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Giant Mealworm (*Zophobas Morio*) as a "Vehicle" to Transport Healthy Nutritional Ingredients from Seaweed (*Ascophyllum Nodosum*) towards Fish Cultured: Amino Acids

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Abstract: ***This study is the first step investigating a new food chain, using Zophobas morio as a potential "vehicle" to transport amino acids (AA) from Norwegian kelp (Ascophyllum nodosum) into the insect body. Additionally, suitability of Z. morio as a dietary protein substitute for fishmeal (FM) in aquaculture feeds, was evaluated.***

Proximate composition (dry matter, ash, protein, fat and energy) and complete (free + hydrolysed + tryptophan) AA profiles were determined for Z. morio fed with kelp granulate or oat meal. Using principle component analysis we identified similarities and differences in essential AA between diets and Z. morio fed with these diets. AA scores were calculated to evaluate protein quality of Z. morio.

Results showed a slight enrichment in several essential AA for Z. morio fed kelp granulate. Protein level of Z. morio in this study fell within the ranges of protein requirements for fish. All essential AA for fish were present in Z. morio. Based on AA scores it was concluded that methionine would be first limiting.

Overall, protein levels and AA profiles showed that Z. morio fed with kelp granulate can be seen as an interesting candidate to replace FM in aquatic diets.

Keywords: Zophobas morio, Ascophyllum nodosum, amino acid composition, fish meal replacement, innovative food chain.

1. Introduction

Today, the world's natural resources are under increasing pressure due to an overgrowing world population, estimated to reach approximately 9.5 billion people in 2050 (Nations 2015). To keep up with this increase, modernization of production methods is required, and advantages of non-utilized plant and animal sources in the present production process should be explored.

Terrestrial agriculture is reaching its limits for quantitative food production (Godfray et al., 2010). With 2/3 of the world covered by oceans, focus shifts more towards mariculture production (Nations 2015). Aquaculture already supplies approximately 50% of the seafood consumed worldwide (FAO 2016). Over the last few decades aquaculture production has increased by nearly 10% per year, making it the fastest growing global food production sector (FAO 2014; FAO 2016; Hall et al., 2011). Fish and shellfish aquaculture represent 11 and 40% of all mariculture production, while seaweed culture contributes 47% (≈28 million tonnes) (FAO 2016).

Fishmeal (FM) is an important protein source in animal feeds, including aquaculture diets (Brown et al., 2011; Tacon and Metian 2008). Unfortunately, as a result of various factors (e.g. overexploitation of fish stocks, uncertain supplies during the year and abundant uses), it has become scarce and costly. At present the fish feed industry is highly dependent on FM, resulting in increased costs for aquaculture feeds (Gümücs et al., 2009). Alternatives to replace FM and reduce costs of aquaculture diets should therefore be investigated.

Although several ingredients have been explored (reviewed in Gatlin et al., (2007)), there are no alternatives yet, that can completely replace FM in aquaculture diets. Most commonly substitution of FM by

soybean meal (SBM) has been studied, however, numerous problems and constraints have been reported. Especially for salmonids, inclusion of SBM in their diets can result in enteritis, which is an inflammatory response of the distal intestinal mucosa (Hu et al., 2016; Urán 2008). The search for alternatives continues, and research efforts focus nowadays on novel, and locally accessible resources (Lenka et al., 2010).

FM substitutes should meet certain requirements, among others for protein levels, fatty acid profiles and amino acids (AA) profiles. Seaweeds, which are abundant but poorly exploited, have been suggested as potential alternative (Pereira et al., 2012; Soler-Vila et al., 2009; Valente et al., 2006; Xu et al., 2011). Most seaweed species are rich in polysaccharides, vitamins and minerals, and they contain all essential amino acids (EAA) (Dawczynski et al., 2007; Holdt and Kraan 2011). Furthermore, seaweeds are known as a good source of polyunsaturated fatty acids (PUFA) (van Ginneken et al., 2011). Several seaweed species, among others Norwegian kelp (*Ascophyllum nodosum*), contain relative high levels of important PUFAs, like eicosapentaenoic acid (EPA, C20:5, n-3) and arachidonic acid (ARA, C20:4, n-6) (Holdt and Kraan 2011; van Ginneken et al., 2011). Studies have already shown that it is possible to at least partly replace FM (up to 10%) with seaweeds in diets of rainbow trout (Pereira et al., 2012; Soler-Vila et al., 2009), Nile tilapia (Pereira et al., 2012; Silva et al., 2014), red sea bream (Nakagawa et al., 1997), European sea bass (Valente et al., 2006) and striped mullet (Wassef et al., 2001). Still, a major inflicting property of seaweeds, is the presence of several anti-nutritional factors, which can affect digestibility and tastiness of diets (Silva et al., 2014).

Insects may also have the potential as a sustainable alternatives for FM, and studies evaluating this alternative have been developed during the last decade (reviewed by Henry et al., (2015)). The common mealworm (*Tenebrio molitor*) showed to be highly palatable for African catfish and replacement of up to 60% FM with this insect did not affect growth and feeding efficiency (Ng et al., 2001). The superworm, *Zophobas morio*, has also been proposed as a successful alternative for FM, due to the richness in proteins and their EAA profile (Henry et al., 2015). Total replacement of FM with *Z. morio* only, has not been successful in Nile tilapia (Abd Rahman Jabir et al., 2012b). Nevertheless optimal growth was observed when up to 25% of FM was replaced by *Z. morio* (Abd Rahman Jabir et al., 2012b), showing the potential to investigate this alternative further.

For commercial production of *Z. morio*, diets based on mixed grains (i.e. oats, soy and corn) are commonly used (Oonincx and De Boer 2012), which still puts pressure on arable land. Therefore here a new food chain is introduced, whereby *Z. morio* is used as a valuable "vehicle" to transport healthy nutrients from Norwegian kelp towards the insect body, and serve as a potential alternative for FM in aquatic diets. This new food chain is suggested as a sustainable and innovative blue technology. In the current study first steps were taken in exploring this new food chain and preliminary results on total AA profiles of *Z. morio* fed with a kelp granulate diet, are shown. Aim of this study was to evaluate protein quality of *Z. morio*, fed with a kelp diet, as potential substitute of FM in aquaculture diets.

2. Materials and Methods

2.1 Experimental animals and setup

Adult *Z. morio* (approximately 50-60 mm) were purchased from a local pet shop (Zoo & Zo, Ede, The Netherlands). The experimental setup consisted of two mouse husbandry boxes (20 x 12 x 12 cm), each covered by a lid with air ventilation holes. Temperature was kept at 23-24 °C. *Z. morio* were equally divided over the 2 boxes.

Each box was provided with one of two diets: (1) kelp granulate (*A. nodosum*, seealgenmehl, Grau, Germany, 1-3 mm) or (2) oat meal (*Avena sativa*, obtained from a local grocery shop, 1-3 mm). The latter diet served as a control. Every two days, each box was cleaned by shaking the whole content (worms with food) through a sieve. After cleaning, new food was added. The whole feeding trial lasted for two months, during which both the control and treatment group were fed at maintenance level (based on a food conversion of 2.2 (Nieuwland 2016)). At the end of the experiment, from each box 100 individuals were randomly selected for further analyses.

2.2 Analyses

Proximate composition of diets and Z. morio

Whole worm samples (n=100) were pooled per tank, and grounded using a coffee bean mill. Oat meal and kelp granulate were grounded using a centrifugal grinding mill (Retsch 200 ZM, 1mm sieve). Proximate analyses

for worm samples, oat meal and kelp granulate were done in triplicates for dry matter (DM; ISO 6469/NEN 3332), ash (ISO 5984/NEN 3329), crude protein (CP; Nx6.25; Kjeldahl, ISO 5983/NEN 3145), crude fat (CF; Soxhlett, ISO-DIS 6492), and gross energy (adiabatic bomb calorimetry, IKA-calorimeter C 7000).

AA profiles of diets and Z. morio
Complete AA profiles were determined for the two diets and *Z. morio* fed with these diets by Ansyth Services BV (Roosendaal, The Netherlands). For a complete AA profile, three different analyses were done (1) free AA analyses, (2) protein bound AA analyses (i.e. hydrolysed AA), and (3) tryptophan analyses. These analyses will be described separately.

Free amino acid analyses
Freeze dried samples (250 mg) were transferred into 25ml volumetric flasks. Loading buffer (Li-citrate pH 2.2) was added and stirred for 1 hour. Hereafter demi water was added. This solution was mixed and filtered. Extracts (750 µl) were mixed with 250 µl internal standard solution (Sigma A-9906). After filtration with a 0.2µm CA filter, 80µl of the filtrate was injected and analysed by a Biochrom20 amino acid analyser. Detection was done at a wavelength of 570nm and 440nm.

Protein bound amino acid analyses
Protein bound AA was analysed by acerbic hydrolyses. Freeze dried samples (100mg) were hydrolysed with 6M HCl (3ml) for a total of 22 hours (temp 105-110 °C). Hydrolysate was transferred in 25ml volumetric flasks. pH was adjusted to 2.2 with LiOH and demi water was added. Of this solution, 250 µl was mixed with 250µl of the internal standard solution (Norleucine 0.5 mM) and filtrated using a 0.2mm CA filter. Hereafter, 40µl of the filtrate was injected and analysed using a Biochrom20 amino acid analyser.

Tryptophan measurement
Tryptophan was analysed by alkaline hydrolyses. Freeze dried samples (80 mg) were hydrolysed with 4.2M NaOH for 22 hours (temp 105-110˚C). Hydrolysate (25 ml) was extracted and analysed using a Beckman System Gold HPLC. Detection was done fluorimetrically (280-340 nm).

For all three types of AA analyses, detection limit for the diets was 9 mg kg^{-1}, while detection limit for the *Z. morio* was 33 mg kg^{-1}.

2.3 Calculations

Protein level of diet and Z. morio
CP was calculated using two different methods. In the first method total nitrogen (N) determined by the Kjeldahl method was multiplied by 6.25, which has been suggested as a good estimate for true protein contents of insects (Finke 2002). In the second method CP was calculated by adding the sum of free AA, the sum of protein bound AA and tryptophan (CP = ∑ free AA + ∑ protein bound AA + tryptophan). In calculations described hereafter it was chosen to use CP calculated by the Kjeldahl method, since this method is mostly used in other studies.

AA scores of Z. morio
To evaluate *Z. morio* as potential protein ingredient in fish diets, AA scores were calculated as described in (Hepher 1988). The following formula was used:

$$AA\ score = \frac{mg\ of\ limiting\ AA\ in\ 1\ gram\ test\ protein}{mg\ of\ limiting\ AA\ in\ 1\ gram\ of\ reference\ protein} \times 100 \quad (1)$$

Minimal requirements for two important aquaculture fish species, i.e. tilapia (*Oreochromis* spp.) and salmon (*Salmo salar*) were used as reference values ((NRC) 2013). AA scores were calculated for arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine, which were considered as the EAA for fish ((NRC) 2013). Fish can only synthesize cysteine and tyrosine from respectively methionine and phenylalanine (Tacon and Cowey 1985), which means that both cysteine and tyrosine

requirement are dependent on methionine and phenylalanine content of the diet. Therefore it was chosen to add the values for methionine and cysteine and add the values for phenylalanine and tyrosine. These added values were used as one, to calculate AA scores. For a comparison of *Z. morio* with FM, AA scores were also calculated for FM based on herring (72% protein). Values for the AA composition of herring based FM were obtained from NRC (2013).

2.4 Statistics

Data of proximate analyses and AA profiles are reported as mean ± standard deviation (SD). Means were taken from three analytical replicas. Data were described using principle component analysis (PCA) on the essential AA concentrations, to identify similarities and differences between diets and *Z. morio* fed with these diets.

3. Results

Proximate composition of diets fed to *Z. morio* is shown in table 1. Notable is the ash content of kelp granulate, which was almost 13 times higher than for oat meal. This high ash content was not reflected in *Z. morio* itself (table 2). CP, CF and energy content of *Z. morio* were comparable, independent of the diets they were fed (table 2).

Table 3 shows complete AA profiles of the two diets. All nine EAA were present in both diets. Oat meal contained more free tryptophan, while free lysine, phenylalanine and threonine were more abundant in kelp granulate. For protein bound AA, kelp granulate was more abundant in lysine, threonine, valine and isoleucine. These results are visualized in figure 2.

Of the non-essential AA, kelp granulates showed to be rich in the combined values of asparagine acid + asparagine, while oat meal was richer in proline. For both diets, total protein content calculated based on AA profiles (table 3) was lower than protein content calculated based on proximate analyses (table 1). Slightly higher protein content was observed for the kelp granulates diet, compared to the oat meal diet. Ratio of EAA/non-essential AA (E/N) was comparable for the two diets (table 3).

Compared to their diets, all EAA increased in *Z. morio* in equivalent proportions (table 4, figure 1). Principle Component Analyses was carried out on the total EAA profiles of both worms and diets (figure 1, table 5). The first principle component (PC) in the analysis explained almost all of the variance (99%), because of the major differences in EAA between the diets and the worms. The second PC represents the differences in EAA profiles between treatments: both in the diets and in the worms. Distribution along this axis shows that the EAA signature profile from the diets was passed on to the worms that fed on these diets, thus resulting in equivalent EAA profiles. Only one replica did not follow this pattern: while it had been fed with oat meal, it showed a profile similar to *Z. morio* fed with kelp (figure 1).

Results were also visualized using a spider web diagram (figure 2). These diagrams show that with the exception of tryptophan, *Z. morio* fed kelp granulate was slightly enriched in the free EAA (figure 2). However, the spider web diagram also showed that protein bound EAA were comparable for *Z. morio* independent of their diets.

Table 1
Analysed proximate composition of the diets, oat meal and kelp granulate.

	Oat meal	Kelp granulate
Proximate composition (g kg dm^{-1}) *		
Ash	19.8 ± 0.09	252.6 ± 0.48
Crude protein	120.7 ± 0.60	141.9 ± 1.29
Crude fat	18.6 ± 0.60	18.5 ± 0.72
Energy (kJ kg^{-1})	18.4 ± 0.87	13.7 ± 0.72

* Values given are the mean of three replicate analysis and are presented as mean ± SD

Table 2
Analysed proximate composition of *Z. morio* fed oat meal or *Z. morio* fed kelp granulate.

	Z. morio fed oat meal	*Z. morio* fed kelp granulate
Proximate composition (g kg dm^{-1})*		
DM (g kg^{-1})	47.3	49.9
Ash	23.1 ± 0.74	24.2 ± 0.30
Crude protein	450.4 ± 8.30	448.1 ± 6.51
Crude fat	402.3 ± 9.78	417.8 ± 2.00
Energy (kJ kg^{-1})	29.6 ± 0.06	29.2 ± 0.19

* Values given are the mean of three replicate analysis and are presented as mean ± SD

Similar as EAA concentrations, ratio of E/N also increased in *Z. morio* compared to their diets. Furthermore E/N ratio was comparable between the *Z. morio* fed with the two diets. Different than observed for the diets itself, for *Z. morio* total protein content calculated based on AA profiles (table 4) was higher than protein content calculated based on proximate analyses (table 2). However for both methods values were comparable between *Z. morio* fed oat meal and *Z. morio* fed kelp granulate (table 2 and table 4).
To evaluate the potential of *Z. morio* as a dietary protein substitute for FM, AA scores were calculated using nutritional requirements of salmon (*Salmo salar*) and tilapia (*Oreochromis* spp.) as reference values. AA scores were also calculated for FM itself, to make a comparison possible. Table 6 shows that independent of the diets fed, the first limiting AA for *Z. morio* was methionine, followed by the combined values of methionine and cysteine, for both salmon and tilapia. AA scores also showed that when using *Z. morio* in a salmon diet lysine might become limiting. Generally AA scores for *Z. morio* fed with kelp granulate were higher that AA scores for *Z. morio* fed with oat meal (table 6).

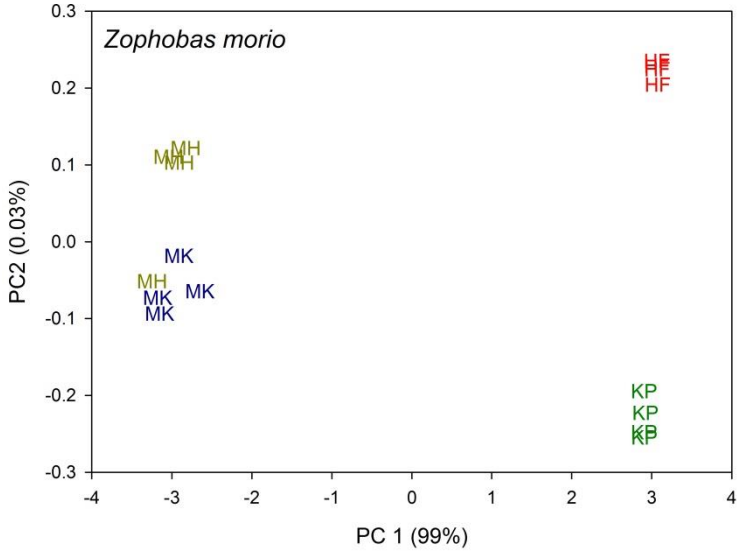

Figure 1
Differences in AA concentrations (analysed by Principle Component Analysis) in kelp granulate (KP), oat meal (HF), and *Zophobas morio* fed with either kelp (MK) or oat meal (MH). The correlations between AA and principle components (PC1 and PC2) are given in table 5.

Table 3
Amino acids profile (total, protein bound and free amino acids) of the diets, oat meal and kelp granulate.

	Total		Protein bound		Free	
	Oat meal	Kelp granulate	Oat meal	Kelp granulate	Oat meal	Kelp granulate
AA (g kg DM^{-1})[*]						
Essential						
Histidine	2.475	1.618	2.48±0.006	1.62±0.022	nd[2]	<0.027
Isoleucine	3.785	4.215	3.79±0.066	4.22±0.053	nd	<0.027
Leucine	7.325	6.747	7.33±0.060	6.71±0.069	<0.027	0.04±0.001
Lysine	3.049	5.502	3.02±0.028	5.36±0.086	0.03±0.001	0.15±0.007
Methionine	1.623	2.188	1.62±0.019	2.19±0.080	nd	nd
Phenylalanine	5.220	4.195	5.22±0.075	4.11±0.071	nd	0.09±0.002
Threonine	3.258	4.658	3.26±0.010	4.57±0.067	<0.027	0.09±0.000
Tryptophan	1.723	1.536	1.46±0.008	1.52±0.026	0.26±0.002	0.02±0.001
Valine	4.733	5.280	4.73±0.043	5.25±0.083	<0.027	0.03±0.001
Non-essential						
Alanine	4.069	5.047	3.97±0.046	6.70±0.050	0.10±0.001	1.07±0.004
Arginine	5.321	5.290	5.21±0.081	4.51±0.128	0.11±0.002	0.08±0.003
Asparagine					0.27±0.001	1.63±0.008
Asparagine acid					0.25±0.002	0.44±0.002
Asparagine acid +	6.084	16.446	5.56±0.069	14.38±0.119		
Cysteine	2.318	2.403	2.32±0.046	2.40±0.088	nd	nd
Glutamic acid					0.19±0.003	1.64±0.008
Glutamine					0.06±0.002	4.52±0.020
Glutamic acid +	33.893	39.805	33.65±0.0722	29.6 ±0.088		
Glycine	4.476	5.571	4.44±0.041	5.28±0.055	0.04±0.001	0.29±0.014
Proline	10.817	3.860	10.79±0.03	3.79±0.103	0.03±0.002	0.07±0.003
Serine	5.240	4.346	5.24±0.047	4.27±0.084	<0.027	0.08±0.001
Tyrosine	2.980	3.457	2.98±0.027	3.42±0.067	nd	0.04±0.001
Ornithine	nd	0.021			nd	0.02±0.001
gamma-aminobutyric	0.061	nd			0.061±0.002	nd
Citruline		0.258			nd	0.26±0.003
Ethanolamine		0.048			nd	0.05±0.002
Taurine	nd	0.052			nd	0.05±0.002
Cystathionine	nd	0.064			nd	0.06±0.001
Total protein	108.48	122.60				
Ratio E/N[3]	0.44	0.41	0.44	0.48	0.27	0.04

[*] Values given are the mean of four replicate analysis and are presented as mean ± SD
[2] nd: not detectable
[3] E: sum of essential amino acids and N: sum of non-essential amino acids

Table 4
Amino acids profile (total, protein bound and free amino acids) of *Z. morio* fed oat meal or *Z. morio* fed kelp granulate.

	Total		Protein bound		Free	
	Z. morio fed oat meal	*Z. morio* fed kelp granulate	*Z. morio* fed oat meal	*Z. morio* fed kelp granulate	*Z. morio* fed oat meal	*Z. morio* fed kelp granulate
AA[*] *(g kg DM*$^{-1}$*)*						
Essential						
Histidine	15.539	15.611	13.90±0.178	13.91±0.449	1.64±0.003	1.70±0.005
Isoleucine	23.040	23.018	21.67±0.446	21.41±0.608	1.37±0.010	1.61±0.015
Leucine	35.378	35.454	32.79±0.809	32.54±1.075	2.59±0.007	2.92±0.045
Lysine	26.791	28.144	24.30±0.996	25.22±0.868	2.49±0.011	2.92±0.012
Methionine	5.612	6.008	5.36±0.245	5.62±0.176	0.25±0.011	0.39±0.008
Phenylalanine	19.024	19.586	17.77±0.506	18.06±0.512	1.25±0.008	1.53±0.014
Threonine	19.819	19.495	18.70±0.447	18.25±0.650	1.12±0.020	1.25±0.029
Tryptophan	7.100	6.626	5.77±0.246	5.38±0.230	1.33±0.004	1.25±0.019
Valine	31.815	31.235	29.68±0.532	28.80±0.957	2.14±0.006	2.43±0.012
Non-essential						
Alanine	45.826	44.280	42.05±2.147	40.00±2.307	3.78±0.003	4.29±0.049
Arginine	23.120	24.258	21.17±0.823	21.69±0.849	1.95±0.006	2.57±0.003
Asparagine	-	-	nd[2]	nd	0.19±0.003	0.12±0.006
Asparagine acid	-	-	-	-	1.29±0.004	1.46±0.005
Asparagine acid	39.244	39.283	37.76±1.104	37.71±1.187	-	-
Cysteine	3.355	3.250	3.36±0.058	3.25±0.074	nd	nd
Glutamic acid	-	-	-	-	2.88±0.002	3.65±0.037
Glutamine	-	-	nd	nd	nd	<0.1
Glutamic acid + Glycine	66.034 24.000	66.755 23.777	63.15±2.043 23.35±	63.11±1.820 23.02±	0.65 ± 0.004	0.75 ± 0.013
Proline	29.715	26.037	25.50±0.468	23.22±0.855	4.22±0.027	2.82±0.024
Serine	20.536	20.401	19.51±0.420	19.19±0.780	1.03±0.011	1.22±0.001
Tyrosine	37.553	38.584	35.48±0.667	35.90±1.148	2.07±0.009	2.68±0.010
Ornithine	0.112	0.124	-	-	0.11±0.005	0.12±0.002
Citruline	2.229	2.316	-	-	2.23±0.009	2.32±0.027
Ethanolamine	0.155	0.256	-	-	0.15±0.004	0.26±0.014
Taurine	<0.1	<0.1	-	-	<0.1	<0.1
Total protein	476.00	474.50				
Ratio E/N[3]	0.63	0.64	0.63	0.63	0.69	0.72

[*] Values given are the mean of four replicate analysis and are presented as mean ± SD
[2] nd: not detectable
[3] E: sum of essential amino acids and N: sum of non-essential amino acids

Table 5
Correlations between essential amino acids and principle components (PCA1 and PCA2).

Amino acid	PC 1	PC 2
Threonine	-0.33	-0.21
Valine	-0.33	-0.09
Isoleucine	-0.33	0.03
Leucine	-0.33	0.20
Phenylalanine	-0.33	0.39
Lysine	-0.33	-0.40
Histidine	-0.33	0.40
Methionine	-0.33	-0.64
Tryptophan	-0.33	0.13

4. Discussion

Alternatives for FM have to be explored to make a persistent growth in aquaculture production possible. Insects have been considered as good candidates, not only because of their richness in AA, lipids, vitamins and minerals, but also because of their small ecological footprint (Henry et al., 2015; Oonincx and De Boer 2012). Seaweeds have also been suggested as potential alternatives, however the presence of anti-nutritional factors make them less applicable as direct replacers of FM in aquatic diets (Silva et al., 2014). Despite of these anti-nutritional factors, seaweeds are a relatively untapped but valuable source of nutrients (Burtin 2003). Potentials to apply these food sources in the food chain should be explored. Therefore, in this study a new food chain is introduced, using *Z. morio* as a "vehicle" to transport valuable nutrients from Norwegian kelp towards the insect body, and serve as a potential alternative for FM. This new food chain fits well in the development of sustainable blue technologies.

Table 6
AA scores of essential amino acids calculated for *Z. Morio* fed oat meal, *Z. Morio* fed kelp granulate and fish meal (herring, data obtained from NRC, (2013)).

	Tilapia			Salmon		
	Z. morio fed oat meal	*Z. morio* fed kelp granulate	Fish meal	*Z. morio* fed oat meal	*Z. morio* fed kelp granulate	Fish meal
AA						
Arginine	124	131	125	103	108	104
Histidine	100	101	62	155	157	96
Isoleucine	148	149	147	167	168	165
Leucine	120	121	99	188	190	156
Lysine	108	114	184	89	94	152
Methionine	52	56	127	64	69	157
Methionine+cystine	58	60	153	65	68	173
Phenylalanine	111	115	98	169	175	149
Phenylalanine+tyrosine	228	235	120	251	260	133
Threonine	116	115	91	144	142	113
Tryptophan	152	143	90	189	177	112
Valine	137	135	88	212	209	136
Limiting AA	Methionine	Methionine	Histidine	Methionine	Methionine	Histidine

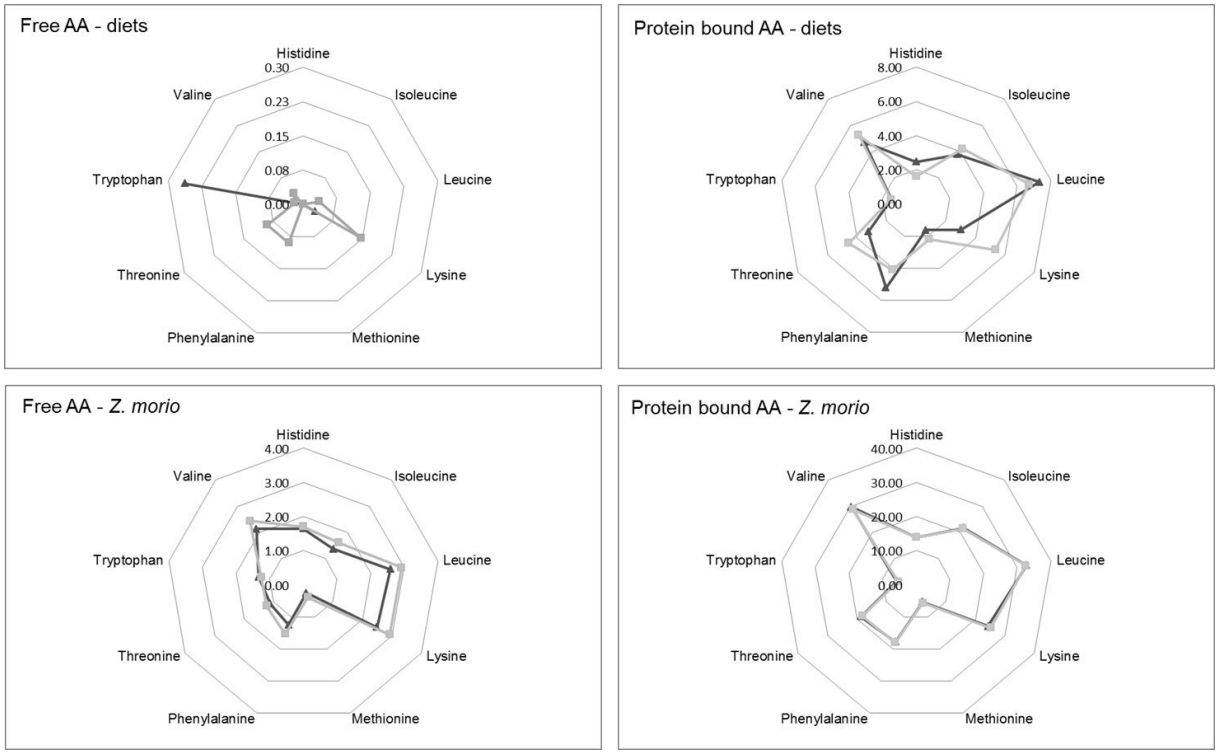

Figure 2
Spider web diagrams for the essential free AA and protein bound AA of the diets

(Oat meal Kelp granulate) and of *Z. morio* fed with these diets (Oat meal Kelp granulate)

FM replacers in aquatic diets should meet several requirements, among others for protein quality and quantity, which was the focus in this study. CP (45% of DM) and CF (40-42% of DM) content of *Z. morio* measured in the current study fall within ranges reported in other studies (43-47% of DM CP and 40-42% of DM CF) (Abd Rahman Jabir et al., 2012a; Barker et al., 1998; Bosch et al., 2014; Finke 2002; Rumpold and Schlüter 2013). CP content of FM is often higher, and range between 63 and 78% of DM ((NRC) 2013). Nevertheless, minimal protein requirements reported for fish vary between 29 and 42% of DM ((NRC) 2013), which is comparable to the CP content measured for *Z. morio* in this study.
FM is not only valuable because of its protein quantity, but also of its quality. Protein quality depends on several factors. EAA profiles can be used to indicate protein quality and alternatives for FM should have EAA profiles which are well balanced for fish (Meer and Verdegem 1996). Independent of diets fed, *Z. morio* in the current study contained all EAA that fish require for their growth ((NRC) 2013). Compared to their diets, all EAA were increased in the worms in equivalent proportions, showing potential for the use of *Z. morio* in aquatic diets.
AA profiles of insects are highly variable across studies (Bosch et al., 2014). Histidine content of *Z. morio* in the present study (15.5 g kg DM^{-1}) was for example lower than in the study of Bosch et al., (2014) (23 g kg DM^{-1}), but fell within the ranges of other studies (13-16 g kg DM^{-1}, (Rumpold and Schlüter 2013)-(Yi et al., 2013)). Phenylalanine on the other hand, was slightly higher in the current study (19 g kg DM^{-1}) than in other studies (15-17 g kg DM^{-1}, (Rumpold and Schlüter 2013)-(Bosch et al., 2014)). Different factors can be responsible for these variations, among others diets fed to the insects (Ramos-Elorduy et al., 2002). Also in the current study, PCA results indicate a difference in EAA profile between *Z. morio* fed oat meal and *Z. morio* fed

kelp granulate. Based on the second PC it can be stated that this difference was a result of the diets fed, and that EAA profiles of the diets was passed on to *Z. morio*. In particular for the free AA, *Z. morio* fed kelp granulate was slightly more enriched in most of the EAA (lysine, leucine, isoleucine, valine, phenylalanine and methionine). This shows the potential for a kelp granulate diet to replace grain diets in the commercial cultivation of *Z. morio* (Oonincx and De Boer 2012), and improve EAA quality of *Z. morio*. With the growing importance of insects in food chains (van Huis 2015), it is essential to get a better understanding of the intrinsic protein accretion in edible insects with varying feed and AA resources. This will contribute to a better control over AA compositions in insects, which is needed for the commercialization of insects as a protein source in feed. Protein accretion in insects is influenced by specialized cell types that interact differently with environmental factors, and for silkworm (*Bombyx mori*) it has been shown that the expression of certain genes and enzymes are organ- and tissue related (Brown et al., 2011; Xia et al., 2007). It is therefore recommended to apply a *genomics* and further *proteomics* approach in order to explain our observations and get a better understanding of influences of varying food sources on AA compositions in insects.

AA scores calculated in the current study showed that the first limiting AA of *Z. morio* is methionine for both tilapia and salmon. The lowest AA score was found for *Z. morio* fed with oat meal diet, due to their slightly lower methionine content compared to *Z. morio* fed with kelp granulate. Methionine is often reported as the first limiting AA, also for other potential candidates to replace FM in aquatic diets, like SBM (Furuya et al., 2004; Meer and Verdegem 1996). Total replacement of FM with only these alternatives may therefore result in impaired growth performances of fish. However, Furuya et al., (2004) showed that 100% replacement of FM with SBM was possible for Nile tilapia, when dicalcium phosphate and several AA were supplemented as well. Further research should therefore focus on a total replacement of FM in aquatic diets by *Z. morio*, supplemented with methionine.

Besides AA profiles, protein quality also relates to E/N ratio and digestibility of the protein fraction in the feed (Green et al., 2002; Henry et al., 2015). Similar as for other animals, fish require an adequate E/N ratio to sustain growth (Green et al., 2002). Several studies have investigated the optimum E/N ratio for different fish species and reported a minimum requirement of 0.6 (Green et al., 2002; Kim et al., 1991; Mambrini and Kaushik 1994). E/N ratio of *Z. morio* in the current study was 0.6, which fulfils this requirement.

Digestibility of the protein fraction of *Z. morio* by fish was not tested in this study, and this should be the next step in the realization of this new food chain. Insects, including *Z. morio*, contain chitin in their exoskeleton. It is the consensus that monogastric animals, like fish, are unable to digest chitin (Halver and Hardy 2002; Henry et al., 2015; Ringø et al., 2012). Three types of enzymes (chitinase, chitobiase and lysozyme) are needed to digest chitin (Fines and Holt 2010). Although the presence of these enzymes have been confirmed for both marine (Fines and Holt 2010; Kurokawa et al., 2004) and freshwater fish species (Lindsay et al., 1984), results on effects of chitin on digestibility are variable. For common carp it was for example observed that the presence of chitin did not affect their growth (Gopalakannan and Arul 2006; Victor et al., 2004), while several other studies showed that performance of salmonids is highly effected by chitin (Buddington 1980; Murai et al., 1980; Olsen et al., 2006). Chitin content is highly variable between insect species and can range from 11.6 to 137.2 mg kg DM^{-1} (Finke 2002). Although for *Z. morio* an moderate chitin content (average of 49.8 mg kg DM^{-1}) has been reported (Finke 2002), it is important that further studies focus on the digestibility of *Z. morio* by fish.

5. Conclusion

Results of this study are promising for the use of *Z. morio* as a "vehicle'' to transport EAA from kelp granulates into the insect body. Based on protein levels and AA profiles, *Z. morio* fed with kelp granulate can be seen as an interesting candidate to replace FM in aquatic diets. Addition of methionine is required. Next steps in the realization of this new food chain should focus on FA profiles of *Z. morio* and digestibility and palatability of *Z. morio* for fish. Special attention should be given to the effect of chitin on the digestibility of *Z. morio* by fish. Together, these aspects will determine if *Z. morio* can be used as alternative for FM in aquatic diets. Furthermore, other options using the new and untapped food sources introduced in this study (i.e. kelp granulate and *Z. morio*), as replacement for FM in aquatic diets, should be explored. FA extracted from kelp granulates, might be directly used as ingredient in aquatic feeds, while proteins left from these 'fat extracted' kelp, can be fed to *Z. morio*, which in turn can be used in aquatic diets as protein source.

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