Protocol M - Available methods for sexing and tagging sea bass [Pratical guide of protocols: sexing and tagging]

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I - Introduction

Fish tagging and sexing are important tools for both research and management of fish. Thus, understanding of genotypic sex and the availability of methods for tagging fish are basic tasks in aquaculture. The ability for distinguishing sex at early developmental stages could facilitate breeding techniques and improve the production of farm fish species. However, most fishes are not phenotypically different and males are undistinguishable from females. Although several approaches have been addressed for sexing fish, finding a reliable, fast and easy methodology is more complicated than expected.

The development of DNA marker technologies has resulted in a variety of genetic markers, including monolocus and multilocus markers, with potential utility in the characterization of DNA fragments associated with traits of interest in aquaculture, including the identification of DNA sex-specific markers (Woram *et al*., 2003; Liu and Cordes, 2004; Felip *et al*., 2005). Although DNA markers have not proved to be useful tools for examining sex in all species analysed (Li *et al.*, 2002; Khamnamtong *et al.*, 2006; Wuertz *et al.*, 2006). In the sea bass, a subtractive hybridization approach was conducted in order to identify sex-linked markers in this species but no polymorphic markers were found (Martínez *et al*., 1999). A comparison of genome size in the sea bass by flow cytometric determination has revealed the lack of sex-specific differences in DNA content between males and females (Peruzzi *et al*., 2005).

To date, a routine activity in the laboratory, based on abdominal massage of sexually mature fish during the reproductive season (December-March), is used to identify males and females in broodstocks. In the Mediterranean area and under culture conditions, sexual maturation in sea bass males is reached during the second year of life, although some males reach an early puberty near the first year of life. In contrast, females usually reach puberty near the third year of life (Carrillo *et al.*, 1995). Sexually mature sea bass may also be identified macroscopically by gonadal morphology (i.e., colour and shape) and microscopically by standard histological procedures (i.e, gonadal structure and type of germ cells). Gonadal cell stages can be identified and characterized according to several morphological traits previously described in female (Mayer *et al.*, 1988) and male sea bass (Roblin and Bruslé, 1983; Begtashi *et al.*, 2004). Recently, a rapid and effective method for sexing juvenile sea bass has been described based on gonad squash mounts (Menu *et al*., 2005). It has resulted in a practical use for research purposes, mainly in those studies where a large number of experimental fish need to be sexed. Currently, the early identification of sex in the sea bass by a non-invasive approach with practical use in the industry is still a challenge in this species.

For practical reasons, in sea bass, when fish are sexed during the reproductive season they are tagged. Thus, males and females are individually identified for further breeding schemes and estimation of reproductive parameters. There are many tagging methods that have been used in fish. External systems include freeze branding, fin clipping and colour tagging among others. Internal systems include Carlin tags, visible implant elastomer (VIE) marking, coded wire tags (CWT), and passive integrated transponder (PIT) tags. Tagging systems need to be tested for each species in order to evaluate their feasibility without affecting growth and mortality (Navarro *et al*., 2006). The PIT tag system has been used in sea bass in the abdominal cavity by excision, when fish are small, and in the dorsal muscle, when they are larger. The PIT tagging has shown that it is feasible in this species without affecting growth or mortality and showing a low tag loss rate in animals under experimentation.

II - Purpose

The main objective of this Protocol is to present three available methods for sexing sea bass. Acording to the age or the size of the animals and the season period, different methods can be used. These methods include: (i) stripping procedures and cannulation of sexually matured fish during the reproductive season; (ii) macroscopic and microscopic examination of the gonads are commonly applied independently of age (juveniles or adults) or season period (reproductive or resting period); and (iii) a shortcut method based on a gonad squash mount technique is usually used for sexing of juvenile fish.

In addition, a tagging protocol for PIT tagging of sea bass broodstock is here described.

III - Procedure

1. Stripping, cannulation and tagging

(i) Sexually mature fish can be sexed during the reproductive season (December-March) by abdominal massage and cannulation (see Protocol *B*). Fish must be previously anaesthetized with MS-222 or 2-phenoxyethanol. The use of gloves is recommended during this process.

(ii) Males are usually sexually mature and release sperm after gentle abdominal massage. Fish that do not release sperm are probably females. They can be gently cannulated with a plastic catheter in order to obtain a subsample of oocytes that can be visualized under a light microscope. The presence of male or female gametes unequivocally confirms the sex of these animals. They can be electronically tagged by using PIT-tags for future broodstock management purposes.

(iii) All fish must be anaesthetized prior to tagging with PIT tags. PIT tags must be previously immersed in alcohol and introduced horizontally into the fish using a syringe. Iodine is applied after injection to avoid putative pathological lesions or infections. Generally, sea bass broodstock are tagged in the dorsal muscle.

2. Macroscopic and microscopic gonadal examination

(i) Under experimental conditions, at the laboratory, mature and immature fish are usually sexed by macroscopical and microscopical examination of the gonads. For this purpose, fish are previously slaughtered and the gonads removed.

(ii) For macroscopical examination, the gonads are characterized using morphological traits such as colour and shape.

(iii) For microscopical examination, the gonads are removed and fixed in 4% formaldehyde: 1% gluteraldehyde buffered saline (McDowell and Trump, 1976) for at least 24 hours, dehydrated in a 70-96% ethanol series and embedded in glycol methacrylate resin. Serial sections are obtained at a thickness of 4 µm on a microtome using disposable blades. After drying, slides are stained with methylene blue/ azure II/ basic fuchsin (Bennett *et al*., 1976) and examined with a light microscope. The gonads are characterized based on morphological and structural traits that are differential between males and females (Roblin and Bruslé, 1983; Mayer *et al.*, 1988; Begtashi *et al.*, 2004).

3. Gonad squash mount technique

(i) Alternatively, a shortcut method based on a gonad squash mount technique can be used for sexing juvenile fish (over a period of 2 months, from 109 to 227 mm total length and 11-122 g of weight). For this use, the gonads are removed, mounted on a clean microscope slide and stained with few drops of aceto-carmine (Guerrero and Shelton 1974).

(ii) The tissue gonad is squashed with a cover slip and examined with a light microscope.

IV - Materials and equipment

- Plastic catheter
- Glass slides
- Cover slips
- Forceps
- Fixer solution
- Glass vials
- Specific apparatus for histological preparations (Histology laboratory)
- Stain solutions
- A light microscope
- PIT tags
- PIT tag reader
- PIT tag injection kit (syringe)
- Gloves and lab coat

V - Reagents and solutions

- Anaesthetic: MS-222 (0.1 g l^{-1} of sea water) or 2-phenoxyethanol (0.5 ml l^{-1} of seawater). Alternatively, the induction of anaesthesia can be carried out using clove oil. Clove oil has been evaluated as an effective anaesthetic in sea bass and it can be used at almost 10-fold lower doses than 2-phenoxyethanol.
- Fixer: Formaldehyde (1% gluteraldehyde buffered saline)
- Ethanol series (70-96%)
- Glycol methacrylate resin
- Stain solution for histological procedure: methylene blue/ azure II/ basic fuchsin
- Stain solution for gonad squash mount technique: aceto-carmine
- Iodine

VI - Results and discussion

The development of methods for sexing fish is an important task for aquaculture. The identification of males and females during early developmental stages may be useful for breeding programmes and farming conditions. It could avoid sexual dimorphism that affects commercial traits of aquacultural fish such as growth, sexual maturation or disease resistance among others. In sea bass, males are indistinguishable from females, although males are usually smaller than females at the first maturity. Currently, stripping procedures and cannulation are effective for sexing when fish are sexually mature during the reproductive season (Fig. M.1. A, B, C). At this time, broodstock are usually tagged for future individual identification for research and management purposes (Fig. M.2).

On the other hand, males and females exhibit distinctive testicular and ovarian differentiation,

which occurs between 9 and 11 months of age or standard length of 13.8-27 cm in females and 11.2-17.5 cm in males (Roblin and Bruslé, 1983; Blázquez *et al*., 1995). Accordingly, the macroscopic examination of the gonads, which is based on morphological traits such as colour and shape, can be a reliable method for sexing. Female gonads usually have an orange colour and a rounded section in shape whereas male gonads have a transparent colour and triangular section (Fig. M.1.D). By microscopical examination, the morphological and structural traits are crucial for sexing (Fig. M.1.E-F). Female gonads exhibit the ovarian lamellae projecting into the lumen, while male gonads show the formation of cysts in the testicular tubules. Nevertheless, microscopical examination requires the use of histological procedures that, although they are standardized, are laborious.

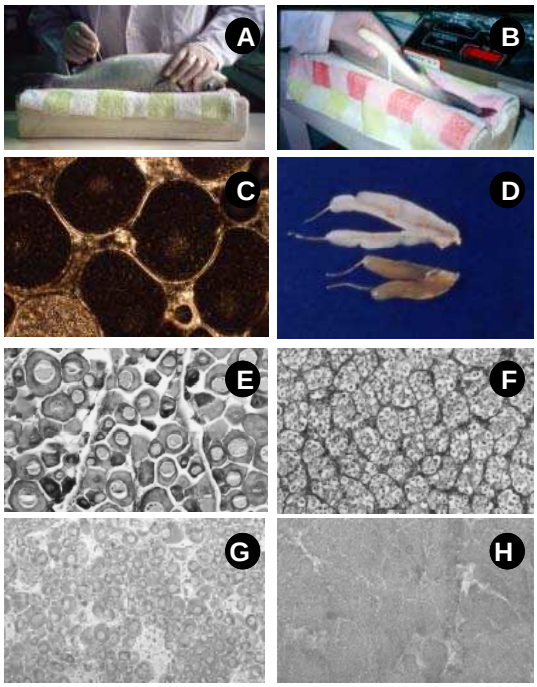

Fig. M.1. Sex identification in sea bass. Cannulation (A) and stripping (B) of sexually mature female and male fish, respectively. (C) Sample of oocytes obtained by intraovarian cannulation and then observed under the microscope. (D) Photomacrograph of a testis (above) showing a pale white colour and a triangular section in shape and an ovary (below) showing a pale orange colour and a rounded shape. (E) Histological section of an ovary with oocytes located in the ovarian lamellae (arrowhead). (F) Histological section of a testis showing spermatogonia in the testicular tubules (arrowhead), which are well formed. (G) Wet squash preparations of an ovary with previtellogenic oocytes and a testis (H) showing the typical testicular tubules. Photographs A, B, C supplied by A. Felip. Photograph D modified from Felip *et al*. (2001), and G and H modified from Menu *et al*. (2005).

An alternative to this procedure is the application of a gonad squash mount technique that has resulted to be a fast and effective method of sexing juvenile sea bass (Menu *et al*., 2005). Squashed ovaries exhibit different sizes of previtellogenic oocytes, while squashed testis show the well-developed testicular tubules (Fig. M.1.G-H). Unfortunately, the techniques based on the gonadal examination require the sacrifice of fish that can be a limiting factor for some purposes when it is necessary to keep fish alive.

Although these techniques are useful for many studies in the laboratory and can be successfully applied to the aquaculture industry, their use is limited when the identification of the sex is required during early developmental stages. Currently, the characterization of markers associated with sexual determination and differentiation in this species deserves more attention in order to achieve a simple, fast and valuable application for sexing sea bass.

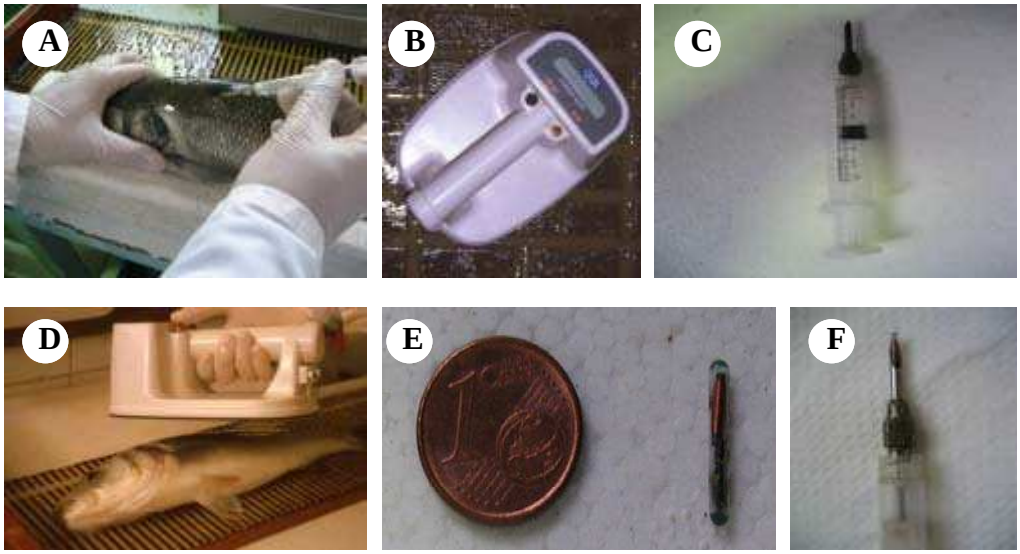

Fig. M.2. **Fish tagging. (A) Tagging sea bass broodstock. (B) PIT tag reader. (C) Syringe for PIT-tag injection. (D) Reading PIT tags in tagged fish previously anaesthetized. (E) A Electronic tag ,compared with a 1 cent euro coin (16 mm). (F) Detail of syringe with the electronic tag inside for injection.**

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