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Research Article

Digestive Enzyme Activity and Soy Bean Meal Digestion of Marbled Rabbitfish (*Siganus Rivulatus*)

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Abstract

The present work was performed to assess digestive enzyme presence and activity in the digestive system of wild marbled rabbitfish (*Siganus rivulatus*), and also to try to understand why *S. rivulatus* do not efficiently digest soybean based diets. We also investigated how digestive enzyme activity differed between wild and aquacultured fish. Wild rabbitfish were trapped off the Beirut beach in June 2018 and transported to the aquaculture research facility at the American University of Beirut. An initial gut sample of the fish was removed and freeze-dried within 24 hours post catch. The remainder of the fish were cultured in outdoor round tanks and their guts collected after one month and one year of being fed a commercial formulated diet. Activities of protein, lipid and carbohydrate digestive enzymes were detected in the wild caught fish. Activity of trypsin was particularly high. Activity of some digestive enzymes in the foregut, midgut and hindgut were significantly altered after the fish were offered a commercial formulated feed for a month. Trypsin and chymotrypsin activities in fish fed the commercial diet increased significantly as compared to wild fish after one month of feeding, but amylase and lipase activity were not significantly affected even after one year of feeding. Digestive enzyme activity varied among gut sections, with trypsin and chymotrypsin being relatively high in the mid and hindgut sections and aminopeptidase higher in the foregut.

Results suggest that digestive enzyme activity in the various gut sections of marbled rabbitfish change with diet and time. Furthermore, enzymes needed for the digestion of soybean meal and most proteins including soy protein concentrate are present in the gut of *S. rivulatus*.

Keywords: Aquaculture; Digestive enzymes; Fish nutrition; Rabbitfish; Soy digestibility

Introduction

Sustainable aquaculture necessitates a rational and critical choice of species, preferably one that requires little to no fish products in their diets [1]. This is mainly because fishmeal production is not sustainable at the present rate of fish extraction and demand, resulting in fishmeal being ever more expensive [2]. Accordingly, an algaevorous fish that does not require fishmeal in its diet, such as the marbled rabbitfish *Siganus rivulatus* is a good candidate for marine aquaculture [3]. Rabbitfishes are native of the Indo-West Pacific region [4]. *S. rivulatus* got established in the Eastern Mediterranean region after migrating from the Red Sea through the Suez Canal in 1869 [5]. In its natural environment, *S. rivulatus* feeds on macrophytes but in captivity the fish can easily be accustomed to artificial feeds and adapt to omnivorous feeding habits [6]. Rabbitfish are of economic importance and good candidates for aquaculture because they are tasty, herbivorous, euryhaline, eurythermal marine fish that can tolerate high stocking densities and are easy to rear [3,7-9].The marbled spinefoot has good commercial demand in local markets around the Red Sea and have gained importance in Greek fisheries [10,11].

Aquaculture of rabbitfishes is in its infancy and their digestive physiology in farms is not well understood. For most aquacultured fishes, aquaculture scientists attempt to substitute dietary fishmeal with high protein plant-based ingredients such as soybean meal (SBM) [12-16]. Depending on the fish species and the type of SBM, replacing fishmeal with SBM is accompanied by a change in the activity of digestive enzymes. Upon increasing levels of soybean in tilapia feed, the activity of intestinal proteases decreases while the activity of intestinal amylases does not vary significantly [14]. The activity of trypsin decreases in the proximal segments of the intestine when Atlantic salmon is offered feed with high inclusion levels of soybean [17]. Obviously, animals are capable of adjusting their digestive physiology to adapt to a change in their diets [14] and will perform well as long as their energetic and amino acid requirements are met. Surprisingly, inclusion of soybean in *S. rivulatus* diets was not found suitable even at low inclusion levels [18]. Marbled spinefoot did not grow well even when fishmeal was partially replaced with pure Soybean Protein Extract (SPE) (unpubl. data). A possible reason for the unsuitability of soybean-based ingredients could be a lack of the proper digestive enzymes in the fish gut. Also, SBM contains antinutritional factors for fish (i.e. saponins, lectins, phytic acid, alkaloids, oligosaccharides, glucosinolates, antigens) that are associated with damage of mucosal integrity, decreased pancreatic and mucosal enzymes and reduced palatability of feed [19].

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The present work was performed with three goals in mind. First, we wanted to study digestive enzyme activity in the various gut sections of *S. rivulatus* in order to better develop diets for the species. Second, we wanted to assess whether enzyme activity varies between wild and aquacultured fish. Finally, we attempted to understand why *S. rivulatus* do not tolerate SBM in their diet. The present work investigated the presence of some of the main digestive enzymes found in the various parts of the digestive tract of *S. rivulatus* and how enzyme activity changes over time when feeding on a manufactured compound diet.

Materials and Methods

Juvenile *S. rivulatus* (initial weight 40 g to 80 g) were captured in traps off the Beirut beach on June 10, 2018 and transferred alive to the aquaculture research laboratory at the Department of Biology at the American University of Beirut (AUB). The research methodology and animal handling procedures were approved by the Institutional Animal Care and Use Committee of the university. Fish were divided into three groups; the first group (wild fish, WF) was fasted for 12 hours then 12 fish were dissected on a glass pane placed on ice to maintain enzyme integrity. Intestines were removed and those of four fish were placed in Eppendorf tubes while the rest were divided into three sections: foregut, midgut and hindgut, then placed in Eppendorf tubes. Samples in Eppendorf tubes were submerged in liquid nitrogen for 1 hr. All samples were then freeze-dried and stored at -80 ºC until analysis. The second and third groups were maintained for one month (AM1) and one year (AC1), respectively, in an outdoors recirculation system composed of six 1m^3 round tanks connected to a biofilter and a sedimentation tank. Three tanks per treatment were used. Fish were offered a 32% protein, 6% lipid soy based Rangen commercial tilapia diet (Rangen Inc., Buhl, Idaho) till apparent satiation, twice daily. Water temperature was maintained at 23 °C, salinity at 35 ppt, Total Ammonia Nitrogen (TAN) < 0.1 mg.L^{-1}, NO_2-N< 0.05 mg.L^{-1}, oxygen > 5.5 mg.L^{-1} and 7.8 ≤ pH ≤ 8.2. At the end of the assigned culture period, fish were starved for 24 hours and the intestines of 12 fish from each group were extracted and treated as described above.

To obtain digestive enzyme extracts, samples were cut into pieces and the tissues were homogenized for 2 minutes in 5 mL cold distilled water using a tissue grinder (POLYTRON® PT 1200, Kinematica AG, Switzerland). Samples were then centrifuged for 45 minutes at 21,000g, 4 °C (5417R, Eppendorf, USA) and supernatants collected and frozen at -80 °C as 0.5 mL aliquots. All aliquots were used within 48h after thawing at 4 °C. Analyses estimated specific enzyme activity (U mg^{-1} protein) for the foregut (pyloric caeca and start of intestine), midgut, and hindgut. The experimental protocol was adjusted to perform spectrophotometric measurements on a plate reader (Varioskan Flash; Thermo Scientific, USA). Software SkanIt RE 2.4.5 (supplied with the plate reader) was used to analyze the data collected. A blank sample made only from distilled water and a positive control composed of commercial enzymes at a concentration of 1 mg mL^{-1} were used in each assay.

Trypsin activity was estimated using the method described by Erlanger et al. [20]. Briefly, 1 mM of BAPNA (Nα-benzoyl-DL-arginine-p-nitroanilide hydrochloride, Sigma B-4875) was dissolved in 500 µL DMSO and used as a substrate. The reaction was allowed for 30 minutes at 37 °C and a pH 8.2 using 50 mM Tris-HCl buffer and 20 mM $CaCl_2$. Thirty percent (30%) acetic acid was added to stop the reaction. Samples were allowed to stabilize for 10 minutes before recording absorbance at 410 nm.

The method of Hummel [21], as modified by Applebaum et al. [22], was used to determine activity of chymotrypsin. BTEE (N-Benzoyl-L-tyrosine ethyl ester, 0.56 mM, Sigma 13110-F) was dissolved in 100 mM Tris-HCl buffer with 25 mM $CaCl_2$ at pH 7.8 and methanol 2.5 % was considered the substrate. The test was performed in a 96-well plate at 37 °C and the absorbance (at 256 nm) was recorded every minute for 30 minutes.

Activity of Leucine Aminopeptidase (LAP) was measured according to the method of Appel [23].

L-leucine-P-nitroanilide (1.2 mM, Sigma, L-9125) was used as a substrate in 50 mM HCl-Tris buffer, pH 8.0 at 37 °C. After 30 minutes, 30% acetic acid was added to stop the reaction and samples were left 10 minutes for stabilization. The absorbance was measured at 405 nm.

The method of Sarath et al. [24] was used to assess total alkaline proteinase activity. Azocasein (2 %, Sigma, A2765) was used as a substrate in 50 mM HCl-Tris buffer, 10 mM $CaCl_2$, 9.0 pH at 37 °C. Samples were incubated for 10 minutes before stopping the reaction using 10% trichloroacetic acid. After centrifugation at 21000g, 4 °C for 5 minutes, the absorbance of the supernatants was measured at 440 nm.

Amylase activity was determined using the method described by Worthington [25]. One percent (1 %) starch (Sigma, S9765) mixed in 20 mM sodium phosphate buffer and 6 mM NaCl at a pH of 6.9 was used as a substrate. The homogenate was mixed with the buffer containing the substrate at fixed time intervals and incubated at 25 °C. At 3 minutes, 1 % dinitrosalicylic acid (coloring reagent) was added. After incubation for 5 minutes in a boiling water bath, all tubes were left at room temperature to cool, mixed well and the absorbance measured at 540 nm. Micromoles of maltose obtained from each sample were calculated using the regression equation of a standard maltose curve. Amylase activity was calculated using the formula: Unit/mg = (micromole maltose released / mg enzyme in reaction mixture x 3 (min)). To perform the readings within the range of the standards, all samples were subject to a 10 times dilution in a final reaction volume of 1.2 mL.

The method described by Gjellesvik et al. [26] was used to estimate lipase activity. 0.56 mM of 4-Nitrophenyl myristate (Sigma 70124) was dissolved in 0.5 mL DMSO and was used as a substrate. The reaction was performed using 150 mM Tris-HCl buffer, 15 mM sodium taurocholate, at a pH 8.5 and 37 °C. Absorbance was measured at 405 nm, every minute for 30 minutes.

For all the various enzymes, one unit of enzyme activity (U) was considered to be the amount of enzyme needed to increase absorbance by 1 unit per minute [27].

Statistical analysis was performed using SAS (V.9.2, SAS Institute Inc., Cary, North Carolina, USA). Results are reported as mean values ± standard error of the mean and compared using a one-way ANOVA. No factorial design was used to analyze the data as the interaction of time of feeding on a formulated diet and gut section were not considered in the experimental design. Significant differences among means were analyzed using Student Newman- Keuls (SNK) mean separation test. Differences among treatment means were considered significant at $P < 0.05$.

Results

Presence and activity of digestive enzymes in wild and cultured fish

Specific activity for total alkaline proteases, trypsin, chymotrypsin, bile-salt dependent lipase and amylase were detected in wild caught rabbitfish and values presented in (Table 1). Activities of all enzymes evaluated were present in the various regions of the intestine in wild fish collected from their natural environment . No significant differences in digestive enzyme specific activity was found among the measured enzymes in the various regions of the digestive system in wild specimens (Table 2). Nonetheless, trypsin activity tended to increase from the foregut to the hindgut, while lipase activity decreased towards the end of the gut.

Trypsin specific activity was significantly higher in the hindgut compared to the foregut and midgut in one moth cultured fish. In contrast, chymotrypsin activity was significantly higher in the foregut compared to the midgut and similar to the activity found in the hindgut. A similar trend to trypsin activity was observed for amylase where the activity was significantly greater in the hindgut compared to mid and fore gut. After one year under culture conditions and feeding on a formulated diet, total alkaline proteases, trypsin, and chymotrypsin activity were greater in the hindgut but not statistically different compared to the foregut and midgut.

Time	Proteases	Trypsin	Chymotrypsin	L- aminopeptidase	Lipase	Amylase
W	199.1 ± 15.9	195.8 ± 10.5.3	195.8 ± 105.3	254.9 ± 53.8^{ab}	73.1 ± 30.3	120.4 ± 33.8
AM1	164.9 ± 11.1	379.9 ± 213.6	224.7 ± 10.4	324.7 ± 18.5^{a}	63.6 ± 72.3	87.0 ± 62.9
AC1	188.2 ± 35	484.5 ± 140.3	345.5 ± 114	232.6 ± 17.9^{b}	71.4 ± 42	82.1 ± 17
One way ANOVA (*p*)	0.3	0.2	0.2	0.0	1	0.5

Table 1: Digestive enzyme activity (U/mg protein) in the entire gut of *Siganus rivulatus* (W = wild fish; AM1 = fish offered a formulated diet for one month; AC1 = fish offered formulated diet for one year).

Effect of duration of feeding on a formulated diet on digestive enzyme activity in entire gut

Duration of feeding on a formulated diet significantly affected L-aminopeptidase specific activity in the entire gut, where activity increased after 1 month of culture and then significantly decreased after one year of culture to levels similar to those observed in wild fish. Although not statistically significant, trypsin and chymotrypsin activity increased as early as one month of feeding on a formulated diet and continued to increase until the end of the feeding trial. Duration of the time of feeding on a formulated diet did not significantly affect lipase or amylase activity.

Effect of duration of feeding on a formulated diet on enzymatic activity in gut sections

LAP activity in the foregut was significantly greater in wild fish and one month cultured fish than in fish offered a formulated diet for one year. However, LAP activity was not affected by the time of feeding on a formulated diet in the midgut or hindgut (Table 3). Contrastingly, trypsin activity was high in the foregut and midgut for wild fish and decreased with feeding on the formulated diet after one month but returned to previous levels after one year. Similarly, amylase activity in the foregut decrease after one month of feeding on a formulated diet but increase towards the end of the experimental period. Chymotrypsin activity in the midgut significantly increased with time of feeding on a formulated diet, with higher activities at the end of the experimental period.

Time	Section	Proteases	Trypsin	Chymotrypsin	Aminopeptidase	Lipase	Amylase
W	Foregut	62.7 ± 17.4	165.6 ± 71.5	78.5 ± 15.7	89.0 ± 5.4	29.8 ± 13.1	49.1 ± 38.1
	Midgut	62.8 ± 3.3	234.4 ± 111.3	49.2 ± 36.7	93.4 ± 30.9	19.6 ± 11.3	33.7 ± 27.6
	Hindgut	73.6 ± 4.8	324.1 ± 43.3	68.2 ± 18.8	72.5 ± 17.8	23.8 ± 24.7	50.1 ± 7.9
One way ANOVA (*p*)		0.4	0.3	0.7	0.5	0.8	0.1
AM1	Foregut	46.9 ± 11.7	59.5 ± 17.7^{b}	109.7 ± 32.1^{a}	99.1 ± 7.6	13.8 ± 14.1	6.0 ± 3.8^{b}
	Midgut	59.9 ± 10.8	64 ± 12.4^{b}	34.5 ± 12.5^{b}	115.8 ± 12.5	19.1 ± 24.1	11.3 ± 2.5^{b}
	Hindgut	58.1 ± 4.1	297 ± 24.4^{a}	80.5 ± 12.7^{ab}	109.7 ± 8.7	30.7 ± 34.8	71.5 ± 7^{a}
One way ANOVA (*p*)		0.3	0.0	0.0	0.2	0.7	0.0
AC1	Foregut	52.8 ± 10.4	164.3 ± 53.3	138.2 ± 74	39.7 ± 21.7	15.6 ± 18.4	19.8 ± 15.5
	Midgut	64.2 ± 14	212.7 ± 84.7	82.1 ± 19.4	99.5 ± 25.6	26.6 ± 25	30.4 ± 6.3
	Hindgut	71.1 ± 11.9	223 ± 132	125.2 ± 39.9	93.4 ± 28.7	29.2 ± 10.8	31.9 ± 18.5
One way ANOVA (*p*)		0.3	0.8	0.4	0.1	0.6	0.9

Table 2: Digestive enzyme activity (U/mg protein) in gut sections of wild and cultured rabbitfish Siganus rivulatus. (W = wild fish; AM1 = fish offered a formulated diet for one month; AC1 = fish offered formulated diet for one year).

Total alkaline protease in the foregut, midgut and hindgut were not significantly affected by duration of feeding on a formulated diet. Duration of time feeding on a formulated diet did not significantly affect lipase activity in the various parts of the gut.

Discussion

As expected, enzymes for the digestion of the major nutrients (i.e., proteins, lipids and carbohydrates) were present in the digestive tract of rabbitfish. In general, the activities of the digestive enzymes measured were greater in the foregut, in particular for chymotrypsin and lipase. Interestingly, although not significantly different, trypsin activity tended to increase towards the midgut and hindgut. The same trend was observed after one month or one year of aquaculture conditions. Previous research suggests that protease activity is greater

Section	Time	Proteases	Trypsin	Chymotrypsin	Aminopeptidase	Lipase	Amylase
Foregut	W	62.7 ± 17.4	165.6 ± 71.5	78.5 ± 27.3	89 ± 5.4[a]	29.8 ± 13.1	49.1 ± 38.1
	AM1	46.9 ± 11.7	59.5 ± 17.7	109.7 ± 32.1	99.1 ± 7.6[a]	13.8 ± 14.1	6 ± 3.8
	AC1	52.8 ± 10.4	164.3 ± 53.3	138.2 ± 74	39.7 ± 21.7[b]	15.6 ± 18.4	19.8 ± 15.5
One way ANOVA (*p*)		0.4	0.1	0.4	0.0	0.4	0.2
Midgut	W	62.8 ± 3.3	234.4 ± 111.3	12.4 ±1[b]	93.4 ± 3.9	19.6 ± 11.3	49.5 ± 5.3
	AM1	59.9 ± 10.8	113.5 ± 86.3	34.5 ± 12.5[b]	115.8 ± 12.5	28.1 ± 25.6	32 ± 5.9
	AC1	64.2 ± 14	212.7 ± 84.7	82.1 ± 19.4[a]	99.5 ± 25.6	26.6 ± 23.3	30.4 ± 6.3
One way ANOVA (*p*)		0.9	0.3	0.0	0.5	0.9	0.6
Hindgut	W	73.6 ± 4.8	324.1 ± 43.3	68.2 ± 19.8	72.5 ± 17.8	23.8 ± 247	50.1 ± 7.9
	AM1	58.1 ± 4.1	206.8 ± 157.1	80.5 ± 12.7	109.7 ± 8.7	30.7 ± 34.8	49.0 ± 39.3
	AC1	71.1 ± 11.9	223.0 ± 132	125.2 ± 39.9	93.4 ± 287	29.2 ± 10.8	31.9 ± 18.5
One way ANOVA (*p*)		0.1	0.6	0.1	0.2	0.9	0.6

Table 3: Digestive enzyme activity (U/mg protein) for gut sections of marbled rabbitfish *Siganus rivulatus*. (W = wild fish; AM1 = fish offered a formulated diet for one month; AC1 = fish offered formulated diet for one year).

in the foregut and midgut of most cultured fish species and tends to decrease towards the hindgut [27-29]. Accordingly, results from the present study suggests that algaevorous fish might have a different mode of protein digestion compared to piscivorous fishes and that entails further investigation among rabbitfishes and other algaevorous fishes.

Culturing fish using a commercial feed caused changes in some but not all of the digestive enzyme activities assessed in the various parts of the gut of marbled rabbitfish. An interesting change observed is that trypsin activity in the gut of the fish increased in cultured fish compared to wild fish after just one month of feeding and continued increasing even after a year of culture. The same trend but to a lesser degree was observed for chymotrypsin activity after feeding on a formulated diet. These results suggest that rabbitfish are able to adapt to a commercial formulated diet with increase protein content by increasing activity of their proteases. Another, possibility is that as dietary protein increased or changed, digestion efficiency of proteins decreased thus fish had to adapt by increasing protease production to meet nutritional requirements although it appears that the fish were still getting enough nutrients since fish tended to grow well. Interestingly, amylase activity tended to decrease with time feeding on a formulated diet, additionally suggesting and adaptation to the new diet. More controlled studies manipulating specific nutrients (i.e., proteins and carbohydrates) are warranted to elucidate more precise changes and adaptation of this interesting algaevorous species.

Results of the present work also suggest that the inability of rabbitfish to digest SBM as observed in previous work in our lab, is not related to a lack of adequate digestive enzymes. In fact, protein rich solvent extracted SBM contains mostly glycinin and β-conglycinin [30] which should be readily digestible by rabbitfish since they have all proteases necessary for the digestion of these compounds. Although soybean isolates are not properly digested when hydrolyzed by single proteases (i.e., trypsin or chymotrypsin) [31], better hydrolysis is achieved when multiple proteases such as commercially acquired flavourzyme and alcalase are added together to a diet [32]. This suggests a complementary role for proteases in the digestion of complex proteins such as those in soybean. Present results show that a set of protease complements are present in the gut of rabbitfish and thus would invalidate our suspected hypothesis with respect to the lack of digestive enzymes necessary to digest soybean meal.

Another major component of SBM that could possibly affect digestion is the carbohydrate components of SBM. However, marbled rabbitfish has amylase in its gut and thus should be able to digest some of the long chain carbohydrates. Nevertheless, when we tried offering rabbitfish a diet containing soy protein extract instead of SBM, we still observed a decrease in fish growth rate correlated to increasing dietary SPE. SPE is a protein concentrate made by industrially removing many of the carbohydrates present in SBM. Accordingly, we believe that the lack of suitability of soybean for rabbitfish is not related to its carbohydrate content. Interestingly, many of the necessary enzymes for SBM digestion are expressed in the gut of marbled rabbitfish and yet reduced growth rate was observed upon feeding the fish soybean or pure SPE ([18]; unpubl data from our lab). Such observations suggest that soy product digestion and/or assimilation are hampered by an as yet undetermined factor.

Another possible reason for the unsuitability of SBM as a dietary ingredient for rabbitfish is that the SBM might not have been properly processed prior to being offered to the fish. Better carbohydrate and protein digestibility are achieved when SBM is heated at high humidity [33]. For example, heating SBM to more than 95°C denatures β-conglycinin and 11S glycinin [34,35] thus making the proteins more bioavailable to the fish. Moreover, exposure of SBM to heat and high humidity results in total hydrolysis of antinutritional factors such as trypsin inhibitor and lectins [30,33], thus allowing better digestion and availability of the SBM nutrients. In previous work by Monzer et al. [18], feed was not exposed to high temperature and high humidity because it was produced using a meat grinder where the temperature never exceeded 70°C. Apparently, cold-pelleted feed is not appropriate for *S. rivulatus* and warrants the evaluations of cold-pelleted feeds against extruded diets. In addition to protein digestibility and denaturation of antinutritional factors, heat and high humidity exert a beneficial effect on carbohydrate digestibility. Thus, in the Monzer et al. [18] study, cold extrusion may have also reduced carbohydrate digestion by the fish. Combined together, these three factors could have contributed to the reduced growth rate of rabbitfish. Present results thus suggest that having necessary digestive enzymes is not

sufficient to digest the proteins in the diet. We recommend preparing experimental SBM based diets in an extruder to increase temperature to more than 110 °C at relatively high humidity to help make soybean nutrients more bioavailable to the fish.

To conclude, the present work demonstrates that marbled rabbitfish have a good complement of digestive enzymes. A change in diet and duration of feeding affects the activity of these enzymes in particular during the first month of feeding if fingerlings are sourced from the wild. Present results would probably be different when growing hatchery produced fish. Finally, reduced growth of rabbitfish observed upon inclusion of soybean to the feed as observed in previous studies does not seem to be caused by the lack of adequate complement of digestive enzymes. A possible explanation could be the low digestibility and hydrolysis of the main components of SBM when the feed is not exposed to high heat at high humidity such as the extrusion process and warrants further evaluation.

Declarations

Ethical approval

The present work was reviewed and approved by the Institutional Animal Care and Use Committee of the American University of Beirut.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author's contribution

The research was designed and directed by Drs. Lazo and Saoud. They wrote the grant proposal, designed the research, trained and supervised the students and wrote the manuscript. Eliasid Nogueda performed the biochemistry and statistics. Christine Khalil and Razan Zeineddine collected fish, maintained them for a year, extracted guts and processed them and wrote the first draft of the manuscript.

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Availability of data and materials

Data can be obtained by contacting Juan Pablo Lazo at jplazo@cicese.mx

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