Opportunistic feeding experiment in a captive franciscana (*Pontoporia blainvillei*): assessing gastrointestinal transit time and gastric digestion

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A variety of methods have been used to draw inferences on marine mammal diet, such as direct observations, past and current traditional methods (examination of food remains present in scats, stomachs, intestines, vomits), and actual disseminated novel tools (*e.g.* stable isotopes, fatty acids, molecular identification of prey) (Barros and Clarke, 2009). The traditional methods still in use require low cost and simple equipment, samples can be collected from carcasses in advanced stage of decomposition, and it is possible to assess size classes of many prey species. From these methods, the undigested hard parts of prey, mainly fish otoliths and cephalopods beaks, have been widely collected and their distinctive morphology and meristic relationships have been used to identify the species, number, size, and weight of prey items eaten by the predator (Fitch and Brownell, 1968; Pierce, 1986; Clarke and Boyle, 1991). Although the analysis of stomach contents for diet interpretation was widely used and still is a practical method, some studies indicate that the recovery of partly digested hard parts will bias results (*e.g.* Prime and Hammond, 1987; Tollit *et al.*, 1997; Santos *et al.*, 2001; Ross *et al.*, 2016). Otoliths are composed of calcium carbonate and would be expected to be completely or partially digested when exposed over time to the gastric acids of marine mammals' stomachs. Consequently, an underestimation of the prey sizes and weights consumed is to be expected (Jobling and Breiby, 1986; Pierce *et al.*, 1993; Cortes, 1997). Cephalopods beaks are retained in the stomachs for much longer periods, from weeks to several months, and otherwise predators can vomit the beaks (Clarke, 1986*a,b*; Xavier *et al.*, 2016). Therefore, otoliths and beaks recovered from the stomach may not be representative of a single feeding episode (Murie and

Lavigne, 1986). To understand those biases, animal feeding experiments have been conducted for pinnipeds (*e.g.* Prime, 1979; Murie and Lavigne, 1986; Krockenberger and Bryden, 1994; Arim and Naya, 2003), and information from a few such results for cetaceans are available (Kastelein *et al.*, 1993; Walker *et al.*, 1986). Some *in vitro* experiments for prey and otolith digestion rates were also performed (McMahon and Tash, 1979; Sekiguchi and Best, 1997; Wijnsma *et al.*, 1999; Pusineri *et al.*, 2003).

Information on digestive tract passage time for franciscana dolphin (*Pontoporia blainvillei*) is not available, but it is for some other marine mammals[1, 2] (*e.g.* Eastman and Coalson, 1974; Helm, 1984; Kastelein *et al.*, 1993; Krockenberger and Bryden, 1994; Grellier and Hammond, 2006). Usually, this information is obtained from controlled experiments with captive animals. Currently, no methods are available to conduct such research using animals in their natural habitat.

This study was carried out opportunistically with a franciscana dolphin in captivity, and the objectives were (1) to investigate the gastrointestinal passage time of the dolphin; (2) to determine prey hard parts (fish otoliths and cephalopods beaks) condition after gastric exposure for each particular meal; (3) to compare differences in the reduction of otolith measurements among prey species, as a function

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[1]Helm, R.C. and Morejohn, G.V. (1979) Initial defecation time and intestinal length of three species of pinnipeds: *Phoca vitulina, Zalophus californianus* and *Mirounga angustirostris*. Page 27 *in* Abstracts, *III Biennial Conference on the Biology of Marine Mammals*, 7-11 October, Seattle, USA.
[2]Waldemarin, H.F. and Colares, E.P. (1994) Tempo de passagem de alimento pelo trato digestivo de *Otaria flavescens* jovem e adulto em cativeiro. Page 41 *in* Abstracts, *6ª Reunião de Especialistas em Mamíferos Aquáticos da América do Sul.* 24-28 October, Florianópolis, Brazil.

Table 1. The captive franciscana dolphin's feeding regime. The rows indicate the order of the meals. The last column shows the recovery and conditions of the otoliths and beaks found on each meal (FL = fish length, ML = mantle length).

Meal	Hours in stomach	Feeding regime Species	Common name	N	FL/ML (mm)	Conditions of the prey structures found in the stomach contents
1	50	*Cynoscion guatucupa*	Striped weakfish	8	120-125	Otoliths not found
		Cynoscion guatucupa	Striped weakfish	1	215	Otoliths found very digested (still indentifiable by the shape, no lobation, and sulcus hardly visible)
		Loligo sanpaulensis	Long-finned squid	1	110	Beaks in very good conditions
2	44	*Engraulis anchoita*	Anchovy	14	130-140	Otoliths not found
		Loligo sanpaulensis	Long-finned squid	2	70-75	Beaks in very good conditions
3	38	*Macrodon ancylodon*	King weakfish	4	195	Otoliths not found
4	32	*Micropogonias furnieri*	White croaker	2	205	All otoliths in median condition (no lobation on margins and sulcus less evident)
5	26	*Cynoscion guatucupa*	Striped weakfish	6	175	All otoliths in good condition (partial lobation on margins but sulcus well defined)
6	20	*Paralonchurus brasiliensis*	Banded croaker	4	170-175	All otoliths in median condition (no lobation on margins and sulcus less evident)
7	14	*Micropogonias furnieri*	White croaker	6	175	All otoliths in very good condition (clear lobation on margins and sulcus well defined)
8	8	*Cynoscion guatucupa*	Striped weakfish	11	120-140	All otoliths in very good condition (clear lobation on margins and sulcus well defined)
		Macrodon ancylodon	King weakfish	1	155	All otoliths in good condition, but not as good as those of C. guatucupa of this meal (partial lobation on margins but sulcus well defined)
9	2	*Micropogonias furnieri*	White croaker	8	140-145	All otoliths were inside the skulls in original conditions, bodies were very digested ~70% (skin and viscera gone and muscle reduced), and head lightly digested ~10%

of time after feeding; and (4) to estimate the rate of otolith digestion and approximate time for complete dissolution.

A juvenile female franciscana dolphin (TL = 112 cm) was found stranded alive in Rio Grande, southern Brazilian coast. The animal was transported to the Marine Animals Rehabilitation Centre (CRAM/MO-FURG), Rio Grande, Brazil. The dolphin remained in captivity for about four days, from 9 October 1998 to its death on 13 October 1998. The animal died, probably of pneumonia and injuries caused by the stranding, although the dolphin may have been sick before the stranding event[3] It was maintained in a plastic paddling pool (1.5 x 2.0 m, 0.5 m deep), filled with salt water at about 35 psu, and monitored during all its time in captivity. The dolphin was fed after the second day, four times a day at six hours intervals (total of nine meals) with different prey species (fish and squid) and sizes for each meal (Table 1). The prey species used are commonly observed in the diet of franciscana for this area (Pinedo, 1982; Bassoi, 1997; 2005).

Gastrointestinal passage time

The first phase of this experiment, with the dolphin alive, was carried out to measure the transit time through the gastrointestinal tract of the dolphin. From the third to the sixth meal, ten small (5 mm) coloured plastic circle markers were introduced via the mouth into the digestive tract of one of the fish used to feed the animal. The weights of those meals were 145 g (with white plastic circles), 280 g (red), 115 g (green), and 195 g (yellow). The respective fish species (12-13 cm) were white croaker (*Micropogonias furnieri*), banded croaker (*Paralonchurus brasiliensis*), king weakfish (*Macrodon ancylodon*), and striped weakfish (*Cynoscion guatucupa*); and the interval between the feeding trials was six hours. The times that the plastic colour markers were evacuated in the scats were recorded. After each observation, all the plastic circles in the pool were removed. No other hard parts (bones, otoliths or beaks) were found, neither in the animal scats or the pool.

Using the statistical software R (R Development Core Team, 2018), a linear mixed effects model for time series data using the number of observations and hours after feeding as variables were used to analyse the passage of the plastic circles through the digestive tract of the dolphin. The evacuation
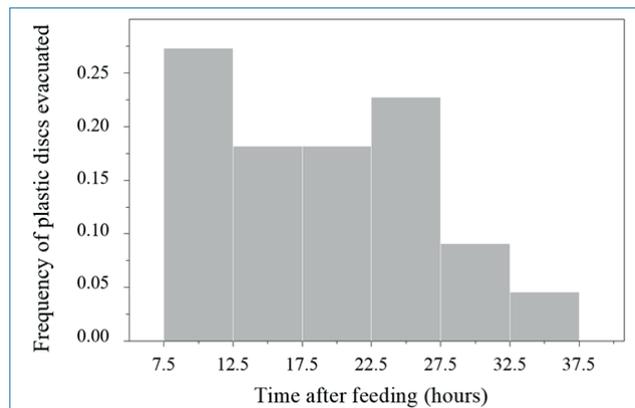
times of plastic circles from four meals is shown in Figure 1 (range = 7.5-35.2 h, mean = 18.7 h, *sd* = 8.0 h). Linear mixed effects model for time series data indicated significant differences among passage times in the experiments (N = 22, *F* = 6.1, *df* = 3, *p* = 0.009). This high variability in the passage of digestion of the captive dolphin may be the result of different amounts of food within the meals. Passage times from consumption to defecation are likely to be highly variable, depending on the amount of food consumed, the time interval between the meals and individual activity and physiology[4] (Bigg and Fawcett, 1985; Jobling, 1987). Initial recovery times in elephant seals (*Mirounga angustirostris*) varied from 4.8 hours (Helm, 1984) to 9.1 hours (Krockenberger and Bryden, 1994), and for South American sea lion (*Otaria flavescens*) varied from 5 to 14 hours[2]. Helm and Morejohn[1], studying three species of pinnipeds, reported that for 22 individuals the average of first defecation time was about five hours. For cetaceans, Kastelein *et al.* (1993) fed captive Commerson's dolphin (*Cephalorhynchus commersonii*) with fish where gelatine capsules containing red dye had been inserted and found that only 40 to 155 minutes elapsed before dye appeared in the faeces, and for our experiment it was 7 to 14 hours for the first appearance of plastic markers in the faeces. It is possible that the liquid marker, such as a dye, would produce a greater passage rate than a solid and adherent material such as plastic (see Harvey, 1989). Besides, there are uncertainties in comparing these results owing to possible differences in passage times related to different marine mammal species. To conclude, this captive franciscana dolphin showed slower gastric evacuation times than found for other marine mammals that used similar markers, but the first time of evacuation did not differ greatly.

Gastric digestion of prey

The second phase of this experiment, after the animal died, was to examine its stomach contents in order to assess the condition, reduction and dissolution of prey hard parts after gastric digestion. The stomach was removed and weighed (520 g full and 270 g empty). The franciscana dolphin stomach can be seen as three compartments: forestomach, connecting channel (tube-like passage) and pyloric chamber (Yamasaki *et al.*, 1974). Remains of fish bones (eight skulls), partly digested tissue fragments, otoliths (N = 78), and cephalopods beaks (N = 6) were recovered from the forestomach, and more cephalopod beaks (N = 123) from pyloric chamber contents. No remains of otoliths or beaks were found in the intestine[5]. Our findings, with no recoveries of otoliths in the intestine analysis, and no remains in the scat or in the pool, could

Figure 1. The frequency histogram of plastic discs passage times from four feeding events of the captive franciscana dolphin from this experiment.

suggest that franciscana dolphin fully digests otoliths in the stomach. Moreover, it is not usual to find them in the pyloric chamber (Bassoi, 1997; 2005). Other pinnipeds studies also suggest that otolith digestion occurs entirely within the stomach (Frost and Lowry, 1980; Harvey, 1989).

The condition of the hard parts and soft tissues, like skin and muscle, of the prey were assessed through observation, and digestion percentage estimated (Table 1). The bodies of the white croakers were very digested (~70%), and the skulls lightly digested (~10%) after two hours of their ingestion. Similar to these findings, *in vitro* experiment using a digestive solution with a pH of ~2.3 reported that for an equivalent fish size the skull began to be digested (15%) at 2-3 hours (Sekiguchi and Best, 1997).

Cephalopod beaks identification and measurements were made in the *Laboratório de Recursos Pesqueiros e Demersais, Universidade Federal do Rio Grande* (LRPD/FURG) and the regressions for cephalopods mantle sizes estimations from the beaks recovered are given in Table 2. There were only three upper and three lower squid beaks of long-finned

[4]Pusineri, C., Meunier, L., Spitz, J. and Ridoux, V. (2003) Differential digestion rates of dolphin prey: observational and experimental bases for an improved dietary analysis procedure. Page 194 *in* Abstracts, *XVII Conference of the European Cetacean Society*, 9-13 March 2003, Las Palmas de Gran Canaria, Spain.
[5]A. Andrade, Universidade Federal de Pelotas, Pelotas, March 1999, pers. comm.

Table 2. Regressions of otolith and fish lengths (mm), where LO = total otolith length; and cephalopod beaks and cephalopod mantle lengths, where URL/LRL = upper/lower beak rostral length, UHL/LHL = upper/lower beak hood length.

Fish species	Fish length (mm)	N	R2
Cynoscion guatucupa	$13.799LO^{1.2007}$	78	0.9894
Paralonchurus brasiliensis	$26.005LO^{-21.709}$	80	0.9847
Macrodon ancylodon	$1.725LO^2+1.9196LO^{31.348}$	61	0.986
Micropogonias furnieri	$18.343LO^{1.0987}$	149	0.9935
Engraulis anchoita	$32.803LO^{1.088}$	39	0.9757
Cephalopod species	Mantle length (mm)	N	R2
Argonauta nodosa	$4.923UHL^{1.2933}$	163	0.9481
	$9.5338LHL^{1.2314}$	164	0.9507
Loligo sanpaulensis	$14.408e^{1.1418URL}$	185	0.9294
	$13.497e^{1.0836LRL}$	185	0.9441

Source: Demersal Fishing Resources Laboratory, University of Rio Grande, Brazil.

squid (*Loligo sanpaulensis*) found on the forestomach, which were not broken and still retained the original colour, and the estimated prey lengths were 72 mm, 75 mm, and 108 mm. They were probably from the three squids given to the dolphin (see Table 1). In addition, we found in the pyloric chamber 123 beaks not belonging to the meals, and they were broken and in poor conditions. Other beaks found broken and darker, not from the meals while in captivity, represented one knobbed argonaut (*Argonauta nodosa*) (ML = 6.15 mm) and 62 long-finned squids (mean ML = 113.23, *sd* = 2.67 mm). No differences of reduction in length of the beaks were found. Recovery rates for cephalopod beaks are highly variable (see Tollit *et al.*, 1997), and it is known that they can be retained in the stomach of marine mammals for long periods (Pitcher, 1981; Big and Fawcett, 1985; Clarke, 1986*b*; Santos *et al.* 2001), and our findings confirm long retention time of the beaks in the pyloric chamber is common for franciscana dolphin. The squid beaks showed no obvious signs of having been digested, corroborating other studies (*e.g.* Hawes, 1983; Harvey, 1989; Tollit *et al.*, 1997). Therefore, beaks could be used to estimate reliably the size of cephalopod eaten by franciscana dolphin, but not when this prey was ingested.

All the recovery times and conditions of the otoliths found are given in Table 1. White croaker, striped weakfish and banded croaker are demersal species with robust otoliths. Pelagic species, such as king weakfish and anchovy (*Engraulis anchoita*), have much thinner otoliths when compared with demersal species. The recoveries results show similar trends from the literature, where recovery rates of otoliths in stomach and scat are higher for species with robust otoliths[6] (Murie and Lavigne, 1986; Harvey, 1989; Tollit *et al.*, 1997). Henceforth, dietary studies of franciscana could underestimate the numerical importance of fish with thinner otoliths, such as the Engraulidae group and other pelagic species. Our results also imply that recovered otoliths could represent more than one day's feeding. Sekiguchi and Best (1997), using *in vitro* digestibility experiment, recovered otoliths of maasbanker (*Trachurus trachurus*) after 27 hours. In contrast, other authors stated that less than one day is needed for complete otolith dissolution in marine mammal stomachs (McMahon and Tash, 1979; Jobling and Breiby, 1986; Murie, 1987), henceforward otolith dissolution analyses were assessed in this study.

Original (initial) otolith sizes, length and width were estimated from fish length using regressions derived by the LRPD/FURG (Table 2). The prey species sizes are presented in Table 1. The digested (final) length and width were measured with a microscope equipped with an ocular micrometre (0.1 mm scale), and the thickness with a calliper (0.2 mm). The otoliths of striped weakfish, banded croaker,

and white croaker were considered for the gastric dissolution analysis because their otoliths are similar in morphology, robustness and size ranges. Otoliths from anchovy were not recovered and the exact time of dissolution is unknown, so they were not considered for the analysis. King weakfish was also discarded since there was just one pair. Statistic analyses were performed using R (R Development Core Team, 2018).

One-way analysis of variance (ANOVA) was used to evaluate the null hypothesis that there was no difference between digested otolith length with respect to thickness and width, and to assess differences between length and width regression equations for original and digested otoliths. The linear regressions for the relationships between digested otolith length with respect to thickness and width are presented in Figure 2. There were significant differences between the species for length and width (N = 78, F = 211.7, df = 3, p < 0.001), meaning differences in shapes. The regressions for length and thickness do not differ significantly between species (N = 78, F = 26.9, df = 3, p = 0.319), implying similar thickness. Comparing both linear regressions (length and width) for the size of the original and digested otoliths, no significant difference was found (N = 78, F = 0.13, df = 2, p = 0.875). It seems that the shape of the otoliths remains
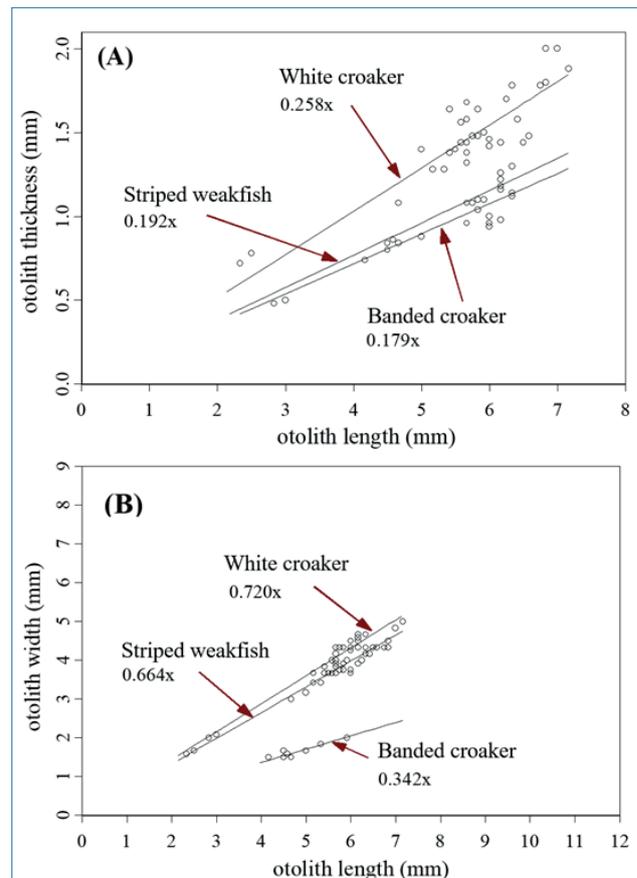
Figure 2. Linear relationships between digested otoliths measurements of three fish species (N = 12 Banded croaker; N = 24 White croaker; N = 42 Striped weakfish) from the feeding meals: (A) length and thickness and (B) length and width.

[6]Prime, J.H. (1979) Observations on the digestion of some gadoid fish otoliths by a young common seal. *Marine Mammal Committee. International Council for the Exploration of the Sea* CM 1979/N:14.

constant during the digestion process. Therefore, the biases for length and width might be similar, and otoliths with long shapes may not differ in time of dissolution from oval or circle shapes. The important characteristics in terms of digestion rate might be the otolith size and thickness, and not its shape, which concurs with the results of *in vitro* digestion experiments (Wijnsma *et al.*, 1999). The next analyses discuss this hypothesis.

The percentage mean size reduction (MSR) (see Tollit *et al.*, 1997) was calculated for otolith length, for each meal (hours after feeding), using the original and the digested length. For a given meal, the MSR = 100*(1- (mean size digested/original mean size)). The MSR values were used to define if there were differences in the rates of digestion, according to hours after feeding and different original length. Table 3 shows the MSR from otolith original and their digested lengths after various feeding times, and the analysis emphasises the previous hypothesis with lower MSR values for larger original otolith ranges whilst longer exposed to gastric digestion (see Table 1).

General linear models (LM) were used to test if there were differences in the otolith length reduction rate among species and original length ranges. Therefore, the digested otolith length was the response (predictor) variable, and hours after feeding, species and original otolith length were the explanatory variables: LM (digested length ~ hours after feeding + original length + fish species). Using LM to explore otolith size reduction due to digestion, there were no significant differences among the species (N = 78, F = 1.5, df = 3, p = 0.215), but the effects of original otolith length (N = 78, F = 2703.9, df = 1, p < 0.001) and hours after feeding (N = 78, F = 295.1, df = 1, p < 0.001) were both highly significant. Similarly, these results support the previous hypothesis that the original length is a significant explanatory variable within LM analysis. Clearly, estimates of fish length from otoliths found in stomach contents are likely to be subject to biases of differential digestion rates for different otolith sizes (McMahon and Tash, 1979; Treacy, 1981; Murie and Lavigne, 1986; Pierce and Boyle, 1991; Pierce *et al.*, 1993). Assessments of the time that the otolith was exposed to gastric digestion according to the condition found (to correct the prey original size) remain dubious, and from our results, it seems that we cannot use otolith shape as an indicator. Besides, otolith features (*e.g.* sulcus and margin) also can remain in good condition after the otoliths have been digested for several hours (see Table 1). The otoliths of the same species recovered at different times up to 26 hours did not show visual evidence of the time for which they had been exposed. Also, after 32 hours in the stomach, otoliths were still with similar features as those found eight and 14 hours before, despite evidence of greater size reduction. It is therefore not easy to establish levels of otolith digestion as a result of the significant variability and uncertainties of otolith features digestion rates. Despite this study and other prediction models for digestion time and prey reduction

Table 3. The percentage of size reduction from original to digested otolith lengths (mm) for different times (hours) after feeding.
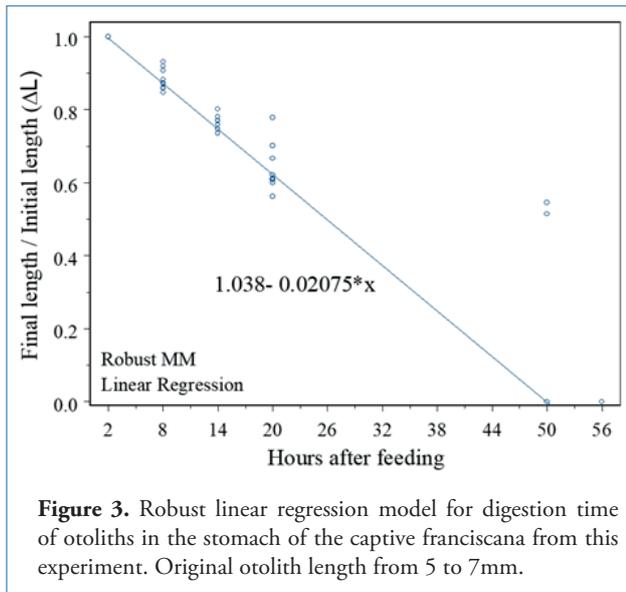
Range original otolith length	Mean original otolith length	Hours after feeding	Mean digested otolith length	N	MSR* %
5 to 7	6.16	2	6.16	6	0.00
5 to 7	6.53	8	5.70	20	12.71
7 to 8	7.83	14	5.96	12	23.88
7 to 8	7.50	20	4.83	8	35.60
8 to 10	8.30	26	6.55	12	21.08
8 to 10	9.05	32	5.23	4	42.21
5 to 7	6.68	50	0.88	16	86.83

Mean Size Reduction=100(1-(mean digested length/mean original length))

reported (*e.g.* Bigg and Fawcett, 1985; da Silva and Neilson, 1985; Sekiguchi and Best, 1997; Tollit *et al.*, 1997; Staniland, 2002; Ross *et al.*, 2016), the appropriate correction factors are very uncertain, as some otoliths could be from considerably greater original fish lengths that have been digested for longer periods.

Finally, to estimate the time of complete otolith dissolution our previous model was simplified to explanatory variables being the estimated original length and hours after feeding, as they were the significant variables. Stepwise regression analysis using ANOVA and Akaike Information Criterion (AIC) values were considered to seek a better LM to predict otolith length reduction. Furthermore, a robust regression model (Maronna *et al.*, 2018) with hours after feeding and the relationship digested length/original length was fitted and the model predictions were applied to estimate complete otolith dissolution. Robust regression models are useful for fitting linear relationships when the data contain significant outliers (Rousseeuw and Leroy, 2005). The data considered for the model were within the original length ranging from 5 to 7 mm. The original lengths range 8 to 10 mm was discarded, owing to the small sample size and the different MSR values (see Table 3). The final linear model formula is: digested length = 3.113 + (0.551)*original length – (0.119)*hours. The predicted times for complete dissolution of an otolith with the original length of 5 mm are between 49 and 50 hours, with the original length of 6 mm are from 54 to 55 hours, and for 7 mm the complete dissolution is between 58 and 59 hours. The robust regression model shows the time for complete otolith digestion (5 to 7 mm) at about 50 hours (Figure 3). These predicted times for complete dissolution differ from those found for other studies (Murie, 1987; Sekiguchi and Best, 1997; Grellier and Hammond, 2006), whereas prey digestibility and otolith length digestion rates among these studies are similar.

More research is required on categorising otolith digestion condition to assess time incurred since feeding in order to determine accurate correction factors to estimate prey sizes, meal compositions and frequency of feeding.

Figure 3. Robust linear regression model for digestion time of otoliths in the stomach of the captive franciscana from this experiment. Original otolith length from 5 to 7mm.

Although this experiment was conducted with a captive franciscana in poor health, the results may nevertheless be useful in elucidating the characteristics of the prey fragments condition found in stomach contents that cannot be obtained from free-ranging franciscana dolphins.

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