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# Sex determination in blue mussels: Which method to choose?

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ABSTRACT

Sexing methods of blue mussels are mostly based on the presence or absence of gametes, and do not take into account reproductive cycle stages. Exposure effects can be affected by the sex of mussels, thus the aim of this study is to determine an efficient sex determination protocol taking into account the reproductive cycle stage. Eight mussel sexing methods were compared. This study demonstrates that the first step in discerning sex in blue mussels should be assessing the reproductive stage, which can be done by mantle histology. During gametogenesis, histology allows the differentiation of males from females by the observation of gametes. However, when mussels are in sexual rest, the only method that should be used is the sex-specific gene method.

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## 1. Introduction

Not taking into account the sex differences in environmental and toxicological studies could lead to misinterpretation of results (Mergler, 2012; Ritz et al., 2014; Weiss, 2011). In bivalves, contaminants can bioaccumulate differently between sexes (Richir and Gobert, 2014; Sokolowski et al., 2004), and some biological functions differ between male and female (Banni et al., 2011; Brown et al., 2006). For example, the serotonin system, involved in sexual differentiation, gametogenesis and spawning (Cubero-Leon et al., 2010; Fong et al., 2003; Gagné and Blaise, 2003; Gibbons and Castagna, 1984), can be altered by contaminants (Almeida et al., 2003; Cubero-Leon et al., 2010; Salanki and Hiripi, 1990). Also, Dang et al. (2012) demonstrated that the percentage of hemocyte cell types fluctuates between sexes in *Saccostrea glomerata* and *Pinctada fucata*, especially for small agranulocytes. In *Mytilus edulis*, cyclooxygenase (COX) expression is higher in male gonads compared to female gonads (Cubero-Leon et al., 2010). Thus, the biological response pattern can diverge between sexes when mussels are exposed to xenobiotics such as endogenous estradiol which affects the COX expression in a sex-specific manner (Cubero-Leon et al., 2010).

Although there are a few studies that include bivalve sex in their analyses (Brown et al., 2006; Cappello et al., 2015; Falfushynska et al., 2013; Lazzara et al., 2012; Ruiz et al., 2011), it is still often neglected (Chandurvelan et al., 2012; Fraser et al., 2014; Klouche et al., 2015; Martinez Bueno et al., 2014). Several methods exist to determine mussel sexes (Hines et al., 2007; Jabbar and Davies, 1987; Mikhailov et al., 1995; Ruiz et al., 2011; Sedik et al., 2010). Most of them are based on the presence or absence of gametes in the mantle. This tissue is the main site of gonad development and stores gametes until spawning (Mikhailov et al., 1995). The mussel reproductive cycle is divided coarsely in four stages in which gametes are not always noticeable, especially in late post-spawning and sexual rest (Lubet, 1959; Ruiz et al., 2011; Seed, 1976). Briefly, the cycle starts by a stage without gametes where a rapid regeneration of the mantle takes place (post-spawning resting stage; stage 0). It is followed by the gamete formation (stage I). In the ripe stage (stage II), the storage vesicles abound in spermatozoon or oocytes: spawning is imminent. In the spawning and post-

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spawning stage (stage III), gametes are emitted in the water. This is followed by the degradation and reabsorption of the remaining gametes until the cycle starts over (Lubet, 1959; Ruiz et al., 2011; Seed, 1976). Thus, sex determination methods based on gametes are not effective and cannot be used at every reproductive cycle stages of mussels (Petes et al., 2008; Sedik et al., 2010). The aim of this study is to compare eight mussel sex determination methods in order to establish an efficient sex determination protocol that includes the reproductive cycle stage.

## 2. Materials and methods

### 2.1. Mussels handling

Blue mussels (>90% *Mytilus edulis*, and <10% *Mytilus trossulus* and hybrid) were collected from the *Baie de Plaisance* located in the *Îles de la Madeleine* region (47°29′N, 61°87′W) in early June 2013 by the mussel farm "La moule du large". Since mussels of this region are known to spawn around this period, individual mussels will be at slightly different stages of the spawning process, but the population would be regarded as not having fully spawned or to have entered into a stage of atresia (Cartier et al., 2004). Mussels were placed in an aerated tank filled with artificial seawater (Instant Ocean®, Reef Crystal, Cincinnati, OH, USA) (15 °C, salinity 31 ± 1 PSU) for 28 days, then sacrificed, and the mantle dissected on ice. Spawning was not observed during the acclimation period.

Smears of the mantle were collected on microscope slides. A small piece of the mantle (3 $mm^2$) was collected for histology. The rest of the tissue was homogenized with a Teflon pestle tissue grinder in a conservation buffer (Hepes-NaOH buffer (pH 7.4) containing 100 mM NaCl, 0.1 mM dithiothreitol and 1 μg/mL aprotinin) as described by Gagné et al. (2011c). Proteins in the homogenate were dosed with the Pierce bicinchoninic acid assay (BCA) according to the manufacturer's instructions (Pierce Biotechnology, CA). All samples were kept at −80 °C until analysis.

### 2.2. Sex determination analysis

All the sexing methods were applied on the 120 mussels to save time and cost. Two experimenters were assigned to establish mussels' sexes using random and blinded ID to decrease subjectivity. Each experimenter had to establish the sex of each mussel twice.

#### 2.2.1. Mantle color

Sex was determined according to color of the mantle: creamy white for males and orange for females (Mikhailov et al., 1995).

#### 2.2.2. Fresh smear

Two singular slide smears of the mantle were made on a microscope slide and immediately observed by optical microscopy at a magnification of 10× in order to observe the presence of moving spermatozoon to determine the sex of the mussel (Gagné et al., 2001). Mussels were considered female when spermatozoon was absent, even though this could cause bias when mussels are in resting stage.

#### 2.2.3. Stained smear

Slides used for fresh smear observations (section 2.2.2) were dried at room temperature overnight and fixed and stained with the Kwik Diff Kit (Thermo Fisher Scientific, MA). The presence or absence of spermatozoon (purple dots) was observed by optical microscopy at a magnification of 10× to establish the sex of the mussel. Mussels were considered female when spermatozoon was absent.

#### 2.2.4. Histology

Mantle samples (3 $mm^2$) were fixed in Bouin solution (Sigma-Aldrich, ON, Canada) for 24 h, dehydrated in a series of ethanol dilutions and embedded in paraffin. Six sections of 5 μm thickness were stained with Harris hematoxylin and eosin solutions (Thermo Fisher Scientific, MA). The reproductive cycle stage and the sex of each mussel, when gametes (spermatozoa and ova) could be observed, were determined by microscopy at a magnification of 10× according to Lubet (1959), Seed (1976) and Ruiz et al. (2011).

#### 2.2.5. Chemical coloration

The chemical method described by Jabbar and Davies (1987) was used with minor modifications. Briefly, 75 μL of the homogenized tissue was mixed with 1 mL of 20% trichloroacetic acid (TCA) and 0.25 μL of 0.75% thiobarbituric acid (TBA) in a 1.5 mL tube. Mixtures were heated during 20 min at 95 °C. According to the color of the resultant solution, a sex was associated: yellow for males and pink for females.

#### 2.2.6. Spectrophotometric analysis

The solutions obtained in 2.2.5 were centrifuged to eliminate tissue suspensions. A volume of 200 μL of each solution was placed in a clear 96 well plate and scanned over the 430–600 nm range with a spectrophotometer (Spectra Max M5, Molecular devices). A peak of absorbance at 460 nm reflects the relative abundance of DNA and oxidizable lipids (Hines et al., 2007; Jabbar and Davies, 1987). This is associated with male mussels.

#### 2.2.7. Male associated polypeptide (MAP39)

The male associated polypeptide (MAP39) was found in the mantle of *Mytilus galloprovincialis* lmk (Mikhailov et al., 1995). Briefly, proteins were extracted from the mantle homogenate solution with radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing protease and phosphatase inhibitors cocktails (Pierce, ThermoScientific). After sonication, solutions were centrifuged (14,000× *g*) at 4 °C during 10 min for protein extraction. Protein concentrations were determined in the supernatant with the BCA kit using the manufacturer's instructions (Pierce Biotechnology, Rockford, CA). Forty micrograms of proteins were separated on 10% SDS-PAGE gel with a running buffer (10% Sodium dodecyl sulfate; *7.2% glycine and 1.5% Tris*) and stained with Coomassie blue. The

**Table 1**
PCR conditions for the sex-specific gene method.

| Gene (genbank) | Sequence | | Annealing | Product (bp) | Reference |
|---|---|---|---|---|---|
| *verl* (FM995161.1) | F | 5′-CTGCAATGGTTTTGGTTGTG-3′ | 50 °C | 350 | Hines et al. (2007) |
| | R | 5′-CCGAAGGAAATGGAACTGAA-3′ | | | |
| *Vcl* (FM995162.1) | F | 5′-TTGCGTTTCACATGGTTGAT-3′ | 55 °C | 250 | Sedik et al. (2010) |
| | R | 5′-AGAGCTGTTTTGGCCACAGT-3′ | | | |

*verl*: vitelline envelope receptor for lysine; *vcl*: vitelline coat lysine.
F: Forward; R: Reverse.

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