

Gastric Emptying and the Enzymatic Activity in the Stomach of *Amphiprion ocellaris* Fed on Artificial Diet

(Pengosongan Perut dan Aktiviti Enzim dalam Perut *Amphiprion ocellaris* yang Diberi Diet Buatan)

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ABSTRACT

This study aims to elucidate the gastric emptying process of clownfish fed on artificial diet using two gastric evacuation models and to determine the pepsin activity in the digestion process in relation to feeding time. Regression analysis was used to evaluate the adequacy of 2 models; Anderson's $[S_t = S_0(1 - S_0^{(\alpha-1)}\rho(1-\alpha)t)^{1/(1-\alpha)} + \xi]$ and Grove's model $[S_t = (S_0^\alpha - \alpha Kt)^{1/\alpha}]$, in describing the gastric emptying rate. Grove's model provided a better fit with higher r^2 value, with the calculated parameters of maximum meal size at time 0 (S_0) = 0.195 g and gastric emptying rate (K) = 0.0165 g h⁻¹. There was no initial delay phase as predicted and the evacuation followed a curve. Pepsin activity in the stomach showed rapid responses to food intake, where activity was detected at 1 h after feeding and reached its peak at 2 h after feeding. Pepsin activity decreased since then until the 12th h after feeding where it reached the lowest point. An increase of pepsin activity was detected later, where a small boost was detected at 24 h after feeding to digest the remaining food item in the stomach before the pepsin secretion decreased and maintained at pre-feeding level. Fast response of digestive enzyme in stomach implied that clownfish is equipped to utilize infrequent and irregular meals effectively.

Keywords: *Amphiprion ocellaris*; evacuation model; gastric digestion, pepsin activity

ABSTRAK

Kajian ini bertujuan untuk menjelaskan proses pengosongan perut ikan badut yang diberi diet buatan dengan menggunakan dua model pengosongan gastrik dan untuk menentukan hubungan antara aktiviti enzim pepsin dalam proses pencernaan dengan masa selepas makan. Analisis regresi digunakan untuk menentukan ketepatan dua model; model Anderson $[S_t = S_0(1 - S_0^{(\alpha-1)}\rho(1-\alpha)t)^{1/(1-\alpha)} + \xi]$ dan model Grove $[S_t = (S_0^\alpha - \alpha Kt)^{1/\alpha}]$, dalam penentuan kadar pengosongan perut selepas makan. Model Grove lebih sesuai kerana mempunyai nilai r yang lebih tinggi dengan parameter yang dikira merupakan saiz hidangan maksimum pada masa 0 (S_0) = 0.195 g dan kadar pengosongan perut (K) = 0.0165 g per jam. Tiada fasa lewat pada permulaan proses penghadaman seperti yang dianggarkan dan pengosongan perut adalah sejajar dengan lengkungan. Aktiviti enzim pepsin dalam perut menunjukkan tindak balas yang pantas terhadap pengambilan makanan dengan aktiviti pepsin dikesan seawal 1 jam selepas makan dan mencapai kemuncaknya pada masa 2 jam selepas makan. Aktiviti pepsin kemudiannya semakin menurun sehingga mencapai tahap terendahnya pada jam 12 selepas makan. Selepas itu, aktiviti pepsin meningkat semula dan terdapat rangsangan kecil dalam rembesan enzim yang dikesan pada 24 jam selepas makan. Aktiviti pepsin kemudiannya menurun semula kepada tahap sebelum makan. Tindak balas yang cepat terhadap pengambilan makanan menunjukkan ikan badut mampu menghadapi keadaan pembekalan makanan yang tidak menentu dengan berkesan.

Kata kunci: Aktiviti pepsin; *Amphiprion ocellaris*; model pengosongan; penghadaman gastrik

INTRODUCTION

The members of the clownfish (*Amphiprion* spp.) are one of the most demanding ornamental fish species in the aquarium trading (Wabnitz et al. 2003). In Malaysia, the supply of the clownfishes is dependently on the wild catch, thus increasing the potential of the fish being over-exploited (Cato & Brown 2003; Chapman 1997). It is unfortunate that the studies on Malaysian pomacentrids are lacking, or only focused mainly on the reproduction biology (Liew et al. 2006; Sin et al. 1994). Studies in fish digestive physiology are of primary importance to set up nutritional protocols responsive to the metabolic capabilities of feed

utilization of targeted species (Smith 1989). Digestion of the fish depends upon both the physical state of the food and the kind and quantity of enzymes in the digestive tract (Fänge & Grove 1979), where the ability of fish to digest a particular component of diet can be ascertained by investigating the complement of digestive enzymes present along the digestive tract (Palanisamy 1989).

Clownfish *Amphiprion ocellaris* is omnivorous in nature and consume a wide variety of larva prey and algae (Fautin & Allen 1992; Myers 1999; Sano et al. 1984). To achieve optimal digestion and utilization of fish feed for this species, the food need to be palatable and be

size appropriate corresponding to its natural food items. Understanding the rate of digestion allows the prediction of return of appetite under given conditions and diets in captivity (Riche et al. 2004). Investigation of the secretory response time would help establish a better insight of its temporal digestive abilities, which in turn allows optimal feeding frequency to be predicted; crucial information for developing suitable regimes with maximum food intake and feed efficiency (McCarthy et al. 1993; Uys et al. 1987; Windell et al. 1972). Therefore, this paper aims to describe the digestion process of clownfish which focused on initiating the gastric emptying rates and the temporal changes in the enzyme pattern during digestion.

MATERIALS AND METHODS

THE EXPERIMENTAL FISHES

Thirty two fishes were bought, quarantined and maintained at condition close to their natural habitat (temperature 27°C, salinity 28-30ppt) (Fautin & Allen 1992; Maison & Graham 2015) and were acclimatized with feeding of artificial diet (Sanyu Marine XP, containing 42% of crude protein and 5% of lipid) for a week. Subsequently, the fishes were deprived for food for 72 h to completely empty the stomach contents. The fishes were then fed to satiation and the exact amount of pellets eaten by each fish was recorded. They were slaughtered at selected time. i.e. 0, 1, 2, 4, 8, 12, 16, 20, 24, 28, 32 and 36 h post feeding and their stomach were separately removed. The contents of the stomach were weighed to the nearest 0.001 g and the data was used to model for gastric emptying rates. The stomach tissue were rinsed and then kept in the freezer at -20°C for further enzyme studies.

MODELLING OF GASTRIC EMPTYING RATES

Collected data on the gastric evacuation are described by a range of mathematical models namely the power equation model based on Jones (1974):

$$dS/dt = -\rho S^\alpha \quad (1)$$

S is the weight of total stomach content; and ρ and α are the parameters to be estimated. ρ was extended as a function of explanatory variables, where the effects of the variables are explored separately.

The equation was further developed to describe the emptying curve for a whole meal which forms a bolus or a simple item with similar shape but varying in sizes by Grove et al. (1985):

$$S_t = (S_o^{(1-\alpha)} - \alpha Kt)^{1/(1-\alpha)} \quad (2)$$

where S_t is total stomach content at time t after ingestion of meal size S_o , and $0 \leq S_o \leq S_{max}$.

Anderson (1999, 1998) then discovered that gastric evacuation can be described as a simple power model with an exponent of 0.5, which is unaffected by meal size and expanded the model by complete integration of (1) from time 0 to t as;

$$S_t = S_o(1 - S_o^{(\alpha-1)}\rho(1-\alpha)t)^{1/(1-\alpha)} + \xi \quad (3)$$

where S_t is total stomach content at time t after ingestion of meal size S_o ; and ξ is the random error term. In their study, the square root model version is incorporated in (3) and the gastric emptying rate constant, ρ (GER) was estimated by non-linear regression of (3). These two equations (2 & 3) are used for the comparative study of the digestion process in this study to discover the best fit for the gastric emptying of the clownfish.

Non-linear regression was used to estimate parameters of a non-linear model using MicroCalOrigin™ software programme for this study. The programme uses the Levenberg-Marquardt iterative method (Hashim et al. 2018; Mazlan 2001), utilizing non-linear procedures to examine the starting value specifications of the parameters and evaluate the Chi square value at each combination of values to determine the best set of values to start the iterative algorithm.

MEASUREMENT OF ENZYME PEPSIN ACTIVITY

Stomach samples obtained from the method described earlier were homogenized individually in ice cold 50 mM tris-HCL buffer; pH7.4. The homogenate was then centrifuged at 5500 g for 5 min at 4°C before removing the supernatant to be stored at -20° prior to analysis. Total protein content of supernatant was determined using Bradford assay (Bradford 1976), using bovine serum albumin as the standard. The standard curve of BSA protein standard prepared was $y = 0.0144x - 0.0234$ (Figure 1), which was used to calculate the mg protein from the gastric samples.

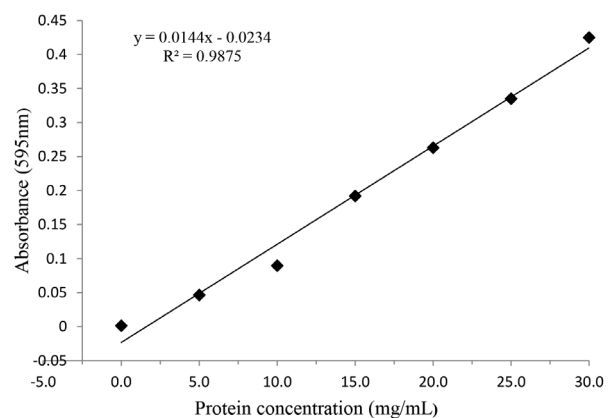


FIGURE 1. Standard curve for BSA protein assay

The pepsin activity assay was determined using the Worthington (1993) assay, where pepsin activity was estimated using 2% haemoglobin in 0.06 N.HCl as substrate. A total of 100 μ L enzyme extract in 0.01 N.HCl was incubated at 37°C with 500 μ L of substrate for 10 min. The reaction was terminated by adding 1 mL of 5% trichloroacetic acid (TCA) and left for 5 min before centrifuging at 12000 \times g for 5 min. Absorbance was recorded at 280 nm. For blank reading, TCA was added to substrate prior to the addition of enzyme extract. Specific activity (U) was expressed as:

$$\frac{\text{Absorbance value at } 280 \text{ (supernatant)} - \text{Absorbance value at } 280 \text{ (blank)}}{10 \text{ minutes} \times \text{mg protein}}$$

RESULTS

MODELLING OF GASTRIC EMPTYING RATES

All stomachs were emptied within 36 h as predicted by Khoo and Mazlan (2014). Gastric evacuation rates (GER) for both equations were curvilinear and closely similar as shown in Figure 2, therefore both models are applicable. The Anderson model predicted a slightly smaller size of maximum food intake and slower emptying rate where the maximum meal size of 0.177 g with gastric emptying rate (ρ) 0.012 gh^{-1} , whereas the Grove model predicted

the maximum meal size of 0.196 g with gastric emptying rate (K) of 0.016 gh^{-1} . Since the r^2 value was higher, the simple power model from Grove et al. (1985) seems more accurate (Table 1).

ENZYMATIC ACTIVITY IN THE STOMACH OF CLOWNFISH

The patterns of specific pepsin activity are as shown in Figure 3. Total pepsin activity recorded in the stomach ranged from 8.77 to 52.36 Umg^{-1} protein with average value of 30.64 Umg^{-1} . Pepsin activity was detected on the first hour after feeding, increases and reached its peak at 2 h after feeding before undergoing a sharp decline to its lowest peak at 12 h after feeding. It then recovered and reached a second but lower peak at the 24th h after feeding before the activity was maintained at a rather constant level after 28 h prior to feeding. The secretory patterns resembled the digestive enzyme activity in African catfish, *Clarias gareipinus* (Uys et al. 1987) where pepsin activity was too detected as early as 1 h after feeding and reached its maximum at about 2.5 h prior to feeding before decreasing progressively after.

DISCUSSION

Gastric emptying process started earlier as predicted by previous works (Khoo & Mazlan 2014). In studies where artificial feeds are concerned, a delay phase is usually

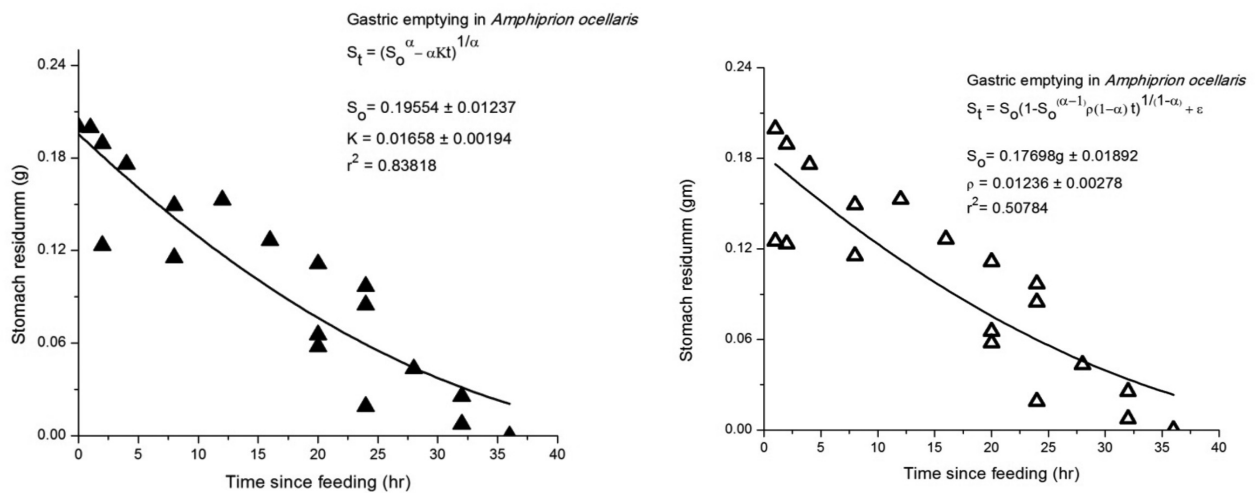


FIGURE 2. Gastric emptying curve of clownfish feeding on pellets using Grove's model (left) and Anderson's model (right)

TABLE 1. Comparison of gastric emptying rates between the two models used for clownfish fed on pellets

Equation	S_{\max} or S_0 (g)	K or ρ (g hr^{-1})	r^2
$S_t = S_0(1 - S_0^{(\alpha-1)}\rho(1-\alpha)t)^{1/(1-\alpha)} + \xi$ (Anderson model)	0.17698 \pm 0.01892	0.01236 \pm 0.00278	0.50784
$S_t = (S_0^\alpha - \alpha K t)^{1/1-\alpha}$ (Grove model)	0.19554 \pm 0.01237	0.01658 \pm 0.00194	0.83818

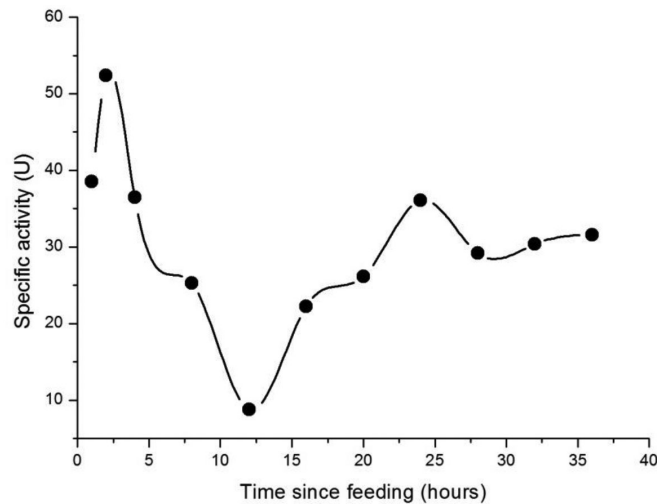


FIGURE 3. Changes in pepsin activity in the stomach contents of clownfish fed on pellets at selected time after feeding

detected as the food ingested needed to be softened before digested and this imposes a demand of water intake which delays the digestion rate (Grove et al. 2001; Hughes & Barrows 1990; Ruohonen et al. 1997). Dry pellets usually contained water level of less than 10% in contrast to the natural preys (70-80%). Therefore, food needed to be moistened before digestion can start (Hughes & Barrows 1990; Kristiansen & Rankin 2001; Ruohonen et al. 1998). However, this is not the case in our studies where digestion was detected as early as 1 h after feeding. This might be due to the pellets absorbing moisture as soon as in contact with water, therefore fishes are likely taken moistened pellets before digestion takes place. The gastric emptying time of 36 h were longer than the hybrid grouper, *Epinephelus* spp. (~15 h) (Moumita et al. 2016, 2014) and the tropical snapper (~20 h) (Mazumber et al. 2015). Although these fishes are of tropical origin, the difference in their feeding niches is probably affecting the gastric emptying time where evidence was reviewed that fish which have dissimilar food in nature have dissimilar gastric emptying time (Fänge & Grove 1979).

This is the first report on the enzymatic activity in clownfish fed on artificial diet. The pepsin activity also showed no delay in the early phase similar to the gastric emptying curve, which indicated that the enzyme secretory appears to be induced by food intake. The gradual decrease after the 2nd h after feeding may be caused by denaturation of enzymes, or most enzymes were being absorbed or bound to substrates and being actively evacuated from the stomach into the anterior intestine. This indicated that enzyme secretion in stomach of clownfish was limiting and not continuous. The small boost of enzyme secretion after 24th h prior to feeding to finish off the remaining food implied that enzyme secretion might be regulated by stomach distension as proposed by Smit (1968) in frogs (*Rana* sp.), and Western and Jennings (1970) in cottids (*Cottus gobio*) and brown trout (*Salmo trutta*). A smaller

bolus of food created less distention of stomach wall which resulted in less enzyme secretion. Storage of secretory enzymes was reported by Caruso et al. (2008), Einarsson et al. (1996) and Uys et al. (1987) in starved Atlantic salmon, European eel and yearling catfish respectively at pre-feeding time (T_0) despite absence of food for 24 h. The enzyme activity was not determined in this study however, the pepsin activity remained constant at 30 U mg^{-1} after the 30th h after feeding and that might suggest the pre-feeding level of enzyme activity in the stomach of the clownfish.

The pepsin activity measured in this study is compared among others, non-congeneric to non-marine species as studies on the temporal changes in enzyme pattern during digestion are limited to only a few species namely, carp (*Cyprinus carpio*), Japanese eel (*Anguilla japonica*), African catfish (*Clarias gariepinus*) and European eel (*A. anguilla*). The changes in the pepsin activity in stomach of clownfish followed closely to the protease activity in the stomach of African catfish with the maximum level of secretion at 2.5 h after feeding before decreasing as reported by Uys et al. (1987). Onishi et al. (1976, 1973a, 1973b) and Palanisamy (1989) reported a peak protease activity after 5 h prior to feeding for carp and grey mullet (*Liza parsia*), respectively, while Takii et al. (1985) discovered that maximum protease activity in Japanese eel is achieved only 12 h after feeding. The slow secretory response time in the species aforementioned studies will be at disadvantage should there be any irregular supply of food item or infrequent meals. In comparison, the clownfish has a more rapid digestive enzyme secretory response which may imply that clownfish is equipped to cope with infrequent and irregular meals effectively.

CONCLUSION

Gastric emptying of clownfish followed a curve, with no delay phase detected. The pepsin activity was at its peak

at 2 h after feeding before a gradual decrease and a small boost of enzyme secretion as observed at 24th h later feeding to finish off the remaining food in the stomach. The results in this study provided basic information on the gastric emptying process and its enzymatic activity on clownfish fed on artificial diet which will eventually give a better insight on how to manage this fish species in captive water. The underlying patterns of feeding and digestion rate found can be further improved to be used for future research aquaculture and fisheries management.

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