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Investigating the impacts of heterotrophic nutrition on *Acropora microclados*

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Abstract

The Scleractinian coral *Acropora microclados* is a commercially important species that functions as both an autotroph and heterotroph. In ex situ coral aquaculture, elucidating an optimal diet to improve growth and resilience is critical in improving the sustainability and success of rearing corals for trade and conservation. To investigate the impacts of diet on *A. microclados*, three groups of 15 nubbins were kept under three different feeding regimes for three months at the National Marine Aquarium, Plymouth, UK. One group was fed nothing, the control (CTL), one group fed *Artemia salina* nauplii (ART) and one group fed a mixed diet of *Artemia* nauplii and microalgae (AA). They were kept in the same water system to maintain homogeneity of water quality between groups. Over the course of the study, their masses, volumes, and cross-sectional areas were measured to determine estimates of biomass and skeletal growth, along with qualitative observations of polyp coverage (e.g. bleaching, encrusting), used as an indicator of health. Nubbins in ART experienced significant growth from day 1 to 84, increasing in mass ($p=0.033$) and volume ($p=0.035$), while nubbins in CTL and AA did not display any increased growth. Over the course of the study, bleaching occurred in all groups, CTL and AA displaying equal levels while ART experienced a much lower proportion. These results align with previous studies that suggest that feeding on live *Artemia* nauplii can improve both growth and resilience in *A. microclados*, and that starved corals more easily succumb to stress-induced bleaching when relying on solely autotrophic pathways. ART had a higher proportion of nubbins with encrusting growth, suggesting that encrusting can be used as a sign of health in corals, though there are no previous studies to confirm or dispute this, only hobbyist opinion. This study demonstrates that *Artemia* can be an effective diet to improve *A. microclados* growth and resilience in aquaria, while highlighting the ample work still necessary to evaluate the feasibility of microalgae as live food for corals.

Keywords: Coral, Nutrition, *Acropora*, Aquaculture, Growth, Heterotrophy

Introduction

Acropora is a genus of small polyp corals with stony skeletons (SPS) in the order Scleractinia. In the wild, the hermatypic *Acropora spp.* are known for their ecosystem services including promoting biodiversity and protecting coastlines from wave action (Wallace and Rosen, 2006). Coral reefs have been identified by experts as one of the most vulnerable marine ecosystems to anthropogenic threats (Halpern *et al.*, 2007), suffering due to climate change, coastal development, eutrophication, habitat destruction and, more recently, the marine ornamental trade (MOT) (Grottoli *et al.*, 2006, Precht *et al.*, 2002). The global MOT is valued at US\$300 million per year, with *Acroporas* being the second most imported coral in the EU, and fourth in the US (Palmtag, 2017, Wabnitz *et al.*, 2003). Optimising sustainable aquaculture practices involving SPS corals can maximise production, helping prevent overexploitation of wild corals and assisting reef restoration efforts (Rhyne *et al.*, 2012).

SPS corals are notoriously sensitive to environmental conditions including light, flow, temperature, salinity, water chemistry and food availability (Osinga *et al.*, 2012). Determining the parameters that enhance growth and survivorship in corals has revealed valuable insights into coral biology, in particular the species-specific nature of adaptations to environmental niches. In *ex situ* aquaculture, conditions can be manipulated to maximise production and boost desirable characteristics like resistance, growth rate and colour (Delbeek, 2001). Several species of coral have been observed to survive outside of their usual range however, repeated fluctuations are shown to cause bleaching and death (Anthony, 2000).

Most Scleractinians are photoautotrophs, receiving energy via the zooxanthellae living in their tissue. *Acroporas* use multiple nutritional pathways, functioning as both autotrophs and heterotrophs, an adaptation thought to help survival in oligotrophic, deep or turbid environments (Anthony, 2000, Ferrier-Pages *et al.*, 2003). *Acropora* also use combination of mucus entanglement and tentacle capture to prey on planktonic organisms (Tagliafico *et al.*, 2018), providing the additional carbon and essential nutrients that are not supplied by zooxanthellae. Predation helps initiate healing and tissue maintenance and allows recovery from bleaching events by providing an alternative energy source (Anthony, 2000, Grottoli *et al.*, 2006., Burmester *et al.*, 2018). This increases the corals resilience during such events. Fed corals have higher skeletal growth rates than starved corals and can synthesise organic matrices twice as fast (Ferrier-Pages *et al.* 2003; Houlbreque and Ferrier-Pages, 2009; Conlan *et al.*, 2019). Coral heterotrophy is highly species specific, as well as depending on life history and environmental conditions. For example, wild corals exhibit higher rates of predation at night, when zooplankton density is higher, while captive corals feed just as well in the day, having adapted to the change in availability of food (Houlbreque and Ferrier-Pages 2009; Tagliafico *et al.*, 2018). A previous study showed several zooxanthellate corals were able to survive for as long as 15 months without food, whilst *Acropora spp.* only lasted 3 months, suggesting a higher dependency on heterotrophy than other coral species (Bakus *et al.*, 1973). Corals feed on a range of dissolved and particulate organic matter, both living and detrital, including zoo- and phytoplankton (Grover *et al.*, 2006 and 2008; Houlbreque and Ferrier-Pages, 2009).

In aquaculture, live foods are preferred to artificial or dry foods as they are more digestible, with higher nutritional value, and provide an optimal diet, representative of

one in the wild. The most popular of these is *Artemia*, also known as brine shrimp. Benefits of using *Artemia* in aquaculture include low cost, relatively long shelf-life of dry cysts, and ease of culture. Drawbacks, however, include sustainability concerns due to the harvest of natural resources and limitations due to supply chain issues (Sorgeloos, Dhert and Candreva 2001). Additionally, while providing important nutrients like nitrogen and phosphorus, *Artemia* is lacking in polyunsaturated fatty acids (PUFAs) like eicosapentaenoic acid (EPA), arachidonic acid (ARA) and docosahexaenoic acid (DHA), which are considered crucial in feed as most animals are not able to synthesise them internally (Imbs *et al.*, 2010). This is mitigated in aquaculture by enriching *Artemia* cultures with emulsions, microalgae and yeast. During the first larval stage (Instar I), *Artemia* does not consume exogenous matter, but after this (Instar II onwards), *Artemia* is not a selective feeder so can be manipulated to become a vehicle for nutrients to the target species. Radhakrishnan (2019) found a greater than 2-fold increase of fatty acids in *Artemia* after enrichment. Therefore, enriched cultures of *Artemia* would provide the required EFAs to *Acropora* that would be absent in non-manipulated cultures

Microalgae are also popular as live food in aquaculture, widely used to feed juvenile crustaceans and molluscs at all life stages, as well as being used to enrich *Artemia* and rotifers (Radhakrishnan *et al.*, 2019). Increasing numbers of studies have shown the ability of corals to feed on microalgae and other plant matter, even seagrass (Lai *et al.*, 2013). Leal *et al.*, (2013) found that three out of the five species studied captured microalgae, in particular the haptophyte *Isochrysis galbana*. Of these three, two were symbiotic Scleractinians but none were of the genus *Acropora*. The herbivory was shown to be highly species specific and was unrelated to the algal size or taxonomy. Conlan *et al.*, 2019 found that three species of *Acropora* (*A. loripes*, *A. millepora* and *A. tenuis*) have significantly higher growth rates when fed a diet of *I. galbana* compared with several other live and artificial diets. There has also been evidence of border brush enzymes in the digestive system of another SPS coral, *Stylophora pistillata*, indicating pathways which have been observed in vertebrates to help digest the carbohydrates and peptides found in plant matter (Osinga *et al.*, 2012; Raz-Bahat, 2017). Different species of algae have different nutritional compositions of carbohydrates and lipids, but all have high levels of protein and EFAs, as well as being good sources of ascorbic acid and riboflavin (Brown *et al.*, 1997). Algae can therefore be used to provide nutrients and vitamins via multiple pathways - through direct herbivory or indirectly as part of the *Artemia* diet.

Previous studies have shown the importance of exogenous food sources to captive corals, especially live *Artemia* (Ferrier-pages, 2003; Houlebreque *et al.*, 2004; Lavorano *et al.*, 2008). Few have focussed on the commercially important and threatened genus *Acropora*, even though heterotrophy has been shown to be highly species specific. These studies have also focused on diets consisting of only one 'ingredient', when it may be more successful to try a mixed diet since this is more representative of one a coral would consume in the wild. As such, an optimal diet for captive *Acropora* corals has not yet been elucidated. EFAs are an invaluable source of energy for coral larvae and other marine organisms, but little research has been conducted into their role in the growth of adult Scleractinians (Sorgeloos, Dhert and Candreva 2001; Figuerdo *et al.*, 2012). Microalgae increases the nutritional value of *Artemia*, so it can be postulated that a diet of mixed *Artemia* and microalgae would

provide nutrition through multiple pathways. Diet is already commonly manipulated in aquaculture and aquaria, both public and private, as a means to improve coral growth rates and survivorship, however, much of the information surrounding this derives from observations from hobbyists and grey literature.

A novel mixed algae-*Artemia* diet could provide a more cost-effective, sustainable and nutritionally beneficial diet for corals in *ex situ* aquaculture. This study intends to evaluate the impact that three different feeding regimes would have on the growth of *Acropora microclados* nubbins. These included popular live food *Artemia salina* (ART), a mixed diet of cultured microalgae and *Artemia salina* (AA) and no food, the control (CTL). In order to test the effectiveness of the three diets, measurements of cross-sectional area, volume and mass were made to consider changes in polyp cover, skeletal growth and biomass. From these metrics, net growth and growth rates were calculated. Observations were also made of any bleaching or encrusting growth, using this to evaluate the quality of polyp coverage on the coral. It was hypothesised that net growth would be highest in corals fed a mixed *Artemia*-algae diet.

Methodology

Experimental set-up

The experiment was carried out at the National Marine Aquarium (NMA, Plymouth, UK) starting in April 2021. All procedures adhered to protocols already in place at the NMA or using the CORALZOO book of protocols (Leewis *et al.*, 2009). Forty-five samples of *Acropora microclados*, ranging in length from 1.40 – 4.70cm (mean 2.77 ± 0.68cm), were cut from an already established colony in the aquarium in order to eliminate variations in growth and survival due to genotype (Protocol 2.2.3.2, Leewis *et al.*, 2009). These nubbins were each dipped in a solution of 'ReVive Coral Cleaner' (Two Little Fishies Inc., Miami Gardens, Florida, USA) to remove any pests and boost immunity, then attached to a ceramic plug using cyanurate glue. Each plug was assigned to group CTL, ART or AA, labelled accordingly and placed on a stand in their respective tanks (Fig. 1).

Two tanks were used to house the three groups of corals, one of them split in half using perforated plastic sheets covered in micromesh (to prevent particle transport) to create three 65L areas of the same length, width and depth supplied by the same life support system (LSS) (Fig. 1). The water flow in each area was created by an MP40 pump in 'Reef Crest' mode, the manufacturer-recommended mode for SPS corals (EcoTech LLC., Bethlehem, Pennsylvania, USA). This minimised growth and survival rate differences between groups due to water quality or flow. The CTL and ART tanks were lit by a Radion G4 light (EcoTech LLC., Bethlehem, Pennsylvania, USA) and the AA tank was lit by two AquaBeam LED lights (Tropical Marine Centre Ltd., Chorleywood, UK) with a lighting period of 10 h day⁻¹.

Plankton Culture and Harvest

Artemia salina and two species of microalgae (*Tetraselmis suecica* and *Isochrysis galbana*), were cultured and harvested daily at the NMA. Each day, the *A. salina* was cultured from decapsulated cysts (EG Artemia, INVE Aquaculture, Salt Lake City, USA) in an 30L aerated vessel at 28°C for 24 hours before harvest (Protocol 3.5.3.1, Leewis *et al.*, 2009). At this point, the *A. salina* was in its first larval stage

(Instar I) and was rinsed with clean saltwater, then diluted to 3.75L (400,000 *Artemia* L⁻¹). The vessel was thoroughly cleaned and rinsed daily and was bleached twice a week to avoid bacterial contamination. 0.6L of the harvested *A. salina* was designated for this project and the rest used elsewhere in the aquarium. 0.3L of this was left unenriched, kept lightly aerated at room temperature and fed to ART that day. The other 0.3L of the harvested was enriched with live microalgae (AA). Concentrations of 2000 *Artemia* L⁻¹ were chosen as this was previously found to be an optimal density (Lavorano *et al.*, 2008).

The microalgae, *T. suecica* and *I. galbana*, were cultured at 21°C under a photoperiod of 18h on, 6h off using protocols already in place at the NMA. The algae starter cultures were sourced from the Plymouth Marine Laboratory (PML) (Plymouth, UK). Cell counts of the cultures were performed each week using a haemocytometer and microscope. The algae cultures were harvested daily, topped up with saltwater and fed with 1ml F/2 fertiliser per L saltwater added (Cell-Hi F2P, Varicon Aqua, Worcester, UK). *T. suecica*, *I. galbana* and *A. salina* were mixed in a ratio of 1:1:2 with 0.15L of each alga added to 0.3L of *A. salina*.

This *Artemia*-algae mix was kept at room temperature under light aeration and an illumination period of 18h day⁻¹. The next morning, the *Artemia* had grown to its second larval stage (Instar II) a water change was performed on the mix, to remove any detritus that could negatively impact the coral tank water quality and the clean mix was subsequently kept aerated until the feed.

Feeding and Husbandry

The nubbins were allowed an acclimation period of 30 days, which ensured that they were able to adjust to any conditions differing from their previous tank without affecting growth rate calculations. It also allowed any tissue damaged during the fragging to regrow. During the acclimation period each group was fed 7ml of live *A. salina* enriched with Shellfish Diet 1800® (Reed Mariculture, Campbell, CA, USA) twice a day.

After the acclimation period, each group was assigned a feeding regime:

CTL: The control, received no food.

ART: 0.3L each day, 0.15L in AM, 0.15L in PM, Instar I *A. salina* nauplii, hatched daily, unenriched.

AA: 0.6L each day, 0.3L in AM, 0.3L in PM, *T. suecica*, *I. galbana*, Instar II *A. salina* mixed ~24h before feed.

During the feed, the pumps in all tanks were switched onto 'feed mode', a much lower speed, for 10 minutes to decrease flow and increase the efficiency of polyp prey-capture. Incoming water flow was kept constant during feeding. All groups were also fed 5ml of AcroPower amino acid formula (Two Little Fishies Inc., Miami Gardens, Florida, USA) twice a week to help skeletal growth.

When required, tanks were spot siphoned (which uses gravity and partial pressure to vacuum water and any detritus out of aquaria, without removing any substrate) for no more than 10 minutes, to remove detritus and excess food, and a thorough siphon

was done once a week alongside a water change on the LSS. This was done to limit fluctuations in the water chemistry. To control algal growth, turban snails (*Tectus fenestratus*) were kept alongside the coral and the tank walls were scrubbed when necessary. A refugium full of *Caulerpa* macroalgae was attached to the same water system to help limit algal growth by removing nutrients (Fig. 1).

The physical and chemical parameters of the water were measured and controlled carefully to remain constant and various filtration mechanisms were used in the LSS to ensure water quality had no negative impact on the study.

Biological Measurements

Methods of growth measurement were selected to obtain data about the survival, biomass, and polyp coverage of the colonies in a non-destructive way. Changes in biomass, an indicator of skeletal and polyp growth, were determined by mass and volume, while changes in polyp coverage showed the growth of the living coral tissue and were determined using photo analysis of the cross-sectional area and qualitative observations. All measurements were taken using protocols from Leewis et al., 2009. Net growth of the nubbins was expressed as a percentage of the original physiological parameters.

Once the acclimation period was over, the mass and volume of each coral nubbin were recorded. Volume was determined using the volume replacement technique and the mass was taken as the drip-dry mass (Protocol 1.3.2 and 1.2.3 respectively, Leewis *et al.*, 2009). In the volume replacement technique, the nubbin was placed into a beaker filled to a specific volume. Any water over this line was removed using a syringe and emptied into a measuring cylinder, giving the volume of the nubbin (to nearest 0.1 cm³). For the mass, the nubbin was removed from the tank, gently shaken until no more drops fell off, and then weighed (to the nearest 0.01g, Brifit KA8 Series).

To estimate the cross-sectional area, two photos of each nubbin were taken against a background with a 1cm² grid, one along the coronal plane and one along the sagittal (Fig. 2). The photos were then uploaded into the imaging analysis software, ImageJ (Schneider, Rasband and Eliceiri, 2012) (Protocol 1.3.1, Leewis *et al.*, 2009). For each photo, pixel spacing was calibrated to a known distance using the 1cm² background. The image was then converted to a 16-bit type and the threshold adjusted to include only the coral. The wand tracing tool was then used to select the coral and measure the area from the number of pixels enclosed. Any bleached sections of the nubbin were not included, and the measured area was taken to the nearest 0.01 cm² to account for partial pixels. The total cross-sectional area of each specimen was represented by a sum of the two cross-sectional areas of the nubbin (sagittal and coronal) to give results absent of error caused by replicate data.

To assess level of polyp coverage on the nubbins, qualitative observations were recorded each week. Coverage was categorised into full (F), encrusting (E), bleaching (B) and dead (M). Encrusting growth was noted when the polyps grew covering the ceramic plug to which they were attached, and bleaching noted when the polyps lost colour, exposing the skeleton beneath. All nubbins were categorised 'F' in week 1.

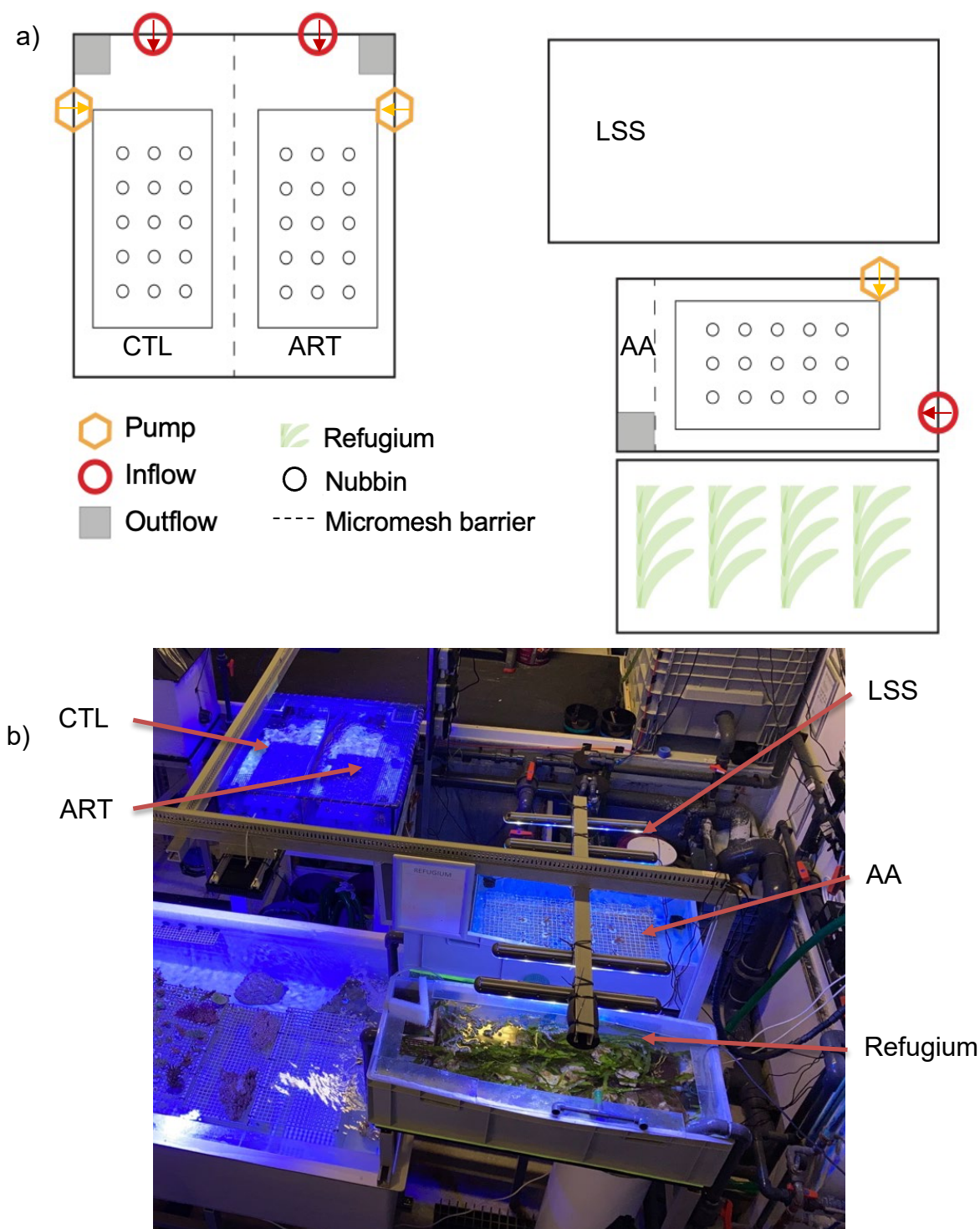


Figure 1: a) Schematic diagram of the tanks used in this study (not to scale). Three 65L tanks (0.9m x 0.45m x 0.2m) containing 15 *Acropora microclados* nubbins on egg-crate stands, a refugium containing macroalgae and a LSS (life support system) Nubbins in CTL were fed nothing, ART *Artemia* nauplii, and AA an *Artemia*-microalgae mix. Each tank contained an inflow (supplying water from the LSS), a pump (supplying flow) and an outflow (water sent back into LSS to be cleaned and recirculated). Direction of flow from pump and inflow indicted with arrows within symbol. b) Photo of ‘Coral Prop’ area at the NMA, Plymouth (Photograph by author).

Photos were taken on day 1 and day 84, observations were made each week, and the mass and volumes were measured every other week. Removal of the nubbins from the water could be detrimental to their health; therefore, this was limited to up to 4 minutes out of the water per nubbin when taking measurements. Observations on coverage were made when the coral were in their tanks; hence this was not included in the timings.

For the cross-sectional area, volume and mass, the growth rate (G , % day⁻¹) was calculated using the following equation from Ferrier-Pages *et al.*, (2003).

$$G = (M_{t+1} - M_t) / (M_t * (T_{t+1} - T_1))$$

where M_{t+1} is the mass (g), volume (ml) or area (cm²) at the end (T_{t+1}) and M_t is the mass, volume or area at the start (T_1).

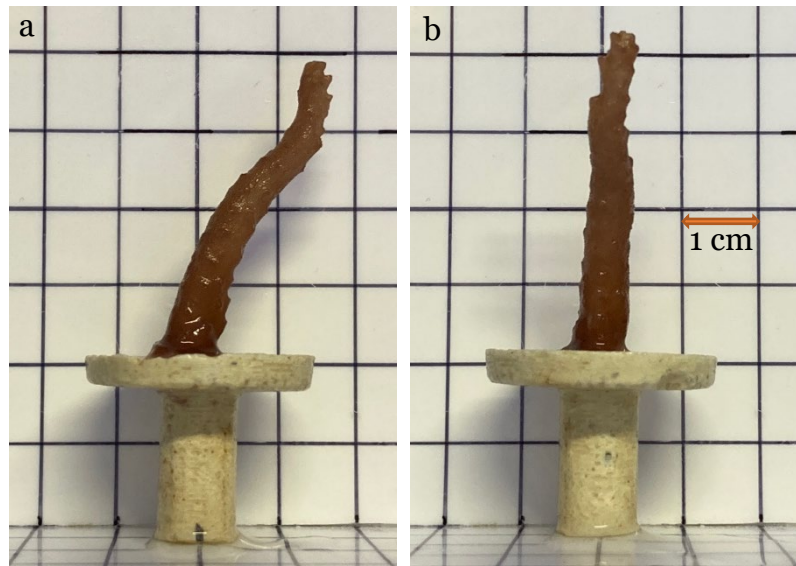


Figure 2: Photos of Nubbin 'A1' in group CTL along its (a) coronal plane and (b) sagittal plane against a 1cm² grid. A pen mark on the ceramic plug was made using waterproof marker before the day 1 photos were taken to align nubbin consistently with grid throughout study.

Statistical Analysis

All data analyses and visualisations were performed using RStudio (R Core Team, 2021). Each treatment used one tank; therefore, each nubbin was considered a pseudo-replicate (Heffner *et al.* 1996). The data were firstly tested for normality (Shapiro-Wilks) and equal variance (Levene's). Wilcoxon signed-rank tests were performed to compare cross-sectional area, volume and mass at the start (day 1) with those at the end (day 84). Since net growth and growth rate data was found to be parametric (Shapiro-Wilks, $p > 0.05$), a one-way ANOVA was performed to test the effect of diet on the change in the physiological parameters between groups. If results were found to be significant, a Tukey post hoc test was run to see where the significance lay. Spearman's rank correlation was used to test the relationship between time and each size metric and growth rate. The impact of diet on the polyp

coverage was assessed using a Pearson χ^2 test. The area lost due to bleaching was evaluated using a Mann Whitney U test. The confidence level for all tests was taken at 95% and data in tables were reported as mean \pm standard deviation.

Results

Survival

During the study, only one mortality occurred (<5%), which was deemed negligible. In order to ensure this was a death and not just severe bleaching, the nubbin was not declared as dead until there was algal growth over much of the coral skeleton, therefore showing no polyp growth in those areas.

Net Growth

After 84 days, the masses and volumes of nubbins in CTL were not significantly different than at the start but the cross-sectional areas were (Wilcoxon signed-rank, Table 1). The masses and volumes of nubbins in ART were significantly different than at the start, while the cross-sectional areas were not (Table 1). Nubbins in the group AA had significantly different volumes at day 84 than day 1 but their masses and areas were not significantly different. Where there were significant differences, the mass of ART increased, volume of ART and AA increased, and area of CTL decreased (Figure 3).

Table 1: Results of Wilcoxon signed-rank sum tests on the mass, volume and cross-sectional area of *Acropora microclados* nubbins on day 1 and day 84 of the study. The corals were maintained under one of three feeding regimes (CTL - control, ART – fed *Artemia* nauplii, and AA – fed an *Artemia* and microalgae mix). Means reported \pm the standard deviation. N = 15 per treatment. Confidence interval of 95% (significant $p < 0.05$ denoted by *)

Variable	Treatment	Mean \pm SD	V	p
Mass (g)	CTL	8.49 \pm 1.89	26	0.055
	ART	8.39 \pm 1.03	18	0.033 *
	AA	8.26 \pm 1.01	89	0.11
Volume (cm ³)	CTL	4.2 \pm 0.7	31	0.11
	ART	4.4 \pm 0.7	18.5	0.035 *
	AA	4.2 \pm 0.7	8	0.0034 *
Area (cm ²)	CTL	4.79 \pm 2.13	96	0.041*
	ART	5.69 \pm 2.13	22	0.058
	AA	5.14 \pm 2.30	75.5	0.39

At the end of the study, 13 nubbins (29%) had increased in cross-sectional area by >5%, 28 (62%) in volume and none in mass. Between groups, the net change (%) was tested using a one-way ANOVA. No significant difference was found between treatments in the net change of volume, but there was a significant net change of cross-sectional area and of mass (Table 2). positive and the net change in area of CTL and AA were both negative while ART was positive (Figure 4).

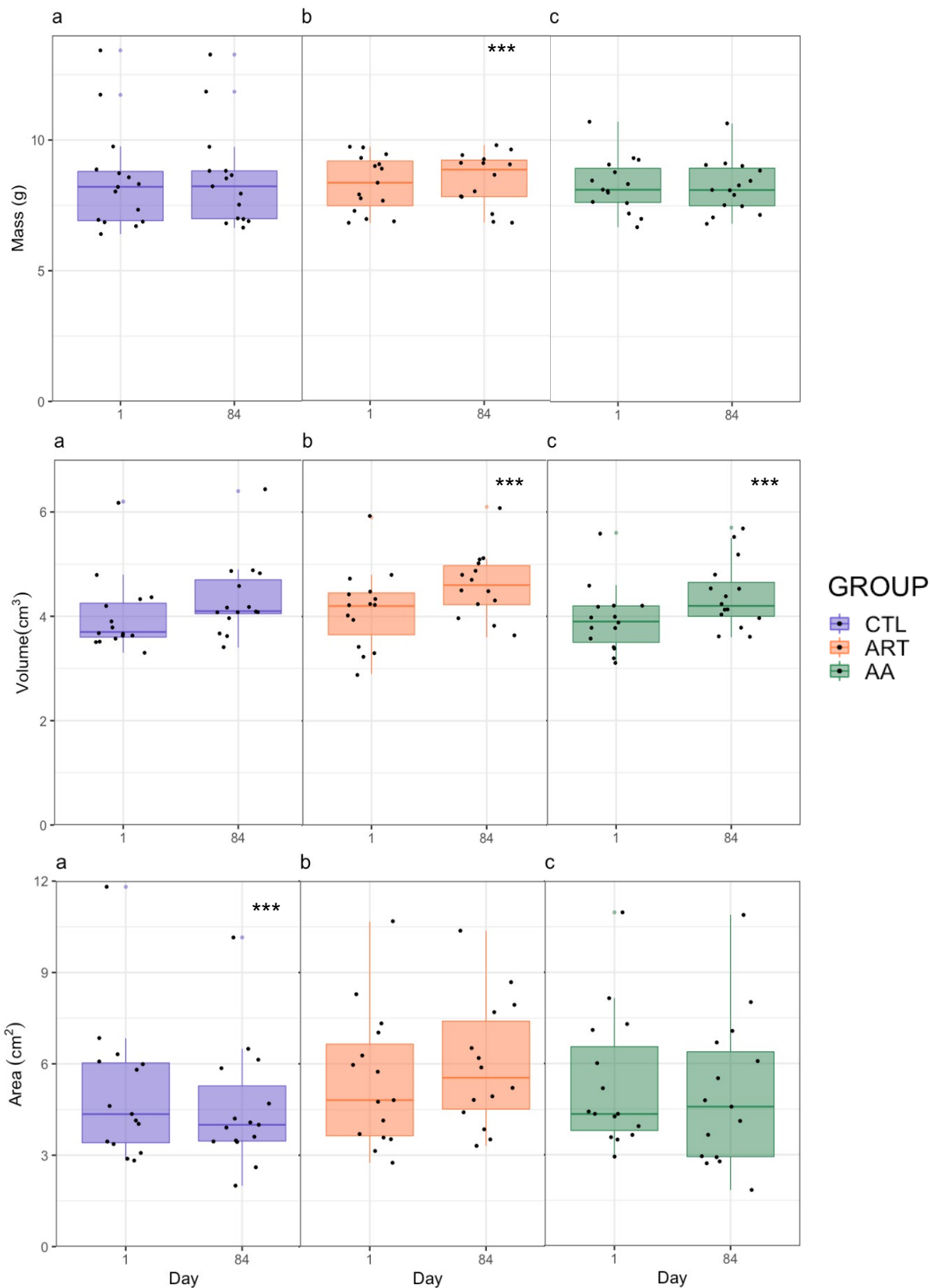


Figure 3: Masses (g), volumes (cm³) and cross-sectional areas (cm²) of *A. microclados* nubbins on day 1 and day 84 fed 3 different diets. a) CTL – control (fed nothing), b) ART – fed live *Artemia salina* nauplii, c) AA – fed a live mixed *Artemia* and microalgae diet. Boxes' limits are the 25th and 75th percentiles. N = 15 per treatment. Individual masses are represented by black dots, and outliers of the box and whisker plot are represented as coloured dots. Graphs where a significant difference was found in the data between day 1 and day 84 are denoted by '***' in the top right corner (Wilcoxon signed-rank, Table 1).

Table 2 - Results of one-way ANOVA tests on the % change in mass, volume and area of *Acropora microclados* nubbins maintained under three feeding regimes (CTL - control, ART – fed *Artemia* nauplii, and AA – fed an *Artemia* and microalgae mix). Means reported \pm the standard deviation. N = 15 per treatment. Confidence interval of 95% (significant $p < 0.05$ denoted by *)

Variable	df	Mean \pm SD	F	p
Mass (g)	2	0.44 \pm 1.51	5.5	0.0077 *
Volume (cm ³)	2	11.4 \pm 15.1	0.16	0.856
Area (cm ²)	2	-3.38 \pm 16.47	4.6	0.016*

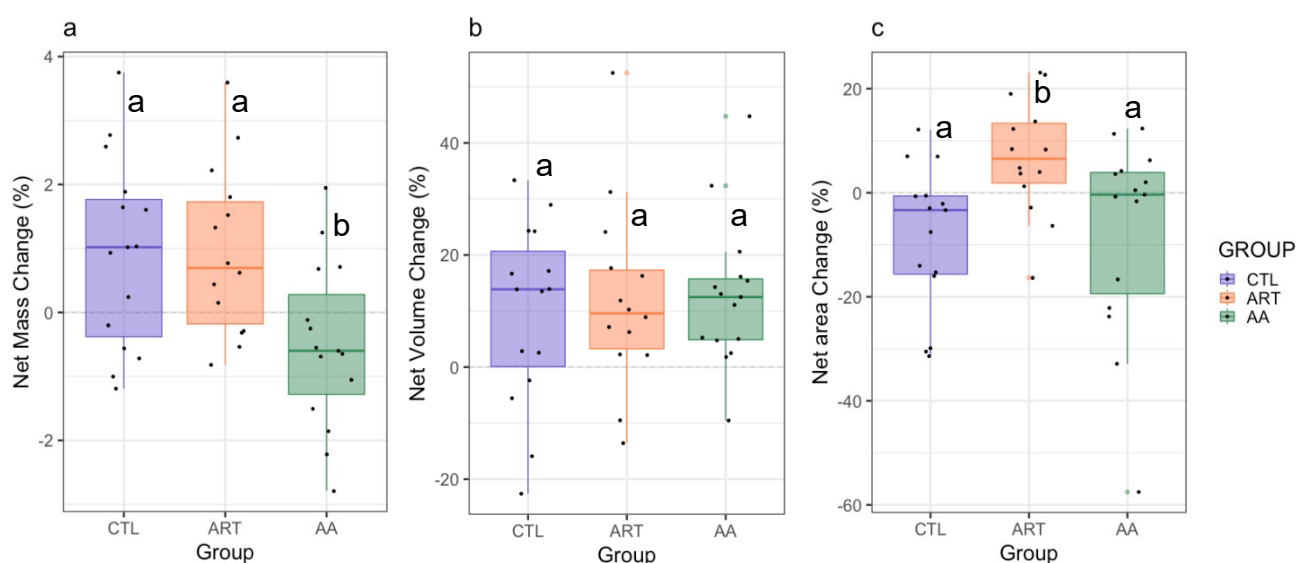


Figure 4: Effect of three different feeding regimes on the a) net mass b) net volume c) net cross-sectional area change (%) of *Acropora microclados* nubbins from day 1 to day 84. Day 84 was the final day of the study. Boxes' limits are the 25th and 75th percentiles. Individual nubbins' net differences are represented by black dots. Outliers are represented by coloured dots. Different superscript letters denote values in the same facet that are significantly different (ANOVA, $p < 0.05$). N = 15 per treatment. CTL = control (fed nothing), ART = *Artemia salina* nauplii, AA = mixed *Artemia*-Microalgae diet. Note the y axis of a, b and c are different scales.

Growth Trends

Post hoc testing showed that the net change in mass of AA was significantly different to CTL and ART and the net change in area was significantly different in ART than AA and CTL (Tukey's HSD, Table 3). The net change in mass of AA was negative, whereas CTL and ART were both

Table 3: Results of Tukey’s post hoc tests on the net change (%) mass, and area of *Acropora microclados* nubbins over the 84 days of the study. Tests were performed after finding significant difference between groups for mass and area using a one-way ANOVA. The corals were maintained under one of three feeding regimes (CTL - control, ART – fed *Artemia* nauplii, and AA – fed an *Artemia* and microalgae mix). N = 15 per treatment. Confidence interval of 95% (significant $p < 0.05$ denoted by *)

Variable	Treatment	Difference	Lower	Upper	p adjusted
Mass (%)	ART-CTL	0.024	-1.21	1.26	0.10
	AA-CTL	-1.43	-2.65	-0.22	0.018*
	AA-ART	-1.46	-2.69	-0.22	0.018*
Area (%)	ART-CTL	15.38	1.61	29.15	0.026*
	AA-CTL	0.85	-12.68	14.38	0.99
	AA-ART	-14.53	-28.30	-0.76	0.037*

Trend analysis using Spearman’s R test showed no significant correlation between period and mass or volume for nubbins in CTL. In ART and AA there was significant correlations between volume and period but not mass and period (Table 4, Figure 5).

Table 4: Results of Spearman’s R rank correlation tests on the mass, and volume of *Acropora microclados* nubbins over the 84 days of the study. The corals were maintained under one of three feeding regimes (CTL - control, ART – fed *Artemia* nauplii, and AA – fed an *Artemia* and microalgae mix). Means reported \pm the standard deviation. N = 15 per treatment. Confidence interval of 95% (significant $p < 0.05$ denoted by *)

Variable	Treatment	Mean \pm SD	S	p	Rho (ρ)
Mass (g)	CTL	8.49 \pm 1.89	190000	0.92	0.0096
	ART	8.39 \pm 1.03	180000	0.65	0.045
	AA	8.26 \pm 1.01	200000	0.68	-0.040
Volume (cm ³)	CTL	4.4 \pm 1.0	170000	0.28	0.0096
	ART	4.4 \pm 0.6	150000	0.027*	0.22
	AA	4.3 \pm 0.6	140000	0.0049*	- 0.27

Growth Rate

Trend analysis of G showed that none of the groups had significant correlation between mass or volume growth rates and time (Spearman’s R rank correlation, Table 5). Significant differences in the overall G were the same as the net growth (%) as G is equal to the net growth (%) divided by a constant (84).

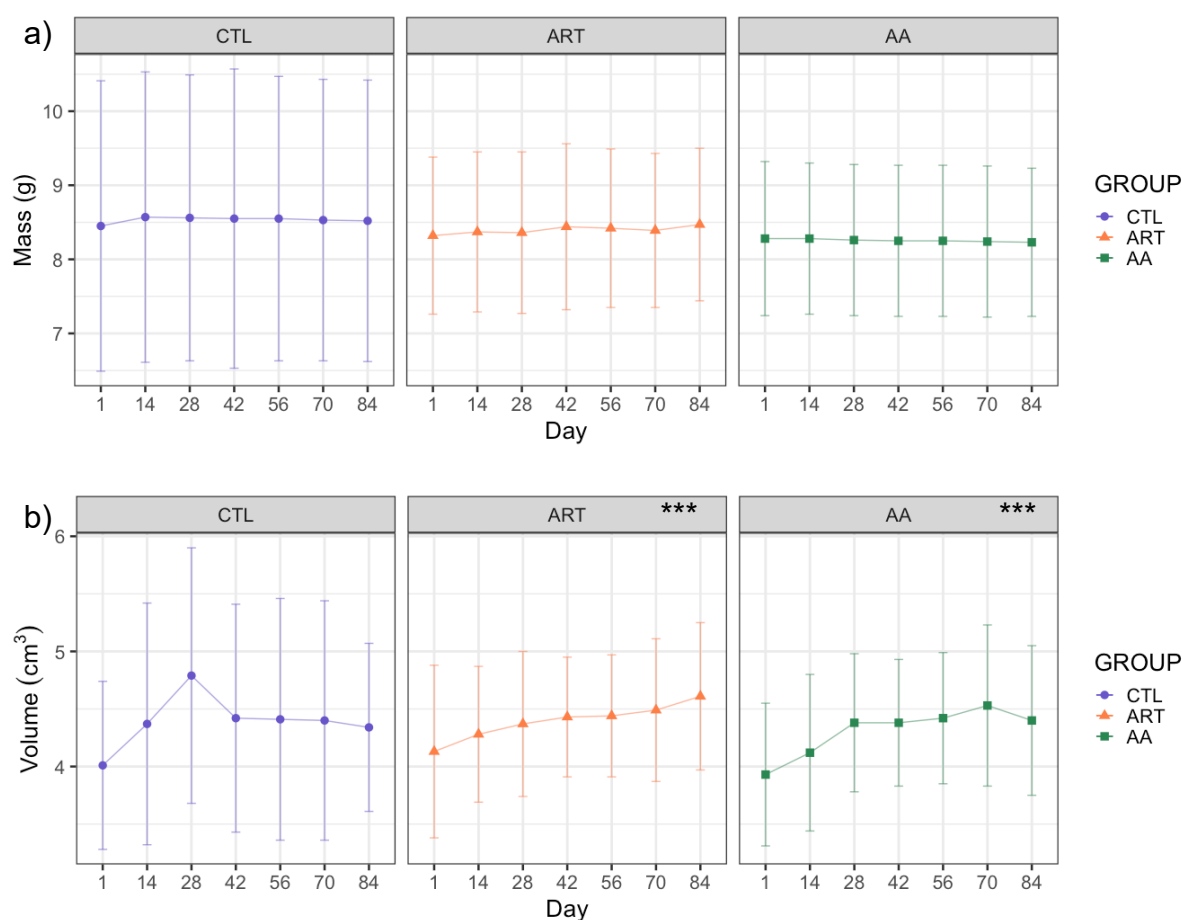


Figure 5: Mean weekly a) mass and b) volume of *Acropora* microclados over 84 days of the study. Error bars represent standard deviation. N = 15 per treatment. CTL = control (fed nothing), ART = *Artemia salina* nauplii, AA = mixed *Artemia*-Microalgae diet. *** show significant trend in growth (Spearman’s R, $p < 0.05$). Note y-axis for a and b do not start at zero.

Table 5 - Results of Spearman’s R rank correlation tests on the growth rates (G , % day^{-1}) mass, and volume of *Acropora* microclados nubbins over the 84 days of the study. The corals were maintained under one of three feeding regimes (CTL - control, ART – fed *Artemia* nauplii, and AA – fed an *Artemia* and microalgae mix). Means reported \pm the standard deviation. N = 15 per treatment. Confidence interval of 95% (significant $p < 0.05$ denoted by *)

Variable	Treatment	Mean \pm SD	S	p	Rho (ρ)
Mass (% day^{-1})	CTL	0.00012 \pm 0.00051	52	0.33	-0.49
	ART	0.00013 \pm 0.00037	36	0.40	-0.43
	AA	-0.00006 \pm 0.00009	36	0.96	-0.03
Volume (% day^{-1})	CTL	0.001 \pm 0.005	54	0.27	-0.54
	ART	0.002 \pm 0.001	62	0.07	-0.77
	AA	0.002 \pm 0.003	60	0.11	-0.71

Polyp Cover Type

On day 84, across the treatment groups, 13 (29%) displayed full coverage of polyps, 15 (33%) exhibited encrusting growth (Fig. 6) and 16 (36%) showed signs of bleaching (Fig. 7) These observations were not distributed homogenously across the treatment groups (χ^2 test df = 6, p = 0.0001, Fig. 8). Post hoc testing revealed higher proportions of bleaching nubbins were found in CTL (p=0.0006) and AA (p=0.009).

12 nubbins displayed >10% loss of area attributed to bleaching, 6 in CTL, 1 in ART, and 5 in AA (Figure 8). A Mann Whitney U test was done on the mean % of area lost to bleaching in CTL compared to AA. The bleaching between the two groups was not significantly different (p = 0.32). The same test was unable to be performed on CTL~ART or AA~ART as there was only one nubbin in ART that bleached >10%. 6 nubbins displayed >10% increase in area due to encrusting, 1 in CTL, 5 in ART and none in AA (Fig. 9).

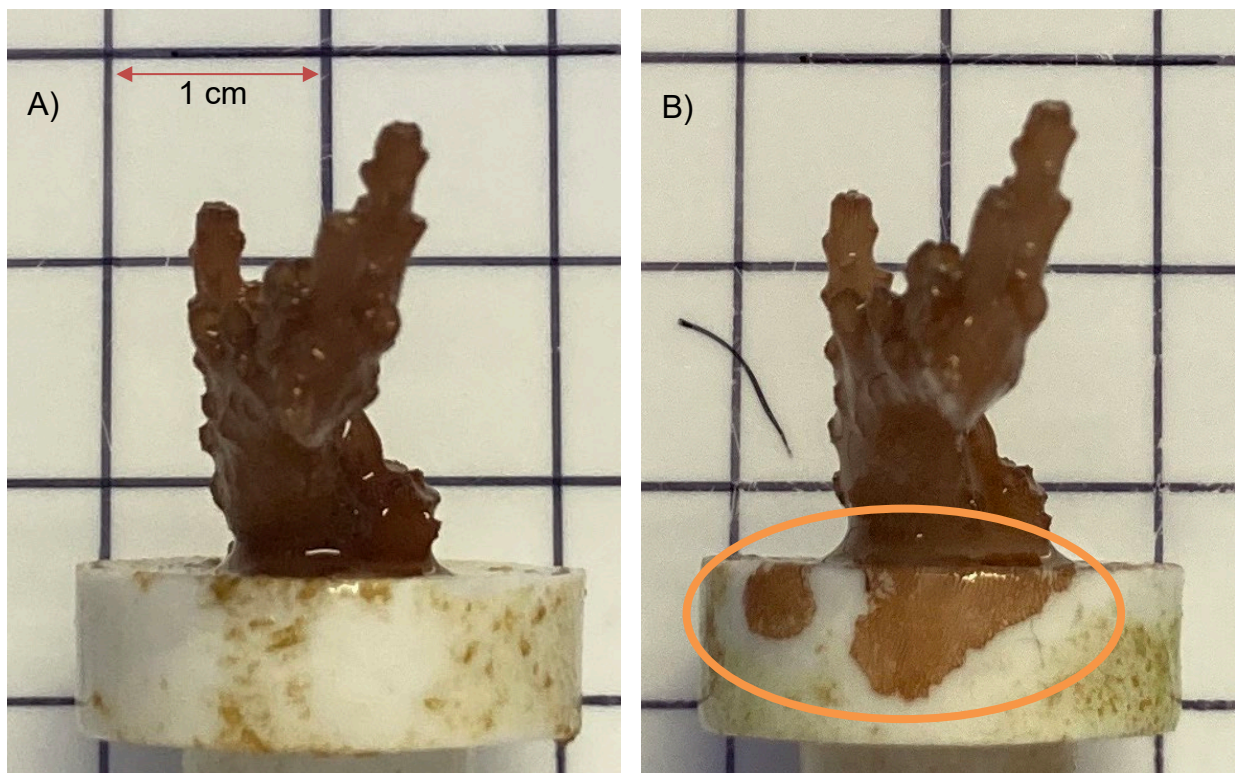


Figure 6: *Acropora microclados* nubbin 'B10' showing encrusting growth (orange circle). Treatment group = ART, fed *Artemia* nauplii. A) Day 1 of the study B) Day 84. Photos taken of the coronal plane of the nubbin in front of a 1cm² grid.

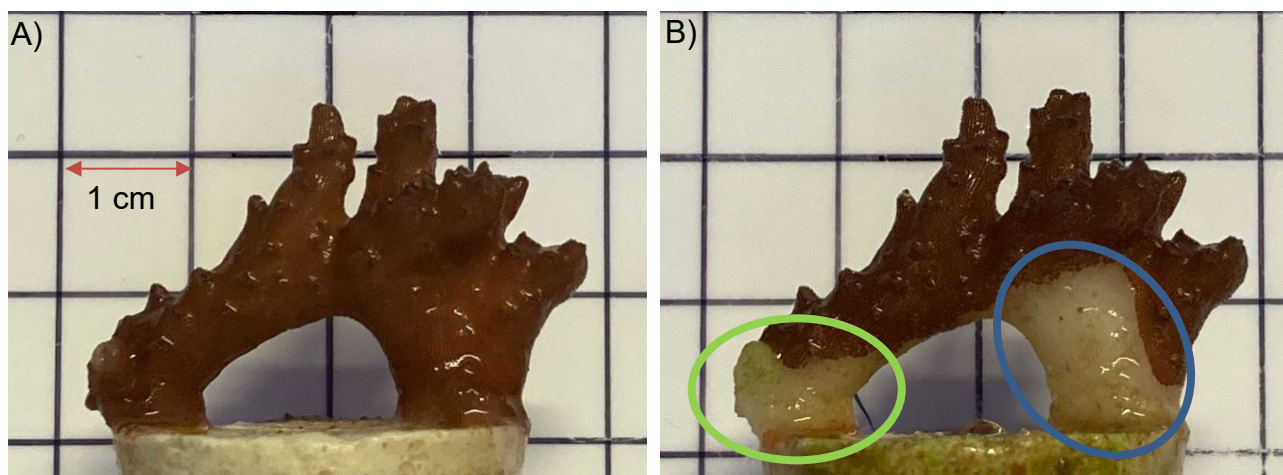


Figure 7: *Acropora microclados* nubbin 'A4' showing signs of bleaching. Blue circle shows bleaching on the coral skeleton. Green circle showing areas of the skeleton that polyps are no longer occupying, so have been colonised by algae, giving the light green colour. Treatment group = CTL, fed nothing. A) Day 1 of the study B) Day 84. Photos taken of the coronal plane of the nubbin in front of a 1cm² grid.

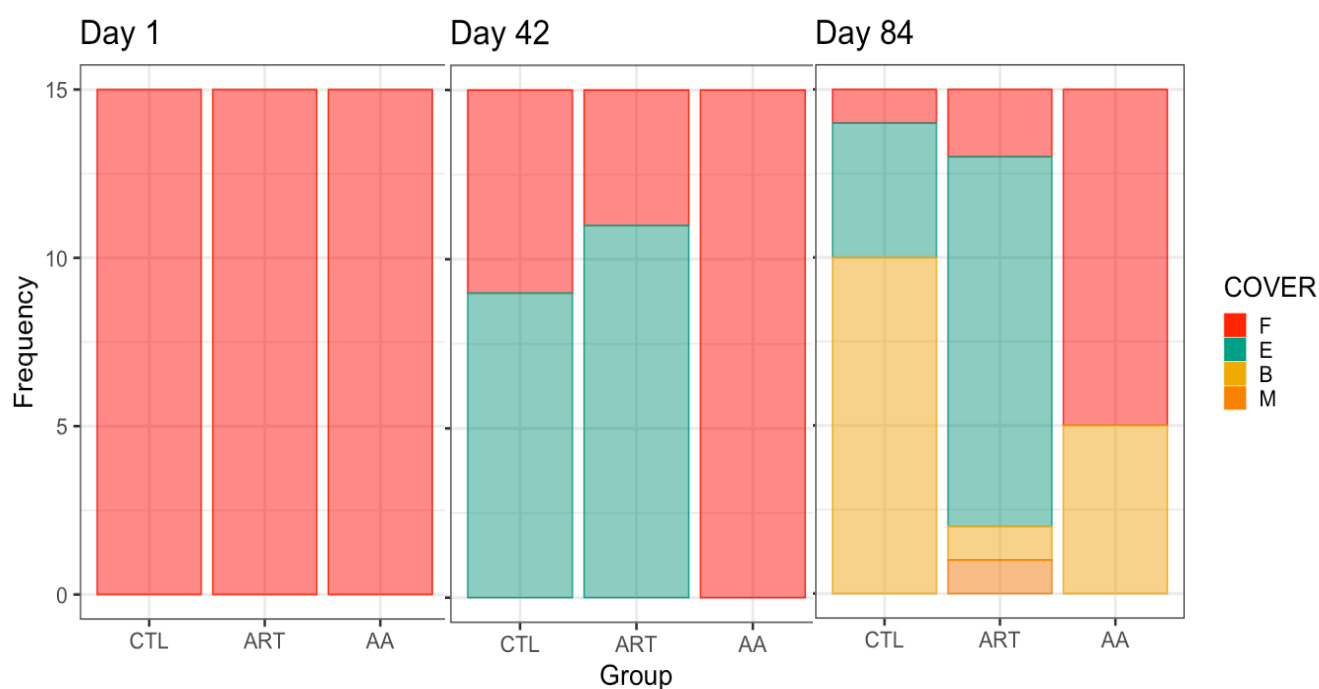


Figure 8: Frequency of polyp cover type on each *Acropora microclados* nubbin observed per diet group on Day 1, 42 and 84. F = Full, E = Encrusting, B = Bleaching, M = Dead. N = 15 per treatment. CTL were fed nothing, ART were fed *Artemia Salina*, and AA were fed a mixed *Artemia*-Microalgae diet.

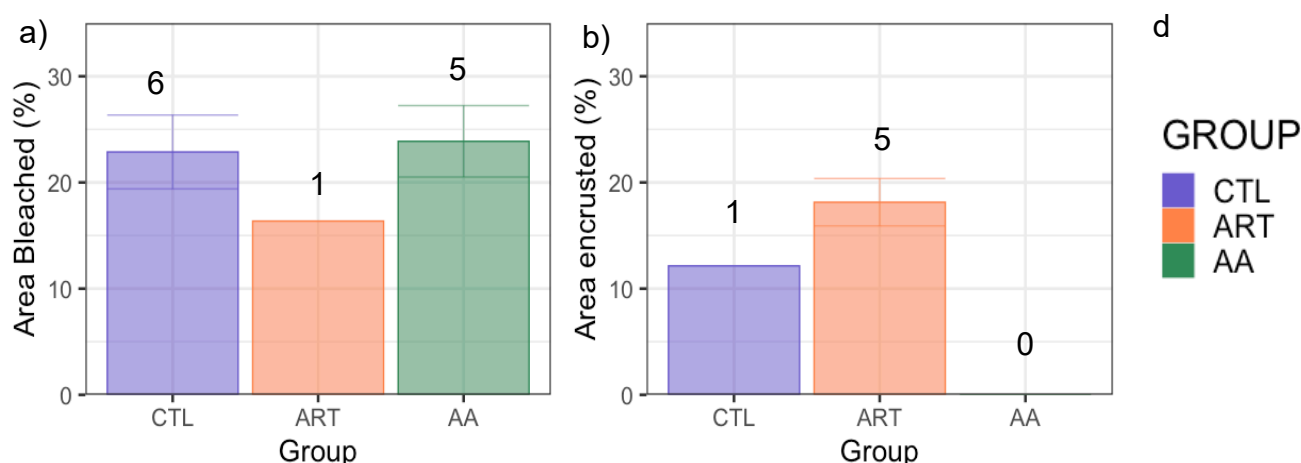


Figure 9: Mean area a) lost to bleaching and b) gained from encrusting as a % of the original cross-sectional area in CTL, ART and AA. CTL were fed nothing, ART were fed *Artemia Salina*, and AA were fed a mixed *Artemia*-Microalgae diet. Only includes nubbins that lost/gained >10% area. Error bars show standard deviation. N shown in super script above bars.

Discussion

Research outcomes

The results of this study revealed that the growth in mass, volume and area of *A. microclados* is affected by diet. Nubbins in CTL displayed no significant growth in mass or volume and decreased in area, with 60% of the nubbins that showed signs of bleaching losing more than 10% of their cross-sectional area. In ART there was significant growth in mass and volume indicating a significant increase in biomass and skeletal growth (Table 6). There was also a large proportion of nubbins displaying encrusting growth. In AA the only significant growth exhibited was volume, therefore biomass did increase but no significant skeletal growth. This suggests that the *Artemia*-algae diet may not be as nutritionally beneficial as the *Artemia* nauplii diet, or that AA were not taking up as much food as ART. This could be because of the size of the instar ii *Artemia* nauplii or negative selective feeding on algae. In CTL there was no trends in growth, whilst in ART and AA there was a significant positive trend in volume over time but not mass. It may also suggest that the growth in mass of ART and AA was too small over the period measured to be significant. The growth rate showed no significant change throughout, indicating that these trends would have continued if the study were to continue for longer. This, along with net growth results, shows that there was no growth in CTL and most likely would not have been.

Diet was also shown to affect the polyp coverage of *A. microclados*. A much higher proportion of corals in ART exhibited encrusting growth than AA and CTL (Table 6). Since there was no evidence of AA displaying higher growth than CTL, other than volume, it could be suggested that nubbins in AA weren't taking up as much exogenous food as ART and were more heavily relying on their symbiotic hosts, just like CTL.

Table 6: An overview of growth and polyp coverage results of the study. Net growth shows the significant changes in mass, volume and cross-sectional area in each group from day 1 to 84 (Wilcoxon rank sum, $p < 0.05$). - = no growth, +ve = increase, -ve = decrease. Polyp coverage shows the proportions of nubbins in each group that displayed full coverage (F), encrusting, bleaching (B) and dead (M). N = 15 per treatment.

Metric	Variable	CTL	ART	AA
Growth from day 1-84	Mass	-	+ ve	-
	Volume	-	+ ve	+ ve
	Area	- ve	-	-
Polyp coverage type (%)	F	6.7	13.3	67
	E	26.7	73.3	0
	B	66.7	6.7	33
	M	0	6.7	0

Comparison to similar research

The significantly higher growth in ART than in CTL illustrates the well-documented nutritional importance of zooplankton to Scleractinia corals. *Artemia* diets have previously been found to increase growth in other captive SPS species such as *Pocillopora damicornis* (Lavorano *et al.*, 2008) and *Stylophora pistillata* (Ferrier-pages, 2003, Houlebreque *et al.*, 2004). These results, and the much lower growth in group AA, are in agreement with Peter and Laterveer, (2008), who found that Instar I *Artemia* increased growth in juvenile *Acropora tenuis* while the microalgae *Phaeodactylum tricornutum* didn't promote growth in another SPS coral, *Favia fragum*.

The comparatively low number of bleaching corals in ART illustrates previous findings that suggest heterotrophy can improve resilience to bleaching (Anthony 2000, Grottoli *et al.*, 2006; Hughes and Grottoli; 2013). Burmester *et al.* (2018), found that exogenous food sources aid specifically with tissue maintenance and healing initiation of *S. pistillata*, in concurrence with some of the results of this study. However, the bleaching in AA was not found to be significantly different to the starved corals in CTL suggesting that, conversely, the Artemia-algae mix was not as nutritionally beneficial in maximising healing potential as *Artemia* nauplii.

The lower growth in AA, comparable to that in CTL also disagrees with literature showing herbivory in SPS corals. Previous studies have shown that microalgae are a suitable diet for SPS corals (Leal *et al.*, 2013), and though herbivory was shown to be species specific, Conlan *et al.* (2019) found that an *Isochrysis galbana* diet increased growth in three different *Acropora* species studied. The high growth of ART nubbins also contradicts Conlan *et al.* (2019), who suggested that a lipid-rich diet such as *Artemia* would not be optimal for *Acropora* compared to a microalgae diet high in EFAs. These discrepancies could be a result of the algal diets used. For example, Conlan *et al.*, (2019) used *I. galbana*, whilst a mix of *I. galbana* and *T. suecica* was used within this study. *T. suecica* is known to contain fewer or lower levels of important long-chain PUFAs, such as EPA (20:5n-3) and DHA (22:6n-3) (Brown *et al.*, 1997), which support corals' stress resistance and recovery (Seeman

et al., 2012). Therefore, by reducing the quantity of these PUFAs accessible to the nubbins during cultivation, their growth may have been limited. This theory also aligns with Osinga *et al.*, (2012) reporting that nutritional benefits are algal-species specific.

The low growth may also be attributable to herbivory characteristics and a paucity of Instar II *Artemia* ingested in AA. Before the study took place AA had only been fed Instar I *Artemia* and former studies show that heterotrophy is heavily influenced by life history and genotype (Houlebreque and Ferrier-pages, 2009; Tagliafico *et al.*, 2018). A longer period of study or use of various mother colonies could provide a remedy to if this is the case.

There is no published scientific literature regarding encrusting growth in captive corals related to health or diet and the limited information that is available is found in anecdotal reports from aquarium hobbyists. Some say they will only purchase nubbins which display encrusting growth on their plug as they believe it is a sign of a healthy nubbin, however some suggest that encrusting could be attributed to sub-optimal environmental conditions such as excessive flow. The lack of bleaching in ART compared to CTL and AA suggests that the nubbins in this group are generally healthier and since the amount of encrusting is significantly higher in ART, a correlation between health and encrusting is likely to exist.

Consequences for *ex situ* coral aquaculture

Further study is required to understand the interactions that must be manipulated to maximise growth of *Acropora microclados* in aquaria. While some factors cannot be adjusted, for example genotype and life history, diet is a crucial aspect that is relatively easy to influence. The positive impact of *Artemia* on *Acropora* growth, as well as its relatively low cost, have made it a popular live food for many aquarists, but its impact on resistance to stressors and tissue maintenance are just as essential to furthering the sustainability and success of coral aquaculture for the MOT and reef restoration efforts. Although the present study did not provide evidence that a mixed diet of microalgae and *Artemia* is a feasible alternative to the popular *Artemia* diet, further research is encouraged in order to understand why heterotrophy is so variable between species and if a diet of just algae would be preferable. Research on nutritional benefits, including high PUFAs and lower lipid content (Brown *et al.*, 1997; Osinga *et al.*, 2012), suggests algae is a viable diet option and further research on this topic could elucidate the optimum ratios for maximum growth. This research is also important to the growing field of coral reproduction, which has been difficult to replicate in aquaria. Heterotrophy is vital for juvenile corals as they often settle in crevasses where light, and therefore autotrophy, is limited.

It has been accepted and shown repeatedly that *Artemia* is an effective food source for SPS corals, though its sustainability has been called into question, as it involves the constant harvest of natural resources, often in remote locations requiring intensive transport (Sorgeloos *et al.*, 2001). Algae, however, can be produced without the emission of any greenhouse gases, does not cause environmental pollution and can be cultured on an industrial scale anywhere in the world (Hemaiswarya *et al.*, 2010; Forján *et al.*, 2014). Therefore, it is essential to continue efforts to study interactions between algae and coral, even though the results of the present study were not in agreement with much literature supporting this.

Research limitations

Due to practical constraints, this study was unable to test the impact that different concentrations of live feed might have. While ensuring the concentration of food was high enough to ensure feeding (>2000 *Artemia* L⁻¹, Lavorno *et al.*, 2008) this study did not specifically determine the quantity of food consumed each day. The level of photosynthetic activity was not measured either, so the effect different exogenous food sources have on energetic pathways cannot be inferred. An analysis of food consumed at different concentrations would give a better understanding of how rate of intake relates to growth rate.

The nubbins began to bleach after 42 days; therefore, it is unlikely that the cause was flow, diet or light as these were constant throughout. The cause may have been water quality, due to its potential to spike and cause acute stress. While every effort was made to carefully monitor the water chemistry, it is possible something was missed in testing, or the issue was with a parameter that was not monitored. For example, total dissolved solids (TDS), organics and iron concentrations were not monitored due to practical constraints and their assumed unlikelihood of impact (Borneman, 2008).

A replication of this study using several different genotypes per treatment could yield results more applicable to wider coral aquaculture practices, as growth variability among genotypes has been shown to be, in some cases, greater than variability among treatments (Osinga *et al.*, 2012). Furthermore, since this study focuses solely on *Acropora microclados*, results should not be applied to corals with different dependencies on trophic pathways without further testing. However, it does broadly emphasize the importance of understanding the impact of exogenous energy sources and provide a foundation for continued research on the importance of heterotrophy for both growth and resilience.

For the scope of this study, qualitative observations alongside quantitative growth measurements were found to be a valuable means to track nubbins health, however if further study were to be completed quantitative measurements of both could be used for more accurate assessments.

Conclusions

The present study demonstrates that a diet of *Artemia* nauplii can improve growth and resilience in captive *A. microclados*. A mixed diet of *Artemia* and algae, showed some growth of biomass but little difference in resilience to starved nubbins. Future investigations may seek to elaborate on this by manipulating density and frequency of feeds as well as investigating purely algal-based feeds. Algal feeds should not be written off as an option as their cost-effectiveness, sustainability and high nutritional value provide an attractive option for coral aquaculture if successfully administered. Due to the species-specific nature of coral heterotrophy, the elucidation of an optimal feeding regime for *Acropora microclados* requires significant work but in order to attain the long-term goal of sustainable SPS culture for the MOT and reef restoration efforts.

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