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Digestive proteinases of red shrimp *Pleoticus muelleri* (Decapoda, Penaeoidea): partial characterization and relationship with molting

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Abstract

The present study describes the activity and some characteristics of proteinases in the hepatopancreas of red shrimp *Pleoticus muelleri* during the different stages of the molting cycle. Proteolytic activity was highest between pH 7.5 and 8. The hepatopancreatic protein content in the premolt stage was higher than in the other stages of the molting cycle ($P < 0.05$). No significant differences were found in total proteolytic activity in the hepatopancreas when comparing molting stages. The proteolytic activity of the *P. muelleri* hepatopancreas enzyme preparations is the main responsibility of serine proteinases. TLCK, a trypsin inhibitor, reduced azocasein hydrolysis between 26% (intermolt) and 37% (premolt). TPCK, a chymotrypsin inhibitor, did not decrease hydrolytic activity, except for in postmolt. Low trypsin and chymotrypsin activities were found during intermolt, and increased in postmolt. The electrophoretogram of the enzyme extracts shows 12 bands of activity during intermolt (from 16.6 to 53.1 kDa). Some fractions were not detected in the postmolt and premolt stages. Three low molecular weight trypsin forms (17.4, 19.1 and 20 kDa) were found in all molting stages. One band of chymotrypsin (21.9 kDa) was observed in all molting stages. High molecular mass active bands (66–205 kDa) could not be characterized with inhibitors. Comparison of the protease-specific activity of the hepatopancreas of some species indicated a relationship between digestive enzyme activity and feeding habits of the shrimp. Omnivorous shrimp, such as *Penaeus vannamei* (syn: *Litopenaeus vannamei*) and *Penaeus monodon*, showed higher protease activity than the carnivorous shrimp, *Penaeus californiensis* (syn: *Farfantepenaeus californiensis*) and *P. muelleri*. In fact, the enzymatic activity in the hepatopancreas of *P. muelleri* showed variations in relation to feeding habit and molting cycle.

Keywords: Digestive enzymes; Molting; *Pleoticus muelleri*; Protein digestion; Proteases

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1. Introduction

The understanding of basic digestive physiology is important for investigations on nutrition and feeding ecology of marine invertebrates. Studies on the kinetic and structural characteristics of invertebrate enzymes and their functions may also help elucidate evolution pathways (Jiwani and Liebman, 1994). Digestion has been one of the more intensely studied areas of shrimp nutrition with emphasis on the enzymes. In several cases, assays have been carried out only with the hepatopancreas, in others, the whole gut has been used. Studies on the characteristics of digestive enzymes in crustaceans (Maugle et al., 1982; Galgani and Nagayama, 1987; Fang and Lee, 1992; García-Carreño et al., 1994; García-Carreño and Hernández-Cortés, 1995), variations in isozyme activities during larval development (Pan et al., 1991; Lemos et al., 1999), and change of enzyme activity by feeding, size, molting stage, ovarian maturation and other factors (Lee et al., 1984; Le Moullac et al., 1994; Fernández et al., 1997) are available.

The red shrimp *Pleoticus muelleri* from Argentina seawaters, is a species of high commercial value. It is distributed from 20°LS in Espirito Santo, Brazil, to 50°LS in Santa Cruz, Argentina (Boschi, 1986), in waters with temperatures ranging between 6 and 22°C and salinity from 31.5 to 33.5‰. This organism is mainly carnivorous, feeding on small benthic animals (Boschi, 1963). There is a lack of information on the digestive system of *P. muelleri*, therefore the present study describes the activity of proteinases, during different molting stages, for a better understanding of the digestive physiology of wild shrimp. This knowledge can eventually be used as a reference for the culture of the species.

2. Materials and methods

Organisms were obtained from a commercial fishery in the coastal waters of Mar del Plata, Argentina (38°LS) and transported during the sea and terrestrial journey on ice chest at low temperature. The hepatopancreas of five adult individuals in different molting stages were aseptically removed from decapitated animals. The molting stage of each individual was determined by microscopic examination of uropod setae (Díaz and Petriella, 1990). Organisms in postmolt, intermolt and premolt stages were selected for the study. Samples from individuals in the same molting stage were immediately pooled and frozen for freeze drying. Dried hepatopancreas were homogenized in chilled distilled water and centrifuged for 30 min at $10\,000 \times g$ and at 4°C. Lipids were eliminated and the soluble protein was evaluated in the supernatants (Bradford, 1976), with bovine albumin as the standard.

The effect of pH on proteinase activity was evaluated by using pH 6.5–10 Universal Buffer (Stauffer, 1989). The substrate (1% azocasein) was dissolved in each of the pH buffer solutions. Triplicates of 5 μl of enzyme extracts were mixed with 0.5 ml of buffer and 0.5 ml of substrate solution. The reaction mixtures were incubated for 10 min at 25°C. Proteolysis was stopped by adding 0.5 ml of 20% trichloroacetic acid (TCA), and the mixture was centrifuged in Eppendorf tubes for 5 min at $14\,000 \times g$. The supernatants were separated from the undigested substrate and the absorbance at 366 nm was recorded for the released dye.

Total proteinase activity of the samples was assayed using 1% azocasein as the substrate in 50 mM Tris·HCl, pH 7.5 (García-Carreño, 1992a). Trypsin and chymotrypsin activities were evaluated by the rate of hydrolysis of synthetic substrates. Trypsin activity was measured using *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) as a specific substrate. BAPNA (1 mM) was dissolved in 1 ml of dimethylsulfoxide (DMSO) and made to 100 ml with Tris·HCl, pH 7.5, containing 20 mM $CaCl_2$. Triplicates of hepatopancreas samples (5 μl) were added to 0.75 ml of substrate solution at 37°C and the changes of absorbance at 410 nm were recorded during 10 min (Erlanger et al., 1961). Chymotrypsin activity was evaluated using 0.1 mM Suc–Ala–Ala–Pro–Phe–*p*-NA (SAPNA) in 0.1 M Tris·HCl, pH 7.5 containing 10 mM $CaCl_2$. Triplicates of hepatopancreas samples (5 μl) were mixed with 0.75 ml of substrate solution and the absorbance at 410 nm was recorded during 5 min (del Mar et al., 1979). Each assay included blanks and commercial enzymes (1 mg ml^{-1}) as internal controls.

Total proteinase, trypsin and chymotrypsin unit of activities were expressed as the change in absorbance min^{-1} mg^{-1} of protein of the enzyme used in the assays (Δ Abs min^{-1} mg^{-1} $protein^{-1}$). Evaluation of the proteinase class was based on

the method of García-Carreño (1992b). Enzyme preparations were incubated with different specific protease inhibitors. Phenylmethylsulfonyl fluoride (PMSF) and soybean trypsin inhibitor (SBTI) were used as inhibitors of proteinases belonging to the serine class. TLCK and TPCK were used as specific inhibitors of trypsin and chymotrypsin, respectively. Solutions (10 μl) of 10 mM TLCK in 1 mM HCl pH 3, 100 mM PMSF in 2-propanol, 5 mM TPCK in MeOH and 250 μM SBTI in distilled water, were separately mixed with the enzyme extracts (10 μl) and incubated for 60 min at 25°C. Then, 500 μl of a solution of substrate containing 1% azocasein in 50 mM Tris–HCl, pH 7.5, was added. Assays including distilled water were used as zero inhibition controls. The reaction was stopped 10 min later by the addition of 0.5 ml of a solution of 20% TCA and the mixture was centrifuged for 5 min at $14\,000 \times g$. The supernatants were separated from the undigested substrate and the absorbance at 366 nm was recorded for the released dye. Commercial trypsin and chymotrypsin were used as enzyme controls for serine proteinases. Activity in inhibition assays was reported as a percentage, considering the activity measured in the absence of inhibitor as 100%. Assays were done by triplicate.

Separation of proteins in the enzyme preparations was done by sodium dodecyl sulfate 12% polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Enzyme preparations (0.02 mg of protein) of each molting stage (one volume of sample with one volume of the sample buffer) were loaded in each track of a temperature controlled (4°C) vertical electrophoresis device. Molecular weight standards (4 μl), mixed with the sample buffer were loaded on each plate. After electrophoresis, gels were immersed in a solution containing 40% methanol, 7% acetic acid and 0.05% Coomassie brilliant Blue R-250. After at least 24 h of staining period, gels were destained with the same solution without the Coomassie dye.

Proteinase composition was studied after separation of proteins by substrate–SDS-PAGE (García-Carreño et al., 1993) loading 10 mU of activity (evaluated in test tubes as above) in each track. After electrophoresis as above mentioned, gels were immersed in 3% casein in 50 mM Tris–HCl, pH 7.5 for 30 min at 5°C to allow the substrate to diffuse into the gel at low enzyme activity. Next, the gels were allowed to hydrolyze the casein in its vicinity for 60 min at 25°C. Then, the gels were washed with distilled water and stained as above. Clear zones on the blue background indicated proteinase activity bands.

Enzyme preparations containing 10 mU of activity were incubated with proteinase inhibitors to identify trypsin and chymotrypsin activities in the hepatopancreas of organisms at different molting stages. Solutions of TLCK, TPCK, PMSF and SBTI were separately added to enzyme extracts at a ratio of 1:2 (inhibitor/extract) and incubated at 25°C for 60 min. Distilled water replaced inhibitors in zero inhibition controls. Then, the treated enzyme preparations were subjected to SDS-PAGE. After electrophoresis separation, the molecular weight lane was cut apart. It was immediately stained. For digestion of the protein substrate by the active fractions on the SDS-PAGE, the gels were soaked in casein as described above. Next, gels were washed in water and immediately fixed and stained by immersion in a Coomassie blue solution following the described procedures. Bands with and without proteinase inhibitors were compared. A conspicuous reduction of the intensity of the band with activity was recorded as inhibition, identifying the type of enzyme involved. Since TLCK and TPCK are specific for trypsin, and chymotrypsin, respectively, and PMSF and SBTI are for serine proteinases, inhibited bands on substrate–SDS-PAGE could be attributed to the presence of such enzymes.

Data were expressed as mean ± standard deviation. ANOVA and Student's test analyzed differences among means. In all cases, the level of significance was set for $P < 0.05$ (Sokal and Rohlf, 1979).

3. Results

The hepatopancreas of *P. muelleri* weighed 0.07 ± 0.032, 0.07 ± 0.024 and 0.09 ± 0.041 g in postmolt, intermolt and premolt stages, respectively. The total enzymatic activity of the hepatopancreas was highest between pH 7.5 and 8 (Fig. 1). The following evaluations in test tubes and after SDS-PAGE were done at pH 7.5. Table 1 shows the protein content and specific activities in the hepatopancreas at different molting stages, using azocasein as protein substrate. Protein content in premolt was significantly higher than in

Table 1
Protein content and specific protease activity of *P. muelleri* hepatopancreas in different molting stages

Molting stage	Protein concentration	Specific activity (abs min^{-1} mg^{-1})
Postmolt	6.71 *a*	0.63 *a*
Intermolt	7.37 *a*	0.63 *a*
Premolt	14.33 *b*	0.36 *a*

Substrate for protease activity was azocasein. Values are means of triplicate assays. Different italic letters in the same column show statistical differences ($P < 0.05$).

the other stages. No significant differences ($P > 0.05$) were found in proteinase activity of different molting stages.

The three molting stages exhibited trypsin and chymotrypsin activities. The activities of trypsin for BAPNA and chymotrypsin for SAPNA in the hepatopancreas are shown in Table 2. Trypsin and chymotrypsin activities varied significantly during the molting cycle. Activity was lower in intermolt, and higher in postmolt for both enzymes.

Proteinase activity in hepatopancreas extracts of *P. muelleri* for azocasein was inhibited 50.6% (postmolt), 48.9% (intermolt) and 51.4% (premolt) by SBTI. The PMSF reduced the enzymatic activity in 33% (postmolt), 36.8% (intermolt) and 32.5% (premolt). TLCK, a specific inhibitor for trypsin, reduced the azocasein hydrolysis by 35.4% (postmolt), 26.01% (intermolt) and 37% (premolt). TPCK, an inhibitor for chymotrypsin, did not cause an appreciable decrease of the hydrolytic activity of extracts, except in postmolt (Table 3). Samples were analyzed for trypsin and chymotripsin activity using specific synthetic substrates, after incubating with TLCK and TPCK, respectively. Table 4 shows the reduction in activity at different molting stages. Trypsin activity was highly inhibited by the specific substrate TLCK, in all stages (86.3–93.9%) and chymotrypsin activity was reduced by the TPCK at approximately 49.3–62.4%.

The number of proteins and their molecular weight detected by the electrophoretography are shown in Table 5. Twelve protein fractions observed in intermolt were from 16.6 to 53.1 kDa. Some of these bands were not found in postmolt and premolt stages. Zymograms of proteinase activity were done to determine the composition and molecular weight of the fractions with activity in the hepatopancreas extracts. Ten active bands distributed from 17.4 to 66 kDa were detected in all molting stages (data not shown). Their molecular weights were 17.4, 19.1, 20, 20.9, 21.9, 22.9, 26.6, 33.5, 43.7 and 66 kDa. In intermolt, one band of 50.1 kDa and in premolt, one band of 65.3 kDa was found.

There was no inhibitory effect on activity when the extracts were incubated with TPCK. Active bands inhibited with both TLCK and SBTI were considered trypsins, and bands inhibited with PMSF and SBTI were considered chymotrypsin.

[1] Values are means of triplicate assays.

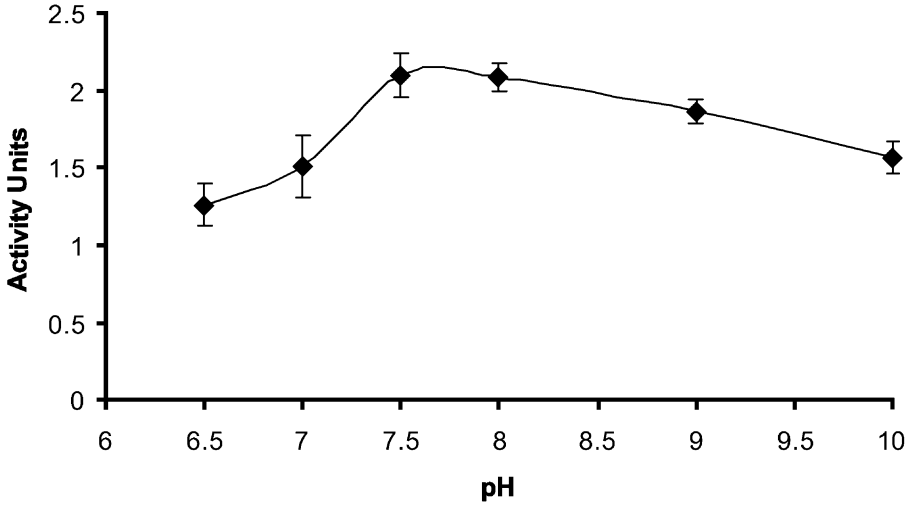

Fig. 1. The effect of pH on protease activity of *P. muelleri*.[1]

Table 2
Variation of the trypsin and chymotrypsin activities in hepatopancreas from *P. muelleri* during molting stages

Specific activity (abs min^{-1} mg^{-1})		
Molting stage	Trypsin	Chemotrypsin
Postmolt	2.4 ± 0.19 *c*	3.7 ± 0.29 *c*
Intermolt	0.9 ± 0.06 *a*	1.2 ± 0.10 *a*
Premolt	1.4 ± 0.07 *b*	2.1 ± 0.07 *b*

Values are means of triplicate assays ± standard deviation. Different italic letters in the same column show statistical differences ($P < 0.05$).

Table 3
The effect of specific inhibitors on proteinase activity of *P. muelleri* hepatopancreas using azocasein as substrate

Inhibitor	Percentage of inhibition		
	Postmolt	Intermolt	Premolt
SBTI	50.6 ± 1.88	48.9 ± 5.48	51.4 ± 8.31
PMSF	33 ± 3.86	36.8 ± 6.02	32.5 ± 6.34
TLCK	35.4 ± 7.19	26.0 ± 7.09	37.0 ± 5.00
TPCK	21.3 ± 3.02	8.4 ± 2.93	7.4 ± 3.00

Values are means of triplicate assays. The percentage of inhibition was calculated using the activity without inhibitor as 100%.

Three trypsin forms (17.4, 19.1 and 20 kDa) were found in all molting stages (Table 6). One band of 20.9 kDa was inhibited by SBTI, but not by TLCK. One intense band of chymotrypsin (21.9 kDa) was observed in all molting stages (Table 6).

4. Discussion and conclusions

This study is the first attempt to understand the function of the digestive system and deals with protein digestion in *P. muelleri*, a high market price species that is being considered for farming. Feeding is one of the constraints in shrimp farming, because of the price and management of natural foods or artificial feeds. Molting is a critical recurrent period for crustaceans, as it represents a physiological crisis, with an important water uptake and the removal of the old exoskeleton. Premolt and postmolt are periods of intense metabolic activity, involving synthesis of some tissues and degradation of others (Skinner, 1985). Since *P. muelleri* is now being evaluated for farming, the understanding of digestive physiology is of relevance to eventually design feed suitable for digestion ability.

Optimum pH for proteinase activity in *P. muel-*

Table 4
The effect of TLCK and TPCK on trypsin and chymotrypsin activities of *P. muelleri* hepatopancreas using specific substrates

Molting stage	Percentage inhibition TLCK	Percentage inhibition TPCK
Postmolt	89.4 ± 1.43	51.0 ± 6.90
Intermolt	86.3 ± 3.73	62.4 ± 4.49
Premolt	93.9 ± 4.92	49.3 ± 3.51

Values are means of triplicate assays. The percentage of inhibition was calculated using the activity without inhibitor as 100%.

Table 5
Protein fractions in SDS-PAGE of *P. muelleri* and variation of these fractions during molting cycle

MW	Molting stages		
	Postmolt	Intermolt	Premolt
53.1	+	+	+
47.3	+	+	+
42.7	−	+	+
22.9	−	+	+
21.9	+	+	+
21.4	–	+	+
20.9	+	+	−
20.4	+	+	+
19.5	+	+	+
19.1	+	+	+
17.4	+	+	+
16.6	+	+	+

MW, molecular weight (kDa). + = present; − = not present.

Table 6
Pleoticus muelleri, schematic representation of proteinase activity bands inhibited by TLCK, PMSF and SBTI in SDS PAGE zymograms during molting cycle

MW	Postmolt	Intermolt	Premolt
17.4	T–S	T–S	T-S
19.1	T–S	T–S	T-S
20	T–S	T–S	T-S
20.9	S	S	S
21.9	P–S	P–S	P-S
22.9	*	*	*
26.6	*	*	*
33.5	*	*	*
43.7	*	*	*
50.1	Not found	*	Not found
65.3	Not found	Not found	*
66	*	*	*

MW, molecular weight (kDa); T, P, S, inhibition by TLCK, PMSF and SBTI; *, no inhibition detected.

leri was 7.5–8. In crustaceans the optimal pH varies, in some cases higher than in vertebrates. Generally, proteases of crustaceans show maximum activity in the range between pH 5.5 and 9.0 (García-Carreño, 1992a; García-Carreño et al., 1994; Ceccaldi, 1997).

P. monodon, *P. japonicus* (syn: *Marsopenaeus japonicus*), *P. penicillatus* (syn: *Fenneropenaeus penicillatus*), *P. vannamei*, *Metapenaeus monoceros* and *Macrobrachium rosenbergii* are decapods that presented serine proteinases, and the hepatopancreas was the organ responsible for the synthesis of the enzymes (Tsai et al., 1986). Digestive enzymatic activities in crustaceans vary with the stage of the molting cycle and ontogeny (Ceccaldi, 1989; Lemos et al., 1999). The proteolytic activity of the hepatopancreas of *P. muelleri* is mainly due to serine proteinases, total proteinase activity was similar in all the molting stages studied. In a recent study of farmed *P. vannamei* (data to be presented elsewhere), the highest activity was found in the intermolt, which was not different than in postmolt. In that study, it was possible to separate organisms in early premolt and late premolt stages. In both stages, the total activity was lower than in previous stages. In *P. notialis* (syn: *Farfantepenaeus notiatis*), maximum values were observed in intermolt and late premolt stages (Fernández et al., 1997).

Trypsin and chymotrypsin activities were found in the hepatopancreas as identified by the hydrolytic activity using specific substrates such as BAPNA and SAPNA. The highest activity for both enzymes was found in postmolt, whereas the lowest was found in intermolt (Table 2). In the hepatopancreas of *P. vannamei*, trypsin activity increased in postmolt, being significantly higher in late premolt, while chymotrypsin did not show differences among molting stages. Comparison among molting stages in shrimp species indicates species-specific regulation of digestive protease synthesis and expression of enzyme activities.

SDS-PAGE analysis of proteins present in the *P. muelleri* hepatopancreas showed several bands corresponding to different molecular weight proteins, some of which are proteases as shown by substrate–SDS-PAGE analysis. Zymogram of *P. muelleri* showed several active bands. According to reduction of activity by specific class- or type-specific inhibitors, three bands corresponded to trypsins. In decapods, trypsin is the main proteolytic enzyme and it is composed of six isoenzymes, with a molar mass of 25 kDa (Galgani et al., 1985). However, Fang and Lee (1992) working on *P. monodon*, found that there were four trypsin isozymes in the hepatopancreas of adult shrimp.

The penaeid chymotrypsins can be classified into two subgroups based on their electrophoretic mobilities. Two purified chymotrypsins from *P. monodon* were found with molecular masses of 27 and 26 kDa, respectively (Tsai et al., 1991). van Wormhoudt et al. (1992), working on *P. vannamei* chymotrypsin found that this enzyme had a molecular weight of 25 kDa by SDS-PAGE. However, Hernández-Cortés et al. (1999), found two bands of activity with chymotrypsin activity in the crayfish *Pacifastacus leniusculus*. One band of chymotrypsin of 21.9 kDa, was found in *P. muelleri* and this band was inhibited by PMSF and SBTI, which was similar to that observed in *P. monodon* (Tsai et al., 1986).

It has been suggested that the digestive enzyme activities would indicate the ability of aquatic animals to utilize feed ingredients (Chuang et al., 1985). Differences among the protein requirements of penaeid species appear to reflect the differences in carnivorous vs. herbivorous feeding habits (Lawrence and Lee, 1997).

Penaeid shrimp feeding habits are difficult to evaluate, partly because identification of gut content material is hampered by the digestive process (Mc Tigue and Zimmerman, 1991). Generally, shrimp have been described as opportunistic omnivores, such as *P. monodon* and *P. vannamei* (Tacon, 1993). However, some species have developed more carnivorous tendencies than others, such as *Penaeus californiensis* and *P. muelleri* which consume benthic invertebrates and organic detritus (Rodríguez de la Cruz and Rosales, 1973; Boschi, 1963).

Comparison of the protease-specific activity of the hepatopancreas of these species indicates a relationship between digestive enzyme activity and feeding habits of the shrimp. The omnivorous shrimp *P. vannamei* and *P. monodon* showed higher protease activity in hepatopancreas (1.57 and 1.20 abs min^{-1} mg^{-1}, respectively) than of *P. californiensis* (1.1 abs min^{-1} mg^{-1}) and *P. muelleri* (from 0.36 to 0.63 abs min^{-1} mg^{-1}; Jiang et al., 1991; García-Carreño et al., 1997).

Reported dietary protein requirements for many species of crustaceans range from 25 to 60%, however, interspecific and intraspecific differences may be partially dependent upon food

sources of protein as well as experimental protocol and data analysis. Colvin and Brand (1977) found that 35% protein gave the best growth for *P. californiensis*; similar protein requirement for *P. muelleri* was determined (Fenucci et al., 1990). Juveniles of *P. monodon* required 40% protein in feeds (Alava and Lim, 1983) and the recommended minimum of this compound in the diets in *P. vannamei* ranges between 30% (Smith et al., 1985) and 40% (Colvin and Brand, 1977; Akiyama et al., 1989). Relationships can be detected among protein requirements, feeding habits and protease activity of these penaeid species. The omnivorous shrimp *P. monodon* and *P. vannamei* have higher protein requirement and protease activity in the hepatopancreas than the carnivorous species *P. californiensis* and *P. muelleri*.

In conclusion, there is strong evidence that the proteolytic enzymatic activity in the hepatopancreas of *P. muelleri* is influenced by factors such as molting cycle, habit feeding and by extension, protein quality and quantity in feeds. This information is relevant for a better understanding of feeding behavior of this species when subjected to farming conditions.

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