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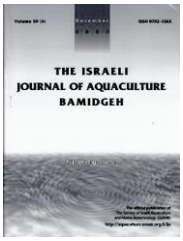

Effects of Housefly Maggot Meal and Earthworms on Growth and Immunity of the Asian Swamp Eel *Monopterus albus* (Zuiew)

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Keywords: Asian swamp eel; immunocompetence; fishmeal replacement; aquaculture; fish; fly larvae; maggot meal

Abstract

Fly larvae (maggot meal) and earthworms are promising potential replacements for increasingly expensive and unsustainable fishmeal. Apart from being a good protein source, fly larvae also contain substances with immunostimulant capacity, however the impact of their inclusion in feed on the immune system remains only partially understood. To address this, we studied the effects of four diets with varying levels of earthworm (40-70%) and maggot meal (0-30%) on growth and immunocompetence of juvenile Asian swamp eels *Monopterus albus* (fish n = 480, duration = 40 days). Maggot meal inclusion resulted in a significantly ($P<0.05$) higher total protein, glutamic pyruvic transaminase activity, anti-oxidizing enzyme and lysozyme activity, and significantly lower triglycerides, in the blood serum. Bacterial (*Aeromonas hydrophila*) challenge test results showed that adding maggot meal to the feed improved survival rate and immunocompetence at all three tested inclusion ratios. The strongest positive impact on most studied parameters (including 50% higher survival and 21.4% higher immunocompetence) was observed in the 20% inclusion group. As positive effects were slightly lower at the 30% level, the optimal combination of the two ingredients was 20% maggot meal and 50% earthworms.

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Introduction

The Asian swamp eel, *Monopterus albus* (Zuiew, 1793), also known as the ricefield eel or rice eel, is an air-breathing freshwater teleost fish (Synbranchidae family; Synbranchiformes order) distributed throughout S.E. Asia. Although it is farmed in several countries, the majority of global production of almost 370,000 tons takes place in China (FAO, 2016). The carnivorous Asian swamp eel requires comparatively high levels of protein in its diet, but the aquaculture industry is under strong pressure (globally, as well as in China) to replace the increasingly expensive and unsustainable fishmeal (traditionally main protein source) with cheaper and more sustainable replacements. As replacement with plant-based protein sources is often fraught with difficulties, such as lower growth rate or negative impact on the health of the farmed fish (Prathomya et al., 2018; Tacon, 1995), it may be necessary to (at least partially) replace fishmeal with alternative animal-based sources. Fly larvae and earthworms are promising replacers, containing adequate levels of digestible protein and essential amino acids (Chen, 2007; Herawati et al., 2018; Hussein et al., 2017; Ogunji et al., 2008). However, although there is one report of Asian swamp eel fed exclusively on earthworms (Herawati et al., 2018), the most suitable ratios of these two replacers for this species remain unknown.

Aside from this problem, with increasing fish production and intensification of aquaculture, the prevalence of diseases among farmed fish has increased severely, creating additional economic and technological pressures to control disease outbreaks as efficiently as possible. Traditionally, antibiotics were used to control (bacterial) diseases, but their application is hampered by numerous difficulties, which include pathogen tolerance, food safety, and environmental issues (Michael et al., 2014; Watts et al., 2017). Thus, there exists a pressing urge to replace antibiotics with more acceptable alternatives. Efficient ways to reduce the use of antibiotics also include prevention and control of diseases by improving the farming environment and strengthening the immune system to improve disease resistance (Chen, 2007). Apart from being a good protein source, fly larvae also contain antibacterial peptides, chitin, and other substances with immunostimulant capacity (Wang et al., 1991), but the impact on the immune system, of the inclusion of fly larvae into animal feed, remains only partially understood.

Therefore, to lay a scientific foundation for the inclusion of fly larvae and earthworms in aquaculture of Asian swamp eel, we designed four different diets, conducting a growth trial which contained a broad range of fly larvae (here referred to as maggot meal) and earthworm inclusion ratios. Furthermore, to advance the understanding of the effects of maggot meal on the immune system of fish, we explored the impact of these four diets on a number of blood serum biochemical parameters, as well as the non-specific immunity and disease resistance of Asian swamp eels. To study the latter, we conducted a bacterial challenge test, calculated mortality rates, and assessed immunocompetence of the fish.

Materials and Methods

Maggot meal and earthworm supply.

The housefly (*Musca domestica*) larvae powder (maggot meal) used in this study (60% protein and 2.6% fat) and frozen earthworms (*Pheretima* sp.) were supplied by the Wuhan Xingwuyuan Biotech Ltd.

Experiment design.

The Asian swamp eels were obtained from the Aquatic Experiment Base of the Yangtze University. Similar-sized, healthy juvenile eels (n=480, average weight = 30±5g) were acclimated in a net cage within a pond for seven days, and then randomly divided into eight net cages (60 specimens per cage, net aperture 2×2mm). These were then assigned to four treatment groups (two cages per each group: 60×2×4=480). The eight cages were kept in the same pond, thus ensuring similar environmental conditions. To test a broad range of maggot meal and earthworm inclusion ratios while maintaining an approximately constant protein ratio, the composition of all four groups was designed to contain an identical maggot meal + earthworms ratio of 70%. As the earthworm inclusion has been studied before, the impact of maggot meal on the immunity of the fish was the main objective of this study, so the diets were named after the maggot meal

inclusion ratios: the control group was designed to contain 0% maggot meal and 70% earthworms, and the experimental groups were named 10% (10/60% maggot meal/earthworms respectively), 20% (20/50%), and 30% (30/40%) (Table 1). The composition of diets varied slightly, but as a general guideline diets were designed to contain: crude protein > 43%, lysine > 2.4%, crude fat > 3%, coarse fiber < 5%, coarse ash < 15%, total phosphorus > 1.1%, moisture < 10%. Ingredients for the four diets were mixed in our laboratory and refrigerated until use. Eels were fed 7-11% of the total fish mass once daily (18:00) for 40 days (6/20/2017 - 7/28/2017). For the duration of the experiment, the water quality was checked periodically, feeding was video-recorded and feed residues were cleaned out. Samples were taken after a 24-h starvation period. All animals were handled and experimental procedures conducted following the guidelines for the care and use of animals for scientific purposes set by the Ministry of Science and Technology, Beijing, China (No. 398, 2006). The animal handling protocol for this study was approved by the Animal Care and Use Committee of Hubei Province (China), Permit number: SCXK(Hubei)2015-0018.

Table 1. Experimental groups and diet composition.

Groups[1]	*Control*	*10%*	*20%*	*30%*
No. of eels	60	60	60	60
Replicates	2	2	2	2
[2]Maggot meal (%)	0	10	20	30
[2]Frozen earthworms (%)	70	60	50	40
[3]Industrial pelleted feed (%)	30	30	30	30

[1] Experimental groups are named according to the maggot meal inclusion ratio.
[2] Producer: Wuhan XingwuYuan Biotechnology Co. Ltd.
[3] Producer: Yueyang Zhitang Biological Science and Technology Co. Ltd., Xinfu brand. Ingredients: fishmeal, soybean meal, fish oil, beer yeast powder, flour, calcium dihydrogen phosphate, copper sulfate, magnesium sulfate, ferrous sulfate, cobalt sulfate, manganese sulfate, sodium selenite, vitamins (A, D3, E, niacin, calcium pantothenate, folic acid, etc.).

Plasma collection.

Twelve Asian swamp eels were randomly chosen from each of the four groups (12×4=48) and their blood samples taken. As this is a relatively small fish, to ensure a sufficient amount of plasma per sample, experimental samples were generated by merging two randomly chosen blood samples from the same group, thereby generating six samples per group. After skin disinfection with 75% alcohol solution, blood was collected from the caudal vein using a sterile syringe. After placing the blood to settle at room temperature, it was stored overnight at 4°C. On the following day, plasma was separated by centrifugation at 4000r/min and stored at -80°C.

Biochemical and immunological parameters.

We determined a total of nine biochemical and immunological parameters: total protein (TP), triglycerides (TG), glutamic pyruvic transaminase (GPT), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), acid phosphatase (ACP), alkaline phosphatase (AKP), and lysozyme (LZM). Reagents and kits were obtained from the Nanjing Jiancheng Bioengineering Institute.

Bacterial challenge.

After concluding the feeding trial, 40 similarly sized eels were chosen from each group, subdivided into two groups (n=20) and placed in pre-marked 60L plastic containers. After a seven-day adjustment period, each fish was injected 0.4ml of *Aeromonas hydrophila* bacterial solution (19CFU/ml) using disposable syringes. The bacteria were supplied by the Aquatic Laboratory of the Central China Agricultural University. Following the injection, the state (behaviour and health signs) of the eels was monitored every two hours over the course of seven days. The number of surviving specimens was recorded. Infection survival rate (SR), mortality, and immunocompetence (RPS) were calculated as follows:

$$SR = (\text{No. of survivors} / \text{initial total No.}) \times 100;$$

$$\text{Mortality} = 100 - SR;$$

$$RPS = [(\text{control group mortality} - \text{experimental group mortality}) / \text{control group mortality}] \times 100$$

Data analysis.

Statistical significance of differences between groups was assessed for blood parameters (6 biological replicates) by one-way ANOVA and Fisher tests using SPSS20.0 program. Differences were considered significant at the $P<0.05$ level. All results are expressed as mean±SD.

Results

Growth and survival rates.

Both the weight gain and survival rate of the four groups were the highest at the 20% maggot meal inclusion ratio, and lowest in the control group: 20% > 10% > 30% > Control (Table 2).

Table 2. Growth and survival rate of *Monopterus albus* during the growth trial.

Group	*W_I (g)*[1]	*W_F (g)*[2]	*Weight gain*[3]	*Survival (%)*
Control	30.95	58.26±12.44	1.88±0.40	55.83±29.20
10%	30.95	65.60±13.90	2.12±0.45	89.44±1.73
20%	30.95	67.95±15.77	2.20±0.51	97.78±32.56
30%	30.95	63.52±12.48	2.05±0.40	81.67±9.46

[1] W_I = initial weight
[2] W_F = final weight
[3] Weight gain = W_F / W_I

The effect of maggot meal on the blood serum biochemical parameters.

The addition of maggot meal to the feed resulted in increased total protein levels in the serum (Table 3). The highest total protein level was observed at the 20% inclusion level: 8.06% higher than the control group ($P<0.05$). The addition of maggot meal to the feed decreased the triglyceride levels. The largest decrease was observed at the 20% maggot meal inclusion level: 29.93% lower than the control group ($P<0.05$). The addition of maggot meal resulted in a strong increase of the of glutamic pyruvic transaminase (GPT) activity in the blood serum. The largest increase was observed at the 20% maggot meal inclusion: 255% higher than the control group ($P<0.05$).The activity of lysozyme was also the highest in the 20% group: 39.37% higher compared to the control group. When the maggot meal inclusion ratio was increased to30%, both lysozyme and GPT activity decreased.

Table 3. Biochemical analysis of the serum.

Group	*TP (mg/ml)*[1]	*TG (mMol/ml)*[2]	*GPT (U/L)*[3]	*LZM (U/mL)*[4]
Control	[5,6]35.44±1.21^{a}	1.57±0.23^{a}	7.87±1.21^{a}	2.21±0.11^{a}
10%	36.08±1.55^{ab}	1.45±0.21^{ab}	14.39±3.64^{b}	2.48±014^{b}
20%	38.55±1.48^{b}	1.10±0.29^{b}	20.07±2.9^{c}	3.08±0.69^{c}
30%	38.10±2.22^{ab}	1.21±0.29^{b}	15.30±4.81^{b}	2.85±0.17^{d}

[1] total protein (TP)
[2] triglycerides (TG)
[3] glutamic pyruvic transaminase (GPT)
[4] lysozymes (LZM).
[5] Data are expressed as mean±SD (n = 6).
[6] Data in the same column with different superscript letters are significantly different ($P<0.05$).

The effect of maggot meal on phosphatases and antioxidases in the blood serum.

Maggot meal significantly increased the activity of both analysed phosphatase (Table 3). The activity of alkaline phosphatase (AKP) was the highest when the maggot meal ratio was 30%, an increase of 38.80% compared to the control group. The activity of acid phosphatase (ACP) was highest in the 20% group, which is an increase of 60.4% compared to the control group. As opposed to the AKP, the activity of ACP declined when the ratio of maggot meal was 30%.

The inclusion of maggot meal also had significant effects on all three studied antioxidases: superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase. The activities of all three of these enzymes were highest in the 20% group (Table 4), where the SOD showed a 23.80% increase, GSH-Px a 20.98% increase, and catalase a 30.93% increase compared to the control group (all $P<0.05$). When the

maggot meal inclusion ratio was increased to 30%, the activities of all three oxidases decreased.

Table 4. Effects of dietary maggot meal on antioxidase and phosphatase in the serum.

Group	*SOD (U/ml)*[1]	*GSH-Px (U/ml)*[2]	*Catalase (U/ml)*	*AKP*[3]	*ACP (U/100ml)*[4]
Control	[5,6]126.7±11.52[a]	1140±60.39[a]	1.81±0.09[a]	5.00±0.69[a]	4.88±0.70[a]
10%	137.59±9.38[a]	1256±59.62[ab]	2.15±0.22[b]	5.56±0.76[a]	6.11±0.92[b]
20%	156.85±12.70[b]	1379.2±107.66[b]	2.37±0.08[b]	6.63±0.68[ab]	7.81±0.60[c]
30%	140.41±21.21[ab]	1208.8±155.02[a]	2.16±0.45[b]	6.94±1.32[b]	7.13±1.56[bc]

[1] Superoxide dismutase (SOD)
[2] Glutathione peroxidase (GSH-Px)
[3] Alkaline phosphatase (AKP), expressed in King-Armstrong units: *KAU/100ml*
[4] Acid phosphatase (ACP).
[5] Data are expressed as mean±SD (n=6).
[6] Values in the same column with different superscript letters are significantly different (P<0.05).

The effect of maggot meal on the survival rate and immunocompetence of Asian swamp eels after bacterial infection.

The inclusion of maggot meal into the diet of Asian swamp eel increased the survival rate after the *A. hydrophila* challenge (Table 5). The highest survival rate of 45% was observed in the 20% group, representing a 50% increase in comparison to the control group. Immunocompetence was also improved in all three groups (10.7 to 21.4%), with the highest improvement in the 20% group.

Table 5. Results of the bacterial challenge test.

Group	*Infected*	*Surviving*	*Survival rate (%)*	*RPS*[1] *(%)*
Control	40	12	[2]30.00 ± 7.07	-
10%	40	15	37.50 ± 3.54	10.71
20%	40	18	45.00 ± 7.07	21.43
30%	40	15	37.50 ± 3.54	10.71

[1] RPS is immunocompetence change (%) in comparison to the control group.
[2] Data are expressed as mean±SD (n = 2).

Discussion

Growth and survival rates.

Weight gain and survival rates of the four groups both indicate that high levels of earthworms produced negative effects (control group, 70% of earthworms, had the lowest growth rate and the highest mortality). In a previous study of four different replacers for the fishmeal in the Asian swamp eel, a 100% earthworm-based diet resulted in a comparatively good growth rate, but the survival rate was rather low (66%) (Herawati et al., 2018). Therefore, we can conclude that very high earthworm inclusion ratios (>60%) are likely to have negative effects on survival rate, and therefore is not suitable for the Asian swamp eel. These negative (both on growth and survival) effects were successfully offset by the inclusion of maggot meal, even at a comparatively low ratio of 10%. However, whereas the 20% inclusion level produced further improvements, we observed a drop in these two parameters at the highest studied maggot meal inclusion ratio (30%), which indicates that excessively high ratios of maggot meal can also produce negative effects on growth and survival of the Asian swamp eel.

The biochemical indices in the blood serum of the Asian swamp eel.

Fish possess a relatively rudimentary specific immune system, which means that production of antibodies is rather low and often inefficient, and the non-specific, innate immune system plays a major defense role against pathogens (Saravanan et al., 2013; Tort et al., 2003). In studies of non-specific immune system in fish, biochemical and antioxidant indices from blood serum are widely used as indicators of fish health, nutritional status and adaption to environmental setting (Cerón et al., 1996).

The total protein within the blood serum is mainly made up of globulin and albumin. It maintains the colloid osmotic pressure of the blood, and stabilizes the transport of fatty acids and other substances. The total protein in the blood serum can also be used as an indirect indicator of the non-specific immunity level, and as such is an important indicator of the immunological health of the fish (Poelstra et al., 1997). This study shows

that when 20% to 30% of maggot meal is added to the feed, the total protein level in the blood serum of Asian swamp eels increases significantly ($P<0.05$). As total protein levels were similar in all four diets, this indicates that, within a certain range, maggot meal boosts protein synthesis.

Fish triglycerides are primarily synthesized within the liver and adipose tissue, and are generally contained within the adipose tissue. Serum triglycerides are an important indicator of the normality of an organism's blood lipid level and can reflect the condition of the lipid metabolism (Zhou et al., 2012). This study shows that adding maggot meal to the feed, significantly decreases the level of triglycerides in the blood serum of Asian swamp eels, which indicates that maggot meal can promote lipid metabolism in this species.

Glutamic pyruvic transaminase is an important aminotransferase, mostly stored in the liver of fish. Once the liver is diseased or suffers cell damage, a great quantity of glutamic pyruvic transaminase enters the blood serum. Therefore, the magnitude of glutamic pyruvic transaminase activity in the blood serum is an important indicator when diagnosing the health state of the liver (Breitling et al., 2011; Kim et al., 2008; Prisingkorn et al., 2017). In this study, the groups with supplemented maggot meal exhibited an increase of glutamic pyruvic transaminase activity in the blood serum, which appears to be an indication of negative impact of maggot meal on the health of the liver. However, as this effect was reversed at the highest inclusion ratio (30%), it is an indication that some other mechanisms might be at play here, causing increased levels of this enzyme. Further studies are needed to better elucidate this association.

Effect of maggot meal on antioxidative ability and lysozymes of Asian swamp eels.

Aerobic metabolism can produce reactive oxygen species (free radicals), high levels of which can cause oxidative damage (oxidative stress). The enzymes that clean up free radicals in the organism include superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (Beckman et al., 1990; Lubos et al., 2011; Zhang and Tian, 2007). The results of this study indicate that the addition of maggot meal to the feed significantly increased the activity of these three enzymes in the blood serum of Asian swamp eels, with the highest activity at the 20% inclusion level.

In fish and other lower vertebrates, phosphatases help lysosomes to more effectively accomplish the double functions of digestion and defense. Increased levels of phosphatases can boost growth of the organism and improve non-specific immunity (Haijin et al., 1999). The results of this study show that adding maggot meal to the feed of Asian swamp eels can significantly increase the activity of phosphatases: the highest activity of AKP was observed at the maximum inclusion level (30%), whereas the highest ACP activity was observed at the 20% inclusion level. However, as the increase in the AKP activity observed between 20% and 30% was non-significant, this suggests that very high ratios of maggot meal may also result in the inhibition of AKP activity.

Lysozymes are antimicrobial enzymes produced by animals and are an important component of the non-specific immune system in fish. Lysozymes can compromise the structural integrity of bacterial cell walls, thereby causing lysis of the bacteria. They also have destructive effects on viruses, fungi, and parasites (Zhang et al., 2012). The results of this study indicate that adding maggot meal to the feed significantly increases the activity of lysozymes in the blood serum of Asian swamp eels, with maximum activity observed at the 20% inclusion level.

The effect of maggot meal on the survival rate of Asian swamp eels after bacterial challenge and immunocompetence index.

As the breeding density increases, the water environment deteriorates, which causes the immune system of aquatic animals to suffer long-term stress, and eventually increases the chance of infection (Sakai, 1992). Survival rate after an infection is a comprehensive indicator of disease resistance in aquatic animals. Many studies have found that adding maggot meal to the feed of aquatic animals can increase immunity (Chen, 2007; Cheng et al., 2017). The effects of *A. hydrophila* infection on the Asian swamp eel have previously been studied in detail (He et al., 2010), thus in this study we focused only on the comparison of mortality rates among the four groups. We found that adding 10%, 20%, and 30% of maggot meal to the feed respectively increases the

survival rate by 7.5%, 15%, and 7.5%, with respective increases in immunocompetence of 10.7%, 21.43%, and 10.71%. This indicates that adding maggot meal to their feed can improve resistance to disease of Asian swamp eels and thereby lower the risk of infection. This could be connected with the positive correlation between dietary maggot meal supplementation and the activity of phosphatase, antioxidase, and lysozymes in the blood serum, which thereby strengthens the non-specific immunity of Asian swamp eels. There is also a possibility that maggot meal contains antibacterial peptides, which can have a direct antibacterial effect and/or act as an immunity effector, stimulating the host's immune defense system (Guan et al., 2014).

In conclusion although earthworms are easily available and are a good protein source (64–76%), which makes them a promising fishmeal replacer (Herawati et al., 2018), high inclusion ratios resulted in decreased survival rates in the Asian swamp eel. Intriguingly, these were offset even by relatively low rates of maggot meal (10%). This indicates that even low inclusion rates of maggot meal may produce significant effects on the immunity (survival rate) of this species, which deserves further scientific attention. Under the studied conditions, adding maggot meal to the feed of Asian swamp eels effectively improved the non-specific immunocompetence and disease resistance at all inclusion ratios (1%-30%), but positive effects were slightly reversed at the maximum inclusion ratio. We therefore hypothesise that high inclusion levels are likely to produce some negative effects. The best results in this study were consistently observed at the 20% inclusion ratio. Further studies are needed to determine optimal ratios of these two fishmeal replacers in the Asian swamp eel, but our results indicate that earthworms should not be included in ratios much higher than 50%, and that even low ratios of maggot meal can produce positive effects. The provisionally recommended inclusion levels are 20% for maggot meal and 50% for earthworms.

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