Marine Pollution Bulletin xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Intersex related gene expression profiles in clams *Scrobicularia plana:* Molecular markers and environmental application

Corina M. Ciocan [a,d], Elena Cubero-Leon [b,d], William J. Langston [c], Nick Pope [c], Keith Cornelius [d], E.M. Hill [d], Diana Alvarez-Munoz [d], Paolo Indiveri [d], Adelaide Lerebours [f], Christophe Minier [b], Jeanette M. Rotchell [e,*]

[a] *School of Pharmacy and Biomolecular Sciences, University of Brighton, Lewes Road, Brighton BN2 4GJ, United Kingdom*
[b] *Laboratoire d'Ecotoxicologie, Universite du Havre, 25 Rue Philippe Lebon, BP540, 766058 Le Havre, France*
[c] *Marine Biological Association, The Laboratory, Citadel Hill, Plymouth PL1 2PB, United Kingdom*
[d] *School of Life Sciences, University of Sussex, Falmer, Brighton BN1 9QJ, United Kingdom*
[e] *School of Biological, Biomedical & Environmental Sciences, University of Hull, Cottingham Road, Hull HU6 7RX, United Kingdom*
[f] *School of Biological Sciences, Institute of Marine Sciences, University of Portsmouth, Ferry Road, Eastney, Portsmouth PO4 9LY, United Kingdom*

ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Intersex
Clams
English Channel
Gene expression

ABSTRACT

Intersex, the appearance of female characteristics in male gonads, has been identified in several aquatic species. It is a widespread phenomenon in populations of the bivalve, *Scrobicularia plana*, from the south-west coast of the U.K. Genes previously identified as differentially expressed (*ferritin, testicular haploid expressed gene*, *THEG*, *proliferating cell nuclear antigen*, *PCNA*; *receptor activated protein kinase C*, *RACK*; *cytochrome B*, *CYB*; *and cytochrome c oxidase 1*, *COX1*) in intersex clams relative to normal male clams, were selected for characterisation and an environmental survey of the Channel region. Transcripts were significantly differentially expressed at sites with varying intersex incidence and contaminant burdens. Significant correlations between specific gene expressions, key contaminants and sampling locations have been identified, though no single gene was associated with intersex incidence. The results highlight the difficulty in understanding the intersex phenomenon in molluscs where there is still a lack of knowledge on the control of normal reproduction.

1. Introduction

Xenobiotics can interfere with normal gonad development and potentially alter the population structure (Kidd et al., 2007; Lange et al., 2011). Endocrine disrupting chemicals (EDCs), in particular, disrupt the reproductive endocrine system and may cause various biological impacts such as imposex (Strand and Asmund, 2003; Lima et al., 2011), and intersexuality (Kidd et al., 2007; Jobling et al., 2002). Focussing on molluscs, intersex has been reported in several species worldwide including *Littoraria angulifera* (Costa et al., 2013), *Ruditapes sp.* (Ponurovsky and Yakolev, 1992; Delgado et al., 2001; Lee et al., 2010), *Crassostrea gigas* (Lee et al., 2010), *Plaxiphora aurata* (Scarano and Ituarte, 2009), *Mytilus galloprovincialis* (Ortiz-Zarragoitia and Cajaraville, 2010) and *Scrobicularia plana* (Chesman and Langston, 2006; Langston et al., 2007; Gomes et al., 2009; Fossi Tankoua et al., 2012). There is evidence that intersex is a widespread phenomenon in the bivalve, *S. plana*, populations from the south coast of the U.K. (Chesman and Langston, 2006*;* see also Pope & Langston, this volume) and France (Fossi Tankoua et al., 2012), and also inducible in an experimental exposure regime using EDCs (Langston et al., 2007). In *S. plana* intersex is characterised histologically by the occurrence of oocytes among normal testicular tissue (ovotestes) (Langston et al., 2007).

Understanding the cause of the intersex condition in *S. plana*, and indeed other bivalves, is complicated due to natural sexual differentiation and lack of discreet reproductive organs in such species as follows. Sexual differentiation varies widely among bivalves, from species that are strictly of separate sexes, to those that are almost invariably functionally hermaphroditic. Several mechanisms of sex determination in molluscs are characterised (Vitturi et al., 1998; Guo et al., 1998; Kenchington et al., 2002; Breton et al., 2007, 2011; Ghiselli et al., 2012). Because of these variations in the expression of sexuality, in different species and within different individuals of the same species and at different points in life, the bivalve molluscs represent a challenging group in trying to determine the impact of EDCs. In bivalves, there is also no distinct reproductive organ (male or female) and the germinal cells are in direct contact with the surrounding connective tissue (Osada et al., 2007). The developmental stages of sperm and egg follow a similar

---

* Corresponding author. Tel.: +44 (0) 1482 465333; fax: +44 (0) 1482 465458.
E-mail address: J.Rotchell@Hull.ac.uk (J.M. Rotchell).

http://dx.doi.org/10.1016/j.marpolbul.2015.02.019
0025-326X/© 2015 Elsevier Ltd. All rights reserved.

Please cite this article in press as: Ciocan, C.M., et al. Intersex related gene expression profiles in clams *Scrobicularia plana:* Molecular markers and environmental application. Mar. Pollut. Bull. (2015), http://dx.doi.org/10.1016/j.marpolbul.2015.02.019

morphological pattern as seen in vertebrates (Osada et al., 2007). It has been suggested that the initiation of gametogenesis in bivalves depends on several environmental cues, such as temperature, salinity and food availability (Ginsberger-Vogel and Magniette-Mergault, 1981). It is established that vertebrate-like steroids are present in various tissues of molluscs (Zhu et al., 2003; Mouneyrac et al., 2008). A number of enzymatic activities and regulatory pathways in molluscs have also been characterised (Janer and Porte, 2007). Whilst the occurrence of vertebrate-like sex steroids is not in doubt, their source and role in molluscs is less clear (Scott, 2013). To date, a relatively small number of gonad transcriptomic investigations have been conducted using bivalve molluscs (Ciocan et al., 2011; Boutet et al., 2008; Craft et al., 2010; Banni et al., 2011; Llera-Herrera et al., 2013). In these studies, a number of differentially regulated genes in mussel testes, including testis-specific kinases, vitelline lysin and envelope sequences, have been reported (Ciocan et al., 2011). With complications of sexual differentiation and lack of discrete organs, determining the normal reproductive endocrinology and the underlying molecular level cause of induced intersex remain as challenges.

In recent work, gene transcripts involved in cell signalling, cell cycle control, energy production/metabolism, microtubule assembly, and sperm physiology were highlighted as differentially expressed in intersex male clams (Ciocan et al., 2012). Herein, we further characterise a number of the intersex-associated differentially expressed transcripts and determine their expression in natural populations of clam along the U.K. and French coasts of the English Channel, of known EDC contaminant burden, in order to investigate their potential as biological effects markers of EDCs.

2. Materials and methods

2.1. Animals

Scobicularia plana individuals ($n = 30$) were sampled from more than 100 sites along the Channel coast, between June–July 2009, 2011 and 2012 as part of the DIESE project (see Pope et al., this volume). The sampling sites focussed on in this study were, from west to east, as follows: Avon, Wytch Farm, Parkstone, Totton, St. Denys, Northam, Warsash, Le Havre and Berck respectively (Fig. 1). Sites were chosen to represent a range of endocrine disrupting activities (anti-androgenic and estrogenic activities) present in sediments in the Transmanche region (see Alvarez-Muñoz et al., this volume). At the point of sampling, the sex and reproductive status of individual clams was not externally visible. Clams were depurated in 50% seawater for 2 days (to eliminate sediment), before the gonads were excised. A small sample of gonad from each clam was examined histologically to determine sex and reproductive development stage. Chi-squared tests were performed on sex data to reveal any significant departure from a normal male:female ratio for clams sampled at each location. A piece of gonad (approximately 20 mg) from each clam was fixed in RNALater (Qiagen Ltd., Crawley, U.K.). A subset of individuals (39.1 ± 3.8 mm, SEM, $n = 9$), consisting of mostly normal males with the exception of one normal female at Totton, Warsash and Wytch Farm, were randomly selected from each sampling site for molecular analyses.

2.2. Intersex incidence at sampling sites

The incidence of intersex at each sampling site was determined by histological examination of gonad tissue using criteria as described in Chesman and Langston (2006).

2.3. *In vitro* measurements of endocrine disrupting activities

Sediments were extracted using assisted Accelerated Solvent Extraction and the anti-androgenic activity of extracts was determined as flutamide equivalents using the AR CALUX bioassay as detailed in Alvarez-Muñoz et al. (2014). The estrogenic activity of extracts was measured as 17-βestradiol equivalents using a yeast recombinant estrogen receptor screen (YES) as described in Peck et al., 2004.

2.4. Isolation of candidate genes associated with the intersex condition in male clam gonad tissue

Candidate genes for development as potential molecular markers of the intersex condition were isolated using the suppressive

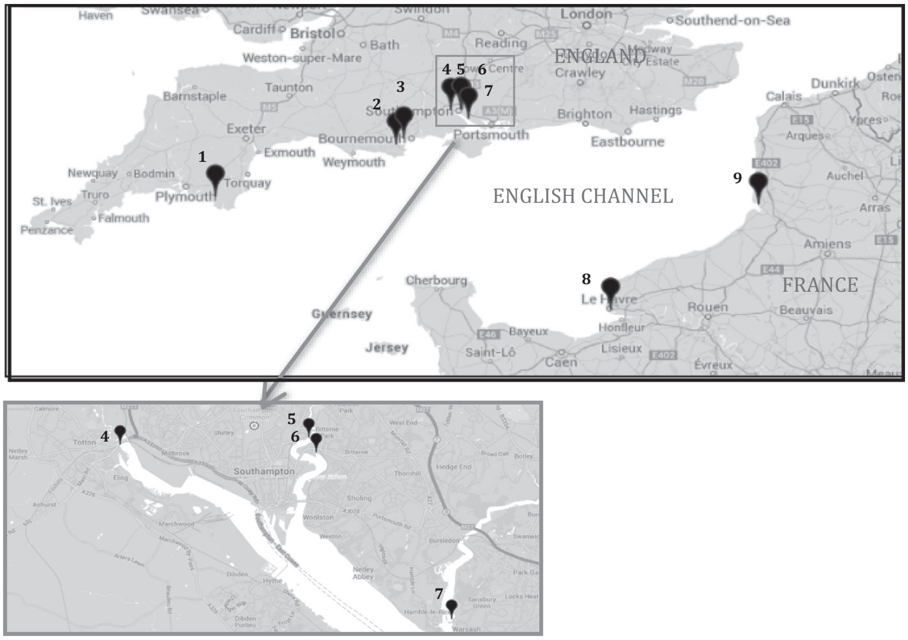

Fig. 1. Map showing the sampling stations (Map data © 2014 Google). 1: Avon; 2: Wytch Farm; 3: Parkstone; 4: Totton; 5: St Denys; 6: Northam; 7: Warsash; 8: Le Havre; 9: Berck.

Please cite this article in press as: Ciocan, C.M., et al. Intersex related gene expression profiles in clams *Scrobicularia plana:* Molecular markers and environmental application. Mar. Pollut. Bull. (2015), http://dx.doi.org/10.1016/j.marpolbul.2015.02.019