A molecular approach to identifying the prey of cephalopod paralarvae

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A molecular method was developed to detect *Artemia franciscana* within *Octopus vulgaris* paralarvae, as a first step towards understanding the diet of octopus during this life stage. Wild eggs were collected from a spawning female in the Ria de Vigo (northwestern Spain) in late summer, and brought to the laboratory. After hatching, paralarvae were reared in 30 l rectangular tanks with an open seawater filtered system. Paralarvae were fed *Artemia*, then immediately fixed in 80% ethanol and preserved at –20ºC. Primers specific to *Artemia franciscana* were designed for the cytochrome c oxidase subunit I gene (COI). A nested PCR was necessary to detect *A. franciscana* within octopus paralarvae. This molecular method provides a new framework for resolving the diet of cephalopod paralarvae in the wild, essential for ecological understanding and increasing survival rates in aquaculture.

Keywords: *Artemia franciscana*, cytochrome oxidase subunit I, diet, *Octopus vulgaris*, PCR.

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Introduction

The identification of prey in the diet of cephalopod paralarvae by visually analysing gut contents is extremely difficult. This gap in understanding their ecology is a consequence of their prey ingestion method. Most paralarvae do not swallow prey whole, but instead bite and administer saliva enzymes that paralyse or kill the prey and cause the muscle to separate from the carapace. The beak and the radula are then used to scrape out the predigested flesh, leaving an empty exoskeleton, which is rejected (Hernández-García *et al*., 2000), although sometimes they ingest small pieces of the carapace or appendages (Vecchione, 1991; Vidal and Haimovici, 1998; Iglesias *et al*., 2006). This ingestion method, together with the relatively short digestion process (1 h estimated for *Octopus vulgaris* paralarvae 12 d old using glass beads encapsulated within *Artemia* nauplii (R. Villanueva, unpublished data), makes the study of stomach contents very difficult.

To date, just four attempts to identify the gut contents of paralarvae have been undertaken. Vecchione (1991) stained *Abralia trigonura* and *Sthenoteuthis oualaniensis* paralarvae with Alcian Blue, and cleared it with trypsin to identify their prey. Passarella and Hopkins (1991) examined the digestive tracts of octopus and squid (paralarvae and juveniles) in the Gulf of Mexico. Vidal and Haimovici (1998) looked at the digestive tract of *Illex argentinus* paralarvae using the cleaning and stained method developed by Vecchione (1991). Song and Sakurai (2009) visually examined the stomach contents of wild paralarvae and juveniles of the common squid *Todarodes pacificus*. Stomach contents of these paralarvae consisted of copepods, ostracods, and crustacean eggs found in zooplankton.

Despite the absence of wild dietary studies of *Octopus vulgaris* paralarvae, the diet has been studied extensively studied in laboratory rearing experiments (Itami *et al.*, 1963; Villanueva, 1994; Shiraki, 1997; Iglesias *et al.*, 2004; Carrasco *et al.*, 2006). These experiments demonstrated the commercial viability the common octopus culture. Iglesias *et al*. (2007) suggested that the lack of a protocol for

rearing paralarvae and nutritional deficiencies in larval diets might account for the high mortality and poor growth observed in the first weeks of life. A suitable diet has not been developed for *Octopus vulgaris* paralarvae, but better understanding of the diet of wild paralarvae is essential for successful commercial culture of the species. To gain this understanding, methods that allow the molecular identification of known prey digested by the paralarvae are needed.

Species-specific polymerase chain reaction (SS–PCR) of DNA is a commonly used molecular tool for resolving trophic links in marine ecosystems (Jarman *et al.*, 2004; Passmore *et al.*, 2006). It has been applied to identify echinoderm larvae (Deagle *et al.*, 2003), to quantify copepod feeding (Nejstgaard *et al.*, 2008), and to analyse the diet of marine vertebrate predators (Jarman *et al.*, 2002). One of the most widely used genes for SS–PCR is the mitochondrial gene cytochrome c oxidase subunit I (COI; Harper *et al.*, 2005). This gene has been used to identify prey in copepod (Vestheim *et al.*, 2005), amphipod (Blankenship and Yayanos, 2005), fish (Smith *et al.*, 2005), and krill (Töbe *et al.*, 2009) diets. We chose mitochondrial DNA (mtDNA) for two reasons. First, hundreds of thousands of copies of mtDNA may be present within each cell (Hoy, 1994), greatly increasing sensitivity and hence the probability of amplifying prey DNA from the predator's gut. Second, the use of the COI gene as a barcode region (Hebert *et al.*, 2003) ensures the availability of prey and predator sequences in databases for designing species-specific primers.

Several studies have reported that prey DNA detection success in gut and faecal samples has been enhanced by targeting short DNA targets (<300 bp). This is because DNA molecules are broken during digestion (Agustí *et al*., 1999; Zaidi *et al*., 1999; Juen and Traugott, 2006). For this reason, we designed a set of primers targeting a 250 bp region within *Artemia franciscana* COI. Because of difficulties amplifying *Artemia* DNA, a two-step nested PCR approach was developed to increase the sensitivity of the molecular method. Using this method, an initial enrichment PCR was conducted with universal primers, and the product of this PCR was used as template for the second specific PCR with the *A. franciscana* species-specific primers designed within the universal region. This method has been observed to improve sensitivity by 100 (Deagle *et al*., 2003) to 10 000 (Miserez *et al*., 1997) times, compared with the standard PCR.

The aim of this work was to develop a molecular method for identifying *A. franciscana* within a single *O. vulgaris* paralarva.

Material and methods

Rearing of octopus paralarvae

To ensure that the digestive tract of *O. vulgaris* paralarvae was empty, egg strings were collected from a wild spawning female in the Ría de Vigo (northwestern Spain, 42º14′N 8º49′W) in late summer 2008. Paralarvae hatched immediately after introducing the egg strings into a bottle, and ~500 recently hatched paralarvae were transferred to an open seawater filtered system (1 μm) consisting of a 30 l rectangular tank with black walls and a white base. Mean water temperature in the culture system was 19.3ºC (17.9–20.2 ºC), and salinity 35 (34.4–35.6). The tank was provided with a light cycle of 24 h. The paralarvae were fed a diet consisting of live *Artemia* EG (Origin: Great Salt Lake, Utah, USA supplied by INVE Aquaculture, Belgium) ranging from 1.1 to 9 mm total length, at a concentration of 0.05–0.1 ind ml^{-1}. Paralarvae fed *A. franciscana* were immediately fixed in 80% ethanol and stored at –20ºC according to the method of Passmore *et al.* (2006). The total lengths (TL) of both paralarvae and *A. franciscana* were measured after fixation, from the apex of the head to the extremity of the arms and from the apex of the head to the end of the abdomen, respectively, with the aid of the image analysis system NIS-Elements D 2.30.

Extracting genomic DNA

Prior to DNA extraction, the surfaces of *O. vulgaris* paralarvae were washed with distilled water to remove possible contaminants. A DNeasy Tissue kit (Qiagen) was used to extract genomic DNA from three sources: newly hatched paralarvae that had not been in contact with *A. franciscana*, individual paralarvae fed with *A. franciscana*, and *A. franciscana* alone. Manufacturer's instructions were followed except that lysis of the samples was performed by adding 12 μl proteinase K and incubating at 56ºC overnight, all reaction volumes were reduced by half, and samples were eluted in 80 μl of AE buffer. Reactions were stored at 4ºC. The purity of the DNA extracted was determined using the

absorbance ratio at 260/280 nm, with values from 1.8 to 2 representing highly purified DNA (Gallagher and Desjardins, 2006).

Amplifying cytochrome oxidase subunit I (COI)

COI was amplified in all three samples using primers HCO 2198/LCO 1490 (Folmer *et al*., 1994; Table 1). PCRs were set up in a 25 µl volume containing 100 ng of template, 2.5 µl of 10X PCR reaction buffer, 0.5 µl of dNTPs, 0.75 µl of each primer, and 0.025 U μl^{-1} Taq polymerase (Roche). PCR amplifications were carried out in a TGradient thermocycler (Biometra), and a negative control was included for each set of PCRs. The cycling protocol consisted of an initial denaturation at 94ºC for 2 min, 40 cycles of 15 s at 94ºC, 30 s at 48ºC, and 45 s at 70ºC, and a final step of 7 min at 70ºC.

Owing to the low product yield obtained by amplifying *A. franciscana* COI with the universal primer pair HCO 2198/LCO 1490 (Figure 1), several tests were taken to optimize PCR yield where *Artemia* DNA was present, testing one variable at a time. First, we improved the purity of the *Artemia* template by extending proteinase K incubation time. Annealing temperature of the primers was optimized using a gradient from 48 to 58ºC. The effects of primer concentration and template quantity of both *Artemia* and *Artemia*-fed *O. vulgaris* were evaluated, along with the effects of adding 0.75 µl of $MgCl_2$ (2.5 mM) and 1.28 µg of bovine serum albumin (BSA) μl^{-1} to the PCR mix (Juen and Traugott, 2006).

Artemia franciscana-specific primer design and nested PCR

Artemia franciscana-specific primers ArteCOIf /ArteCOIr that amplified a 250 bp fragment of the COI gene were designed from *A. franciscana* sequences available on GenBank using the program Primer-3 (Rozen and Skaletsky, 2000; Table 1). PCRs were set up in a 25 µl volume containing 100 ng of template, 2.5 µl of 10X PCR reaction buffer, 0.5 µl of dNTPs, 1.5 µl of each primer, and 0.025 U μl^{-1} Taq polymerase (Roche). PCRs were carried out in a TGradient thermocycler (Biometra), and a negative control was included for each set of PCRs. The PCR protocol consisted of an initial denaturation step of 94ºC for 3 min, 40 cycles of 94ºC for 15 s, 54.6ºC for 35 s, and 70ºC for 45 s, and a final step of 70ºC for 5 min.

Species-specific primers were employed in the PCR with templates of 100 ng *A. franciscana* DNA (positive control) and three different concentrations (75, 150, and 300 ng) of DNA extracted from *O. vulgaris* paralarvae fed on *A. franciscana*. A nested PCR was performed using the universal primer pair HCO2198/LCO1490 for the first round of PCR and the species-specific primers, and ArteCOIf/ArteCOIr for the second round of PCR. For the first round of PCR, 150 ng of *O. vulgaris* paralarvae fed on *A. franciscana* were employed as template, and for the second round, the template used was 2 µl of the amplified product from the first round of PCR (~130 ng). In each set of PCRs, a negative control, consisting of DNA purified from recently hatched *O. vulgaris* paralarvae, was included. All PCR products were separated on 1.75% agarose gel, stained with ethidium bromide, scanned in a GelDoc XR documentation system (Bio–Rad Laboratories), and quantified using Quantity One software (Bio–Rad Laboratories).

Sequence analysis

Bands of the expected size (~250 bp) were cut out and purified with the QIAEX II gel extraction kit (Qiagen). Sequencing reactions were carried out with an automated DNA sequencer (Applied Biosystems 3130), using the BigDyeTerminator V3.1 Cycle Sequencing Kit (Applied Biosystems) with the ArteCOIf primer. Chromatograms were analysed using ChromasPro version 1.32 Technelysium Pty Ltd A. All sequences were searched for similarity using Blast (Basic Local Aligment Search Tool) through the web server of the National Center for Biotechnology Information (NCBI).

Results

A massive mortality of reared paralarvae took place 2 d (actually 50 h) after hatching, with only a few paralarvae still alive, all lying at the bottom of the tank. After that period, only nine paralarvae fed on *A. franciscana*. Total length (TL) of these 9 paralarvae ranged from 2.91 to 3.42 mm (3.16 ± 0.16 mm), whereas captured *A. franciscana* TL varied between 3.1 and 8.2 mm (5.46 ± 1.63 mm).

Amplification of the COI gene fragment with the universal primers HCO2198/LCO1490 in *O. vulgaris* paralarvae, *O. vulgaris* fed on *A. franciscana*, and *A. franciscana* alone produced average yields of 94.5 ± 11.2, 73 ± 6.1, and 42.5 ± 3.5 ng μl^{-1}, respectively (Figure 1). None of the procedures developed to enhance PCR yields using *A. franciscana* as template resulted in significant yield improvement.

Artemia franciscana-specific primers ArteCOIf and ArteCOIr yielded amplicons of the expected size (250 bp; Figure 2). Negative PCR results were observed with the species-specific primers using different DNA concentrations from paralarvae fed on *A. franciscana*. Otherwise, positive PCR results were obtained in samples using nested PCR. The specificity of *A. franciscana* primers was demonstrated by the absence of amplified product in the octopus paralarvae that did not feed on *A. franciscana*, used as negative control. Positive nested PCR results were verified by DNA sequencing of the amplified gene products, and a BLAST search showed high identity values with *A. franciscana*. The amplified *A. franciscana* DNA sequence was registered in GenBank under accession number FN556594.

Discussion

We developed a molecular method to detect a known prey, *Artemia franciscana*, within a single *Octopus vulgaris* paralarvae, providing a new framework for resolving the diet of cephalopod paralarvae in the wild. This is important because knowledge of the diet of paralarvae is crucial for understanding their ecology during this critical period of their life. Prey variety and abundance influence the survival of paralarvae (Itami, 1975), affecting the recruitment of the next generation. Additionally, knowing the diet will allow better understanding of the nutritional requisites of paralarvae, knowledge necessary for increasing the poor rates of survival achieved in feeding experiments and aquaculture (Villanueva and Norman, 2008).

Fixing paralarvae in ethanol is not necessary for the extraction method. However, all samples used in this study were fixed, because the PCRs were not developed just after the ingestion process. The fixation protocol applied to all the samples was 80% ethanol and –20ºC, because Passmore *et al*. (2006) found that such fixation yielded better PCR products than freezing at –80ºC without ethanol. Simply freezing the samples does not destroy the nucleases that denature DNA; it just inactivates them. However, preservation in ethanol precludes the problem of active nuclease, because it permeates the tissue and denatures nuclease enzymes (Flournoy *et al*., 1996). Our results suggest that the use of ethanol is not a drawback of the method, because extracted DNA from paralarvae fixed in ethanol is of excellent quality and realizes good yields during PCRs.

The fact that just 9 paralarvae preyed upon *A. franciscana* and there was a massive mortality 50 h into the experiment could be the result of premature hatching. Mechanical stimulation when collecting egg strings likely stimulated the hatching, as has been observed for *Octopus tetricus* by Joll (1978) and *Enteroctopus dofleini* by Snyder (1986). Unsuccessful feeding experiments with short survival periods suggest that the yolk of paralarva hatchlings provided the metabolic fuel rather than the prey (see Villanueva and Norman, 2008, for a review).

Octopus paralarvae preyed upon *A. franciscana* representing 106–252% of their TL. Paralarvae captured the *A. franciscana* at their posterior end (last parapods and abdomen). Paralysis resulting from the paralarval cephalotoxin extended from the biting point to the head within a few minutes. Feeding of the paralarvae then began before the *A. franciscana* were totally paralysed, by taking small parts with the buccal mass, as observed by Iglesias *et al.* (2006). This strategy allowed paralarvae to capture prey up to 2.5 times larger than their own size, more than twice the size previously observed under experimental conditions.

The length of *A. franciscana* used for rearing *O. vulgaris* hatchlings ranged from 1.1 to 2 mm (Imamura, 1990; Hamazaki *et al*., 1991). Iglesias *et al.* (2006), comparing two sizes of *Artemia* (0.8 and 1.4 mm TL), reported that octopus hatchlings showed a preference for large *Artemia*, representing nearly 50% of octopus total length. Octopus hatchlings used in rearing experiments captured live decapod crustacean zoeae ranging from 1.3 to 3.4 mm, representing 45–118% of octopus total length (Itami *et al*., 1963; Villanueva, 1995; Carrasco *et al*., 2003, 2006; Iglesias *et al*., 2004; Villanueva and Norman, 2008).

It was of note that in every PCR where *Artemia* DNA was present, the bands were of low intensity, despite all the optimization experiments carried out. Therefore, it seems unlikely that PCR conditions

were responsible for the low yields obtained when amplifying *A. franciscana*. A remarkable fact suggesting that something in the *Artemia* DNA extract interferes with the PCR is the reduced yield found in those PCRs where *Artemia* DNA was present. As is clear from Figure 1, band intensity decreases as the quantity of *Artemia* present increases. Given that all the samples have the same quantity of template DNA and were subjected to the same PCR conditions, this result suggests the presence of PCR-inhibitory substances derived from *A. franciscana* extract. Several studies have detected PCR-inhibitory substances when working with animal faeces (Kohn *et al*., 1995) or soil organisms (Juen and Traugott, 2006). However, the tools used in these earlier works to overcome PCR inhibition, such as BSA, were applied in this study with no significant improvement.

Interaction between *Artemia*-derived PCR inhibitors and the low quantities of this prey inside *O. vulgaris* paralarvae rendered impossible the direct detection of *A. franciscana* in the nuclear extract of the paralarvae that fed on them. Even when the quantity of template (nuclear extract of *Artemia*-fed paralarvae) was increased, no positive results were obtained. This false negative result, a problem in many DNA-based techniques for prey detection, would lead to spurious conclusions in field situations. Juen and Traugott (2006) solved this problem by adding BSA to the PCR mix, precluding PCR inhibition and detecting prey within carabids.

A problem associated with the use of molecular methods in diet identification is the low concentration of target DNA. In this regard, the use of mitochondrial genes such as COI should be advantageous compared with nuclear genes, owing to the large number of copies of mtDNA in each cell. However, the number of *Artemia* COI copies inside the paralarvae was not high enough to be detected using standard PCR. To improve the detection of *Artemia*, a nested PCR approach was required. Primary enrichment PCR conducted using the universal COI primers amplified the low number of *A. franciscana* COI genes present inside the paralarval gut contents. The secondary *A. franciscana*-specific PCR carried out with primers ArteCOIf/ArteCOIr enabled us to detect the sequence of *A. franciscana*. This nested approach has been successfully applied before where low concentrations of target DNA were present, e.g. for specific detection of *Mycoplasma mycoides* (Miserez *et al*., 1997) or for detecting *Asterias* larvae in ballast water (Deagle *et al*., 2003).

The difficulties involved in visually examining the gut contents of cephalopod paralarvae as a result of their ingestion behaviour (Hernandez-Garcia *et al*., 2000) mean that few reports exist of the diet of cephalopod paralarvae in the wild (Vecchione, 1991; Passarella and Hopkins, 1991; Vidal and Haimovici, 1998; Song *et al*., 2009). An external digestion process together with a strong digestive ability in *Octopus vulgaris* paralarvae has thus far impeded our understanding of their diet in the wild. We are currently investigating the diet of wild *O. vulgaris* paralarvae using molecular markers that target prey and avoid the amplification of paralarval DNA.

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Figure legends

Figure 1. Agarose gel showing PCR amplification of COI with the universal primers HCO2198/LCO1490. Lanes 1 and 2 contain DNA extracted from *Artemia franciscana*, Lanes 3 and 4 DNA extracted from two recently hatched *Octopus vulgaris* paralarvae that did not feed on *A. Franciscana*, and Lanes 5 and 6 DNA extracted from two paralarvae fed on *A. franciscana*.

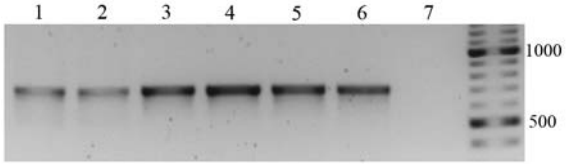

Figure 2. Agarose gel showing PCR results for species specific amplification using different DNA samples. Samples 1–7 were amplified using *Artemia franciscana*-specific primers and samples from 5 and 6 were amplified by nested PCR. Lane 1: DNA extracted from *A. franciscana* (positive control); Lanes 2–4: 75, 150, and 225 ng of DNA extracted from paralarvae fed on *A. franciscana*; Lanes 5 and 6, COI product from paralarvae fed on *A. franciscana*; Lane 7: DNA extracted from recently hatched octopus paralarvae (negative control); Lane 8: 100 bp molecular marker.

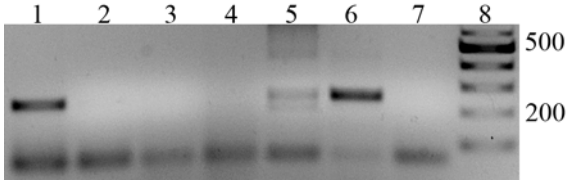

Table 1. Primer sequences used in the study.

Primer	Sequence 5'-3'	Reference	Amplification size
LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al*. (1994)	680 bp
HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al*. (1994)	
ArteCOIf	CTCCTCCTGGCCAGCTCTATG	This study	250bp
ArteCOIr	GGACGGCTGTAATTCCGACTG	This study	