General and Comparative Endocrinology 208 (2014) 116–125

Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Gene expression during ovarian differentiation in parasitic and non-parasitic lampreys: Implications for fecundity and life history types

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ARTICLE INFO

Article history:
Received 8 May 2014
Revised 14 August 2014
Accepted 30 August 2014
Available online 9 September 2014

Keywords:
Apoptosis
Cytochrome c oxidase subunit III
Insulin-like growth factor 1 receptor
Oocyte growth
Sex differentiation
Wilms' tumour suppressor protein 1

ABSTRACT

Lampreys diverged from the jawed vertebrate lineage approximately 500 million years ago. Lampreys undergo sex differentiation much later than most other vertebrates, and ovarian differentiation occurs several years before testicular differentiation. The genetic basis of lamprey sex differentiation is of particular interest both because of the phylogenetic importance of lampreys and because of their unusual pattern of sex differentiation. As well, differences between parasitic and non-parasitic lampreys may first become evident at ovarian differentiation. However, nothing is known about the genetic basis of ovarian differentiation in lampreys. This study examined potential differences in gene expression before, during, and after ovarian differentiation in parasitic chestnut lamprey *Ichthyomyzon castaneus* and non-parasitic northern brook lamprey *Ichthyomyzon fossor*. Eight target genes (17β-hydroxysteroid dehydrogenase, germ cell-less, estrogen receptor β, insulin-like growth factor 1 receptor, daz-associated protein 1, cytochrome c oxidase subunit III, Wilms' tumour suppressor protein 1, and dehydrocholesterol reductase 7) were examined. Northern brook lamprey displayed higher expression of cytochrome c oxidase subunit III, whereas chestnut lamprey displayed higher expression of insulin-like growth factor 1 receptor; these genes may be involved in apoptosis and oocyte growth, respectively. Presumptive male larvae had higher expression of Wilms' tumour suppressor protein 1, which may be involved in the undifferentiated gonad and/or later testicular development. Differentiated females had higher expression of 17β hydroxysteroid dehydrogenase and daz-associated protein 1, which may be involved in female development. This study is the first to identify genes that may be involved in ovarian differentiation and fecundity in lampreys.
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1. Introduction

Sex differentiation is a complex process that coordinates the differential development of males and females, including the development of a previously undifferentiated gonad into a recognizable ovary or testis. The genetic basis of sex differentiation is reasonably well understood in the "higher vertebrates" (e.g., mammals and birds; Chue and Smith, 2011; Wilhelm et al., 2007), but less so in other vertebrates. In fish, some genes involved in sex differentiation have been identified in model species (reviewed in Piferrer and Guiguen, 2008; Piferrer et al., 2012; Sandra and Norma, 2010); however, nothing is known about the genetic factors involved in sex differentiation in lampreys, one of the two surviving lineages of jawless vertebrates.

Although previous studies have identified conserved genes involved in sex differentiation in teleost fishes (e.g., cytochrome p450 aromatase 19a, doublesex and mab-3 related transcription factor 1, forkhead box L2, steroidogenic factor 1; Piferrer and Guiguen, 2008; Piferrer et al., 2012), there are reasons to suspect that the genetic factors involved in lamprey sex differentiation may be somewhat different. Teleosts typically undergo gonadal differentiation within a few weeks to months of hatching (e.g., zebrafish *Danio rerio* are differentiated by 35 days post-fertilization; Wang et al., 2007), and testicular and ovarian differentiation occur at the same time or very close together (Patino and Takashima, 1995; Sandra and Norma, 2010). In contrast, the lamprey gonad remains undifferentiated for up to several years and the differentiation process is asynchronous in males and females: ovarian differentiation occurs in the spring of the first, second, or third year following hatch (Hardisty, 1970, 1969), and testicular differentiation does not occur

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http://dx.doi.org/10.1016/j.ygcen.2014.08.019
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until metamorphosis, several years later (Hardisty, 1965). Although some teleost (e.g., European eel *Anguilla anguilla*; Beullens et al., 1997a,b) and non-teleost (e.g., Siberian sturgeon *Acipenser baerii*; Berbejillo et al., 2012) fishes also display delayed sex differentiation, there are relatively few studies of the genetic factors that may be involved. Lampreys also differ from teleosts in the steroid hormones that they use for sexual development. In teleosts, 17β-estradiol is the most important steroid hormone for ovarian differentiation and 11-ketotestosterone for testicular differentiation (Sandra and Norma, 2010). Lampreys, in contrast, use primarily 15α-hydroxylated sex steroids as adults (Bryan et al., 2008), and the hormonal factors involved in lamprey sex differentiation are unknown. Application of teleost forms of sex steroids, which is effective in controlling sex ratios in teleosts (Piferrer, 2001), does not appear to affect the sex ratio of larval lampreys (Docker, 1992).

One area that is of particular interest is potential differences in gene expression during ovarian differentiation in parasitic and non-parasitic lamprey species (i.e., in lampreys that feed parasitically following metamorphosis and those that do not feed at all after completion of the filter feeding larval stage; Docker, 2009). Ovarian differentiation typically occurs earlier in non-parasitic species (Hardisty, 1970), although this is not always the case (Spice and Docker, 2014). Furthermore, parasitic species (which are larger at sexual maturity) are much more fecund as adults (Vladykov, 1951), but this difference in fecundity likely begins in the larval stage, where parasitic species are thought to produce more germ cells (Hardisty, 1971). This suggests that there may be differences in gene expression between parasitic and non-parasitic species quite early in larval life, when closely related parasitic and non-parasitic species are typically not morphologically distinguishable (and may be genetically indistinguishable as well; see Docker, 2009). These differences may contribute to the vastly different morphology and life history of parasitic and non-parasitic lamprey adults.

This study used publically available lamprey genomic sequence (Smith et al., 2013) and transcriptomic sequence available from previous studies in the Docker laboratory (e.g., Spice, 2013) to identify and amplify eight candidate genes for ovarian differentiation in parasitic chestnut lamprey *Ichthyomyzon castaneus* and non-parasitic northern brook lamprey *Ichthyomyzon fossor*. Expression of these genes (17β-hydroxysteroid dehydrogenase, germ cell-less, estrogen receptor β, insulin-like growth factor 1 receptor, daz-associated protein 1, cytochrome c oxidase subunit III, Wilms' tumour suppressor protein 1, and dehydrocholesterol reductase 7) was examined using quantitative reverse-transcriptase PCR (qRT-PCR) in lamprey larvae prior to, during, and after ovarian differentiation.

2. Methods and materials

2.1. Sample collection and preparation

Chestnut lamprey (n = 31, size range 41–63 mm) were collected from the Rat River in St. Malo, Manitoba (Fig. 1). The primary collection location for northern brook lamprey was the Birch River near Prawda, Manitoba (n = 32, size range 41–68 mm). However, not all required stages were collected at this location; thus, additional samples were used from Canada Creek near Hammond Bay, Michigan (n = 5, size range 48–64.5 mm) and McKinnon Creek near Sault Ste. Marie, Ontario (n = 3, size range 54–66 mm). Details on sample locations and coordinates are given in Table 1. All larvae were collected using a backpack electroshocker (Smith-Root LR-24) between April and August in 2011–2013, in accordance with collection permits (for 2011, SCP 05-11 and SECT 73 SARA C&A 11-012; for 2012, SCP 15-12 and SECT 73 SARA C&A 12-009; for 2013, SCP 28-13 and SECT 73 SARA C&A 13-013).

When possible, larvae were sacrificed within several hours of collection. When this was not possible, larvae were held in aquaria at the University of Manitoba, Department of Biological Sciences Animal Holding Facility until sacrifice, in accordance with Animal Use Protocol F11-019. Lampreys were euthanized within one week of collection in order to minimize the effect of laboratory holding on gene expression. Larvae were anesthetized with an overdose of MS-222 (>400 mg/L) until respiration ceased and they were unresponsive to stimulus. Death was ensured by decapitation prior to dissection. A cross-section that included 5 mm of the elongated gonad was taken from each individual and fixed in formalin for histological analysis (Spice and Docker, 2014). The remaining gonadal tissue was then removed, and was flash-frozen in liquid nitrogen and stored at −80 °C. For individuals from Canada Creek, the gonad was removed and stored in RNA Later solution (Ambion) at −30 °C until use.

In age-classes 1 and 2, lampreys experience rapid growth, which produces a length-frequency distribution with clear peaks corresponding to each age-class (Hardisty, 2006). Length-frequency distributions were prepared separately for each location (data not shown; see Spice and Docker, 2014) and individuals were classified according to age-class. Individuals whose lengths fell outside of the clearly defined peaks for each age-class were not used in this study.

Histological cross-sections of each individual were examined using a compound microscope (Leica Galen III) to determine gonadal stage (Spice and Docker, submitted). Four main stages of gonadal development were considered in this study (Table 2). Up to seven individuals of each stage were randomly selected from each age-class for both species; fewer individuals were available for some stages. Sample sizes are given in Table 1.

Total RNA was extracted from each gonad using the Qiagen RNeasy Mini Kit and following the manufacturer's instructions. A DNase digestion step, with the RNase-free DNase Set (Qiagen), was incorporated in order to remove contaminating genomic DNA. RNA concentrations were measured using a NanoVue Spectrophotometer (GE) and RNA was stored at −80 °C until use.

Complementary DNA (cDNA) was synthesized from approximately 100 ng RNA using the Quantitect Reverse Transcription Kit (Qiagen), following the manufacturer's instructions. Tenfold dilutions of cDNA were prepared and stored at −30 °C until use.

2.2. Selection of target and reference genes

Potential target and reference genes were identified and amplified using sequence data from the sea lamprey *Petromyzon marinus* genome (Smith et al., 2013) and transcriptome data for northern brook and chestnut lampreys generated in a previous project in the Docker laboratory (Spice, 2013; C.T. McFarlane, unpublished data). Target genes were chosen because they were known to be involved in sex differentiation and other processes of interest in other vertebrates (e.g., 17β-hydroxysteroid dehydrogenase, germ cell-less, daz-associated protein 1; Hsu et al., 2008; Labrie et al., 1997; Maekawa et al., 2004) or because they were found to be differentially expressed in a small sample of chestnut and northern brook lampreys using RNA sequencing (e.g., cytochrome c oxidase subunit III, dehydrocholesterol reductase 7; Spice, 2013).

For all 24 potential target and reference genes (see Spice, 2013 for a list of these genes), protein sequences of other vertebrates were downloaded from the NCBI database. These sequences were queried against the sea lamprey genome and/or northern brook and chestnut lamprey transcriptomes in Geneious v. 5.6.6 (Biomatters, available from www.geneious.com) using a tblastn method with an e-value threshold of 1.0×10^{-5}. Contigs with high

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