A comparison between marine and terrestrial invertebrate meals for mirror carp (Cyprinus carpio) diets: impact on growth, haematology and health

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DOI: 10.1111/are.13318

A comparison between marine and terrestrial invertebrate meals for mirror carp (*Cyprinus carpio*) diets: Impact on growth, haematology and health

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Abstract

Invertebrate-meals (e.g. polychaetes and insects) present novel and sustainable high quality nutrient sources for use in fish feed formulations. To test this innovative source, an eleven-week feeding trial was conducted evaluating the effects of replacing the fishmeal (FM) component as an example of a superior protein source (FM CTRL) with ragworm meal (RW, *Nereis virens*), or/and silkworm pupae (SWP, *Bombyx mori*) in mirror carp (*Cyprinus carpio*) diets. Three experimental diets with partial replacement of FM (diets: RW+FM, SWP+FM, and RW+SWP+FM) were formulated. All diets were formulated to be *iso*-nitrogenous, *iso*-lipidic, and *iso*-energetic. Growth performance and feed utilisation indices were assessed, and the feeding trial concluded with the analysis of haematological parameters to provide an indication of carp physiological and health status. Mean weight gain was greatest in mirror carp fed RW+FM (60.83 fish^{-1} day^{-1}; $P<0.05$ vs all other diets) followed by SWP+FM (40.62 g fish^{-1} day^{-1}; $P<0.05$ vs all other diets). The least weight gain was achieved in fish fed FM+SWP+RW+ and FM CTRL (34.34 g fish^{-1} day^{-1} and 33.96 g fish^{-1} day^{-1}, respectively; not significantly different from each other). Fish fed on RW+FM diet had significantly lower plasma ammonia concentrations than any other dietary groups ($P=0.04$). Mirror carp fed on SWP+FM diet (111.52 units mL^{-1}) were observed to have a marked enhancement in alternative complement activity than FM CTRL (79.21 units mL^{-1}, $P=0.041$). Both ragworm and silkworm pupae meal present attractive sustainable functional feed component in carp diets, with benefits on enhancing growth performance and specific physiological parameters.

Keywords: Invertebrate meal; Carp; Silkworm pupae; *Bombyx mori*; Ragworm; *Nereis virens*.

Introduction

Finfish aquaculture has expanded to become one of the largest and fastest sectors in the food production industry. In 2013, 70.5 million tonnes of farmed food fish were produced, and since 1980 the production level of world aquaculture has increased at an annual rate of 8.6% (FAO, 2014). Consequently, the demand for aquafeeds and its main protein constituent, fishmeal, has also dramatically increased. In 2009, it was estimated that 30% of capture fisheries production was processed into fishmeal and fish oil (Olsen & Hasan, 2012). However, with dwindling wild fish stocks, fishmeal is now considered to be a finite protein source and to be used strategically. Responding to the bottleneck in aquaculture production, many governments, researchers and aquafeed manufacturers have now sought to evaluate possible alternatives that are deemed more sustainable.

Previous studies on fishmeal alternatives include various fisheries by-products (Toppe, Aksnes, Hope, & Albrektsen 2006; Lee, Powell, Barrows, Smiley, Bechtel & Hardy 2010), terrestrial animal by-products (Davies, Gouveia, Laporte, Woodgate & Nates 2009), single-cell organisms (Lunger, Craig & McLean 2006; Zerai, Fitzsimmons, Collier & Duff 2008), algae (Soler-villa, Coughlan, Guiry & Kraan 2009; Xu, Zhang, Wu, Liu, Wang, You & Li 2011) and plant meals (Opstvedt, Aksnes, Hope & Pike 2003; Torstensen, Espe, Sanden, Stubhaug, Waagbø, Hemre, Fontanillas, Nordgarden, Hevrøy, Olsvik & Berntssen 2008). However, in some instances fishmeal replacement candidates can cause a variety of physiological problems in the fish, e.g. depressed growth rates, nutritional health problems or reduced palatability (Deng, Mai, Qinghui, Zhang, Wang, Xu, Liufu 2006; Hardy, 2010). These are often attributed to the presence of Anti-Nutritional Factors (ANF's), reduced nutrient availability, and/or the deficiencies in essential nutrient(s), e.g. amino acid, vitamins and trace metals (Francis,

71 Makkar & Becker 2001; Opstvedt *et al*. 2003; Fasakin, Serwata & Davies 2005; Hansen
72 Rosenlund, Karlsen, Wolfgang & Gro-Ingunn 2007).
73
74 In recent years, the use of invertebrate meals has gained much interest as a sustainable
75 alternative to fishmeal (Barroso, De Haro, Sánchez-Muros, Venegas, Martínez-Sánchez
76 & Pérez-Bañón 2014; Henry, Gasco, Piccolo, & Fountoulaki 2015). Invertebrate meals,
77 such as black soldier fly, housefly, and silkworm pupae have been tested in a range of
78 dietary inclusion levels (5 to 30%) in African catfish (Ng, Liew, Ang & Wong 2001),
79 channel catfish (Bondari & Sheppard, 1987), chum salmon (Akiyama, Murai, Hirasawa
80 & Nose 1984), common carp (Nandeesha, Gangadhara & Manissery 1999), rainbow
81 trout (St-Hilaire, Sheppard, Tomberlin, Irving, Newton, McGuire, Mosley, Hardy &
82 Sealey 2007), and blue tilapia (Bondari & Sheppard, 1987). These studies have shown
83 that invertebrate meals have promising attributes, providing a sustainable protein rich
84 alternative, an adequate amino acid profile, highly digestibility, and improving fish
85 growth performance (Barroso, De Haro, Sánchez-Muros, Venegas, Martínez-Sánchez,
86 & Pérez-Bañón, 2014).
87
88 Ragworm (*Nereis virens*) is a marine polychaete worm found in the benthic strata of
89 northern hemisphere estuarine habitats. Preliminary studies have indicated its suitability
90 as a feed ingredient for both crustacean and finfish production (Day, Howell & Jones
91 1997, Salze, McLean, Battle, Schwarz & Craig 2010). Similarly, meals derived from the
92 silkworm (*Bombyx mori*) pupae have been well documented as suitable fishmeal
93 alternatives in diets of meat-producing terrestrial species (Ijaiya & Eko, 2009; Medhi,
94 Nath, Gohain, & Bhuyan 2009). Growth studies performed on carp species (Nandeesha,
95 Gangadhara, Varghese & Keshavanath 2000; Rangacharyulu, Giri, Paul, Yashoda, Rao,

Mahendrakar, Mohanty & Mukhopadhyay 2003) and walking catfish (*Clarias batrachus*, Habib, Hasan, Akand & Siddiqua 1994) have shown that replacement of fishmeal by silkworm pupae meal maintained or exceeded the growth performances compared to the dietary reference treatment group. More recently however, Ji, Zhang, Huang, Cheng & Liu (2015) tested the feasibility of replacing fishmeal with silkworm meal for juvenile Jian carp in multi-ingredient formulated diets containing just 10% fishmeal. The study reported reduced growth rate and impaired anti-oxidant enzyme status, decreased digestive function and unfavourable changes in hepatic and intestinal morphology for these carp fed higher silk worm pupae meal dietary inclusion.

Cyprinids (carp) are an important freshwater farmed fish in Eastern Europe, India and China where demand is high. In 2010, China has produced over 15 million tonnes of farmed cyprinids, ranging from grass carp (*Ctenopharyngodon idella*) to common carp (*Cyprinus carpio*) (Chiu, Li, Guo, Bai, Fedor & Naylor 2013). Furthermore, Koi are the genetic variant of the domesticated farm carp, and are highly prized in the ornamental pet fish trade as companion animals (FAO, 2014) in many parts of the world. It is therefore important to assess the potential of novel feed ingredients for this species in the context of sustainability with respect to the needs of both the aquaculture and aquatics industries. For this reason, an investigation was directed to assess a commercially produced marine invertebrate (polychaete worm meal) and a commercially produced terrestrial invertebrate (silkworm meal) tested singly and in a combination, in experimental diets for mirror carp substituting the fishmeal component in formulated experimental diets for mirror carp.

Materials and Methods

The present study examined the effects on both growth performance and haematological parameters (standard, biochemical and immunological), when ragworm or/and silkworm pupae meal was assessed against a single primary protein (fishmeal) in balanced diets for juvenile mirror carp (*Cyprinus carpio*) as a standard protein of defined Biological Value (BV).

Fish and experimental facilities

An eleven week feeding trial was conducted at the Aquaculture and Fish Nutrition Research Aquarium, University of Plymouth. Mix-sex mirror carp (*n*=240) were sourced from Hampshire Carp Hatcheries (Bowlake fish farm, UK) and acclimatised for 5 weeks in an experimental re-circulated system. During acclimatisation fish were fed on a commercial EWOS micro 50P diet (EWOS Ltd., UK). The experimental re-circulated system comprised 12 (38 cm x 38 cm x 50 cm) fibreglass tanks suspended over a 900 L filter sump tank. The core experimental design consisted of four test diets each with three replicate test groups randomized over the twelve tanks in the holding system. Fish were graded and stocked at a density of 20 fish per tank, with an initial mean fish weight of 14.9 ±0.13 g (± S.E.M, *n*=20). Each tank had an aerated flow rate of 4 L min^{-1} and was maintained at 24.6 ±0.2 $^{\circ}$C with a dissolved oxygen level of >89%. Nitrogenous waste levels were monitored weekly and maintained to values of (means ≤, mg L^{-1}, ± S.E., *n*= 8) ammonium, 0.1 ±0.02; nitrite 0.03 ±0.01 and nitrate 38 ± 6 [Hach Lange, Salford, UK]. Photoperiod was set on a diurnal cycle of 12 h light and 12 h darkness. Diets were given at a ration level of 3% of body weight per day. Batch group

143 weights of each tank were measured on a weekly basis to determine the growth
144 performance:
145 Weight gain: Final weight [g] - Initial weight [g]
146 Specific growth rate (SGR): ln(final body weight [g]) - ln(initial body weight [g]) / time
147 [days] x 100
148 Food conversation ratio (FCR): feed fed [g] / live weight gain [g]
149 Protein efficiency ratio (PER): weight gain [g] / protein ingested [g].
150 At the conclusion of the feeding experiment, six fish from each tank were randomly
151 sampled for blood analysis that included basic haematology, biochemical and
152 immunological haematology parameters. All work was conducted according to the 1986
153 Animal Scientific Procedures Act (UK Home Office) regulations of the UK, the
154 University of Plymouth's ethical approval process, and the Ethics Committee of the
155 WALTHAM Centre for Pet Nutrition.
156

157 ## Diets

158 Three experimental diets were formulated with a protein replacement level of 6.33% for
159 silkworm pupae meal (SWP+FM diet), 7.52% ragworm meal (RW+FM diet), 3.30%
160 and 4.06% of silkworm pupae and ragworm meal, respectively (SWP+RW+FM diet).
161 Calcium chloride (2.98%) was added to SWP+RW+FM diet to maintain the calcium
162 content due to low calcium levels in this specific diet mixture compared to the reference
163 diet. This reference diet was produced with no invertebrate inclusion and providing a
164 superior basal protein BV consisting of LT (Low Temperature) fishmeal. The
165 formulation and preparation process included the use of a commercial food processer
166 [HL1400-10STDA, Hobart Food Equipment, Australia] to blend the feed materials into
167 a dough consistency and cold extruded through a PTM P6 feed extruder system

168 [Plymouth Marine Ltd., UK]. A final pellet size of 2 mm in diameter and 5 mm in
169 length was produced. Diets were dried in a dehumidifying oven at 46 °C for 48 h.
170

171 Proximate analysis

172 Moisture, crude protein, crude lipid, and ash in feed ingredients (Table 1) and finished
173 diets (Table 2) were determined by following AOAC (1995) standard methods. Briefly,
174 moisture was determined by heating samples to 105 °C, until a constant weight was
175 achieved. Crude protein was calculated from the nitrogen content (N x 6.25) using the
176 Kjeldahl method. Samples were first acid digested using a Kjeldahltherm microsystem
177 40 [C. Gerhardt GmbH & Co. KG, Germany] and distilled in a Vapodest 40 [C.
178 Gerhardt GmbH & Co. KG, Königswinter, Germany]. Crude lipid content in samples
179 was determined through the petroleum ether extraction method, using a Soxtec extractor
180 HT 1043 extraction unit [Foss Tecator AB, Hoganas, Sweden], while crude ash content
181 was determined by igniting samples at 600 °C for 16 h and weighing the residual ash.
182 Energy values were measured using a bomb calorimeter Parr 1356 [Parr Instrument
183 Company, Illinois, US].
184

185 Amino acid analysis

186 Amino acid composition (Table 2) was performed on hydrolysed weighed samples of
187 feed in sealed ampoules in *vacuo* with 6N HCl at 110°C for 22 hrs. Excess acid was
188 removed by a flash evaporator under reduced pressure at below 40°C. Samples were
189 reconstituted in buffer and amino acid analysis conducted on a Biochrome 30 Amino
190 Acid Analyser [Biochrom, Cambridge, UK]. Methionine was determined separately
191 using a performic acid oxidation. Tryptophan was recovered in a separate alkaline

192 hydrolysis step according to standard protocols (Longvah, Mangthya & Ramulu, 2011;
193 Davies & Gouveia, 2000).
194

195 Blood sample collection

196 At the conclusion of the experiment, blood samples were collected from the
197 experimental fish to examine possible modifications in the components of carp blood,
198 which could give indications on physiological health and nutritional status (Kaushik &
199 Seiliez, 2010). Samples were collected by lightly anaesthetising fish in an aerated
200 anaesthetic bath (120 mg L^{-1}, tricane methane sulphonate, Pharmaq, Fordingbridge,
201 UK). Six fish from each tank were sampled for blood at the caudal ventral vein, using a
202 25-gauge needle and 1 mL syringe. Needles and syringes used for plasma collection
203 (n=3) were heparinised prior to collection (Walencik & Witeska, 2007). Blood samples
204 were immediately centrifuged at 13,000 rpm for 11 mins and collected supernatant was
205 subjected to a further 1 min centrifuge at 13,000 rpm and aliquot for storage at -80 °C.
206 For serum collection (n=3), blood was allowed to clot for 24 h at 2-4 °C and centrifuged
207 at 13,000 rpm for 11 mins. Supernatant was centrifuged for a further 1 minute. Samples
208 were stored at -80 °C for later analysis.
209

210 Basic haematology parameters

211 Freshly collected whole blood was immediately used in the following haematological
212 parameters. Pack cell volume was determined using a micro hematocrit method, as
213 described by Dacie & Lewis (1984). Haemoglobin determination was performed
214 through the cyanmethaemoglobin method (Dacie & Lewis, 1984) using a commercial
215 Drabkin's cyanide-ferricyanide solution [Sigma Alderich, Poole, UK]. For total

erythrocyte, 20 µL of whole blood was fixed in 1 mL of Dacies fluid (Dacie & Lewis, 1984) and counts carried out by the method described in Handy & Depledge (1999).

Haematological and related immune parameters

Serum haemolytic complement activity through the alternative pathway was performed as described by Yano (1992), using washed rabbit red blood cells (2 x 10^8 cells mL^{-1}, RaRBC) as the target cells [TCS, Botolph Claydon, England]. Briefly, the three serum samples from the same tank were pooled together (250 µL) and diluted 20 fold using Mg^{2+}-EGTA-GVB buffer (20 mL of 4.15 g NaCl, 0.51 g $C_4H_3N_2NaO_3$, 1.75 mL 1 N HCl, dissolved in 100 mL dH_2O, pH 7.5; 10mL of 3.8 g EGTA, 2.03 g magnesium chloride hexahydrate, 0.7 g NaOH, dissolved in dH_2O, pH7.5; 70 mL of 0.1 g gelatin, dH_2O, pH 7.5). Diluted serum was mixed with 100 µL of RaRBC suspension and incubated for 90 mins (20 °C), with occasional shaking. The reaction was ceased by adding 3.15 mL cold phosphate buffer saline to the suspension and centrifuged at 10,000 rpm for 5 min. Supernatant was measured for its maximal absorbance at 414 nm. Total haemolysis was carried out by adding 100 µL RaRBC suspension to 3.4 mL dH_2O. The extent of haemolysis for each serial dilution was calculated by following the equation: Haemolysis (ACH_{50}) = 1/k x (reciprocal of initial dilution) x 0.5.

Serum lysozyme activity determination was achieved using the turbidimetric method as described by Ellis (1990). Briefly, 50 µL of serum sample was mixed into 1.95 mL suspension of 0.2 mg mL^{-1} *Micrococcus lysodeikticus* [Sigma Alderich, Poole, UK] in 0.05 M sodium phosphate buffer (pH 6.2). Reduction of turbidity was measured (450 nm) after mixing from time 0 to 5 mins at 20 °C. Lysozyme activity is defined as one unit of enzyme producing an absorbance decrease of 0.001 min^{-1}.

Haematological biochemistry

Total serum protein was assayed on a 96 multi-well plate, using commercial Bradford's dye solution [Sigma Alderich, Poole, UK]. Four microlitres of diluted serum sample (1:40) was mixed with 200 µL of Bradford solution. The mixture was agitated for 30 seconds before incubation at 25 °C for 10 mins and was read on a microplate reader (595 nm). Albumin was determined using a bromocresol green dye- binding method (Spencer & Price, 1977). Serum samples (20 µL) were mixed with 4 mL of buffered dye solution and incubated at 25 °C for 10 mins before absorbance was measured at 630 nm. Linear standardisation of both methods was carried out using known concentrations of bovine serum albumin and human serum albumin [Sigma- Aldrich, Poole, UK], respectively. Serum globulin was estimated as the difference between total serum protein and serum albumin; thereafter albumin and globulin ratio was subsequently calculated.

Plasma glucose was measured through the oxidase-peroxidase method (Trinder, 1969). Plasma samples (20 µL) were incubated with 3 mL of oxidase-peroxidase stock solution (16 mg 4-aminoantipyrine, glucose oxidase (1800 units, source: fungal, EC 1.1.3.4) [Sigma-Aldrich, Poole, UK.], peroxidase (100 units, source: horseradish, EC 1.11.1.7) [Sigma-Aldrich, Poole, UK.], 105 mg phenol, and 0.01% (v/v) Tween-20, made up to 100 mL phosphate buffer (100nm, pH 7.0) for 15 mins at 37 °C. This reaction was ended by rapid cooling and absorbance (505 nm) was immediately measured. A linear calibration curve was created using known concentrations of glucose standards [Sigma-Aldrich, Poole, UK],

Plasma sodium and potassium measurements were carried out on a Corning 480 clinical flame photometer [Corning, New York, USA]. Plasma samples (20 μL) were diluted fivefold using pure water (18.3 ΩM) before being analysed. Plasma triglyceride levels were measured using a commercial colorimetric assay kit [Cayman chemicals Co., MI, USA]. A commercial assay kit was used [Sigma-Aldrich, Poole, UK] to determine plasma ammonia content.

Lipid peroxidation activity in the blood plasma was determined using the Gerard-Monnier, Erdelmeier, Regnard, Moze-Henry, Yadan & Chaudiere (1998) 1-methyl-2-phenylindole colorimetric method [Sigma-Aldrich, Poole, UK]. Briefly, 650 μL reacting agent (64 mg 10.3mM 1-methyl-2-phenylindole was dissolved in 30 mL acetonitrile, mixed with 0.04408g 2,6-Di-*tert*-butyl-4-methylphenol dissolved in 10mL methanol) was added to a 200 μL plasma sample, together with 100 μL 37% hydrochloric acid. This was vortex mixed and incubated in a water bath at 45 °C for 40 mins. The reaction was ended through immediate cooling in ice and centrifuged at 13,000 rpm for 10 mins. The supernatant was spectrophotometrically read at 586 nm. A standard curve was created through serial dilutions of diluted 1,1,3,3-tetraethoxypropane standard solution (16.5 μL of 10 mM 1,1,3,3-tetramethoxypropane in 20 mM Trizma-Base buffer pH 7.4, added to 10 mL Trizma-HCL buffer).

Statistical analysis

Results were expressed as mean values and reported with pooled standard error (S.E.). Datasets were analysed using nested (Treatment diet (replicate tank)) or one-way analysis of variance (ANOVA) and significant differences among treatment means were determined using *post-hoc* Tukey test. Statistical significance was only considered when

290 $P<0.05$. All statistical analysis was performed on Statistical Package for the Social
291 Sciences-SPSS v16.0 [Manugistics Inc. Rockville, MD, USA].
292

293 Results

294 Growth performance

295 Fish fed on the ragworm inclusion (RW+FM) diet demonstrated a significantly superior
296 growth performance than those on all other diets ($P<0.001$). This was shown by a
297 fivefold increase in weight gain, attainment of the lowest FCR, and the highest SGR and
298 PER (Table 3). Compared to the FM CTRL diet, the difference between the final mean
299 weight on RW+FM diet was 34% higher ($P<0.001$). The silkworm pupae inclusion
300 (SWP+FM) di*et al*so resulted in a significant increase in weight gain, producing a 12%
301 higher final mean weight ($P<0.001$) when compared to the control diet. A combined
302 diet with both invertebrate meals (SWP+RW+FM) did not demonstrate any enhanced
303 morphometric parameters or growth performance indices when compared with the
304 control treatment (*Post-hoc Tukey*, $P>0.05$).
305

306 Basic haematological and immunological parameters

307 Carp fed with either SWP+FM, RW+FM or SWP+RW +FM diet, produced higher
308 packed cell volume values than those on the control diet (Table 4). However, this was
309 found not to be significantly different ($P=0.425$). Similarly, other basic haematological
310 parameters in the experimental carps were unaffected by the addition of invertebrate
311 meals into their diets ($P>0.05$). Likewise, immunological parameters-lysozyme activity

and alternative complement pathway activity (ACH_{50}) showed no apparent significant differences between the dietary groups. The exception was the SWP+FM dietary treatment group, in which ACH_{50} was significantly enhanced by approximately 41% compared to the control diet (FM CTRL, P=0.041).

Haematological biochemistry parameters

Examination of total protein, albumin, globulin and albumin:globulin levels and plasma Na^+ and K^+ concentrations showed no significant differences between treatment groups (P>0.05, Table 5) . Although, all experimental invertebrate meal diets were associated with marked increases in fish glucose levels, there were no significant differences compared to the control treatment (P=0.333). Blood ammonia levels showed no statistically significant differences between the FM CTRL, SWP+FM and SWP+RW +FM diets (P>0.05). However, experimental carp fed on RW+FM diet responded with 30% lower blood ammonia levels when compared to other dietary treatments (P=0.009). Plasma triglyceride levels were significantly higher in fish that were fed either the SWP+FM or RW+FM diets when compared to other experimental diets (P=0.004). Levels of malondialdehyde in the blood were found to be highly variable between sampled individuals (Table 5). Consequently, these differences were statistically insignificant (P=0.454).

Discussion

The present study demonstrates that in comparison with a high BV protein in the form of fishmeal, either ragworm or silkworm pupae meals can support good growth in

addition to enhancing growth performance in mirror carp under laboratory conditions. Most notably inclusion of ragworm meal led to the highest growth performance amongst the diet treatments. The results in the current investigation were favourably comparable to those obtained by Salze *et al*. (2010) with juvenile Cobia (*Rachycentron canadum*), who reported improvement in growth performance and feed efficiency when fish were fed with 30% inclusion of ragworm diet. Rangacharyulu *et al*. (2003) reported a 13% higher weight gain in *Catla catla*, mrigal (*Cirrhinus mrigala*) and rohu (*Labeo rohita*) when 100% fishmeal was replaced by silkworm pupae silage. A previous study in common carp (*Cyprinus carpio*) revealed it was possible to maintain growth performance with a 50% silkworm pupae inclusion diet (Nandeesha, Srikanth, Keshavanath, Varghese, Basavaraja, Das 1990). However, higher dietary inclusion levels had negative impacts on juvenile Jian carp (*Cyprinus carpio*) physiology, such as enhanced oxidative stress, occurrence of irregular-shaped intestinal structure, and decreased protease and liver enzyme activities as reported recently by Ji *et al*. (2015).

Studies using other species of invertebrate meals have also resulted in improvements in growth parameters measured for fish. These include the partial replacement of fishmeal with mealworm (*Tenebrio molitor*) fed to African catfish (*Clarias gariepinus*) (Ng *et al.* 2001), and earthworm meal (*Eisenia foetida*) in rainbow trout (*Oncorhynchus mykiss*) (Velasquez, Ibanez, Herrera & Oyarzun 1991). The rising costs associated with the increasing demand for fishmeal could potentially result in the use of invertebrate meals in fish feed production becoming economically viable (Sánchez-Muros *et al*. 2014).

An imbalance of the amino acid profile in fish diets can often result in decreased protein retention efficiency and reduction of growth performance (Kaushik & Seiliez. 2010).

Moreover, excess supply of even a single amino acid can lead to enhanced amino acid deamination and oxidation. This is in the case with glutamic acid, which is abundantly found in wheat gluten based diets, resulting in a measurable increase liver glutamate dehydrogenase activity and consequently raised ammonia production and ammonia plasma concentration (Robaina, Corraze, Aguirre, Blanc, Melcion, & Kaushik, 1999; Moyano, Cardenete & De La Higuera 1991). Furthermore, excessive supply of protein relative to energy content can trigger protein catabolism to meet the energy demand (McGoogan & Gatelin, 1999). Blood ammonia is the main excretory nitrogenous product in fish, and in turn is excreted into the environment through the gills (Weihrauch, Wilkie & Walsh 2009). The present study on carp resulted in a significant reduction in ammonia production in fish fed the diet with the ragworm inclusion. From this it can be inferred that there would be a reduction in ammonia concentrations in the tank water, although these were not specifically measured. The implication is that ragworm could be considered as a less polluting feed ingredient than fishmeal for mirror carp under intensive rearing conditions and of significance to related ornamental fish kept in tanks. The decrease in plasma ammonia and increase in protein efficiency ratio suggest that the ragworm inclusion diet has an amino acid profile that is more compatible with the dietary requirements of fast growing mirror carp than that provided by the reference fishmeal-based diet, and thus better meets the profile for enhanced protein accretion and consequently growth performance. The amino acid composition of the diets (Table 2) indicated that a number of Essential Amino Acids, (EAA) such as histidine, lysine, valine, methionine, and isoleucine were all lower for the reduced fishmeal dietary treatments when substituted with both invertebrate based meals and a blend. Fishmeal can present an excessive level of specific essential amino acids and might not be as close to the ‘ideal protein’ concept for carp. For instance, in the

385 fishmeal control diet, lysine was determined as 3.01% and the NRC requirement for
386 common carp is stated as being 2.2% of the diet (5.7% of the Crude Protein CP, NRC,
387 2011). The invertebrate containing diets were closer in lysine (2.3-2.7%) to the
388 requirement of carp and may have been better assimilated. This may be true of other
389 amino acids and the overall requirement pattern compounded by the individual apparent
390 amino acid digestibility coefficients for each ingredient. These were not measured in the
391 present study due to the technical difficulties in acquiring faecal material from carp.
392 Longvah *et al.* (2011) used a rat model to assess protein quality and essential amino
393 acid score for silkworm meals and found that leucine scored low for this ingredient
394 compared to standard reference proteins such as hen egg and casein. However, leucine
395 did not appear to be rate limiting in the present diets for carp.
396
397 Both silkworm and ragworm meals have a higher lipid content than fishmeal. The lipid
398 content in the silkworm meal found in this study was similar to that previously reported
399 (Frye & Calvert, 1989; Rao, 1994; Hossain, Nahar, & Kamal 1997; Hertrampf &
400 Piedad-Pascual, 2000; Pereira, Ferrarese-Filho, Matsushita & De Souza. 2003).
401 Elevated lipid content in a feed component can make it difficult to formulate high
402 protein diets with a low to moderate lipid content, and would be a constraint for the low
403 oil diets required for carp production.
404
405 Triglyceride (TG) is one of the primary lipid storage compounds in fish and a principal
406 energy reserve in carnivorous species; it is also deemed important in overwintering carp
407 (Guillaume, Sadisivam, Bergot & Metailler, 2001). As well as being found within major
408 organs, such as the gastro-intestinal tract and liver, it is also abundantly located in the
409 blood lipid fraction, reflecting dietary status and history (Groff & Zinkl, 1999).

Although diets produced in this study were formulated to be *iso*-lipidic and *iso*-energetic, haematological analysis revealed elevated blood plasma TG levels in mirror carp fed on either of the single invertebrate meal inclusion diets. The increased growth may explain the increased plasma TG levels in these fish, as the TG may have been mobilised into the bloodstream via lipolysis from adipose tissue to be utilised to support fish growth. Conversely, TG levels were not elevated in fish fed the mixed invertebrate diet potentially because they did not display an associated enhanced growth. As dietary lipid levels may be reflected in both whole body and fillet composition, it would be prudent to carry out follow-up studies to examine whether the observed blood triglyceride increase would significantly affect the fish fillet lipid composition of larger carp reaching harvest size (Ferreira, De Araujo, Costa, Rosa, Figueiredo & Murgas 2011).

In many marine invertebrates, lipid composition is primarily comprised of the omega-3 series, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Busarova & Isay, 1984; Bischoff, Fink, & Wallera 2009), and in comparison, silkworm pupae are rich in omega-3 α-linolenic acid (ALA) (Tomotake, Katagiri & Yamato 2010). The combination of both high oil content and polyunsaturated fatty acid rich profile can lead to increased susceptibility to lipid peroxidation in the invertebrate meal (Chen, Zhu, Han, Yang, Lei, & Xie 2011). It can be assumed from the current study that the change in lipid source did not in fact alter the level of lipid peroxidation as levels of the by-product malondialdehyde, indicative of oxidative stress, remained unchanged. This implies that lipid quality in both meals is relatively stable in terms of lipid oxidation within these experimental diets, although the fatty acid profiles were not directly measured in the present study.

435

436 The quality of lipids present in fish feeds can alter fillet quality, through physical and
437 organoleptic attributes (Ng & Bahurmiza, 2009). Besides lipids and their oxidation
438 products, other compounds present in feed ingredients can further modify quality. For
439 example, silkworms (*Bombyx mori*) are able to sequester flavenoids and terpenoids
440 compounds from their primary diet of mulberry (*Morus alba*) leaves (Zhou, Yang,
441 Chen, Lou, Zhang, Chen, Wang, Yu, Yu, Li & Zhong 2008). These compounds have
442 been linked with the strong odours associated with the pupae meal and can potentially
443 accumulate in fish muscle tissues. Moreover, a previous study has cited measureable
444 changes in fillet characteristics and in particular the organoleptic qualities leading to
445 'off flavour' in common carp fed experimental diets containing silkworm pupae meal
446 (Hora & Pillay,1962). On the other hand, the presence of low molecular weight
447 bromophenol compounds in ragworms may serve as a functional feed component.
448 Found naturally in marine fish and shellfish, several of these compounds have been
449 shown to contribute to their distinct marine/ocean like flavour, as well as enhancing
450 existing flavours (Whitfield, Drew, Helidoniotis & Svoronos 1999; Ma, Chung, Ang &
451 Kim 2005). Further studies should be conducted in evaluating whether this novel meal
452 would have beneficial organoleptic properties for farmed fish. This would be
453 particularly relevant to freshwater fish such as carp that are typically grown in closed
454 systems (e.g. recirculated aquaculture systems). These systems are often rich in
455 cyanobacteria and actinomycetes, and metabolic by-products (e.g. geosmin) originating
456 from these organisms can accumulate in the fish muscle tissue causing a muddy flavour
457 (Robertson, Jauncey, Beveridge & Lawton, 2005). Through the use of ragworm meal in
458 the diet, it may be possible to mask this undesirable flavour by the presence of
459 bromophenols.

460

461 The current investigation with mirror carp showed enhanced alternative complement
462 pathway activity when fish were fed with the silkworm pupae inclusion diet. A possible
463 explanation for the improved immunity is the presence of long-chain polysaccharides in
464 the pupae meal. Like many invertebrates, the pupae of the silkworm have a cuticle
465 comprised mainly of chitin and chitosan, which gives rise to its structural integrity.
466 When the present study is compared to research conducted by Gopalakannan & Arul
467 (2006) and Lin, Pan, Luo & Luo (2011) with inclusion of extracted chitin and chitosan
468 in common carp (*Cyprinus carpio*) diets, an opposite trend was found, where lysozyme
469 activity was increased and alternative complement activity was decreased. However,
470 chitin was not directly measured in the present study, but would have been of interest to
471 help explain these effects.
472

473 Conclusion

474 Invertebrate meals may offer a sustainable feed material for farmed and ornamental
475 cyprinids such as carp, and furthermore, it can be sourced from waste-streams such as
476 silk production in Asia and polychaete culture in temperate zones. In the present
477 investigation, inclusion of either silkworm pupae or ragworm meal in diets for mirror
478 carp provides preliminary evidence that these novel raw materials can enhance growth
479 performance. In summary, this study has demonstrated that up to 7.5% of the dietary
480 protein content (24% of the fishmeal in experimental diets) can be replaced with either
481 terrestrial or marine derived invertebrate meals in order to sustain growth performance
482 of mirror carp (*Cypinus carpio*). The results also show there was no obvious detrimental
483 effect on selected blood parameters associated with health status. However, favourably

484 modulated components of the alternative complement pathway and decreased plasma
485 ammonia levels were indicative of reduced excretion of ammonia. The functional
486 effects of marine invertebrate meal on fish performance and health may advocate their
487 role as beneficial supplements to provide further 'added value' to the diet. Future work
488 should be directed towards characterising the effects of such novel protein sources for a
489 wider range of fish species within different production systems and phases of
490 development. Further, it may be possible to isolate the specific components and
491 metabolites in these novel ingredients responsible for their bioactive roles to optimise
492 utilisation in commercial diets for both aquaculture and the ornamental fish industry.

Acknowledgments

The research study was part funded by WALTHAM Centre for Pet Nutrition (Mars Petcare Inc.). The authors would like to acknowledge Ben Eynon and Andrew Atfield for their assistance during the feed trial and haematological analysis. Furthermore, acknowledgement is also given to Liz Preston and Natalie Sweet for their technical assistance in the nutritional analysis. The authors would to thank Dr Richard Fitzgerald and Dr Majbritt Bolton-Warberg for their editorial assistance.

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Table 1: Proximate composition of the major feed components used in the experimental diets (*n*=4, %, dry matter).

Parameters	**Herring Fishmeal** (*Clupea harengus*)	**Silkworm pupae meal** (*Bombyx mori*)	**Ragworm meal** (*Nereis virens*)
Moisture	6.4	6.2	6.1
Crude protein	66.3	47.3	50.8
Crude Lipid	7.8	33.9	15.0
Crude Ash	13.0	3.1	7.9
Gross energy; MJ Kg^{-1}	18.8	25.9	21.8

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Table 2: Diet composition and proximate analysis of experimental diets (%).

Parameters	Diets			
	FM CTRL	**SWP+FM**	**RW+FM**	**SWP+RW+FM**
Diet composition				
Fish meal[a]	44.34	33.51	32.00	32.11
Silkworm pupae[b]	-	13.39	-	6.98
Ragworm meal[c]	-	-	14.80	8.00
Corn starch[d]	35.72	35.10	33.99	31.93
Lysamine® pea protein[e]	7.00	7.00	7.00	7.00
Glutalys® wheat gluten[e]	7.00	7.00	7.00	7.00
Mineral premix[f]	2.00	2.00	2.00	2.00
Sunflower oil[g]	2.00	2.00	2.00	2.00
Fish oil[h]	1.94	-	1.20	-
Calcium chloride[d]	-	-	-	2.98
Antioxidant[i]	0.50	0.50	0.50	0.50
Proximate composition[1]				
Moisture	5.4	5.7	6.0	5.6
Crude protein	38.0	36.5	37.2	39.7
Crude Lipid	8.3	9.4	8.5	9.6
Crude Ash	7.3	6.5	6.8	7.4
Gross energy; MJ Kg^{-1}	19.4	20.0	19.5	19.3
Essential Amino Acid composition				
Lysine	3.01	2.30	2.72	2.44
Methionine	1.11	0.85	0.99	0.89
Met + Cys	1.25	0.90	0.90	0.85
Arginine	2.74	2.56	2.43	2.48
Histidine	0.97	0.78	0.92	0.84
Threonine	1.68	1.56	1.60	1.58
Tryptophan	0.42	0.40	0.43	0.42
Leucine	3.46	3.41	3.22	3.32
Isoleucine	1.82	1.72	1.70	1.71
Phenylalanine	1.79	1.77	1.78	1.79
Valine	2.39	2.17	2.25	2.20

FM, Fishmeal (*Clupea harengus*); SWP, Silkworm pupae (*Bombyx mori*); RW, Ragworm (*Nereis virens*)

[a] Scottish Fish meal 70, United fish products Ltd., UK.

[b] Freeze dried silkworm pupae, Medikoi silkworm pupae, NT laboratories, UK.

[c] Freeze dried ragworm meal, Seabait Ltd., UK.

[d] Laboratory grade, Sigma –Aldrich Company Ltd., UK.

[e] Roquette Frères, France.

[f] Vitamin/Mineral premix, Premier Nutrition Products Ltd., UK. (Manufacturer analysis: Ca-12.09 %, Ash-78.71 %, Na-8.86 %, Vitamin A-1.0 µg kg^{-1}, Vitamin D3 0.10 %, Vitamin-E 7.0 g kg^{-1}, Cu-250 mg kg^{-1}, Mg 15.6 g kg^{-1} and P 5.2 g kg^{-1})

[g] Sunflower oil, Costcutter Supermarkets Group Ltd., UK.

[h] Epanoil, Sevenseas Ltd., UK.

[i] Barox plus liquid (Antioxidant: Ethoxquine and BHT), Kemin Europa N.V., Belgium.

Table 3: Growth performance and feed utilisation of mirror carp (*C. carpio*), fed on different experimental diets (± S.E. *n*= 3).

	Diets				
Parameters	**FM CTRL**	**SWP+FM**	**RW+FM**	**SWP+RW+FM**	*P-Value*
Initial mean weight; g fish $^{-1}$	14.93 ±*0.19*	14.63 ±*0.09*	15.07 ±*0.13*	14.90 ±*0.05*	*0.132*
Final mean weight; g fish $^{-1}$	48.89 ±*0.99*^{a}	55.25 ±*0.69*^{b}	74.38 ±*1.07*^{c}	49.23 ±*0.51*^{a}	*<0.001*
Mean weight gain; g fish $^{-1}$	33.96 ±*0.77*^{a}	40.62 ±*0.72*^{b}	60.83 ±*2.57*^{c}	34.34 ±*0.48*^{a}	*<0.001*
Specific Growth Rate, SGR; % day^{-1}	1.78 ±*0.04*^{a}	1.99 ±*0.02*^{b}	2.36 ±*0.04*^{c}	1.81 ±*0.01*^{a}	*<0.001*
Feed Conversion Ratio, FCR; g g $^{-1}$	1.92 ±*0.02*^{a}	1.68 ±*0.02*^{b}	1.36 ±*0.2*^{c}	1.83 ±*0.01*^{a}	*<0.001*
Protein Efficiency Ratio, PER	0.85 ±*0.02*^{a}	1.03 ±*0.02*^{b}	1.38 ±*0.03*^{c}	0.90 ±*0.01*^{a}	*<0.001*

Fish meal, FM; silkworm pupae (*Bombyx mori*), SWP; ragworm (*Nereis virens*), RW. Alternative complement activity, ACH50. Different superscript letters in the same row indicates significant difference (P<0.05).

Table 4: Basic haematological and immunological parameters of mirror carp (*C. carpio*), when fed different experimental diets (± S.E. *n*= 3).

	Diets				
Parameters	**FM CTRL**	**SWP+FM**	**RW+FM**	**SWP+RW+FM**	*P-value*
Packed cell volume; %	36.06 ±*1.95*	43.00 ±*2.23*	41.44 ±*2.00*	39.33 ±*0.71*	*0.425*
Haemoglobin; g dL^{-1}	11.24 ±*0.38*	12.56 ±*0.44*	13.43 ±*0.38*	12.63 ±*0.22*	*0.839*
Erythrocytes; 10^6 μL^{-1}	1.14 ±*0.08*	1.14 ±*0.04*	1.16 ±*0.12*	1.00 ±*0.15*	*0.771*
Lysozyme activity; units mL^{-1}	28.44 ±*3.97*	28.44 ±2.70	27.56 ±3.43	32.89 ±4.26	*0.888*
ACH50; units mL^{-1}	79.21 ±*7.39*[a]	111.52 ±*9.70*[b]	89.80 ±*15.96*[a]	84.28 ±*17.62*[a]	*0.041*

Fish meal, FM; silkworm pupae (*Bombyx mori*), SWP; ragworm (*Nereis virens*), RW. ACH50, alternative complement activity. Different superscript letters in the same row indicates significant difference (P<0.05).

Table 5: The biochemical haematology of mirror carp after being fed with experimental diets (± S.E., *n*= 3).

	Diets				
Parameters	**Control**	**SWP+FM**	**RW+FM**	**SWP+RW+FM**	P-value
Total Protein; g dL^{-1}	1.36 ±*0.02*	1.39 ±*0.03*	1.51 ±*0.03*	1.37 ±*0.02*	*0.161*
Albumin; g dL^{-1}	0.42 ±*0.03*	0.46 ±*0.03*	0.48 ±*0.01*	0.48 ±*0.01*	*0.219*
Globulin; g dL^{-1}	0.97 ±*0.04*	0.87 ±0.04	0.92 ±*0.06*	0.89 ±*0.03*	*0.529*
Albumin/globulin ratio	2.50 ±*0.34*	2.02 ±*0.27*	1.92 ±*0.15*	1.86 ±*0.10*	*0.232*
Glucose; mg dL^{-1}	79.86 ±*1.75*	93.31 ±*2.97*	93.50 ±*3.33*	90.41 ±*1.93*	*0.333*
Na^{+}; mmol L^{-1}	116.39 ±*0.25*	125.00 ±*1.71*	118.98 ±*1.47*	116.94 ±*0.72*	*0.901*
K^{+}; mmol L^{-1}	2.05 ±*0.001*	2.06 ±*0.05*	2.15 ±*0.03*	2.13 ±*0.02*	*0.198*
Ammonia; mg dL^{-1}	21.37 ±*2.24*^{a}	20.60 ±*1.41*^{a}	14.56 ±*1.21*^{b}	24.37 ±*2.01*^{a}	*0.004*
Triglyceride; mg dL^{-1}	75.37 ± *7.10*^{a}	99.67 ± *13.21*^{b}	102.64 ±*17.81*^{b}	64.62 ±*3.72*^{a}	*0.009*
Malondialdehyde; µM mL^{-1}	0.50 ± *0.03*	0.52 ± *0.04*	0.58 ± *0.05*	0.51 ± *0.01*	*0.454*

Fish meal, FM; silkworm pupae (*Bombyx mori*), SWP; ragworm (*Nereis virens*), RW.
Different superscript letters in the same row indicates significant difference (P<0.05).