Life history responses to covariance between temperature and food availability in *Daphnia magna*

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Master Thesis in Ecology and Evolution

60 p

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15.10.2021

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1 Introduction

Freshwater systems are important for a wide variety of organisms. They provide water uptake for terrestrial organisms, breeding grounds, temporary and permanent habitats, feeding grounds and nutrient distribution, to mention a few. The anthropogenic impact on freshwater systems has been significant, for example, through various types of pollution (Häder *et al.*, 2020), eutrophication (Yan *et al.*, 2016) and overfishing (Allan *et al.*, 2005). With the addition of increased mean temperatures, increased frequency of extreme weather events and changes in precipitation patterns brought upon by climate change, organisms in freshwater systems face many perils. Ecological systems at all levels can be resistant to change and adverse conditions, but one should not underestimate the risk of synergistically damaging effects of multiple stressors (Ormerod *et al.*, 2010). In a future lined with increased unpredictability it is important to identify both which stressors that will be prompted for different organisms and to test how the organisms might respond to those stressors, to promote predictions in all ecological levels and assess the necessity of conserving measures.

Most freshwater organisms are ectothermic and are vulnerable to changes in external temperatures, as their body temperatures reflect that of the environment. The response of ectothermic organisms to temperature is often expressed as a Thermal Performance Curve (TPC). The TPC includes a thermal optimum, in which the level of trade-offs has the potential to be at its lowest between life history traits like survival, growth and reproduction, with declining performance below or above the optimum. One reason for such a decline in performance is that different temperatures change internal, physiological rates such as metabolism (Brown *et al.*, 2004) and, extendedly, food requirements. However, food intake will not necessarily match an increase in requirements because of factors such as trophic mismatches (Edwards and Richardson, 2004) or anthropogenic interference. For example, Medvigy and Beaulieu (2012) investigated the trend of variability of day-to-day solar radiation over the years 1984-2007 and found an increase of variability around the equator, which would likely have a negative effect on photosynthesising primary producers, and a possible decrease of solar radiation variability at higher latitudes. Additionally, predicted and observed increasing precipitation patterns and variability in mid- and high latitudes (Trenberth, 2005; O'Gorman and Schneider, 2009) may lead to increased eutrophication

(Jeppesen *et al.*, 2009). Besides increased mean temperatures, climate change will bring upon changes in unpredictable temperature fluctuations in some parts of the world on both a day-to-day basis (Moberg *et al.*, 2000) and on longer time scales (Bathiany *et al.*, 2018). To those ectothermic organisms who are adapted to fluctuations through a high degree of plasticity, such changes may have a small effect (Seebacher, White and Franklin, 2014), but for others, sudden fluctuations in temperature can be detrimental (Paaijmans *et al.*, 2013).

This study will examine the life history responses of the ectotherm crustacean *Daphnia magna* to interactions between temperature and food availability. The genus contains around 100 keystone species of the freshwater food chain, consuming primary producers and other organic particles through filter feeding and serving as food for many other species. *D. magna* is one of the largest daphnids and is therefore rarely found in water bodies with fish predators. It is widely distributed in the Northern Hemisphere, but can be found scattered in the Southern Hemisphere as well, and inhabits most types of small, still freshwater bodies, where the main predators are invertebrate larvae. Predation defence and avoidance strategies include a switch from parthenogenetic reproduction to sexual reproduction (explained in more detail in the next paragraph) (Ślusarczyk, 1999), morphological changes (Rabus and Laforsch, 2011) and diel vertical migration (DVM) (Glaholt *et al.*, 2016). DVM allows the individuals to avoid being detected by predators in the darker waters in the hypolimnion during the day and migrating to the epilimnion during the night. DVM is stimulated by predator kairomones and light intensity (van Gool and Ringelberg, 1997) and can so forth be present also in habitats where predation pressure is lower. Moving from the epilimnion to the hypolimnion usually causes individuals to also move between different temperatures and food concentrations, mostly in a positively covarying manner, which can be associated with trade-offs with other traits (Loose and Dawidowicz, 1994). Depending on the habitat, the temperature and food availability gradient between the epilimnion and hypolimnion may vary and as such also the magnitude of the contrasts in the fluctuations.

DVM has shown direct and indirect effects on phytoplankton communities, depending on the migrating species, the initial phytoplankton composition and environmental factors it promotes increased diversity, changes in composition and both positive and negative effects on biomass (Reichwaldt and Stibor, 2005; Haupt *et al.*, 2009; Petzoldt *et al.*, 2009) and

phytoplankton diversity has been proven important to reversely maintain the diversity among zooplankton (Striebel *et al.*, 2012), which presumably would also translate into higher trophic levels. Things like changes in nutrient availability, temperature and solar radiation mediated by climate change and anthropogenic activities will on the other hand have a negative effect on the diversity of many phytoplankton communities (Delgado-Molina *et al.*, 2009; Schabhüttl *et al.*, 2013; Lewandowska *et al.*, 2014).

D. magna and other daphnids are model organisms and are well suited for laboratory testing because of such features as their short generation time, being transparent and sensitivity to external stimuli, with both physiological (Rabus and Laforsch, 2011), behavioural (Rivetti, Campos and Barata, 2016) and genetic (Harris, Bartlett and Lloyd, 2012) responses. Additionally, *D. magna* and some other daphnids exercise cyclic parthenogenesis, a reproductive strategy where the females reproduces asexually to produce diploid eggs that develop into females when conditions are favourable and males when conditions are unfavourable, alternated with the production of haploid eggs in response to unfavourable conditions. The haploid eggs are fertilised internally by males and then encapsulated in extra layers, becoming what's called an ephippia, which is then deposited and remain in diapause until the conditions become favourable again. The hatched individuals from an ephippia are always female (Fig. 1). The environmental cues to signal unfavourable conditions, such as high population density, changing of seasons or increased predation risk (Pijanowska and Stolpe, 1996), and are different for the development of males and the production of resting-eggs (Hobaek and Larsson, 1990).

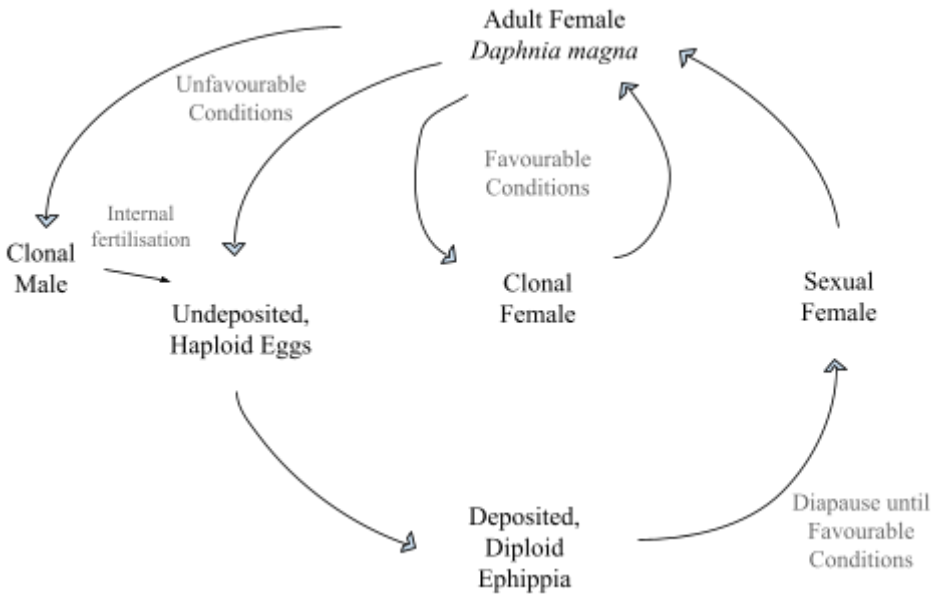

Figure 1. A schematic representation of the life cycle of *D. magna*. Under favourable conditions, females reproduce parthenogenetically to produce clonal females. Under unfavourable conditions they can also produce clonal males or haploid eggs, depending on the environmental cues. The haploid eggs are internally fertilised by males and deposited as ephippium. The ephippium will remain in diapause until conditions are favourable again, at which point they hatch as females.

The life history responses of daphnids and *D. magna* to different temperatures and food availability have been thoroughly investigated. Studies have found that higher temperatures result in faster maturation and therefore smaller sizes of mature individuals relative to lower temperatures (Mc Kee and Ebert, 1996; Hoefnagel *et al.*, 2018). Further, Giebelhausen and Lambert (2001) looked at the responses of juvenile growth rate, age at first reproduction, size at first reproduction and clutch size at first reproduction to combinations of several, constant temperatures and food concentrations. They found that juvenile growth rates and clutch size at first reproduction were at their highest, and age at first reproduction was at its lowest, at 20°C in combination with the highest food concentration, and size at first reproduction was at its biggest at the two highest food concentrations, at both 15 and 20°C, but declined steeply at higher temperatures, showing that size plays an important role in reproduction.

The effects of sudden, frequent fluctuations of temperatures and food concentrations, such as those induced by DVM, have only scarcely been studied and the covariance of the two, even less so. Ona weekly time scale, Adamczuk (2020) conducted a study where both weekly

stochastic fluctuations and stable fluctuations every second week between 5 and 25°C were investigated in relation to constant cold (5°C) and warm (25°C) regimes. She found that stochastic thermal fluctuations had a negative effect on population density, mostly due to decreased reproduction frequency, in addition to a high degree of sexual reproduction, while stable fluctuations had a similar population density as individuals under a constant cold regime, but higher asexual reproduction then the other treatments. Weekly thermal fluctuations may have a relevance in relation to climate change induced heat waves or cold spells. On a shorter time scale, fluctuating temperatures with constant, unlimiting food levels have yielded different responses of *D. magna* and other daphnids. For example, 12 h fluctuations between 12 and 19°C had a negative effect on somatic growth rate (Reichwaldt, Wolf and Stibor, 2005) in relation to the expected mean response. The spectrum of temperature fluctuations is also an important factor in life history responses. Fluctuations at lower temperatures (10-20°C) at unlimiting food conditions made *D. parvula* reproduce more frequently, but no negative effects were found, while fluctuations at higher temperatures (15-25°C) made the reproduction frequency slow down and the clutch sizes smaller (Orcutt and Porter, 1983). Sudden shifts in temperature, without continuous fluctuations, have also been shown to cause a negative response on filtration rates, making nutrient uptake less efficient (Müller, Colomer and Serra, 2018). A new study of another Daphnia species, *D. commutata*, found that growth rate was higher under fluctuating temperatures (10-20°C) in comparison to constant temperature (15) during limiting food conditions (Balseiro *et al.*, 2021). A study of the effects of sudden, frequent fluctuations in food availability during constant temperatures could not be found, but Koussoroplis and Wacker (2016) investigated the effects of covariation between food availability and temperature, similar to this study, mimicking the temperature fluctuations daphnids would experience naturally during diel vertical migration, namely fluctuations every 12 hours between 14 and 24°C. They found that negative covariance had a negative effect on measured life history traits relative to both positive covariance and constant treatment of mean values between high and low food availability and temperatures, while the mean responses of positive covariance was slightly lower than that of a constant treatment, but some overlap in the confidence intervals.

1.1 Aims and hypotheses

Considering that *D. magna* regularly experience fluctuating temperature and food availability through DVM, in addition to predicted changes in environmental variability and food availability provided by climate change and anthropogenic activities, this study aims to further test the responses of reproductive rate, reproductive success, growth and development of different genotypes of *D. magna* to negatively and positively covarying, daily fluctuations between high and low temperature and food availability, in relation to constant mean temperature and food availability. The levels chosen in this experiment represent relatively high temperatures and steep temperature gradient in regards to what they experience in nature, but not unrealistic (Kessler, 2004), and the food levels chosen represent relatively low levels in relation to other studies, but was shown in the pilot experiment to allow for growth at both levels and reproduction at high levels (Appendix A). Although DVM is characterised by 12 hour cycles and environmental fluctuations rarely consistently occur on a daily time-scale, this experiment will be performed in 24 hour cycles, out of practicality. The results can therefore only serve as indicators of the effects of DVM and other cyclic fluctuating events at short time-scales.

The three treatments are expected to elicit different responses in the experimental individuals (Fig. 2), based on metabolic rates changing in response to different temperatures in ectotherms and previous findings that rapidly changing temperatures lowers the filtration rates of *D. magna* and has a negative effect life history traits, and that individuals living in fluctuating conditions at these levels have a lower performance than individuals living in constant mean conditions. The hypotheses of this study can therefore be stipulated as such:

1. The individuals exposed to a positive covariance between high and low temperature and food availability will utilise the resources better than the individuals exposed to a negative covariance, as the food level will coincide with the changes in filtration and metabolic rates.

2. Individuals exposed to constant temperature and food availability at a mean level will best utilise the resources and express higher performance in the life history traits measured than the individuals exposed to covariance treatments.

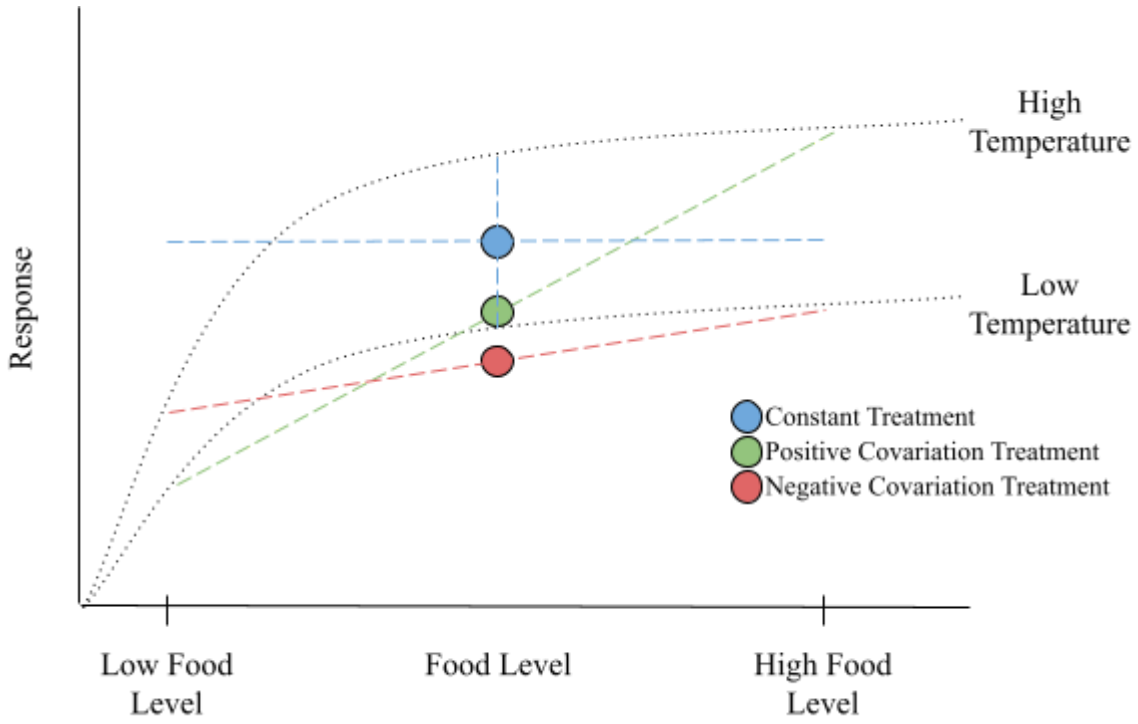

Figure 2. The black, dotted lines represent hypothetical response curves to increasing food levels at low or high temperatures. The filled circles represent the predicted response values of individuals exposed to either negative and positive covariance between temperature and food availability, or a constant mean between high and low temperature and food availability.

2 Methods

2.1 Daphnid Stock Cultures

Individuals of *D. magna* of three different genotypes were used, the specific ones henceforth referred to as clones. One clone originating from a rockpool in Gräsö Island in Sweden, henceforth referred to as Pippi, and one clone originating from an unknown habitat in Morocco, henceforth referred to as Aicha, both provided by professor Dieter Ebert (University of Basel, Switzerland) and arrived at the University of Oslo in March 2020. The third clone originated from a larger pond in Denmark, originally obtained from DHI Water & Environment (Hørsholm, Denmark) and was provided by the Norwegian Institute for Water Research (NIVA), henceforth referred to as Niva. Prior to the experiments, 2-3 stock cultures of each clone were kept in 400 ml jars containing around 8-12 individuals, at 20°C. The medium was changed approximately every three days and they were fed *Chlamydomonas reinhardtii* batch culture (see section 2.2.1) and RotiGrow Nanno (see section 2.2.2) ad libitum. All three clones were used in the pilot experiment (Appendix A), but NIVA was not used in the main experiment due to issues with reproduction (see section 2.5.3).

2.2 Algae Cultures

2.2.1 *Chlamydomonas reinhardtii*

Several *Chlamydomonas reinhardtii* batch cultures were prepared for use in both the pilot experiment and the main experiment. The cultures were kept in 500 ml plastic containers with WC-medium as per the original recipe provided by (Guillard and Lorenzen, 1972) (1972), with the buffer modification made by Makulla (2000), and kept at room temperature. The containers were continuously kept above red and blue light prior and during the pilot experiment and in front of white light prior and during the main experiment. The algae was aerated by an air pump with a filter, which pumped air into a container of distilled water, for further filtration, and the air was from there conducted by a silicon tube into the containers with algae. The optical density was aimed to be maintained below 0.6 Absorbance Units (AU) for all cultures, to keep the *C. reinhardtii* cultures in the growth phase of their growth curve.

2.2.2 RotiGrow

RotiGrow Nanno (Reed Mariculture, henceforth referred to only as RotiGrow) was used in combination with *C. reinhardtii* to feed the stock cultures and in the main experiment. It was not used in the pilot experiment (Appendix A). RotiGrow is a whole-cell microalgae feed of algae of the genus *Nannochloropsis*, developed for rotifers. RotiGrow was diluted with ADaM-medium in a 1:10 ratio and kept at 5°C.

2.3 ADaM Medium

The daphnids were kept in the artificial freshwater medium ADaM, as per the modified version, by the Ebert Group at the University of Basel, Dep. of Environmental Sciences, of the original recipe provided by (Klüttgen *et al.*, 1994) (1994). The medium was aerated by an air pump with a filter, through silicone tubes and into air stones, at least 24 hours within the last 48 hours before use. The air stones were placed at the bottom of either 5L glass bottles or 60L plastic tanks, filled with medium.

2.4 Measuring Length

Daphnid length was measured with a microscope with a millimetre eyepiece reticle and divided by the magnification to calculate actual size. Measurements were made on daphnids placed on their side, from the base of the apical spine to the tip of the head above the eye (Fig. 3). Different microscopes were used for the pilot experiment and the main experiment.

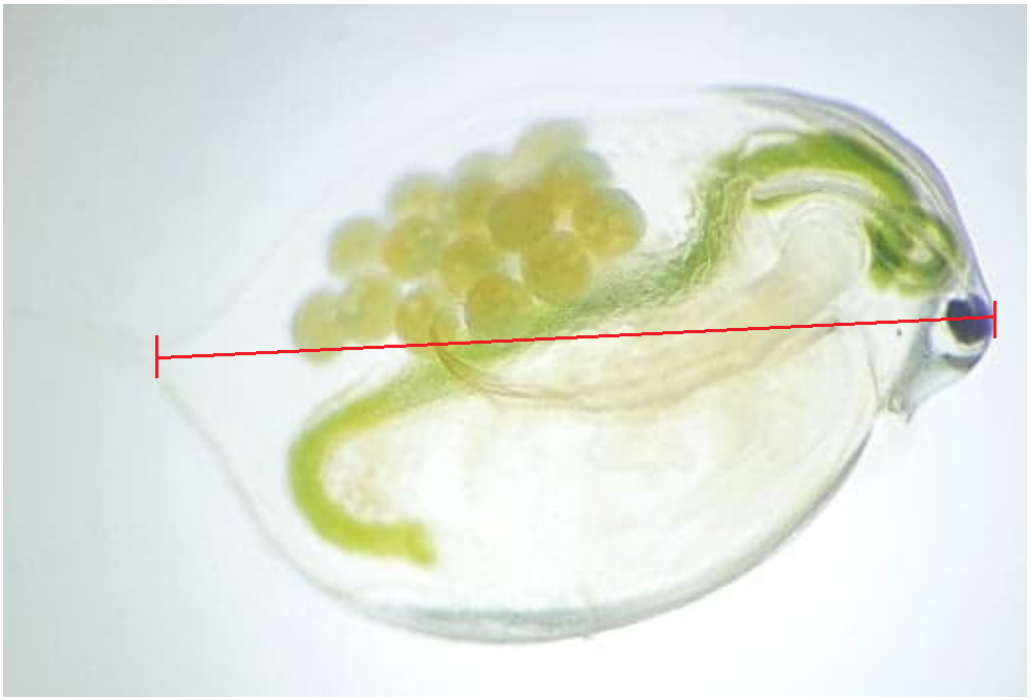

Figure 3. A photo demonstrating the method of measuring length in *D. magna* in a microscope with a millimetre eyepiece reticle. *Photo: A. Olsson*

2.5 Main Experiment

2.5.1 Treatments

Ten individuals of each clone of *D. magna* were exposed to one of three different treatments, one with constant food availability and temperature, one with a positive covariance between food availability and temperature and one with a negative covariance between food availability and temperature. The experiment was performed on a total number of 60 individuals. All individuals received 16 hours of daylight, starting at 06.00 am, and 8 hours of darkness and were kept in 100 ml glass jars containing 80 ml of ADaM medium.

The individuals subjected to constant treatment were kept in a temperature controlled room at a constant 20°C (20.39±0.26) and received 0.155 mg of C per individual, each day. The individuals subjected to covariance treatments were kept in a temperature controlled cabinet fluctuating between 15°C (14.82±0.47) and 25°C (24.87±0.55) every 24 hours, at 08.30 am. The individuals received either 0.01 mg of carbon per individual or 0.3 mg of carbon per individual, depending on the temperature and the treatment (Fig. 4). Food levels were chosen

on the basis of the results from a pilot experiment (A. Olsson and Y. Vindenes, May/June 2020, Appendix B).

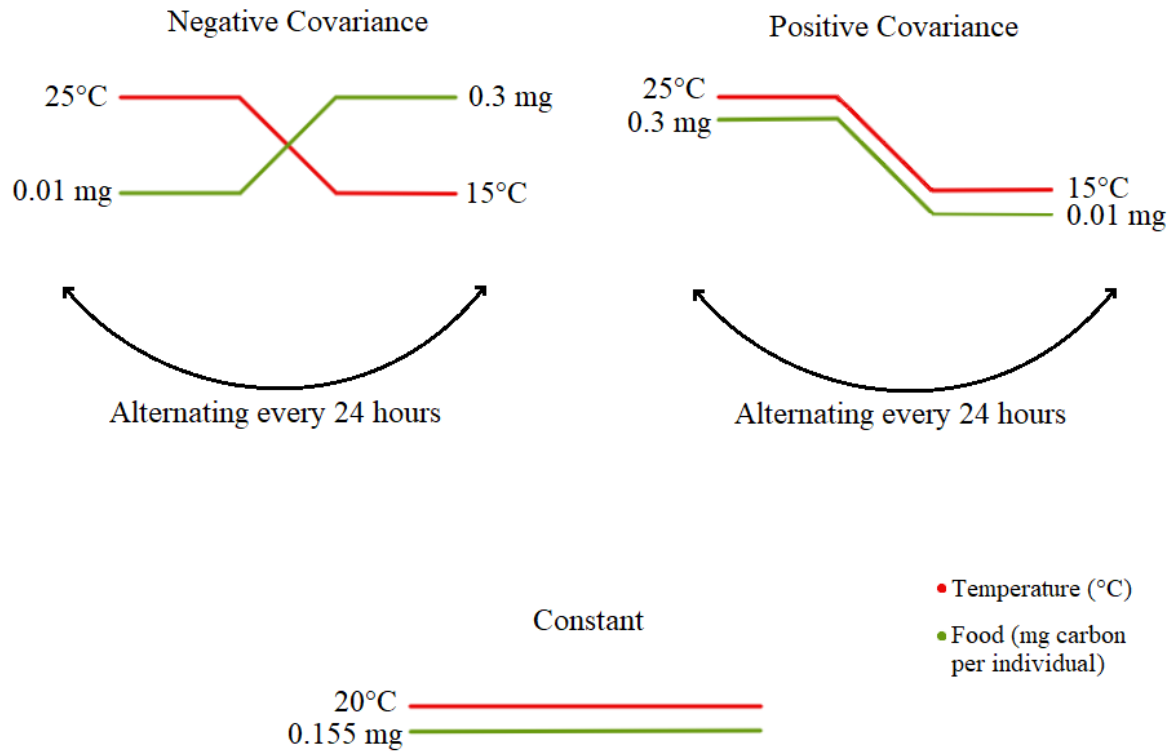

Figure 4. A schematic representation of the three treatments in this experiment. Negative Covariance alternated between receiving 25°C and 0.01 mg C/individual and receiving 15°C and 0.3 mg C/individual every 24 hours. Positive Covariance alternated between receiving 25°C and 0.3 mg C/individual and 15°C and 0.01 mg C/individual every 24 hours. Constant received 20°C and 0.155 mg C/individual without alternation.

2.5.2 Preparations

Between the pilot experiment and the main experiment a change in the spectrophotometer used for the daily measuring of optical density of *C. reinhardtii* was needed, due to malfunction. The relationship of carbon content and AU measured on the old instrument (estimated response curve by Raoul Wolf, see Fig. A-1) was

C. reinhardtii carbon content (g/L) = $-0.02+0.3385x \cdot 1.38$ (2)

As a result, the relationship between optical density of *C. reinhardtii* and its carbon content would need to be modified. Measuring carbon content was not possible until after the experiment and therefore an estimate was made, based on the relationship of carbon content and AU on the old instrument and adjusted to the new one based on the difference in AU measures alone,

Adjusted *C. reinhardtii* carbon content (g/L) = -0.003362+0.1666x (3)

where x is the measured AU. To calculate how much RotiGrow that should be mixed with *C. reinhardtii* to achieve the correct proportion of carbon from each, and how much of the mix the daphnids should receive to get the correct amount of carbon per individual, an equation was first used to calculate how many ml of *C. reinhardtii* that would be needed in the desired proportion to achieve the target mg C/individual for each treatment

$$ml\ C.\ reinhardtii\ per\ treat. = \frac{target\ mg\ C/individual}{(0.1666 \times mean\ AU - 0.003362) \times desired\ proportion\ of\ C.\ Reinhardtii}$$

(4)

where the mean AU of *C. reinhardtii* was taken from three consecutive measurements. The carbon content of RotiGrow had been measured to be 9.3177 mg C/ml (October 2020, Y. Vindenes), so to calculate the how many ml of RotiGrow that would be needed in the desired proportion another equation was used

$$ml\ RotiGrow\ per\ treatment = \frac{target\ mg\ C/individual}{desired\ proportion\ of\ RotiGrow \times 9.1377}$$

(5)

To then calculate how much RotiGrow to mix with the total volume of *C. reinhardtii,* the following equation was used

$$ml\ RotiGrow\ to\ mix\ with\ C.\ reinhardtii = \frac{ml\ RotiGrow\ per\ treatment\ z}{ml\ C.\ reinhardtii\ per\ treatment\ z \times total\ volume\ of\ C.\ reinhardtii}$$

(6)

where z is any of the three treatments. To get the final volume of the mix to give to each individual of each treatment, the calculated ml *C. reinhardtii* (Eq. 4) and RotiGrow (Eq. 5) per treatment were simply added together. This succession of equations was put into a digital conversion sheet, so that the only thing that needed to be entered to receive an output for the amount of RotiGrow to mix with *C. reinhardtii* and the amount to give to each individual at each treatment was the three AU measurements and the total volume of *C. reinhardtii.*

Six individuals of Pippi and Aicha stock cultures were removed and kept separately in 100 ml jars with 80 ml of ADaM medium at 20°C and fed ad libitum with an unspecified ratio of *C. reinhardtii* and RotiGrow. Six offspring of each clone were removed from clutch number four or five, placed in 50 ml jars at 20°C and the medium was added 0.3 mg C/individual with a 50/50 ratio of *C. reinhardtii* and RotiGrow and changed every day. However, because of a change in the formula in the conversion sheet explained in the previous paragraph, the individuals were fed 0.45 mg of C/individual, at a proportion of ⅔ *C. reinhardtii* and ⅓ RotiGrow, each day until one day before Aicha had the second clutch, and the day Pippi had the first clutch. After the first clutch was produced, 3 individuals of each clone were moved into a temperature controlled cabinet fluctuating between 15°C and 25°C every 24 hours, while receiving the same food rations. At the time of the move the temperature in the cabinet was 15°C. The remaining three individuals were kept in the same conditions.

Ten experimental individuals per clone and treatment were randomly picked after combining all offspring from the three individuals per clone in the cabinet and separately combining all offspring from three individuals of the Aicha clone and combining all offspring from two individuals of the Pippi clone at 20°C, from the second clutch within 24 hours of birth. To minimise the amount of stress put on the experimental individuals, their siblings of the same clutches were measured and their averages were used as the measurement for age 0 in the analyses. 30 siblings of Aicha mixed from both the cabinet and the 20°C room were measured (0.656 mm ± 0.034), 20 siblings of Pippi from cabinet were measured (0.749 mm ± 0.024) and 10 siblings of Pippi from the 20°C room were measured (0.767 mm ± 0.017).

2.5.3 Complications

When algae carbon content was measured after the experiment (Y. Vindenes, March 2021), the response curve for the new spectrophotometer was calculated to be

$$\text{carbon content (g/L)} = -0.001+0.185x \quad (7)$$

where x is the measured AU. As a consequence, carbon content had been overestimated during the entirety of the experiment (Tab. 1). In addition, the first time the carbon content of RotiGrow was measured (October 2020, Y. Vindenes), it was mixed with WC medium and was measured to be 9.318 mg C/ml. The RotiGrow used in the main experiment was instead mixed with ADaM medium and the measurements made after the experiment showed the carbon content in that mix to be 6.724 mg C/ml.

Problems also arose during the experiment with the batch cultures of algae. The fresh algae cultures started collapsing a couple of days into the experiment and by the time Aicha was 7 days old and Pippi was 5 days old the fresh algae had such a low optical density that only RotiGrow was used as a carbon provider. Except for when Aicha was 8 days old and Pippi was 6 days old, when *C. reinhardtii* was providing a proportion of 0.2 of the carbon content, RotiGrow was the sole provider of carbon for the remainder of the experiment (Tab. 1). The likely reason for the collapse of the *C. reinhardtii* cultures was an infection of unknown kind. While these issues lead to uncertainty in the exact carbon level used for each treatment, all individuals within each treatment received the same food level at all times during the experiment.

When Aicha was 8 days old it was confirmed that all the individuals subjected to the two covariance treatments and two individuals subjected to the constant treatment were males. The sex was confirmed by looking at the individuals in a microscope and observing the specialised, elongated first antennae, specific for males. The males were measured and the experiment was terminated for those individuals. Eight individuals of Aicha subjected to the constant treatment and all individuals of Pippi were females.

Table 1. The Absorbance Unit (AU) of *C. reinhardtii* for each day it was used, the proportion of *C. reinhardtii* for each day it was used and the actual amount of carbon that was given per individual in each food level, calculated from the actual relationship between the AU and the spectrophotometer (Equation 3). RotiGrow was the other source of carbon and the proportion each day can be calculated as 1 - Proportion of *C. reinhardtii.*

Age of Aicha	Age of Pippi	AU	Proportion of *C. reinhardtii*	Actual amount of carbon given per individual		
				High food	Constant	Low food
0	-	0.182	0.5	0.1836	0.0949	0.0061
1	-	0.23	0.5	0.1795	0.0928	0.0060
2	0	0.127	0.5	0.1940	0.1003	0.0065
3	1	0.108	0.5	0.2017	0.1042	0.0067
4	2	0.263	0.5	0.1777	0.0918	0.0059
5	3	0.16	0.5	0.1866	0.0964	0.0062
6	4	0.092	0.5	0.2128	0.1100	0.0071
7	5	-	0	0.2165	0.1119	0.0072
8	6	0.058	0.2	0.2555	0.1320	0.0085
9	7	-	0	0.2165	0.1119	0.0072
10	8	-	0	0.2165	0.1119	0.0072
11	9	-	0	0.2165	0.1119	0.0072
12	10	-	0	0.2165	0.1119	0.0072
13	11	-	0	0.2165	0.1119	0.0072
-	12	-	0	0.2165	0.1119	0.0072
-	13	-	0	0.2165	0.1119	0.0072
-	14	-	0	0.2165	0.1119	0.0072
-	15	-	0	0.2165	0.1119	0.0072
-	16	-	0	0.2165	0.1119	0.0072
-	17	-	0	0.2165	0.1119	0.0072
-	18	-	0	0.2165	0.1119	0.0072
-	19	-	0	0.2165	0.1119	0.0072

After the experiment's conclusion it was discovered a likely bacterial infection in the *D. magna* stock cultures. The infection was most likely responsible for disruptions in

reproduction and, to a lesser extent, mortality. This was noticeable already in the stock cultures, where NIVA had variable reproduction and a high degree of ephippia production, and was therefore chosen not to be included in the main experiment. It is feasible that the experimental individuals were exposed to the unidentified infection and it should therefore be taken into account when considering the results. However, all experimental individuals originated from the same stocks and the comparisons between treatments and clones are still justifiable.

2.5.4 Experimental implementation

Food ration was calculated by performing three independent measurements of the optical density of *C. reinhardtii* with a spectrophotometer PV4 (VWR) and entering the AU's in a conversion sheet. The conversion sheet used equation 2 with the average value to calculate the amount of carbon in the *C. reinhardtii* culture, the amount of RotiGrow that should be added to these cultures in regards to the desired proportions of total carbon, and how much of the final mixture should be given to the individuals of each treatment. The ADaM medium was kept together with the individuals for which treatment it was to be used 24 hours beforehand, to achieve minimum temperature differences before and after the transfer. The ADaM medium and food ration needed for all individuals in each treatment was mixed together and 80 ml was then distributed into clean glass jars. Each day at 10-12 am the individuals were transferred by plastic pipettes into the clean jars. As the individuals grew, the tips of the pipettes were removed to create a bigger opening, to avoid any damage to the animals. The used glass jars were washed in a dishwasher with laboratory detergent at 60°C for 80 minutes.

2.5.5 Logging data

Each day the age and the absence or presence of moults, eggs and offspring was recorded for each individual. When the individuals had offspring, the number of offspring was counted and the length of up to 24 offspring originating from 8-10 individuals per treatment was measured. The adult individuals were measured after the first moult subsequent to the first and the third clutch had been released, at which time the experiment was terminated for those

individuals. The experiment was concluded on day 21 and individuals who had not yet had a third clutch were then measured and terminated.

Temperature and light loggers (HOBO) took measurements every 10-30 minutes throughout the experiment. There were two in the temperature controlled room, one taking measurement from the air and one submerged in water, and one taking measurements from the air in the temperature controlled cabinet.

2.6 Statistical analyses

All analyses were executed in R version 3.6.1 (R core team 2019) and the packages ggplot2 (Wickham *et al.,* 2016), gridExtra (Auguie 2017) and ggpubr (Kassambara 2020) were used for the graphical visualisation of the data.

To assess the contrasts between the variables for the different treatments, the 95% confidence interval for all variables was calculated through linear regression. The measurement was the dependent variable and treatment was the independent variable when comparing treatments and clone was the independent variable when comparing clones. The degree of overlap in the 95% confidence intervals was used to determine the distinctness of differences between the treatments. R-squared was used to estimate the fit of the linear model, in addition to Residuals vs. Fitted Values plots (Appendix 2).

Length was measured at three points. Length at age 0 was estimated through the average length of the siblings of the same clutch of the experimental individuals at the time of birth, length at the first clutch and the third clutch was measured on the experimental individuals. Growth rate was then calculated using the linear formula

$$\text{Linear Growth Rate (mm/day)} = \frac{(L_2 - L_1)}{(A_2 - A_1)} \quad (8)$$

where L is the length at measurement 1 and 2 and A is the age at measurement 1 and 2. The growth rate was then calculated for each individual and separated into juvenile growth rate, where the average length of the siblings at age 0 was L_1 and length at first clutch was L_2, and adult growth rate, where length at first clutch was L_1 and length at third clutch or termination was L_2.

Moulting rate was measured at each age a moulting event occurred and was separated into juvenile and adult moulting rates. In contrast to juvenile growth rate, which was calculated using the length at the age at first clutch as the second measurement, moulting rate was calculated using the moult before the age at maturation to define the juvenile stage. The moulting rate similarly calculated through the linear formula

$$\text{Linear Moulting Rate (moults/day)} = \frac{(M_2 - M_1)}{(A_2 - A_1)} \tag{9}$$

where M is the amount of moults at measurement 1 and 2 and A is the age at measurement 1 and 2. For the juvenile moulting rate, moults at age 0 was used as M_1 and was by default 0, and M_2 was the amount of moults at the age of maturation. For the adult moulting rate, the amount of moults at the age at the first clutch was used as M_1 and the amount of moults at the age of the third clutch or termination as used as M_2.

3 Results

Because all 20 individuals of the Aicha clone subjected to both of the covariation treatments and 2 individuals subjected to the constant treatment were males, the analyses were divided into three groups. The first group, including female individuals of the Pippi clone, compared the effects of the treatments. The second group, including females of the Pippi and Aicha clones subjected to the constant treatment, compared the responses of the clones to the constant treatment. The third group, including male individuals of the Aicha clone, compared only the responses to the negative and positive covariance treatments. The two male individuals of the Aicha clone subjected to the constant treatment were not included in any analysis.

3.1 Comparing treatments

All individuals subjected to the Constant and the PosCov treatment had a third clutch and were measured and terminated after the first moult, after the third clutch was released, except for one individual in the PosCov treatment, who was accidentally killed at 7 days of age, the age at which it matured and could therefore be included in the analysis of age at maturation and juvenile moulting rate. All individuals of NegCov released the first clutch, but only 6 individuals produced a second clutch and no individuals produced a third clutch. In three instances an individual produced eggs which didn't develop into offspring and were discarded together with the next moult, which was then recorded as a clutch release with zero offspring. One of the individuals of NegCov died at day 19 and became unmeasurable, why the experiment was concluded at day 20.

3.1.1 Growth and Development

The Constant individuals were the longest at both the first and the third clutch, PosCov individuals were the second longest and NegCov individuals were the shortest even though they were several days older than the other treatments at the first clutch and, since no

individuals produced a third clutch, were instead measured at experiment termination (Fig. 5a). The differences within each clutch were distinct, with no overlap in the 95% CI (Fig. 5b).

The Constant individuals had a higher juvenile growth rate than the other treatments, and PosCov had a higher juvenile growth rate than NegCov (Fig. 6a). The adult growth rate was similar for Constant and PosCov and higher than the adult growth rate of NegCov (Fig. 6b). The moulting rate followed the same pattern, with the difference that PosCov, in contrast with the other treatments, had no variation in moulting rate at either the juvenile or the adult stage (Fig. 7a&b). The differences were distinct in both the juvenile growth and moulting rate, with no overlap in the 95% CI, while in both the adult growth and moulting rate there was no overlap in the 95% CI between NegCov and the other treatments, but overlap between Constant and PosCov (Tab. 2).

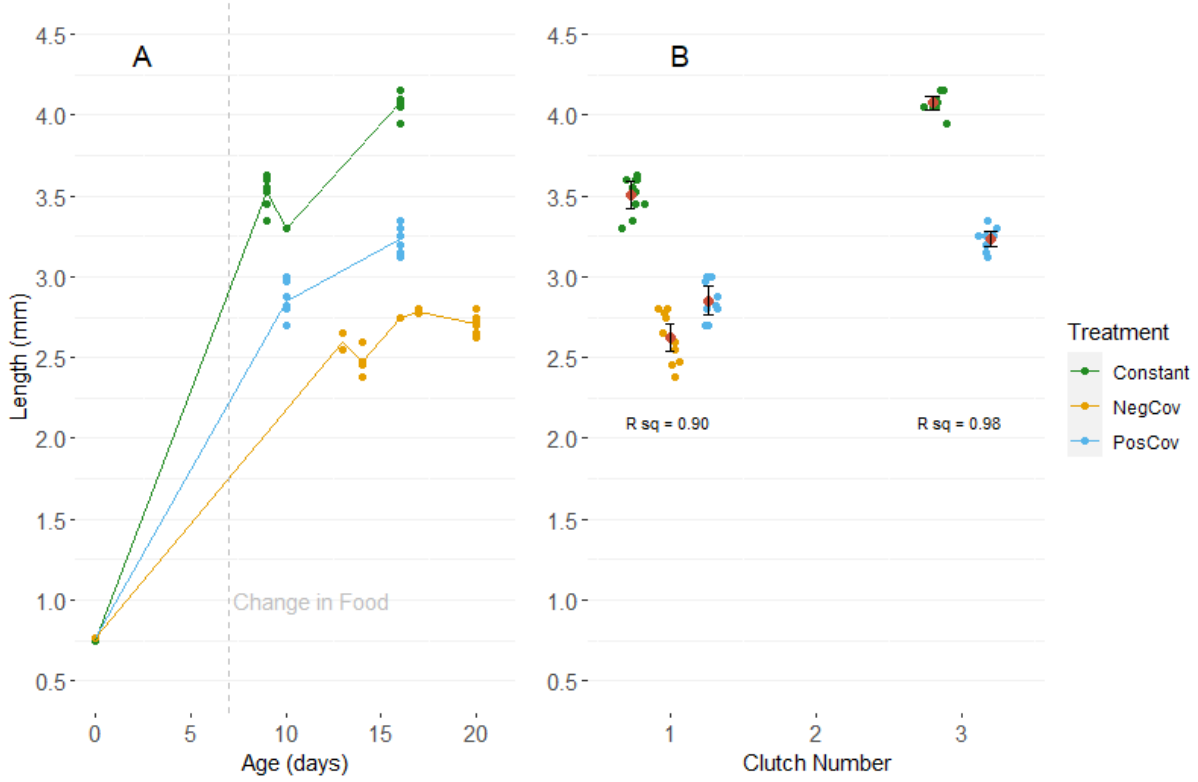

Figure 5. (A) The average length (mm) of the siblings of the experimental individuals at age 0, the length of each individual at the first and the third clutch or termination, plotted over the age (days) of the individuals. The vertical, dashed line represents the day at which no more *C. reinhardtii* was used and RotiGrow became the sole provider of carbon in the experiment. (B) The length (mm) of each individual at the first and third clutch and the mean and 95% confidence interval for each treatment and clutch number plotted over the clutch number, and the R squared of each regression analysis.

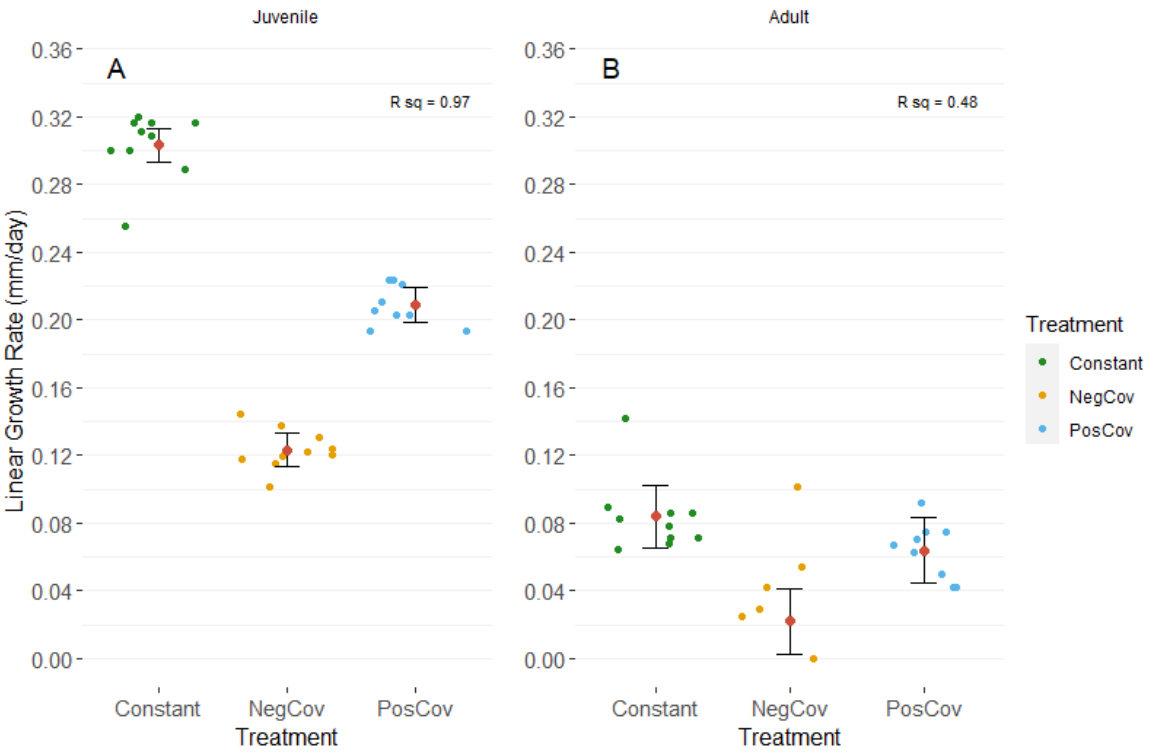

Figure 6. The growth rate (mm/day) for each individual at the juvenile (A) and adult (A) stage and the mean and 95% confidence interval for each treatment plotted over treatment, and the R squared of each regression analysis.

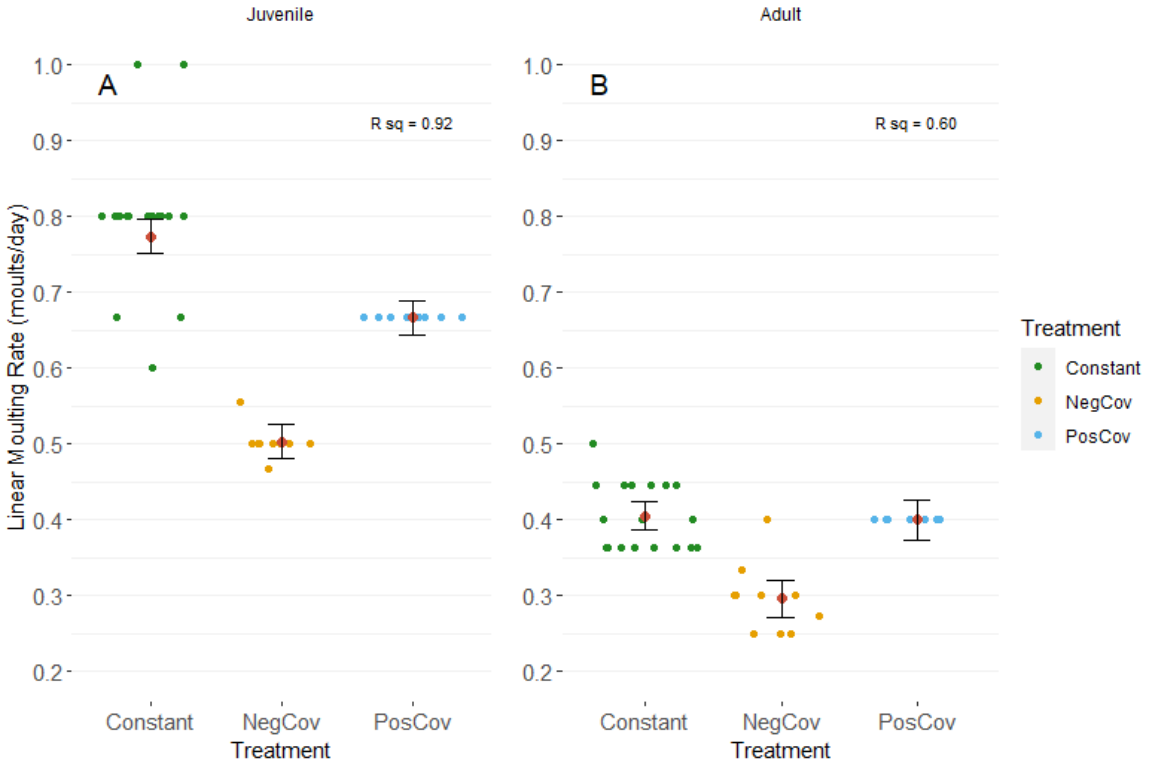

Figure 7. The moulting rate (moults/day) for each individual at the juvenile (A) and adut (B) stage and the mean and 95% confidence interval for each treatment, plotted over treatment, and the R squared of each regression analysis

Table 2. The growth (mm/day) and moulting (moults/day) rate mean and 95% confidence interval for each treatment divided into a juvenile and an adult stage, and the R squared of each regression analysis. The juvenile stage was defined as before the release of the first clutch for growth rate and as before maturation for moulting rate.

		Constant	NegCov	PosCov	R sq
Growth rate		n = 10	n = 10	n = 9	
Juvenile (mm/day)	95% CI, lower	0.29	0.11	0.20	0.97
	Mean	0.30	0.12	0.21	
	95% CI, upper	0.31	0.13	0.22	
		n = 10	n = 9	n = 9	
Adult (mm/day)	95% CI, lower	0.07	0.00	0.04	0.48
	Mean	0.08	0.02	0.06	
	95% CI, upper	0.10	0.04	0.08	
Moulting rate		n = 10	n = 10	n = 10	
Juvenile (moult/day)	95% CI, lower	0.75	0.48	0.64	0.92
	Mean	0.77	0.50	0.67	
	95% CI, upper	0.80	0.52	0.69	
		n = 10	n = 10	n = 9	
Adult (moult/day)	95% CI, lower	0.36	0.27	0.38	0.60
	Mean	0.38	0.30	0.40	
	95% CI, upper	0.40	0.32	0.42	

3.1.2 Reproduction

Measurements of reproduction were divided into Age At Maturation (AAM), age at each clutch, clutch size and length of the offspring. The ability to produce a clutch can also be seen as a measurement.

The AAM was later for NegCov than the other treatments and AAM was similar for Constant and PosCov (Fig. 8). The difference in AAM between NegCov and the other treatments was distinct with no overlap in the 95% CI, while the difference in AAM between Constant and PosCov was not as distinct with overlap in the 95% confidence intervals (Tab. 3).

Table 3. The Age At Maturation (days) mean and 95% confidence intervals for each treatment, and the R squared of the regression analysis.

		Constant	NegCov	PosCov	R sq
		n = 10	n = 10	n =10	
AAM (days)	95% CI, lower	5.5	11.3	6.3	
	Mean	6.2	12.0	7.0	0.87
	95% CI, upper	6.9	12.7	7.7	

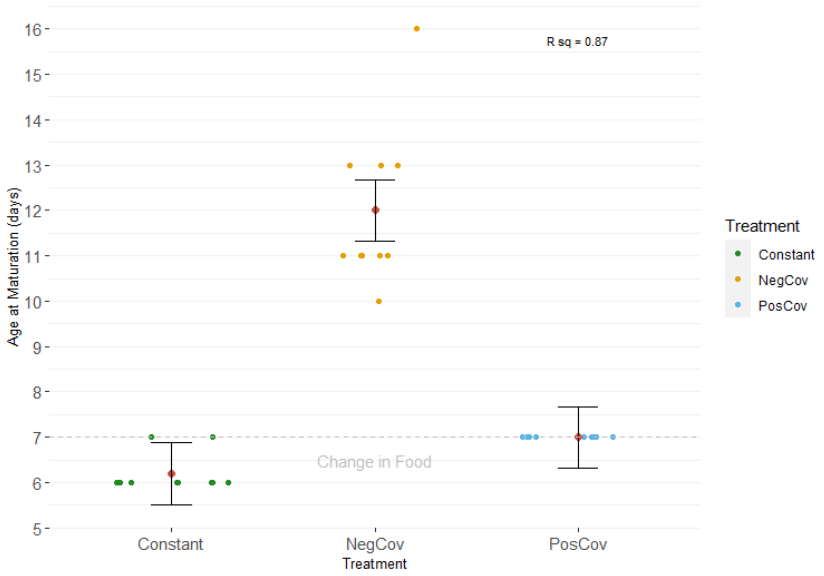

Figure 8. The Age At Maturation (days) for each individual and the mean and 95% confidence interval for each treatment plotted over treatment, and the R squared of the regression analysis. The horizontal, dashed line represents the day at which no more *C. reinhardtii* was used and RotiGrow became the sole provider of carbon in the experiment.

The age at the first and second clutch releases was later for NegCov than the other treatments and it had a lot of variation in age at the first clutch, in contrast to the other treatments which had no variation in the first and second clutch, but at different ages (Fig. 9a&b). All but one individual of the Constant treatment had the third clutch at the same age as PosCov (Fig. 9c). The difference in age between NegCov and the other treatments at both the first and second clutch and the difference in age between Constant and PosCov at the second and third clutch was distinct with no overlap in the 95% CI. There was overlap in the 95% CI in age between Constant and PosCov at first clutch, however, because of heteroscedasticity (see Fig. 24b in Appendix B) the CI for those treatments does not represent their variation.

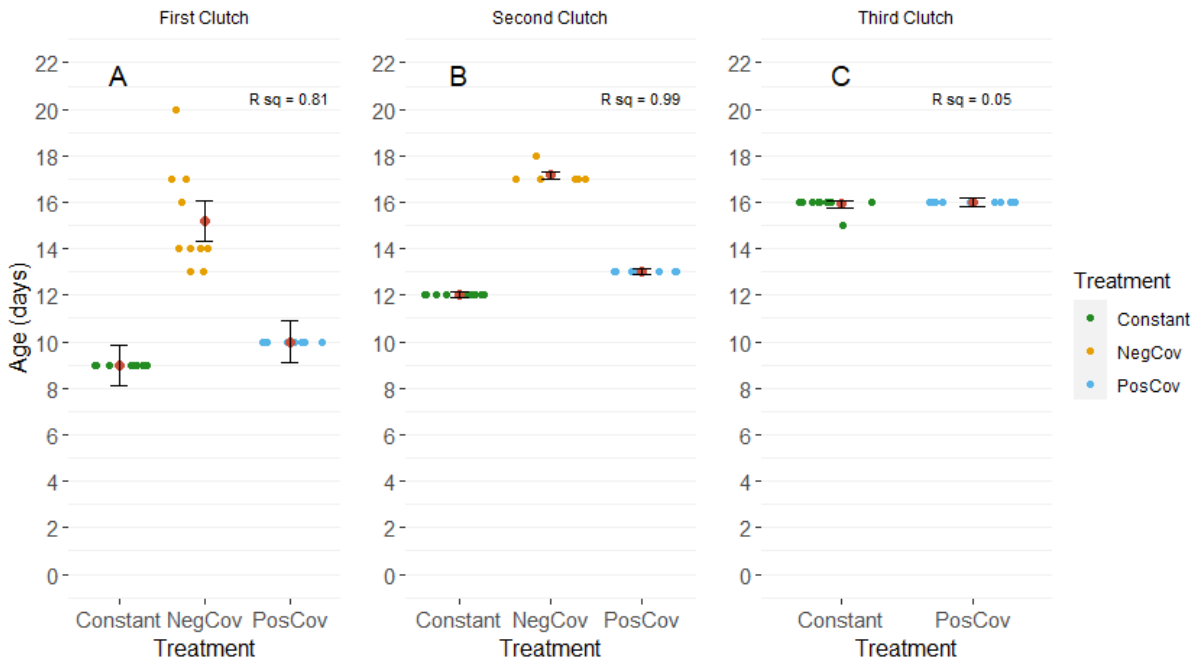

Figure 9. The age (days) of each individual at the first (A), second (B) and third (C) clutch and the mean and 95% confidence interval for each treatment plotted over treatment, and the R squared of each regression analysis. NegCov never produced a third clutch.

Table 4. The age (days) mean and 95% confidence intervals of the first, second and third clutch for each treatment, and the R squared for each regression analysis. NegCov never produced a third clutch.

		Constant	NegCov	PosCov	R sq
First clutch		n = 10	n = 10	n = 9	
	95% CI, lower	8.3	14.2	9.1	
Age (days)	Mean	9.1	15.0	10.0	0.81
	95% CI, upper	9.9	15.8	10.9	
Second clutch		n = 10	n = 6	n = 9	
	95% CI, lower	11.9	17.0	12.9	
Age (days)	Mean	12.0	17.2	13.0	0.99
	95% CI, upper	12.1	17.3	13.1	
Third clutch		n = 10	n = 0	n = 9	
	95% CI, lower	15.8	NA	15.8	
Age (days)	Mean	15.9	NA	16.0	0.04
	95% CI, upper	16.1	NA	16.2	

The clutch size was larger for Constant than for the other treatments in all three clutches and PosCov had larger clutch sizes than NegCov in the first and second clutch (Fig. 10a-c). The differences in clutch size between Constant and the other treatments and the difference between all treatments was distinct with no overlap in the 95% CI, while there was overlap between PosCov and NegCov in the first clutch and between Constant and PosCov in the third clutch (Tab. 5) However, there were three outliers, individuals where part of the clutch was released as undeveloped eggs, in the Constant treatment disrupting the analysis (see Fig. 26c in Appendix B).

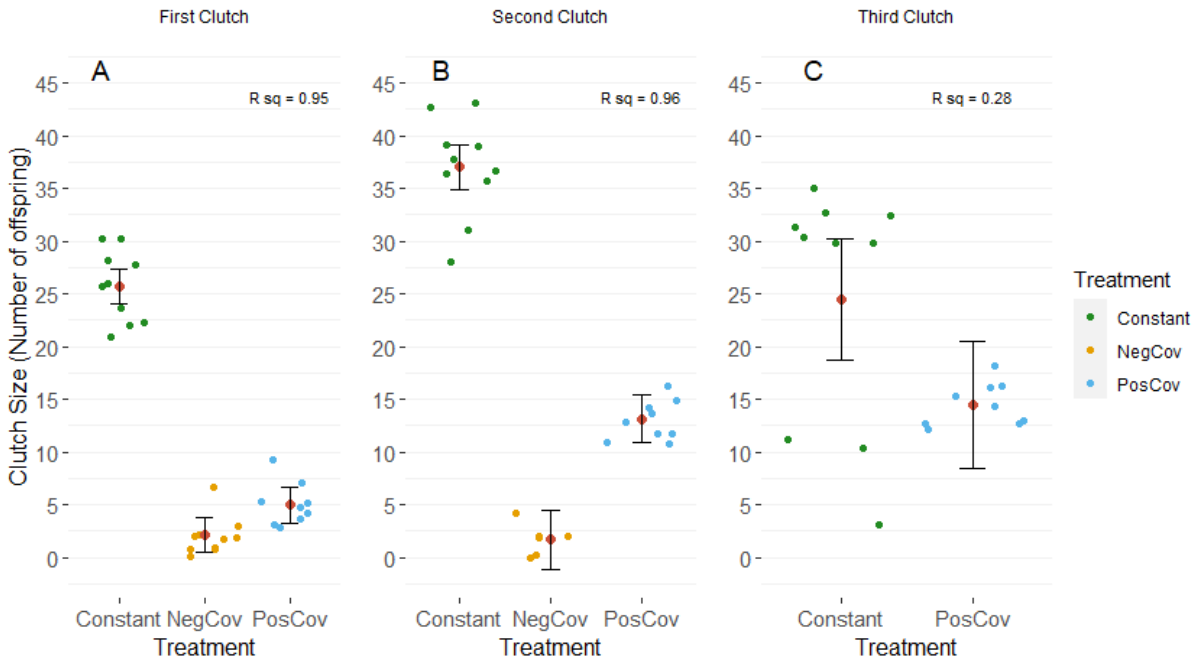

Figure 10. The clutch size (number of offspring) of each individual at the first (A), second (B) and third (C) clutch and the mean and 95% confidence interval of each treatment plotted over treatment, and the R squared of each regression analysis. NegCov never produced a third clutch.

Table 5. The clutch size mean and 95% confidence interval of the first, second and third clutch for each treatment, and R squared of each regression analysis. NegCov never produced a third clutch.

		Constant	NegCov	PosCov	R sq
First clutch		n = 10	n = 10	n = 9	
	95% CI, lower	24.1	0.5	3.3	
Clutch size	Mean	25.7	2.1	5.0	0.95
	95% CI, upper	27.3	3.7	6.7	
Second clutch		n = 10	n = 6	n = 9	
	95% CI, lower	34.9	-1.1	10.9	
Clutch size	Mean	37.0	1.7	13.1	0.96
	95% CI, upper	39.3	4.4	15.4	
Third clutch		n = 10	n = 0	n = 9	
	95% CI, lower	18.8	NA	8.4	
Clutch size	Mean	24.5	NA	14.4	0.28
	95% CI, upper	30.2	NA	20.5	

In the first clutch, the offspring length was longer for NegCov than the other treatments, while the offspring length was similar for Constant and PosCov, and in the second and third clutch the offspring length was similar between all treatments (Fig11a-c). The difference in offspring length between NegCov and the other treatments in the first clutch was distinct with no overlap in the 95% CI, while there was overlap in offspring length between Constant and PosCov in the first clutch and between all treatments in the second and third clutch (Tab. 6).

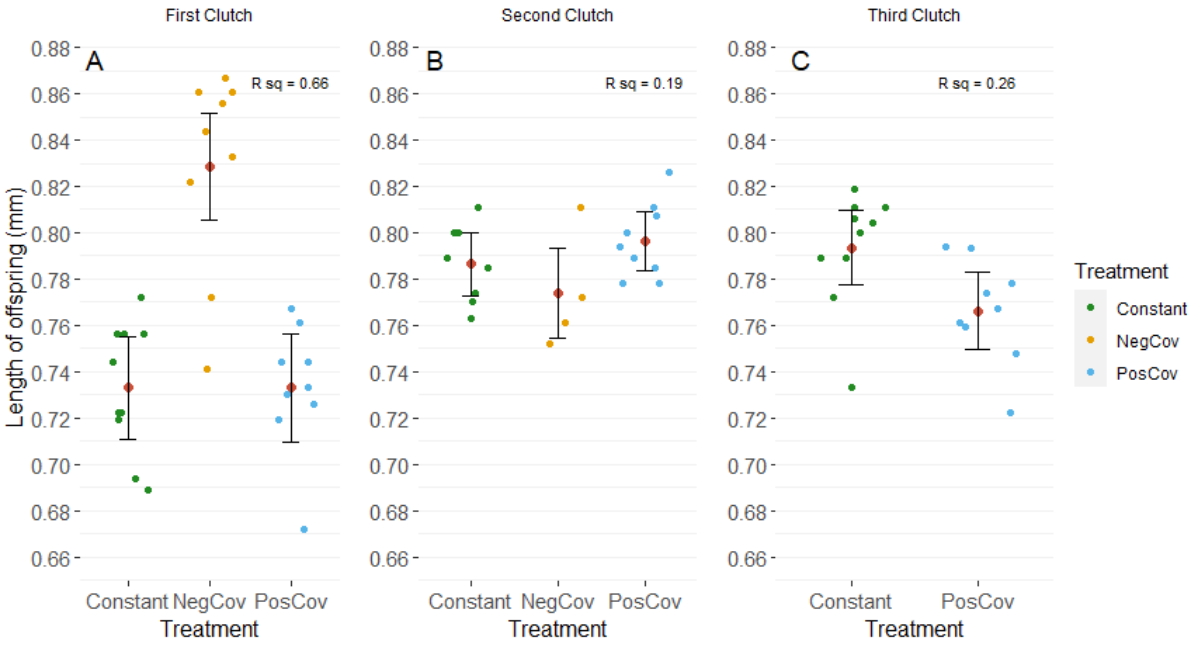

Figure 11. The length (mm) of randomly selected offspring from the first (A), second (B) and third (C) clutch and the mean and 95% confidence interval of each treatment plotted over treatment, and the R squared of each regression analysis. NegCov never produced a third clutch.

Table 6. The length of offspring (mm) mean and 95% confidence interval of the first, second and third clutch for each treatment, and the R squared of each regression analysis. NegCov never produced a third clutch.

		Constant	NegCov	PosCov	R sq
First clutch		n =24	n = 17	n = 23	
Length of offspring (mm)	95% CI, lower	0.71	0.81	0.71	0.66
	Mean	0.73	0.83	0.73	
	95% CI, upper	0.76	0.85	0.76	
Second clutch		n = 24	n = 10	n = 23	
Length of offspring (mm)	95% CI, lower	0.77	0.76	0.78	0.19
	Mean	0.79	0.77	0.80	
	95% CI, upper	0.80	0.79	0.81	
Third clutch		n = 24	n = 0	n = 23	
Length of offspring (mm)	95% CI, lower	0.78	NA	0.75	0.26
	Mean	0.79	NA	0.77	
	95% CI, upper	0.81	NA	0.78	

3.2 Comparing clones

3.2.1 Growth and Development

The length of Pippi was longer than that of Aicha at both the first and the third clutch (Fig. 12a&b). The difference in length was distinct at both clutches with no overlap in the 95% CI and with increasing difference from the first to the third clutch (Fig. 12b).

Figure 12. (A) The average length (mm) of the siblings of the experimental individuals at age 0, the length of each individual at the first and the third clutch or termination, plotted over the age (days) of the individuals. The purple vertical, dashed line represents the day at which Pippi stopped receiving *C. reinhardtii* and RotiGrow became the sole provider of carbon. The red vertical dashed line represents the same change in food for Aicha. (B) The length (mm) of each individual at the first and third clutch and the mean and 95% confidence interval for each clone and clutch number plotted over the clutch number, and the R squared of each regression analysis.

Aicha had a higher juvenile and adult growth rate than Pippi (Fig. 13a&b) and the juvenile moulting rate was similar between the clones, while the adult moulting rate was higher for Aicha than for Pippi (Fig. 14a&b). The difference in juvenile growth rate and adult moulting rate between the clones was distinct with no overlap in the 95% CI, while the adult growth rate had a slight overlap and the juvenile moulting rate was not distinct, with a lot of overlap in the 95% CI (Tab. 7). The juvenile growth rate was influenced by the different average lengths of the siblings to the experimental individuals of Aicha and Pippi (0.656 mm and 0.749 mm respectively), used as age 0 in the calculations and the adult growth and moulting rate by that Pippi had a longer instar before the third clutch, which gave Pippi one day more to grow and one day more in the linear rate calculations (Eq 8 & 9).

Table 7. The growth (mm/day) and moulting (moults/day) rate mean and 95% confidence interval for each clone, divided into a juvenile and an adult stage, and the R squared of each regression analysis. The juvenile stage was defined as before the release of the first clutch for growth rate and as before maturation for moulting rate.

		Pippi	Aicha	R sq
Growth rate		n = 10	n = 8	
Juvenile (mm/day)	95% CI, lower	0.29	0.32	0.47
	Mean	0.30	0.33	
	95% CI, upper	0.31	0.35	
		n = 10	n = 8	
Adult (mm/day)	95% CI, lower	0.07	0.04	0.41
	Mean	0.08	0.05	
	95% CI, upper	0.10	0.07	
Moulting rate		n = 10	n = 8	
Juvenile (moult/day)	95% CI, lower	0.71	0.75	0.08
	Mean	0.77	0.83	
	95% CI, upper	0.84	0.90	
		n = 10	n = 8	
Adult (moult/day)	95% CI, lower	0.36	0.41	0.38
	Mean	0.38	0.43	
	95% CI, upper	0.40	0.46	

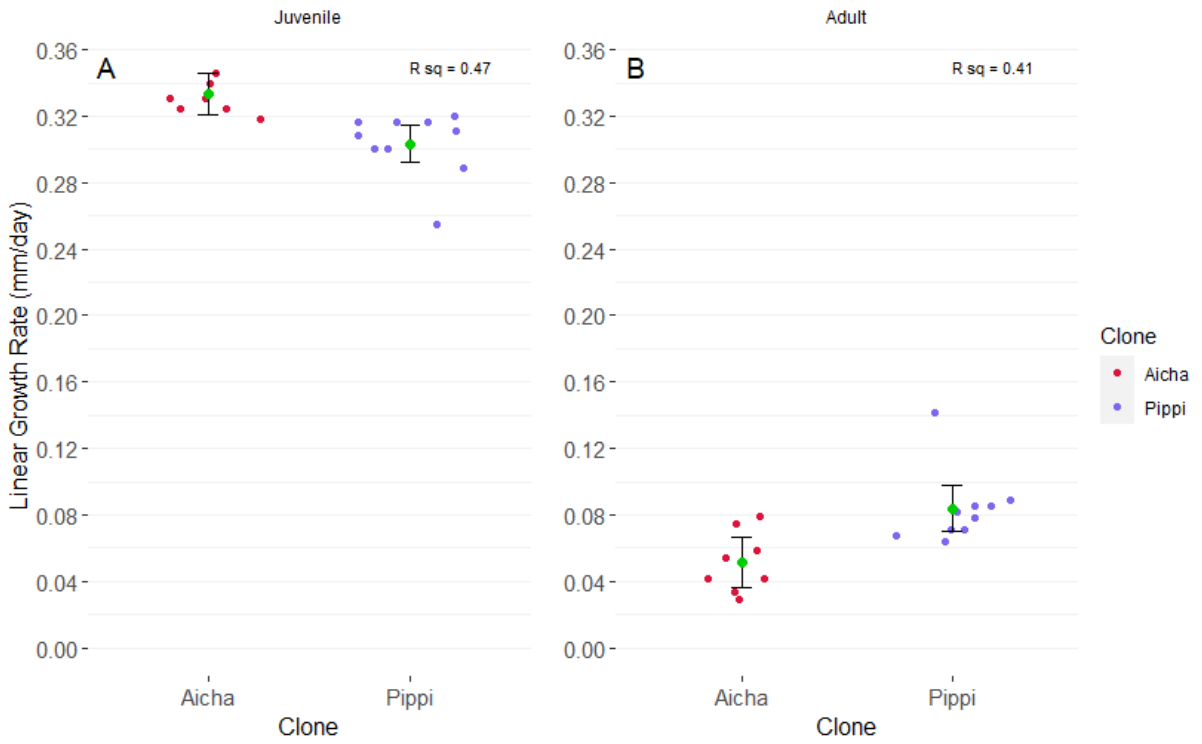

Figure 13. The growth rate (mm/day) for each individual at the juvenile (A) and adult (A) stage and the mean and 95% confidence interval for each clone plotted over clone, and the R squared of each regression analysis.

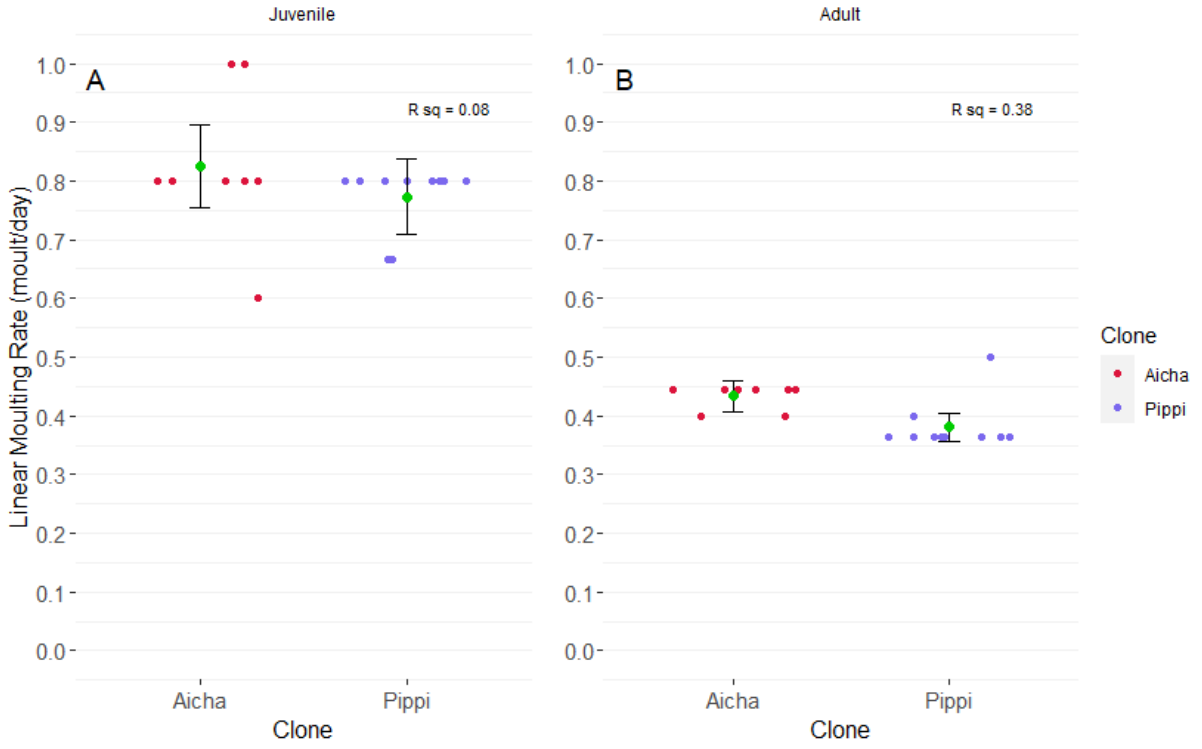

Figure 14. The moulting rate (moults/day) for each individual at the juvenile (A) and adult (A) stage and the mean and 95% confidence interval for each clone plotted over clone, and the R squared of each regression analysis.

3.2.2 Reproduction

The AAM was similar between the clones, with overlap in the 95% CI (Tab. 8). Eight out of the ten Pippi individuals matured at 6 days of age and two individuals matured at 7 days of age. Six out of eight Aicha individuals matured at 6 days of age and two individuals matured at 5 days of age (Fig. 15).

Table 8. The Age At Maturation (days) mean and 95% confidence interval for each clone, and the R squared of the regression analysis.

		Pippi	Aicha	R sq
		n = 10	n = 8	
AAM (days)	95% CI, lower	5.9	5.4	0.23
	Mean	6.2	5.8	
	95% CI, upper	6.5	6.1	

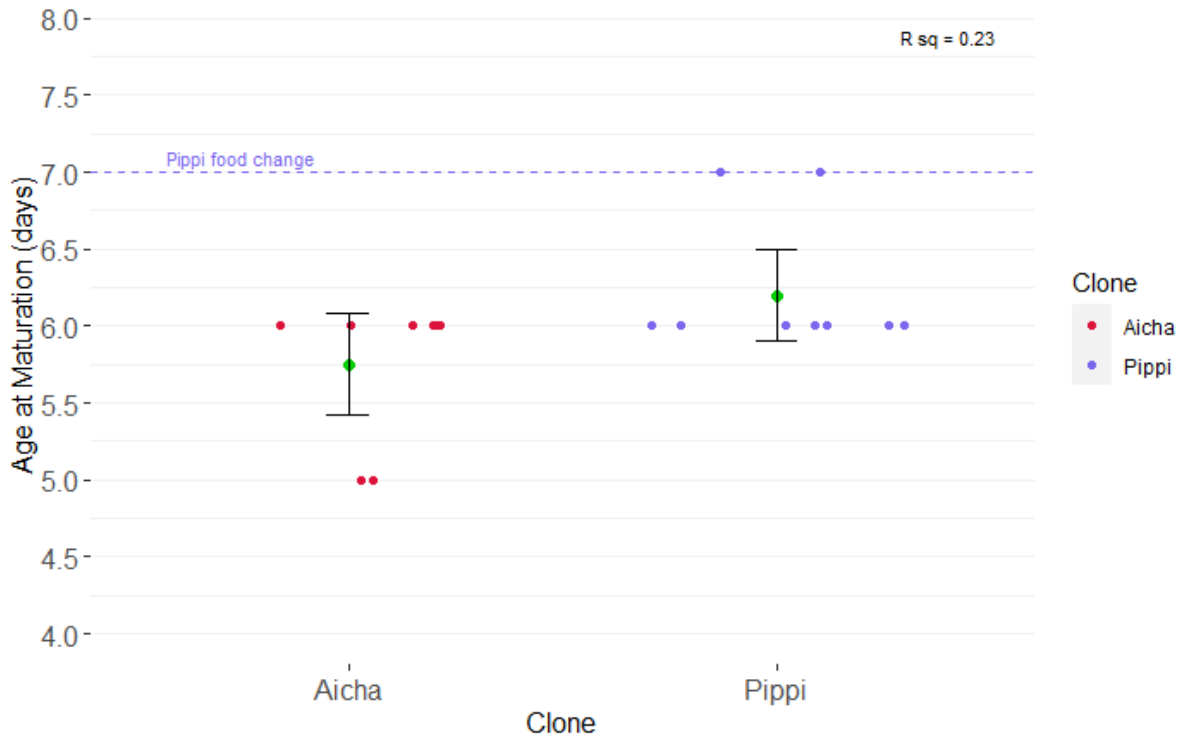

Figure 15. The Age At Maturation (days) for each individual and the mean and 95% confidence interval for each clone plotted over clone, and the R squared of the regression analysis. The purple horizontal line represents the day at which Pippi stopped receiving *C. reinhardtii* and RotiGrow became the sole provider of carbon in the experiment.

The age at each clutch was earlier for Aicha than for Pippi (Fig. 16a-c). The individuals within each clone were very synchronised, with only one individual of the Pippi clone which reproduced one day before the majority in the third clutch. The instar before the first clutch for Aicha was two days and three days for Pippi individuals. The instar before the second clutch was three days for both clones, but the instar before the third clutch was three days for Aicha and four days for Pippi, meaning that Pippi took two days longer than Aicha from maturity to the release of the third clutch. The difference in age at each clutch was distinct, with no overlap in the 95% CI (Tab. 9).

Figure 16. The age (days) of each individual at the first (A), second (B) and third (C) clutch and the mean and 95% confidence interval for each clone plotted over clone, and the R squared of each regression analysis.

Table 9. The age (days) mean and 95% confidence interval of the first, second and third clutch for each clone, and the R squared of each regression analysis.

		Pippi	Aicha	R sq
First clutch		n = 10	n = 8	
Age (days)	95% CI, lower	9.0	8.0	1
	Mean	9.0	8.0	
	95% CI, upper	9.0	8.0	
Second clutch		n = 10	n = 8	
Age (days)	95% CI, lower	12.0	11.0	1
	Mean	12.0	11.0	
	95% CI, upper	12.0	11.0	
Third clutch		n = 10	n = 8	
Age (days)	95% CI, lower	15.8	13.8	0.95
	Mean	15.9	14.0	
	95% CI, upper	16.1	14.2	

The clutch size was similar between the clones in the first clutch (Fig. 17a), Aicha had a slightly bigger clutch size in the second clutch (Fig. 17b) and a much bigger clutch size in the third clutch (Fig. 17c). The differences in clutch size between the clones in the first and second clutch was not distinct, with overlap in the 95% CI, but the difference in clutch size at the third clutch between the clones was distinct with no overlap in the 95% CI of the clutch size at the third clutch (Tab. 10). While the same outliers of Pippi as in the comparisons between the treatments are present (see section 3.1.2 and Fig. 31 in Appendix B), the difference between the clones without the outliers was still big (Fig. 17c).

Table 10. The clutch size (number of offspring) mean and 95% confidence interval for the first, second and third clutch for each clone, and the R squared of each regression analysis.

		Pippi	Aicha	R sq
First clutch		n = 10	n = 8	
	95% CI, lower	23.8	24.0	
Clutch size	Mean	25.7	26.1	0.01
	95% CI, upper	27.6	28.2	
Second clutch		n = 10	n = 8	
	95% CI, lower	34.0	38.7	
Clutch size	Mean	37.0	42.1	0.26
	95% CI, upper	40.0	45.5	
Third clutch		n = 10	n = 8	
	95% CI, lower	18.4	49.8	
Clutch size	Mean	24.5	56.6	0.77
	95% CI, upper	30.6	63.5	

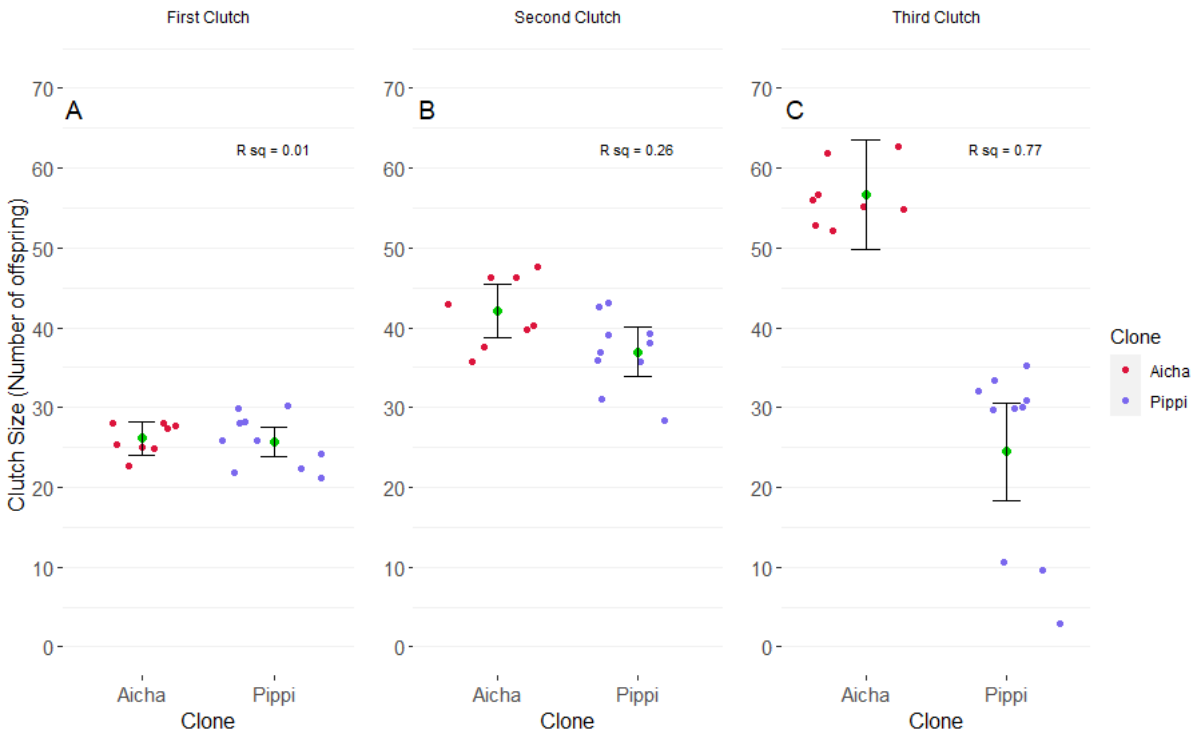

Figure 17. The clutch size (number of offspring) of each individual at the first (A), second (B) and third (C) clutch and the mean and 95% confidence interval for each clone plotted over clone, and the R squared of each regression analysis.

The offspring of Pippi was longer than the offspring of Aicha in all three clutches (Fig. 18a-c). The offspring length in the first clutch mirrored the length of the experimental individual's siblings at age 0, but was longer in the second and third clutch for both clones. The difference in offspring length between clones was distinct for all clutches, with no overlap in the 95% CI (Tab. 11).

Table 11. The length of offspring (mm) mean and 95% confidence interval for the first, second and third clutch for each clone, and the R squared for the regression analysis.

		Pippi	Aicha	R sq
First clutch		n = 24	n = 24	
Length of offspring (mm)	95% CI, lower	0.72	0.64	0.71
	Mean	0.73	0.66	
	95% CI, upper	0.75	0.68	
Second clutch		n = 24	n = 24	
Length of offspring (mm)	95% CI, lower	0.77	0.73	0.45
	Mean	0.79	0.75	
	95% CI, upper	0.80	0.77	
Third clutch		n = 24	n = 24	
Length of offspring (mm)	95% CI, lower	0.78	0.73	0.45
	Mean	0.79	0.75	
	95% CI, upper	0.81	0.77	

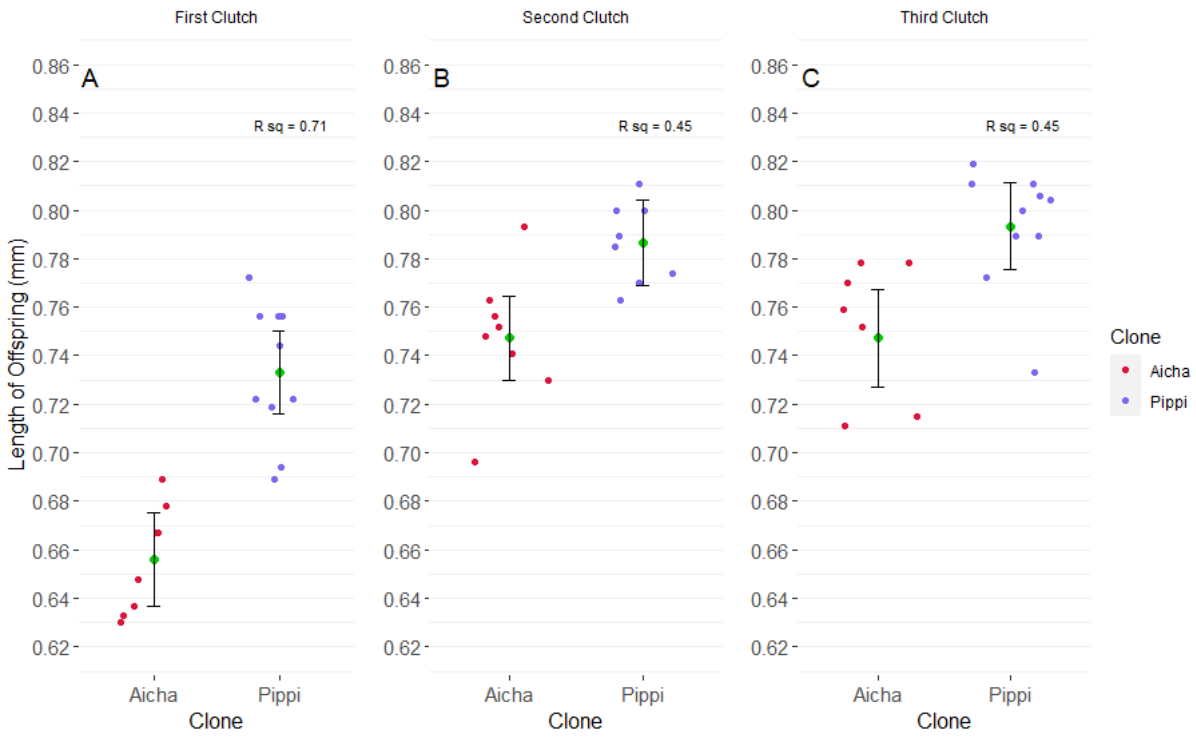

Figure 18. The length (mm) of randomly selected offspring from the first (A), second (B) and third (C) clutch and the mean and 95% confidence interval for each clone plotted over clone, and the R squared of each regression analysis.

3.3 Comparing males

3.3.1 Growth and Development

The male individuals, who were only subjected to the two covariance treatments, were terminated and measured at 8 days of age. The growth rate was higher for PosCov, but the moulting rate was almost identical (Fig. 19a&b). The difference in growth rate between the treatments was distinct with no overlap in the 95% CI, while the moulting rate had identical confidence intervals.

Table 12. The growth (mm/day) and moulting (moults/day) rate mean and 95% confidence interval for each treatment, and the R squared of each regression analysis.

		NegCov	PosCov	R sq
Growth rate		n = 10	n = 10	
	95% CI, lower	0.10	0.14	
(mm/day)	Mean	0.11	0.15	0.89
	95% CI, upper	0.11	0.15	
Moulting rate		n = 10	n = 10	
	95% CI, lower	0.60	0.60	
(moult/day)	Mean	0.63	0.63	0.00
	95% CI, upper	0.65	0.65	

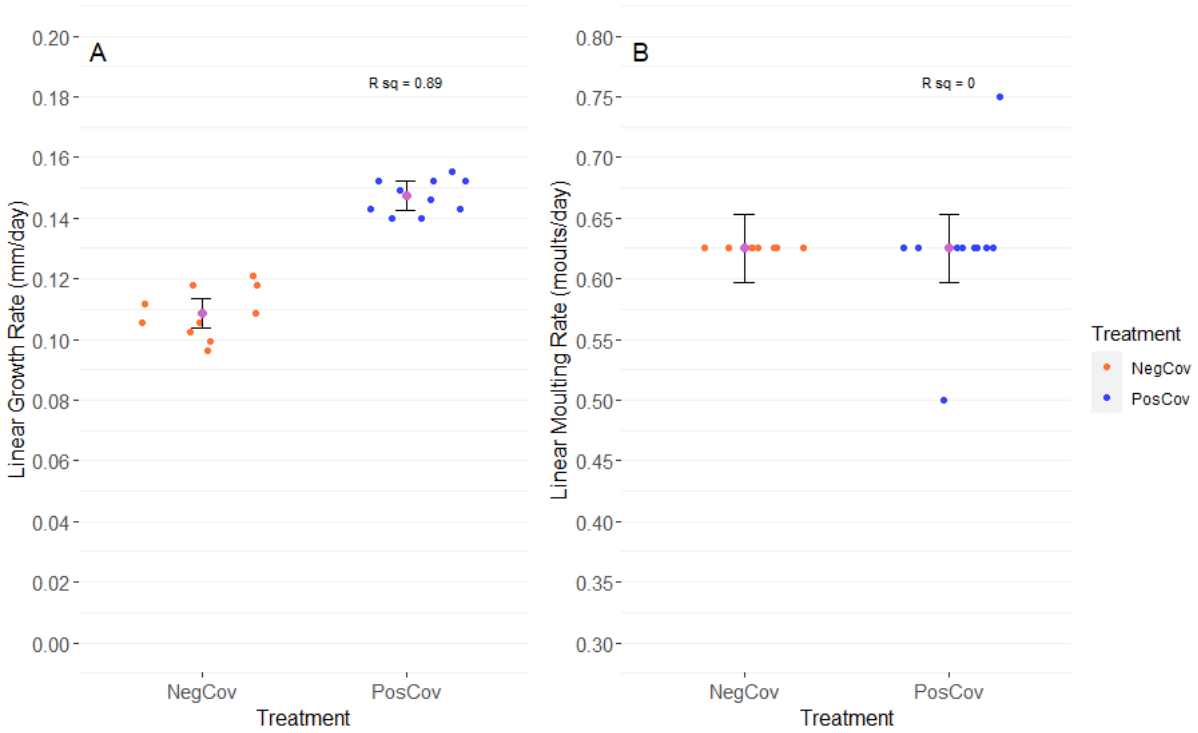

Figure 19. (A) The growth rate (mm/day) of each individual and the mean and 95% confidence interval for each treatment plotted over treatment, and the R squared of each regression analysis. (B) The moulting rate (moults/day) of each individual and the mean and 95% confidence interval for each treatment plotted over treatment, and the R squared of each regression analysis.

4 Discussion

Although this experiment experienced complications, with lower food levels than intended, male production and a suspected bacterial infection, it is important to note that all individuals within the treatments received the same food level and it is likely that all individuals had approximately the same degree of infection. However, the infection might have enhanced or diminished the responses which may make them difficult to compare to other studies even if they would have temperature and food levels equal to the actual levels used in the experiment.

4.1 Comparing Treatments

It was not possible to compare the responses of covarying temperature and food availability on two different genotypes, due to all individuals of the Aicha clones subjected to those treatments being male. It would have been interesting to see if the covariance treatments yielded different responses from genotypes from habitats with different temperature averages, but the results provided from the female individuals of the Pippi clone were, at least, quite clear and corresponded with the hypotheses proposed in the study. Individuals subjected to constant mean temperature and food availability expressed higher performance in the life history traits measured, in relation to covarying food and temperature, and individuals subjected to a positive covariance between temperature and food availability utilised the carbon better with resulting higher performance in the life history traits than those subjected to negative covariance. However, the differences between NegCov and the other two treatments were larger than the differences between PosCov and Constant, a response somewhat bigger than expected. The differences between NegCov and the other treatments were prominent in almost all measurements, except the clutch size at first clutch (Fig. 10a) and the length of offspring in the second clutch (Fig. 11b). Only in juvenile growth rate (Fig. 6a), juvenile moulting rate (Fig. 7a), the age at the second clutch (Fig. 9) and the clutch size of the first and second clutch (Fig. 10b), did PosCov and Constant have no overlap in the 95% confidence intervals. However, the 95% CI of the age at first clutch was influenced på heteroscedasticity and of the clutch size at the third clutch was influenced by outliers.

One explanation for the great variability of responses in reproduction of NegCov and greater difference to the other treatments is that the conditions were so close to the toleration limits of *D. magna* that the differences between the individuals in the treatment became more pronounced. For example, compared with the age at the first clutch, the age at the second clutch of NegCov didn't have a lot of variation, but it was only the individuals who had their first clutch early (age 13 or 14 days) that ever reached the second clutch (Fig. 9). The resilience of those specific individuals were therefore the only ones contributing to the data. Although NegCov had much larger offspring than the other treatments in the first clutch, in the second clutch NegCov individuals were slightly smaller than in the first clutch and the offspring of the other treatments were slightly bigger, resulting in similar lengths (Fig. 11), but there was no individual dependency as there was for age at the second clutch. In an experiment with a longer time frame, the weight of such dependencies could be mitigated. In this experiment, however, NegCov never reached the third clutch and were terminated at 20 days of age due to fear that the individuals would die and become unmeasurable, as had happened with one individual at 19 days of age. Except for the apparent extra investment in offspring size in the first clutch for NegCov, an expected response to low food availability (Ebert, 1993; Boersma, 1997), the investment was low in all traits measured and one can assume that there has been a trade-off between the reproduction, growth and development measured in this experiment, and the maintenance of basal functions for survival.

The differences in growth rate were most prominent during the juvenile stage (Fig. 6a). It is natural that the biggest differences in growth would be seen during the juvenile stage, as *D. magna* has a sigmoid growth curve (Martínez-Jerónimo, 2012) and exerts the highest investment in growth and development at that stage. The general, fundamental processes of reproduction and growth are energy intensive and trade-off between them is present in most organisms, also in *D. magna* (Enserink *et al.*, 1995), but could also be expressed as a trade-off between maturation time and lifetime fecundity where individuals have a size threshold to start reproduction (Kozłowski, 1992; Stearns, 2000). Considering the lower growth rate and longer maturation time of NegCov (Fig. 8), in relation to that the clutch mass of *D. magna* has been shown to be positively correlated with maternal size (Glazier, 1992), it is reasonable that such a trade-off has been observed in this experiment. One common way of estimating fitness of an organism is the intrinsic rate of population increase (*r*), since it

describes both reproduction and survival, and Lampert and Trubetskova (1996) showed that the juvenile growth in *D. magna* can be used as an estimator for *r* and such as an extension to estimate the relative fitness. Although they transformed the differences in length through the natural logarithm before calculating the growth rate, making the growth rate in itself incomparable in the two studies, the differences between the treatments in this experiment are still consistent with their findings.

Moulting is both connected to growth and reproduction. Moulting is needed in order for the individuals to grow and there is always a moulting event subsequent to clutch releases. The frequency of clutch releases will accordingly influence the frequency of moults. The adult moulting rate of Constant and PosCov therefore followed the synchronous behaviour of their clutch releases (Fig. 7b). For NegCov, however, reproduction was disturbed and the responses were variable. Some individuals matured much later than the other individuals in the same treatment and some individuals never produced a second or third clutch and the moulting rate was accordingly mirrored as distinctly lower than the other treatments in both the juvenile and adult stages (Fig. 7a&b). Sumiya et al. (2014) found moulting and ovulation to be affected by the same regulatory mechanism, but also found that treating individuals with exogenous ecdysteroid resulted in 13% of the experimental individuals moulting without ovulating. Similarly, several individuals in NegCov did not ovulate after the moulting subsequent to a clutch release and one individual in Constant released a clutch and moulted without ovulation, but moulted again the next day with a coupled ovulation. This suggests that ovulation also is affected by other regulatory mechanisms, of which I could not find any literature.

The release of the clutches generally followed the AAM for Constant and PosCov in a synchronised manner, with three days between each clutch, up until a one day delay of Constant between the second and third clutch (Fig. 9). The delay also coincided with a sharp decline in clutch size for Constant, which was due to undeveloped eggs that were released together with developed offspring (Fig. 10c). The reason for the undeveloped eggs and delayed clutch release of Constant is unclear, but could be a response to the suspected infection in addition to food stress, and comparing those measurements should therefore be done with caution. It is, though, interesting that PosCov, who had the third clutch the same

day as Constant and therefore experienced the food change the same amount of time, had consistently increasing clutch sizes. *D. magna* has shown to express maternal effects (Tsui and Wang, 2005; Garbutt and Little, 2014), however, the move of the mothers of the covarying treatment individuals from 20°C to 15°C inside the temperature controlled cabinet is very unlikely to have yielded such effect because the exposure was very short and elevated pathogen resistance has been found when mothers have been exposed to high temperatures (Garbutt *et al.*, 2014; Im, Na and Jung, 2020). Mitchell et al. (2005) showed a decrease in virulence of a bacterial pathogen when individuals of *D. magna* when they were kept at colder temperatures (10 and 15°C) and indeed one possibility is that the temperature fluctuations for PosCov actually slowed down the effects of the suspected infection, while getting an appropriate food level for the immune system to function well during high temperature periods, in contrast to NegCov.

DVM is probably a realistic scenario which can closest resemble the conditions in this experiment and in habitats where the hypolimnion has a higher food availability, a deep chlorophyll maximum, than the epilimnion it is conceivable that individuals would choose to stay in the colder waters, as it would also provide a constant predatory detection protection. Considering the findings of Koussoroplis and Wacker (2016), who also investigated the effects of negative covariance of temperature and food availability, but also included a treatment of constant low temperature and high food, that would indeed be a viable option. Deep chlorophyll maximum development is influenced both by light attenuation and thermal stratification, following the depth of the thermocline (Leach *et al.*, 2018), but depending on the species composition, the food present in the hypolimnion might actually be of a lesser quality and may affect growth and survival (Cole *et al.*, 2002). Also, without the presence of predators, the steepness of the temperature gradient has been proven to be a stronger determinant in the migrating behaviour of another daphnid species than food concentration (Lampert, McCauley and Manly, 2003; Kessler and Lampert, 2004), so it leads to questioning if predatory presence, which is a strong stimuli for DVM, would change the migratory behaviour into spending more time in cold, nutrient rich waters, even in waters with a steep temperature gradient that could maybe be made more common by increased air temperatures induced by climate change.

Due to it being widespread and residing in water bodies with different qualities, *D. magna* naturally experiences different thermal and food regimes at different times and so the levels of temperature and food availability used in this experiment will not be relevant for all *D. magna* populations or genotypes. Understanding what these response levels mean in situ requires many variables, the complex interactions between them and the interactions between those variables and the treatments, to be taken into consideration. Although the world is facing more variability of due to climate change (Screen, 2014; Bathiany *et al.*, 2018), the day-to-day covariation tested in this experiment is not very likely to naturally occur in such a synchronised manner, with these contrast levels and over such a long time. There are, however, many factors that act as stressors and influence the life history traits of *D. magna*, both directly and indirectly. For example, over longer time scales, there is a positive covariance between global warming and the prevalence of cyanobacteria which is of low nutritional value (O'Neil *et al.*, 2012), and a cyanobacteria diet has shown to have an effect on life history traits in *D. magna* (Bednarska, Pietrzak and Pijanowska, 2014). This experiment has shown the impact of negatively covarying metabolic rates and nutrient intake, which can be applied to a wide array of organisms and on different time scales.

4.2 Comparing Clones

Since the comparison of the female individuals of the Aicha and Pippi clone could only be done through the constant treatment, it cannot compare the differing responses of the two genotypes to covarying treatments. Therefore, this experiment is more of an ecological comparison of the two genotypes to see how the life history traits differ under favourable conditions. The experiments show indications that there are some specific differences between the clones, however, it is important to consider that the experiment started two days earlier for Aicha, which meant that the change from a combination of *C. reinhardtii* and RotiGrow to only RotiGrow as a food source came two days later for them than for the Pippi individuals, in addition to possibly living two extra days with the suspected infection. Whether RotiGrow was of less quality for the daphnids is unknown, but because the margins are much smaller comparing two genotypes to the same treatment, than to different treatments, it is worth noting.

The habitat of the Aicha clone, and therefore many of the environmental conditions it may have been adapted to, is unknown. However, originating in Morocco, which mostly has a Mediterranean climate, it would likely have warm average temperatures and livable conditions all year round. Pippi, on the other hand, originates from a rock pool in Sweden that might experience a more fluctuating environment and unlivable conditions parts of the year. It could be imagined that Aicha, originating from a warmer climate, is adapted to higher and more stable temperatures and could therefore grow and reproduce more efficiently than Pippi. The experiment showed that Aicha had a slightly higher juvenile growth rate than Pippi (Fig. 12) and the clones matured approximately at the same age (Fig. 14), but the first and the last instar of Aicha was shorter than Pippi. It took Pippi 10 days from AAM to reach the release of the third clutch, while Aicha only took 8 days. That, and the distinct difference in clutch size at the third clutch (Fig. 17), could however have been influenced by the fact that Pippi had lived longer with the food change and the suspected infection, as discussed previously. The offspring length of both clones was distinctly different between the clones, with Aicha having shorter offspring (Fig. 18), which was also the case for the length of the siblings of the experimental individuals at age 0, a natural consequence of the Aicha individuals being slightly smaller than Pippi, while having similar clutch sizes.

Based on the results of Aicha individuals in relation to Pippi individuals at constant temperature and food levels, I speculate that Aicha would have performed better under positive covariance than Pippi, but worse under negative covariance, because of more efficient utilisation of the food during days with high food levels. Due to uncertainties regarding the food quality and pathogen exposure, however, such speculations do not hold any weight.

4.3 Comparing Males

The male individuals of the Aicha clone, subjected to the PosCov treatment, clearly had a higher growth rate than those subjected to the NegCov treatment, without any overlap in the 95% confidence intervals, but there was no difference in moulting rate (Fig. 19). Since there was no reproduction coupled to moulting, the NegCov treatment couldn’t affect the moulting rate as heavily as it did with the females of the Pippi clone. Male and female *D. magna*

become sexually mature approximately at the same age, under the same conditions (Mitchell, 2001), so it is intuitive that development would be prioritised over growth under adverse conditions, to ensure the correct timing of sexual reproduction. In fact, when you compare the moulting rate of the male individuals to the moulting rate of the females of Pippi and Aicha you see that the moulting rate of all Pippi individuals, regardless of treatment, is identical (0.625±0), but the majority of the females of Aicha, who also matured earlier than Pippi, had a higher moulting rate (one extra moult, 0.75±0.06) than all the others (results not shown).

4.5 Conclusion

In regards to the hypotheses stipulated in this study, the results corresponded well with the expectations. Individuals exposed to negative covariance between temperature and food availability were not able to grow and reproduce at the same levels as individuals exposed to a positive covariance or a constant mean between the two temperature and food levels. The performance of individuals exposed to positive covariance was similar to that of those exposed to constant levels, but was clearly investing less in juvenile growth and development, and in the amount of offspring per clutch. The relatively high temperature levels and low food levels in this study, made even lower by changes in the carbon content calculations, in addition to a mismatch between metabolic and feeding rate and food availability in the individuals exposed to the negative covariance treatment, seem to have created a trade-off rather between survival and other life history traits, rather than a trade-off between the traits measured in this experiment. The suspected infection might also either have amplified or condensed the responses in all treatments, but the differences between the treatments were nonetheless clear. This study has also made some interesting observations. Firstly, the individuals in the constant treatment experienced a negative effect on clutch size and instar in the third clutch, while the individuals exposed to positive covariance did not, which might be an effect of the colder part of the temperature fluctuations slowing down the effect of the suspected infection. A targeted experiment of infection response during a longer time frame could be of future interest. It was also shown apparent differences in regulatory mechanisms of ovulation and moulting in *D. magna*, since several individuals exposed to

negative covariance kept moulting throughout the experiment, but would at several occasions moult without ovulating.

These results show that diel vertical migration (DVM) can be detrimental to *D. magna* and similar organisms in instances with a deep chlorophyll maximum and a steep temperature gradient in the thermal stratification. Daphnids have been shown to choose migration in the absence of predators even if the food availability in the warmer waters are low, to promote growth and development, but when faced with the additional variables of predator presence and a steep food gradient it is possible that the organisms would choose to reside in the colder waters more permanently. Although climate change is set to increase the variability of climatic variables like temperature, precipitation and solar radiation that may make living conditions more variable, the conditions in this experiment are not easily translatable to natural weather conditions, the general finding of the highly detrimental effect of low nutrient uptake, either through availability, competition or physiological restraints, in combination with increased metabolic rate is applicable to many organisms in many environments.

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Appendix A

Pilot experiment

Treatments

Four individuals of each clone were kept in glass jars containing 80 ml of ADaM medium and received one of six food treatments. The pilot experiment was performed on a total of 72 individuals. Each individual received either 0.001, 0.005, 0.01, 0.05, 0.1 or 0.3 mg of carbon per day, exclusively from *C. reinhardtii*. The individuals were kept in a temperature controlled room at a constant 20°C and received 12 hours of daylight and 12 hours of darkness.

Preparations

Prior to the experiment, stock cultures of each clone were kept in large jars, at 10°C. Experimental individuals of Aicha were collected directly from the stock cultures at 0-2 days of age. Individuals of Pippi and Niva were taken out of the stock cultures and kept at 20°C, and experimental individuals were collected from their second clutch at 0 days of age.

The relationship between carbon content and AU (800 nm), previously measured and calculated on the spectrophotometer by Raoul Wolf, was linear and had the formula

$$\text{carbon content (g/L)} = -0.02+0.3385x, \quad (1)$$

where x is the AU of *C. reinhardtii* (Fig. A-1).

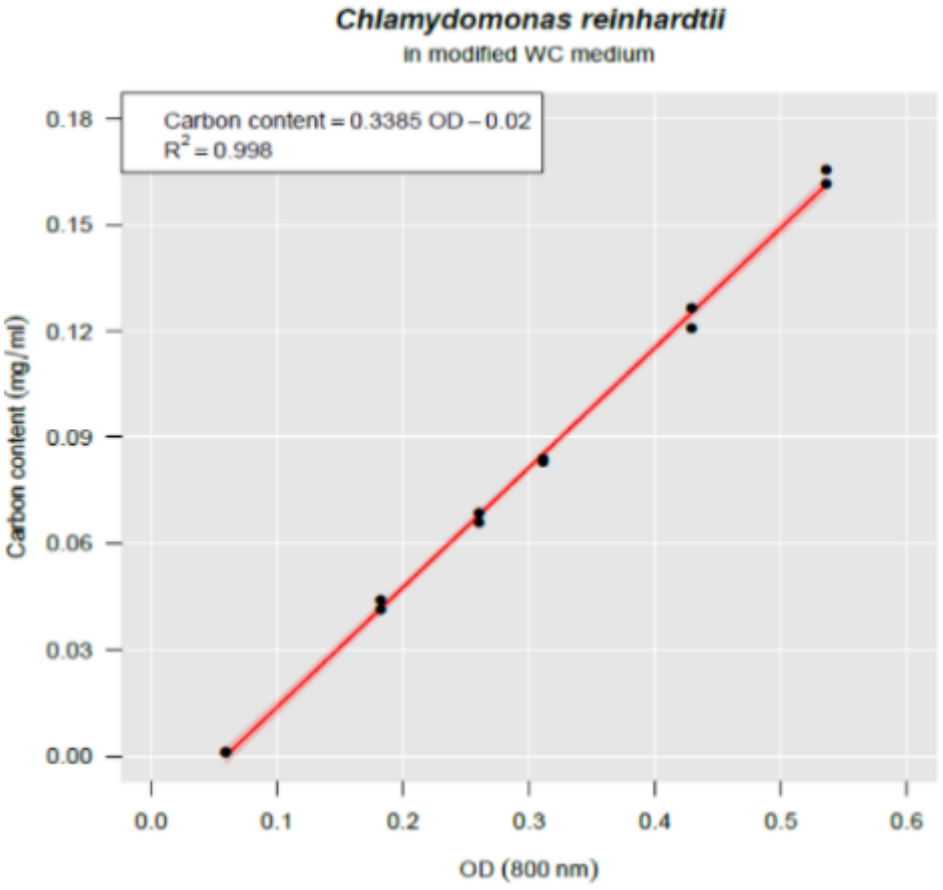

Figure 20. A graph showing the linear relationship between Optical Density (OD, 800 nm, OD corresponds to Absorbance Unit, AU, used as denotation in the this study) and Carbon Content (mg/ml) of *Chlamydomonas reinhardtii,* based on previous measurements made by Raoul Wolf on the spectrophotometer used during the pilot experiment.

Experimental implementation

Each day an amount of *C. reinhardtii* was removed from the aerenated cultures and optical density was measured with a spectrophotometer. The Optical Density Unit (ODU) was typed into a conversion sheet, which calculated the amount of algae needed per individual to achieve the desired carbon addition for each treatment, as per formula (1). 80 ml of ADaM medium was added to clean glass jars and the food amount corresponding to the treatment was added. The individuals were transferred from the old jars to the new jars by plastic pipettes. As the individuals grew, the tips of the pipettes were removed to create a bigger opening, to avoid any damage to the animals. The used glass jars were washed in a dishwasher with laboratory detergent at 60°C for 80 minutes.

The variables moulting, eggs present, amount of offspring, death and length and width at termination or death were recorded each day for each individual. Individuals were followed until the first clutch or, in the case of no clutch being produced, until the termination of the experiment at day 17.

Results

All individuals that received 0.05 mg C/individual or more reproduced. None of the individuals who received 0.01 mg C/individual reproduced, but were alive at the termination of the experiment at day 17, except for two of the Aicha clone who died at age 2 and 4 and one of the Pippi clone who died at age 16. Only one individual from each clone who received 0.005 mg C/individual survived until age 17 and no individuals who received 0.001 mg C/individual survived beyond 12 days of age. (Fig. A-2)

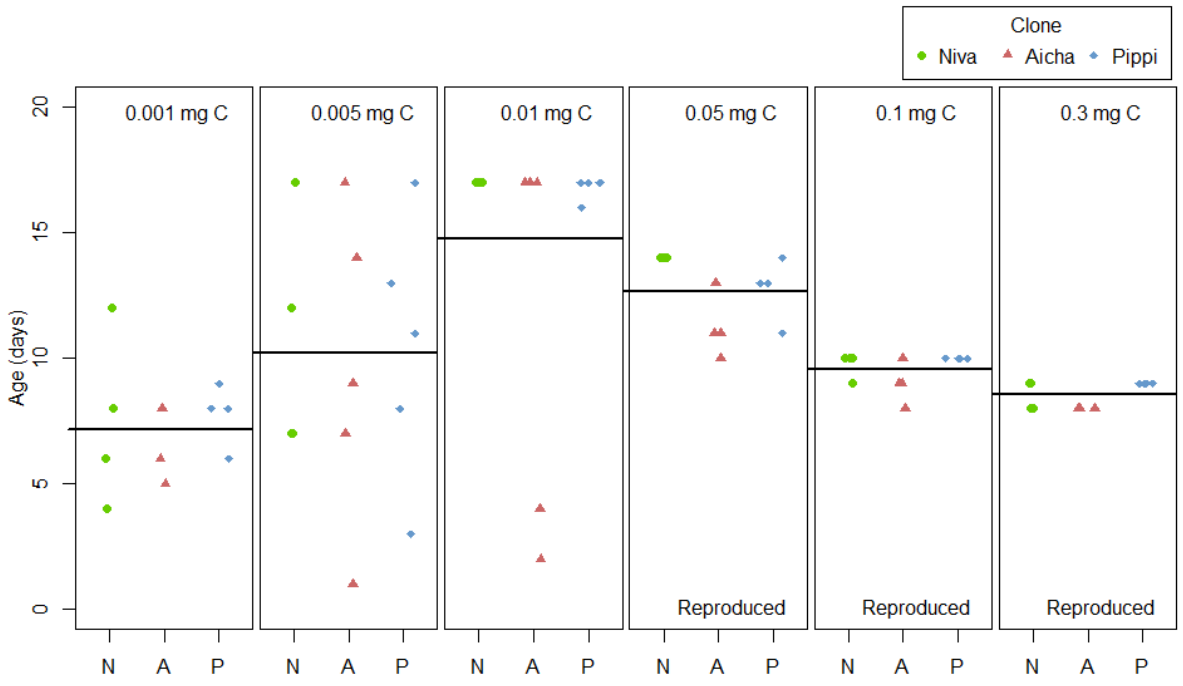

Figure 21. The age at death for each clone at each food level. Age at death is the same as the age at first clutch for the individuals in the treatments that reproduced (food levels 0.05-0.3 mg C/ind.), the age when the experiment was terminated (17 days, food level 0.005 mg/Cind. and 0.01 mg C/ind.) or the day individuals died naturally without reproducing (food level 0.001-0.01 mg C/ind.). The horizontal black lines represent the mean age at death for all clones combined at each food level. (*Y. Vindenes, Jun 2020 and A. Olsson, Oct 2021*)

Out of the three treatments that reproduced, the treatment with the highest food level had the biggest clutch sizes, varying from 6 to 16. The other two treatments were more similar and generally had 2 to 5 offspring in their clutches (Fig. A-3)

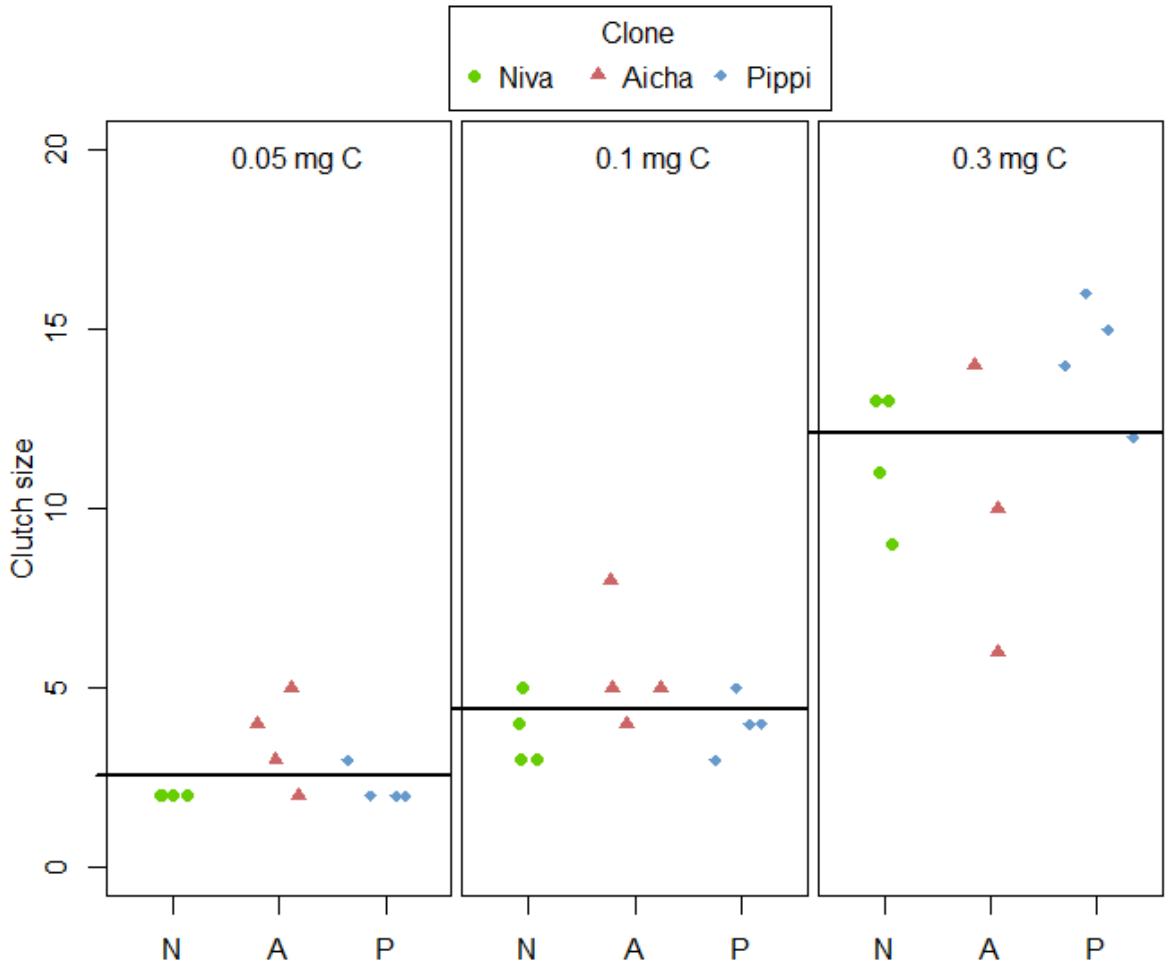

Figure 22. The clutch sizes for each clone, receiving 0.05, 0.1 or 0.3 mg C/individual. The horizontal black lines represent the mean clutch size for all clones combined at each food level. (*Y. Vindenes, Jun 2020 and A. Olsson, Oct 2021*)

The clones generally responded similarly to the food treatments. Aicha could seem to have more variability in the treatments with higher food levels than the other clones and to produce slightly bigger clutches at the two lower food levels of those that reproduced, but since those individuals were collected from the stock cultures kept at 10°C and the other clones were kept at 20°C prior to the experiment, and since the sample sizes were small, the differences are not useful.

Appendix B

Residuals vs. fitted values plots for each variable

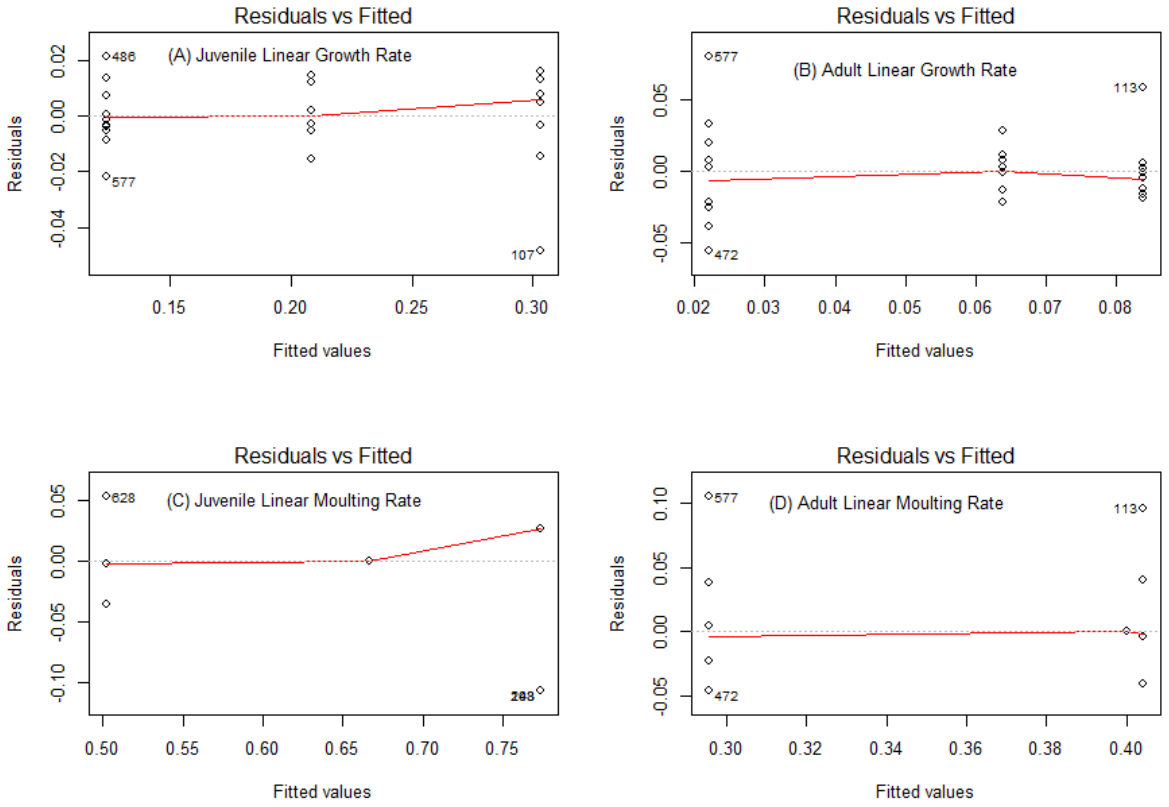

Figure 23. (A) The residuals plotted over fitted values from the linear regression analysis with juvenile growth rate (mm/day) as the independent variable and treatment as the dependent variable, only including individuals of the Pippi clone. The R squared of the analysis is 0.97 and diagnostic plots indicate that the model is a good fit. (B) The residuals plotted over fitted values from the linear regression analysis with adult growth rate (mm/day) as the independent variable and treatment as the dependent variable, only including individuals of the Pippi clone. The R squared of the analysis is 0.46 and the diagnostic plots indicate heteroscedasticity, but relatively equal leverage. (C) The residuals plotted over fitted values from the linear regression analysis with juvenile moulting rate (moults/day) as the independent variable and treatment as the dependent variable, only including individuals of the Pippi clone. The R squared of the analysis is 0.92 and the diagnostic plots indicate heteroscedasticity, but constant leverage. (D) The residuals plotted over fitted values from the linear regression analysis with adult moulting rate (moults/day) as the independent variable and treatment as the dependent variable, only including individuals of the Pippi clone. The R squared of the analysis is 0.60 and the diagnostic plots indicate heteroscedasticity, but a few unequal leverages.

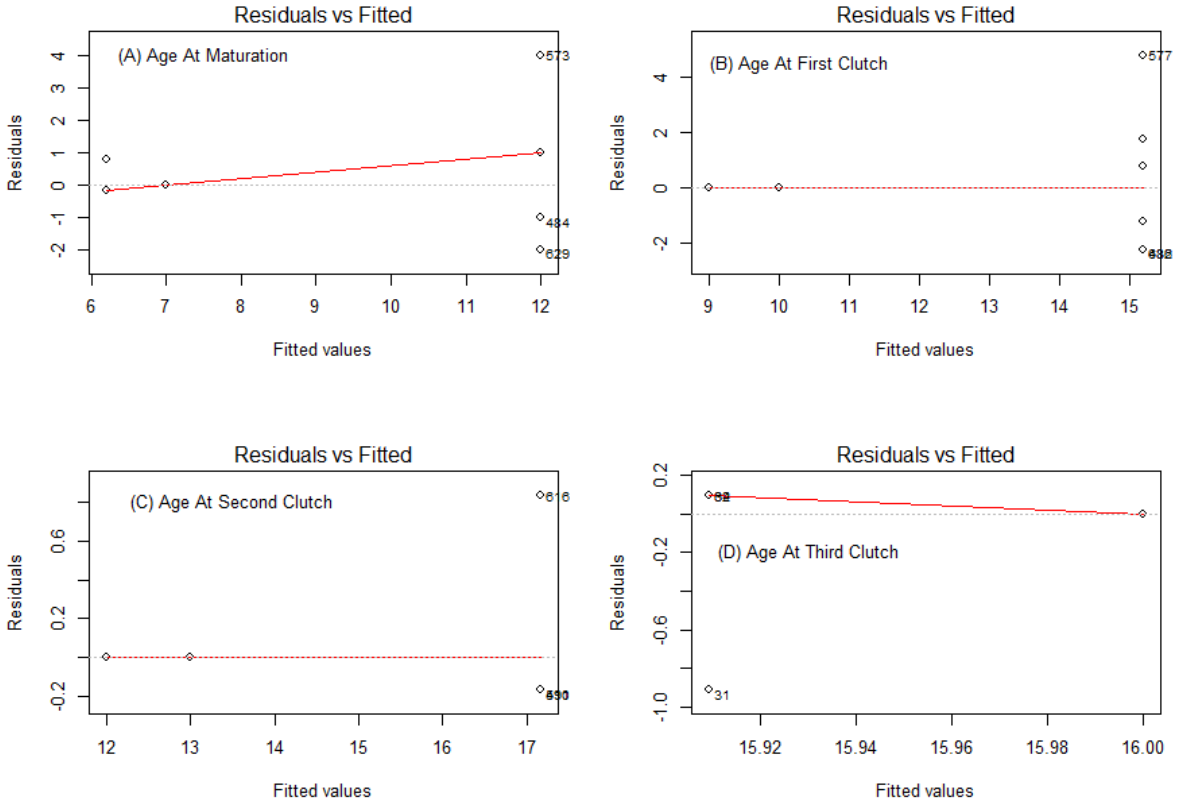

Figure 24. (A) The residuals plotted over fitted values from the linear regression analysis with age at maturation (days) as the independent variable and treatment as the dependent variable, only including individuals of the Pippi clone. The R squared of the analysis is 0.87 and the diagnostic plots indicate heteroscedasticity (NegCov had more variation), but constant leverage. (B) The residuals plotted over fitted values from the linear regression analysis with age at the first clutch (days) as the independent variable and treatment as the dependent variable, only including individuals of the Pippi clone. The R squared of the analysis is 0.81 and the diagnostic plots indicate heteroscedasticity, but relatively equal leverage, except one influential point (NegCov had variation, the other treatments did not). (C) The residuals plotted over fitted values from the linear regression analysis with age at the second clutch (days) as the independent variable and treatment as the dependent variable, only including individuals of the Pippi clone. The R squared of the analysis is 0.99 and the diagnostic plots indicate uneven distribution of the residuals (one individual of NegCov had the second clutch one day later, otherwise all treatments were fully synchronised). (D) The residuals plotted over fitted values from the linear regression analysis with age at the third clutch (days) as the independent variable and treatment as the dependent variable (only including Constant and PosCov because NegCov never produced a third clutch), only including individuals of the Pippi clone. The R squared of the analysis is 0.05 and the diagnostic plots indicate uneven distribution of the residuals (one individual of Constant had the third clutch one day earlier, otherwise all individuals of both treatments had the third clutch at the same age).

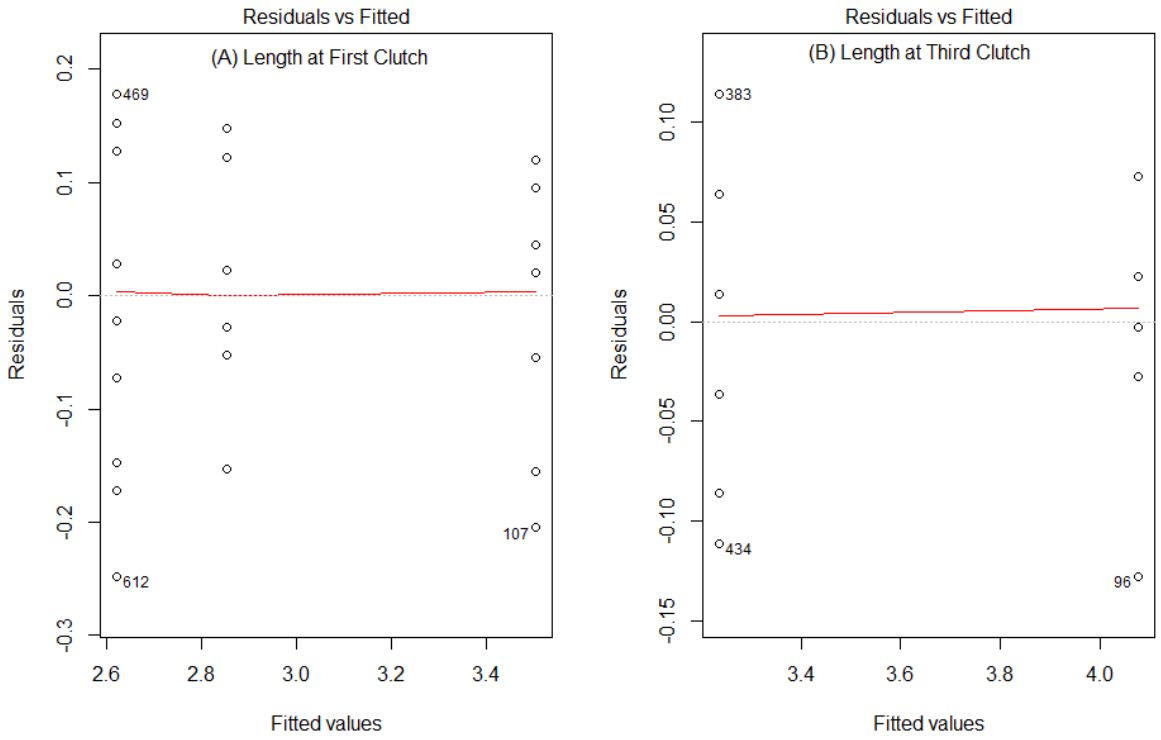

Figure 25. (A) The residuals plotted over fitted values from the linear regression analysis with length at the first clutch (mm) as the independent variable and treatment as the dependent variable, only including individuals of the Pippi clone. The R squared of the analysis is 0.90 and the diagnostic plots indicate that the model is a good fit. (B) The residuals plotted over fitted values from the linear regression analysis with length at the third clutch (mm) as the independent variable and treatment as the dependent variable (only including Constant and PosCov because NegCov never produced a third clutch), only including individuals of the Pippi clone. The R squared of the analysis is 0.98 and the diagnostic plots indicate that the model is a good fit.

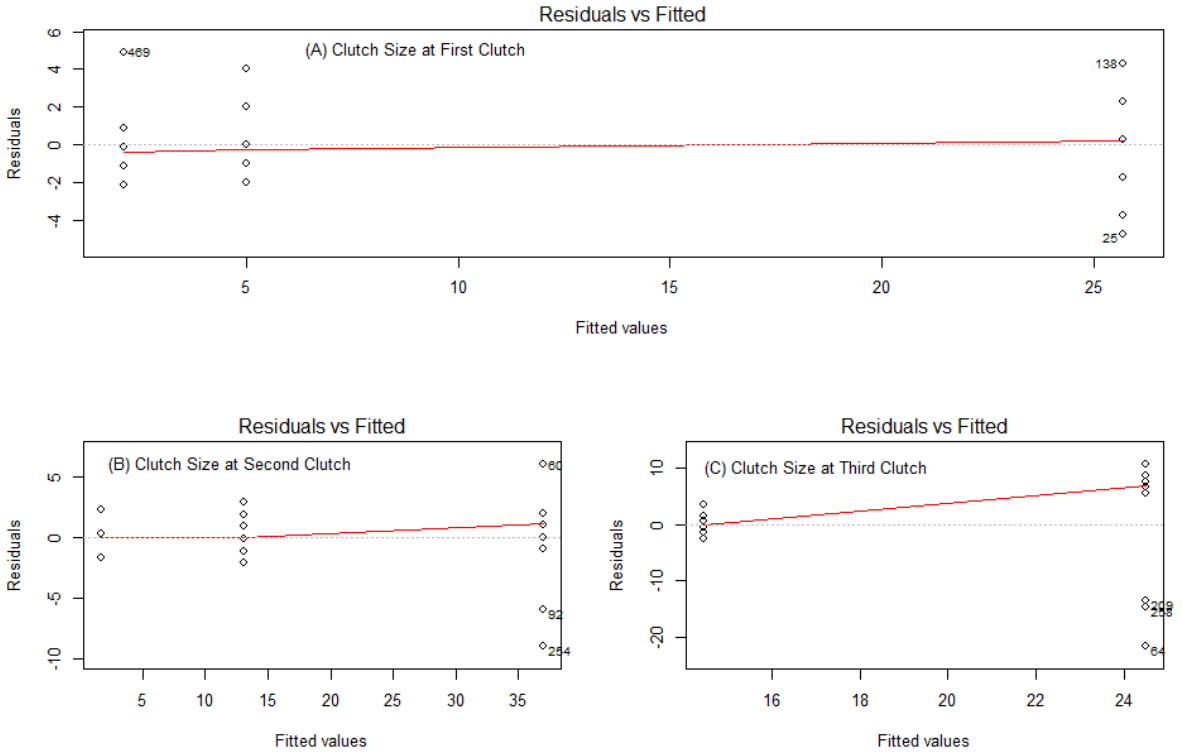

Figure 26. (A) The residuals plotted over fitted values from the linear regression analysis with clutch size at the first clutch (number of offspring) as the independent variable and treatment as the dependent variable, only including individuals of the Pippi clone. The R squared of the analysis is 0.95 and the diagnostic plots indicate heteroscedasticity (Constant had more variation), but relatively equal leverage. (B) The residuals plotted over fitted values from the linear regression analysis with clutch size at the second clutch (number of offspring) as the independent variable and treatment as the dependent variable, only including individuals of the Pippi clone. The R squared of the analysis is 0.96 and the diagnostic plots indicate heteroscedasticity (Constant had more variation), but relatively equal leverage. (C) The residuals plotted over fitted values from the linear regression analysis with clutch size at the third clutch (number of offspring) as the independent variable and treatment as the dependent variable, only including individuals of the Pippi clone. The R squared of the analysis is 0.28 and the diagnostic plots indicate heteroscedasticity and a few points with higher leverage (three individuals of Constant had smaller clutches because of undeveloped eggs).

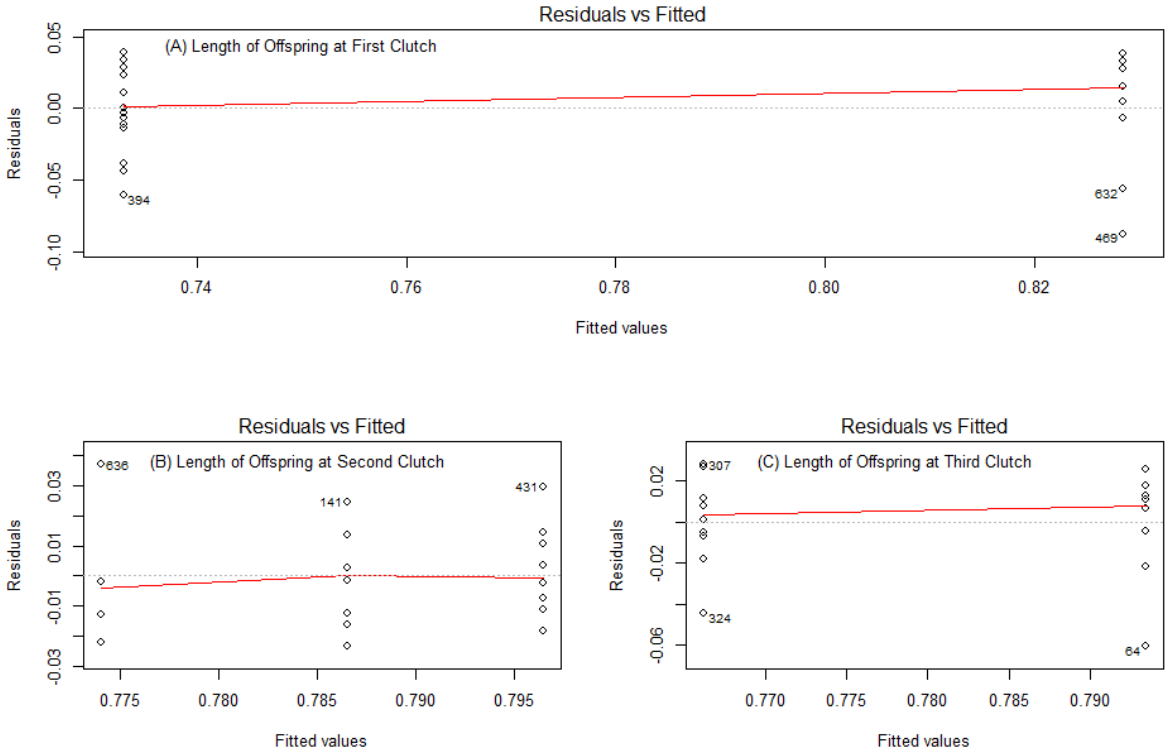

Figure 27. (A) The residuals plotted over fitted values from the linear regression analysis with length of offspring at the first clutch (mm) as the independent variable and treatment as the dependent variable, only including individuals of the Pippi clone. The R squared of the analysis is 0.66 and the diagnostic plots indicate that the model is a relatively good fit. (B) The residuals plotted over fitted values from the linear regression analysis with length of offspring at the second clutch (mm) as the independent variable and treatment as the dependent variable, only including individuals of the Pippi clone. The R squared of the analysis is 0.19 and the diagnostic plots indicate one influential point (one offspring from NegCov was longer than the majority from that clutch). (C) The residuals plotted over fitted values from the linear regression analysis with length of offspring at the third clutch (mm) as the independent variable and treatment as the dependent variable (only including Constant and PosCov because NegCov never produced a third clutch), only including individuals of the Pippi clone. The R squared of the analysis is 0.26 and the diagnostic plots indicate unequal leverage (one offspring from each treatment was shorter than the majority from that clutch).

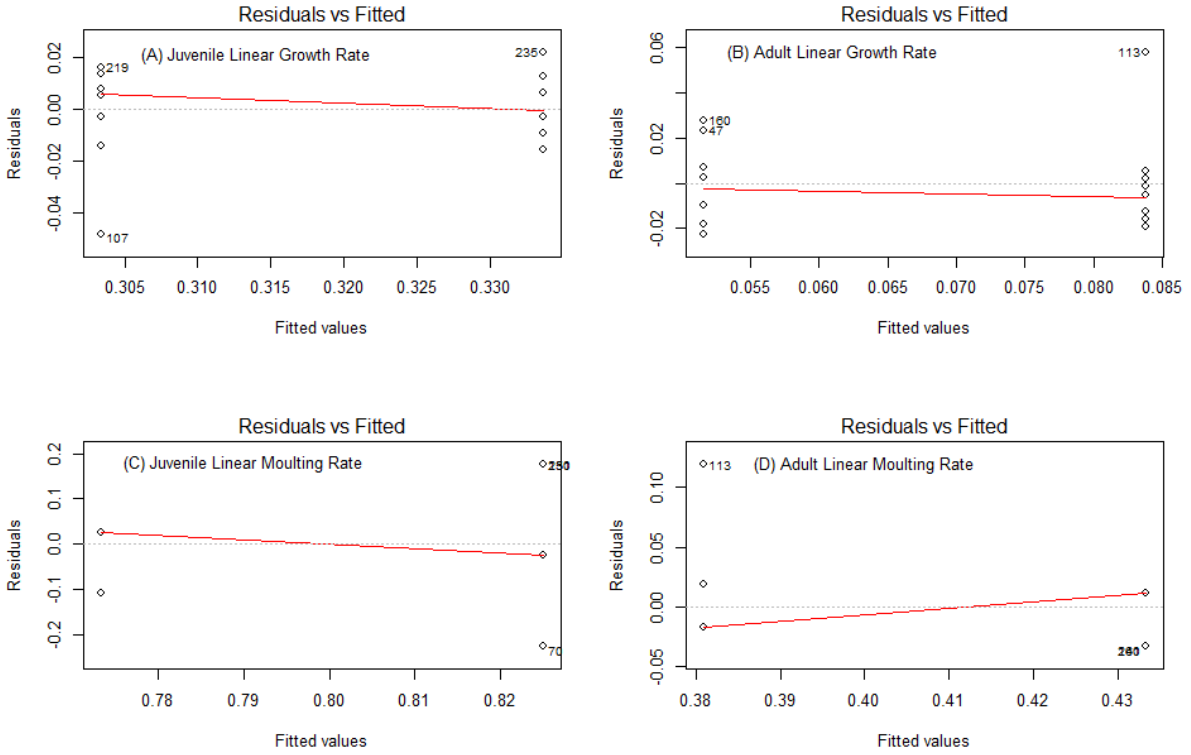

Figure 28. (A) The residuals plotted over fitted values from the linear regression analysis with juvenile growth rate (mm/day) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 0.47 and the diagnostic plots indicate one influential point (one individual of Pippi had a lower growth rate than the rest). (B) The residuals plotted over fitted values from the linear regression analysis with adult growth rate (mm/day) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 0.41 and the diagnostic plots indicate one influential point (One individual of Pippi had a higher growth rate than the rest). (C) The residuals plotted over fitted values from the linear regression analysis with juvenile moulting rate (moults/day) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 0.08 and the diagnostic plots indicate heteroscedasticity and somewhat unequal leverage (both clones had individuals with lower moulting rest than the majority, but only Aicha had individuals with higher than the majority). (D) The residuals plotted over fitted values from the linear regression analysis with adult moulting rate (moults/day) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 0.38 and the diagnostic plots indicate one influential point (one individual of Pippi had a higher moulting rate than the rest).

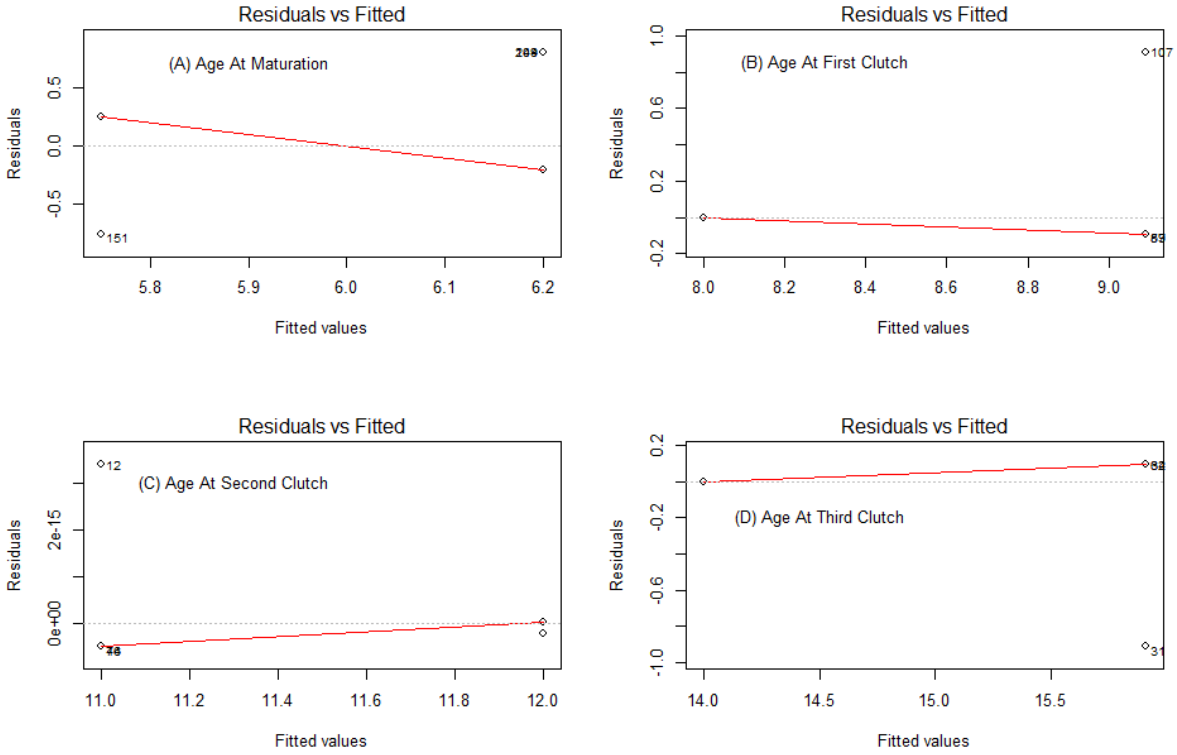

Figure 29. (A) The residuals plotted over fitted values from the linear regression analysis with age at maturation (days) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 0.23 and the diagnostic plots indicate unequal leverage (two individuals matured one day earlier than the majority for Aicha and two individuals matured one day later than the majority for Pippi) . (B) The residuals plotted over fitted values from the linear regression analysis with age at the first clutch (days) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 1 and the diagnostic plots are unreliable because of perfect fit (all individuals of each clone had the first clutch completely synchronised). (C) The residuals plotted over fitted values from the linear regression analysis with age at the second clutch (days) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 1 and the diagnostic plots are unreliable because of perfect fit (all individuals of each clone had the first clutch completely synchronised). (D) The residuals plotted over fitted values from the linear regression analysis with age at the third clutch (days) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 0.95 and the diagnostic plots indicate one influential point (one individual of Pippi had the third clutch one day earlier than the rest).

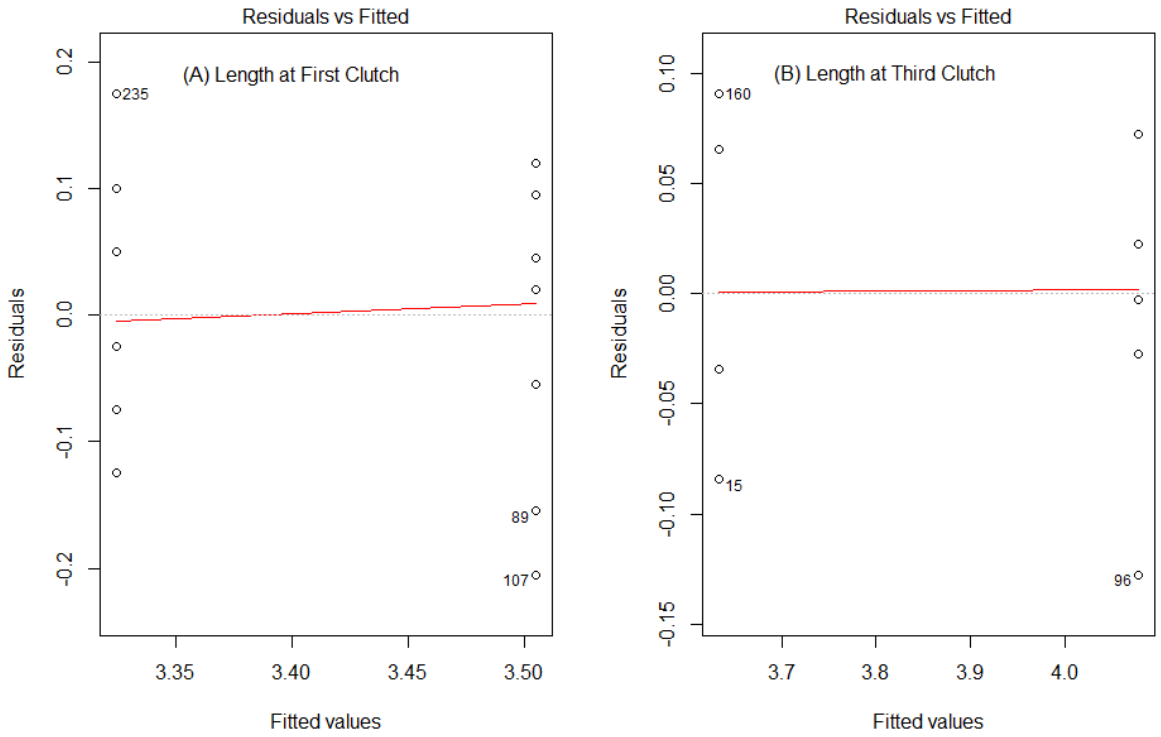

Figure 30. (A) The residuals plotted over fitted values from the linear regression analysis with length at the first clutch (mm) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 0.43 and the diagnostic plots indicate that the model is a good fit. (B) The residuals plotted over fitted values from the linear regression analysis with length at the third clutch (mm) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 0.92 and the diagnostic plots indicate that the model is a good fit.

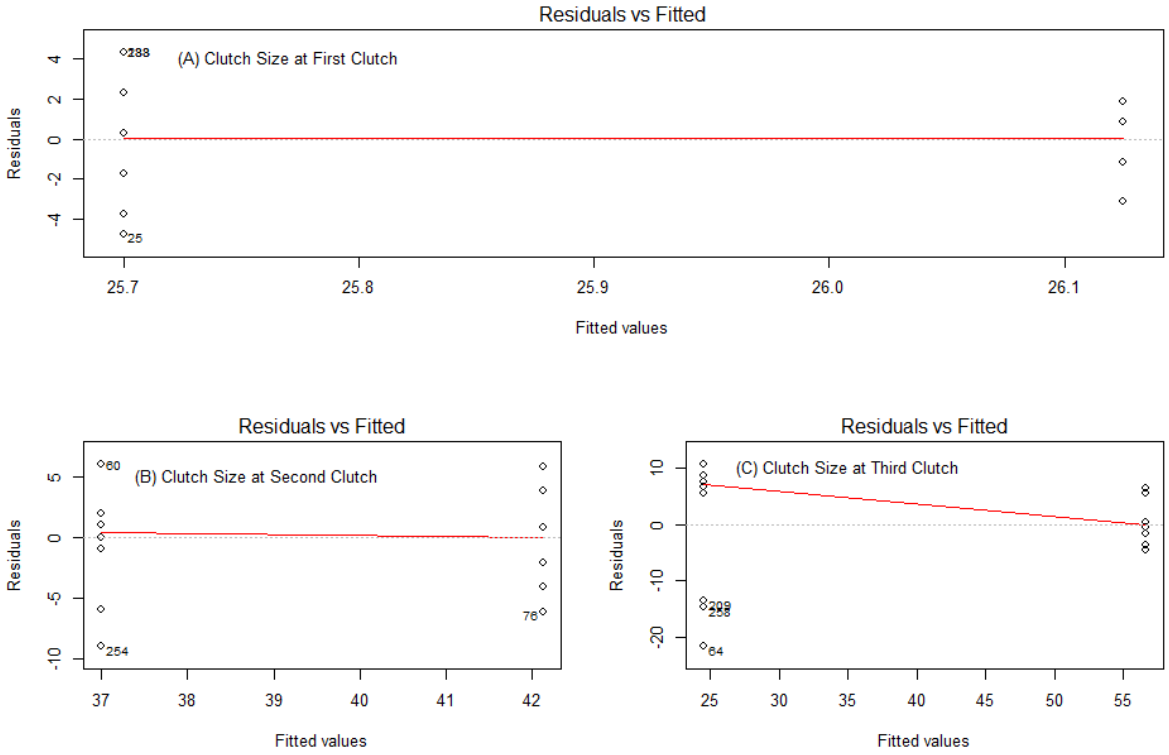

Figure 31. (A) The residuals plotted over fitted values from the linear regression analysis with clutch size at the first clutch (number of offspring) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 0.01 and the diagnostic plots indicate heteroscedasticity, but equal leverage. (B) The residuals plotted over fitted values from the linear regression analysis with clutch size at the second clutch (number of offspring) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 0.26 and the diagnostic plots indicate that the model is a good fit. (C) The residuals plotted over fitted values from the linear regression analysis with clutch size at the third clutch (number of offspring) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 0.77 and the diagnostic plots indicate heteroscedasticity and unequal leverage (Constant had three individuals with smaller clutch sizes due to undeveloped eggs).

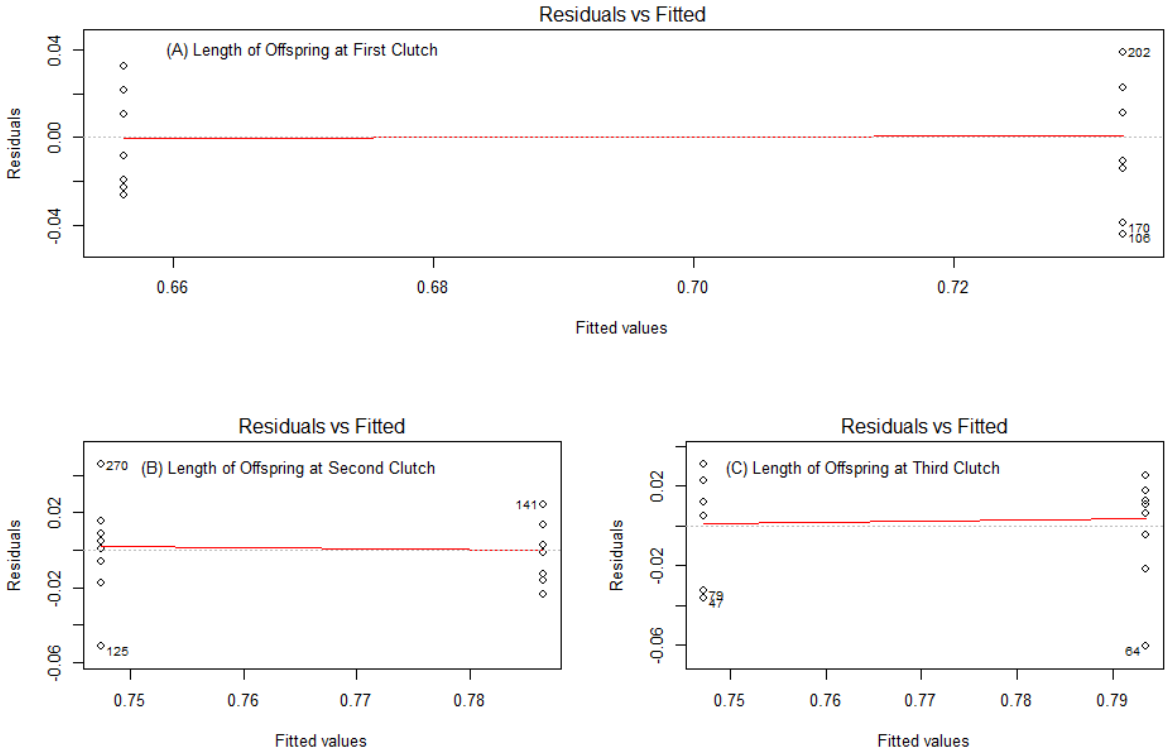

Figure 32. (A) The residuals plotted over fitted values from the linear regression analysis with length of offspring at the first clutch (mm) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 0.71 and the diagnostic plots indicate that the model is a good fit. (B) The residuals plotted over fitted values from the linear regression analysis with length of offspring at the second clutch (mm) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 0.45 and the diagnostic plots indicate heteroscedasticity, but constant leverage (Aicha had more variation). (C) The residuals plotted over fitted values from the linear regression analysis with length of offspring at the third clutch (mm) as the independent variable and clone as the dependent variable, including individuals of the Pippi and Aicha clone. The R squared of the analysis is 0.45 and the diagnostic plots indicate somewhat unequal leverage (two offspring from Aicha and one offspring from Pippi were shorter than the majority from that clutch).

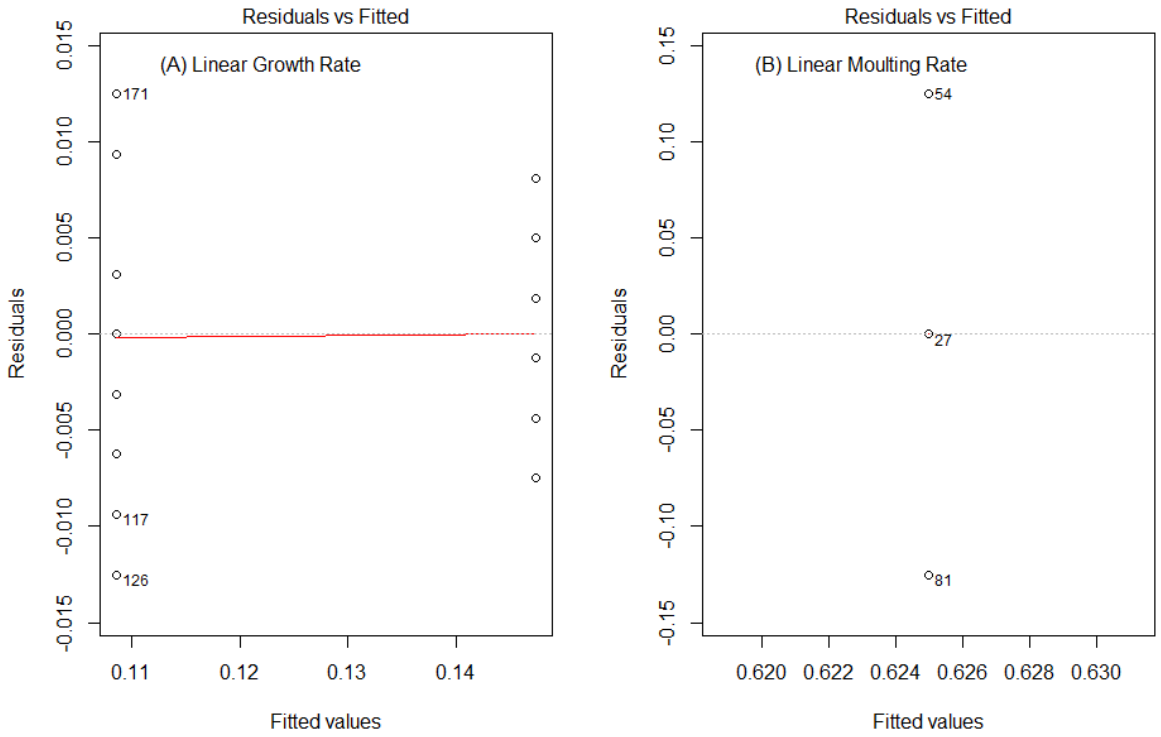

Figure 33. (A) The residuals plotted over fitted values from the linear regression analysis with growth rate (mm/day) as the independent variable and treatment as the dependent variable, only including male individuals of the Aicha clone. The R squared of the analysis is 0.89 and the diagnostic plots indicate weak heteroscedasticity, but constant leverage. (B) The residuals plotted over fitted values from the linear regression analysis with moulting rate (moults/day) as the independent variable and treatment as the dependent variable, only including male individuals of the Aicha clone. The R squared of the analysis is 0 because the mean moulting rate was exactly the same for the two treatments so there could be no linearity between the variables.