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The freshwater macroalga *Oedogonium intermedium* can meet the nutritional requirements of the herbivorous fish *Ancistrus cirrhosus*

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ABSTRACT

The macroalga *Oedogonium intermedium* has a diverse elemental profile, high energy potential, high lipid content, a high proportion of essential amino acids and a total concentration of protein with the potential to meet the nutritional requirements of herbivorous fishes. The aim of this study was to assess growth, condition, colouration and reproductive output in an herbivorous ornamental fish. The catfish *Ancistrus cirrhosus* was fed one of three pelletised experimental feeds, *Oedogonium*, a formulated commercial feed and a 50:50 mix of the two, over a 10 month trial. After 10 months, fish that were fed exclusively on *Oedogonium* had equivalent growth rates, body condition, colouration, fecundity and fertilisation rates compared to those fed the commercial and mixed feeds. The absence of any major differences of these characteristics between feeds, especially between *Oedogonium* and the commercial feed, demonstrates the viability of *Oedogonium* as a feed, or significant feed ingredient, for this herbivorous fish. As such, linking the cultivation of *Oedogonium* with the production of herbivorous fish may provide an ideal application for *Oedogonium*.

1. Introduction

Marine and freshwater macroalgae can effectively recover residual nutrients from nutrient-rich wastewater streams from aquaculture [1,2], agriculture [3–5] and the treatment of municipal waste water [6,7]. This has environmental benefits for receiving waters by improving the quality of the effluent, and economic benefits through the provision of macroalgal biomass for value-added products [7,8]. A targeted species for this process is the green freshwater macroalga *Oedogonium intermedium* (henceforth referred to as *Oedogonium* [9]). *Oedogonium* is well-suited to bioremediation due to its competitive dominance over other algal species [10], its high uptake rates of nitrogen and phosphorus, its high biomass productivity, and its ability to be cultured across a range of environmental conditions [7,11–14]. The *Oedogonium* biomass produced from the treatment of waste water has a nutrient profile which reflects growth conditions, resulting in a diverse elemental profile, high energy potential, high lipid content and a high proportion of essential amino acids [7,14]. This establishes *Oedogonium* as a potential feed for terrestrial animals such as poultry and swine [15,16], and aquatic animals, in particular fish, where there are strong drivers for alternative sources of feed [17–19]. However, despite the ability of *Oedogonium* to rapidly convert nitrogen into proteins, the total protein content of *Oedogonium* only reaches 26.5% [20], which is likely to be too low for use as a complete feed for non-ruminant animal production systems such as finfish and crustacean aquaculture [21].

Aquaculture production systems rely on maximum growth rates and this requires high protein feeds, which vary between 30 and 60% dietary protein for finfish and crustacean aquaculture [22,23]. However, for animals where maximum growth rates are not required, the total protein concentration is less important than the correct ratio of amino acids [24], which provides positive health effects over longer periods [25]. Notably, for fish and crustaceans in the aquatic ornamental industry, growth rates are less important than condition, colour intensity and breeding health [26], where condition and breeding health are affected by food type, ration size and frequency, nutrient profiles, dietary lipid, protein and carbohydrate content, and essential fatty acid compositions [27–29], while colouration is affected by carotenoid content [30,31].

Globally, over 1 billion ornamental fish are traded annually, including approximately 4000 freshwater species and 1400 marine species, at a total industry value of $25 billion each year and an annual growth of 14% [32,33]. A large proportion of this valuation is due to commercially-available feeds, which are tailored for individual species, or functional species types [34–36]. However, much of the

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commercially-available feed currently used for ornamental fish is formulated on the dietary requirements of aquaculture species, where high protein is essential, and fishmeal is the main source of protein [26]. For example, the 'algal' feeds marketed towards suckermouth catfish or plecos (Loricariidae), a popular species of herbivorous ornamental fish [37–39], have low proportions of algae and higher proportions of fishmeal. In their natural habitat, algae, including *Oedogonium*, make up a large proportion of the suckermouth catfish diet [40]. Consequently, alternative feeds, which use macroalgae as either a base component or as a full ration, may be suitable as a feed for this, and related species of, herbivorous ornamental fishes.

Therefore, the aims of this study were to determine whether pelletised *Oedogonium* could be used as a dietary component or complete feed and meet the nutritional requirements of an herbivorous freshwater ornamental fish, by assessing (1) pellet stability in the water, (2) growth and condition of juveniles, (3) colouration and (4) fecundity and fertilisation in adults.

2. Materials and methods

Three treatment feeds were given to the herbivorous freshwater catfish *Ancistrus cirrhosus* (henceforth referred to as *Ancistrus*). All experiments were done in 25 L opaque plastic experimental tanks (400 mm [l] × 300 mm [w] × 300 mm [h]) maintained with a constant flow of 1.5 L/min or 107 tank volumes per day. The experimental tanks were part of a recirculating aquaculture system consisting of one 10,000 L underground sump, a propeller bead filter (PBF-50S; Aquaculture Systems Technologies, New Orleans, LA, USA), a high-output UV steriliser (E150S; Emperor Aquatics, Pottstown, PA, USA) and a water chiller (DHP603-R; EvoHeat, Seventeen Mile Rocks, QLD, Australia) to maintain water quality. The mean water temperature, salinity, pH, O_2-content and conductivity (Pro Plus; YSI Inc., Yellow Springs, Ohio, USA) in the tanks throughout the experiment was 26.01 ± 0.23 °C, 0.14 ± 0.00 ppt, 8.39 ± 0.06, 65.01 ± 1.74 mg/L and 316.00 ± 22.70 μS/cm, respectively.

2.1. Treatment feeds

Feed 1 (commercial) was a high-quality commercial algal feed (Hikari Algae Wafers; Kyorin Co. Ltd., Hachioji, Tokyo, Japan) specially formulated for herbivorous fishes, which included *Spirulina* sp., *Chlorella* sp. and an unidentified seaweed meal (Supp. A). Feed 2 (mix) was a 50:50 mix of the commercial feed and *Oedogonium*. Feed 3 was *Oedogonium*, cultivated at the Cleveland Bay municipal waste water treatment plant (Townsville, QLD, Australia) using the treated discharge effluent intended for release to the environment (see [7] for full culture details), harvested, and dried at 60 °C for 48 h prior to processing. To prepare the feeds and ensure consistency of the physical form of the feed, all material was milled to a fine powder (Wiley Mill Model 4; Thomas Scientific, Swedesboro, NJ, USA), pelletised (N-Micro; Nova Pellet, Cremona, Italy) and extruded through a 5 mm die. The pellets were air dried, cut to a standardised size (~12 mm) with a guillotine and stored in air tight plastic tubs with silica packets. Pellets from each treatment feed (n = 100) were randomly selected and used to quantify the mass (g), length (mm) and density (g/cm^3) of the pellets (Table 1).

Samples from each treatment feed (n = 3 for each set of analyses) were also analysed for proportion of dry matter content, ash content, total lipids, total protein (sum of amino acids), total carbohydrates and elemental composition (Table 1). The dry matter content was determined by drying samples at 60 °C to constant weight (Ultra FD1000; Ezidri, Moorabbin, Victoria, Australia) and the ash content was determined by the combustion of 1 g samples at 550 °C (152C; S.E.M. (S.A.), Magill, SA, Australia) over 8 h. Total lipid concentrations were quantified gravimetrically by extracting samples with a dichloromethane:methanol (2:1, v/v) solution [41]. Crude protein was estimated by the sum of amino acids which were determined using the Water AccQTag protocol at the Australian Proteome Analysis Facility (Sydney, Australia) according to [14]. The amino acids included histidine, serine, arginine, glycine, aspartic acid, glutamic acid, threonine, alanine, proline, lysine, tyrosine, methionine, valine, isoleucine, leucine and phenylalanine (Table 2), of which arginine, lysine and methionine are the most important [42]. Cysteine, tryptophan and taurine were not accounted for as they only represent < 2% of the total amino acids found in macroalgae and require a different set of analytical methods [43,44]. Total carbohydrate concentrations were calculated by subtracting moisture (100% - dry matter content), ash, total lipids and crude protein from 100% [8] and pooling the standard error using Satterthwaite's approximation. Approximate metabolisable energy was estimated from the crude protein, total lipids and carbohydrates using the Atwater factor [45]. Concentrations (mg/kg) of ten elements (Ca, K, Mg, Na, Fe, Al, Cu, Zn, P and Mn) were quantified externally (Advanced Analytical Australia Pty. Ltd., North Ryde, NSW, Australia) using an inductively coupled plasma atomic emission spectrometer.

2.2. Pellet stability

The physical stability and nutrient leaching rates of the three treatment feeds were tested by quantifying degradation over time upon submergence [46]. Ten pellets from each feed treatment (approximately 4 g) were weighed and placed into 100 mm polyvinyl chloride end caps each with a 10 mm hole covered in fine mesh (300 μm), to keep the pellet biomass contained and facilitate draining the water while preventing the loss of any particulate matter. The end caps were placed into experimental tanks (one dish per tank) and removed after 0, 2, 4, 8, 16, 24, 36 and 48 h (n = 3). At each time point, the end caps were slowly lifted out of the tanks, drained and photographed. The pellet biomass was then used to determine changes in dry matter content, ash content and total lipids over time as described above. In addition, nitrogen was quantified at each time point externally using an elemental analyser (OEA Laboratories Ltd., Callington, Cornwall, UK) and crude protein was calculated using the protein:N ratio as determined by the amino acid profile (Table 2).

2.3. Experimental design

Seventy-eight randomly-selected *Ancistrus* juveniles (3–4 cm) were obtained from a wholesale distributor (Aquarium Industries, Epping, VIC, Australia) and transported to James Cook University (JCU). During an initial two week conditioning period, fish were kept communally (no > 15 individuals per tank) and fed all three treatment feeds together in equal portions *ad libitum*. After the conditioning period, the fish were separated into the three treatment groups (n = 26) based on a randomised block design for size (Table 3) to control for any significant size differences at the beginning of the trial. The fish were randomly placed into one of 78 experimental tanks (one individual per tank) with a ceramic cave (14 cm [l] × 3 cm [w] × 4 cm [h] with an opening at one end) for refuge. The fish were fed manually each day at 4 pm *ad libitum* to prevent any restriction of growth due to a lack of food availability. To limit stress on the fish, which can have detrimental effects on health and productivity [47,48], each tank was covered with shade cloth and excess feed was removed twice weekly using a siphon. Within each treatment group 20 fish were used for growth measurements and colour assessment and 6 fish were used for post growth trial liver assessment. Of the 20 fish used in each group in the growth trial, 7 males and 7 females went on to be used in breeding trials and the remaining 6 fish were used for post growth trial whole fish body condition assessment.

2.4. Growth and body condition

The growth trial was conducted over a period of 27 weeks (~6 months) and during that time each fish (n = 20) was weighed and

Table 1
Pellet characteristics, proximate analysis and mineral composition of the three treatment diets (commercial, mix and *Oedogonium*).

	Commercial	Mix	*Oedogonium*	Pseudo-$F_{(df)}$	*p*-Value
Pellet characteristics					
Mass (g)	0.46 ± 0.01	0.45 ± 0.01	0.45 ± 0.01	$0.27_{(2,299)}$	0.775
Length (mm)	12.43 ± 0.16	12.35 ± 0.19	12.40 ± 0.16	$0.20_{(2,299)}$	0.828
Density (g/cm^3)	1.86 ± 0.01	1.86 ± 0.01	1.85 ± 0.02	$0.71_{(2,299)}$	0.491
Proximate analysis (%)					
Dry matter	88.76 ± 0.21^a	89.19 ± 0.12^a	90.71 ± 0.35^b	$17.47_{(2,6)}$	0.011*
Ash	10.43 ± 0.00^a	10.61 ± 0.02^b	10.23 ± 0.02^c	$123.12_{(2,6)}$	0.004*
Lipids	9.06 ± 0.08^a	8.87 ± 0.06^a	9.74 ± 0.07^b	$39.32_{(2,6)}$	0.007*
Protein^	24.28 ± 0.06^a	22.21 ± 0.06^b	18.81 ± 0.33^c	$157.59_{(2,6)}$	0.005*
Carbohydrates	44.99 ± 0.08^a	47.50 ± 0.03^b	51.93 ± 0.25^c	$110.34_{(2,6)}$	0.004*
ME (MJ/kg)	15.01 ± 0.01^a	15.01 ± 0.03^a	15.51 ± 0.02^b	$145.91_{(2,6)}$	0.036*
Elemental analysis (mg/kg)					
Ca	14,667 ± 333^a	10,667 ± 33^b	4333 ± 33^c	$397_{(2,7)}$	0.001*
K	9567 ± 384^a	15,333 ± 333^b	27,667 ± 333^c	$266_{(2,7)}$ (2,7)	< 0.001*
Mg	2600 ± 100 ^{a}	3800 ± 58^b	5300 ± 58^c	$154_{(2,7)}$ (2,7)	< 0.001*
Na	8167 ± 393^a	5233 ± 120^b	1267 ± 33^c	$448_{(2,7)}$	0.001*
Fe	637 ± 33^a	533 ± 22^b	257 ± 3^c	$136_{(2,7)}$	< 0.001*
Al	120 ± 0^a	303 ± 3^b	553 ± 7^c	$5011_{(2,7)}$	0.001*
Cu	10 ± 0^a	11 ± 0^b	12 ± 0^c	$345_{(2,7)}$	< 0.001*
Zn	89 ± 1^a	100 ± 0^b	130 ± 0^c	$2367_{(2,7)}$	0.001*
P	11,667 ± 333^a	9733 ± 219^b	6300 ± 58^c	$123_{(2,7)}$	< 0.001*
Mn	98 ± 0^a	166 ± 1^b	359 ± 1^c	$31,932_{(2,7)}$	0.001*

^ protein quantified from the sum of amino acids in Table 2; ME, metabolisable energy estimated using the Atwater factor; * denotes a significant effect of treatment diet; superscript letters represent significant groupings within each row, where applicable; all data presented as mean ± standard error.

Table 2
The composition and quantity of amino acids (g/100 g DW) of the three diet treatments (commercial, mix and *Oedogonium*).

	Commercial	Mix	*Oedogonium*	Pseudo-$F_{(df)}$	*p*-Value
N (%)	5.51 ± 0.04	4.95 ± 0.08	4.25 ± 0.02	$174.5_{(2,6)}$))	0.003*
Amino acids (g/100 g)					
Histidine[1]	0.603 ± 0.003^a	0.493 ± 0.003^b	0.357 ± 0.009^c	$293.4_{(2,6)}$	0.004*
Serine	1.173 ± 0.003^a	1.113 ± 0.003^b	0.983 ± 0.017^c	$76.8_{(2,6)}$	0.005*
Arginine[1]	1.677 ± 0.007^a	1.443 ± 0.003^b	1.070 ± 0.021^c	$378.3_{(2,6)}$	0.003*
Glycine	1.370 ± 0.006^a	1.243 ± 0.003^b	1.023 ± 0.017^c	$222.7_{(2,6)}$	0.005*
Aspartic acid	2.523 ± 0.009^a	2.400 ± 0.021^b	2.133 ± 0.037^c	$56.5_{(2,6)}$	0.004*
Glutamic acid	3.963 ± 0.009^a	3.387 ± 0.018^b	2.540 ± 0.046^c	$411.2_{(2,6)}$	0.004*
Threonine[1]	1.163 ± 0.003^a	1.117 ± 0.003^b	1.020 ± 0.020^c	$33.3_{(2,6)}$	0.004*
Alanine	1.537 ± 0.003^a	1.467 ± 0.007^b	1.337 ± 0.023^c	$45.1_{(2,6)}$	0.004*
Proline	1.273 ± 0.003^a	1.160 ± 0.000^b	0.970 ± 0.015^c	$220.4_{(2,6)}$	0.002*
Lysine[1,2]	1.723 ± 0.003^a	1.523 ± 0.009^b	1.233 ± 0.022^c	$237.8_{(2,6)}$	0.004*
Tyrosine	0.680 ± 0.012	0.673 ± 0.012	0.620 ± 0.017	$5.6_{(2,6)}$	0.057
Methionine[1,2]	0.560 ± 0.012^a	0.427 ± 0.007^b	0.327 ± 0.020^c	$46.1_{(2,6)}$	0.003*
Valine[1]	1.487 ± 0.003^a	1.420 ± 0.000^b	1.280 ± 0.025^c	$44.3_{(2,6)}$	0.004*
Isoleucine[1]	1.197 ± 0.003^a	1.097 ± 0.003^b	0.907 ± 0.013^c	$253.2_{(2,6)}$	0.004*
Leucine[1]	2.077 ± 0.003^a	1.993 ± 0.003^b	1.820 ± 0.030^c	$48.7_{(2,6)}$	0.004*
Phenylalanine[1]	1.277 ± 0.003^a	1.257 ± 0.003^b	1.190 ± 0.020^c	$13.7_{(2,6)}$	0.004*
Total EAA	11.763 ± 0.035^a	10.770 ± 0.015^b	9.203 ± 0.158^c	$148.0_{(2,6)}$	0.004*
Total NEAA	12.520 ± 0.029^a	11.443 ± 0.041^b	9.607 ± 0.168^c	$163.4_{(2,6)}$	0.003*
Proportion of total protein concentration (wt%)					
EAA	48.44 ± 0.02^a	48.49 ± 0.06^a	48.93 ± 0.07^b	$24.4_{(2,6)}$	0.020*
NEAA	51.56 ± 0.02^a	51.52 ± 0.06^a	51.07 ± 0.07^b	$24.4_{(2,6)}$	0.023*
Lysine	7.10 ± 0.01^a	6.86 ± 0.02^b	6.56 ± 0.02^c	$190.6_{(2,6)}$	0.004*
Methionine	2.31 ± 0.04^a	1.92 ± 0.03^b	1.74 ± 0.11^b	$12.7_{(2,6)}$	0.004*
Ratios					
Protein:N	4.41	4.49	4.43	–	–

EAA, essential amino acids; NEAA, non-essential amino acids; [1] EAA; [2] EAA important in fishes [34]; all data presented as mean ± standard error; * denotes a significant effect of treatment diet; superscript letters represent significant groupings within each row, where applicable; all data presented as mean ± standard error.

measured every 3 weeks. At the end of the trial, growth performance and condition of each individual was assessed by the determination of the following:

$$weight\ gain\ (g) = FW - IW$$

$$body\ condition\ index = \frac{FW/IW^*}{FL^3}\ 100$$

where *FW* is individual final weight (g), *IW* is the individual initial weight (g) and *FL* is the individual final length (mm) [49,50]. At the end of the growth trial, six fish were euthanised and used for analysis of

Table 3
Analysis of growth, condition and whole body composition of *Ancistrus* fed the commercial, mix and *Oedogonium* diets at the end of the 27 week growth trial.

	Commercial	Mix	*Oedogonium*	pseudo-$F_{(df)}$	*p*-Value
Growth and body condition					
Initial weight (g)	0.51 ± 0.02	0.50 ± 0.02	0.50 ± 0.02	$0.02_{(2,58)}$	0.995
Initial length (mm)	36.13 ± 0.47	35.97 ± 0.49	36.02 ± 0.54	$0.03_{(2,60)}$	0.974
Initial width (mm)	8.99 ± 0.22	8.92 ± 0.28	8.76 ± 0.24	$0.23_{(2,35)}$	0.792
Final weight (g)	5.73 ± 0.20	5.61 ± 0.20	5.40 ± 0.18	$0.74_{(2,52)}$	0.482
Final length (mm)	79.61 ± 0.89	78.69 ± 1.03	78.32 ± 0.94	$0.70_{(2,52)}$	0.504
Final width (mm)	17.93 ± 0.20	17.79 ± 0.33	17.66 ± 0.21	$0.32_{(2,54)}$	0.735
Weight gain (g)	5.25 ± 0.20	5.12 ± 0.19	4.92 ± 0.17	$0.75_{(2,54)}$	0.473
BCI	0.0023 ± 0.0001	0.0025 ± 0.0001	0.0023 ± 0.0001	$0.48_{(2,54)}$	0.625
HSI	1.59 ± 0.23	1.58 ± 0.19	1.57 ± 0.17	$0.03_{(2,15)}$	0.995
Hepatic lipids (%)	35.85 ± 2.97	27.06 ± 5.89	22.59 ± 2.48	$2.75_{(2,15)}$	0.087
Whole body analysis					
DM (%)	29.28 ± 0.16	28.98 ± 1.28	30.14 ± 0.76	$0.52_{(2,12)}$	0.626
Ash (%)	17.45 ± 0.47	17.90 ± 1.00	17.38 ± 0.92	$0.10_{(2,12)}$	0.916
Lipid (%)	24.64 ± 2.21	26.76 ± 3.76	29.31 ± 2.62	$0.64_{(2,12)}$	0.543
Protein (%)	35.61 ± 1.08	35.03 ± 1.35	32.84 ± 1.23	$1.39_{(2,12)}$	0.285

SGR, specific growth rate; BCI, body condition index; HSI, hepatosomatic index; DM, dry matter.

the whole fish. For whole fish analysis, the dry matter content of each individual was determined by vacuum freeze-drying (VirTis Benchtop 2K; Warminster, Pennsylvania, USA) to constant weight at − 55 °C and 120 μbar for 48 h. Subsequently, the fish were individually homogenised (Magic Bullet MB1001; Pacoima, California, USA) and one third of each fish was used to determine ash, total lipid and crude protein content as described above.

For liver analysis, livers (n = 6) were excised from each fish and weighed to determine the hepatosomatic index:

$$hepatosomatic\ index = \frac{LW_{(final)}{}^{*}}{FW}\,100$$

where $LW_{(final)}$ is the weight of the liver at the end of the growth trial [51]. The livers were then immediately fixed in 4% phosphate buffered formaldehyde prior to histological analysis. The livers were dehydrated in ethanol, transferred to a tissue processor (Intelstint EFTP; Pangalark Pty. Ltd., Alexandra Hilss, QLD, Australia) and embedded in paraffin wax (Histocentre 3; Thermo Fisher Scientific Pty. Ltd., Scoresby, VIC, Australia). The samples were then sliced to 4 μm using a rotary microtome (HM 325; Thermo Fisher Scientific Pty. Ltd., Scoresby, VIC, Australia), mounted onto slides and stained with hematoxylin and eosin using standard histological procedures [52]. Images of the samples were taken at 40 × magnification using a digital camera (DP73; Olympus, Notting Hill, VIC, Australia) attached to a microscope (BX53; Olympus, Notting Hill, VIC, Australia). The images were converted to a black (liver) and white (lipid stores) pixel threshold (Photoshop v. 15.0.1) and the percent surface area coverage of hepatic lipid stores was quantified using Image J (v. 1.42; National Institute of Health, USA). The surface area coverage of hepatic lipid stores from each fish was then used for analysis.

2.5. Colouration

At the end of the growth trial, each fish was photographed to quantify any differences between the intensity of colouration of the fish fed the three treatment feeds. Prior to taking the photographs, each fish was left in a clean experimental tank with no cave and no shade cloth for 10 min. After 10 min, the fish was placed into a small dish filled with water positioned between two halogen lamps (HL254 250 W; Arlec, Blackburn North, Vic, Australia) angled at 45° [53]. The fish was photographed immediately using a digital camera (EOS 600D; Canon, Macquarie Park, NSW, Australia) with a macro lens (SP 60 mm F/2.0; Tamron Co. Ltd., Hasunuma, Saitama Prefecture, Japan) mounted onto a tripod to capture an aerial view of the fish. A colour chart (ColorChecker passport photo; X-Rite Pantone, Grands Rapids, MI, USA) was included in each photograph to standardise the images prior to analysis. This was done by adjusting the temperature, tint, exposure and contrast to obtain RGB values of two neutral greys (3.5 and 8) that matched the predefined values of the colour chart [54,55]. All colour standardisation and analysis was done using Adobe® Photoshop Lightroom (v. 2015.1.1). After the images were standardised, the RGB values of the five lightest spots on the head of each fish were recorded along with the RGB values of the dark areas adjacent to each of the lightest spots to determine the intensity of colouration as follows:

$$Intensity\ of\ colouration = \frac{(R_{light} + G_{light} + B_{light})}{(R_{dark} + G_{dark} + B_{dark})}$$

where a higher number represented a more intense fish colouration [56]. The median intensity of colouration was obtained for each fish and used for analysis.

2.6. Fecundity and fertilisation

After the growth trial, the 14 remaining fish from each treatment were randomly paired off into breeding pairs (n = 7 pairs per feed) based on a randomised block design for size to control for any significant size differences between individuals within a pair. The pairs were kept in the experimental tanks and provided with two ceramic spawning caves (14 cm [l] × 3 cm [w] × 4 cm [h]) with an opening at one end and a removable lid. The pairs were kept on the same feeds as in the growth trials and fed *ad libitum* (four pellets daily) while excess feed was removed twice weekly. The caves were checked each morning for eggs, and the first spawning event after pairing marked time zero for each pair to standardise start times. When eggs were found, they were immediately removed, counted, digitally photographed to calculate egg diameter (mm; n = 20 randomly selected eggs), immersed in a 2% betadine solution (2 × 1 min dip-rinses separated by a 30 s freshwater dip-rinse) and placed into an egg tumbler (Hatching Hope; QLD Fish Breeders, Sunshine Coast, QLD, Australia) taking care never to expose the eggs to air. The tumblers were kept on the same system as the experimental tanks with constant flow and aeration and the eggs were checked daily for evidence of fertilisation. Fecundity was estimated by the number of eggs produced per spawn and the number of days between spawns. Fertilisation success was estimated by quantifying embryonic survival (defined as hatched individuals) compared to the number of eggs produced [57] and reported as % survival. Prior to their release, free-swimming juveniles that had completely resorbed their yolk-sac were also digitally photographed to obtain length (mm) and

width (mm) measurements (n = 20 randomly selected juveniles). All images were taken using a digital camera (Lumix DMC-FT5; Panasonic, Macquarie Park, NSW, Australia) and subsequently analysed using Image J (v. 1.42; National Institute of Health, USA). Each subsequent spawning event was processed in the same way, until each breeding pair spawned four times (~4 months). Data obtained from the first spawn was discarded to mitigate the effects of fish inexperience and for the second, third and fourth spawns, median values for egg diameter and juvenile size were obtained and used for analysis.

2.7. Statistical analysis

Separate one-factor permutational analysis of variances (PERMANOVA) were used to test for significant differences in the physical characteristics of the pellets (mass, length and density of the pellets), and the proximate analysis, amino acid composition and elemental composition of the three treatment feeds (fixed factor). A two-factor repeated measure PERMANOVA was used to test for significant differences in pellet degradation (dry matter content, ash content, total lipids and crude protein) over time between the treatments (fixed factor). In addition, to predict the relationship between proximate analysis factors over time, the data were fitted with generalised additive models (GAMs).

One-factor PERMANOVAs were used to test for significant differences in the initial (0 weeks) and final (27 weeks) weight, length and width of *Ancistrus* fed the three treatment feeds (fixed factor). In addition, to predict the relationship between *Ancistrus* growth (weight, length and width) over time, the data were fitted with generalised additive models (GAMs). One-factor PERMANOVAs were also used to test for significant differences in the weight gain, body condition index, hepatosomatic index and whole fish body analysis (dry matter content, ash content, total lipids and crude protein), between the three treatment groups (fixed factor) at the end of the trial. A two-factor PERMANOVA was used to test for significant differences in the intensity of colouration between the males and females (fixed factor) given the three treatment feeds (fixed factor). One-factor PERMANOVAs were used to test for significant differences in the size and number of eggs, spawning interval, fertilisation rates and the length and width of viable offspring between the three treatment feeds (fixed factor).

All PERMANOVA analyses were performed using PRIMER 6 (v. 6.1.13 [58]) and PERMANOVA + (v. 1.0.3. [59]). For PERMANOVA, Bray-Curtis similarity matrices were produced using the untransformed raw data and dummy variables (0.0001) were used to account for zero values. The p-values were calculated from 9999 random permutations and pair-wise *a posteriori* comparisons were used to determine significant groupings, where applicable. For PERMANOVA, differences were considered significant if $p < 0.05$ and Monte Carlo p-values were used when the number of permutations was low. All data are presented as mean ± standard error unless otherwise indicated. GAMs were produced using the mixed GAM computational vehicle 'mgcv' package within R (v. 3.0.1 [60]) and the data were fitted with cubic regression spline smoothers to examine differences in total degradation and growth as well as differences in the rate of change of degradation and growth between each treatment over time. Models were validated using k-values, normality was tested using Q-Q plots, homogeneity was tested by visual examination of the residuals and differences were considered significant if $p < 0.001$. Goodness-of-fit of the individual smoothers was quantified using the hydroGOF package within R (v. 3.0.1 [60]) and was assessed from the proportion of variance in the data that was accounted for by the model (r^2; [61]).

3. Results

3.1. Treatment feeds

The mass, length or density of the pellets did not differ significantly between the commercial, mix and *Oedogonium* treatment feeds (Table 1). However, there were significant differences in the proximate analysis, the largest of which was in the total concentration of protein (sum of amino acids), which was 24.28 ± 0.06%, 22.21 ± 0.06% and 18.81 ± 0.33% in the commercial, mix and *Oedogonium* feeds, respectively (Table 1). Although the dry matter, ash content, total lipids and total carbohydrates also differed statistically between the feeds, the differences were never > 9% with *Oedogonium* having the highest concentrations of dry matter (90.71 ± 0.35%), lipids (9.74 ± 0.07%) and carbohydrates (51.93 ± 0.25%), and the lowest ash content (10.23 ± 0.02%) compared to the commercial and mixed feeds (Table 1). In addition, although statistically *Oedogonium* had the highest metabolisable energy (15.51 ± 0.02 MJ/kg), it was only 3.2% higher than the commercial and mix feeds. Similar to the total protein concentrations, the quantities of each amino acid tested were consistently highest in the commercial feed and lowest in the *Oedogonium* feed (Table 2). In particular, the concentrations of lysine and methionine as a proportion of the total concentration of protein in each feed were 7.10 ± 0.01, 6.86 ± 0.02 and 6.56 ± 0.02% (lysine) and 2.31 ± 0.04, 1.92 ± 0.03 and 1.74 ± 0.11% (methionine) in the commercial, mix and *Oedogonium* feeds, respectively.

The five most abundant elements in the three feeds were Ca, K, Mg, Na and P, accounting for between 97 and 98% of the elements quantified. Although the total concentration of these five elements was similar across feeds, the ratios varied (Table 1). The commercial feed had the highest concentrations of Ca (14,667 ± 333 mg/kg), Na (8167 ± 393 mg/kg) and P (11,667 ± 333 mg/kg), and *Oedogonium* had the highest concentrations of K (27,667 ± 333 mg/kg) and Mg (5300 ± 58 mg/kg) while the mix feed had concentrations of elements approximately half those of the commercial and *Oedogonium* feeds. The remaining elements quantified (Fe, Al, Zn and Mn) comprised between 2 and 3% of the elemental composition in total with concentrations ranging from 10 ± 0 to 637 ± 33 mg/kg (Table 1).

3.2. Pellet stability

There was a clear distinction between the visible degradation of the pellets after only 8 h of submergence and these effects remained consistent for all three feeds until the end of the trial at 48 h (Fig. 1). The commercial feed had completely broken down into a fine particulate matter within 8 h covering the entire base of the experimental dish, and by 48 h most of the material had dissolved. Likewise, the mix feed had also completely broken down within 8 h, although the material was visibly coarser and it did not dissolve into the tank. Notably, the *Oedogonium* feed, which expanded to approximately twice the size, remained intact for the entire 48 h.

The pellet biomass collected from the end caps was subsequently used for proximate analysis, where there were significant feed*time interactions for dry matter content (PERMANOVA, pseudo-$f_{(14,48)} = 10.16$, $p < 0.001$; GAM, $F = 9.01_{(2)}$, $p = 4e^{-04}$, $r^2 \geq 0.93$; Fig. 2A), ash content (pseudo-$f_{(14,47)} = 8.70$, $p < 0.001$; GAM, $F = 8.14_{(2)}$, $p = 0.0009$, $r^2 \geq 0.97$; Fig. 2B), total lipids (pseudo-$f_{(14,48)} = 7.60$, $p < 0.001$; GAM, $F = 23.06_{(2)}$, $p = 3.9e^{-8}$; $r^2 \geq 0.84$; Fig. 2C) and crude protein (pseudo-$f_{(14,48)} = 7.31$, $p < 0.001$; GAM, $F = 10.77_{(2)}$, $p = 0.0001$, $r^2 \geq 0.88$; Fig. 2D). At the beginning of the submergence trial until 8 h, the rates of decrease of proximate analysis factors were either similar across feeds (dry matter, total lipids and crude protein) or the mix and *Oedogonium* feeds had faster rates of loss compared to the commercial feed (ash content). However, after 8 h of submergence, the rate of loss for the commercial feed increased for all factors quantified, and at 48 h, the commercial feed had 59.4 and 67.1% less dry matter, 53.9 and 40.0% less ash content, 50.0 and 51.9% less total lipids and 53.7 and 43.6% less crude protein compared to the mix and *Oedogonium* feeds, respectively. There were also differences between the mix and *Oedogonium* feeds at 48 h where the mix feed had 18.8% less dry matter and 17.9% more protein.

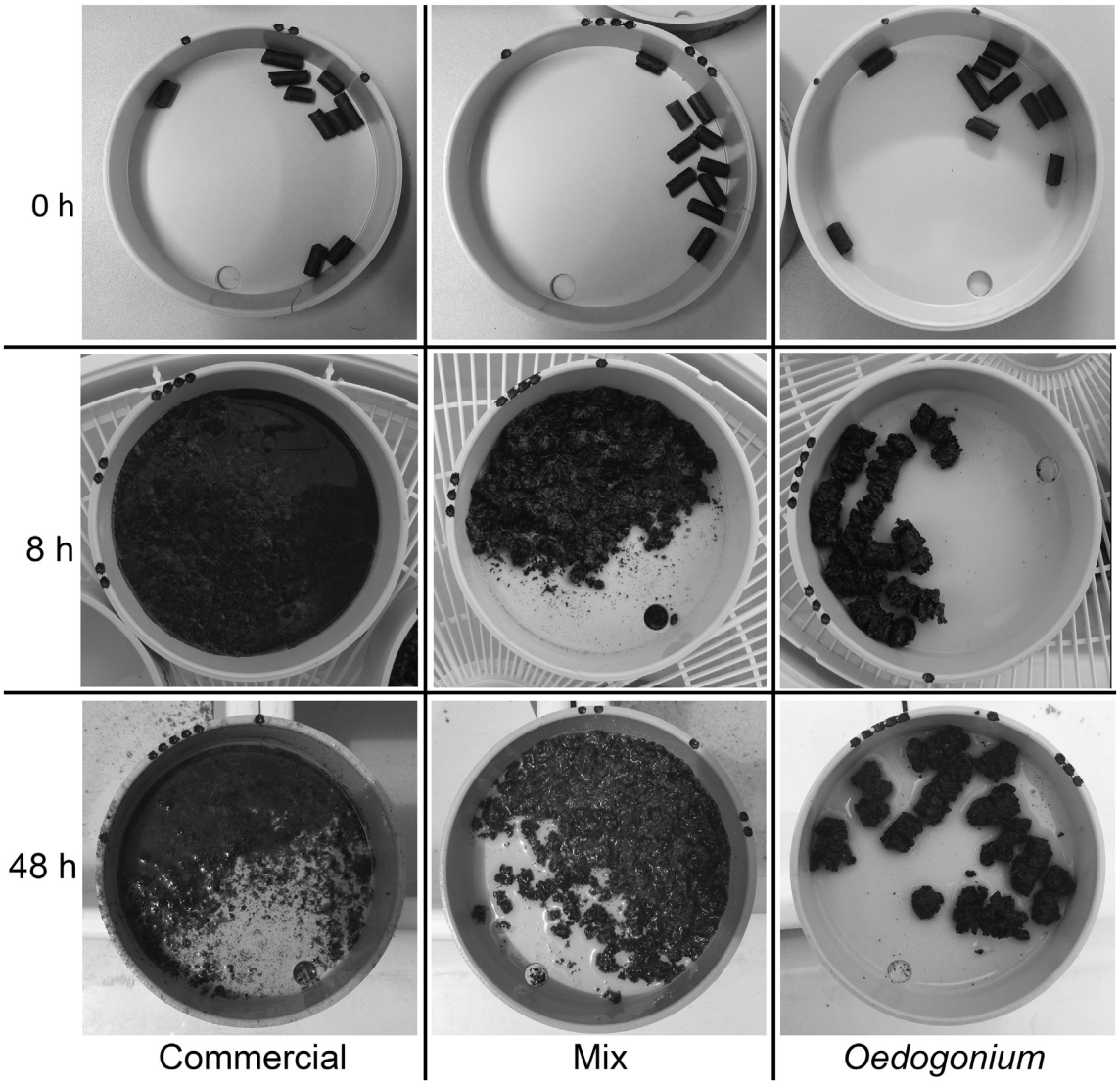

Fig. 1. Ten pellets from each treatment feed (commercial, mix and *Oedogonium*) were placed into 100 mm polyvinyl chloride end caps and submerged to determine their physical stability over time (presented here at 0, 8 and 48 h).

3.3. Growth and body condition

There were no significant differences in weight, length and width of *Ancistrus* between the three treatment feeds at the start and at the end of the 27 week growth trial (Table 3). There were also no significant differences in *Ancistrus* weight gain, body condition index, hepatosomatic index or whole body analysis factors (dry matter, ash, lipids and protein) between the three feeds at the end of the trial (Table 3). Importantly, the commercial feed had the largest surface area coverage of hepatic lipid stores (35.85 ± 2.97%) followed by the mix feed (27.06 ± 5.89%), while *Oedogonium* had the lowest coverage (22.59 ± 2.48%), however, there were no significant differences between the three feeds (Table 3). In addition, despite the lack of significant effect of feed on size of *Ancistrus* at the end of the 27 week growth trial, between weeks 9 and 21, the fish on the commercial feed grew at a faster rate than those on the mix and *Oedogonium* feeds. This was true for weight (GAM, $F = 23.30_{(2)}$, $p = 1.9e^{-10}$, $r^2 \geq 0.89$; Fig. 3A), length ($F = 183.60_{(2)}$, $p < 2e^{-16}$, $r^2 \geq 0.93$; Fig. 3B) and width ($F = 122.80_{(2)}$, $p < 2e^{-16}$, $r^2 \geq 0.87$; Fig. 3C). However, these differences were never > 15% during that time and by the end of the trial were not significant due to a reduction in the rate of growth of *Ancistrus* fed the commercial feed.

3.4. Colouration

There were no effects of feed treatment on the intensity of colouration (pseudo-$f_{(2,48)} = 0.03$, $p = 0.990$) and no interaction between sex and feed (pseudo-$f_{(2,48)} = 0.15$, $p = 0.881$). The intensity of colouration of female *Ancistrus*, which was 1.89 ± 0.11, 1.86 ± 0.10 and 1.85 ± 0.12 for the three treatment feeds (commercial, mix, *Oedogonium*), respectively, was significantly lower (PERMANOVA, pseudo-$f_{(1,48)} = 33.47$, $p < 0.001$) than the intensity of colouration of the male *Ancistrus*, where the intensity of colouration was 2.42 ± 0.13, 2.51 ± 0.17 and 2.52 ± 0.13 for the three treatment feeds, respectively.

3.5. Fecundity and fertilisation

At the end of the breeding trial, after each pair had spawned four times, pairs fed the commercial feed had smaller eggs (2.65 ± 0.03 mm) than pairs fed the mix (2.73 ± 0.02 mm) and *Oedogonium* (2.75 ± 0.02 mm) feeds (PERMANOVA, pseudo-$f_{(2,60)} = 5.44$, $p = 0.006$; Fig. 4a) but had more eggs per spawn (135.19 ± 5.76) than pairs fed the mix (113.43 ± 4.96) and *Oedogonium* (118.76 ± 2.36) feeds (pseudo-$f_{(2,60)} = 5.54$, $p = 0.005$;

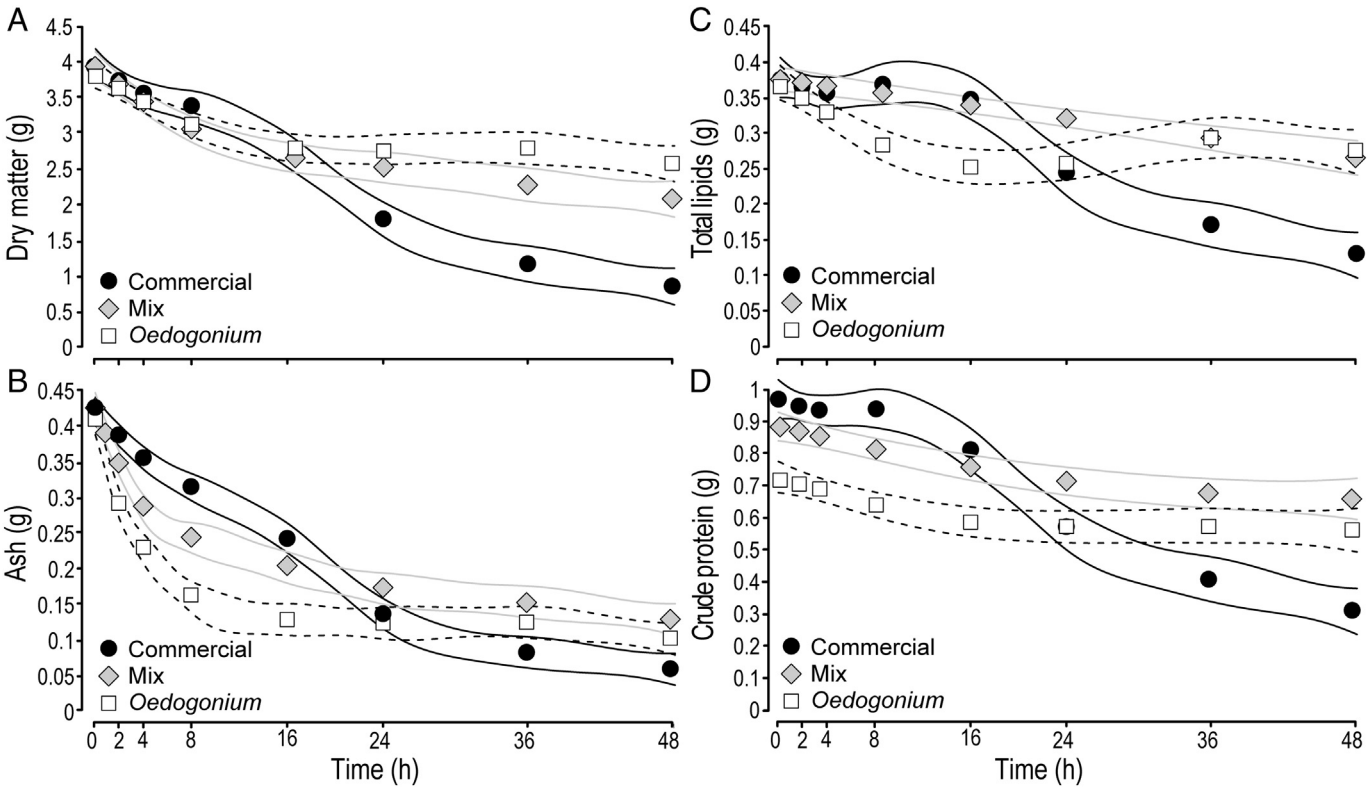

Fig. 2. Pellets from each treatment feed (commercial, mix and *Oedogonium*) were submerged to quantify changes in (A) dry matter content; (B) ash content; (C) total lipids; and (D) crude protein over 48 h. Black, grey and dashed lines represent confidence intervals for each treatment as predicted by the generalised additive model.

Fig. 4b). There were also differences between the spawning interval (pseudo-$f_{(2,60)} = 5.54$, $p < 0.014$; Fig. 4c), however, these differences never exceeded more than four days, where pairs fed the mix feed had the shortest interval between spawns (24.14 ± 0.57 days) and pairs fed the *Oedogonium* feed had the longest interval between spawns (27.95 ± 0.91 days), while pairs fed the commercial feed were similar to both (25.62 ± 1.33 days). Conversely, there were no effects of treatment feed on fertilisation rates (pseudo-$f_{(2,60)} = 2.43$, $p = 0.096$; Fig. 4d) which ranged from 97.49 ± 0.84% survival (commercial feed) to 99.48 ± 0.22% survival (mix feed). In addition, there were also no effects of treatment feed on the length (pseudo-$f_{(2,60)} = 0.13$, $p = 0.872$; Fig. 4e) and width (pseudo-$f_{(2,60)} = 0.41$, $p = 0.664$; Fig. 4f) of free-swimming juveniles which had completely resorbed their yolk-sacs, which ranged from 11.96 ± 0.12 mm (commercial feed) to 12.02 ± 0.10 mm (*Oedogonium* feed) and 2.68 ± 0.03 mm (mix feed) to 2.71 ± 0.03 mm (commercial feed), respectively.

4. Discussion

This study has established the suitability of *Oedogonium* as a complete feed for all life-stages of the herbivorous catfish, *Ancistrus*. Over the 10 month trial, *Ancistrus* that were fed exclusively on *Oedogonium* had equivalent growth rates, body condition, colouration, fecundity and fertilisation rates compared to those fed the mix feed and the commercial feed, which was formulated to meet all the requirements of herbivorous fishes. The absence of any major differences of these characteristics between feeds, especially between *Oedogonium* and the commercial feed, demonstrated that *Oedogonium* can meet all of the nutritional requirements of this herbivorous ornamental fish. This is a positive outcome, especially considering the sustainability of *Oedogonium* as a feed source. The *Oedogonium* biomass used in this study was cultivated in the treated discharge water from a municipal wastewater treatment plant, where it acted as a tertiary treatment system and recovered residual nutrients, transforming them into algal protein.

The crude protein concentration of *Oedogonium* (18.81 ± 0.33%) was 22.5% lower than that of the commercial feed (24.28 ± 0.06%), however, there is strong evidence that the protein supplied in all three treatment feeds was adequate, as growth, condition and reproductive output were equivalent in all fish across the treatments. The success of any feed is linked to not only the crude protein content, but also the energy concentration, digestible energy and amino acid composition [62]. Here, although *Oedogonium* had a lower concentration of crude protein compared to the commercial feed, it also had higher total lipids and carbohydrates and, therefore, an equivalent metabolisable energy content to the mix and commercial feeds. In addition, in their natural habitat, algae make up a large proportion of the *Ancistrus* diet [40] and like all herbivorous fish, *Ancistrus* has the necessary physiology to access and digest the carbohydrates and amino acids in plant-based materials [63]. Furthermore, *Oedogonium* had a well-balanced ratio of EAA to NEAA as seen previously [8,14,20], and although the concentrations of each individual amino acid tested were lower in *Oedogonium* than the commercial feed, they were not low enough to become deficient in *Ancistrus* and cause detrimental effects on growth and health. This is especially important for lysine and methionine, which are the first limiting amino acids in plant-based diets [64–66], and establish the maximum potential threshold of a feed [21]. Notably, all the EAA tested, in particular arginine, lysine, methionine, histidine, threonine, valine, isoleucine, leucine and phenylalanine, were found in high enough concentrations as a proportion of the total protein content to, not only satisfy the requirements of *Ancistrus* in this study, but also be within the range of amino acid requirements for some commercially-important fishes from each trophic level [67,68]. The results from this study are promising and have implications for both the aquatic ornamental industry and finfish aquaculture.

The aquatic ornamental industry could benefit from the use of *Oedogonium* as-is. Contrary to aquaculture where high growth rates are essential, the most important factors for keeping ornamental fishes are

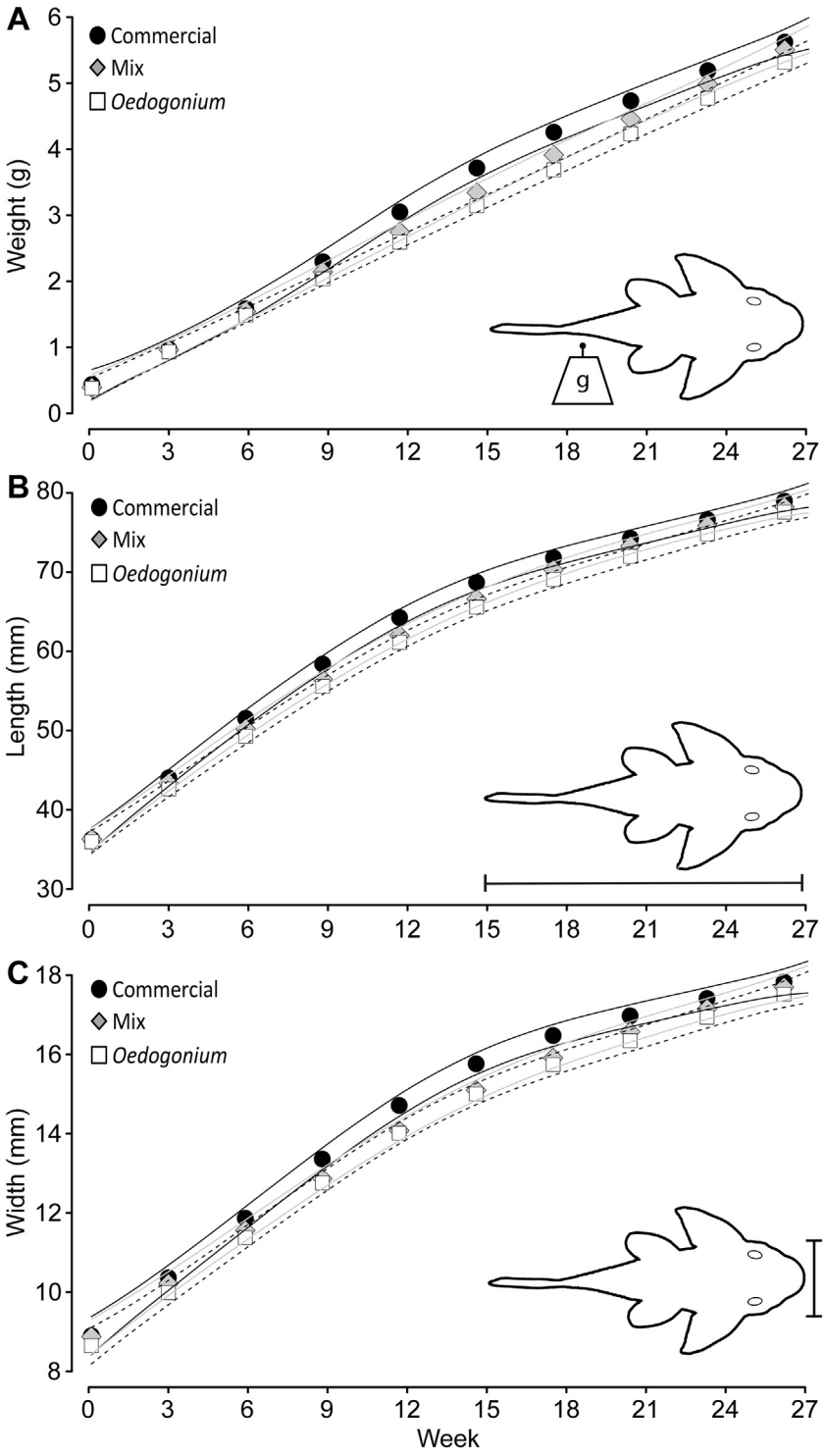

Fig. 3. Change in (A) weight; (B) length; and (C) width of *Ancistrus* fed three feeds (commercial, mix and *Oedogonium*) for 27 weeks. Black, grey and dashed lines represent confidence intervals for each treatment as predicted by the GAM.

condition, colour intensity and subsequently, breeding health [26]. *Oedogonium* used as a complete feed would be able to meet the nutritional requirements of suitable herbivorous fishes within this industry. Notably, the *Oedogonium* pellets used in this study were physically more stable than the mix and commercial pellets, which would reduce the occurrence of fine particulate matter, and minimise tank fouling.

Herbivorous and omnivorous finfish aquaculture comprises over 60% of the production of fish for human consumption [69,70]. These species, from lower trophic levels, in particular milkfish and grass carp (herbivores), and channel catfish, tilapia and common carp (omnivores), generally have total protein requirements of between 23–40% and 25–44%, respectively [22,68,71]. However, the optimum concentration of dietary protein can be directly or indirectly dependent on, for example, the size of the fish, where larger fish require less protein [72], and culture conditions, such as temperature and salinity, where higher temperatures and salinities reduce the amount of protein required [42,68,73]. As such, several studies have reported that dietary protein concentrations as low as 24% for channel catfish [74], 20% for tilapia [75] and 24% for milkfish [71] can sustain an equivalent rate of growth to that of control feeds, with higher concentrations of protein. *Oedogonium* has the potential to meet these demands as the culture of the algal biomass can be manipulated such that the total amino acid

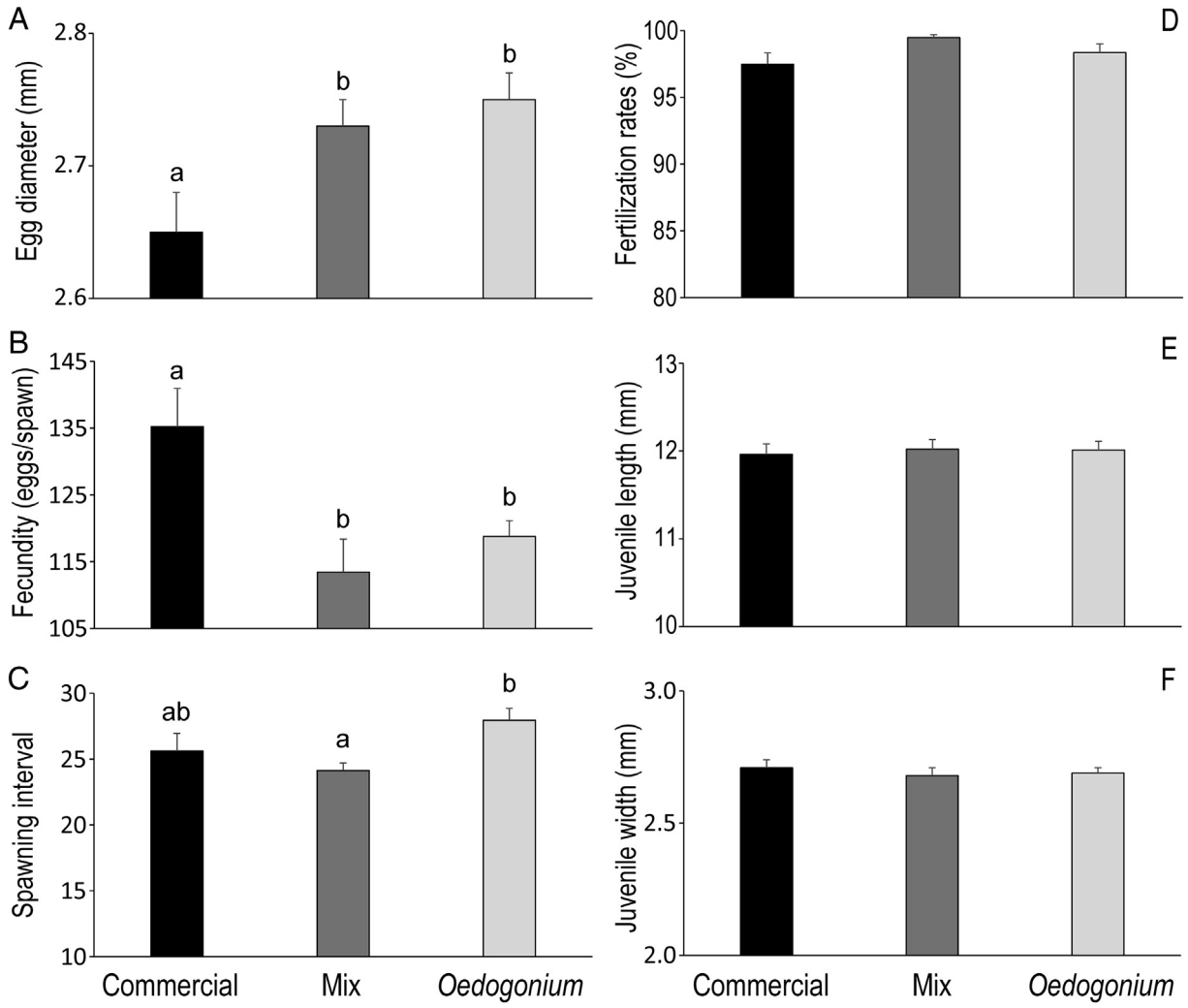

Fig. 4. Breeding trial with *Ancistrus* fed three feeds (commercial, mix and *Oedogonium*) characterising (A) egg diameter; (B) fecundity; (C) spawning interval; (D) fertilisation rates; and (E) length and (F) width of juveniles. Superscript letters represent significant groupings; data displayed as mean ± SE.

content can be increased to as high as 26.5% [20]. In addition, *Oedogonium* has a suitable carbohydrate to lipid ratio (4.4:1–5.3:1 [7,8]) for herbivorous and omnivorous fishes, including channel catfish, grass carp and tilapia which have optimal carbohydrate to lipid ratio requirements of 4.5:1 [76], 4.7:1 [77] and 5.0:1 [78], respectively. Even a partial addition of *Oedogonium* into the feeds of these commercially-important species will have the added advantage of reducing the dependence of the aquaculture industry upon a limited set of feed ingredients [70,79,80]. Feed represents up to 66% of the costs associated with aquaculture [81,82], putting pressure on the industry to find cost-effective alternatives, particularly in developing economies [83]. However, extensive dietary experiments will be required on a per-species basis to optimise growth and product quality. As such, if the right fish is paired with the appropriate rearing conditions and algal biomass feedstock, then *Oedogonium* has the potential to become a beneficial feed ingredient for commercially-important herbivorous and omnivorous fishes.

Oedogonium would not be suitable as a complete feed for carnivorous finfish aquaculture due to the protein requirements of fishes from higher trophic levels. Species such as salmon, trout and seabass, generally have total crude protein requirements of between 34 and 55% [22,68] and lack the necessary physiology to access plant-based protein [63,84,85]. However, the biochemical properties of macroalgae, including protein content, can be manipulated using post-production processing techniques to obtain biomass with specific end-use characteristics [86–91]. More specifically, to extract or concentrate protein from algal biomass, the most commonly used methods are milling, osmotic shock, ultrasonication, microwave assisted extraction, digestion with enzymes and the use of alkaline solutions and reducing agents [92–94]. The success of those methods is dependent on their ability to break down cell wall polysaccharides which bind the protein molecules and their effectiveness at minimising the quantity of non-protein fractions in the final product thereby maximising the concentration of protein. To date, the most effective methods can extract approximately 60% of the original protein content from the algal biomass with approximately 29% of the final product as non-protein fractions [95]. If these methods can be modified to extract or concentrate the high-quality protein in *Oedogonium*, there is potential for *Oedogonium* to be used as a protein component in a formulated feed, with the caveat that the digestibility of the concentrated extract must be demonstrated. Notably, *Oedogonium* could then be used to offset the need for fishmeal in the culture of higher trophic level species, since, although carnivorous finfish comprise < 17% (~5 M tonnes) of the production of fish for human consumption, the inclusion rates of fishmeal in the feeds of these fishes can be up to 65%, representing a substantial proportion (~45%) of the global demand [69,80].

In conclusion, this study demonstrated the viability of *Oedogonium* as a feed for herbivorous ornamental fish based on its amino acid and nutrient profile, its effects on growth, condition, colouration and reproductive output, and its physical stability in the water. As such,

linking the biomass produced with the specific diet demands of herbivorous ornamental fish may provide an ideal application for *Oedogonium*.

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