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PREDICTING THE IMPACTS OF ANTHROPOGENIC STRESS ON AQUATIC POPULATIONS:

A CASE STUDY IN RESOURCE-CONSUMER-TOXICANT DYNAMICS

by

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THESIS APPROVAL PAGE

This is to certify that the thesis prepared by Tim Woo entitled *Predicting the impacts of anthropogenic stress on aquatic populations: a case study in resource-consumer-toxicant dynamics* has been approved by the thesis committee as satisfactorily completing the thesis requirements for the degree of Master of Science in Environmental Science.

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Abstract

The overarching theme of this research was bridging the gap between individual- and population-level effects of anthropogenic stressors under the current paradigm of ecological risk assessment. There is a general dissonance in scales of biological organization and energetic complexity between ecotoxicological studies and the natural environments in need of protection. Traditional toxicity tests assess stressor effects on individuals, while protection goals are generally concerned with the population level and above. Additionally, these tests largely ignore the impacts that the resource environment and the physiological state of a given individual have on toxicological outcomes. As such, the main objective of this research was to explore effects of – and interactions between – resource availability, individual physiology, and stress response at the individual and population levels using *Daphnia magna* as a model aquatic organism.

The research began with an exploration of how manipulations to quantitative and qualitative aspects of the resource environment altered the physiology and stress response of laboratory *D. magna*. Among the scenarios tested, per capita food level driven by intraspecific competition in density-stressed populations was deemed the most influential on *D. magna* stress response. Thus, the next study assessed the outcomes of pulse toxicity of the fungicide pyraclostrobin to *D. magna* populations, with the hypothesis that density-dependent changes in food level per capita at various phases of population growth would result in different population stress responses. Differences in population mortality and recovery were observed at each of four population growth phases. A multi-age acute toxicity study, determining the 48h LC50 for *D. magna* and pyraclostrobin at three age classes and two food levels, suggested that pyraclostrobin toxicity was food- and size-dependent. Lastly, food- and size-dependent toxicity were

incorporated into a bioenergetic modelling framework (DEB-IBM) as an exercise in refining model output of population-level impacts of anthropogenic stressors.

In summary, the results of this research demonstrate the importance of considering the resource environment and physiology of individuals when attempting to predict the impacts of anthropogenic stressors on natural populations. While traditional toxicity tests generate general toxicity benchmarks, the use of more 'non-traditional' methods may greatly improve the informative capacity of experiments and the predictive capabilities of energetic models. And as the goal of ecological risk assessment is to protect populations, communities, and ecosystems from anthropogenic stressors, it is important that our methods first be able to robustly bridge the gap between individual- and population-level effects.

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Chapter I.

Exploring effects of the resource environment on *Daphnia magna* life history, physiology, and stress response

Introduction

The cladoceran *Daphnia magna* is used extensively as a model aquatic organism in functional ecology, physiology, and evolution (Green 1954, Schindler 1968, Porter et al. 1983, Lynch 1984). This species also plays an important role in ecotoxicology and environmental management for its use in the commonly applied invertebrate life cycle test (EPA 1987, OECD 2012). Because of its long and widespread use, much is known about how *D. magna* life history traits (i.e. survival, growth, reproduction) are influenced by a suite of environmental factors. Resource depression is one such prevalent factor, relating to decreases in both food quantity and food quality. Food quantity describes the availability of consumable energy, usually in terms of the concentration of ingestible carbon (Urabe & Sterner 2001). Food quality describes a makeup of biochemical content (with an emphasis on lipids), elemental nutrient stoichiometry, and physical characteristics such as cell size and digestion resistance (Sterner & Schulz 1998, Urabe & Sterner 2001). For the ease of differentiating terms here, 'quantity' will be interchangeable with 'level' (a quantitative measure) - and 'quality' will be interchangeable with 'type' (a qualitative distinction). In aquatic environments, both aspects of food are dynamic (McCauley & Murdoch 1987, Arts et al. 1997, Anneville et al. 2004, Hartwich et al. 2012), and periods of 'low food' conditions can drive zooplankton dynamics and sensitivities to pollutants (Chandini 1989, Antunes et al. 2003, Smolders et al. 2005). Yet, the effects of low or dynamic food conditions are rarely considered in applications such as toxicity testing, and most studies that do address the

resource environment do so with respect to food level and not food type (Chandini 1989, Antunes et al. 2003, Reyes et al. 2015, Stevenson et al. 2017).

The effects of low food level on *Daphnia* are largely understood, generally involving shifts in relative energy allocation from somatic growth and reproduction to structural maintenance (Glazier & Calow 1992, Lynch 1989, Ingle et al. 1937). Low food level can also induce maternal effects where the reproductive strategy shifts from an emphasis on neonate quantity to quality (Cleuvers et al. 1996, Gabsi et al. 2014, Tessier & Consolatti 1991). Mothers raised in low food levels tend to have a larger relative investment into fewer eggs per brood; they produce neonates with higher dry mass and lipid content compared to mothers raised in high food levels (Glazier 1992, Cleuvers et al. 1996, Gabsi et al. 2014). These effects are suggested to be adaptive responses to emphasize survival over reproduction in scarce conditions (Glazier & Calow 1992, Pietrzak et al. 2010). Food level is largely conceded to be the most influential driver of *Daphnia* life history, the availability of dietary carbon being largely predictive of growth and reproduction (Lynch 1989, Glazier 1992, Hessen et al. 2002, Persson et al. 2007). More recently, however, the importance of food quality is being increasingly emphasized for its impact on carbon transfer efficiency, and is an active area of research (Müller-Navarra et al. 2000, Hessen et al. 2002, Darchambeau & Faerovig 2003, Martin-Creuzburg et al. 2005, Persson et al. 2007).

Poor food quality generally has the effect of decreasing the efficiency of carbon transfer from food item to *Daphnia* (Müller-Navarra 1995b, Urabe & Sterner 2001, Hessen et al. 2002, Von Elert et al. 2003, Persson et al. 2007). For instance, *Daphnia* maintain relatively homeostatic elemental ratios and therefore cannot utilize, or must expend, excess carbon from foods with high C:N or C:P ratios (Darchambeau & Faerovig 2003). Also, like most animals,

Daphnia cannot synthesize essential polyunsaturated fatty acids (PUFAs) or sterols *de novo* and must obtain them from their diet; reductions in both lipid types can therefore limit *Daphnia* growth (Müller-Navarra 2000, Becker & Boersma 2003, Hartwich et al. 2012, Wacker & Martin-Creuzburg 2007, Martin-Creuzburg et al. 2014). In terms of maternal effects, the available literature on food type suggests that there is no adaptive effect at low food quality conditions (Martin-Creuzburg et al. 2005, Wacker & Martin-Creuzburg 2007, Frost et al. 2010). Allocation of lipids and sterols between mother and offspring remains equal, relative to the amounts of these biomolecules available in the diet (Wacker & Martin-Creuzburg 2007, Martin-Creuzburg et al. 2005). This means that mothers raised in high food quality environments produce neonates with higher lipid content that are more resistant to periods of low food quality (Brett 1993, Becker & Boersma 2003).

Because the resource environment has direct effects on daphnia life history traits, there are important consequences for laboratory studies using these endpoints and aiming for environmental relevance. Toxicity tests, for example, have guidelines for feeding high levels, using one type of food, and focusing on individual neonates (OECD 2012). The standardized protocol for a *D. magna* toxicity test requires 0.1 to 0.2 mg C/*Daphnia*/day sourced from *Chlorella sp., Raphidocelis subcapitata* (formerly *Pseudokirchneriella subcapitata* and *Selenastrum capricornutum*), or *Desmodesmus subspicatus* (formerly *Scenedesmus subspicatus*) (OECD 2012). In a culture beaker of 10 daphnia/L, this corresponds to roughly 1 to 2 mg C/L. During the summer, average lake algal densities are orders of magnitude lower, ranging from 0.009 to 0.07 mg C/L (McCauley & Murdoch 1987, Murdoch et al. 1998, Stevenson et al. 2017). Furthermore, it is unlikely that natural daphnia populations would occupy an environment with only one type of food available – phytoplankton communities are dynamic and vary based on

season, lake successional stage, and nutrient availability (Hartwich et al. 2012; Sommer et al. 1986; Anneville et al. 2004). For instance, lake oligotrophication and eutrophication alter the taxonomic composition of phytoplankton, which in turn alter the quality of food available to zooplankton (i.e. dominance of high quality cryptophytes and diatoms, or of low quality cyanobacteria; Hartwich et al. 2012). Concurrently, *Daphnia* and phytoplankton population abundances are tied together in a cyclical nature, and the available food per capita is therefore dependent on the state of either population (Sommer et al. 1986, McCauley & Murdoch 1987, Hartwich et al. 2012). Individual-based laboratory studies lack this natural feedback regulation of *Daphnia* and phytoplankton population densities (OECD 2012, Grant 1998, Stark & Banken 1999). Furthermore, literature on maternal effects of the resource environment suggests that neonate quality is variable upon the food conditions of the mothers, which has broad implications for *Daphnia* culture techniques and variability in neonate sensitivity.

The lack of variability in laboratory resource environments thus has direct and indirect influences on results. Directly, feeding high levels of one type of food promotes particular life history endpoints. Indirectly, individual-based studies exclude population-level feedback of intraspecific competition, and the resource environment of mothers (i.e. *Daphnia* culture) could bear maternal effects that influence the starting conditions of neonate test subjects and how they interact in their own resource environment. These physiological effects, in turn, can alter individual stress response (i.e. resistance to starvation or toxic exposure; Gliwicz & Guisande 1992, Enserink et al. 1990, Smolders 2005, Reyes 2015, Stevenson et al. 2017). For instance, in a study on food level maternal effects, offspring from mothers grown in low food levels were larger and more resistant to starvation (Gliwicz & Guisande 1992).

Effects of the resource environment thus have broad implications on applied fields and practices such as ecotoxicology and ecological risk assessment, which aim to protect natural populations from anthropogenic stress. A better understanding of how both food level and food type affect *Daphnia* physiology and stress response could inform the design of toxicity studies and models that aim to predict the impact of stressors on natural populations. However, few studies explore this causal chain in full, and most studies manipulate either food level or type, but not both. The effects of food type on *Daphnia* physiology and stress response is in need of more exploration; to our knowledge, there are currently no studies on the effects of food type on laboratory populations of *Daphnia*. In addition, there is a lack of understanding of population-level effects and how they feed back to individual physiology and stress response. The primary objective of this study was therefore to assess changes to *D. magna* physiology and stress response by means of manipulations to the resource environment. In addition, neonate quality is often judged by length or dry mass (Enserink et al. 1990, Gliwicz & Guisande 1992), but it has been suggested that these are not good indicators of neonate quality, as similar sized neonates can differ in quality (Becker & Boersma 2007, Tessier et al. 1983). A secondary objective was therefore to identify a biological marker of individual physiology (i.e. individual carbon and lipid content, dry mass, body size) that translated effects of the resource environment to individual stress response.

Methods

The resource environment was manipulated in two ways: a) directly through different food types (two species of algae – *Raphidocelis subcapitata* and *Nannochloropsis sp.*), and b) indirectly through population density stress on food level. These algae species were chosen because, while they are both 'high quality' foods for daphnia, *Nannochloropsis* is known for its particularly high lipid content (Wacker & Martin-Creuzburg 2007, Sperfeld & Wacker 2015). Many studies on food quality have use extreme differences in food quality, such as with a cyanobacteria lacking PUFAs and sterols compared against a high quality, standard algae (Martin-Creuzburg et al. 2005). In this study, the use of two suitable food types with less drastic differences in lipid content emphasized the importance of, even subtle, variations in food quality. To manipulate population density effects on the energetic resource environment, two study designs were run consecutively. In one, a 'population study', daphnia populations were allowed to bloom for 18 days until they reached peak density above carrying capacity and were 'density stressed'. In the other, a 'culture study', daphnia were cultured for 18 days and all neonates were culled before every feeding, thus eliminating intraspecific competition and creating 'non-density stressed' conditions. Physiological effects were explored by sampling daphnia at the end of the study and measuring lipid content, carbon content, dry mass, and length. Lipid-, carbon- and length-to-dry mass models incorporating data from all age classes (neonates, subadults, adults) were used to assess general effects of food type and density stress on daphnia physiology. Lipid, carbon, and dry mass in neonates were assessed against individual length to assess trends in these physiological markers that relate to the resource environment of the mother and to the stress response of the neonate. The relative stress response of neonates from each treatment was tested

by pulling neonates from each treatment at the end of the study into a neonate starvation test. A diagram of experimental methods is shown in **Appendix, Figure A1**.

General Daphnia magna culture & care

Daphnia magna were purchased from Aquatic BioSystems and cultured in standard synthetic freshwater (moderately hard; formula in appendix; EPA 2002) with 10 individuals per 1L beaker. Cultures were kept under a 16:8 hour light:dark cycle at 20°C. They were fed the microalga *Raphidocelis subcapitata* (formerly *Pseudokirchneriella subcapitata* and *Selenastrum capricornutum*) three times a week at a concentration of 1.15 mg C/L (1.85E+08 cells/L). Neonates were removed at least three times a week, or daily near the start of experiments using transfer pipets prior to feeding. Cultures received a 100% water change once a week, and were turned over to a new generation by replacing adults with neonates (<24 hours old) once adults reached 25-30 days old.

Diet analysis & preparation

Two species of microalgae were purchased by the liter and analyzed for carbon and lipid content to arrange diet treatments. *Raphidocelis subcapitata* (RAP) were purchased from Aquatic BioSystems and *Nannochloropsis sp.* (NAN) were purchased from Carolina Biological Supply. Cell counts were performed using a hemocytometer.

We chose to focus on lipid content as the qualitative difference in food, rather than elemental nutrient stoichiometry because storage of PUFAs is more important than storage of phosphorus for daphnia growth in poor food quality environments (Becker & Boersma 2005).

Additionally, P limitation appears to be an indirect effect of changes in biochemical composition (Müller-Navarra 1995a).

Lipid analysis was performed using a modified Bligh & Dyer (1959) method, using a chloroform/methanol mixture to extract lipids into the chloroform layer, and incubation with sulfuric acid to prepare the samples for colorimetric analysis. 15 ml of algae stock were centrifuged in falcon tubes at 2000G for 1 hour. Supernatant was removed, leaving 5 ml of solution, which was vortexed to resuspension. The concentrated stock was homogenized on ice for 1 min, and 1 ml of concentrated stock was transferred to conical vials. Chloroform and methanol were added at a ratio of 8:4:3 chloroform:methanol: sample (2.66 ml $CHCl_3$, 1.33 ml MeOH). Samples were vortexed for 1 min, then centrifuged at 2000G for 10 min to separate the layers. To capture lipid molecules that may not have made it into the chloroform layer, a second extraction was performed by transferring the methanol layer to new conical vials, adding 1 ml chloroform, and repeating the vortex and centrifugation steps. From the chloroform layer, 200 µl of each sample were transferred to glass vials, acidified with 0.5 ml H_2SO_4, and vortexed for 1 min. A standard curve was prepared using a serial half-dilution of the standard, tripalmitin, in chloroform starting at 0.75 mg/ml. Tripalmitin was weighed on a microbalance and transferred to a 10 ml volumetric flask to make the stock standard solution. In an effort to increase absorbance readings from the algae samples relative to the standard, the standard curve was acidified with 1 ml H_2SO_4. All samples were baked at 200°C for 15 min, cooled for 15 min, then plated on 96-well plates with 150 µl of purified sample per well. Plates were run on a microplate reader (Gen5, BioTek) at 340 nm.

For carbon analysis, 50 ml of algae stock were centrifuged in falcon tubes at 2000G for 1 hour. Supernatant was removed, leaving 3 ml of solution, which was vortexed to resuspension.

600 µl of concentrated stock was pipetted into pre-weighed 8x8x15 mm tin boats and dried overnight in an oven at 60°C. Samples were then reweighed and run on a CHNOS elemental analyzer (vario EL III Element Analyzer).

Per cell, NAN had higher carbon and lipid content (mg/cell) than RAP (NAN:RAP being 2.72 and 4.22, respectively; **Appendix, Table 1**). Diet treatments were thus normalized to mg C fed/L/day to maintain comparability of food quantity (carbon content) while altering food quality (lipid content). NAN-fed daphnia thus received, on average, 1.5 times the dietary lipid content as RAP-fed daphnia, while both received the same quantity of carbon (**Appendix, Table 1**). In both the population and culture study, *Daphnia* were fed either NAN or RAP at a level of 0.6 mg C/L/day. Stock algae was aliquoted and sealed into whirlpacks and wrapped in foil for each feeding day to reduce algal growth.

Population (density stressed) study

Populations were fed either NAN or RAP at a rate of 0.6 mg C/L/day. There were three replicate populations per treatment. Populations were each started with five neonates (<24 hours old from cultures acclimated to each respective food type for three generations; see below) and maintained under constant resource conditions until populations reached peak density. It was determined in a previous experiment that populations would peak around day 18. Each population was maintained in 800 ml of moderately hard water, which was renewed with 90% water changes three times a week. Water changes were conducted using a fishnet and siphon to slowly draw down water without harming or removing any daphnia. While the water level was at 10%, a ruler was placed under the jars and digital images of populations were taken from the top of each jar. The populations were then fed and refilled with fresh water.

Digital images of populations were processed in ImageJ (Version ij150, 2016) for daphnia count and length. All daphnia in each replicate were counted. Daphnia length was obtained by calibrating to a known length (10 mm) on the ruler in the image, and measuring a line drawn from the eyespot to the base of the tail. Lengths were taken for approximately 25% of the daphnia in a given replicate. This was achieved by zooming in on the image to 150%, creating a square view of a small section of the image which could be centered around a group of daphnia - one 'plot'. Every daphnia in the plot that showed its entire length was measured, and then that plot was marked off and the viewer was moved to a different section and the process repeated until at least 25% of the population had been measured. Because early in population growth, the populations were dominated by neonates, the first plot area was always centered on a group of daphnia where an adult was present. This ensured that the top end of the length distribution was always captured. On day 18, water was drawn down for the last digital images, and daphnia were prepared for lipid and carbon analysis or a neonate starvation test (described below).

Culture (non-density stressed) study

Five replicate cultures of *D. magna* were established for each diet, NAN or RAP, with five individuals housed in 0.5 L of moderately hard water. Cultures were fed three times a week at a rate of 0.6 mg C/L/day, and otherwise maintained in the same way as general cultures described above. The cultures were maintained for three generations with no diet crossover to acclimate cultures fed the NAN diet. The third generation (F2) was deemed sufficient to negate any physiological impacts from either direct feeding (F0) or indirect maternal effects (F1) of a different food type. On the third generation, adults were reared until day 18 and then adults and

neonates (<24 hours old) were prepared for lipid and carbon analysis. To ensure enough neonates were obtained for both physiological sampling and the starvation test, the neonate starvation test started on day 17 to ensure enough neonates (<24 hours old) would be available.

Daphnia lipid and carbon analysis

Daphnia from every treatment from the population and culture studies were sorted by length into age classes of neonates, subadults, and adults. Length ranges for each age class were as follows: neonates <1.2mm, subadults 1.2 to 2.5 mm, adults >2.5 mm. Subadults were absent from the culture study, as neonates were consistently culled. Adults were further sorted into pregnant and non-pregnant, as the state of brood development dramatically alters *Daphnia* lipid content (Tessier & Goulden 1982). Age class length ranges were chosen based on previous experimental data in which the largest observed neonates less than 24 hours old were ~1.2 mm, and length at first reproduction was ~2.5 mm.

To attain sufficient sample masses for lipid and carbon analysis, individual daphnia were pooled into each sample. Neonate, subadult, and adult samples contained 15, 5, and 1-3 individuals, respectively. We sought to maximize the number of replicate samples per treatment, with the aim of three replicates per analyte, per age class.

Daphnia were first pipetted onto labelled glass slides and dried on a light table. A digital image of each daphnid was taken so lengths of individuals could later be measured using ImageJ. Lipid samples were then transferred to glass conical vials, and carbon samples were transferred into preweighed 8 x 5 mm tin capsules. Samples were stored overnight at -20°C and processed within the next few days.

Lipid extraction and quantification followed the same method as described above for algae, but using different volumes of solvents. In the conical vials, 1.07 ml $CHCl_3$ and 0.53 ml MeOH were added, and the sample was homogenized for 30 sec. The homogenizer was rinsed into the vial with 0.4 ml DI water. Two standard curves were generated with six serial half-dilutions in effort to capture all of the readings, one starting at 0.074 mg lipid/ml $CHCl_3$ (R^2 = 0.993) and the other starting at 0.67 mg lipid/ml $CHCl_3$ (R^2 = 0.998). Lipid content was calculated as mg lipids/mg dry mass. For lipid samples, which were not directly weighed, dry mass was obtained from length-to-dry mass models fitted by the carbon samples using the equation:

$$dry\ mass = a(length)^3 \qquad \text{Eq. 1}$$

This model was fitted to data using R (Version 1.0.143, R Core Team, 2016).

For carbon analysis, samples were dried overnight in an oven at 60°C to ensure dryness prior to weighing. Samples were processed on a CHNOS elemental analyzer (vario EL III Element Analyzer). Carbon content was calculated as mg C/mg dry mass.

Neonate starvation tests

To assess the effects of maternal resource condition on offspring stress response, neonates from adults (17-18 days old) from the culture and population studies were used in neonate time-to-starvation tests. Neonates from the population and culture studies were transferred to clean jars with 50 ml moderately hard water and no food. There were five neonates per jar and six replicates per treatment. Jars were checked for mortality (immobilization) twice daily until all individuals had starved. Dead individuals were extracted at the time of observation.

Statistical analyses

Data analyses were conducted in R (Version 1.0.143, R Core Team, 2016). A t-test was used to compare population size between NAN- and RAP-fed populations and cultures on days 16 and 18. Lipid- and carbon-to-dry mass models were linear regressions, and length-to-dry mass models were nonlinear regressions. All models were parameterized in R (Version 1.0.143, R Core Team, 2016). The Wilcoxon signed-rank test was used to compare neonate carbon, lipids, and dry mass between density and non-density stressed treatments, and we compared 95% confidence intervals (Newman 2008) to determine whether these endpoints could be explained by length. T-tests were used to compare neonate length data between density stress conditions and between food types. For comparing neonate starvation time, censored survival data was expressed using the {survival} package in R (Version 1.0.143, R Core Team, 2016) and Kaplan-Meier curves. Differences in survival curves were tested using *G-rho* family tests (Harrington and Fleming 1982).

Results

Daphnia reproduction and population dynamics

Populations fed NAN had faster population growth and larger peak sizes than those fed RAP (**Fig. 1**). At day 18, populations fed NAN had mean counts of 216 (± 11 SD) individuals, while those fed RAP had mean counts of 185 (± 22 SD) individuals (rounded to nearest whole number). Notably, NAN-fed populations were beginning to decline by day 18; they actually peaked earlier on day 16 with a mean count of 241 (± 26 SD) individuals, while RAP-fed populations on day 16 had 124 (± 26 SD) individuals. T-tests on population counts between NAN- and RAP-fed populations showed significant differences on day 16 (t-test; $t_4 = 5.55$, $p<0.01$), but not day 18.

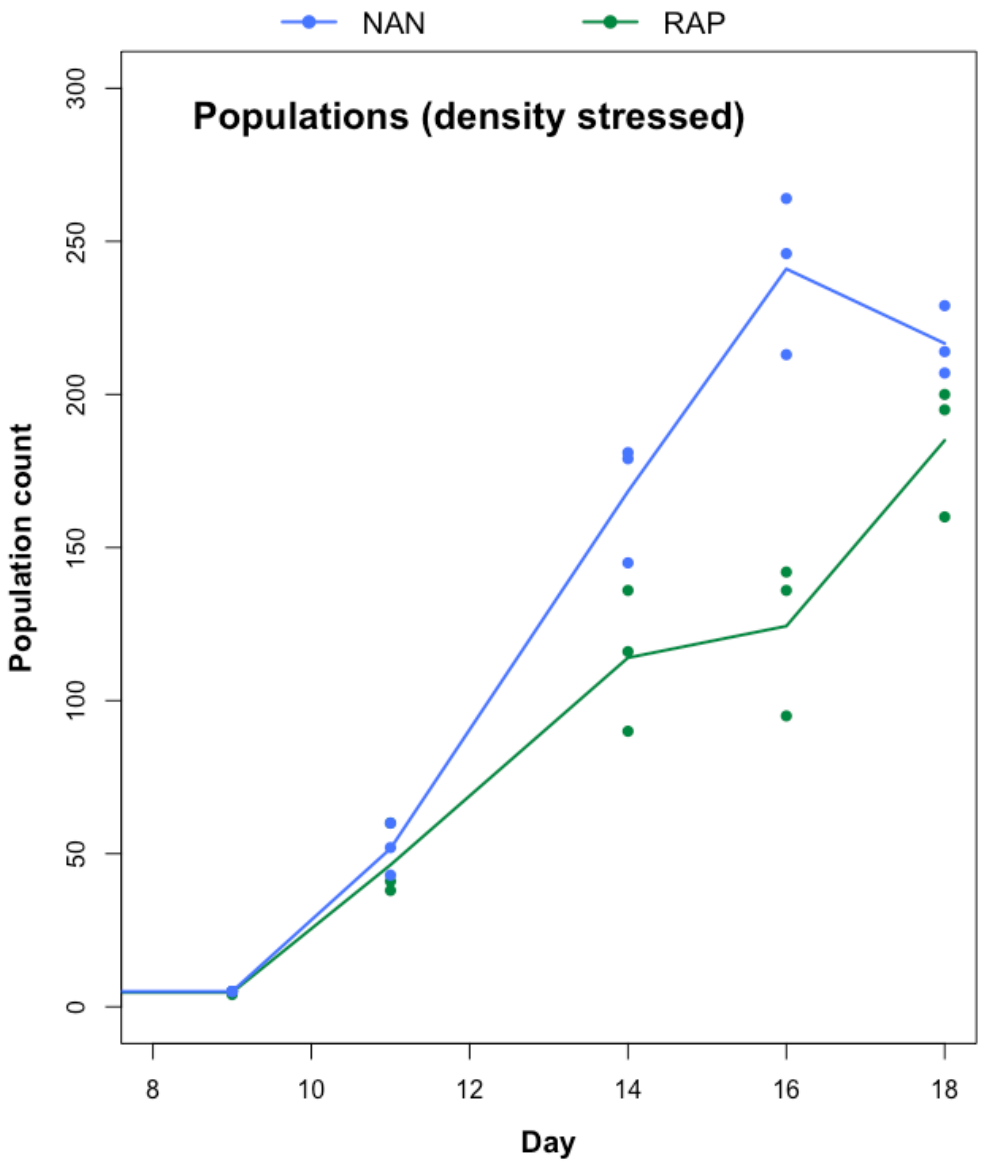

Figure 1. Plot of abundance over time in *Daphnia* populations. Lines are means, and points are actual values of each replicate. NAN-fed treatments are in blue and RAP-fed treatments are in green.

D. magna cultures fed NAN or RAP were reared for three generations. In the third generation, daphnia fed NAN had significantly lower cumulative reproduction than those fed RAP (**Fig. 3**; t-test; t_8=-6.17, p <0.001). By day 18, NAN-fed cultures produced a mean of 17 (±

8 SD) neonates per individual, while RAP-fed cultures produced a mean of 48 (± 8 SD) neonates per individual.

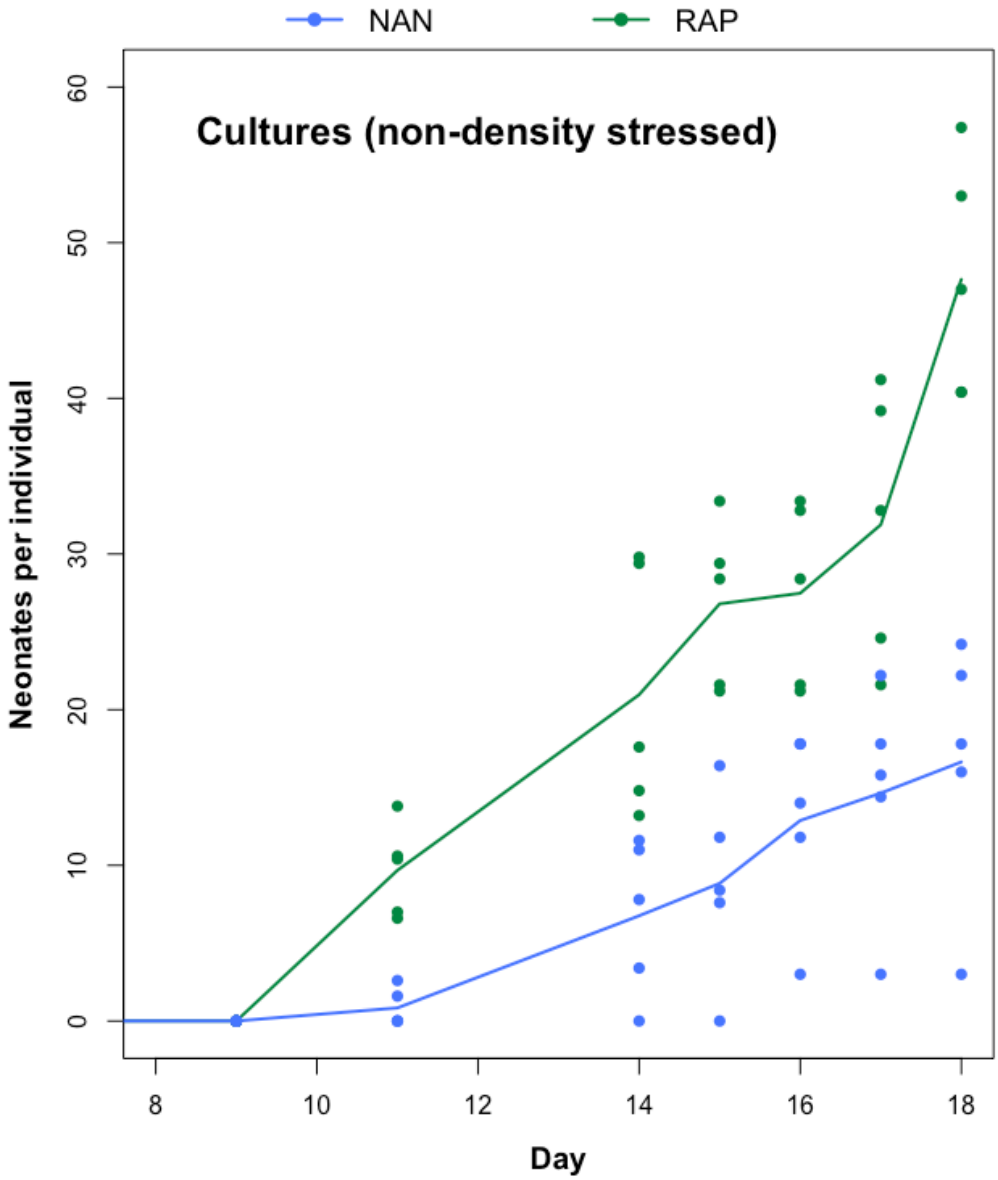

Figure 2. Plot of cumulative reproduction over time in *Daphnia* cultures. Lines are means, and points are values for each replicate. NAN-fed treatments are in blue and RAP-fed treatments are in green.

Daphnia physiology - all age classes

All daphnia from both the population and culture study were sampled on day 18 and measured for carbon and lipid content, dry mass, and length. Data on carbon, lipids, and length from samples at each daphnia age class were plotted and modelled against dry mass in **Figure 3** to assess the impact of food type and density stress on these biological endpoints as daphnia grew.

Lipid-to-dry mass showed the largest effect of both food type and density stress; daphnia individuals fed RAP accumulated lipids at the same rate whether they were density stressed (slope=0.1044) or non-density stressed (slope=0.1095). But individuals fed NAN had higher accumulation of lipids in non-density stressed conditions (slope=0.1719), and lower accumulation of lipids in density-stressed conditions **(**slope=0.0370; **Fig. 3)**.

Daphnia carbon content was similar with both food types and in density and non-density stressed conditions. When fed NAN, non-density stressed daphnia accumulated slightly more carbon than density-stressed daphnia (slopes=0.4345, 0.3636, respectively). When fed RAP, the opposite occurred and density stressed daphnia accumulated slightly more carbon than non-density stressed daphnia (slopes=0.4012, 0.3438, respectively; **Fig. 3**).

Daphnia length-to-dry mass was greater under density stressed conditions compared to non-density stressed conditions, regardless of food type. Like with the accumulation of lipids, NAN-fed daphnia again showed greater difference in length between density stressed and non-density stressed conditions than RAP-fed daphnia **(Fig. 3)**.

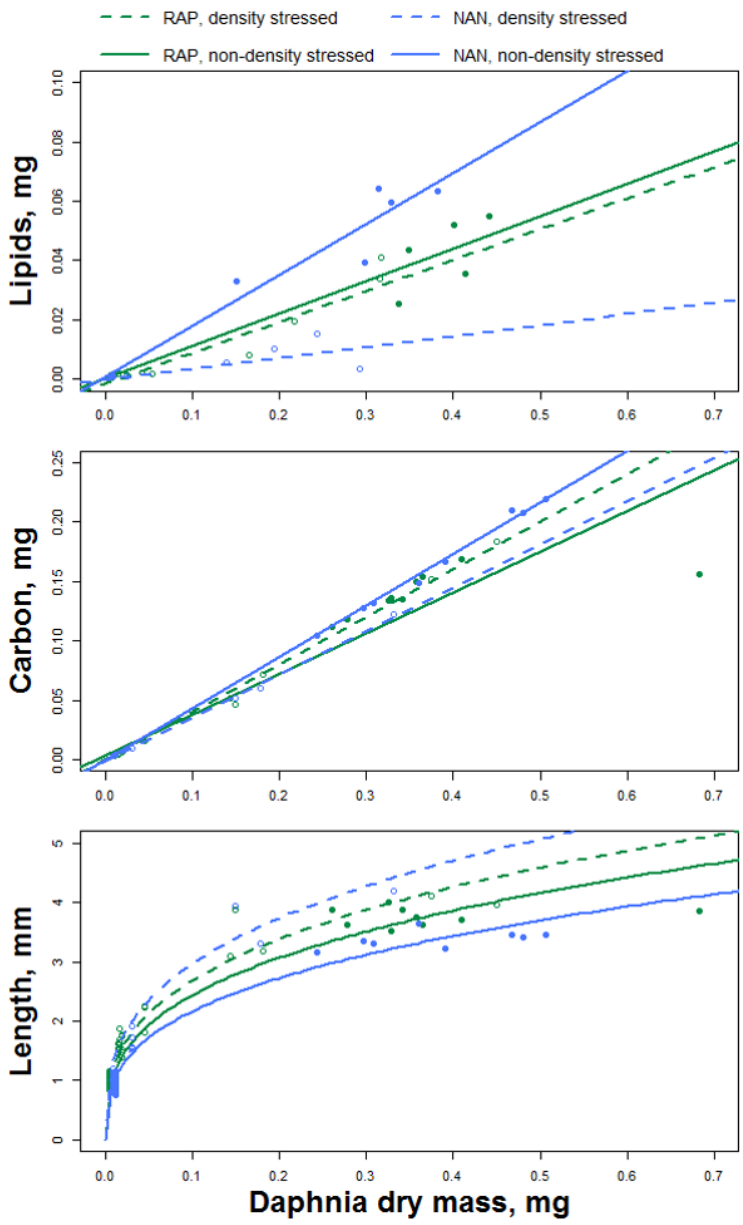

Figure 3. Models of lipid-, carbon-, and length-to-dry mass for daphnia of all age classes from density stressed populations or non-density stressed cultures fed NAN (blue) or RAP (green). Points show experimental data (open = populations, solid = cultures), and lines show fitted models (dashed = population, solid = cultures).

Daphnia physiology - neonates

Neonate percent lipids, percent carbon, and dry mass were plotted against the lengths (mean and 95% confidence intervals) of neonates in each sample (15 neonates per sample; **Fig. 4**). When comparing the effects of density stress, neonate percent carbon was significantly higher in non-density stressed conditions, regardless of food type (Wilcoxon; W=0, p=2.0e-08; W=0, p=2.8e-13; NAN and RAP, respectively). Neonate percent lipids were significantly higher in non-density stressed conditions when neonates were fed RAP (Wilcoxon; W=0, p=0.00394), but not NAN. Neonate dry mass was significantly greater in non-density stressed conditions when mothers were fed RAP (Wilcoxon; W=0, p=6.13e-14).

When comparing effects of food type among non-density stressed cultures, neonate percent lipids was higher in RAP-fed cultures relative to NAN-fed cultures (t-test; t_6=-3.12, p=0.02069; **Fig.4**). There were no differences found in neonate percent carbon or dry mass between food types. When comparing effects of food type among density stressed populations, neonate percent carbon was higher in RAP-fed populations relative to NAN-fed populations (Wilcoxon; W=0, p=6.934e-08; **Fig. 4**). Neonate dry mass was lower in RAP-fed populations relative to NAN-fed populations (Wilcoxon; W=492, p=7.225e-09; **Fig. 4**).

When combining neonate length data from all neonate samples (lengths from lipid and carbon samples), there were significant effects of density stress on neonate length at either food type; neonate length was greater in density-stressed populations relative to non-density stressed cultures when fed both NAN (t-test; $t_{176.93}$=6.49, p<0.0001) and RAP (t-test; $t_{101.29}$=5.80, p<0.0001; **Appendix, Fig. A9**). Food type also had a significant effect on neonate length; NAN neonates had greater lengths than RAP neonates in both density stressed populations (t-test; $t_{226.08}$= 2.7347, p<0.01) and non-density stressed cultures (t-test; $t_{78.84}$=2.5809, p=0.01171;

Appendix, Fig. A9). The 95% confidence intervals (Newman 2008) of neonate length data were used to test for differences in neonate length at different values of neonate percent lipids, percent carbon, and dry mass. No significant relationships between neonate length and the other measured physiological endpoints were determined by comparison of the 95% confidence intervals.

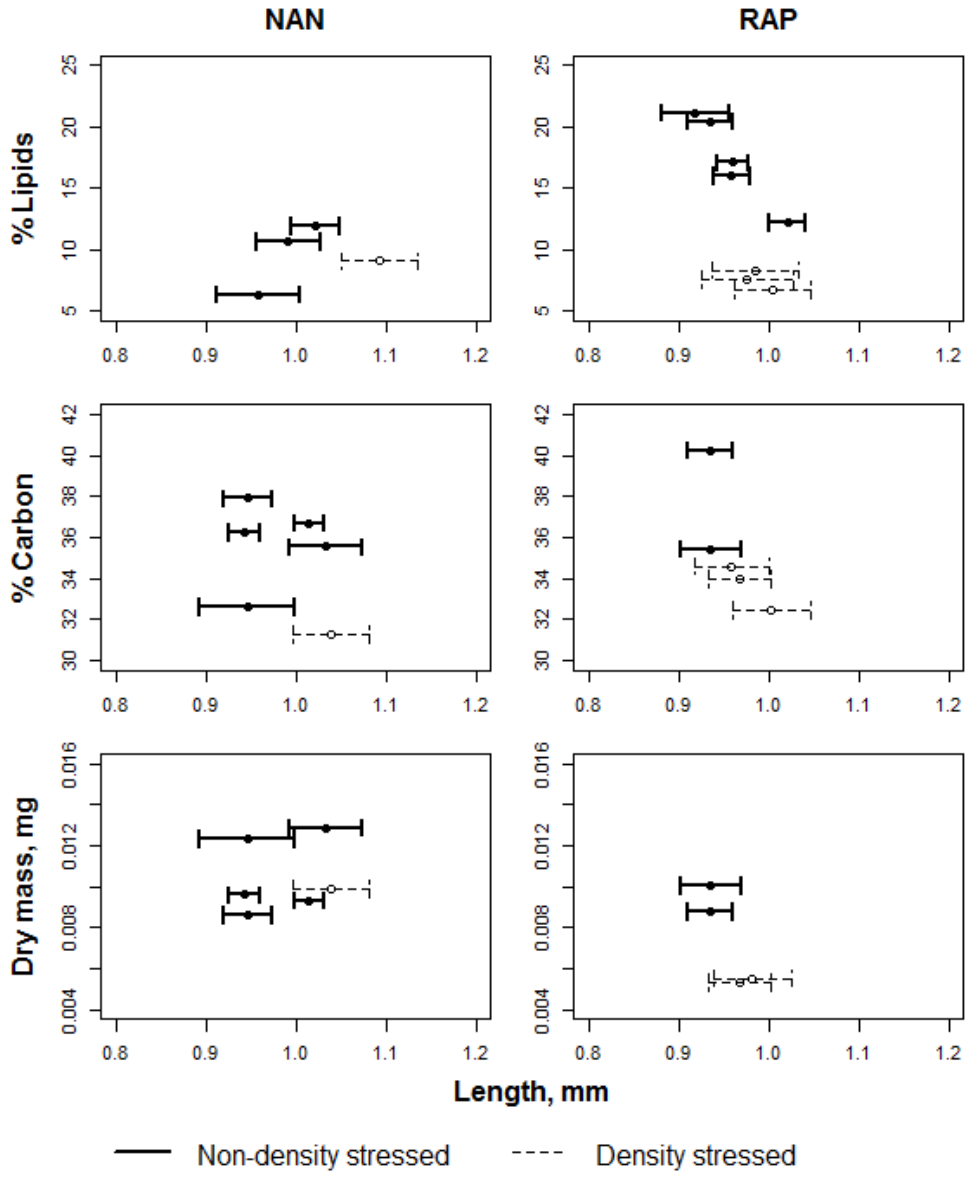

Figure 4. Lipid-, carbon-, and dry mass-to-length for neonates from density stressed populations (dashed) or non-density stressed cultures (solid) fed NAN (left) or RAP (right). Length data is expressed as means w/ 95% confidence intervals.

Neonate starvation tests

Neonates from non-density stressed cultures survived for longer periods than those from density-stressed populations, when fed both NAN (*G-rho*; chi-squared=33.4, df=1, p=7.65e-09) and RAP (*G-rho*; chi-squared=33.6, df=1, p=6.9e-09; **Fig. 5**).

Among neonates from density stressed populations, there was a significant effect of food type on starvation time (*G-rho*; chi-squared=12.8, df=1, p=0.000338), where neonates from populations fed NAN survived starvation longer (**Fig. 5**). Among neonates from non-density stressed cultures, food type did not have a significant impact on starvation time.

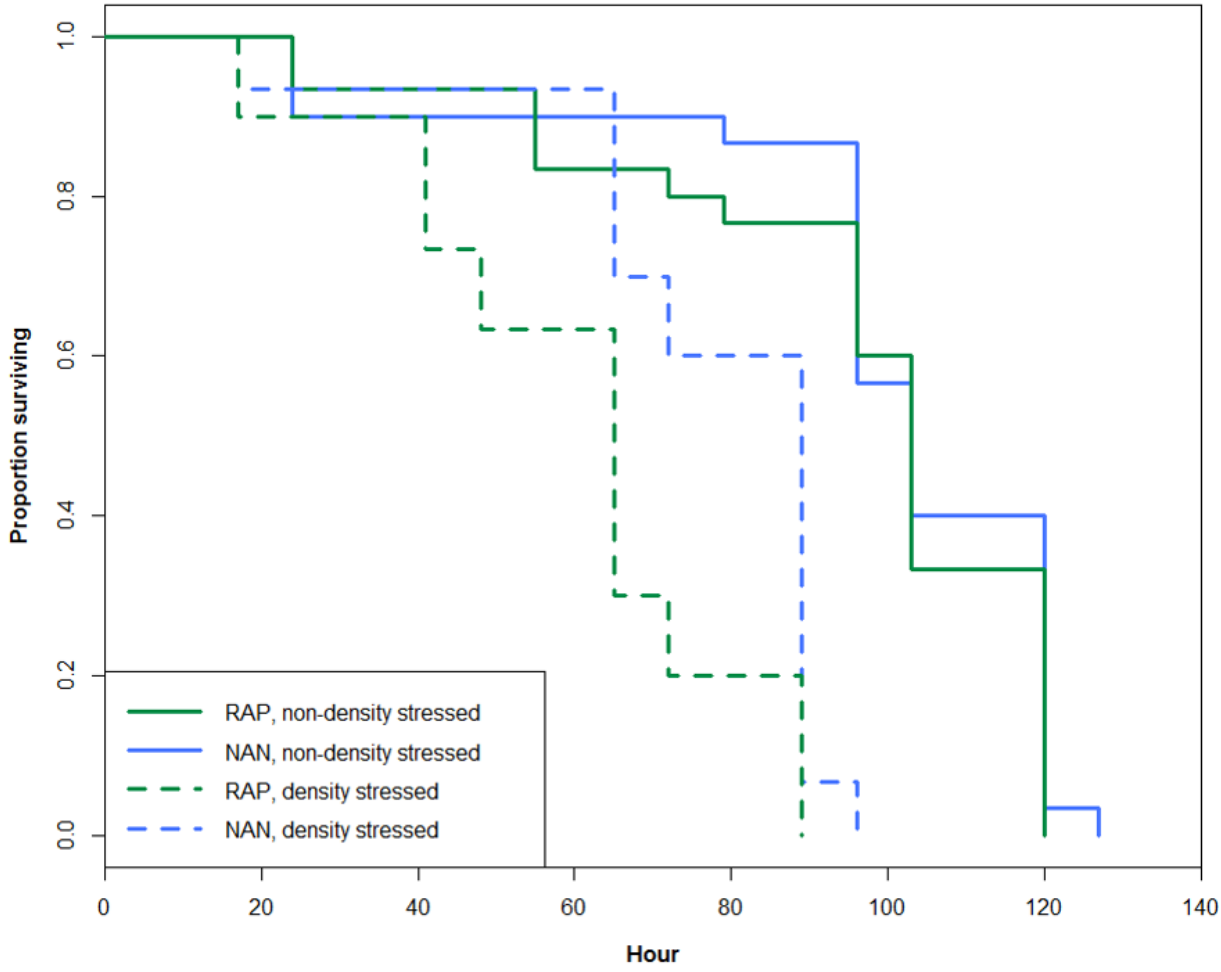

Figure 5. Plot of time-to-starvation for neonates extracted from populations (dashed lines) or cultures (solid lines) from mothers fed NAN (blue) or RAP (green).

Discussion

Daphnia reproduction and population dynamics

A novel observation was made in which *D. magna* population counts were higher when fed a food type with higher dietary lipids, even though diets were normalized for carbon (**Fig. 1**). Dietary lipids in NAN diets averaged 1.5x greater than in RAP diets. This result displays the potential importance of even minor differences in food quality (in this case, lipid content) with regard to population dynamics. These results are consistent with other observations of food quality effects on *Daphnia* life history traits. Becker & Boersma (2007) found EFA-enriched mothers grew larger and had greater reproduction than controls. Martin-Creuzburg et al. (2005) found that somatic and population growth rates increased in nonlinear regression with increasing dietary sterols. In these studies, the differences in food type are far more extreme compared to those used in the current study. Becker & Boersma (2007) enriched *Chladymonas sp.* with three fatty acids and Martin-Creuzburg et al. (2005) compared effects of the green alga *Scendesmus obliquus* and the cyanobacterium *Synechococcus elongatus*. For our study, we used two high quality algae that are known to be sufficient for daphnia growth (OECD 2012, Wacker & Martin-Creuzburg 2007, Sperfeld & Wacker 2015), creating minimally manipulated experimental conditions which emphasized the influence of dietary lipids on *Daphnia* population dynamics and life history.

Interestingly, results from the culture study appeared to contradict those from the population study in that adults fed NAN were smaller (**Fig. 3**) and had lower cumulative reproduction compared to the adults fed RAP (**Fig. 2**). A possible explanation for this discrepancy may be that jars in the population study received water changes three times a week, concurrent with feeding, meaning algae was recirculated in the jars with the new water. Culture

study jars were fed on the same schedule but received water changes only once per week because they were kept at low density. NAN cells were heavier and less abundant in diets than RAP cells (**Appendix, Table 1**), requiring about half the number of cells to reach the same amount of carbon. Although every jar was aerated to reduce algae settling, it is possible that, in the culture study, NAN cells settled to the bottom more quickly than RAP cells, becoming less available for ingestion. This would have larger effects early in the experiment when young neonates had lower ingestion rates and assimilation efficiencies (Vanni & Lampert 1992) and did not eat all of the algae. If RAP was thus more immediately accessible, for longer, in the cultures, these individuals may have had higher 'realized' food levels. This seems plausible because there is evidence of maternal effects of food level between the three generations that were cultured. Reproductive output in the first generation of the culture study (**Appendix, Figure A7**) was similar to the output in the third generation (**Figure 3**), where NAN-fed individuals, compared to RAP-fed individuals, had delayed age at first reproduction and lower cumulative reproduction. In the second generation of the culture study, NAN-fed individuals had earlier and greater cumulative reproduction (**Appendix, Figure A7**). If the first generation of NAN-fed individuals 'sensed' a lower food level, dietary maternal effects suggest that they would decrease clutch size and increase per offspring investment, resulting in fewer neonates (second generation) that were better capable of growth in low food levels (Becker & Boersma 2003, Becker & Boersma 2007). This maternal effect then disappeared when second generation NAN-fed individuals grew larger (relative to the first generation), and therefore increased clutch size and decreased per offspring investment. This hypothesis seems plausible, as Becker & Boersma (2007) found that maternal effects on offspring size and clutch size were mostly attributed to the size of the mother.

While such maternal effects have been observed with regard to food level effects on neonate length (Enserink et al. 1990, Gliwicz & Guisande 1992, Gabsi et al. 2014), and dry mass (Glazier 1992, Gliwicz & Guisande 1992), the current study showed that maternal effects can be driven by food type, even at the same food level. In density stressed populations, those fed NAN had neonates with higher dry mass than those fed RAP (**Fig. 4**) because the rapid growth of the former resulted in a peak at day 16 followed by decline starting on day 18. Thus, density stressed mothers fed NAN experienced low food levels which induced maternal effects increasing offspring size and decreasing clutch size. Gorbi et al. (2011) suggested that *Daphnia magna* mothers respond to population density by either reducing fecundity or increasing neonate starvation resistance, and that relative use of either strategy differs by clones. In this case, mothers in the density stressed populations seem to have focused more on decreasing fecundity, as their respective neonates were more prone to starvation than neonates from non-density stressed mothers (**Fig. 5**).

Daphnia physiology - all age classes

Slopes of daphnia carbon-to-dry mass models were the least variable compared to the variability seen in daphnia lipids and length among treatments (**Fig. 3**), suggesting that the accumulation of carbon in daphnia is relatively stable when feeding at a fixed carbon level. However, the small differences in the slopes of these carbon models are notable given that NAN and RAP diets differed only in lipids by a factor of 1.5. Müller-Navarra (2000) suggested that PUFAs can regulate the transfer of carbon from primary producer to consumer, which may be evident here in the higher accumulation of daphnia carbon in non-density stressed cultures fed NAN (**Fig. 3**). Accordingly, these individuals also accumulated the most lipids (**Fig. 3**).

Interestingly, food type seemed to influence the effect that density stress has on accumulation of lipids. For daphnia fed NAN, density stressed populations had considerably less lipids compared to non-density stressed cultures (**Fig. 3**). However, for daphnia fed RAP, there was hardly any difference in accumulation of lipids between density stressed populations and non-density stressed cultures (**Fig. 3**). This pattern was also seen in daphnia length-to-dry mass models, where lengths between non-density stressed culture and density stressed population daphnia were more similar when fed RAP than when fed NAN (**Fig. 3**). The low lipid accumulation in NAN populations could be due to sampling near the peak population density. Populations fed NAN peaked higher than those fed RAP, resulting in higher density stress and lower per capita food resources. Density stress on food availability would decrease maternal clutch size, reducing the accumulation of lipids that occurs during the intermolt phase before being transferred to eggs (Tessier & Goulden 1982). Lipid storage in daphnia mothers has been positively correlated with clutch size (Tessier & Goulden 1982). However, this does not explain why mothers in NAN cultures had higher lipid accumulation but lower clutch sizes than mothers in RAP cultures (**Fig. 3**). Interestingly, the slope for lipid accumulation was slightly lower in density-stressed populations fed NAN relative to those fed RAP (**Fig. 3**). This could again be symptomatic of the higher density stress in NAN populations.

Length-to-dry mass models (**Fig. 3**) corroborated reproductive patterns in **Figures 1 and 2**. Reproduction is known to be a hyperbolic function of daphnia length (Kooijman 2010). In density stressed populations, models showed greater daphnia length-to-dry mass ratios when fed NAN compared to RAP (**Fig. 3**); accordingly, NAN populations had higher growth rates and peak density (**Fig. 1**). In non-density stressed cultures, models showed greater daphnia length-to-

dry mass ratios when fed RAP compared to NAN (**Fig. 3**); accordingly, RAP cultures had higher cumulative reproduction than NAN cultures (**Fig. 2**).

Neonate physiology and fitness

Neonate starvation resistance was most influenced by density stress under these experimental conditions. Fed either food type, neonates from non-density stressed cultures survived starvation significantly longer than neonates from density-stressed populations (**Fig. 5**). Food type had a significant effect on neonate starvation resistance only in density-stressed populations (**Fig. 5**). This suggests that food quality is relatively more important at low food levels, as here imposed by peak phase population density. Non-density stressed culture daphnia had more access to dietary carbon and lipids and therefore could transfer more energy to offspring to have more reserves to battle starvation.

A secondary objective of this study was to identify a physiological 'marker' in daphnia that translates the effect of the resource environment to an indication of stress response. We analyzed neonates for lipid and carbon content as well as dry mass and length to see which 'marker' correlated the most with differences in starvation resistance. We checked if any of the physiological markers tested held a consistent pattern that may explain the greater starvation resistance observed in neonates from non-density stressed cultures, relative to those from density stressed populations (both food types), as well as in neonates from density stressed populations fed NAN, relative to those fed RAP (**Fig. 5**). Consistent with the effect of density stress on neonate starvation resistance, neonate percent carbon in non-density stressed cultures was higher than in density stressed populations, regardless of food type (**Fig. 4**). However, inconsistent with the effect of food type on starvation resistance in density stressed populations, neonate percent

carbon was actually lower in populations fed NAN than in those fed RAP (**Fig. 4**). Notably, analysis of percent carbon in NAN neonates from density stressed populations was complicated by the fact that only one sample was obtained. Despite this inconsistency, percent carbon was the best indicator of neonate quality pertaining to starvation resistance in density versus non-density stressed conditions.

Other studies have suggested lipid content to be a potential indicator of neonate quality. Lipids are a major storage product for cladocerans (Forkas 1970, Lee 1975, Goulden & Hornig 1980) and are metabolized during periods of starvation to prolong survival (Lemche & Lampert 1975, Goulden & Hornig 1979). Cowgill et al. (1984) found positive correlations between algal fat, adult daphnia fat, and neonate starvation times. Becker & Boersma (2007) observed EFA enrichment in daphnia mothers caused offspring to be less vulnerable to starvation. Tessier et al. (1983) found *Daphnia* lipids to be a better predictor of starvation resistance than dry mass. In our study, neonate lipid content was not consistently greater in neonates more resistant to starvation. Neonates from non-density stressed cultures had higher percent lipids than neonates from density stressed populations, but only when fed RAP, not NAN (**Fig. 3**). This was the same case for neonate dry mass (**Fig. 3**). Nevertheless, lipids as a biomarker cannot be ruled out, as only one sample in the NAN population treatment was obtained.

In any case, other studies concede that the traditional use of offspring size in terms of mass or length (Enserink et al. 1990, Gliwicz & Guisande 1992) is not a good indicator of neonate quality, as similar sized neonates can differ in quality (Becker & Boersma 2007, Tessier et al. 1983). We found that neonates from density-stressed populations were larger than those from non-density stressed cultures, regardless of food type (**Appendix, Fig. A8**), a pattern that

clearly did not translate to greater starvation resistance. Furthermore, there were no trends found in neonate lipids, carbon, or dry mass that were explained by length.

Summary and Conclusions

A major takeaway from this study is that food type, with subtle differences in quality, can result in significant differences in individual-level physiology and life history traits and population-level dynamics. In addition, during periods of low food levels (peak population density), *Daphnia magna* mothers fed food with higher lipid content (NAN) may bear offspring with increased resistance to starvation.

Neonate starvation resistance was most impacted by density stress as imposed by peak population density. Although other studies have observed larger size and greater starvation resistance in neonates born from mothers in low food level conditions (Glazier 1992, Gliwicz & Guisande 1992), our studies showed that neonates from 'low' food mothers (density stressed) were larger, yet less starvation resistant than neonates from 'higher' food mothers (non-density stressed). This observation does not discount insights from the maternal effects literature in any way. We pulled neonates when populations were at peak density (day 18). Mothers simply may not have had enough time during which reproductive strategies may have shifted such that neonates were born with more energy reserves.

Percent carbon was a better indicator of neonate quality than percent lipids, dry mass, or length. Further studies are necessary to better address this question, as sample sizes in this study were limited by the number of neonates available. However, our results reinforce other findings that neonate 'size' is not a suitable indicator of neonate quality (Becker & Boersma 2007, Tessier

et al. 1983), and that more fine-tuned metrics such as carbon or lipid content should continue to be explored.

Overall, the resource environment, as described by food level and type, has significant effects on, and feedbacks between, daphnia physiology, stress response, and population dynamics. In the context of ecotoxicology and ecological risk assessment, these findings suggest that food dynamics and effects at the individual and population level should be considered when designing experiments and models to assess the impact of stressors on aquatic organisms. The primary concern should be with food level, especially with the feedback of population density and intraspecific competition on per capita food level, as this creates the greatest stress on individuals in a population. A secondary concern should be that food type has distinct impacts on population dynamics and individual physiology, even at the same food level. It is important to consider, however, that the effects of diet type are likely even more significant when diets differ substantially in quality.

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Appendix

The figures in this section show data supporting the main document.

Experimental design:

Figure A1 shows a diagram of the experimental design of this study/

Characterization of diets:

Table A1 and Figure A2 show quantitative results of characterizing food types NAN and RAP for elemental and lipid content, and dry mass.

Population study – food level treatments:

There were actually multiple food level treatments (low, med, high) in the population study; low and med treatments were excluded from the main document for clarity and conciseness. Populations were fed either NAN or RAP, at low, medium, or high levels (0.2, 0.4, or 0.6 mg C/L/day, respectively). Figure A3-A6 show results of all food level treatments.

Culture study – first and second generations:

Results from the culture study discussed in the main document were for the third generation of cultures fed each food type. Culture cumulative reproduction results from the first and second generations are shown in Figure A7, supporting the discussion of food maternal effects in the main discussion.

Neonate length data:

Figure A8 shows direct comparisons of all length data from neonates that were sampled in the study, and supports a section of discussion in the main document.

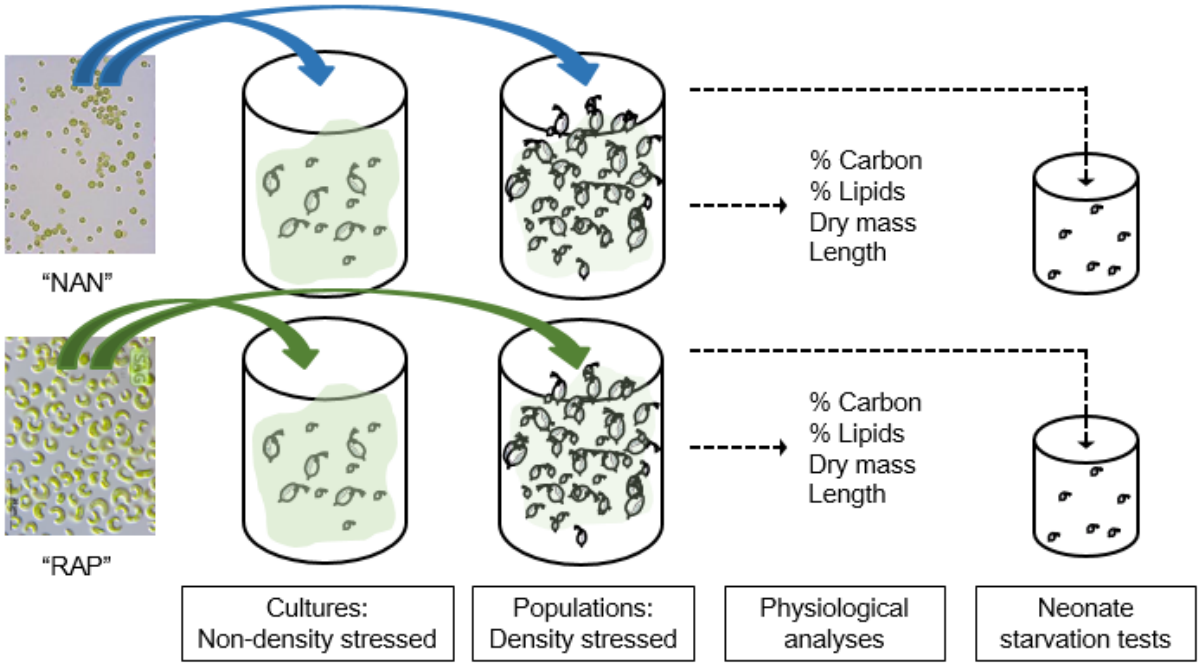

Figure A1. Diagram of experimental methods.

Table A1. Quantitative characterization of food types NAN and RAP. Values are means and standard deviations of all algae bottles used for experiments (n_{NAN}=3; n_{RAP}=2).

Food type	dry mass per cell	% C	% N	C:N	mg C per cell	% lipids	mg lipids per cell	mg lipids per mg C
NAN (*Nannochloropsis sp.*)								
mean	**4.70E-08**	**34.80**	**6.47**	**5.37**	**1.64E-08**	**17.00**	**8.21E-09**	**0.49**
SD	*3.84E-09*	*1.74*	*0.37*		*2.10E-09*	*3.48*	*1.35E-09*	*0.12*
RAP (*Raphidocelis subcapitata*)								
mean	**1.23E-08**	**50.16**	**6.56**	**7.65**	**6.17E-09**	**14.47**	**1.95E-09**	**0.33**
SD	*3.19E-09*	*0.28*	*1.02*		*1.63E-09*	*1.06*	*6.17E-11*	*0.08*
NAN:RAP	**3.82**	**0.69**	**0.99**	**0.70**	**2.66**	**1.18**	**4.22**	**1.50**

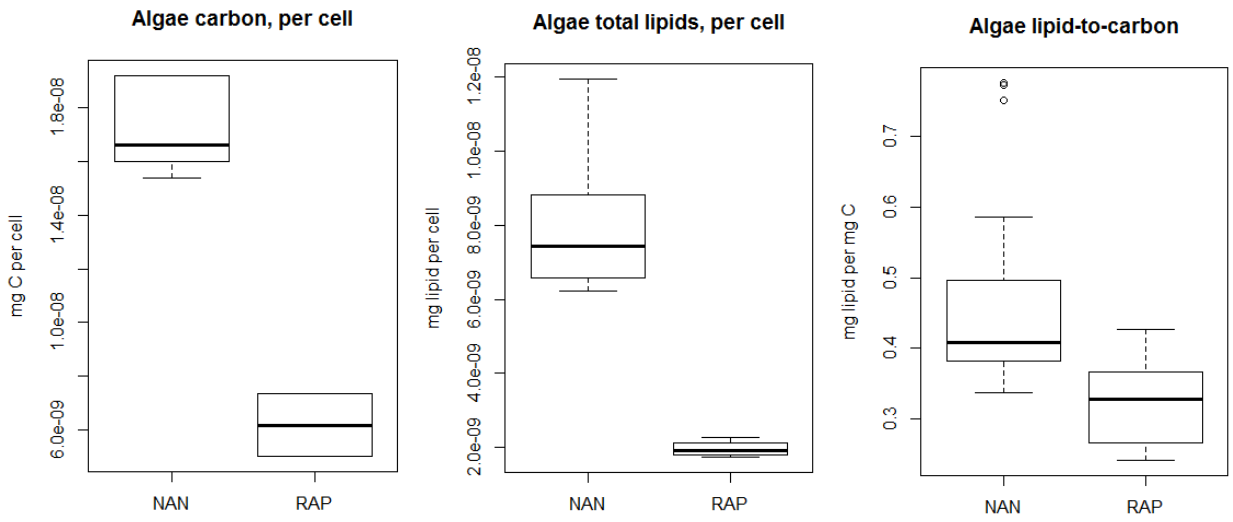

Figure A2. Characterization of food types NAN and RAP by (a) mg C per cell, (b) mg lipids per cell, and (c) mg lipids per mg C.

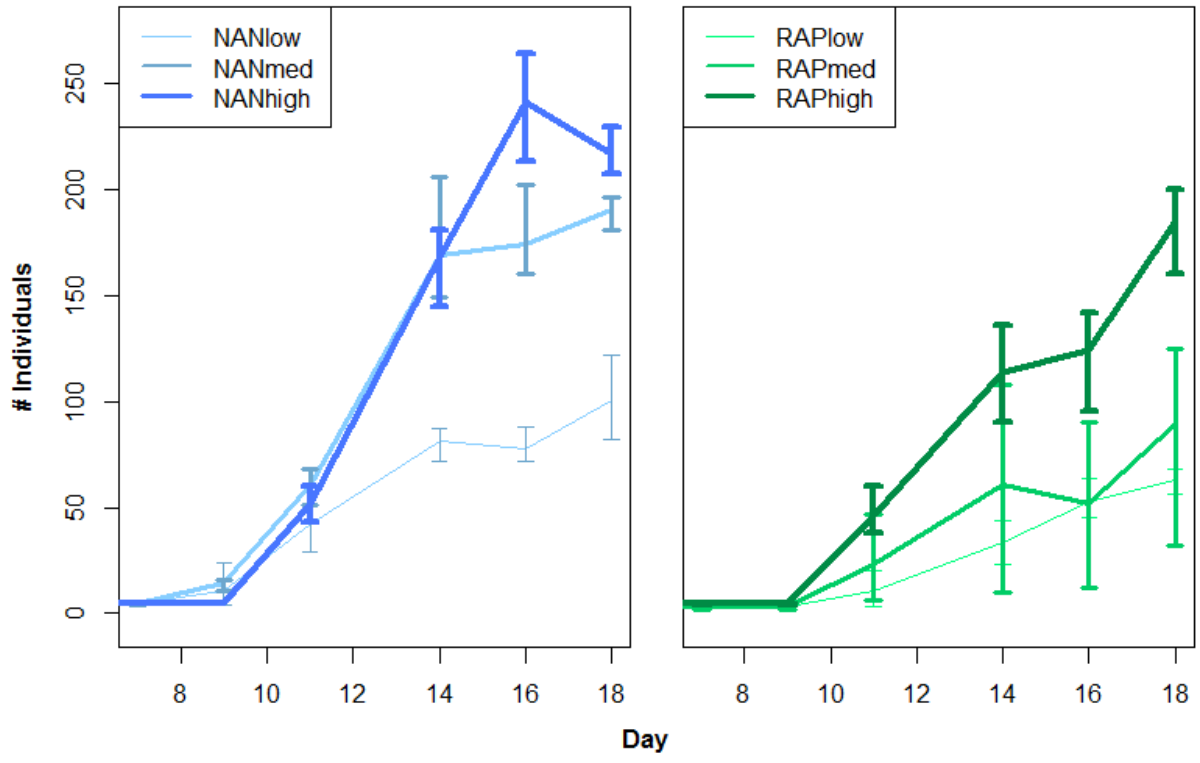

Figure A3. Plot of abundance over time in *Daphnia* populations (all food levels). Lines are mean count, and error bars represent minimum and maximum count.

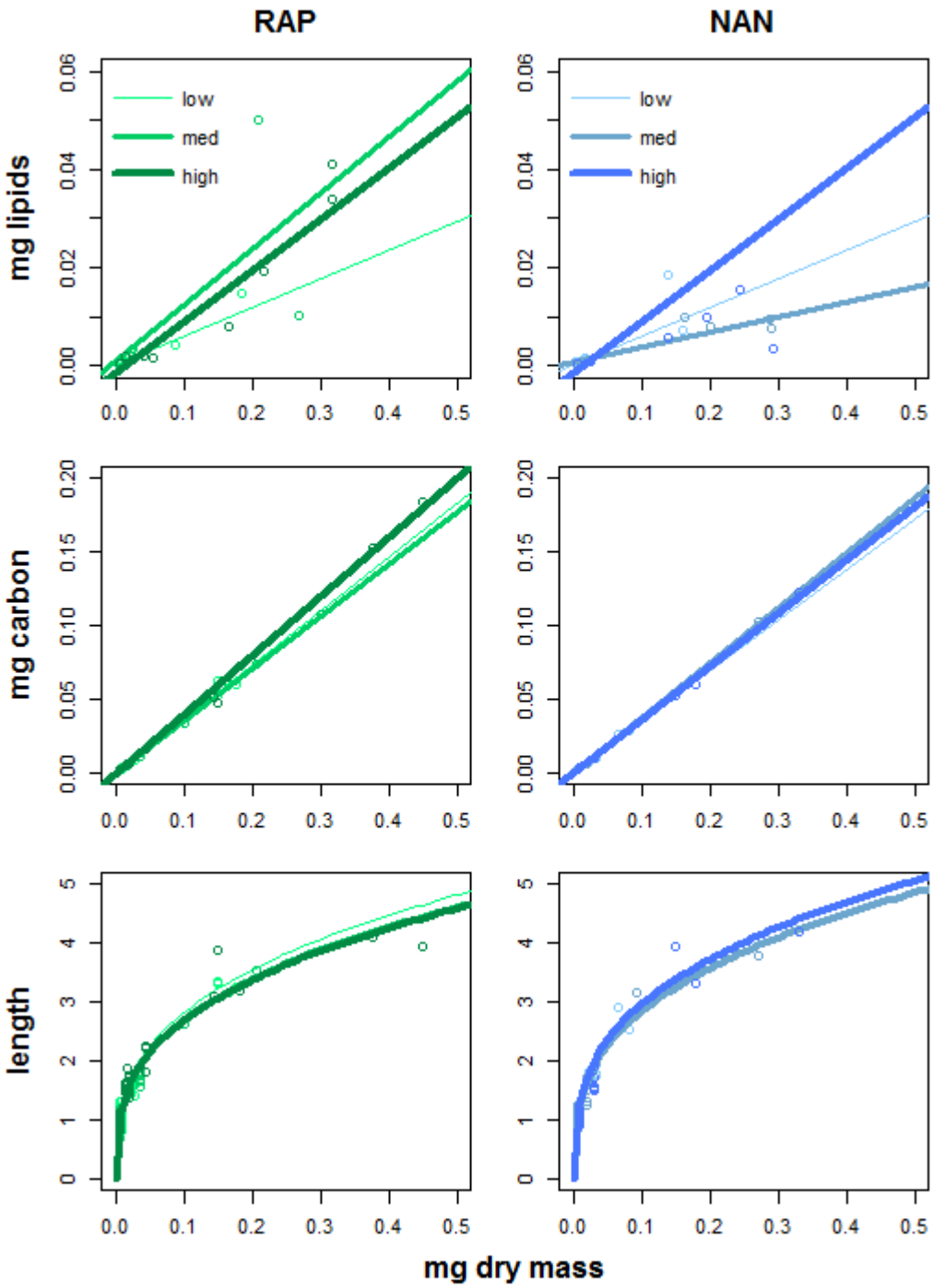

Figure A4. Models of lipid-, carbon-, and length- to dry mass in daphnia of all age classes from density stressed populations fed low, med, and high food levels. Points show experimental data and lines show fitted models.

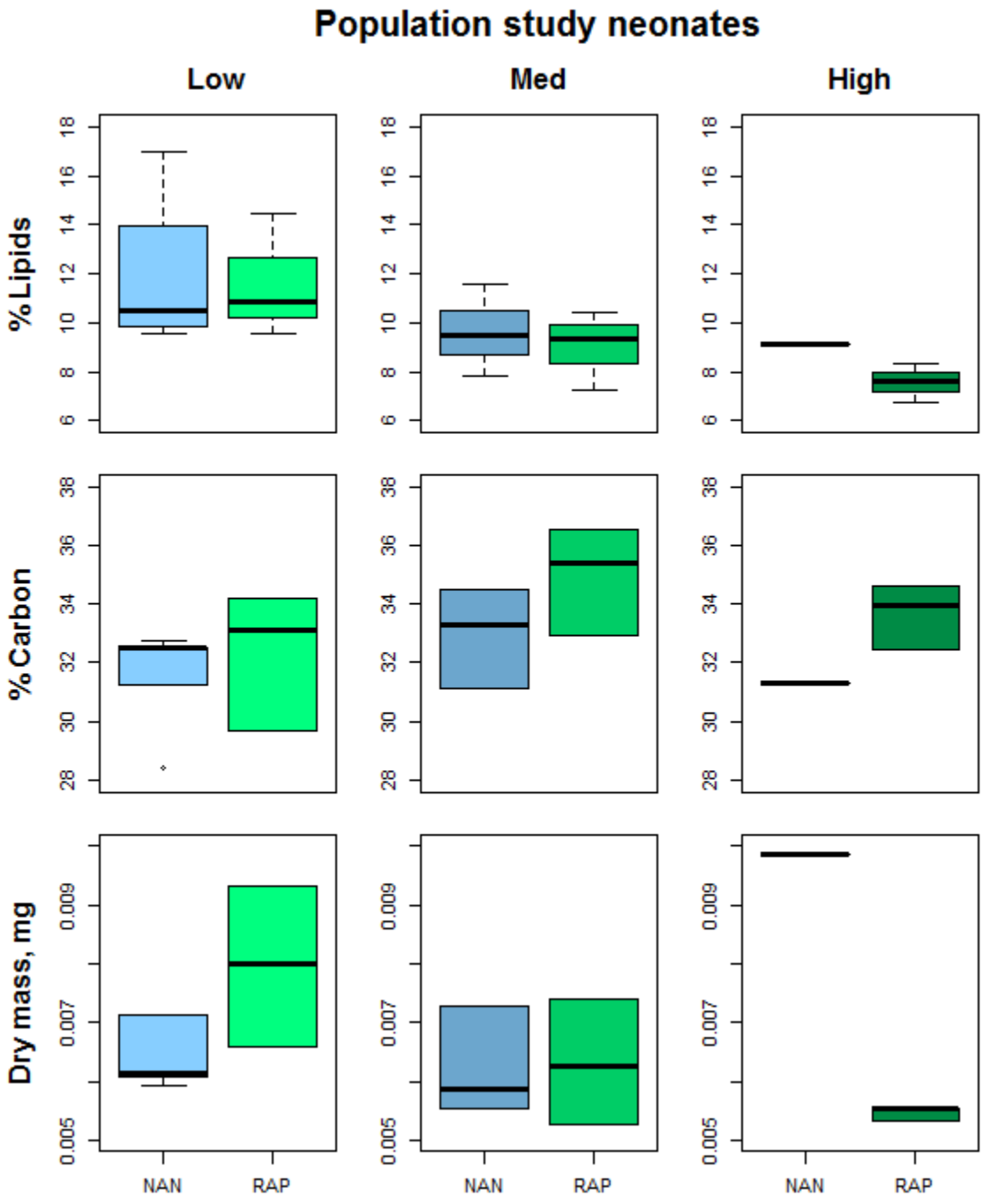

Figure A5. Boxplots of neonate percent lipids, percent carbon, and dry mass for neonates extracted from populations fed NAN or RAP at low, medium, and high levels.

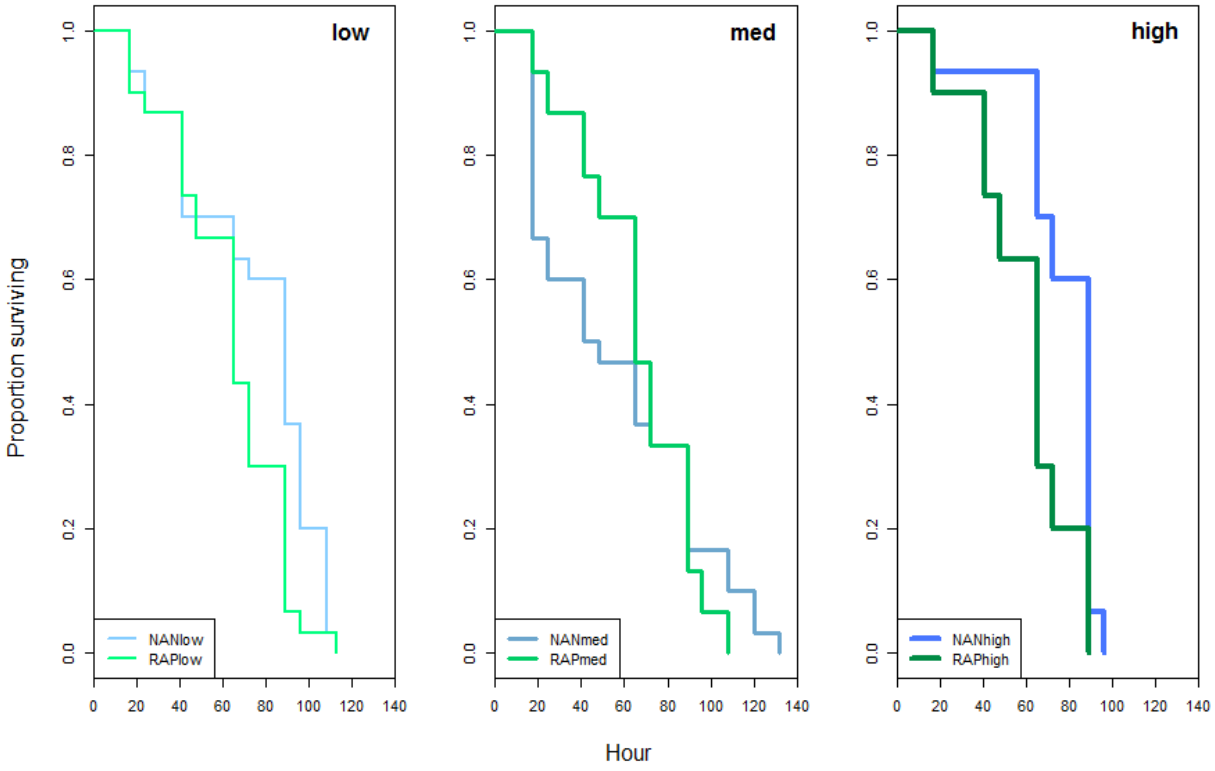

Figure A6. Time-to-starvation for all food level treatments of the population study. Differences were significant at low food (*G-rho*; chi-sq=4.9, df=1, p=0.0265) and high food (reported in main document).

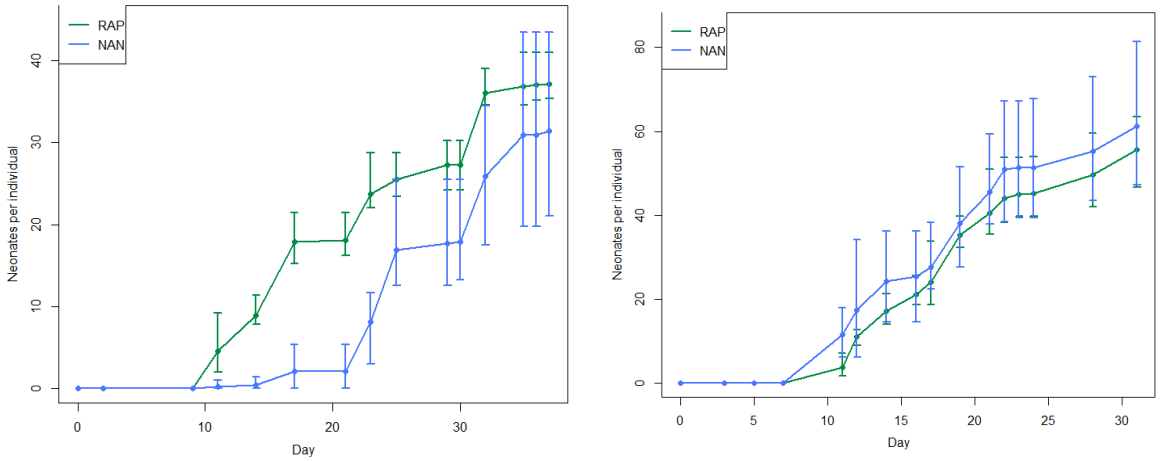

Figure A7. Cumulative reproduction in non-density stressed *Daphnia* cultures in the first generation (left) and second generations (right) of the culture study.

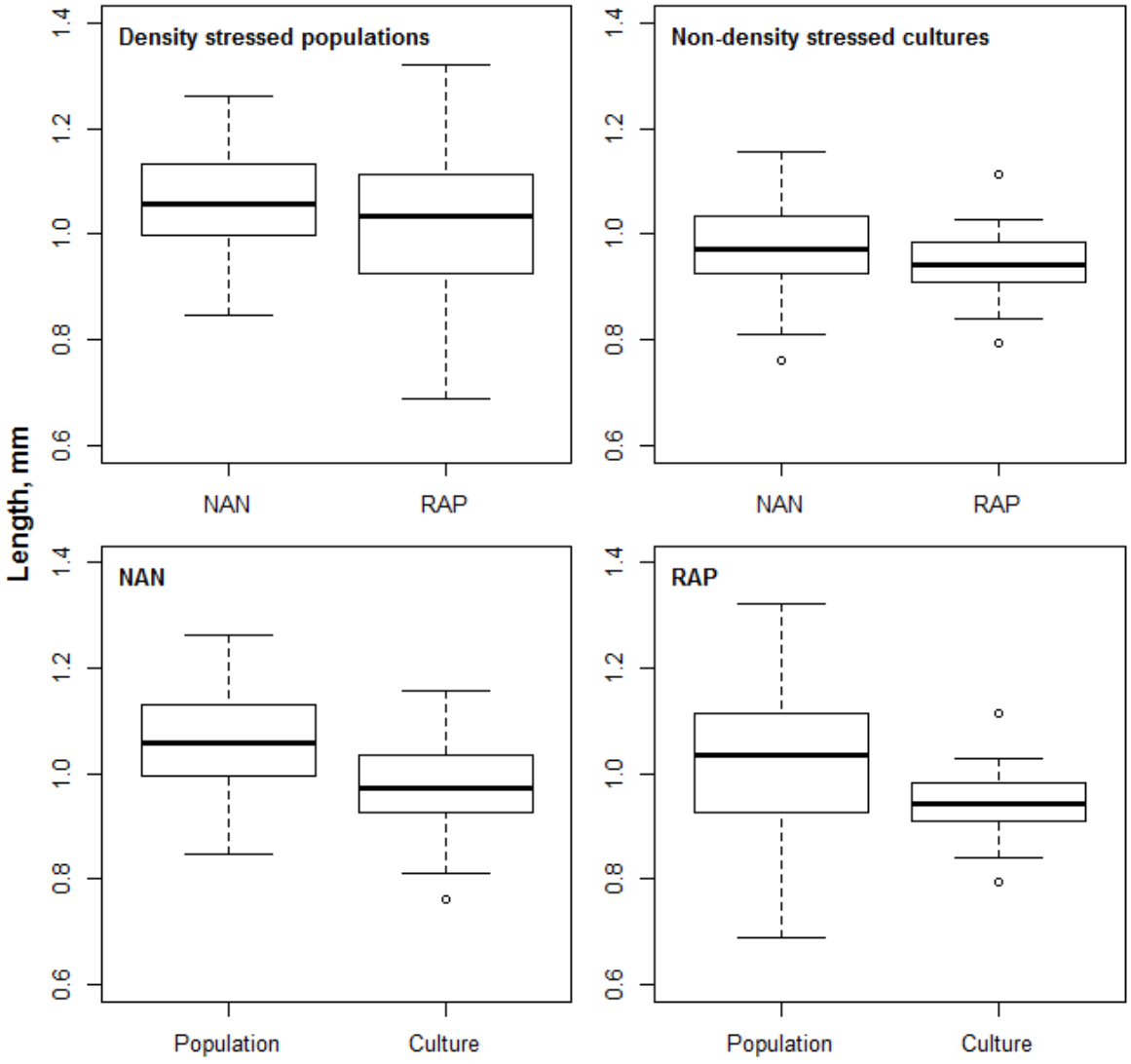

Figure A8. Boxplots of neonate length data by food type in density stressed populations (top left), non-density stressed cultures (top right) and by density stress when fed NAN (bottom left) and RAP (bottom right).

Chapter II.

Intraspecific competition on food resource affects outcomes of pulse toxicity at different *Daphnia magna* population growth phases

Introduction

Guideline toxicity studies are designed to assess the impact of anthropogenic stressors on individuals (US EPA 2002, OECD 2012), despite the general consensus among scientists and regulators that protection goals are focused on higher levels of biological organization such as populations (US EPA 1992, Hommen et al. 2010). It has been suggested that directly assessing the population level is important for ecological risk assessment, as individual- and population-level effects of toxicants can differ (Liess 2002, Agatz et al. 2012, Forbes et al. 2001). Studies focused at the individual-level lack feedbacks such as density-dependent factors that regulate the type of environment and response exhibited at the individual-level.

In *Daphnia* populations, food level per capita is affected by intraspecific competition at different population growth phases (Lampert et al. 1986, McCauley & Murdoch 1987, Foit et al. 2012). Because populations grow toward and then oscillate around carrying capacity, resource availability changes through the cycle of population dynamics and, hence, individuals at different population growth phases have different physiological and energetic states (Slobodkin 1954, Pieters & Liess 2006, Cleuvers et al. 1998, Smolders et al. 2005). These phase-dependent energetic states can then translate to different sensitivities to toxicants (Pieters & Liess 2006, Smolders et al. 2005, Chandini 1989, Antunes et al. 2003, Enserink et al. 1990). Toxicant-induced mortality in populations could also cause feedback by alleviating intraspecific competition and increasing food resources per capita for surviving individuals (Pieters & Liess 2006). Individual-level experiments do not capture these compensatory responses at the

population-level (Forbes et al. 2001). Studies that explicitly address the population-level therefore have the benefit of exploring multiple stressors, both natural (intraspecific competition at various phases of population growth) and anthropogenic (toxicant exposure).

Population dynamics in *Daphnia* have long been described (Slobodkin 1954, Pratt 1943). Initial growth phase during high food and low daphnia density are associated with a high frequency of neonates, as adult fecundity is high (Slobodkin 1954, Gergs 2013). Eventually, the continual production of neonates and growth of young individuals lowers the food supply; adult fecundity decreases because less energy is available to allocate towards reproduction, and the population reaches a peak before individuals become energy-limited and the population declines (Slobodkin 1954). Populations eventually approach stable equilibrium at a point where any mortality in the population only releases density stress and increases fecundity to an amount that maintains the population at a relatively stable density (Slobodkin 1954). Until recently, these population dynamics have been ignored in the context of ecotoxicology; toxicant impacts are often assessed on individuals, or on populations in low density, high food level conditions (OECD 2012, Liess et al. 2006, Forbes et al. 2001).

Previously, our research has determined that density stress when the population enters the peak phase was the most influential aspect of the resource environment affecting neonate stress response in *Daphnia magna* (**Chapter 1**). Specifically, neonates from density stressed conditions (peak phase populations) had significantly lower resistance to starvation relative to neonates from non-density stressed conditions (*D. magna* cultures). Accordingly, the aim of this study was to observe the outcomes of pulse toxicity at different population growth phases, using population dynamics as a 'natural' manipulation of the resource environment, where daphnia population density and intraspecific competition alters food level per capita at each phase, and.

Differences in population mortality and recovery were observed in *D. magna* populations when exposed to the fungicide pyraclostrobin at each of four population growth phases: growth, peak, decline, and stable. Populations of *D. magna* were maintained for 51 days, and different treatments were designated to be pulsed with either 20 or 30 µg/L pyraclostrobin for 48 hours during the growth, peak, decline, or stable phase. Mortality, recovery, and changes to population age/size-class structure were determined. In addition, a 48-hour acute pyraclostrobin toxicity study was conducted on three age-classes of daphnia at two food levels to examine the influence of food level and daphnia size on acute mortality.

Methods

Pyraclostrobin as Model Stressor

The fungicide pyraclostrobin was chosen as a model stressor. Pyraclostrobin belongs to the strobilurin class of fungicides frequently used to combat fungi responsible for Soybean Rust (Ochoa-Acuna et al. 2009). It acts by inhibiting mitochondrial respiration in fungi and other eukaryotes by blocking electron transfer between cytochromes and halting ATP production (Bartlett et al. 2002). Pyraclostrobin has a relatively short half-life (48-72 hours) in the environment suggesting that exposures in the field are also likely to be short (Ochoa-Acuna et al. 2009). This chemical is very acutely and chronically toxic to *D. magna* at environmentally relevant concentrations (Ochoa-Acuna et al. 2009, Cui et al. 2016) and has been used frequently in our laboratory as a model pesticide stressor. Nominal exposure concentrations were 20 and 30 µg/L, and were chosen based on a previous study in the lab (East et al. in preparation) as doses that would cause mortality, but not extinction, of populations.

Population Pulse Study

D. magna populations were started with five neonates (<24 hours old) in glass beakers with 800 ml standard synthetic freshwater (moderately hard; EPA 2002). They were maintained for 51 days and fed the phytoplankton *Raphidocelis subcapitata* at a constant rate of approximately 0.6 mg C/L/day. Feeding levels were informed by mean values of mg C/ml algae stock in 1L bottles purchased from Aquatic Biosystems (mean=0.2252 mg C/ml, SD=0.0574, n=5). Carbon content of algae stock was measured using a vario III Elemental Analyzer (for full description of methods, see **Chapter 1**). Ninety percent water changes were administered three times a week using a siphon in a fish net to draw water down with minimal disturbance to

D.magna populations. While the water level was at 10%, a ruler was placed under the jars, and digital images of each population were taken from the top of each jar. Populations were then fed and refilled with fresh water.

There were nine treatments, consisting of four population phases designated for exposure (growth, peak, decline, stable), two exposure concentrations (20 and 30 µg/L), and one control. The timing of exposure at each phase was estimated from previous population studies conducted in our laboratory. At the designated food level, ranges of each phase were estimated to be days 14-18 for the growth phase, days 18-20 for the peak phase, days 21-30 for the decline phase and days 35-45 for the stable phase. To be consistent with these phases and water changes, exposures were administered on day 14, 19, 26, and 35 for growth, peak, decline, and stable phases, respectively, for an exposure period of 48 hours. The 48-hour exposure duration was chosen due to both experimental relevance to standard acute toxicity test benchmarks (48h LC50) and because of the 48-72 hour half-life of pyraclostrobin in the environment (Ochoa-Acuna et al. 2009).

To administer pulse exposures, pyraclostrobin was weighed on a microbalance, dissolved in acetone, and mixed with moderately hard water to the treatment concentrations. Pulse exposures were administered during water changes on the exposure days, and left for 48 hours until the next water change. The ten percent of water left in the jar during the water change was accounted for in calculating treatment stock concentrations, such that the final concentration in each jar would equal nominal concentrations.

Digital images were processed in ImageJ (Version ij150, 2016) for population density count and length distributions. Counts were made at each water change (three times a week) to track population growth. Daphnia lengths were measured for each population phase, pre- and

post-exposure: days 14 and 16 (growth), 19 and 21 (peak), 26 and 28 (decline), and 35 and 37 (stable). Lengths were obtained by calibrating to a 10 mm length on the ruler in the image and measuring lines drawn from the eyespot to the base of the tail of the daphnia. To obtain 'random' samples, population images were viewed at 150% zoom, which allowed a square view of a small section of the image. The viewer was moved to a group of daphnia, setting up one 'plot'. In each plot, every daphnia showing its entire body length was measured, and then the view was moved to a new plot and the procedure repeated until at least 25% of the population had been measured. The first sample plot always targeted an area where a large adult was present, to capture the top end of the length distributions early in population phases, when few adults were present. Daphnia were categorized into three age classes based on length: neonates were <1.2 mm, subadults were 1.2 to 2.5 mm and adults were >2.5mm. These length ranges were derived from previous lab data indicating that neonates <24 hours old were always less than 1.2 mm, and that daphnia became reproducing adults around 2.5 mm.

Multi-age Acute Toxicity Study

Daphnia were cultured in the laboratory to produce 250 individuals of each age class (neonate, subadult, and adult) for the start of the acute study. Neonates were <24 hours old, subadults were grown to five days old, and adults were grown to 15 days old. Adult test subjects were initiated first, 15 days before the study. They were taken as neonates (<24 hours old) from laboratory cultures (for general *D. magna* culture care, see **Chapter 1**) and initially grown in 1 L glass beakers at densities of 25 individuals/L of moderately hard water for the first five days. After five days, adult test subjects were provided new water and density was decreased to 15 individuals/L to avoid density-dependent effects. Water was renewed for these individuals once

more a week later. Once adults started to reproduce, neonates were culled daily. Subadult test subjects were initiated five days before the study, taken as neonates (<24 hours old) from laboratory cultures and raised at densities of 25 individuals/L. While being raised, subadult and adult test subjects were fed at a rate of 0.6 mg C/L/day. Neonate test subjects (<24 hours old) were obtained as offspring from adult test subjects on the day of the study.

The acute study was a full factorial design with the three *D. magna* age classes, five pyraclostrobin concentrations, and two food levels. Concentrations for the acute study were informed by a preliminary range finder study on *D. magna* of each age class. Subadults and adults were give a larger range of concentrations due to their higher tolerance to pyraclostrobin. For neonates, nominal concentrations were 5, 10, 15, 25, and 50 µg/l. For both subadults and adults, nominal concentrations were 10, 25, 50, 75, and 100 µg/l. Because acetone was used to dissolve pyraclostrobin for treatments, acetone controls were included for each age class and food level. These controls received moderately hard water with the same concentration of acetone as the highest pyraclostrobin treatment (8 µg/l acetone).

The two food levels were chosen based on estimated food levels encountered by *D. magna* populations in the population study at low density (growth phase, day 14) and high density (peak phase, day 21). Feeding rate in the population study (0.6 mg C/L/day) was divided by the average density of all non-exposed daphnia populations on these days to obtain a value for average food level per capita (mg C/daphnia/day). This value was multiplied by five – the number of individuals in each experimental unit of the acute study – to derive the feeding level. Values from the growth phase became the 'high food' treatment at a feeding rate of 0.009 mg C/daphnia/day (0.45 mg C/L/day), and values from the peak phase became the 'low food' treatment at a feeding rate of 0.0028 mg C/daphnia/day (0.14 mg C/L/day). Daphnia were fed at

these rates at the beginning of the 48-hour acute study to mimic feeding and exposure dynamics in the population study.

To initiate the study, daphnia test subjects were pooled from their respective 'growing' jars and distributed randomly into test vessels with the appropriate pyraclostrobin concentrations and food levels. There were five individuals in each jar and four replicates per treatment. All individuals were checked for mortality at 24 and 48 hours. Some adults reproduced during the study, and these neonates were counted and extracted from the test vessels - any neonate mortality was noted.

Statistical Analyses

Data analysis was done in R (Version 1.0.143, R Core Team, 2016). Overlapping or non-overlapping 95% confidence intervals (Newman 2008) were used for population count data to assess when population size was different than controls after exposure, and when exposed populations recovered to control levels. Recovery from exposure was defined as three consecutive observations where 95% confidence intervals overlapped with controls. Kruskal-Wallis rank sum test (Hollander & Wolfe 1973) was used to assess significant differences in proportional changes in population size due to mortality from exposure. After Kruskal-Wallis, a post-hoc Tukey and Kramer (Nemenyi) test (package {PMCMR} in R; Pohlert 2016) was used to isolate significant differences between specific groups. Package {drc} in R was used to generate dose-response curves (three-parameter Weibull functions) for visualizing proportional changes in population size at 0, 20, and 30 µg/l exposures in the population study, and for visualizing dose-response (two-parameter Weibull functions) and estimating LC50s in the multi-age acute toxicity study.

Results

Population Study

Daphnia populations were followed for 51 days and different treatments were designated for a 48-hour pulse exposure to either 20 or 30 µg/l pyraclostrobin on day 14, 19, 26, or 35, corresponding to population growth, peak, decline, and stable phases. At the start of the study, one control replicate had random mortality for three out of five neonates intended to start the population, which reduced its population growth and, I believe, erroneously reduced the control mean. For this reason, this replicate was removed from subsequent analysis.

Control populations grew rapidly from five individuals on day 9 until reaching peak density on day 23 with a mean count of 199 (± 27 SD) individuals (**Fig. 1**; rounded to the nearest whole number). Control populations then declined until stabilizing near day 30 with a mean of 115 (± 9 SD) individuals from day 30-51.

When pulsed during the growth phase (day 14), populations exposed to 20 µg/l pyraclostrobin did not have a net decrease in population size, but halted growth relative to controls which was not statistically significant (95% confidence intervals). The 20 µg/l exposed populations went from 50 (± 9 SD) individuals on day 14 to 51 (± 28 SD) individuals on day 16, then immediately began growing again until peaking on day 23 at 180 (± 56 SD) individuals (**Fig. 1**). Afterwards, these populations declined until stabilizing at a mean count of 119 (± 17 SD) individuals from day 30-51. Growth phase populations exposed to 30 µg/l decreased in population size significantly relative to controls (95% confidence intervals), dropping from 45 (± 20 SD) on day 14 to 15 (± 11 SD) on day 16. They recovered immediately, by the next observation according to our definition of three consecutive overlapping 95% confidence intervals with controls. They grew until peaking on day 23 at 148 (± 23 SD), and stabilizing at a

mean count of 118 (± 20 SD) from day 30-51. One replicate in this treatment crashed to extinction after exposure - these 'zeroes' for the remainder of the study were excluded from mean and standard deviation calculations.

When pulsed during the peak phase (day 19), 20 and 30 µg/l exposures both resulted in significant decreases in population size relative to controls. Populations exposed to 20 µg/l pyraclostrobin dropped from 153 (± 40 SD) individuals on day 19 to 67 (± 15 SD) individuals on day 21 (**Fig. 1**). These populations grew a slight amount after exposure, reaching a second peak of 103 (± 56 SD) on day 23 before stabilizing at a mean count of 86 (± 12 SD) from day 30-51, and recovering to control levels on day 42. Peak phase populations exposed to 30 µg/l dropped from 186 (± 25 SD) individuals on day 19 to 65 (± 16 SD) individuals on day 21, then grew to another peak at 96 (± 31 SD) individuals on day 23 and stabilized at a mean count of 89 (± 11 SD) from day 30-51, recovering to control levels on day 40.

When pulsed during the decline phase (day 26), populations exposed to 20 µg/l pyraclostrobin dropped from 146 (± 30 SD) individuals on day 26 to 86 (± 47 SD) individuals on day 28 (**Fig. 1**). This decrease in population size was not determined significantly different from control levels. However, it should be noted that the wide 95% confidence intervals in the 20 µg/l treatment were due largely to one replicate which had crashed lower than the others, declining to 17 individuals on day 28, then to 4 individuals on day 37 (population count figure showing data from all replicates is shown in **Appendix, Figure A1**). Population size in this treatment was significantly different from controls from days 35-47 (95% confidence intervals). The surviving individuals in this replicate eventually began reproducing causing a secondary growth phase leading to a peak of 119 individuals on day 49. This replicate may be responsible for driving the mean counts up in the 20 µg/l decline phase treatments, resulting in a trajectory towards recovery

(overlapping 95% confidence intervals on the two last observation days). Otherwise, mean population size stayed relatively stable with a mean count of 68 (± 30 SD) from day 30-51. Decline phase populations exposed to 30 µg/l dropped, significantly relative to controls, from 192 (± 34 SD) individuals on day 26 to 79 (± 11 SD) individuals on day 28. These populations never fully recovered and stayed relatively stable and suppressed until the end of the study with a mean count of 73 (± 13 SD) from days 30-51). In this treatment, two replicates continued to drop to lower abundances than the other two replicates, reaching 38 and 43 individuals on day 35, roughly half the abundance of the other replicates on this day (68 and 82 individuals). These two lower abundance replicates increased to meet the levels of the other replicates in the treatment by the end of the study. These two replicates, again, may have been the main drivers behind the slow increase in mean count during the otherwise relatively stable period in populations pulsed at the decline phase.

When pulsed during the stable phase (day 35), population sizes dropped significantly relative to controls but began reproducing and increasing in mean count until 95% confidence intervals overlapped on the last day of the study (day 51; **Fig. 1**). Populations exposed to 20 µg/l pyraclostrobin dropped from 105 (± 23 SD) individuals on day 35 to 86 (± 47 SD) individuals on day 37. These populations continued to drop to 55 (± 14 SD) individuals on day 40, but then mean counts began to increase, reaching 85 (± 15 SD) individuals by day 51. Stable phase populations exposed to 30 µg/l pyraclostrobin dropped from 114.75 (± 19 SD) individuals on day 35 to 52 (± 18 SD) individuals on day 37. These populations continued to drop to 33 (± 2 SD) individuals on day 40, but then mean counts began to increase, reaching 84 (± 28 SD) individuals by day 51. The upward trajectory of population sizes in these exposed populations at the end of

the study could suggest either recovery or overshoot of population size relative to controls if the study had continued.

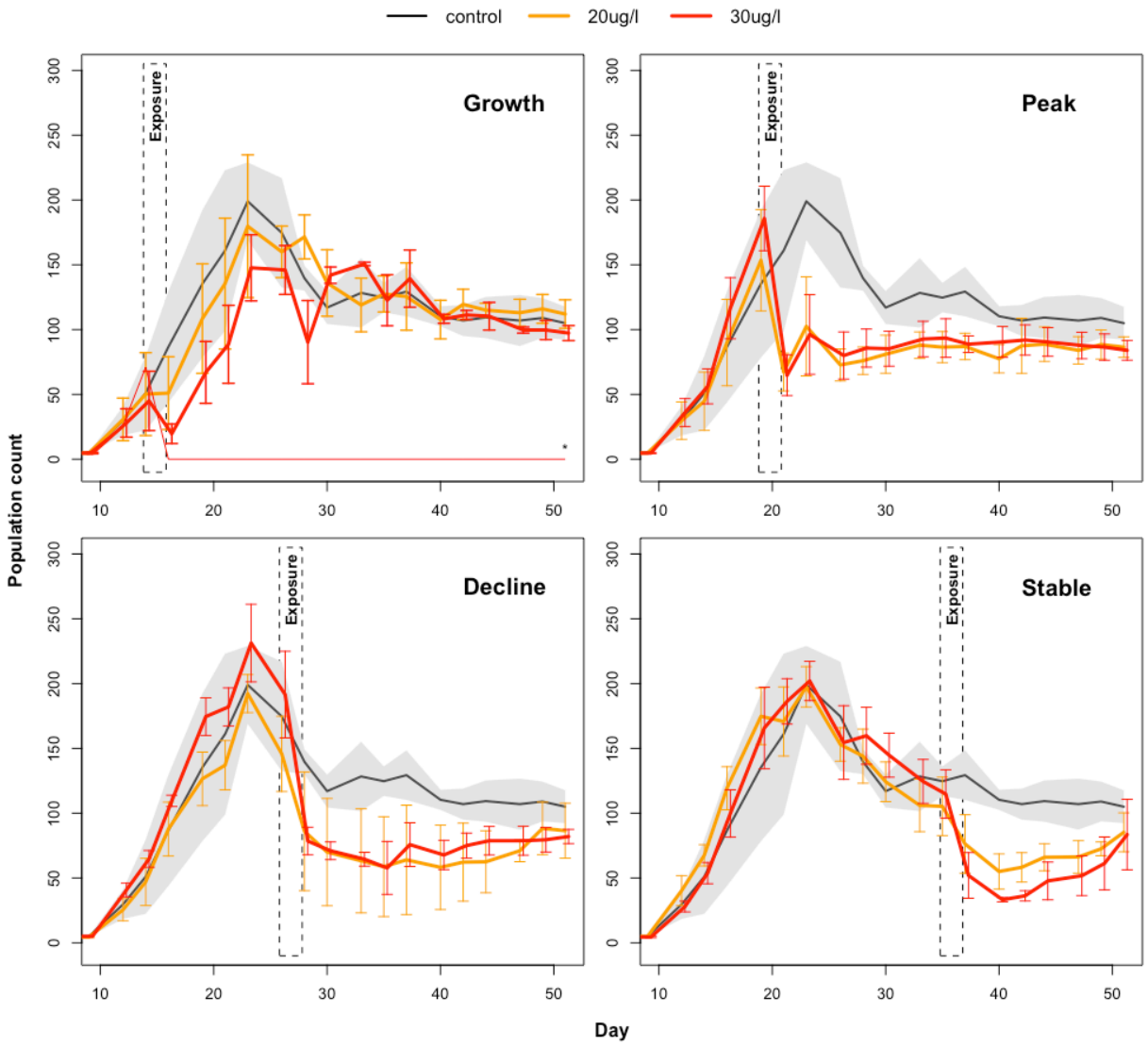

Figure 1. Plots of population count through time exposed either at the growth, peak, decline, or stable phase. Lines indicate mean count (n=4). 95% confidence intervals are indicated by error bars in exposed treatments (orange = 20 µg/l, red = 30 µg/l) and by the extents of gray area for controls (black). Timing of exposures are indicated by dashed boxes. *In the growth phase, one replicate crashed to extinction after exposure, indicated by the single red line flat-lined at zero.

To better evaluate the impact of exposure time (e.g., growth, peak, etc.), proportional changes in population size were plotted against pyraclostrobin exposure concentration (**Fig. 2**). A clear exposure-response relationship was observed between the effect size of mortality on populations at the two nominal concentrations;mortality in populationswas greater in the higher pyraclostrobin concentration. This can be seen in the proportional change in population size in each treatment during periods of exposure (**Fig. 2)**. The proportional change (PC) values can be thought of as an instantaneous population growth rate during the 48 hours of exposure (i.e. $PC_{stable} = N_{day37}/N_{day35}$). PCs above 1 therefore indicate growth of the population, while values below 1 indicate decline. Three-parameter Weibull functions were fit to the data for visualization of the exposure-response. Data points for 0 µg/l are control values at each population phase. Accordingly, control populations were growing (PC = 1.73 ± *0.096*), approaching peak size (PC = 1.22 ± *0.21*), declining (PC = 0.83 ± *0.20*), and stable (PC = 1.03 ± *0.06*) on the days when corresponding pulse treatments were exposed.

When exposed to 20 µg/l pyraclostrobin, there were marked differences between effect size when pulsed at growth, peak, decline, and stable phases (PC = 1.35 ± *0.72*, 0.46 ± *0.17*, 0.61 ± *0.34*, 0.72 ± *0.10*, respectively). At a higher concentration of 30 µg/l, effect size at each population phase converged, with mortality over 50% for each phase (PC = 0.41 ± *0.25*, 0.36 ± *0.11*, 0.41 ± *0.03*, 0.44 ± *0.11* for growth, peak, decline, stable phases respectively; **Fig. 2**). Kruskal-wallis rank sum test showed significant differences in proportional change at different population phases when exposed to 20, but not 30 µg/l (chi-squared=8.4044, df=3, p=0.03835). Post-hoc pairwise comparison using Tukey and Kramer (Nemenyi) test using package {PMCMR} in R isolated the difference between growth and peak phase proportional changes (p=0.02).

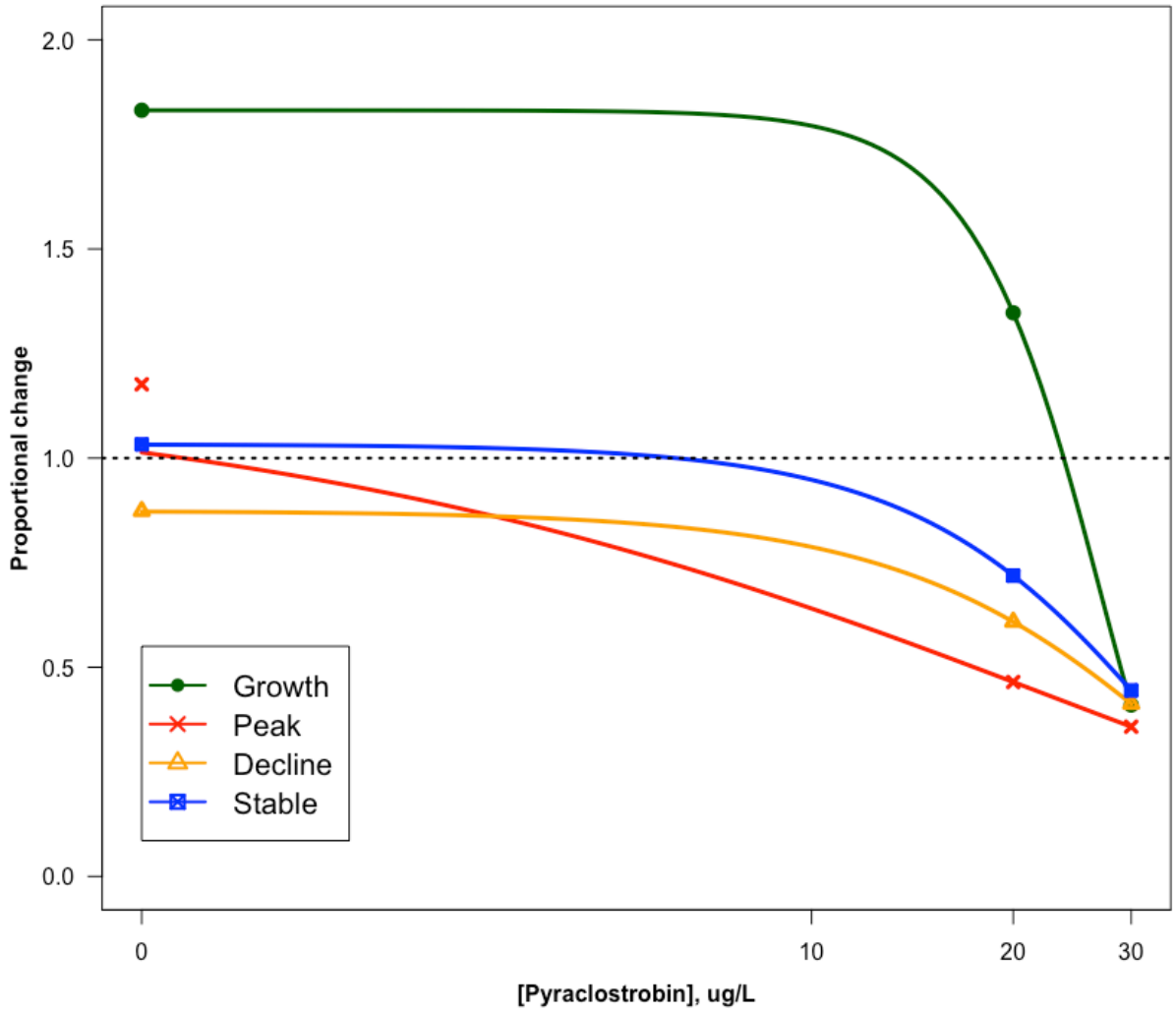

Figure 2. Proportional change in population size by the concentration of pyraclostrobin exposure at growth (green), peak (red), decline (orange), and stable (blue) phases (PC = $N_{post\text{-}exposure}/N_{pre\text{-}exposure}$). Lines are dose-response models (three-parameter Weibull functions) fit to data points.

Length distributions pre- and post-exposure indicated that mortality from pyraclostrobin occurred primarily in smaller individuals **(Fig. 3)**. At each population phase, the left end of the length distributions (smallest neonates) shifted right, indicating a loss of those smaller animals from the population. In the growth phase, this led to lower relative density of small neonates and increased relative density of subadultsIn growth phase populations exposed to 30 µg/l, the

survival of the larger individuals was apparent by the increase in the density peak at the adult age class. In the peak phase exposure, there was less change in the overall structure of the population, indicated by the similar shapes of pre- and post- exposure length distributions. There was, however, still the signature of loss of small neonates and a shift toward subadults in the left peak . The decline and stable phase showed interesting changes to population age class structure indicated by a flattening of the left peak as small neonates were eliminated and surviving subadults thus took more of the relative density than in populations pre-exposure. Notably, in the stable phase populations, this flattening of the left peak encroaches past 2.5 mm as, simultaneously, larger subadults transitioned into adults, and adults that were already present captured more of the relative density of the population. This effect was greater in the stable phase 30 µg/l treatment due to higher mortality of smaller individuals, pushing the peak towards small adults more strongly **(Fig. 3)**.

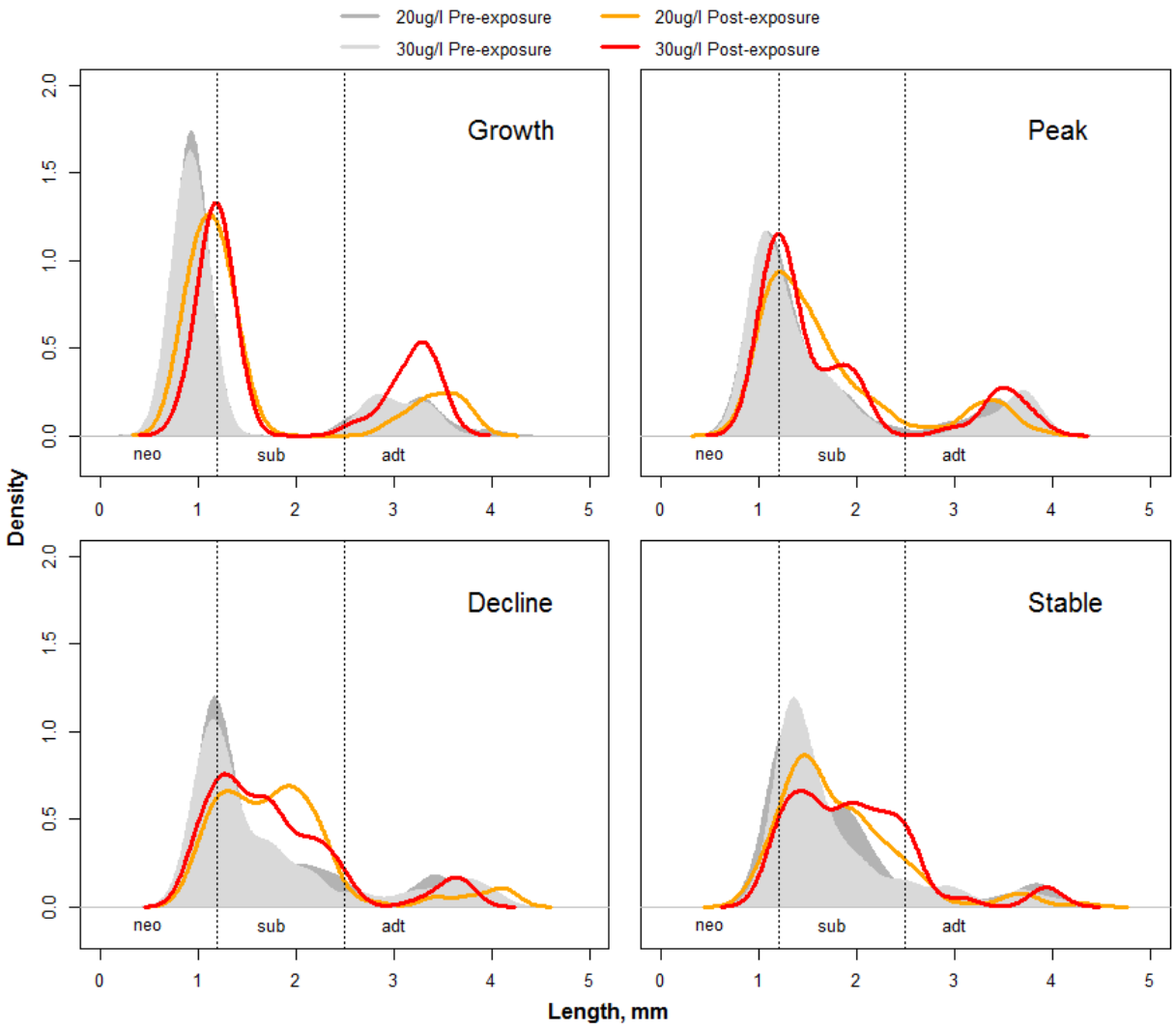

Figure 3. Plots of length distributions in populations pre- and post-exposure for 20 µg/l (pre = dark gray area; post = orange line) and 30 µg/l (pre = light gray area; post = red line) exposure treatments. Distributions are probability density functions with kernel density estimation to smooth the distribution of length data with a bandwidth of 0.15. Dotted lines delineate age classes (neonates: ≤ 1.2 mm, subadults: 1.2 to 2.5 mm, adults: ≥ 2.5mm)

Multi-age Acute Toxicity Study

The effects of acute (48-hour) exposure to pyraclostrobin on three daphnia age classes (neonate, subadult, adult) and two food levels (low, high) suggested pyraclostrobin toxicity was both food- and size-dependent **(Fig. 4)**. Dose-response models (two-parameter Weibull functions) were fit to data using package {drc} in R (Version 1.0.143, R Core Team, 2016). At low food, 100% mortality at the highest concentrations was approached. At high food, adults reached a mean of 50% mortality at the highest concentration (100 µg/l). In high food adults, one replicate in the 10 µg/l treatment had high mortality (80%), while all other replicates had no mortality. This caused a much gentler slope in the dose-response model for high food adults than the rest of the data suggested **(Fig. 4)**. In low food subadults, there was also relatively high mortality in the 10 µg/l treatment (same average mortality as in the 25 µg/l treatment), which seemed to cause the dose-response model slope to decrease (**Fig. 4**). There was also random mortality in control animals (25%) that appeared to be caused by individuals getting stuck in their carapaces while molting, and not due to acetone or other water quality variables. This is supported by the lack of issues in control mortality in the rest of the treatments. The low food subadult control mortality did not affect the respective dose-response model, as there was no change in LC50 by eliminating the two control replicates with the highest mortality.

Food level had an impact on acute toxicity of pyraclostrobin at each age class, where daphnia fed low food at the time of exposure had higher mortality at lower concentrations than daphnia given high food. Dose-response models predicted higher LC50s for daphnia fed high food. For neonates, the LC50 at low food was 13.64 (± *3.37 SE*) versus 30.01 (± *13.11 SE*) at high food. Subadult LC50 at low food was 14.51 (± *8 SE*) versus 35.48 (± *5.46 SE*) at high food. Adult LC50 at low food was 58.18 (± *23.08 SE*) versus 813.72 (± *2961.7 SE*) at high food.

Clearly, the high food adult LC50 is an illogical value and symptomatic of the mortality at 10 µg/l. Otherwise, the data for high food adults show a clean increasing trend in proportional mortality from 50 to 100 µg/l, suggesting the LC50 should be around 100 µg/l. Still, the trend that high food LC50s were higher serves the purpose of showing that higher food level is protective to daphnia under pyraclostrobin toxicity.

There was also a trend in age-size class of daphnia, in that smaller classes of daphnia were more sensitive to pyraclostrobin (**Fig.4**). Subadults were only slightly more tolerant to pyraclostrobin than neonates at both low food (neonate LC50 = 13.64 (± 3.37 SE), subadult LC50 = 14.51 (± 8 SE)) and high food (neonate LC50 = 30.01 (± 13.11 SE), subadult LC50 = 35.48 (± 5.46 SE)). Adults were the most tolerant to pyraclostrobin.

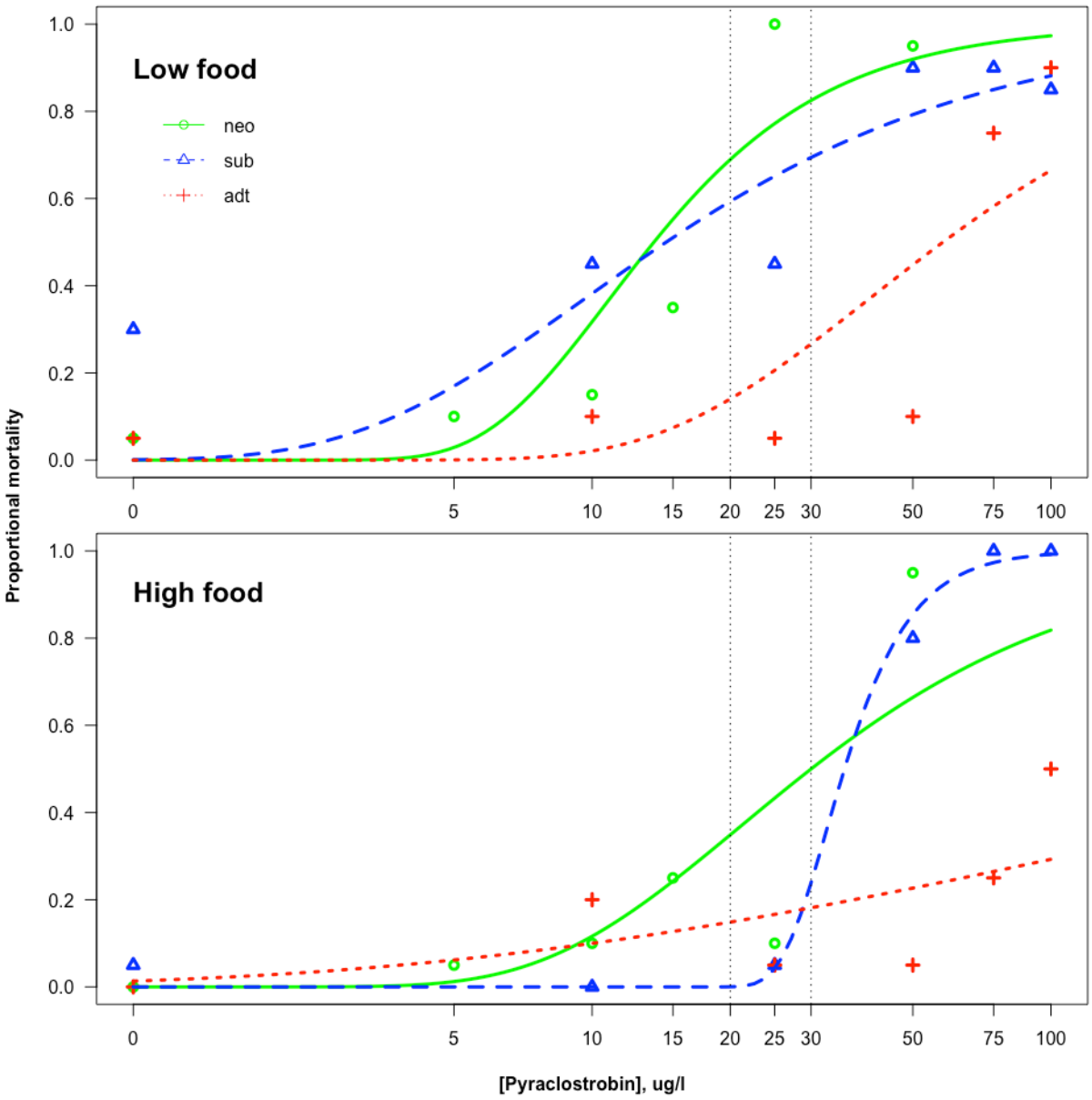

Figure 4. Dose-response models compared by age class. Lines are two-parameter Weibull functions fit to experimental data points from the multi-age acute toxicity study for mortality in neonates (green), subadults (blue), and adults (red) fed low food and high food at the start of exposure. Vertical dotted lines mark concentrations 20 and 30 µg/l, which were used in the population experiment.

Discussion

In the population study, interesting differences in mortality and recovery were observed that were specific to the population phase in which exposure occurred. At the 20 µg/l exposure, differences in population mortality were pronounced; populations exposed during the growth phase were least affected (persisting with net positive growth), followed by populations exposed during the stable phase, then the decline phase, and the peak phase was most affected by pyraclostrobin **(Fig. 2)**. At the 30 µg/l exposure, the differences among when populations were exposed converged, each population phase experiencing roughly 40% mortality **(Fig. 2)**. This suggests that population growth phase (and subsequently, daphnia density, intraspecific competition, and food availability) can influence toxicant-induced mortality, but at higher concentrations these differences matter less to the outcome. These findings are reinforced by the multi-age acute toxicity study. Food treatments in the acute study were designed on a 'per capita' basis from average daphnia densities during the growth (high food) and peak (low food) phases in the population study and thus provide some insight to the mortality in the population study. There was generally higher mortality in individuals fed low food versus high food at the time of exposure, which corroborates the observation that peak phase had the highest mortality, and growth phase had the lowest. When looking at the dose-response models in **Figure 4**, at the concentrations used in the population study (20 and 30 µg/l) it is apparent that toxicity was more food-dependent at the lower concentration. At each age class there appear to be larger differences between predicted mortality at low versus high food at the 20 µg/l mark **(Fig. 4)**. At 30 µg/l, the protective effect of food is lessened, which supports the observation in the population study that differences in mortality at different population phases converged at 30 µg/l exposure. Overall, these results suggest that food availability can reduce pyraclostrobin toxicity

at lower exposure concentrations, altering population responses to pyraclostrobin toxicity at different phases of population growth.

The acute study also confirmed that smaller *D. magna* were more sensitive to pyraclostrobin toxicity. Neonates had the lowest LC50 values at each food level **(Fig. 4)**. This supports the length distribution data in **Figure 3** showing that pulse exposures at each population phase caused mortality to smaller individuals. For exposure at the growth phase, this resulted in the 'left' length density peak (neonates, small subadults) being shortened and shifted right towards subadults, and adults being in greater relative density than pre-exposure **(Fig. 3)**. When exposed at the peak phase, the relative shape of the length distributions had minimal changes **(Fig. 3)**. High mortality rates induced by extreme food shortage during this phase may have resulted in more even loss of age-classes. This type of effect has been previously observed; Takahashi & Hanazato (2007) found that extreme food shortage at peak densities of *Daphnia magna* increased the sensitivity of adults to carbaryl, who were not affected at lower densities. In addition, food scarcity has been observed to cause *D. magna* mothers to have broods with fewer, but larger offspring (Enserink et al. 1990, Gliwicz & Guisande 1992, Gorbi et al. 2011) that have increased tolerance to starvation (Gliwicz & Guisande 1992) and cadmium toxicity as a result (Enserink et al. 1990). This type of maternal effect ('larger' neonates having increased tolerance to pyraclostrobin) might explain why the size-frequency distribution at peak phase was least affected, whereas other phases saw a distinct drop in neonates **(Fig. 3)**. When exposed at decline and stable phases, loss of neonates caused a shortening and flattening of the 'left' peak giving populations relatively similar distributions of neonates and subadults **(Fig. 3)**. At the stable phase, this extended beyond the subadults to adults ('left' peak goes past 2.5 mm adult line; **Fig. 3**). This may be part of the reason why stable phase populations were on trajectory to recovery

relatively quickly, as adults released from density stress could allocate food resources towards reproduction.

Populations exposed at growth phase had instantaneous recovery, with positive growth rates directly after exposure ended **(Fig. 1)**. Exposed populations did not peak as high as controls, but stabilized at the same mean density. This makes sense because, with ample food levels still available during the growth phase, adult fecundity stayed high, quickly replacing neonates that were lost from the exposure. However, the loss of neonates meant that adults in these populations grew larger relative to the smaller individuals in the population, consuming more of the population's food resource capital and resulting in lower peak density. The convergences of exposed and control population densities at their respective stable phase indicated full recovery of the populations. When exposed at the peak phase, populations stayed stable at a lower density than controls, reaching 'recovery' in terms of overlapping 95% confidence intervals by day 40 **(Fig. 1)**. This is probably due to the fact that subadults took over the population at this phase **(Fig. 2)**. Despite the release from high density, the high relative frequency of subadults took up most of the resource capital, which was allocated to growth and not to reproduction as these individuals were not of reproductive age or size. A similar effect was seen at the decline phase **(Fig. 3)**. Some slow upward growth in exposed population mean counts after the decline phase exposure was observed **(Fig. 1)** but these can be attributed mostly to certain replicates that dropped lower than others, and thus had more resource capital for recovery. Populations exposed at the stable phase were on trajectory to either match or overshoot control population sizes, likely due in part to the fact that surviving subadults had begun transitioning to being adults **(Fig. 3)** and thus could take advantage of the release of density and increase in resource capital to allocate towards reproduction. It should be noted that, while stable

phase population density was returning, the population age-class structure was altered as a result. The length distributions of these populations will skew left as they grow and produce neonates. Slobodkin (1954) observed that population size frequency distribution moves towards a larger mean size as populations age. Populations have a unique size distribution at equilibrium, such that the death of one animal increases fecundity only to an amount that density is maintained (Slobodkin 1954). Pulse exposure at the stable phase therefore diverted these populations from equilibrium size distribution and, despite quick return of density, they would require oscillations before returning to equilibrium.

Despite higher sensitivity of smaller individuals to pyraclostrobin, populations dominated by neonates in the growth phase were the least affected by pulse exposure. It was demonstrated that food availability was crucial to the resilience of the populations; growth phase populations had the least mortality and the most complete recovery, and the acute study showed that *D. magna* fed a higher food level were less sensitive to pyraclostrobin. Populations exposed at peak phase were the most affected in terms of mortality similar to observations with carbaryl exposure to populations of *Daphnia pulex* (Hanazato & Hirokawa 2004) and *Daphnia magna* (Takahashi & Hanazato 2007). The acute study demonstrated that low food resources per capita due to high daphnia density and intraspecific competition increased the sensitivity of all daphnia age-classes to pyraclostrobin toxicity. This effect of increased toxicity at low food has been observed before in *Daphnia magna* exposed acutely to carbaryl (Takahashi & Hanazato 2007) and chronically to fenvalerate (Pieters et al. 2005) and silver nanoparticles (Stevenson et al. 2017).

Our results corroborated the hypothesis that recovery from toxicant exposures in populations depends on the age-class structure of the population at the time of exposure (Stark & Banken 1999). Recovery of population density will depend on the level of ‘release’ from

population density stress, and whether the newly available energy will be allocated mostly to growth or reproduction. As seen in the peak phase exposure, remaining populations were mostly subadults, which consumed the excess energy and allocated towards structural growth but not reproduction. Similarly, Pieters & Liess (2006) pulsed *Daphnia magna* populations with fenvalerate at the growth and stable phase and observed that recovery time was longer when exposure occurred during the stable phase, attributed to the time lag for adults to restore body mass before proceeding with egg development (Pieters & Liess 2006, Perrin et al. 1990).

Ultimately, recovery success in populations probably depends on the definition of recovery, and the protection goals in ecological risk assessment. Populations exposed in the growth phase demonstrated the most resilience in terms of recovering population density and probably achieving similar size-frequency distributions to controls at equilibrium. Peak and density exposed populations reached stability shortly after exposure. However, they were held at lower densities than controls, which could theoretically affect future resilience of the populations to further stress. Stable phase exposed populations began recovering relatively quickly, but would require further oscillation of density before reaching equilibrium (Slobodkin 1954). Prolonged studies following population recovery of both density and age/size-class structure would provide more information as to long-term effects of exposure. Studies incorporating multiple pulses on the same population could demonstrate how alterations to population density and structure influence population resilience to future stress.

The results of these studies showcase the importance of ‘non-traditional’ toxicity studies. Acute and chronic toxicity tests provide useful but incomplete sets of information when it comes to informing decisions in risk assessment. It was noted before that individual and population level effects can differ (Liess 2002, Agatz et al. 2012, Forbes et al. 2001) and that simple

feedbacks such as density dependence at various population growth phases create individuals of different physiological and energetic states (Slobodkin 1954, Pieters & Liess 2006, Cleuvers et al. 1998, Smolders et al. 2005) with different responses to toxicants (Pieters & Liess 2006, Smolders et al. 2005, Chandini 1989, Antunes et al. 2003, Enserink et al. 1990). The traditional acute toxicity utilizes one age class (neonates) at one physiological state (<24 hours old from healthy cultures, no feeding during test; OECD 2004). The traditional chronic toxicity test requires feeding high levels of food and constant chemical exposure (OECD 2012). Both tests focus on individuals and thus lack density-dependent factors such as intraspecific competition (Pieters & Liess 2006, Takahashi & Hanazato 2007) and crowding, *per se,* effects on reproduction (Burns 1995, Preuss et al. 2009). Natural populations obviously do not consist of one age class, food level, exposure profile, etc., and just by adding simple variations in these factors in our study designs, we observed a range of insightful results. Our multi-age acute toxicity study showed that age/size-class and food level greatly influenced 'standard' toxicity benchmarks (LC50). Our population study showed that food level and physiological state of individuals, as well as age/size distribution of populations at the time of exposure all significantly influenced effect size of mortality and recovery in exposed populations. These results have broad implications for the current paradigm of ecotoxicological studies under ecological risk assessment, suggesting that standard tests not only stray from ecological relevance, but in doing so provide limited insight into how natural populations may react to stressors. All this said, it is important to note that the highest observed toxicity for pyraclostrobin generally results from acute toxicity studies on unfed neonate daphnia. So, while not reflective of actual effects in more "natural" populations, these traditional results may be protective if applied in ecological risk assessments.

Ultimately, population studies can provide important information on the effects of multiple stressors such as intraspecific competition and toxicant exposure to reach a better understanding of how natural populations may react to anthropogenic stress. Such information can inform population models designed to take standard toxicity data on individuals and create meaningful, accurate population-level output to help with the goals of ecological risk assessment.

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Appendix

The following figures show data supporting the main document.

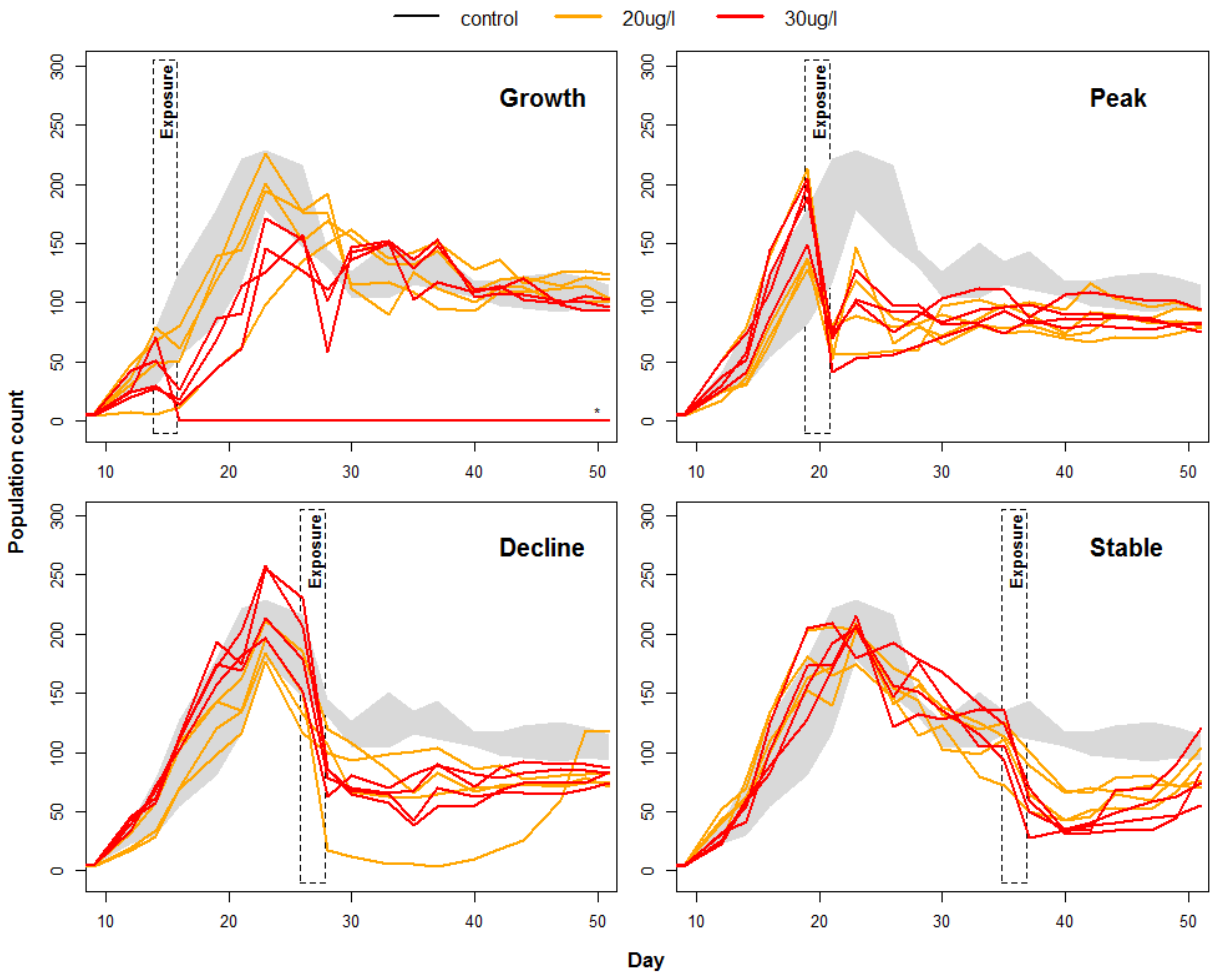

Figure A1. Plots of population count of all replicates through time split by treatments exposed at growth, peak, decline, and stable phase. Each line is one replicate of exposed treatments (orange = 20 µg/l, red = 30 µg/l). Gray area covers min and max count for controls. Timing of exposures are indicated by dashed boxes.

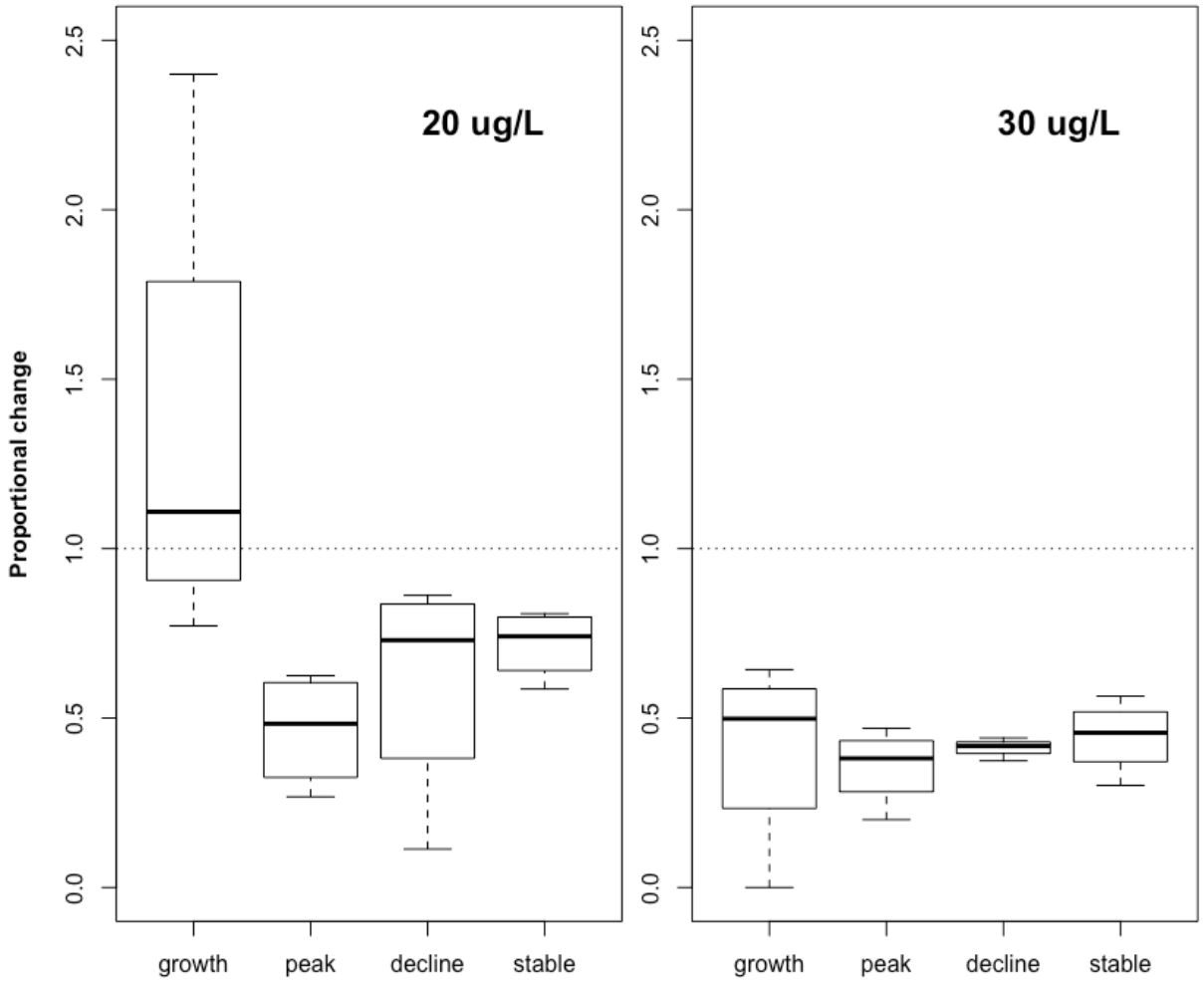

Figure A2. Boxplots of proportional change in population size at 20 µg/l and 30 µg/l exposure ($PC = N_{post\text{-}exposure}/N_{pre\text{-}exposure}$).

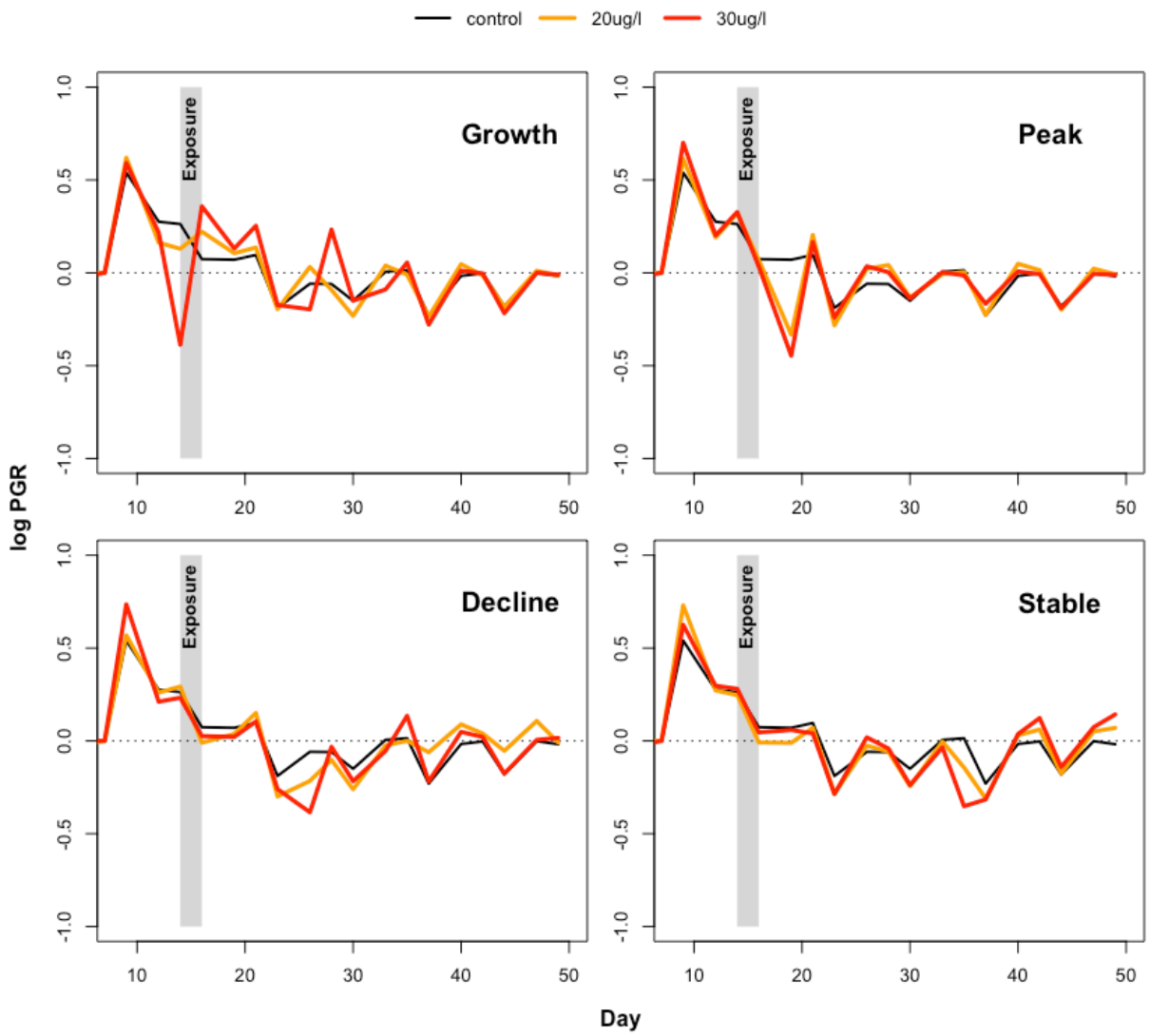

Figure A3. Plots of log population growth rate (PGR = N_{t+1}/N_t) over time for control (black), 20 µg/l (orange) and 30 µg/l (red) populations. Gray box indicates exposure period.

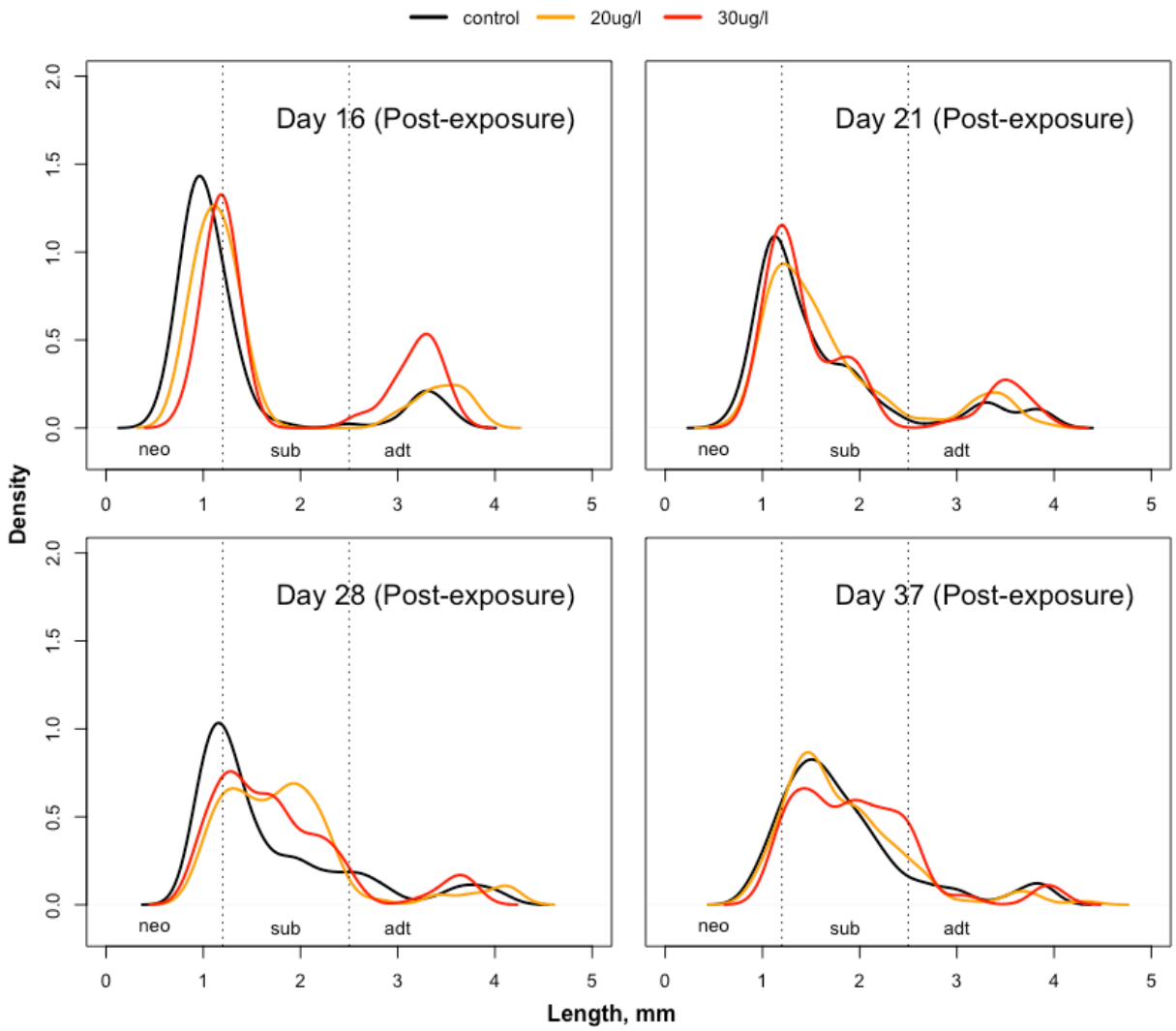

Figure A4. Plots of length-distributions in exposed vs. control populations. Post-exposure length distributions for 20 µg/l (orange) and 30 µg/l (red) exposure treatments, are compared to control populations (black) on the same day. Distributions are probability density functions with kernel density estimation to smooth the distribution of length data with a bandwidth of 0.15. Dotted lines delineate age classes (neonates: ≤ 1.2 mm, subadults: 1.2 to 2.5 mm, adults: ≥ 2.5mm)

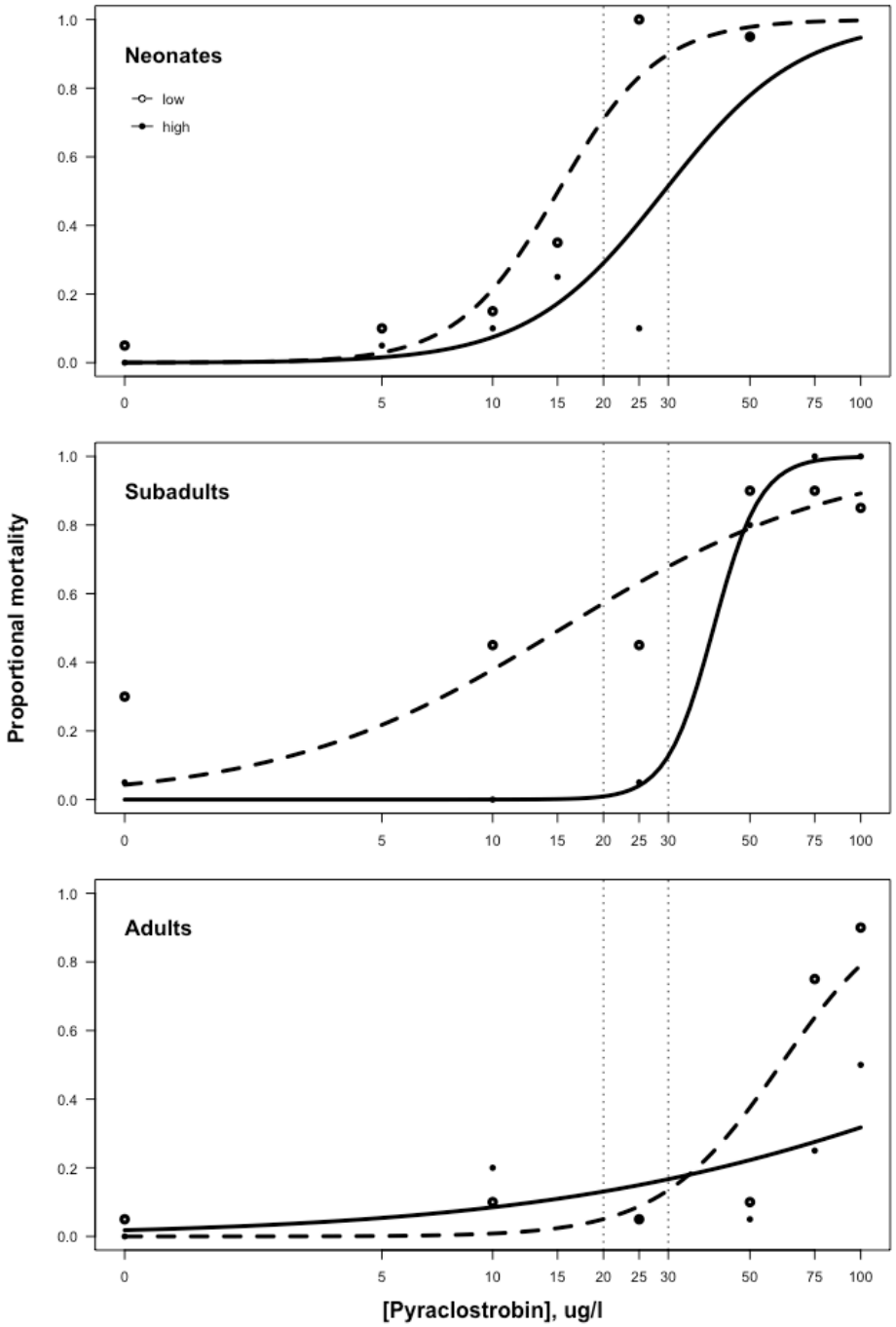

Figure A5. Dose-response models compared by food level. Lines are two-parameter log-logistic functions fitted to datapoints from the multi-age acute toxicity study for mortality in neonates, subadults, and adults fed low food (open circles, dashed lines) and high food (closed circles, solid lines) at the start of exposure. Vertical dotted lines mark concentrations 20 and 30 µg/l, which were used in the population experiment.

Chapter III.

Integrating food- and size-dependent toxicant sensitivity in a bioenergetic modeling framework to predict population level impacts of stressors

Introduction

Within the process of environmental management of manufactured chemicals, standard toxicity studies aim to inform ecological risk assessments for anthropogenic chemical stressors. Traditionally, these studies assess the impact of toxicants on individual organisms (US EPA 2002; OECD 2004, 2012), although protection goals are generally concerned with higher levels of biological organization (e.g. populations, communities, ecosystems; US EPA 1992, Hommen et al. 2010). As such, there remains a need for robust methods that bridge this gap – to use individual-based data to effectively predict impacts at the population level and above. Bioenergetic modeling provides one theoretical framework for this linkage. Energy as a 'universal currency' is a common thread among all levels of biological organization. Energy budget models track the flow of energy over an individual organism's life as it ingests, assimilates, and allocates food energy from its resource environment to processes of growth and reproduction (Kooijman 2010). The energetic 'cost' of stress can then be incorporated into bioenergetic models as changes in energy allocation, such as increasing maintenance rates, thereby integrating the energetics of the resource environment, individual consumers, and tolerance to stress (Sokolova 2012).

Many bioenergetic models exist, but the Dynamic Energy Budget (DEB) Theory for Metabolic Organization (Kooijman 2010) is one with a strong mathematical foundation that has been extensively tested (Nisbet et al. 2000, Kooijman et al. 2001, Sousa et al. 2010) and even incorporated into risk assessment guidance (ISO 2006, OECD 2006). It was developed around

the hypothesis that basic metabolic organization is similar across species. Once a DEB model for a particular species is parameterized, it can be integrated with individual-based models (IBMs) that track the bioenergetics of many individuals in a cohort, hence providing population-level model output (Martin et al. 2012, 2013; Biron et al. 2011; Jager et al. 2013). This DEB-IBM model framework has proved successful for capturing population dynamics and stressor effects in *Daphnia magna* (Martin et al. 2013; East (2016); East et al., in preparation). Martin et al. (2013a) developed a DEB-IBM model that captured population dynamics and effects of 3,4-dichloroaniline exposure in *D. magna*, providing a road map of how the DEB-IBM model framework can be effectively utilized in ecological risk assessment. East (2016) married the framework of the Martin et al. (2013) model with elements of Preuss et al.'s (2009) work on IBM crowding effects in *D. magna* to create a behaviorally and spatially explicit DEB-IBM that captured laboratory observations of *D. magna* populations. East's (2016) model was designed for "virtual toxicity testing", explicitly capturing the spatial arrangement of experimental chambers, resources, organisms, and stressors. A key contribution of East's (2016) work was the incorporation of Preuss et al.'s (2009) crowding effects into what was referred to as the "Neighborhood Effect" – where the movement and reproduction of individual daphnia were impacted by their local density. This was an effect separate from that of local food level; the actual crowding *per se* of daphnia releases chemical signals and alters behavior such that growth and reproduction are influenced (Preuss et al. 2009, Burns 1995). This model effectively captured effects of a pulse of the fungicide, pyraclostrobin, on *D. magna* populations (East et al., in preparation). A key factor in the success of this DEB-IBM model in capturing population effects of pyraclostrobin exposure was the incorporation of food-dependent toxicant-sensitivity (East et al., in preparation). A 48h acute toxicity study with

pyraclostrobin at a gradient of food levels showed protective effects of food on neonate sensitivity (East et al., in preparation). Incorporation of exposure-response as a function of algae concentration into the model allowed estimation of LC50s based on the localized food level of any given individual in the population.

This DEB-IBM iteration was used in the current study to explore the model's ability to capture mortality and recovery in *D. magna* populations exposed to pyraclostrobin at different phases of population growth. Mortality and recovery in populations were experimentally observed to differ depending on whether populations were pulsed at the growth, peak, decline, or stable phase (**Chapter II**). A multi-age acute toxicity study, in which acute mortality was observed in *D. magna* of three age classes (neonate, subadult, adult) at two food levels (low, high), showed reduced sensitivity (i.e. higher LC50s) as both daphnia size and food level increased (**Chapter II**). The main objective of this study was to expand on current methods in using the DEB-IBM to make accurate predictions of stressor impacts at the population level by integrating both daphnia size- and food-dependent toxicity into the working DEB-IBM model. Model performance was observed against experimental observations of pyraclostrobin exposure at each population growth phase. A secondary objective was to identify areas for improvement of model fit. Lastly, the results were discussed in light of ongoing efforts to improve predictive ecotoxicology.

Methods

DEB-IBM

A working DEB-IBM model was provided courtesy of East et al. (in preparation) and conducted in NetLogo (version 6.0.2, Wilensky 1999). Simplified DEB functions were obtained from Kooijman (2010) and Jager & Zimmer (2012) and DEB parameters were obtained from the 'add_my_pet' (http://www.bio.vu.nl/thb/deb/deblab/add_my_pet/index.html) database for *D. magna*. A full list of DEB parameters used in the model can be found in **Appendix, Table A1**.

Model output of population size over 51 days (length of the population experiment) was calibrated to experimental control population data. Populations were started with five neonates. In the model, these neonates tended to grow faster, resulting in reproduction and onset of the 'growth phase' approximately two days earlier than observed in the experiment. This was hypothesized to be attributed to spatial algae dynamics in the experimental chambers – in reality, most of the algae in the first few days of the experiment was not ingested by the five small daphnia and thus settled to the bottom of the jars. This would mean a lower 'realized' food level in these early days despite constant feeding rate. These spatial algae dynamics were not explicitly modelled, but by simply lowering the feed level for the first nine days, the timing of first reproduction and the growth phase matched that of experimental populations. The only parameter values changed for this study were $HSC_{Movement}$ and $HSC_{Reproduction}$, which were half saturation constants for the Neighborhood Effect on movement and reproduction. These parameters were lowered ($HSC_{Movement}$=1, $HSC_{Reproduction}$=10) relative to East (2016) to generate greater impact of local density in order to calibrate the model to the population dynamics of experimental controls. The feeding regime in the model was also altered to match that of the Monday-Wednesday-Friday feeding schedule in the experiment, with Friday receiving 1.5x

(225,000 cells/ml) the amount of algae as on Monday and Wednesday (150,000 cells/ml), such that the average daily rate remained constant (75,000 cells/ml). To explore food effects on model LC50 estimation and response, a second model run was performed with lower feeding rates after day nine (50,000 cells/ml and 75,000 cells/ml on Mondays/Wednesdays and Fridays, respectively). The effects of daphnia length on model LC50 estimation and response was also explored by running a 'half-length' test where daphnia length in the LC50 function was halved.

In general, the procedure of the model in each 'timestep' was to update patch (e.g. local) conditions with algae and pyraclostrobin concentrations, update daphnia DEB parameters according to the patch they occupy, then allow daphnia to grow, reproduce, potentially die, and move.

Estimating size- and food-dependent LC50

To incorporate the results of the multi-age acute toxicity study into the DEB-IBM framework, data from this study were used to create functions that estimated an individual's LC50 based on its length and food level. Notably, some measures were taken to 'clean' up the dataset for better model implementation. First, for adult age class at high food, the 10 µg/l treatment was excluded, due to mortality which disrupted the dose-response model fit. As seen in **Chapter II, Fig. 4,** these data points made the slope of the model too shallow, drastically overestimating the LC50 at 813.72 (± *2961.7 SE*), whereas visual inspections indicated a clean trend of increasing mortality from 50 to 100 µg/l leading to 50% mortality. Removing the 10 µg/l treatment greatly improved model fit with a new LC50 of 103.6 (± *29.22 SE*). This was also conducted for subadults in the low food treatment, which also had relatively high (45%) mortality in the 10 µg/l treatment (mortality was also 45% in the 25 µg/l treatment). The

improvement to the model fit was not as drastic due to random control mortality (see **Chapter II**), but it did shift closer to the data points at the 25 µg/l treatment, changing the LC50 from 14.51 (± *8 SE*) to 20.95 (± *17.61 SE*).

Two-parameter Weibull functions (**Eq. 1**, Ritz et al. 2015) were fitted to dose-response data to estimate LC50 values for each age class and food level:

$$proportional\ mortality = 1 - \exp(-\exp(b(\ln([pyra]) - \ln(e)))) \qquad \text{Eq. 1}$$

where *b* and *e* are the two fitting parameters of the Weibull function returned by {drc} in R. LC50s estimated by the Weibull functions were then plotted against lengths of daphnia in each age class. Daphnia lengths were obtained from digital images taken of a subset of each age class grown for the acute study. Using ImageJ (Version ij150, 2016), measurements were taken from the eyespot to the base of the tail for 10-17 individuals in each age class after calibrating to a known length on a ruler placed in each image. Non-linear regressions were then fitted to data for LC50 by length at low and high food levels using package {nls} in R:

$$LC50 = b + a(length)^c \qquad \text{Eq. 2}$$

where parameters *a*, *b*, and *c* are fitting parameters for nonlinear regression returned by {nls}. To create one function that described the relationship of LC50 and daphnia length, parameters *a*, *b*, and *c* at low and high food levels were averaged.

Next, LC50 values for neonates, subadults, and adults were plotted against algae concentrations in the low and high food levels (cells/ml). In the multi-age acute toxicity study, low food levels were set at 0.14 mg C/L/day which corresponded to 100 µg algae stock/100 ml moderately hard water fed at the beginning of the 48-hour study. The high food level was 0.45 mg C/L/day corresponding to 320 µg algae stock/100 ml moderately hard water. These volumes were converted to concentrations (cells/ml) using data on mean algae stock concentrations for 1L

bottles purchased from Aquatic BioSystems (*Raphidocelis subcapitata*, mean=3.93E+07 cells/ml, *SD=3.28E+06*, n=3). Linear regressions were fitted to data for neonate, subadult, and adult LC50 by algae concentrations. The lowest slope and intercept (neonates) were used to capture the highest sensitivity.

Finally, parameter values obtained from these functions were entered back into a nonlinear regression function for integration into the DEB-IBM model to estimate the LC50 for every daphnia in a population according to its length and the algae concentration within its patch (presented here in simplified Netlogo syntax):

$$set\ LC50\ [(m * [algae] + b) + a * length\hat{}c] \qquad \text{Eq. 3}$$

where *m* is the slope of the linear regressions for LC50~algae concentration in neonates (*m*=8.21e-4), *b* is the intercept of the linear regression for LC50~algae concentration in neonates (*b*=10.29), and *a* and *c* are the averages of the same parameters, *a* and *c*, from the nonlinear regressions for LC50~length of all three age classes (*a*=0.64685, *c*=3.9482). In essence, the parenthetical expression, a linear regression, in **Eq. 3** uses the algae concentration of the patch the daphnia occupies to set the intercept for the nonlinear regression which then uses the daphnia length to find the LC50 along the nonlinear regression curve. Thus, one function effectively incorporates algae concentration and daphnia length to estimate the LC50 of a given daphnia.

To then implement the LC50 into probabilistic mortality in the DEB-IBM, the generic Weibull function from our first estimations of LC50s is recalled to generate a 'response' variable:

$$resp = 1 - \exp\left(-\exp\big(b(\ln([pyra]) - \ln[LC50])\big)\right) \qquad \text{Eq. 4}$$

where *b* is the average slope parameter from the Weibull functions generated for neonate and adult age classes at low and high food levels. The response variable was then used to probabilistically determine mortality in a daphnia:

```
if random-float 1 < (1 - (1 - resp) ^ (1 / timestep)) [die]
```

which in essence means that if the probability of mortality from pyraclostrobin exposure was greater than a random number generated from 0 to 1, the daphnia dies. The (1 / timestep) exponent was used to adjust the resolution of our LC50 values from the lower resolution experimental timeframe (days) to the higher resolution model timeframe (minutes).

Model output

Model populations were given 48h pyraclostrobin exposures at each of the growth, peak, decline, and stable phases (days 14, 19, 26, and 35 as per the experiment). Concentrations were 20 and 30 µg/l with standard deviations of 2. At each timestep in the model, population count was saved as output to track mortality and recovery of populations from pyraclostrobin exposure. Mean algae concentration and mean daphnia length were also saved in order to assess the performance of the LC50 estimation functions in context with model population dynamics and response. Fifty model simulations were conducted for each tested scenario. Model performance was interpreted using 95% confidence intervals around mean population size through time. Model and experimental populations were considered significantly different if 95% confidence intervals did not overlap (Newman 2008). Comparisons were made after day 9, when populations began reproducing, leaving 18 observations in which model and experimental populations could be compared. Model fit was qualified on the percentage of these 18 observations in which model and experimental 95% confidence intervals overlapped: >90%

(model fit very well), 70-89% (model fit well), 50-69% (model fit was moderate), <50% (model did not fit the data).

Results

Functions to estimate LC50

Two-parameter Weibull functions fit dose-response data from the multi-age acute toxicity study well in most cases (**Fig. 1**). For subadults at low food, control mortality made the dose-response curve very shallow. As such, the slope of this value was excluded from calculation of the mean *b* parameter later during model implementation. However, the LC50 values generated by this model were sufficient for plotting LC50 by length (**Fig. 2**) and by algae concentration (**Fig. 3**). Estimations for LC50 at low food were 16.37 (± *3.87 SE*), 20.95 (± *17.608 SE*), and 62.5 (± *11.4 SE*) for neonates, subadults, and adults, respectively. At high food LC50s were 29.75 (± *6.65 SE*), 42.25 (± *6.03 SE*), and 103.55 (± *29.22 SE*) for neonates, subadults, and adults, respectively. Parameter values, *b*, for low food were 1.75, 0.83, and 2.27 for neonates, subadults, and adults, respectively. At high food, *b* values were 2.06, 5.0, and 2.54 for neonates, subadults, and adults, respectively. Parameter values from the subadults were excluded from calculation of the mean *b* because the low food subadult curve had a much gentler slope (b=0.83) and the high food subadult curve had a much steeper slope (b=5.0) than the curves for neonates and adults (mean b=2.15, excluding subadult parameter values).

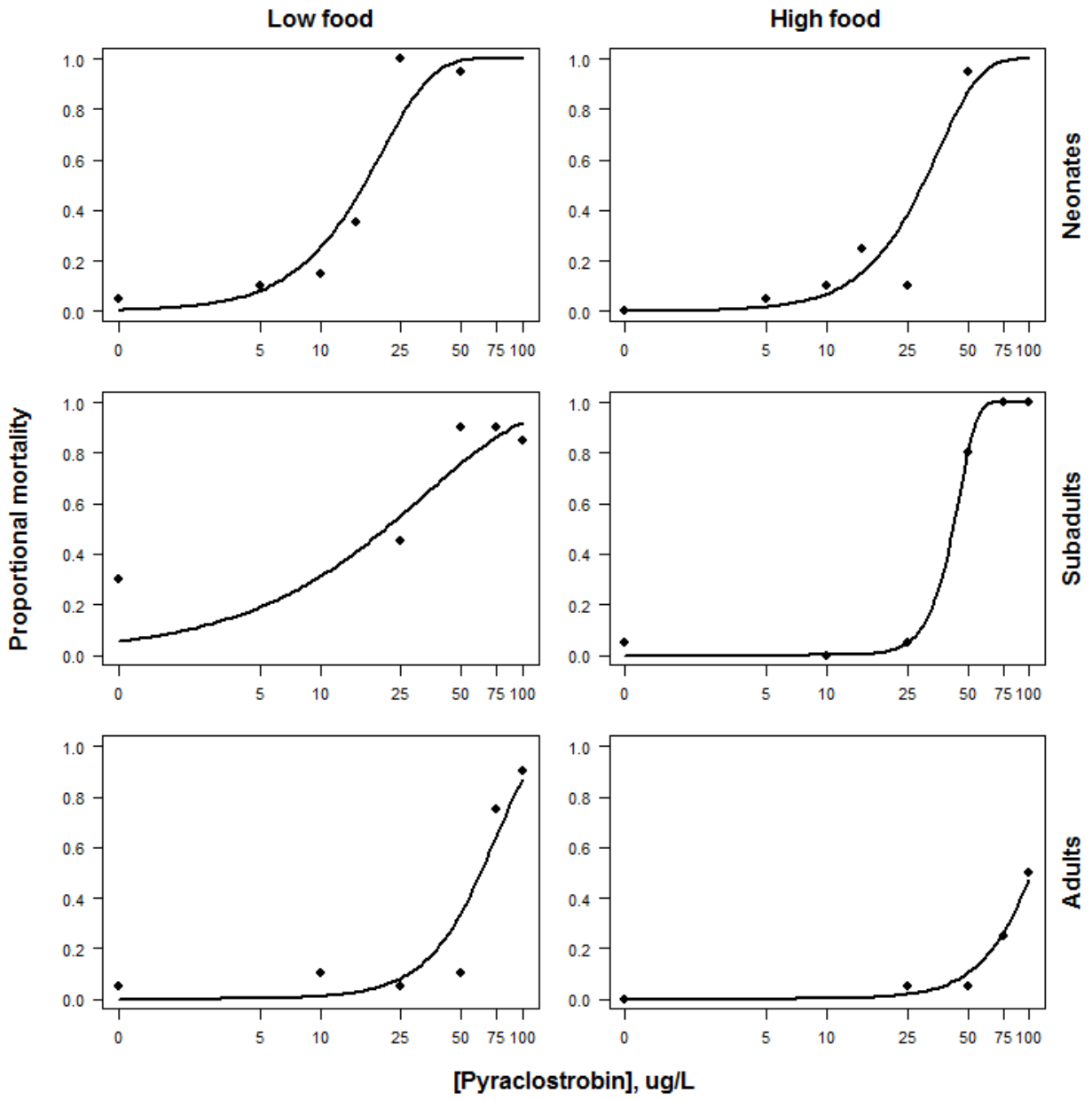

Figure 6. Dose-response curves for pyraclostrobin and daphnia at neonate, subadult, or adult age classes (top, middle, bottom), when fed low or high food (left and right). Points are mean proportional mortality (n=4) and lines are two-parameter Weibull functions.

Plots of LC50 by daphnia length suggested nonlinear regression lines with exponential increase in LC50 as daphnia length increased (**Fig. 2**). Low and high food level data showed similar patterns in terms of this nonlinear regression, with high food level essentially raising the intercept. Nonlinear regressions for low and high food treatments were approximately parallel to each other, with parameters *a* equaling 0.33 and 0.96, and parameters *c* equaling 4.2 and 3.7 for low and high food, respectively. As such, a curve with averaged parameters of the low and high food treatments (a_{avg}=0.65, c_{avg}=3.95) approximately divided the area between the two curves, suggesting it could be used as a generalized function of LC50~length, the intercept of which would be derived from the linear regressions for LC50~algae.

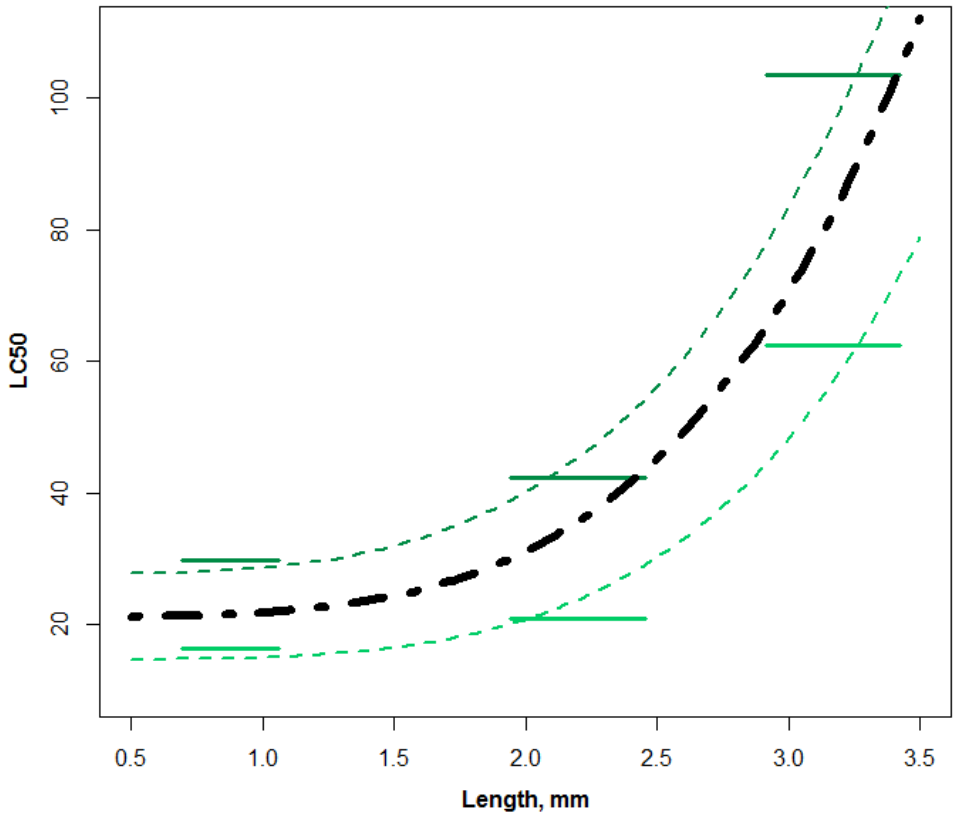

Figure 7. Plot of LC50 by length of daphnia at neonate, subadult, and adult age classes and fed low (light green) or high food (dark green). LC50s were estimated from two-parameter Weibull functions (**Fig. 1**). Horizontal lines represent the variability of lengths of individuals at each age class (n=10-17). Colored dashed lines are models fit to data at each food level. The black dotted-dashed curve represents a function with parameter values "averaged" from the low and high food level curves.

Plotting linear regressions for LC50~algae concentration gave roughly parallel regressions, with slopes of 8.21e-5, 1.31e-4, and 2.52e-4, and intercepts of 10.29, 11.26, and 43.82 for neonates, subadults, and adults, respectively (**Fig. 3**). It should be noted that a previous study in our lab generated acute toxicity data for pyraclostrobin on neonates at five different food levels, and the function for LC50~algae became nonlinear at even higher food concentrations (East et al., in preparation). Linear models were used here because there were only two food levels, and these concentrations were captured on the 'linear' end of the nonlinear function from

East et al. (in preparation). The neonate LC50 at 'no food' (intercept) supported the data and dose-response models, as previous traditional (non-fed) acute studies in our lab found LC50s for pyraclostrobin around 10 µg/l (East et al. in preparation).

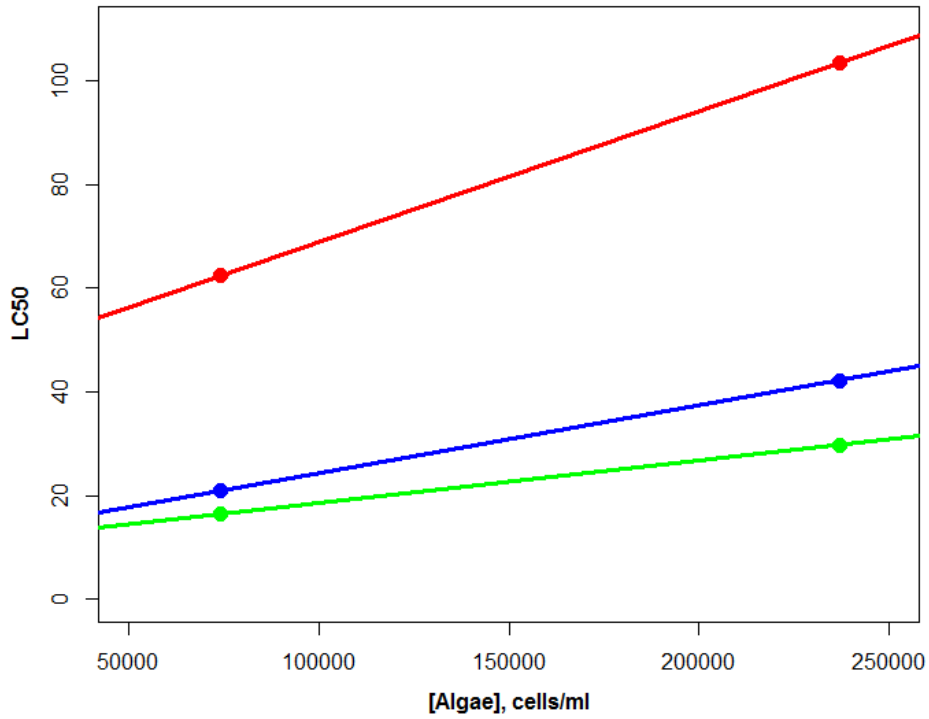

Figure 8. Plot of LC50 by algae concentration. Points are LC50s for neonates, subadults, and adults (green, blue, red) at low and high algae concentrations (7.41E+04 and 2.37E+05 cells/ml) food levels. Lines are linear models fit data points.

These functions and parameters were assembled together in the DEB-IBM in Netlogo to estimate LC50 as a function of daphnia size and algae concentration (**Eq. 3**)

$$set\ LC50\ [(1.55e-4 * [algae] + 10.29) + 0.65 * length\text{^}3.95]$$

and to estimate the response variable (**Eq. 4**).

$$resp = 1 - \exp\left(-\exp\left(2.15(\ln([pyra]) - \ln[LC50])\right)\right)$$

Model output

Altering the DEB-IBM feeding regime and Neighborhood Effect parameters resulted in model output of daphnia population size that fit very well with that of experimental control populations (100% overlapping 95% confidence intervals; **Fig. 1**). **Figures A2** and **A3** (**Appendix**) demonstrate that the functions for estimating LC50 by daphnia length and food level worked well. These plots show LC50 by length (**Fig. A2**) for all timesteps during the control populations (**Fig. 4**). It is evident that the function of LC50~length was being shifted up and down the y-axis based on intercepts from the LC50~algae function. Likewise, **Figure A3** shows general shifts in LC50 based on three different common mean algae concentrations in model patches (corresponding to food levels). The shift up and down in LC50 within respective food levels here were likely due to the range of individual daphnia lengths in the model populations.

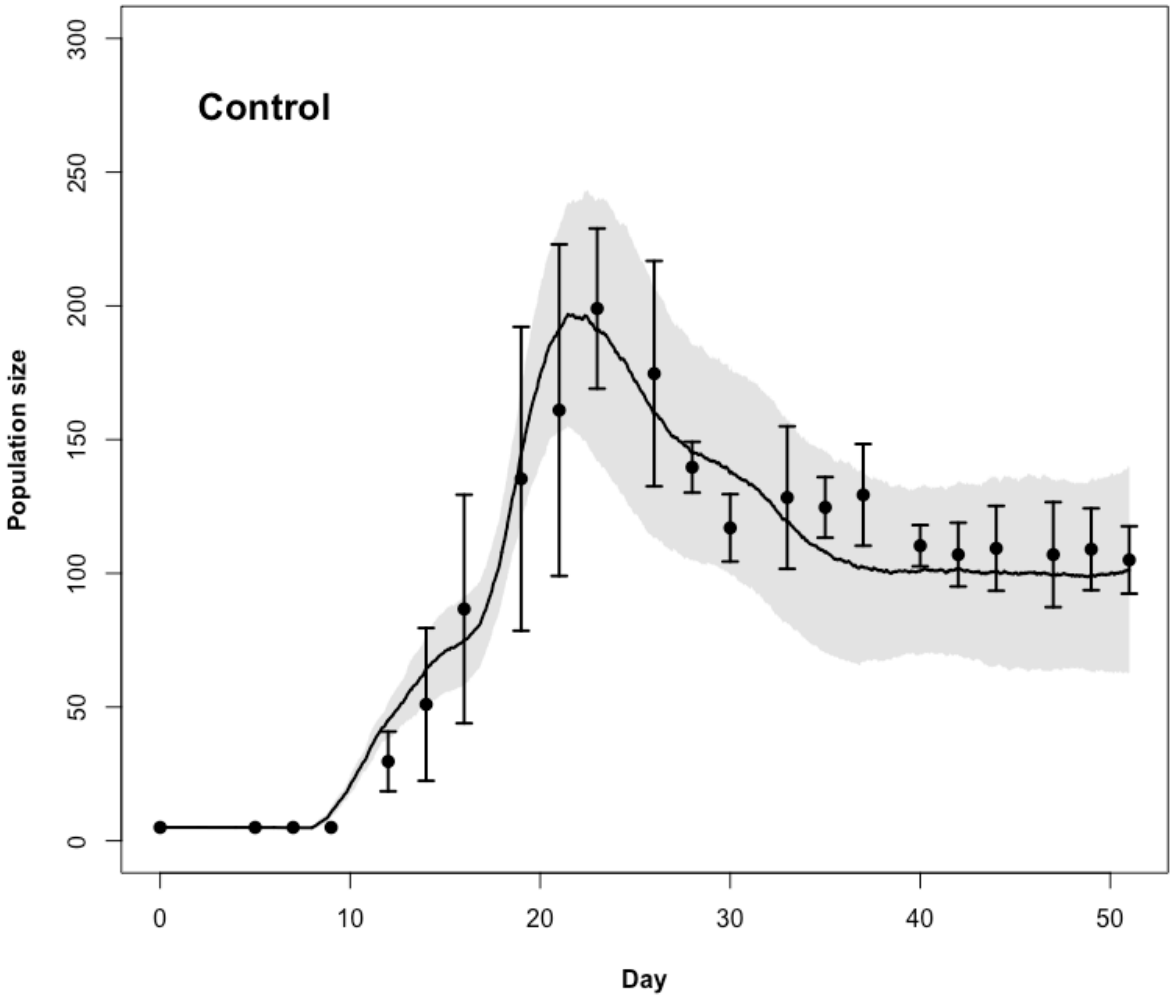

Figure 9. Plot of control population size through time, showing model mean (line) and 95% confidence intervals (gray area) of 50 simulations, and experimental mean (points) and 95% confidence intervals (error bars) of three control populations.

Incorporation of daphnia size- and food-dependent toxicant sensitivity into the DEB-IBM resulted in model output that fit experimental population data in a range from 'very well' to 'moderately'. Model output against experimental data for population size for exposed populations pulsed at the growth, peak, decline, and stable phases is shown in **Figure 5**. Exposures were 20 and 30 µg/l for 48h at days 14, 19, 26, and 35 for the growth, peak, decline, and stable phase, respectively. Quality of model fit was dependent on the phase at which

populations were exposed. Effects of exposure at the growth phase were captured very well by the model (100% and 94.44% for 20 and 30 µg/l exposures, respectively). In contrast, effects of exposure at the peak phase were only moderately captured (66.67% and 61.11% for 20 and 30 µg/l exposures, respectively). A decrease in mean model population size was not observed for 20 µg/l exposure, and only a slight dip was observed in the 30 µg/l exposure. In comparison, experimental populations had the highest proportional change in population size when exposed at the peak phase. In the decline phase, the model fit very well for the 20 µg/l exposure (100%), but only moderately for the 30 µg/l exposure (66.67%). Notably, the lack of model fit here occurred before exposure; during the growth phase, these experimental populations had grown faster than controls, resulting in the model being calibrated less well overall for these treatments. Still, the effect size of pyraclostrobin exposure on mean population size was lesser in the model than in the experiment. In the stable phase, the model fit well (83.33% and 77.78% for 20 and 30 µg/l exposures, respectively). The trajectory of mean model population size was similar to that of experimental populations, but less mortality was seen in the model.

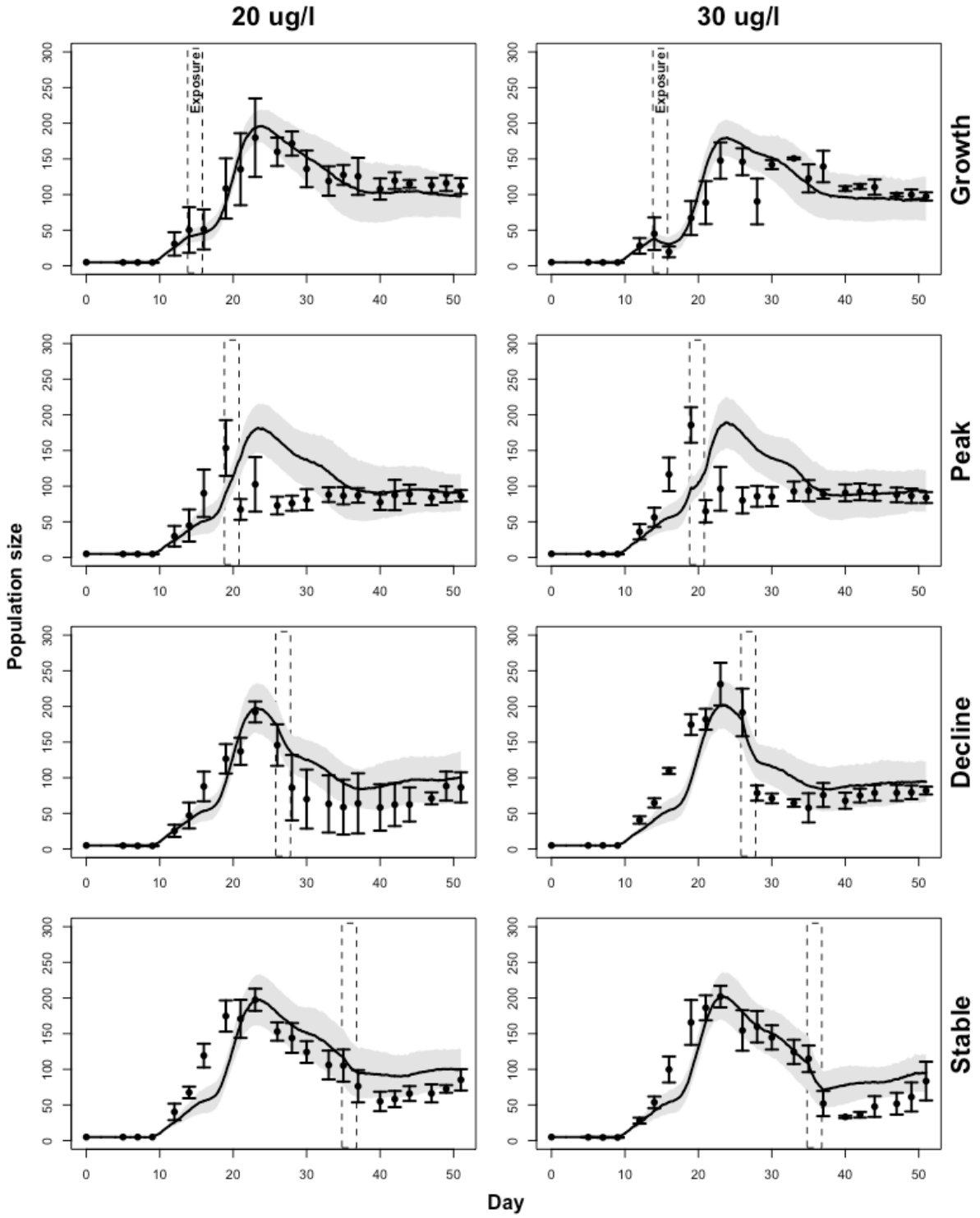

Figure 10. Plots of population size for exposed populations pulsed with 20 and 30 µg/l for 48h at the growth, peak, decline, and stable phases, showing model mean (line) and 95% confidence intervals (gray area) of 50 simulations, and experimental mean (points) and 95% confidence intervals (error bars) of four populations. Model feeding rate after day 9 was 75,000 cells/ml/day on a 3x weekly schedule. Dashed boxes indicate timing of exposures.

Since the model underestimated mortality in the peak, decline, and stable phases, it was assumed that LC50's of daphnia were being overestimated. This could be attributed to overestimation of either daphnia length or algae concentrations in the model. Both have been noted as potential shortcomings in the current model. Daphnia mean, min, and max in model simulations were observed to generally be greater than in experimental observations (Comparison of **Appendix, Fig. A2** and **Chapter II, Fig. 3**). Limitation of spatial algae dynamics was discussed above. In the model, patch algae concentrations are determined by feeding rate minus the sum ingestion rate of daphnia in each patch. In the model, the ingestion rate of daphnia never depleted the algae in a patch in the timeframe between feeding events (**Appendix, Fig. A3**). In contrast, it was visible in the experiment that large daphnia populations would 'clear' all of the algae from the experimental chambers in a day.

To test the effect of food level remaining high in the model simulations, a second model run was performed with lower feeding rates. After day nine, model populations were fed 50,000 cells/ml on 'Mondays/Wednesdays' and 75,000 cells/ml on 'Fridays' – a rate of 25,000 cells/ml/day and a three-fold reduction from experimental conditions. Forcing lower algae concentrations in the model resulted in greater mortality and closer representation to pyraclostrobin effects observed in experimental results (**Fig. 6**). Peak phase populations were still the least responsive in the model, but the amplitude of effect in the decline and stable phases were much closer to experimental observations. General model fit was slightly improved in terms of overlapping 95% confidence intervals. In the growth phase, the model fit very well (100% and 88.88% for 20 and 30 µg/l exposures, respectively). In the peak phase, model fit was moderate (61.11% and 72.22% for 20 and 30 µg/l exposures, respectively). In the decline phase,

the model fit well (88.89% and 77.78% for 20 and 30 µg/l exposures, respectively). And in the stable phase, the model fit well (77.78% and 77.78% for 20 and 30 µg/l exposures, respectively).

Interestingly, attempting to lower LC50 estimation by reducing individual daphnia length by half in the LC50 function resulted in barely noticeable differences in model output (**Fig. A6**).

On day 19 when peak phase populations were exposed, experimental populations had experienced a large boom and thus had high mean population sizes relative to model populations which had been calibrated to control data. To test whether or not this difference in population density at the time of peak phase exposure had an influence on the severe underestimation of effect, model runs were also performed changing the peak phase exposure to day 23 instead of day 19, so that population density more closely matched that of experimental populations when they had been exposed (**Fig. A4, Fig. A5** – 'lower food scenario'). This alteration seemed to yield more pronounced effects of peak phase exposure, though the application of the 'lower food scenario' was still needed to see relevant mortality effect size. The analysis of this altered peak phase scenario was also made complicated by the fact that populations declined after day 23, so it was difficult to interpret the degree to which modeled population decline was toxicant-induced.

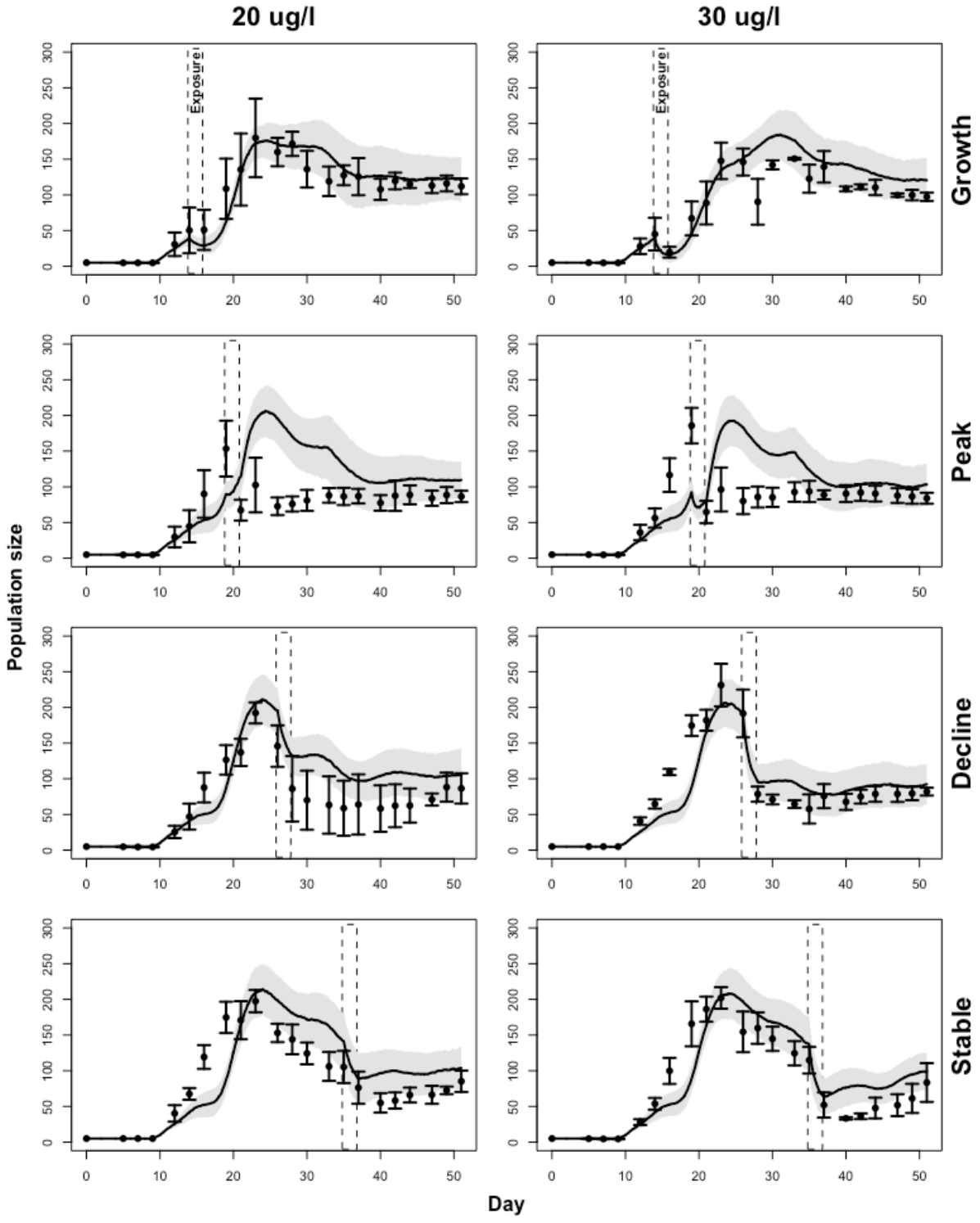

Figure 11. Lowered feeding rate population sizes for exposed populations pulsed with 20 and 30 µg/l for 48h at the growth, peak, decline, and stable phases, showing model mean (line) and 95% confidence intervals (gray area) of 50 simulations, and experimental mean (points) and 95% confidence intervals (error bars) of four populations. Model feeding rate after day 9 was 25,000 cells/ml/day on a 3x weekly schedule. Dashed boxes indicate timing of exposures.

Discussion

The current work has added to this area of refining population modelling techniques for the purpose of predicting population-level impacts of anthropogenic stressors. First, a new dataset where population-level effects of pyraclostrobin were observed at each of four population growth phases enabled the analysis of model performance at different timepoints of exposure. Second, the multi-age acute toxicity study provided data for the estimation of LC50 by daphnia length in addition to food level. Performance of the model showed advances in predicting the impacts of stressors on populations of *D. magna* by capturing dynamic experimental data for populations exposed at four different growth phases 'very well' to 'moderately'. Dose-response data from the multi-age acute toxicity study were effectively translated into functions for estimating LC50 based on daphnia size and algae concentration (**Fig. 2, 3**). These functions were easily integrated into the existing DEB-IBM framework to enable model outputs of stressor exposure on *D. magna* populations that were dependent on individual size and food level (**Fig A2, A3**). The DEB-IBM calibrated very well to data for control populations by simply adjusting the feeding regime and crowding parameters (**Fig. 4**). Using the LC50 functions in the DEB-IBM produced model output that captured experimental data for populations pulsed at different population growth phases reasonably well (**Fig. 5**). The quality of fit of the model to experimental data was dependent on the population phase in which pyraclostrobin exposure occurred. The model fit very well for exposures at the growth phase, moderately for exposures at the peak phase, and well for exposures in the decline and stable phases. Generally, effect size of toxicant-induced mortality was under predicted in the model, suggesting that LC50s were overestimated. This was demonstrated with the lower feeding level regime test, in which lower algae levels increased the effect size of mortality in populations (**Fig. 6**). Interestingly,

attempting to reduce LC50 estimation by halving daphnia lengths in the LC50~length function did not have an impact on model performance. Variability between experimental populations designated for exposure (i.e. 'peak phase populations') and control population sizes at the time of exposure (i.e. day 19 for peak phase exposure) was also identified as influential on model performance (**Fig. A4, A5**).

Adjustment to algae dynamics in the model could improve model fit. Currently, algae concentrations in a patch are never depleted, and mean concentrations remain close to levels given on a feeding day (**Appendix, Fig. 3**). This suggests there is a difference in the dynamics of the resource environment in the model and in the experiments, where *D. magna* populations 'clear' algae from the jars in a day or two. With respect to DEB theory, experimental animals are therefore more exposed to low functional response, *f,* from available food. This parameter dictates much of the organismal growth rate and maximum length, in turn influencing reproductive output (Kooijman 2010). Previous studies have suggested that food availability influences *D. magna* physiology and starvation resistance (**Chapter I**) and toxicant sensitivity (**Chapter II**). Furthermore, high food availability may be the reason that individual lengths are generally overestimated in the current DEB-IBM model. This also theoretically has the effect of increasing individual LC50 estimates, though the lack of change in model output during the 'half length' test did not corroborate this hypothesis (**Fig. A6**).

Under the current paradigm of ecological risk assessment, 'risk' is ultimately estimated using data from standardized tests on individuals (OECD 2004, 2012). The dissonance in levels of biological organization between what is studied and what is to be protected has been a thread of concern in ecotoxicology (Forbes et al. 2011, Rohr et al. 2016). Overall, the results of this study demonstrated the potential that the DEB-IBM has in taking individual-based data and

making accurate predictions of population-level impacts of anthropogenic stressors. Using data from the multi-age acute toxicity study to inform the current model showed that more data from 'non-traditional' or 'modified' toxicity tests are crucial in updating and calibrating these models. Additionally, direct experimentation on populations (**Chapter II**) provides very important data with which to inform, calibrate, and assess the performance of models. Ultimately, it has been demonstrated that the interactions between resources, consumers, and toxicants are crucial components in need of consideration when attempting to refine predictions of the impacts of anthropogenic stressors on natural aquatic populations, and that the DEB-IBM modelling framework is an apt integrative tool for this purpose.

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Appendix

The following tables and figures support the main document.

Table A 2. List of DEB parameters and their units, values, source, function, and description. Courtesy of East (2016, thesis). The only parameters changed for this study were $HSC_{Movement}$ *and* $HSC_{Reproduction}$, which were 1 and 10, respectively.

Parameter	Units	Value	Source	Function	Description
K	$\#/l^{-3}$	1661.539	Martin et al.	--	Half-Saturation Constant
f	unitless	–	–	$f = \frac{[food]}{[food]+K}$	Functional Response Value
$\dot{v}$	$L\ t^{-1}$	0.1584	Martin et al.	--	Energy Conductance
g	unitless	2.44936	Martin et al.	--	Energy Investment Ratio
$\dot{k}_M$	t^{-1}	0.488075	Martin et al.	--	Somatic Maintenance Rate Coefficient
L	L	–	–	$V = L^{1/3}$	Volumetric Length
L_phys	L	–	–	$L\_phys = L * shape\_factor$	Physical Length
$shape\_factor$	unitless	0.2637	Martin et al.	$shape\_factor = V^{1/3} / L$	Shape Coefficient
L_∞	L	–	–	$L_\infty = \frac{f\dot{v}}{g\dot{k}_M}$	Asymptotic von Bertalanffy Length
$\dot{r}_B$	t^{-1}	–	–	$\dot{r}_B = \frac{\dot{k}_M g}{3(f+g)}$	von Bertalanffy Growth Rate
$CrowdCount$	#	variable	–	--	Count of Daphnids in 27mL 'Neighborhood'
$HSC_{Movement}$	#	1.75	East	--	Half-Saturation Constant for Movement Neighborhood Effect
$HSC_{Reproduction}$	#	17.5	East	--	Half-Saturation Constant for Reproduction Neighborhood Effect
$NE_{Movement}$	unitless	–	–	$NE_{Movement} = \frac{CrowdCount}{CrowdCount + HSC_{Movement}}$	Neighborhood Effect on Movement
$NE_{Reproduction}$	unitless	–	–	$NE_{Reproduction} = \frac{CrowdCount}{CrowdCount + HSC_{Reproduction}}$	Neighborhood Effect on Reproduction
a	unitless	–	–	--	Cumulative Reproduction Fitting Parameter
b	unitless	–	–	--	Cumulative Reproduction Fitting Parameter

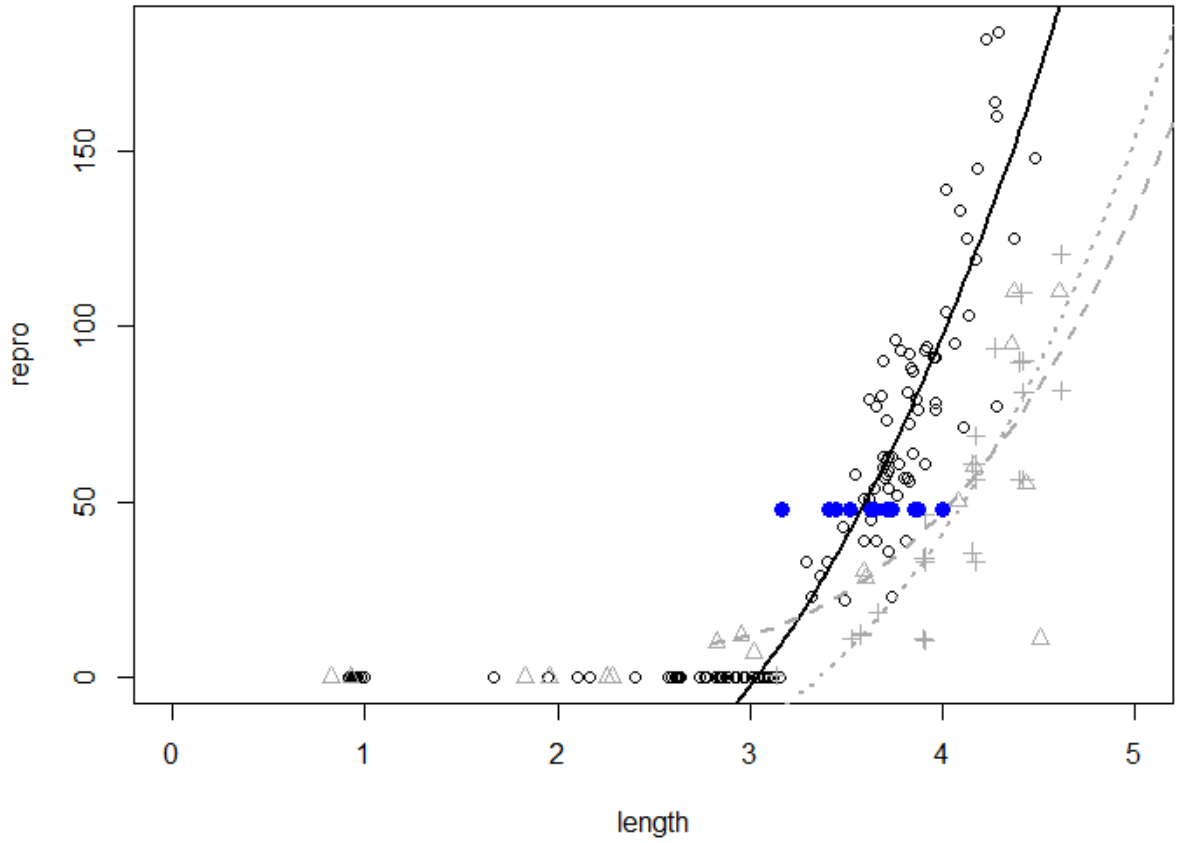

Figure A9. Daphnia cumulative reproduction by length from East (2016, thesis; open circles, solid line), Martin et al. (2013; cross, dashed line), Preuss et al. (2009; triangle, dotted line), and Woo (Chapter I, solid blue circles). Lines are nonlinear regressions fitted by {nls} package in R.

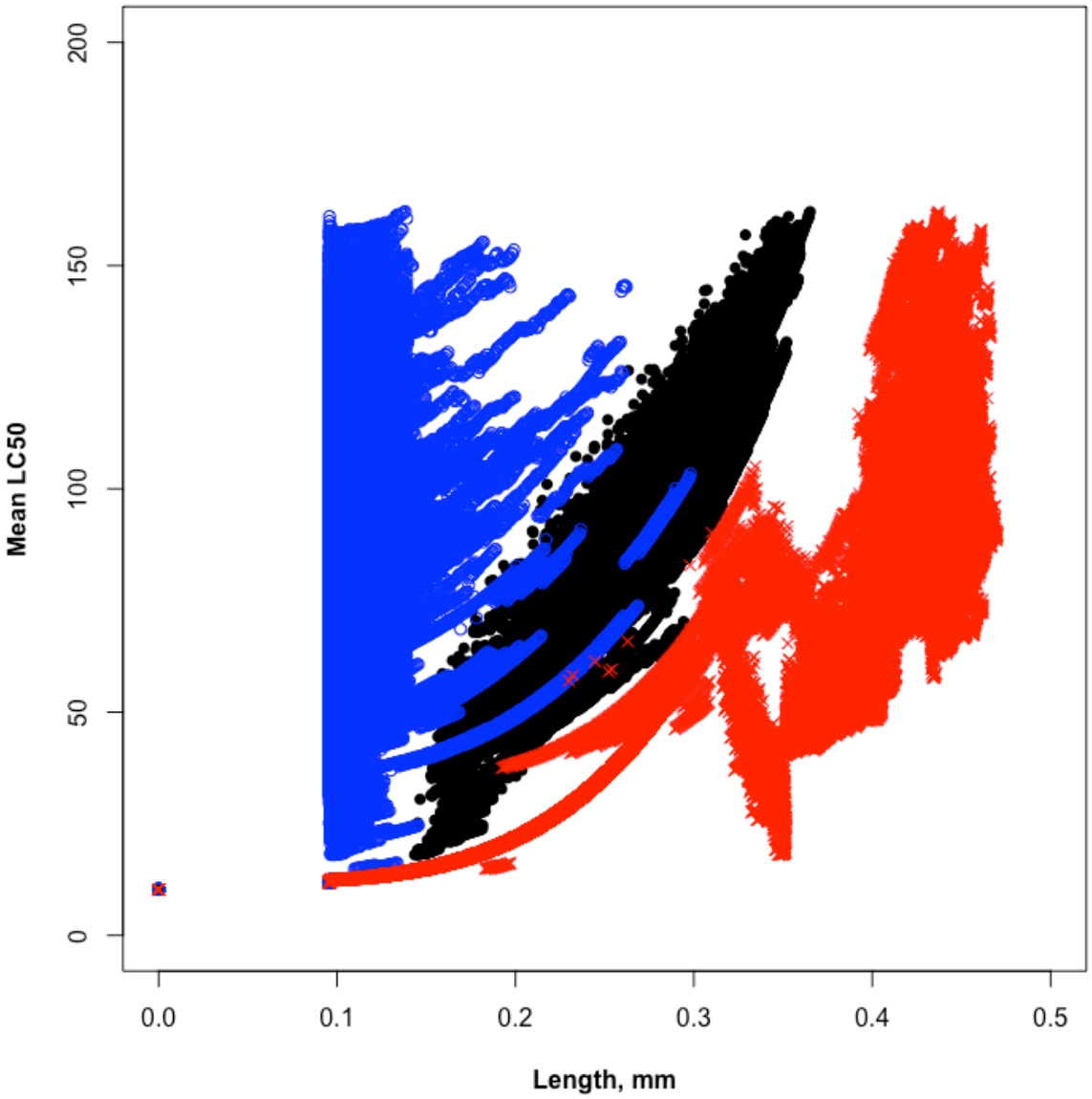

Figure A10. Mean LC50 by length of individual daphnia in model populations (50 simulations, all timesteps). Grouping represents min (blue), mean (black), and max (red) lengths in all model simulations and timesteps.

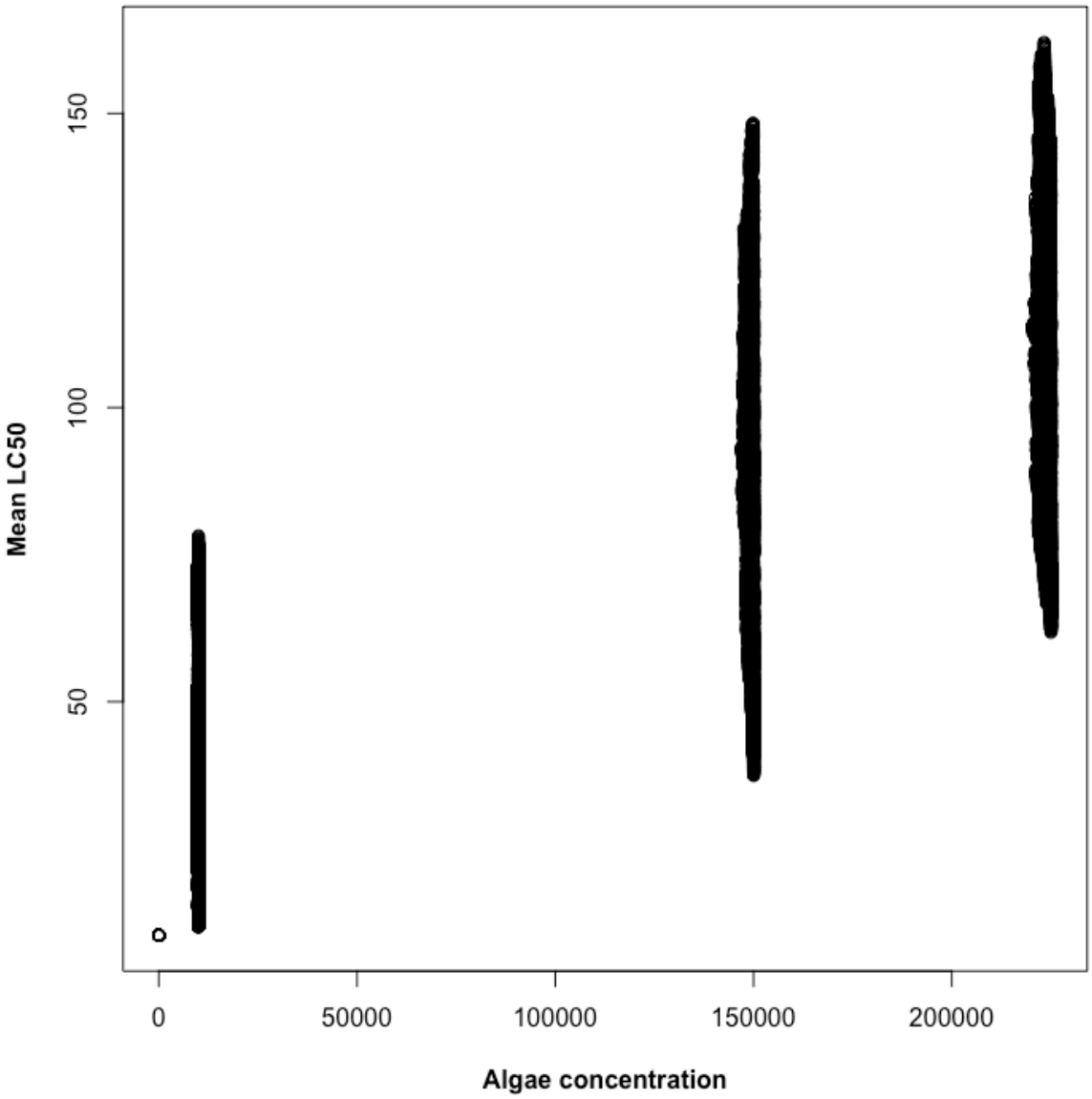

Figure A11. Mean LC50 by mean patch algae concentration (cells/ml) for individual daphnia in model simulations (50 simulations, all timesteps). Groupings are due to feeding levels (10,000 cells for the first nine days, thereafter 150,000 cells/ml every "Monday and Wednesday" and 225000 cells/ml every "Friday" – a rate of 75,000 cells/ml/day.

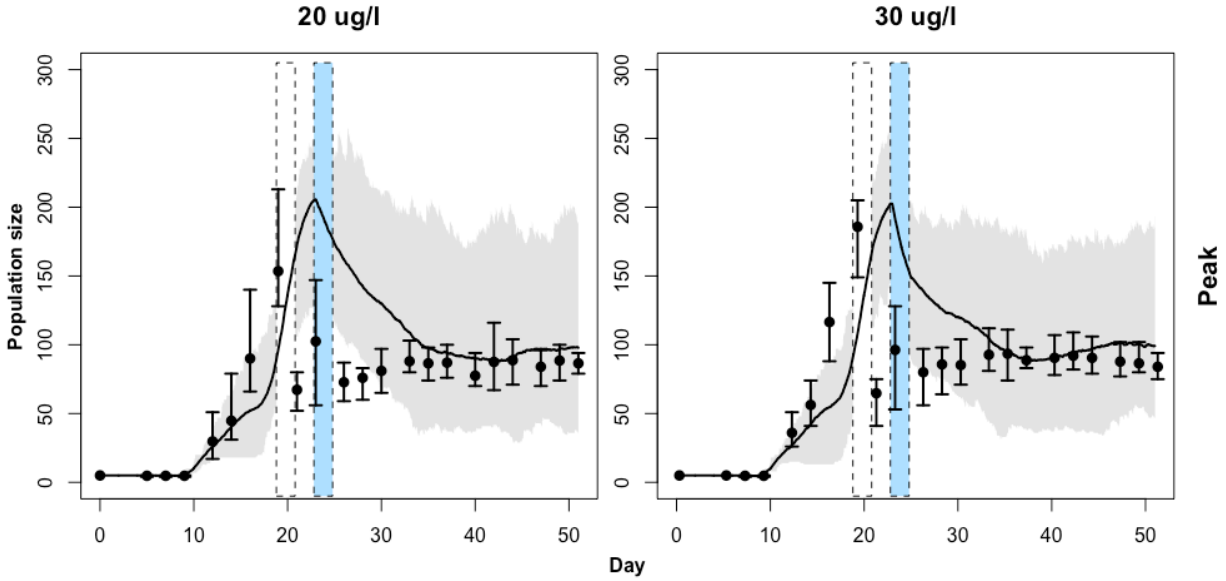

Figure A12. Model output with 'peak' phase exposure at day 23, against experimental data for population size through time. Model feeding rate after day 9 was 75,000 cells/ml/day in a 3x weekly schedule (matched experiment). Lines are means of 50 model simulations (gray area). Points and error bars are mean, min, and max of experimental populations (n=4). White dashed box indicates timing of exposure in the experiment (48h pulse on day 19). Blue dashed box indicates timing of exposure in the model (48h pulse on day 23).

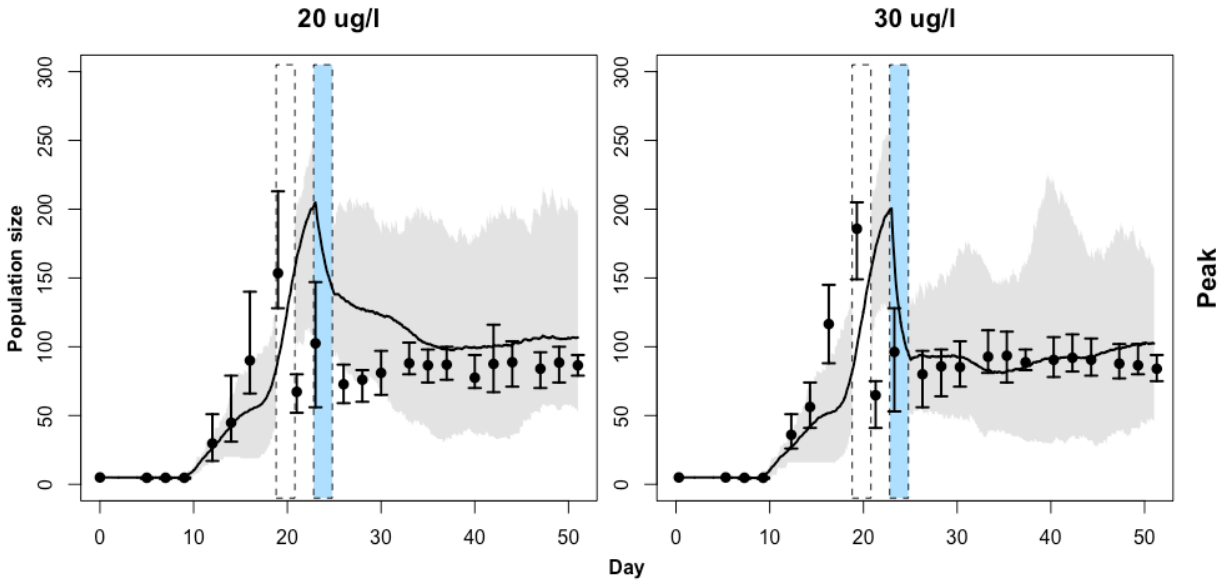

Figure A13. Model output at lowered feeding rates with 'peak' phase exposure at day 23, against experimental data for population size through time. Model feeding rate after day 9 was 25,000 cells/ml/day in a 3x weekly schedule (matched experiment). Lines are means of 50 model simulations (gray area). Points and error bars are mean, min, and max of experimental populations (n=4). White dashed box indicates timing of exposure in the experiment (48h pulse on day 19). Blue dashed box indicates timing of exposure in the model (48h pulse on day 23).

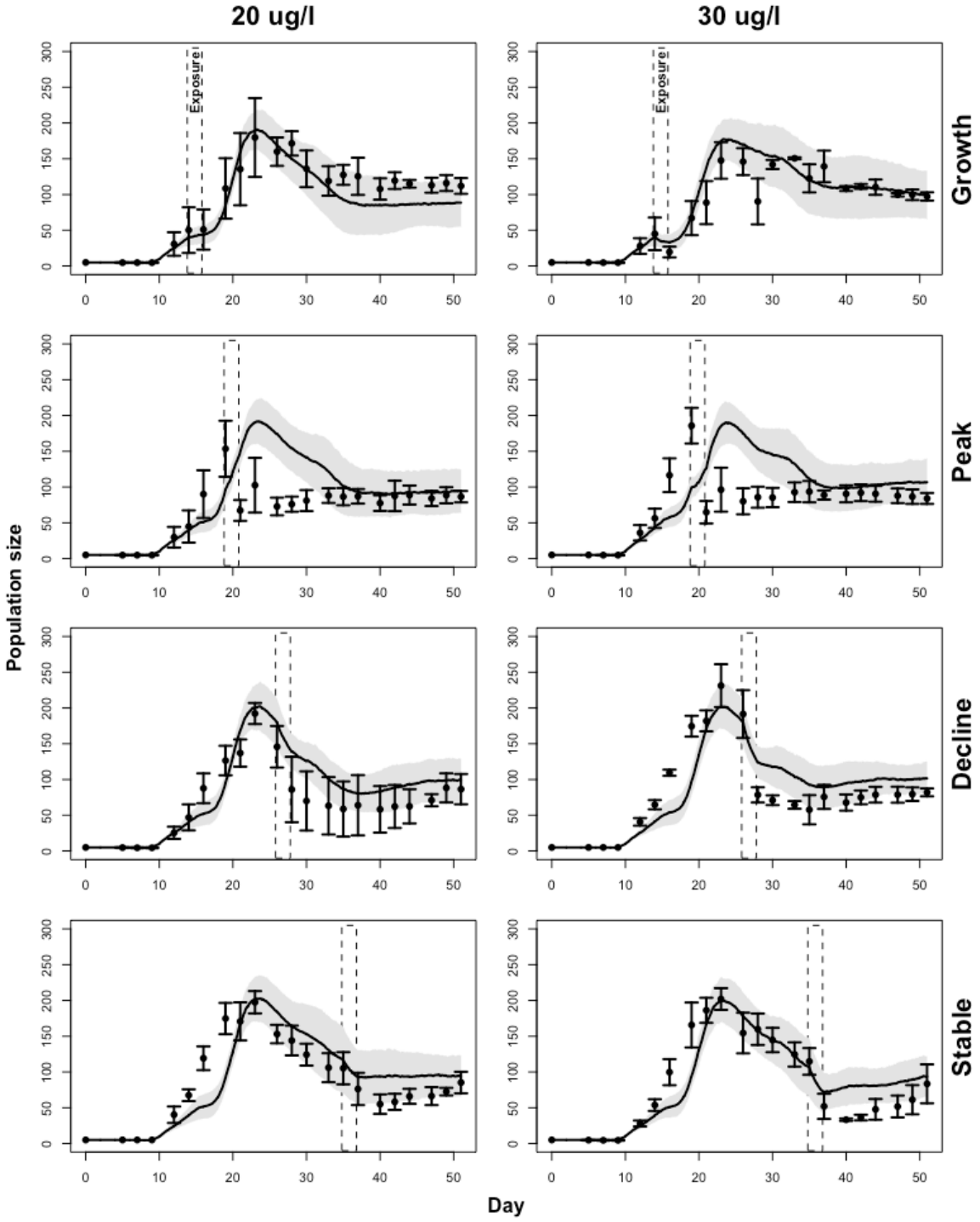

Figure A14. Plots of population size for exposed populations using half-length LC50 functions, showing model mean (line) and 95% confidence intervals (gray area) of 50 simulations, and experimental mean (points) and 95% confidence intervals (error bars) of four populations Model feeding rate after day 9 was 75,000 cells/ml/day on a 3x weekly schedule. Dashed boxes indicate timing of exposures.

Timothy J. Woo

[redacted]

EDUCATION

M.S. Environmental Science *2017*
Environmental Science and Studies Program, Towson University
Thesis advisor: Dr. Christopher J. Salice
Thesis title: Predicting the impacts of anthropogenic stress on aquatic populations: A case study in resource-consumer-toxicant dynamics

B.S. Biology - Organismal Biology and Ecology *2015*
Department of Biological Sciences, Towson University

B.S. Environmental Science - Environmental Biology *2015*
Environmental Science and Studies Program, Towson University

RESEARCH EXPERIENCE

Research Assistant *2015—Present*
Applied Aquatic Ecology and Ecotoxicology Lab, Towson University
PI: Dr. Christopher J. Salice

- Used traditional methods - acute and chronic toxicity tests - and novel methods - population experiments and bioenergetic modelling - to develop frameworks for accurately predicting effects of multiple stressors on aquatic populations.

Tropical Field Ecology in the Peruvian Amazon *2013*
Cocha Cashu Biological Field Station, Peru
Towson University Study Abroad

- Quantified differences in aquatic bird species diversity between two Amazonian habitats

PUBLICATIONS

Woo, T., & Salice, C. (2017). Timing is everything: Pulsed versus constant exposures in assessing effects of road salt on aquatic organisms. *Integrated Environmental Assessment and Management*, *13*(4), 792–794. **invited publication**

PRESENTATIONS

Woo, T. & Salice, C. (2017). Dietary carbon and lipids influence *Daphnia magna* population dynamics, physiology, and resistance to starvation. Society of Environmental Toxicology and Chemistry North America 38th Annual Meeting.

Woo, T., East, A., Salice, C. (2016). Timing is everything: Assessing the effects of pulse exposure patterns on salt toxicity in Daphnia magna. Society of Environmental Toxicology and Chemistry North America 37th Annual Meeting.

East, A., **Woo, T.**, Salice, C. (2016). Framework to predict toxicity of ion pulses in Baltimore region streams. Maryland Water Monitoring Council 22nd Annual Conference.

Woo, T., & Salice, C. (2016). Pulse exposure patterns influence the toxicity of NaCl on Daphnia magna. 2016 Chesapeake-Potomac Regional Chapter of Society of Environmental Toxicology and Chemistry Annual Spring Meeting.

Woo, T., Pereira, V. Salice, C. (2015). Effects of common anthropogenic stressors on the surrogate freshwater invertebrate Daphnia magna. Maryland Water Monitoring Council 21st Annual Conference.

PROFESSIONAL SOCIETIES

Society of Environmental Toxicology and Chemistry (SETAC)
Chesapeake and Potomac Regional Chapter of SETAC
Maryland Water Monitoring Council (MWMC)

CERTIFICATES

Hazardous Waste Generator Training *2016*
Department of Environmental Health and Safety, Towson University

AWARDS

Graduate Student Association Travel Award, Towson University *2017*
Graduate Student Association Travel Award, Towson University *2016*

PROFESSIONAL SERVICE

Volunteer

International Society for Ecological Modelling Global Conference *2016*
East Capitol Urban Farm, University of the District of Columbia *2015*
Tree Planting, Blue Water Baltimore *2014*

TECHNICAL SKILLS

Computation
R, NetLogo, STELLA, MS Office Suite, Windows, Mac OS

Experimental methodology
Standardized aquatic toxicity tests and analytical methods, population dynamic studies, comparative physiological tests

Analytical chemistry
Lipid extraction from microalgae and small aquatic organisms, C:N:S analysis of liquid and solid samples, TOC analysis of field water samples, IC analysis of water samples.

Sampling
Field sampling of benthic macroinvertebrates, water, soil; experimental sampling of aquatic organisms for physiological, elemental, and lipid analysis