture. (Most commercially available sea salt is produced by evaporation of water from sea water. In this case, some of the salts do not solve anymore once put back into water). We buy this sea salt from an aquarium supply company. Atrificial sea salt works fine.

These are the concentrations for the three stock solutions:

Stock solution	Chemical	Concentration [g/l]
A	$CaCl_2 \times 2H_2O$	117.6
B	$NaHCO_3$	25.2
C	SeO_2	0.07

Some notes on our experience with this medium:

- We use 60 liter plastic tanks to prepare the medium (see picture). The salts do not harm the plastic. It is better to use non-transparent material to prevent growth of algae.
- We produce the medium 24 hours in advance, but shorter time periods don't really seem to harm the *Daphnia*.
- The tanks are constantly aerated. The air passes through a filter with 0.2 µm pores. This prevents small particles and microbes to enter the water. Note that this saturated the medium with gas. If the medium warms up you get small gas bubbles in the water, which may cause the *Daphnia* to float on the water surface. If your animals have this problem allow the medium to stand still for one day before you use it.
- We keep the stock solutions in a fridge and had never problems with older solutions.
- If the tanks are used over longer period, you may get some growth of fungi and/or algae. We clean the ADaM tank regularly to keep this down.
- We have good experience with this medium culturing *Daphnia magna*, *pulex*, *longispina*, *galeata* and *hyalina*, as well as hybrids of *galeata* × *hyalina*. The inventors of the medium tested it successfully for other cladoceran species as well.
- If you have problems culturing *Daphnia* or other cladocerans in this medium, try to add some natural water (lake, stream, spring water). Adding 20% spring water we had good experience with clones which were tricky to keep otherwise.

CHLAMYDOMONAS CULTURE TECHNIQUES

Chlamydomonas cultures were cultured in an aerated semi-continuous cultures. Media used for growth was Wright Crypophyte (WC). Samples of algae were spun down with a centrifuge and all WC was removed and replaced with ADaM medium. The optical density was then measured using a spectrophotometer. The optical density (OD) was measured at 800 mm with the spectrophotometer (Shimadzu UV 160-A, Japan). The ratio of algae was then calculated using techniques established by Erik Sperfeld (Unpublished).

WC MEDIUM FORMULA

culturecollection of algae and protozoa

media recipes

MWC (Modified WC Medium)

Freshwater algae

Stocks		**per litre**
(1)	$CaCl_2.2H_2O$	36.80 g
(2)	$MgSO_4.7H_2O$	37.00 g
(3)	$NaHCO_3$	12.60 g
(4)	$K_2HPO_4.3H_2O$	11.40 g
(5)	$NaNO_3$	85.00 g
(6)	$Na_2O_3Si.5H_2O$	21.20 g
(7)	Combined trace elements:	
	$EDTANa_2$	4.36 g
	$FeCl_3.6H_2O$	3.15 g
	$CuSO_4.5H_2O$	0.01 g
	$ZnSO_4.7H_2O$	0.022 g
	$CoCl_2.6H_2O$	0.01 g
	$MnCl_2.4H_2O$	0.18 g
	$Na_2MoO_4.2H_2O$	0.006 g
	H_3BO_3	1.00 g
(8)	Vitamin mix:	
	Thiamine HCl	0.1 g
	Biotin	0.0005 g
	Cyanocobalamin	0.0005 g
(9)	Buffer (add dry when making up medium):	**per litre final medium**
	TES	0.115 g

Medium	**per litre**
Stock solutions 1 - 8	1.0 ml each
Dry Buffer (9)	0.115 g

Combine stock solutions 1-8 and the dry buffer and make up to 1 litre with deionized water. Autoclave at 15 psi for 15 minutes.

Reference

Guillard RRL & Lorenzen CJ (1972) Yellow-green Algae with Chlorophyllide C. J. Phycol. **8**, 10-14.

CCAP (Culture Collection of Algae and Protozoa), Dunstaffnage Marine Laboratory,
Oban, Argyll, PA37 1QA, UK
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7.1.4

NECKTEETH COUNTING TECHNIQUE

We documented neckteeth expression in the 1st and 2nd juvenile instar according to an established neckteeth scoring system (Tollrian, 1993) using a light microscope (Nikon SMZ-U Stereomicroscope with a zoom of 1:10) in combination with a Nikon imaging system. Without kairomone, the young *D. pulex* form a normal, round neck shape. At low kairomone concentrations this shape remains but neckteeth develop (> 5µL).

Average values from experiment

Clone	**Kairomone**		**UV+Kairomone**		**UV**		**Control**	
DAY 1	NT	B	NT	B	NT	B	NT	B
Uni-17	1.81	1.00	1.48	0.98	1.46	0.92	1.30	0.72
Pond 5-17	2.20	0.97	1.02	0.36	1.60	0.52	2.34	1.01

Clone	**Kairomone**		**UV+Kairomone**		**UV**		**Control**	
DAY 2	NT	B	NT	B	NT	B	NT	B
Uni-17	1.89	0.82	0.96	0.25	0.92	0.42	0.00	0.00
Pond 5-17	1.95	0.93	2.20	0.9	0.60	0.00	0.02	0.01

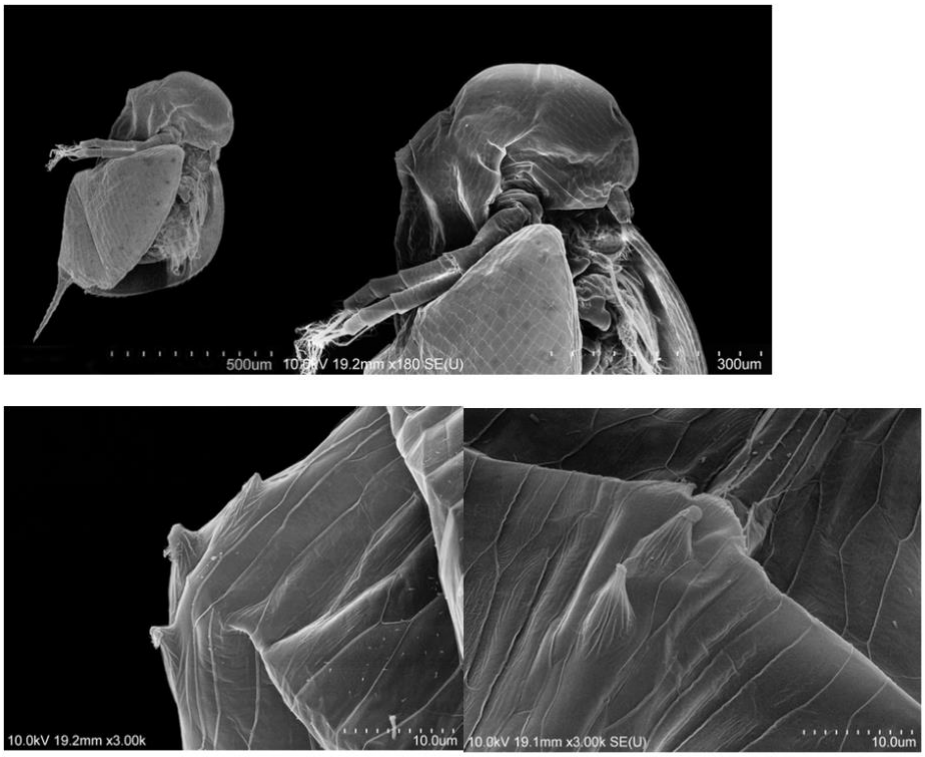


Figure A. Scanning electron microscopy (SEM) images prepared by Jannicke Wiik-Nielsen from the Norwegian Veterinary institute. Image of individual P5 clone offspring in instar 2 used in the main experiment treated with kairomones. Image includes full-body, headshot and close-ups of induced neckteeth with neck-keel type B.

7.1.5

DAPHNIA SIZE PROTOCOL

Protocol for measuring *Daphnia* (full body) *from photos WITHOUT randomized file names*

1) Create folders ‘roi’ and ‘jpg’ and ‘Measurements’ in the folder containing the photos
2) open ImageJ
3) install the macro 'Daphnia_fullBody.ijm' (Menu: Plugins -> Macros -> Install…)
4) open the first picture
5) enlarge if necessary with ‘Magnifying glass’
6) run the macro by pressing the key ‘v’ and follow the instructions
7) first draw a line from spina base to end of head through the middle of the eye → OK
8) set landmarks (5 at the moment) → OK
9) the next picture appears automatically, press ‘v’ again to start the macro and follow instructions (i.e. draw line and set landmarks)
10) proceed with individuals and save the ‘Results’ table containing the coordinates from time to time (replace existing results file) as ‘Results.xls’ in the folder ‘Measurements’
11) after the last individual has been processed, save the ‘Results’ table containing the coordinates a last time
12) chose appropriate micrometer-scale image
13) open this micrometer image with ImageJ AND enlarge if necessary
14) draw a straight line from over the full scale (in the example below: 0 to 2.0)
15) determine the ‘scaled pixel value’: Analyze -> Set Scale and exchange value in ‘Known distance:’ to the length of the scale (here 2) and the ‘Unit of length’ (here mm), see figure below
16) note down the number after ‘Scale:’ (in the example figure below: 438.6991 pixels/mm)
17) open the R-file ‘length calculation_without random naming.r’
18) put name of folder containing the pictures and scaled pixel value in R-file
19) run all code lines

IMAGE J

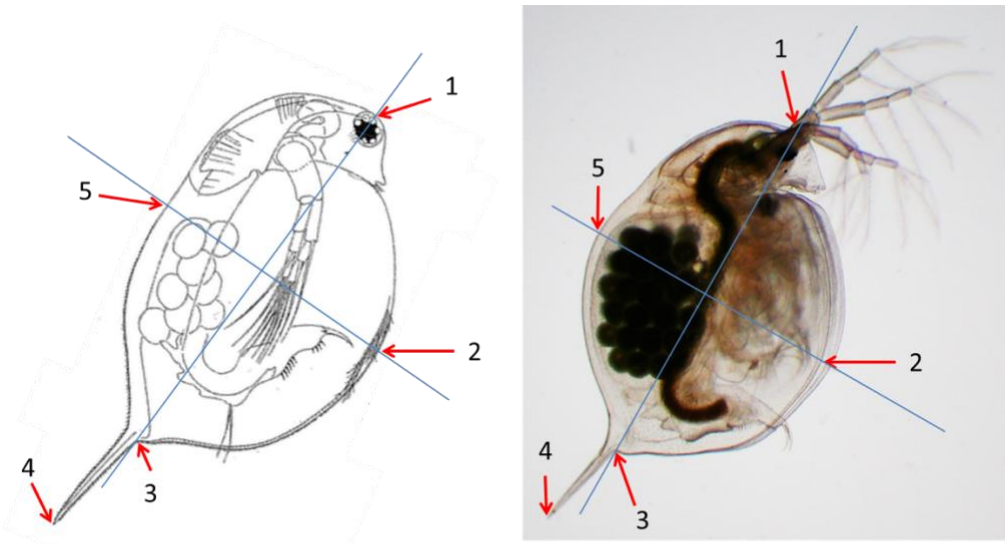
Description of setting landmarks

Command: "Please draw line from spina base to head"

a) start drawing the line from spina base to the end of the head by passing through the middle of the eye – click “ok”
b) dorsoventral axis line will be drawn automatically (90° angle to anterior-posterior axis)

Command: "Please set landmarks"

1) Point where anterior-posterior axis line ends at the head (top of head)
2) Point at ventral side of the dorso-ventral axis line (ventral midpoint)
3) Point at spina base where maximum curvature is observed (spina base)
4) Point at tip of spina (spina end)
5) Point at dorsal side of the dorso-ventral axis line (dorsal midpoint)



Landmarks:
1. Tip of head
2. ventral midpoint
3. Spina base
4. Spina end
5. dorsal midpoint

Calculation of characters:
body_length: 1 to 3
spina_length: 3 to 4
body_width: 2 to 5
relative_width: body_width/body_length
relative_spina_length: spina_length/body_length

7.1.6

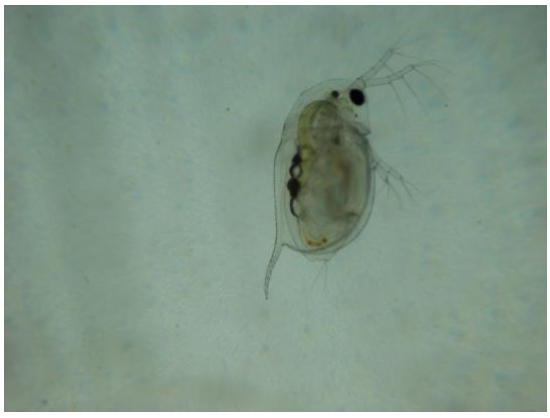
R CODE

Image J code pre-sets

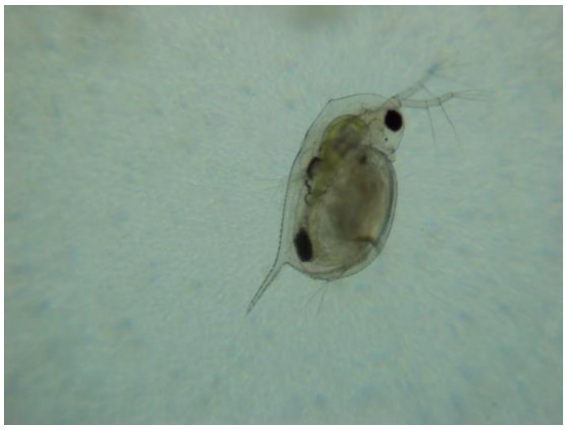
```
macro "Daphnia Full Body Morphometrics [v]"{
        run("Select None");
        setTool("line");
        waitForUser("Click OK to continue.", "Please draw line from spina base to head");
        run("ROI Manager...");
        roiManager("Add");
```

```
count = roiManager("count");
if (count==0) {
exit("ROI Manager line selection required");
}
roiManager("select", count-1);
if (selectionType!=5) {
exit("Straight line selection required");
}
run("Rotate...", "angle=90");
roiManager("Add");
roiManager("Show All without labels");

setTool("multipoint");
waitForUser("Click OK to continue.", "Please set
landmarks");

run("Measure");

roiManager("Add");
roiManager("Select", 2);
fname = replace(getInfo("image.filename"),".tif","");
roiManager("rename", fname);

path = getInfo("image.directory");
saveAs("Selection", path + "roi/" + fname + ".roi");

roiManager("select", 0);
roiManager("select", 0);

run("Flatten");
saveAs("Jpeg", path + "jpg/" + fname +
"_marked.jpg");

roiManager("reset");
close();
run("Open Next");
```

7.1.7 Unidentified foreign body responsible for infected neonates



UNI UV treatment group day 1



P5 UV treatment group day 2

7.1.8 BRMS model development

Figures A and B show the time series and distributions of parameters phi, zoi, and coi which are all quite precisely estimated compared to the other plots. Figure C represents a table of a summary of the Brms model used for interpretation of statistical support for the induction of neckteeth in instar 2. The phi value represents the precision of the beta-distributed mean, values represent in the inverse of the standard error such that high values ~20 mean low error. The zoi value is well constrained around 0.5, meaning there is 50% probability for a value to be drawn from the beta-distribution and 50% of it being either 0 or 1. The coi parameter serves as the conditional probability for being 1 given that the values is non-beta (i.e. 0 or 1) is also well-constrained and quite small (<5%) which conforms with the impression that 100% Tollrian scores are rare. Notice also the good mixing of these time series compared to the other 2 brms figures.

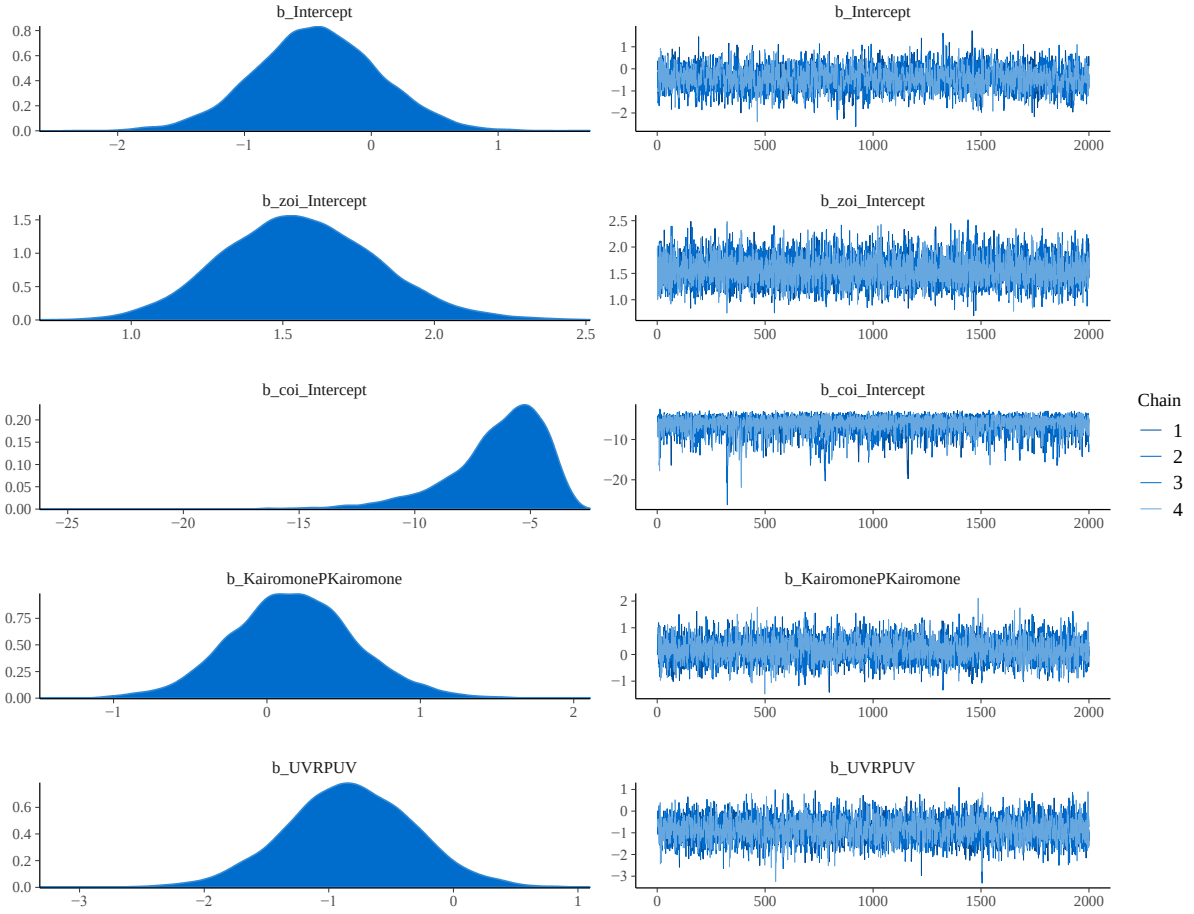


Figure A. Brms model plot of convergence of chains Part 1.

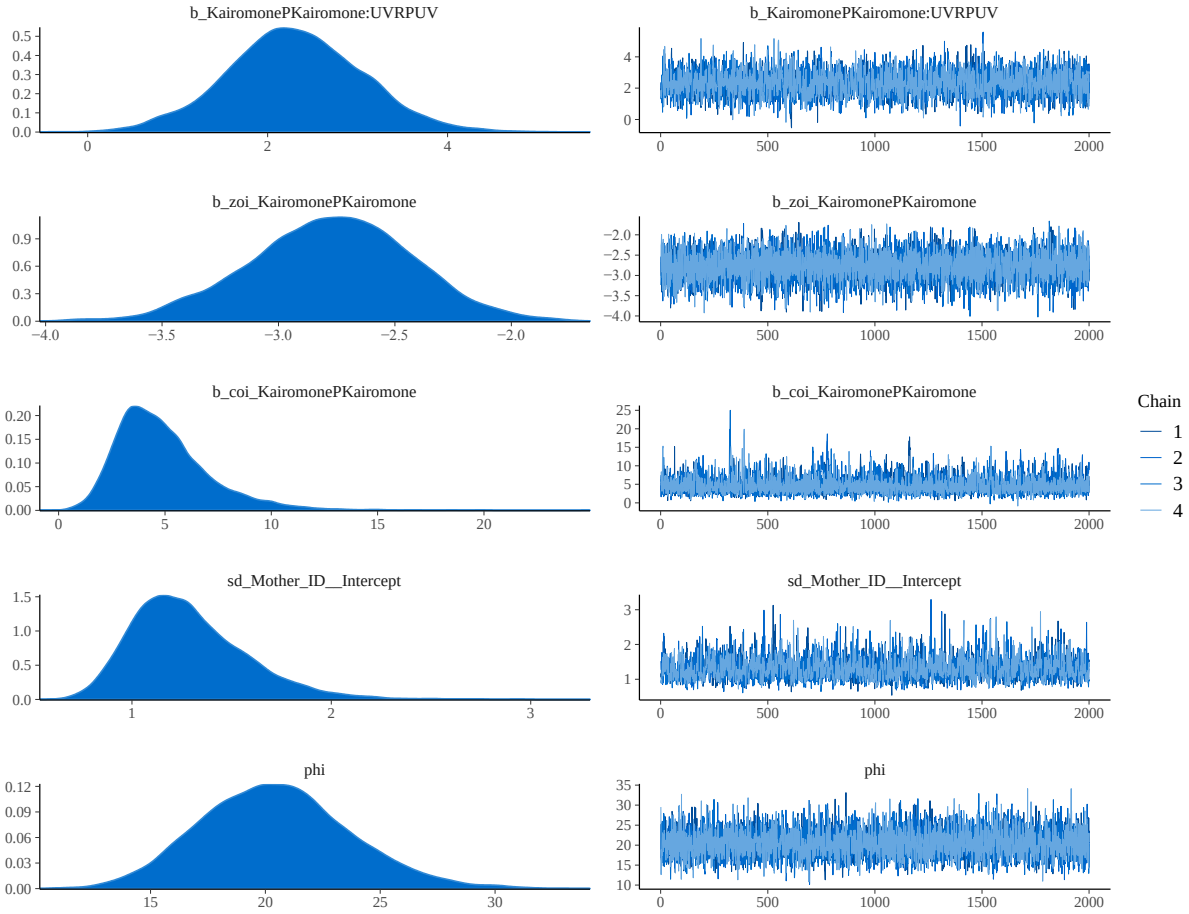


Figure B. Brms model plot of convergence of chains Part 2.

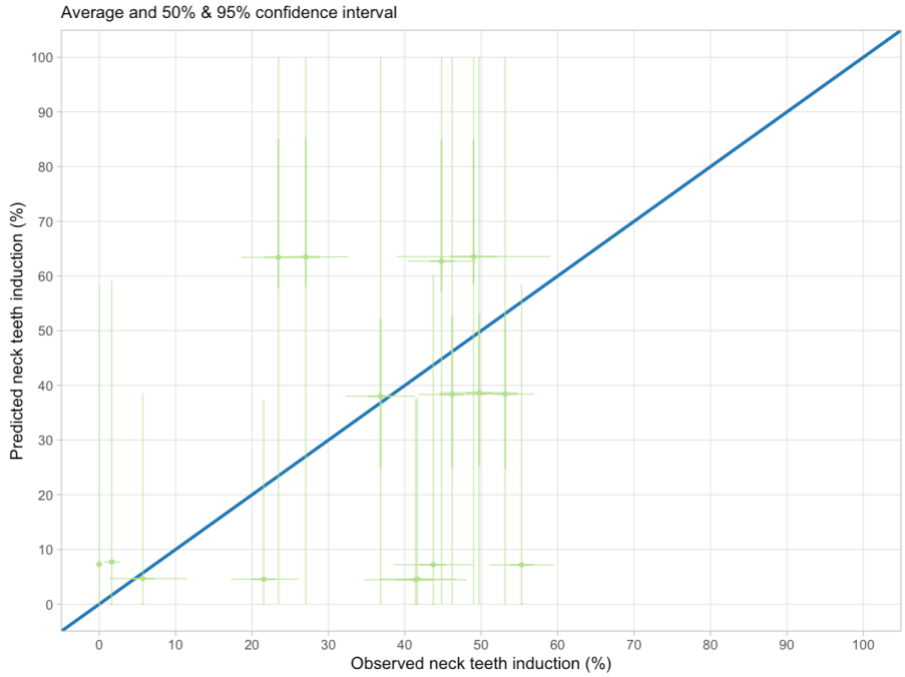


Figure C. Observed vs predicted values from neckteeth induction for all values in Brms model 10 for instar 2.

ShinyStan

	n_eff	Rhat	mean	mcse	sd	2.5%	25%	50%	75%	97.5%
b_Intercept	2970	1	-0.4	0	0.5	-1.4	-0.8	-0.4	-0.1	0.5
b_zoi_Intercept	8000	1	1.6	0	0.3	1.1	1.4	1.5	1.7	2.1
b_coi_Intercept	2560	1	-6.4	0	2.2	-11.8	-7.4	-6	-4.9	-3.5
b_KairomonePKairomone	5350	1	0.2	0	0.4	-0.6	-0.1	0.2	0.4	1
b_UVRPUV	4547	1	-0.8	0	0.5	-1.9	-1.2	-0.8	-0.5	0.2
b_KairomonePKairomone:UVRPUV	4025	1	2.3	0	0.8	0.8	1.8	2.3	2.8	3.8
b_zoi_KairomonePKairomone	8000	1	-2.8	0	0.3	-3.5	-3	-2.8	-2.5	-2.1
b_coi_KairomonePKairomone	2763	1	4.9	0	2.2	1.7	3.3	4.5	6	10.3
sd_Mother_ID__Intercept	2321	1	1.3	0	0.3	0.8	1.1	1.2	1.5	2
phi	8000	1	20.5	0	3.3	14.5	18.2	20.3	22.5	27.3

Figure D. ShineStan Brms model results.

BAYESIAN ANALYSIS FOR BODY LENGTH

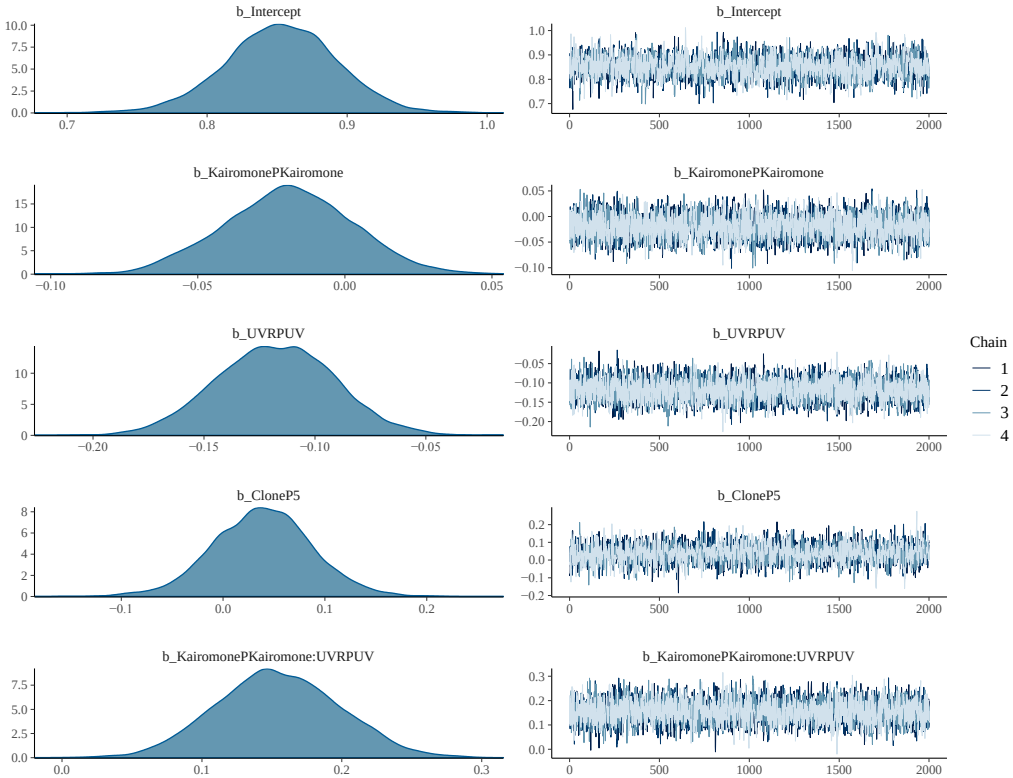


Figure E. Bayesian analysis plot for model BRM.G.

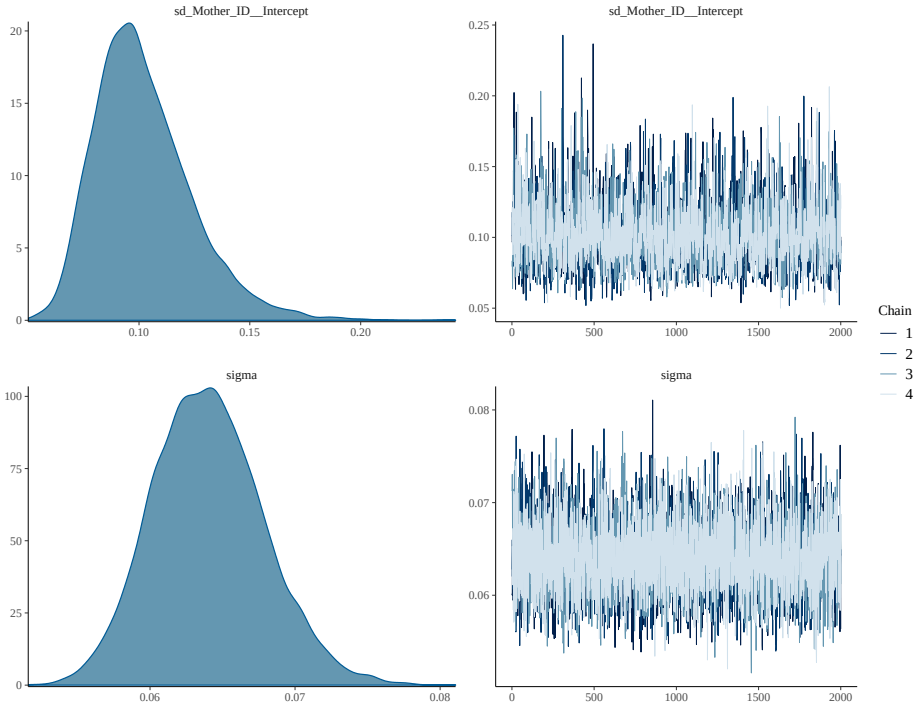


Figure F. Bayesian analysis plot for model BRM.G.

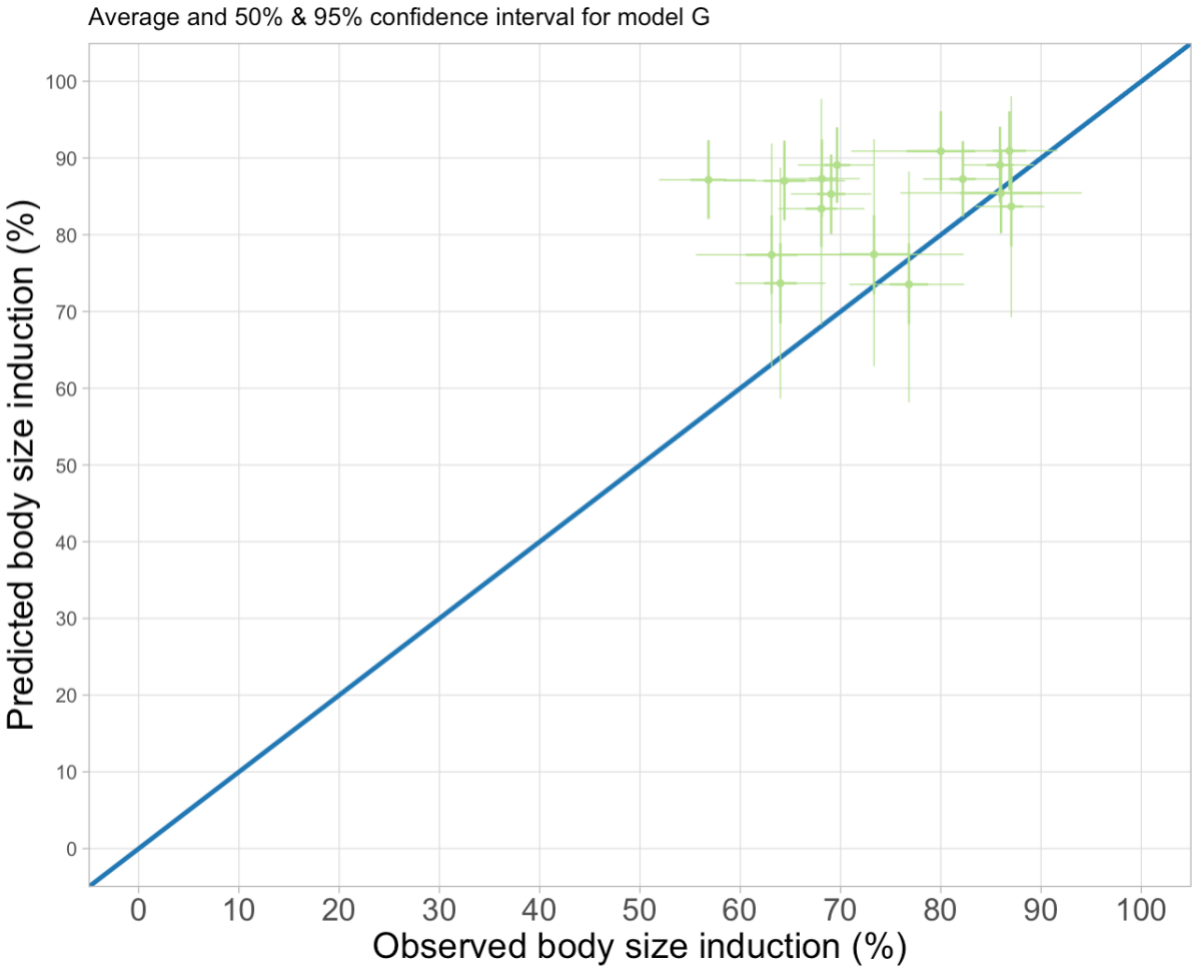


Figure G. Observed vs predicted values for body length for offspring in instar 2 using brms model G.

ShinyStan

	n_eff	Rhat	mean	mcse	sd	2.5%	25%	50%	75%	97.5%
b_Intercept	2748	1	0.9	0	0	0.8	0.8	0.9	0.9	0.9
b_KairomonePKairomone	3979	1	0	0	0	-0.1	0	0	0	0
b_UVRPUV	4213	1	-0.1	0	0	-0.2	-0.1	-0.1	-0.1	-0.1
b_CloneP5	2534	1	0	0	0	-0.1	0	0	0.1	0.1
b_KairomonePKairomone:UVRPUV	3858	1	0.2	0	0	0.1	0.1	0.2	0.2	0.2
sd_Mother_ID__Intercept	2064	1	0.1	0	0	0.1	0.1	0.1	0.1	0.2
sigma	4919	1	0.1	0	0	0.1	0.1	0.1	0.1	0.1
r_Mother_ID[P5_2,Intercept]	2563	1	0	0	0	-0.1	-0.1	0	0	0
r_Mother_ID[P5_8,Intercept]	3483	1	0	0	0	-0.1	-0.1	0	0	0.1
r_Mother_ID[P5_5,Intercept]	2650	1	0	0	0	0	0	0	0.1	0.1

Figure H. ShineStan Brms model G results.