Development of techniques for gender identification in *Holothuria forskali* (Delle Chiaje, 1823)

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Abstract

Holothuria forskali is a widely distributed sea cucumber species in the Atlantic Ocean and Mediterranean. Understanding the reproductive biology study of *H. forskali* is a crucial step towards achieving the sustainable production of this species in aquaculture facilities. Because echinoderms have no sexual dimorphism, it is not possible to determine their gender externally. This study aims to apply four different techniques for determining the gender of holothurians through the collection of a piece of gonad: biopsy, aspiration without incision, a short incision in the dorsal side, and a short cut in the anterior part. The biopsy method showed the highest percentage of accuracy, with 100% of gender identification compared with the other methods. In the two methods with incision, specimens were fully recovered after 21 days, showing no signs of scars or any evidence of the cut made.

Introduction

Echinoderms show no sexual dimorphism, so externally it is not possible to distinguish between males and females (Yahyavi et al. 2012). To improve broodstock conditioning of *Holothuria forskali*, gender identification is an important step. The existing methods of gender identification were only reported in studies of rearing, but with only a few descriptions (Battaglene 1999; Morgan 2000). According to Santos et al. (2015), the tubule shape of the gonads of *Holothuria forskali* is the first way to evaluate gender. When they are mature, it is possible to see (macroscopically) the oocytes inside the tubule walls, and the lumen filled by mature spermatocytes. The gonads of mature females have a distinct orange colour and the males, a strong salmon-pink colour (Keshavarz et al. 2012; Ramofafia et al. 2000). This study aimed to determine gender without causing evisceration or consequential mortality, through the application of four different methods: aspiration of a piece of gonad using a biopsy needle, aspiration of the gonad blunt, cutting the dorsal part with suction, and cutting the anterior part. The regeneration ability of this species was evaluated.

Methods

Sampling

Holothuria forskali were captured in Quebrado beach Peniche (39°22'3"N 9°22'26" W) (Fig. 1), during low tide on the Peniche coast (39°21'14.4 "N and

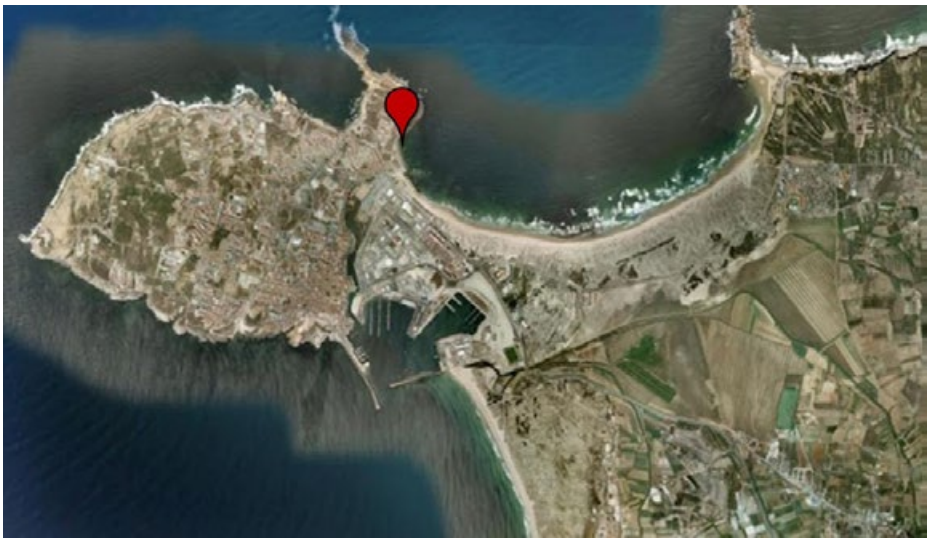

Figure 1. Peniche, Portugal. The defined area corresponding to the geographical area where *Holothuria forskali* were collected.

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9°23′43.7″ W), to obtain a total of 80 organisms. Sea cucumbers were caught by hand and transported in 4 groups of 20 individuals to 20-L plastic containers. Individuals were brought immediately to the Aquaculture Lab of MARE (Marine and Environmental Sciences Centre)-IPLeiria, where they were kept in 60-L tanks with recirculation systems. The methods were applied after a seven-day period of adaptation, to reduce physiological stress.

Conditioning

For each method, 20 individuals were conditioned in two 60-L tanks (10 sea cucumbers in each) in a recirculation system. The first tank was used as a control in which the animals were not subject to any method, and the second tank was the experimental tank. The temperature was regulated by a refrigeration system (FRIMAR 300, Portugal), maintained at 16°C. Holothurians were fed with a mixture of four microalgae species: *Tetraselmis suecica*, *Clorella sp*., *Phaeodactylum tricornutum* and *Isochrysis galbana* (Ivy and Giraspy 2006), provided once a day, in the morning, in the same proportion, according to the tank volume in a concentration approximately of 1.5 x 10^5 cells mL^{-1} throughout the entire experimental period (Battaglene et al. 2002). Water quality parameters such as dissolved oxygen and temperature (INNOVAQUA, Spain), ammonia and nitrites were measured weekly (Hanna Instrument Ammonia and Nitrite Test Kit for Salt Water).

Method 1 — Biopsy method

The first method was based on studies made by Battaglene (1999) and Morgan (2000). The sex ratio was found using a biopsy needle (Fig. 2) to collect some gametes that were observed and photographed with a Leica DM microscope (Leica, Bensheim, Germany).

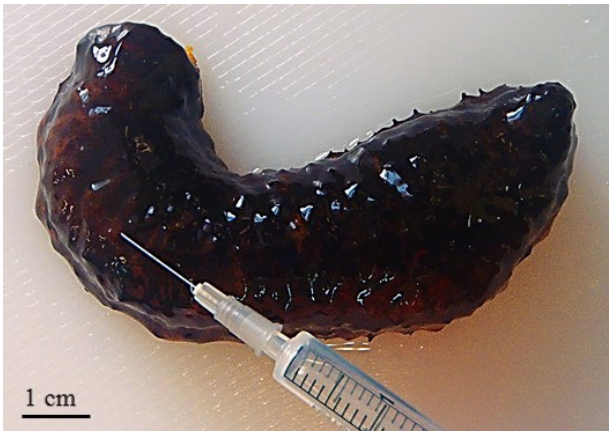

Figure 2. Method 1 — A biopsy needle used for gonad sampling of *Holothuria forskali*.

Method 2 — Aspiration without incision

This method was based on experiments performed by Battaglene (1999) and Al-Rashdi et al. (2007). Each animal was picked up and squeezed on the ventral side in order to push all organs to the anterior end. Afterwards, a Pasteur pipette was placed into the mouth and an attempt was made to pull part out of the gonads, making it possible to identify the gender of the individual. In some animals, it was possible to observe the shape and colour of the gonad and macroscopically identify the respective gender. In individuals where sexual identification was in question, a microscopic (Leica DM (Leica, Bensheim, Germany) observation of the gametes was made.

Method 3 — Incision in dorsal side

The third method emerged as an adaptation of the work described by Menton and Eisen (1973) and Yanagisawa (1998). This method was based on making a small incision in the dorsal area of the body (Fig. 3). The body was placed on a tray by making a small cut in the tegument with a scalpel. Once the section was made, a Pasteur pipette was placed in the opening and part of the gonad was pulled on.

Figure 3. *Holothuria forskali* after a cut was made on its dorsal side (Method 3).

Method 4 — Cut in anterior part

This method is based on previous works (Battaglene 1999; Menton and Eisen 1973; Yanagisawa 1998). It consisted of making a small cut at the forward end of the body. Using scissors, a small incision was made in the mouthpiece, providing a larger opening for collecting part of the gonad. After cutting, a Pasteur pipette was inserted to aspirate part of the gonads, which were then collected by tweezers and a microscopic examination of them was performed.

After the application of each method, individuals were left to recover for 21 days, without manipulation, in recirculation aquaculture systems, although food supply was maintained. Mortality and/or evisceration were recorded daily. In cases of

evisceration, the gonads were collected to determine the maturity stage (Keshavarz et al. 2012; Ramofafia et al. 2003).

Results

During the trial and recovery period, temperature was maintained at 16 ± 1°C, and salinity and dissolved oxygen were maintained at 33 ± 1 ppt and 8.0 ± 0.2 mg $L^{-1,}$ respectively, in all tanks. Neither mortality nor evisceration were recorded using the first, third and fourth methods. It was observed that with the second method, aspiration without incision resulted in evisceration and mortality. The survival rate obtained for the second method was 80% and was 100% in the control tank.

Gender determination

The results of gender determination for all four methods are shown in Figure 4. A success rate of 100% was achieved using the first method, 40% for the second and third methods each, and 70% for the fourth method. For the third and fourth methods, specimens showed a full recovery 21 days after the method was conducted, showing no signs of the incision that was made.

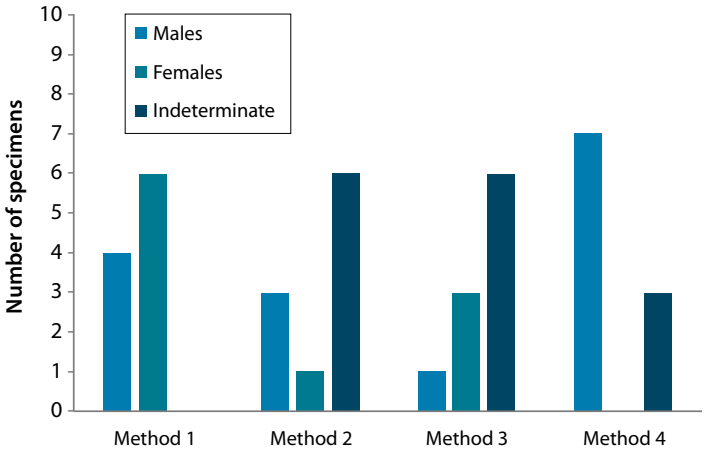

Figure 4. Total number of males, females or indeterminate individuals in each performed method.

Discussion and conclusion

According to Morgan (2000), the extraction of gonadal material is useful in determining the sex of sea cucumbers. Once this technique had been refined, no animals eviscerated after a puncture of the viscera with the biopsy needle. In our study, with the first method, 100% of gender identification was made without any detection of mortality or evisceration.

Mortality and evisceration that occurred in the second method are associated with handling procedures, more specifically, when the animals were squeezed in the ventral side to push all organs to the anterior end. This can be confirmed by 30% evisceration and 20% mortality using this method. Despite these results, the identification had the same success as the third method, in which there was no mortality or evisceration. In the third method where there is an incision in the dorsal side, the organs were exposed through the cut made, but it was not always possible to see the gonads, and therefore better results were obtained using the first method.

Regarding the fourth method, the cut made in the anterior area caused relaxation of the mouth muscles, which made it was possible to insert the tweezers. In this method it was not possible to see the gonads at first, but through compression, it was possible to locate and extract the gonadal tubules. With this method, it was possible to remove a tubule of each individual gonad, thus identifying the gender. This method differs from the second method because it was made by a cut, using tweezers to grab the gonads instead of a Pasteur pipette. This fact may explain the easiness in obtaining the entire gonad tubules.

In all methods, the aspirated content had perceptible oocytes in the case of females, and a lumen filled with mature spermatocytes, in males. There are surprisingly few studies based on skin healing in holothurians (Yanagisawa 1998). Despite the evasive methods that were performed where a cut was applied, *Holothuria forskali* has developed a mechanism capable of recovering from a cut. The described time for complete regeneration of a wound was three to four weeks, including for an organ or an appendage, and the sea cucumber is able to regenerate a missing structure (Cowden 1968; Menton and Eisen 1973; Sun et al. 2011). In the present study, it was observed that specimens had no scar or other traces that indicated a cut had been made within the recovery time of 21 days. In conclusion, getting information on the gender identification can made a successful broodstock collection, thus enhancing the success of breeding programmes with new sea cucumber species.

Acknowledgements

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