Ovarian differentiation in an ancient vertebrate: Timing, candidate gene expression, and global gene expression in parasitic and non-parasitic lampreys

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Abstract

Lamprey adults may be parasitic or non-parasitic, but the genetic basis of life history type is unknown. Although external differences between types are not apparent until metamorphosis, previous studies have suggested histological differences during ovarian differentiation. This study examined potential differences between parasitic chestnut lamprey *Ichthyomyzon castaneus* and non-parasitic northern brook lamprey *I. fossor* before, during, and after ovarian differentiation, using histological examination of the gonad, quantitative reverse-transcriptase PCR, and RNA sequencing. There were no observable differences in the timing or nature of ovarian differentiation. However, there was evidence of differential expression of individual genes associated with growth, apoptosis, and fecundity and of sets of genes associated with energy and lipid metabolism. The sequence resources developed in this project will be useful for future examination of the genetic basis of lamprey life history type and of the genes controlling sex differentiation in these ancient vertebrates.

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CHAPTER 1. GENERAL INTRODUCTION

There is increasing interest in elucidating the genetic basis of phenotypic differences within and among species. Of particular interest is the genetic basis of differences between species in very early stages of divergence (e.g., threespine stickleback *Gasterosteus aculeatus* life history types; Peichel *et al.* 2001; Colosimo *et al.* 2004). With recent advances in technology (particularly genomics and next generation sequencing), it is becoming more possible to address these questions (Hoekstra & Coyne 2007; Elmer & Meyer 2011). This thesis explores the theme of the genetic basis of divergence in an understudied system, divergent life history types in lampreys.

Although external differences between lamprey life history types are rarely apparent before metamorphosis (see Section 1.1.2.2), the earliest known differences between types are thought to occur at ovarian differentiation (see Section 1.1.2.4). Thus, this thesis explores potential differences between **life history types** (note that terms that are bolded on first use within each chapter are defined in the glossary in Appendix 1) before, during, and after ovarian differentiation. Areas of focus include the timing of ovarian differentiation (Chapter 2), expression of individual genes of interest (Chapter 3), and global gene expression during ovarian differentiation (Chapter 4).

1.1 Life history types

In many vertebrates, differences in life history type occur within or among species. One important group of different life history types is alternate feeding types (trophic polymorphisms) within a species or among closely related species. Alternate feeding types may consume different types of food, and each feeding type may have particular morphological specializations related to feeding (Wimberger 1994). For example, different life history types occur in tiger salamander *Ambystoma tigrinum* (cannibal and non-cannibal morphs, Collins & Cheek 1983; paedomorphic and metamorphic types, Shaffer & Voss 1996) and in *Geospiza* ground finches (birds with different bill sizes eat different foods; Ford *et al.* 1973; Grant & Grant 1989).

Trophic polymorphisms and other alternate life history types are particularly common in fishes, especially in different habitats or microhabitats. One of the best-studied groups of life history types occurs in threespine stickleback. In North America, multiple freshwater threespine stickleback populations have been independently derived from ancestral marine populations (McKinnon & Rundle 2002). Freshwater populations have then diverged in life history, morphology, and physiology. There are three major pairings of life history types within the stickleback species complex: anadromous and freshwater forms, lake and stream forms, and benthic and limnetic forms (McKinnon & Rundle 2002). These forms vary in their degree of reproductive isolation (Hendry *et al.* 2009). Benthic and limnetic forms are one of the most recently evolved and well studied groups. These forms have evolved repeatedly in multiple small lakes in British Columbia within approximately 13 000 years, and they are different in morphology, habitat, and

diet (Schluter & McPhail 1992). They are considered to have strong but not complete reproductive isolation: although they may occasionally interbreed, hybrids have reduced fitness (Nagel & Schluter 1998; Gow *et al.* 2007; Hendry *et al.* 2009).

Lake whitefish *Coregonus clupeaformis* also have alternate life history types. In south-eastern Canada and the north-eastern United States, dwarf- and normal-sized whitefish have diverged in sympatry independently in multiple lakes. Normal (benthic) individuals are much larger, mature later, and live longer than dwarf (limnetic) individuals (Chouinard *et al.* 1996; Lu & Bernatchez 1999). Dwarf fish feed mostly on zooplankton, whereas normal fish feed mostly on benthic prey (Bernatchez *et al.* 1999). At maturity, the dwarf form can be distinguished primarily by its much smaller size and the larger number of gill rakers it possesses (which assist in retention of its smaller prey; Bernatchez *et al.* 2010). In the most well studied system, the St. John River drainage, lakes were colonized after glaciation (about 12 000 years ago) by phenotypically similar forms from different glacial refugia. These forms later diverged phenotypically and became reproductively isolated (Bernatchez *et al.* 2010).

Understanding life history types is complicated by the tendency for freshwater fish species to repeatedly and independently diverge into different life history types, making taxonomic classification difficult (Taylor 1999). Even in model systems such as the stickleback, there is debate regarding whether different life history types should be considered the same or different species and whether morphological differences are caused by genetic differences or by phenotypic plasticity (Wimberger 1994). These questions have implications for the evolution, conservation, and management of these species and other species with alternate life history types.

1.1.1 Life history types in lampreys

Lampreys are primitive fishes that diverged from the rest of the vertebrate lineage prior to the evolution of jaws (Meyer & Zardoya 2003; see Figure 1.1), approximately 510 million years ago (Donoghue *et al.* 2000; Near 2009). Adult lampreys spawn in fast-flowing riffle areas of streams and rivers in spring or early summer. The eggs hatch into worm-like larvae, which then burrow into the sediment downstream of the spawning area. Lamprey larvae filter-feed on algae and detritus for several years prior to metamorphosis (Hardisty 2006).

At metamorphosis, different life history types—parasitic and non-parasitic—become evident. Parasitic adults migrate downstream to larger bodies of water, where they feed on fish for up to several years. Some parasitic species (e.g., anadromous sea lamprey *Petromyzon marinus*; Beamish 1980) spend their feeding phase in marine environments, whereas others (e.g., chestnut lamprey *Ichthyomyzon castaneus*; COSEWIC 2010) remain in fresh water, and sometimes even in the same river as the larvae. All parasitic species return to smaller freshwater rivers and streams to spawn. As adults, non-parasitic species (collectively called "brook lampreys") do not feed at all after metamorphosis and have no substantial migration (i.e., they remain in fresh water close to their larval habitat). Instead, non-parasitic species undergo rapid **sexual maturation** and spawn within 6–10 months after metamorphosis (Docker 2009). All lampreys die after spawning.

In seven of the ten lamprey genera, there are closely related parasitic and non-parasitic species which are believed to have evolved from a parasitic common ancestor; this is a dramatic example of parallel evolution (Salewski 2003). These related species

can be referred to as paired species (in the case of one parasitic species and one non-parasitic derivative; Zanandrea 1959) or satellite species (in the case of one parasitic species and multiple non-parasitic derivatives; Vladykov & Kott 1979).

1.1.2 Differences between lamprey life history types

1.1.2.1 Heterochrony

Non-parasitic lampreys are believed to have evolved through a shift in the timing of sexual maturation relative to metamorphosis. This type of change in the timing of life cycle events is referred to as heterochrony (Smith 2001). Gonads of parasitic and non-parasitic species are similar at metamorphosis. After metamorphosis, however, sexual maturation occurs within several months in non-parasitic species, whereas this process may not be complete for up to several years in parasitic species (Hardisty & Potter 1971; Hardisty 2006; Docker 2009).

Some non-parasitic species may also have a longer larval period. Analysis of the age-classes of parasitic European river lamprey *Lampetra fluviatilis* and its non-parasitic counterpart European brook lamprey *Lampetra planeri* indicates that the larval stage is two years longer in the brook lamprey (Potter 1980; Hardisty 2006). With the increased length of the larval period and decreased length of the adult period in the brook lamprey, European river and brook lampreys have the same overall life-span (Hardisty 2006). This is the only species pair in which this pattern has been clearly demonstrated. However, at metamorphosis, non-parasitic species are generally larger than their parasitic counterparts (Hardisty 2006; Docker 2009); this suggests that non-parasitic species may also have a longer larval period in other species pairs. For example, the parasitic Pacific lamprey

Entosphenus tridentatus may reach a maximum larval size of 142 mm (Beamish & Richards 1982; Beamish & Levings 1991). Its non-parasitic derivative the Pit-Klamath brook lamprey *E. lethophagus* reaches a larger maximum larval size of 155 mm (Hubbs 1971).

1.1.2.2. Morphological differences

Lampreys lack many of the morphological characteristics that are normally used to classify other fish (e.g., scales and paired fins; Hubbs & Potter 1971); therefore, parasitic and non-parasitic lampreys are distinguished based on other features. During the adult feeding phase, parasitic species have a well-developed oral disk, a functional foregut, and sharper, more keratinized, and more frequently replaced teeth, whereas non-parasitic adults (which do not feed) have a poorly developed oral disk, a non-functional digestive tract, and poorly developed, more variable teeth (Hardisty & Potter 1971; Vladykov & Kott 1979; Youson 1980; Hardisty 2006). Parasitic species continue to grow after metamorphosis, whereas non-parasitic species decrease in length (Vladykov & Kott 1979); thus, parasitic adults are larger (often much larger) than non-parasitic adults. Parasitic Pacific lamprey (mentioned above) has a maximum adult size of 850 mm (Orlov *et al.* 2008), whereas non-parasitic Pit-Klamath brook lamprey has a maximum adult size of 116 to 142 mm (Hubbs 1971).

Despite their large morphological differences as adults, larvae of some paired species may be difficult or impossible to distinguish (Hardisty 2006). Differences occurring prior to metamorphosis are very subtle, if present at all. Most morphological characteristics used to identify lampreys are useful only after metamorphosis (e.g.,

dentition and velar tentacles; Hubbs & Potter 1971; Gill *et al.* 2003). In larvae, the oral disk and teeth often used to identify adult lampreys have not yet developed, the eye is rudimentary and located under the musculature, and the dorsal and caudal fins are not as clearly differentiated as in the adult lamprey (Hardisty 2006). Although pigmentation may be used to distinguish some species, this is especially difficult for small larvae (Hardisty 2006), and pigmentation differences do not occur for all species pairs (Docker 2009). Subtle differences in body proportions and/or myomere (muscle segment) counts may also be used for identification (Vladykov & Kott 1980; Richards *et al.* 1982); however, these differences are not usually diagnostic (Docker 2009). Some non-parasitic species have a lower number of trunk myomeres than their parasitic counterparts, presumably corresponding to smaller adult body size. However, these differences are often not diagnostic. In some species pairs, such as European river and brook lampreys, average myomere counts are slightly different but the ranges overlap; in other pairs, such as silver lamprey *Ichthyomyzon unicuspis* and northern brook lamprey *I. fossor*, there is no reported difference (Potter 1980; Docker 2009). The trend for much lower myomere counts in non-parasitic species is most apparent in the relict species (e.g., least brook lamprey *Lampetra aepyptera*; Vladykov & Kott 1979). Relict species are assumed to have diverged from their parasitic ancestors a very long time ago and are often found at the southernmost edges of the distribution of the presumed parasitic ancestor (Hubbs and Potter 1971). These "old" non-parasitic species (in contrast to the recently-derived paired species) may provide valuable information about trends in the evolution of non-parasitism in lampreys. However, this information is difficult to interpret in many cases where the parasitic ancestor is no longer extant, and is especially problematic where the

closest parasitic relative is difficult or impossible to determine (Hubbs & Potter 1971). Morphological classification of several relict species has been questioned by molecular data in at least two studies (Docker *et al.* 1999; Lang *et al.* 2009).

1.1.2.3 Genetic differences

In some species pairs, there are fixed genetic differences between the parasitic and non-parasitic species (see Table 1.1). Arctic lamprey *Lethenteron camtschaticum* and Siberian brook lamprey *Le. kessleri* can be distinguished using allozyme alleles, even when the species occur sympatrically (Yamazaki & Goto 1998). Mitochondrial DNA (mtDNA), however, was not capable of distinguishing between Arctic lamprey and Siberian brook lamprey, possibly due to the different evolutionary rates of nuclear and mitochondrial genomes (Yamazaki *et al.* 2006; Okada *et al.* 2010). Although mtDNA is unable to distinguish between most species pairs (Docker 2009), it has been used to distinguish between allopatric Arctic lamprey and American brook lamprey *Le. appendix* populations (Docker *et al.* 1999). Studies using allozyme allele frequencies (Schreiber & Engelhorn 1998) and mtDNA sequence (Blank *et al.* 2008) were unable to genetically distinguish European river and brook lampreys; however, genome-wide fixed single nucleotide polymorphism (SNP) differences between these species have very recently been documented at the extreme southern limit of their range in Portugal (Mateus *et al.* 2013). At least some of these fixed SNPs are in functional genes such as vasotocin, which is involved in osmoregulation; this sequence difference may contribute to life history differences between the anadromous European river lamprey and the freshwater resident European brook lamprey (Mateus *et al.* 2013).

Other species pairs are genetically indistinguishable at all loci examined to date (Docker 2009). Beamish & Withler (1986) found no significant differences in allozyme allele frequencies between the North American river lamprey *Lampetra ayresii* and its non-parasitic derivative the western brook lamprey *Lampetra richardsoni.* As well, the parasitic silver lamprey and non-parasitic northern brook lamprey are genetically indistinguishable using both microsatellite loci (McFarlane 2009; Docker *et al.* 2012) and mtDNA (Docker *et al.* 2012). Mitochondrial DNA and microsatellites are presumably neutral markers (Avise *et al.* 1987; Selkoe & Toonen 2006); thus, it is possible that these markers have failed to detect divergence at functional loci. However, the absence of genetic differentiation at neutral loci is unusual for "good" species and for life history types in fishes. For example, approximately 90% of North American freshwater fishes can be distinguished using mtDNA barcoding (April *et al.* 2011), and life history types of the lake whitefish show significant differences in allele frequency at microsatellite loci (Lu & Bernatchez 1999).

The large size differences between parasitic and non-parasitic adults are generally thought to prevent interbreeding. When lampreys spawn, the male attaches to the female's head, wraps around her body, squeezes the eggs out, and fertilizes them (Hardisty 2006). Size differences greater than about 20% prevent successful spawning and were thought to be a mechanism producing reproductive isolation between paired species (Beamish & Neville 1992). However, "sneaker male" fertilizations between anadromous European river lamprey, freshwater-resident European river lamprey, and European brook lamprey have recently been documented. In this situation, a male of a different life history type releases gametes in close proximity to a spawning pair; this

may be a mechanism for gene flow between life history types (Hume *et al.* 2013). Chestnut lamprey and southern brook lamprey *Ichthyomyzon gagei* have also been observed spawning together (Cochran *et al.* 2008); similarly, different life history types of Arctic lamprey have been observed in the same spawning aggregations (Kucheryavyi, Savvaitova, Gruzdeva, *et al.* 2007).

1.1.2.4 Gonadal differences

Given the pronounced differences in adult body size, parasitic and non-parasitic lamprey species differ considerably in their fecundity. As adults, non-parasitic species have reduced oocyte numbers relative to their parasitic counterparts (Vladykov 1951). For example, the parasitic chestnut lamprey has an adult fecundity of 10 144 to 18 563 oocytes, whereas its non-parasitic counterpart the southern brook lamprey has an adult fecundity of 934 to 2454 oocytes (Beamish & Thomas 1983). In species pairs where the parasitic member is anadromous (and consequently even larger than freshwater-resident parasitic species), this difference is even more pronounced; the adult fecundity of the parasitic Arctic lamprey is 62 936 to 119 180 oocytes, whereas the adult fecundity of the Siberian brook lamprey is 1387 to 2125 oocytes (Yamazaki, Konno, *et al.* 2001).

Reduced actual (adult) fecundity in non-parasitic species may occur through oocyte degeneration at metamorphosis and/or through reduced potential fecundity prior to metamorphosis (e.g., Hardisty 1961; Hughes & Potter 1969; Beamish & Thomas 1983). In some species pairs, non-parasitic larvae produce far more oocytes than they are able to bring to maturity. These oocytes degenerate at metamorphosis in a process called atresia, which has been documented in European brook lamprey (Hardisty 1971), Far Eastern

brook lamprey *Lethenteron reissneri* (Fukayama & Takahashi 1983), and precocious lamprey *Mordacia praecox* (Hughes & Potter 1969). In other species pairs, non-parasitic females may produce fewer oocytes (i.e., have lower potential fecundity) than parasitic females and have no detectable (e.g., southern brook lamprey; Beamish & Thomas 1983) oocyte atresia at metamorphosis.

As well, **sex differentiation** and oocyte development appear to occur one year earlier in non-parasitic species than in parasitic species (Hardisty 1960a). Lampreys spawn in the spring and their young develop as larvae in streams for several years. In at least some paired species, female sex differentiation occurs in the spring of the first year following hatching for the non-parasitic species and in the spring of the second year following hatching for the parasitic species (Hardisty 1970). As non-parasitic lampreys undergo oocyte development earlier in larval development (i.e., at a smaller body size), they may be less able to produce large numbers of oocytes. The correlation between age at sex differentiation and adult fecundity is reinforced by the observation that anadromous sea lamprey, which do not undergo ovarian differentiation until the third year following hatch, have higher adult fecundity than landlocked sea lamprey, which undergo ovarian differentiation in the second year (Hardisty 1969a; Barker & Beamish 2000).

It has been suggested that non-parasitic species that have been diverged from their parasitic ancestor for a long period of time (i.e., relict species) have experienced selection against production of oocytes that will degenerate (i.e., selection against having much higher potential fecundity than actual fecundity; Docker 2009). This would result in the evolution of lower potential fecundity and reduced atresia (Beamish & Thomas 1983).

This trend is evident in the relict least brook lamprey which has little or no oocyte atresia during metamorphosis (Docker & Beamish 1991). It is important to note that this trend may not hold true for all species pairs. The southern brook lamprey has reduced potential fecundity compared to the parasitic chestnut lamprey and no detectable atresia at metamorphosis (Beamish & Thomas 1983); however, morphological and genetic similarities suggest that these two species are relatively recently diverged (Hubbs & Trautmann 1937; Beamish 1982; Page & Burr 1991; Docker 2009; Lang *et al.* 2009).

There is no clear pattern linking the extent of atresia to the time since divergence of paired species. This may be due to methodological differences among studies. Some researchers have quantified atresia by comparing estimates of potential and actual fecundity obtained from cross-sections of larval ovaries and samples of adult ovaries, respectively (e.g., Beamish & Thomas 1983), whereas others have used the relationship between absolute fecundity and length to estimate atresia (e.g., Beamish *et al.* 1994), and others have examined cross-sections of the ovaries of post-metamorphic lampreys and counted atretic oocytes (e.g., Hughes & Potter 1969). Studies indicating a lack of atresia in both relict and recently diverged non-parasitic species suggest that the extent of atresia may vary among species pairs; however, this may be confounded by differences among individuals (e.g., body condition) or populations (e.g., environmental conditions). Hardisty (1971) suggested that oocyte atresia may provide an important source of energy for non-parasitic species during metamorphosis, when they do not feed. Further examination of atresia in multiple large populations, using standardized methods, is required.

1.2 Gonadal development in lampreys and other vertebrates

Three important events in gonadal development are **sex determination**, **sex differentiation**, and **sexual maturation**. For the purposes of this study, these terms will be defined as follows: a) sex determination is the event that "predisposes a bipotential gonad to develop as an ovary or a testicle" (Sandra & Norma 2010); b) sex differentiation is the process by which the phenotypic sex of an individual is produced (Piferrer & Guiguen 2008) and may be subdivided into ovarian differentiation and testicular differentiation; and c) sexual maturation (a term that is inconsistently applied and often confusing; Patiño & Sullivan 2002) will be defined as the final changes in a gonad prior to reproduction. Each of these processes will be discussed in turn.

1.2.1 Sex determination

In fishes, both genetic sex determination (GSD) and environmental sex determination (ESD) have been identified. Each of these will be discussed below, in both teleosts (e.g., anguillid eels, salmonids, tilapia) and non-teleost fishes (e.g., cartilaginous fishes, sturgeons, gars; see Figure 1.1 for phylogenetic details).

Genes involved in sex determination are highly variable among and even within fish taxa (Piferrer *et al.* 2012). Only one single "master" sex-determining gene has been identified in any fish: the *DMY* gene in medaka *Oryzias latipes* (Matsuda *et al.* 2002); however, genetic regions involved in sex determination and sex-linked markers have been identified in several other species (reviewed in Piferrer *et al.* 2012). Most teleosts do not have morphologically distinct ("heteromorphic") sex chromosomes (Sandra &

Norma 2010); however, there are some exceptions. Systems with heteromorphic males (e.g., medaka) and heteromorphic females (e.g., blue tilapia *Oreochromis aureus*) have both been found (Sandra & Norma 2010). Non-teleost fish are less well studied, but some information on sex determination is available. In elasmobranchs, some species with heteromorphic sex chromosomes have been identified; most have males heteromorphic, but females are heteromorphic in some species (Maddock & Schwartz 1996). In sturgeons, a sex-determining system with heteromorphic females has been suggested, based on breeding experiments with white sturgeon *Acipenser transmontanus* (Van Eenennaam *et al.* 1999); however, further tests have failed to identify sex-determining genes in any sturgeon species (e.g., lake sturgeon *Acipenser fulvescens*, among others; McCormick *et al.* 2008). In some fish, sex is determined by the combined effects of multiple loci (polygenic sex determination; Bull 1985). This may occur for European seabass *Dicentrarchus labrax* (Vandeputte *et al.* 2007).

Environmental sex determination occurs in many species of fish. In temperature-dependent sex determination (TSD), sex is determined by the water temperature during development. It is common for male development to occur at higher temperatures and female development at lower temperatures (reviewed in Baroiller *et al.* 2009). Temperature can also affect sex ratios in fish with GSD; for example, many species of tilapia are affected by temperature, despite having heteromorphic sex chromosomes (Desprez & Mélard 1998; Baroiller *et al.* 2009; Wang & Tsai 2000). Similarly, in two species of silversides (*Menidia menidia* and *M. peninsulae*), GSD occurs, but population sex ratios may also be affected by temperature. In this genus, TSD is most common at intermediate latitudes, where the length of the growing season and thus adult size can be

accurately predicted from water temperature during larval growth (Yamahira & Conover 2003). Environmental factors other than temperature may also affect sex determination. In some species of fish, high pH causes female-biased sex ratios (e.g., the cichlid genus *Apistogramma*; Römer & Beisenherz 1996); however, the effect of pH is generally not as strong as the effect of temperature (Sandra & Norma 2010). In the European eel *Anguilla anguilla*, high population density causes a higher proportion of individuals to undergo male development (Davey & Jellyman 2005). In some other species, including the Midas cichlid *Amphilophus citrinellus* (Francis & Barlow 1993), the coral reef goby *Gobiodon erythrospilus* (Hobbs *et al.* 2004), and the false clown anemonefish *Amphiprion ocellarus* (Iwata *et al.* 2008), interactions with other individuals influence sex determination.

There are no definitive answers about the nature of sex determination in lampreys. Northern Hemisphere lampreys have upwards of 160 chromosomes (Potter & Robinson 1981), making it difficult or impossible to identify sex chromosomes, if any exist. Furthermore, there has been no research into the genetic basis of sex determination and differentiation in lampreys. Thus, most information about sex determination in lampreys comes from histological studies of lamprey gonads and studies of population sex ratios.

There is evidence that sex in lampreys is affected by environmental factors. Multiple studies support density-dependent sex determination (Torblaa & Westman 1980; Beamish 1993; Docker & Beamish 1994), and other factors such as growth, temperature, and pH may also have an effect (Beamish 1993). Increasing population density appears to increase the proportion of males in the population in multiple lamprey species (Torblaa and Westman 1980; Beamish 1993; Docker and Beamish 1994). However, some studies do not show density-dependent sex determination in lampreys (e.g., Hardisty 1960b).

Sex in lampreys may be determined by a combination of genetic and environmental factors; further research into both elements is required.

1.2.2 Sex differentiation

The timing of sex differentiation (i.e., the phenotypic differentiation of the gonad into ovary or testis) varies among fish taxa. In many teleosts, sex differentiation occurs quite early in development. In the zebrafish *Danio rerio* (Wang *et al.* 2007), sex differentiation is complete by 35 days post-fertilization. It is relatively common in teleosts for testicular differentiation to occur slightly later than ovarian differentiation (Patino & Takashima 1995; Sandra & Norma 2010). For example, in the masu salmon *Oncorhynchus masou*, ovarian differentiation occurs between 13 and 35 days post-hatch, and testicular differentiation begins at 35 days post-hatch (dates post-hatch are, of course, slightly later in the life cycle than dates post-fertilization; Nakamura 1984). In the cichlid *Cichlasoma dimerus*, ovarian differentiation occurs between 42 and 100 days post-fertilization, whereas testicular differentiation begins at 72 days post-fertilization and is not yet complete at 100 days (Meijide *et al.* 2005). In at least some non-teleost fish, sex differentiation occurs later in development. In Siberian sturgeon *Acipenser baerii*, sex differentiation occurs around 16–18 months post-hatch (Berbejillo *et al.* 2012). In longnose gar *Lepisosteus osseus*, testes begin to differentiate at about two months post-hatch; however, the rate of gonadal development after the onset of testicular differentiation varies widely, and testicular development is not complete in some individuals at more than a year post-hatch (Jaroszewska *et al.* 2010). The authors suggested that the timing of sex differentiation in longnose gar may be intermediate

between sturgeons (late in development) and teleosts (early in development), reflecting the phylogeny of these organisms (Jaroszewska *et al.* 2010; see Figure 1.1; but also see below regarding delayed sex differentiation in anguillid eels).

Lamprey sex differentiation is substantially different from sex differentiation in most other fishes in that female lampreys undergo ovarian differentiation at one or two years of age (and later, probably at three years of age, in the anadromous sea lamprey; Hardisty 1969, 1970), whereas future male lampreys have undifferentiated gonads until metamorphosis (i.e., during the entire larval period of several years; Hardisty 1965). One of the only teleost groups with a similarly long sexually indifferent stage is the anguillid eels. In European eel, ovaries develop directly from the undifferentiated gonad at about two years of age, whereas testicular differentiation occurs (following an intersexual stage) with metamorphosis to the silver eel stage, at around three years of age (Beullens, Eding, Gilson, *et al.* 1997; Beullens, Eding, Ollevier, *et al.* 1997). Delayed sex differentiation has also been documented in American eel *Anguilla rostrata* (Krueger & Oliveira 1999) and Japanese eel *A. japonica* (Chiba *et al.* 1997).

An additional difference between lampreys and many other vertebrates is that most vertebrates use "classical" sex steroids such as 17β-estradiol, progesterone, and testosterone, whereas lampreys use 15α-hydroxylated forms of these hormones (Lowartz *et al.* 2004; Bryan *et al.* 2008), and application of classical sex steroids may not affect the sex ratio of larval lampreys (Docker 1992).

In contrast to sex determination, where the genes involved are highly variable, the genetic basis of sex differentiation is much more conserved among fish taxa (Piferrer *et al.* 2012). This makes it easier to study sex differentiation in lampreys, despite their long

divergence from many fish model species. Relatively conserved and important genes in sex differentiation in teleosts and tetrapods include *sf1*, *sox9*, and *dmrt1* (steroidogenic factor 1, sex-determining region Y-related high mobility group containing box 9, and doublesex and mab-3 related transcription factor 1), which are involved in testicular development, and *cyp19a* and *foxl2* (cytochrome p450 aromatase 19a and forkhead box L2), which are involved in ovarian development (Bulun *et al.* 2003; Wilhelm *et al.* 2007; Sandra & Norma 2010). Some of these genes may also play a role in lamprey sex differentiation; however, the lengthy divergence time between lampreys and gnathostomes suggests that the molecular basis of lamprey sex differentiation will be somewhat different. To date, no studies have examined genes involved in lamprey sex differentiation.

In lampreys, germ cells first appear during gastrulation and then migrate to the future site of the single median gonad, below the aorta. This region then develops into a median genital ridge containing a small number of germ cells covered by peritoneal cells (Lewis & McMillan 1965; Hardisty 1971). At this point, the germ cells may be referred to as protogonia if they occur singly. Protogonia are generally each surrounded by follicle cells (Hardisty 1965, 1971). The germ cells may also divide to form groups of two to four deuterogonia (Hardisty 1965). Besides the occasional mitosis of protogonia to produce deuterogonia, there is little development of the gonad for six months to two years following hatch (Hardisty 1971). During this time, the gonad of most species is in an "intersexual" state, in which undifferentiated germ cells and a few oocytes may be present (Hardisty 1971); these few oocytes appear to originate directly from protogonia (Hardisty 1965). However, the intersexual state is less pronounced in some species. For

example, in the pouched lamprey *Geotria australis*, oocytes are rare in individuals other than future females (Hardisty *et al.* 1986). The reasons for this difference are uncertain.

Ovarian differentiation occurs in the spring of the first, second, or third year following hatch (Hardisty 1969, 1970; the timing of ovarian differentiation is discussed in more detail in Chapter 2). The protogonia or deuterogonia divide by mitosis to produce cysts of multiple germ cells surrounded by thin fibrous tissue and follicle cells (Lewis & McMillan 1965; Hardisty 1971). Germ cell cysts have been reported as less common in Far Eastern brook lamprey, suggesting that oocytes develop directly from protogonia in this species (Fukayama & Takahashi 1983). In other species, the germ cells in these cysts then enter meiotic prophase, and they will remain arrested in prophase throughout larval development (Hardisty 1971). Many of these germ cells will then undergo atresia before or during future cytoplasmic growth. In future ovaries, approximately 25 to 30% of germ cells survive the cystic stage and meiotic prophase (Hardisty 1971; Hardisty *et al.* 1986). Cysts of germ cells then enter the cytoplasmic growth phase, during which time the germ cells enlarge and become more basophilic. The cysts are gradually invaded and broken up by follicle cells that stretch to enclose each germ cell (Lewis & McMillan 1965). Different authors consider germ cells to be oocytes at different times (e.g., at meiotic prophase in Lewis & McMillan 1965; at cytoplasmic growth in Hardisty 1971); for this study, germ cells will be considered oocytes at the onset of cytoplasmic growth. Cytoplasmic growth is quite rapid while cysts are breaking up and much slower throughout the remainder of larval life (Hardisty 1965). Generally, ovarian differentiation in lampreys can be considered complete after

the rapid growth of oocytes ceases (Hardisty 1971). Final oocyte growth and vitellogenesis occur after metamorphosis (Hardisty 1971).

Hardisty (1965), examining European brook lamprey, said that "potential male ammocoetes [larvae] are those in which the gonads are composed predominantly of meiotic cysts, or in which regression is taking place at various phases of oogenesis." It appears that many if not all gonads destined to develop into testes will nevertheless enter the cystic stage (Hardisty 1971). However, future testes appear to develop directly from undifferentiated gonads in some individuals of the Far Eastern brook lamprey, where the cystic stage is rare (Fukayama & Takahashi 1983). Most of the germ cells in these cysts will degenerate shortly after entering meiotic prophase, and the remainder will degenerate during the cytoplasmic growth phase. Because of the extent of atresia, there is a reduction in the size of the testis, with only small numbers of undifferentiated germ cells that have not entered meiotic prophase remaining. Little growth occurs until just before or during metamorphosis, when these germ cells undergo mitosis to produce primary spermatogonia (Hardisty 1971). This pattern was also observed in Arctic lamprey, where germ cell cysts were found in all individuals of the size where cysts would be expected to occur (Fukayama & Takahashi 1982). Development of future testes appears to occur through degeneration of cysts in most individuals; however, in multiple lamprey species, about 10–20% of future testes develop through the degeneration of oocytes during or after the cytoplasmic growth phase (European brook lamprey, Hardisty 1965; Arctic lamprey, Fukayama & Takahashi 1982; Far Eastern brook lamprey, Fukayama & Takahashi 1983). Not all lamprey species exhibit oocyte production and degeneration in some future males: in the pouched lamprey, oocyte development appears to occur only in

future female larvae (Hardisty *et al.* 1986). In Arctic lamprey, oocytes in presumptive testes were often smaller than those in presumptive ovaries, suggesting that the future sex of the individual was already determined as male, even though female development was occurring (Fukayama & Takahashi 1982). Likewise, Hardisty (1965) believed that some future testes could be distinguished by the shape and other characteristics of the gonad, and he considered these to be "morphologically differentiated testes," even if female development was occurring in the germ cells. However, as the germ cells themselves do not develop into spermatogonia until metamorphosis but instead remain undifferentiated (Hardisty 1971), this thesis does not consider male sex differentiation to occur until metamorphosis.

The occasional degeneration of ovaries and later development of testes suggests that initial sex differentiation is not definitive in all lampreys. Atypical gonads may be relatively common in both landlocked and anadromous sea lamprey (Barker & Beamish 2000). In landlocked sea lamprey, larvae with atypical gonads have been documented to develop into males, and larvae with typical male and female gonads (monitored following gonadal biopsy) have undergone sex reversal (Beamish & Barker 2002). Adult male Pacific lamprey have also been documented to have a few previtellogenic and vitellogenic oocytes present in the gonad along with extensive testicular tissue, although this was rare (two of 427 individuals; Clemens *et al.* 2012).

1.2.3 Gonadal changes after metamorphosis and during maturation

The condition of the ovaries is relatively stable between ovarian differentiation and metamorphosis, although some atresia and/or slow cytoplasmic growth may occur

(Hardisty 1971). In early metamorphosing females, oocyte diameter varies from 43.1 μm (in pouched lamprey *Geotria australis*; Hughes & Potter 1969) to 200 μm (in southern brook lamprey; Beamish & Thomas 1983). In non-parasitic species, vitellogenic growth proceeds rapidly after metamorphosis; in parasitic species, vitellogenesis proceeds much more slowly (Hardisty 1971; Hardisty and Potter 1971). Final oocyte diameter varies from as low as 385 μm (in one population of pouched lamprey; Hughes & Potter 1969) to more than 1200 μm (in Far Eastern brook lamprey; Yamazaki *et al.* 2001); a clear relationship between oocyte diameter and adult body size has not yet been established. Ovulation occurs shortly before spawning (Hardisty 1971). For the purposes of this study, the oocytes will not be considered "eggs" until ovulation.

In future male gonads, there are small numbers of germ cells remaining in late larval life. Groups of primary spermatogonia (pre-meiotic cells) will develop from mitosis in these germ cells shortly before or during metamorphosis (Hardisty 1971). Primary spermatocytes (meiotic cells) may remain arrested in meiosis I for some time (Potter & Robinson 1991). In non-parasitic species, meiosis is complete by late winter following metamorphosis (Hardisty 1971). The timing of the onset of meiosis varies among parasitic species; for example, meiosis begins more than one year prior to spawning in pouched lamprey (Potter & Robinson 1991) but less than nine months prior to spawning in European river lamprey (Lanzing 1959; Larsen 1973). Development of sperm appears to be asynchronous, with pre-meiotic, meiotic, and post-meiotic germ cells (spermatids and developing sperm) present in the testes of upstream migrating pouched lamprey (Potter & Robinson 1991).

1.3 How is lamprey feeding type determined?

Earlier ovarian differentiation in non-parasitic lampreys may be one of the first observable differences between paired species. This raises several questions: When does an individual "commit" to a particular feeding type? Is feeding type determined prior to or during ovarian differentiation in females? If so, is feeding type determined at the same time or later in future males? Is feeding type determined the same way in each species pair? Are different feeding types truly different species? None of these questions have yet been answered.

Along with the lack of genetic differentiation observed to date in most species pairs (see Section 1.1.2.3 and Table 1.1), there also appears to be flexibility of feeding type in some species. In the 19th century, there were reports of European brook lamprey feeding as adults (Hardisty 2006). Some European brook lamprey individuals have been reported to have an open gut (Morris 1972), even though non-parasitic species are expected to have closed guts and thus be unable to feed (Youson 1980). Open guts after metamorphosis (Gage 1928) and "giant" adults (Manion & Purvis 1971; Cochran 2008) have also been reported in American brook lamprey. These giant adults are suspected to have fed after metamorphosis because of their large size and because their hindguts contained parasites that have previously been found only in lampreys that have fed after metamorphosis (Cochran 2008). The American brook lamprey more closely resembles parasitic species than many other non-parasitic lampreys, as it possesses a large oral disk and sharp teeth (Manion & Purvis 1971). As well, the histology of its hindgut resembles that of the parasitic silver and sea lampreys (Battle & Hayashida 1965). It is possible that

European and American brook lampreys are typically non-parasitic species in which some individuals retain the ancestral ability to feed after metamorphosis. Alternatively, if there is no genetic basis for parasitism in these species, parasitism may be possible under certain, as yet unknown, environmental conditions.

Additional evidence for the flexibility of feeding type comes from the production of both parasitic and non-parasitic lampreys by a single population of the typically non-parasitic western brook lamprey (Beamish 1987). The parasitic and non-parasitic life history types had identical allozyme allele frequencies, suggesting gene flow between the life history types (Beamish 1987). The parasitic or "marifuga" form (so named because it is incapable of osmoregulating in sea water) is distinguished from the non-parasitic form by its well-developed teeth, functional gut, and silver colouration. It was capable of feeding in the laboratory; however, there are no indications that feeding occurs in nature (Beamish 1985).

About 80% of the marifuga individuals are male, and the female gonad is atypical in appearance, although viable eggs have been produced in the laboratory (Beamish 1985; Beamish & Withler 1986). Similarly, in Arctic lamprey, 92% of individuals of the anadromous praecox form (which spends less time feeding at sea than the typical anadromous form) are male (Kucheryavyi, Savvaitova, Pavlov, *et al.* 2007). This suggests that alternative life history types may occur first or more frequently in males (Docker 2009). Possibly, males may retain flexibility of feeding type longer than females; this may be related to the later timing of testicular differentiation.

Some authors have suggested that life history type could be determined in a given individual by factors such as body condition (in lampreys; Kucheryavyi, Savvaitova,

Pavlov, *et al.* 2007) or size at maturity (e.g., in salmonids; Garant *et al.* 2003). Kucheryavyi, Savvaitova, Pavlov, *et al.* (2007) suggested that the food available to the larvae determines the adult life history type in the Arctic lamprey and its derivatives. They suggested that larvae that have sufficient energy reserves at metamorphosis become non-parasitic residents, whereas those with lesser energy reserves become parasitic and feed in order to acquire more energy (Kucheryavyi, Savvaitova, Pavlov, *et al.* 2007). This is consistent with the general observation that non-parasitic lampreys, especially females, are larger at the time of metamorphosis (Docker 2009); the larger size of non-parasitic larvae at metamorphosis may assist them in undergoing sexual maturation without feeding after metamorphosis. Different life history forms of the Arctic lamprey also spawn together (Kucheryavyi, Savvaitova, Gruzdeva, *et al.* 2007), suggesting a lack of reproductive isolation between life history forms. The determination of life history type by factors such as body condition at maturity would mean that life history type is not necessarily "set" at fertilization (i.e., heritable). Instead, variation in body condition (or other factors) may signal for the expression of different genes, leading to phenotypic and life history differences. The flexibility of feeding type in some species suggests that feeding type may not be irreversibly set at the time of ovarian differentiation; however, much more investigation is needed.

1.4 Gene expression

The growing body of evidence showing lack of genetic divergence and intraspecific polymorphism in some lamprey paired species suggests that it may not be the genes each individual possesses, but rather the timing and extent of the expression of these genes, that determines life history type. Modification of gene expression has been involved in rapid evolution of different life history types in other species (e.g., anadromous steelhead versus landlocked rainbow trout forms of *Oncorhynchus mykiss*; Aykanat *et al.* 2011). In lampreys, differences in gene expression (particularly heterochronic differences) between parasitic and non-parasitic species might explain the repeated, rapid evolution of non-parasitism.

1.4.1 Analysis of gene expression in divergent life history types in other species and potential applications in lampreys

As stated above, at least some non-parasitic species show earlier ovarian differentiation and reduced potential fecundity compared to parasitic species (see Section 1.1.2.4). This suggests that differences between life history types may begin during or prior to ovarian development. The phenotype of an organism is not determined solely by the genes it possesses (i.e., the DNA sequence of these genes) but also by which genes are turned "on" or "off" and when. In species pairs with no known differences in gene sequence, differences in the timing and/or extent of gene expression may account for their developmental (i.e., timing of ovarian differentiation) and morphological (i.e., number of oocytes produced) differences.

Various methods of gene expression analysis have been used in other animals with more than one life history type (these methods are discussed in more detail in Section 1.4.2). Many studies of sex differentiation in other fish have used quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) to look at expression of individual candidate genes (e.g., Baron *et al.* 2005; Jørgensen *et al.* 2008; Berbejillo *et al.* 2012). Other techniques can be used to examine a larger number of genes. In the typically anadromous Atlantic salmon *Salmo salar*, some males may remain in fresh water and mature quickly into small "sneaker" forms, in contrast to large anadromous males which mature more slowly. Microarray analysis of gene expression in the brain indicated that about 15% of genes are differentially expressed between mature sneaker males and age-matched immature anadromous males, and individuals of the same life history type have similar gene expression profiles (Aubin-Horth *et al.* 2005). Next generation sequencing (NGS) techniques are currently a popular method of **transcriptome** analysis in non-model organisms (Ekblom & Galindo 2011), including alternative life history types of other fish species. For example, RNA sequencing (RNA-Seq) determined that adults of the dwarf form of the lake whitefish overexpressed more genes related to energy metabolism, whereas the adults of the normal form overexpressed more genes related to protein synthesis; these differences correspond to metabolic and growth differences between the life history types (Jeukens *et al.* 2010). The success of gene expression analysis in other species with closely related life history types suggests that this may be a suitable technique for examining differences between parasitic and non-parasitic lamprey species.

1.4.2 Methods for analysis of gene expression

There are multiple methods that may be used to analyze gene expression (i.e., the relative abundance of messenger RNA for a particular gene). The choice of method is dependent on the question being asked, the genomic resources available for the study species, and financial considerations (Huestis & Marshall 2009).

Microarrays are a popular method for analysis of gene expression where complementary DNA (cDNA) fragments of many different genes of interest are fixed on a microscope slide or other surface. Each messenger RNA (mRNA) sample to be analyzed is reverse-transcribed to cDNA (which is more stable) and hybridized to the fragments on the slide. When the sample cDNA binds to the microarray cDNA for a gene of interest, fluorescence is given off and relative gene expression among genes and samples can be compared by measuring the intensity of this fluorescence (Nygaard & Hovig 2009). A major advantage of microarrays is that they can measure the expression of thousands of genes at one time (Nygaard & Hovig 2009). In order to design a microarray, however, a great deal of sequence information is required, and specific target genes must be known (Shiu & Borevitz 2008). It is also difficult to compare results between separately constructed microarrays (Nygaard & Hovig 2009), reducing the repeatability of this technique. The amount of sequence information required for microarray construction makes microarrays less suitable for analysis of gene expression in non-model species such as lampreys (although an unpublished microarray has been constructed for sea lamprey; http://www.glfc.org/research/reports/Li_microarray.html). However, some microarrays developed for one species may be used in other, closely related species; for example, one study of gene expression in dwarf and normal lake

whitefish used a microarray originally developed for Atlantic salmon (Derome *et al.* 2006); lake whitefish and Atlantic salmon are members of different subfamilies in the family Salmonidae.

With recent advances in technology, NGS is becoming a popular method of gene expression analysis. Next generation sequencing techniques vary; however, the basic method is the same for many. In NGS studies of gene expression, mRNA is reverse-transcribed into cDNA, and the cDNA is sheared into fragments. These fragments are attached to some type of solid support and amplified using PCR. After amplification, thousands or millions of different fragments are sequenced directly on the support, at the same time. These sequence fragments can then be assembled into longer sequences (Hudson 2008; Tucker *et al.* 2009; Ekblom & Galindo 2011). Conventional Sanger sequencing produces sequence lengths of about 1 kilobase at best, whereas some NGS technologies produce 10–28 gigabases of sequence (Tucker *et al.* 2009); this is a massive increase in the amount of sequence data that is available. Sequencing an entire eukaryote genome is often still difficult and costly; however, sequencing the transcriptome of an organism is a more manageable task and often very informative (Hudson 2008). When a **non-normalized sequence library** is used for NGS, the relative abundance of transcripts can be identified which allows comparison of gene expression among samples (Ekblom & Galindo 2011). In addition to allowing comparisons of transcript abundance, NGS generates massive amounts of sequence data, which can be used to develop microarrays or select candidate genes for further analysis (Ekblom & Galindo 2011).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) is also a common method of gene expression analysis. In qRT-PCR, specific target genes are

amplified from cDNA, producing fluorescence that increases as the PCR product amplifies. The fluorescence is measured over approximately 40 cycles of PCR, and the expression of the target gene is inferred from the time required for the amount of fluorescence to cross a threshold (Jozefczuk & Adjaye 2011). Some sequence information is required to design primers for qRT-PCR; however, a much smaller number of genes are analyzed with qRT-PCR than with microarrays, so the amount of sequence data required is smaller (although the amount of information gained is also smaller; Nygaard & Hovig 2009). qRT-PCR is particularly useful for confirming differences in gene expression that have been identified using other techniques, because it is less expensive (allowing the analysis of more samples) and more sensitive to small differences in gene expression (Huestis & Marshall 2009; Nygaard & Hovig 2009). Confirmation of results from other techniques is valuable both because the repeatability of other techniques varies (e.g., microarrays; Nygaard & Hovig 2009) and because gene expression will be different in different tissues, life stages, and individuals (Ekblom & Galindo 2011).

1.5 Choice of study species and area

Manitoba contains one lamprey species pair: parasitic silver lamprey and non-parasitic northern brook lamprey (Stewart & Watkinson 2004). Silver and northern brook lampreys are one of the best studied lamprey species pairs in terms of the level of genetic differentiation (e.g., McFarlane 2009; Docker *et al.* 2012); they are

indistinguishable at all loci examined to date and are thought to be very recently diverged species or even different ecotypes of the same species (Docker *et al.* 2012). Greater plasticity of life history type is expected in more recently diverged species pairs (Docker 2009); thus, silver and northern brook lampreys may be a system in which gene expression, rather than gene sequence, is particularly important for determining life history type.

The original intention of this study was to examine ovarian differentiation in silver and northern brook lampreys. However, although feeding-phase silver lamprey are frequently encountered in Manitoba, no spawning or larval silver lamprey have been observed in the province. Attempts to collect silver lamprey larvae for this study from the Winnipeg River in Manitoba were unsuccessful over a two-year period. Studies in other areas have indicated that silver lamprey sometimes spawn in deep water (Lamsa 1980; Cochran & Lyons 2004); this may also occur in the Winnipeg River. Spawning in deep water could result in larvae being located in deep water downstream of the spawning grounds, making sampling for silver lamprey larvae difficult or impossible. Sampling efforts for parasitic lamprey were therefore re-focused on chestnut lamprey, which also occurs in Manitoba (Stewart & Watkinson 2004). Although chestnut lamprey and northern brook lamprey are not a species pair, they are in the same genus, and in some ways, chestnut lamprey is a more suitable parasitic species for this study than silver lamprey. Silver and northern brook lampreys cannot yet be distinguished genetically (Docker *et al.* 2012), and although there are populations containing exclusively northern brook lamprey, there are no known Canadian populations containing exclusively silver lamprey. However, chestnut and northern brook lampreys are easily genetically

distinguished (Neave *et al.* 2007), even when they occur together. Furthermore, there are known locations for both chestnut and northern brook lampreys in Manitoba (Case 1970; Stewart & Watkinson 2004).

The Great Lakes and Upper St. Lawrence populations of the northern brook lamprey are of Special Concern, whereas the Saskatchewan-Nelson population (which includes northern brook lamprey in Manitoba) is listed as Data Deficient (COSEWIC 2007). The chestnut lamprey is divided into the same groupings, both of which are considered Data Deficient (COSEWIC 2010). The ecological impact of this study was reduced by targeting younger larvae (which would experience more mortality in nature) and collecting the minimum number required.

1.6 Objectives

As the first observable differences between some closely related paired species appear to be in the timing of ovarian differentiation, the first objective of this study was to confirm that the "rule" of earlier ovarian differentiation in non-parasitic species is broadly applicable, through examination of the timing of histological ovarian differentiation in chestnut and northern brook lampreys (Chapter 2). The second objective was to use qRT-PCR to determine whether there are differences in the timing or extent of expression of five target genes that play a role in sex differentiation in teleost fishes or other vertebrates (Chapter 3). The final objective was to examine global gene expression during ovarian differentiation, using NGS, in order to "cast the net more

widely" when trying to identify genes involved in the ovarian differentiation process and genes that may be differentially expressed in parasitic and non-parasitic species (Chapter 4).

1.7 Relevance

As many studies on other topics have used lampreys as a model for the ancestral vertebrate (McCauley & Bronner-Fraser 2006; Takio *et al.* 2007; Bajoghli *et al.* 2009; Sower *et al.* 2009a; Heimberg *et al.* 2010), it is important to understand whether lamprey ovarian differentiation is similar to that of the ancestral vertebrate. To do this, lamprey ovarian differentiation must be more fully understood; identifying genes involved in ovarian differentiation is important to understanding the process. If genes involved in ovarian differentiation are shared between lampreys and gnathostomes, this suggests that these genes were also present (and possibly involved in ovarian differentiation) in the ancestral vertebrate. Identifying differentially expressed genes may lead to investigations into genetic (e.g., promoter regions, transcription factors) or epigenetic (e.g., environmental influences) control of the expression of genes related to feeding type.

As well, analysis of gene expression in paired species may reveal when adult life history type is determined and identify genes involved in determining life history type. If sex differentiation is a relevant process in the determination of life history type, the differential timing of ovarian and testicular differentiation raises the question of whether life history type is determined at the same time in males and females. Clear differences

in gene expression in parasitic and non-parasitic lampreys at the time of ovarian differentiation would suggest that feeding type (at least of females) is determined prior to or during ovarian differentiation, possibly as early as fertilization. If this is the case, one might also find that parasitic and non-parasitic lampreys have similar gene expression profiles relative to their developmental stage, but that the timing of ovarian differentiation is different in paired species. This would suggest that there are differences in the sequence or expression of a gene or genes regulating the time of ovarian differentiation; this would be a topic for further study.

1.8 Literature cited

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1.9 Tables and Figures

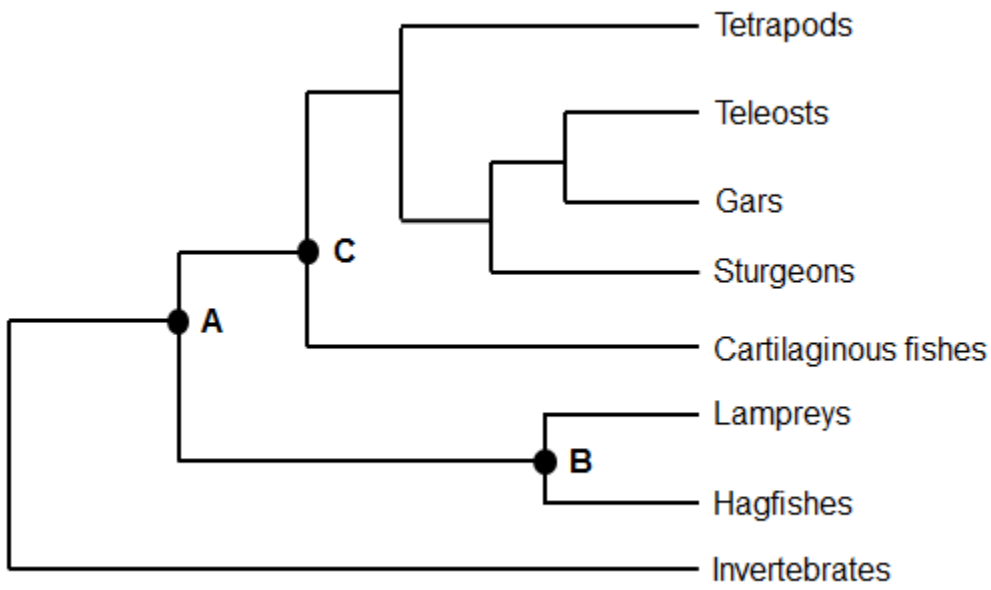


Figure 1.1. Highly simplified phylogenetic tree of vertebrates, highlighting the taxa that are most relevant to this study (inferred from Meyer & Zardoya 2003; Heimberg *et al.* 2010). For nodes: A = vertebrates; B = agnathans; C = gnathostomes. The single branch "Invertebrates" does not imply monophyly of invertebrates.

Table 1.1. Genetic differentiation among paired and satellite species of lampreys. The degree of genetic differentiation identified using allozymes, mitochondrial DNA (mtDNA), microsatellites, and single nucleotide polymorphisms (SNPs) is given for each species pair, when available.

Parasitic species		**Non-parasitic species**		**Genetic differentiation**			
Scientific name	Common name	Scientific name	Common name	Allozymes	mtDNA	Microsatellites	SNPs
Entosphenus tridentatus	Pacific lamprey	*Entosphenus lethophagus*	Pit-Klamath brook lamprey	-	None[1,2,3]	-	-
Ichthyomyzon castaneus	Chestnut lamprey	*Ichthyomyzon gagei*	Southern brook lamprey	-	None[3]	-	-
Ichthyomyzon bdellium	Ohio lamprey	*Ichthyomyzon greeleyi*	Mountain brook lamprey	-	None[3,4]	-	-
Ichthyomyzon unicuspis	Silver lamprey	*Ichthyomyzon fossor*	Northern brook lamprey	-	None[3,5,6]	None[5,7]	-
Lampetra ayresii	River lamprey	*Lampetra richardsoni*	Western brook lamprey	None[8]	None[1,9]	-	-
Lampetra fluviatilis	European river lamprey	*Lampetra planeri*	European brook lamprey	None[10]	None[11,12,13]	-	Fixed sequence differences[14]
Lethenteron camtschaticum	Arctic lamprey	*Lethenteron appendix*	American brook lamprey	-	Fixed sequence differences[1]	-	-
Lethenteron camtschaticum	Arctic lamprey	*Lethenteron kessleri*	Siberian brook lamprey	Distinct alleles[15]	None[16,17]	-	-
Lethenteron camtschaticum	Arctic lamprey	*Lethenteron alaskense*	Alaskan brook lamprey	-	None[3]	-	-
Lethenteron camtschaticum	Arctic lamprey	*Lethenteron camtschaticum*	unnamed	-	-	Distinct populations[18]	-

1. Docker *et al.* 1999
2. Lorion *et al.* 2000
3. April *et al.* 2011
4. Docker 2006
5. Docker *et al.* 2012
6. Lang *et al.* 2009
7. McFarlane 2009
8. Beamish & Withler 1986
9. Boguski *et al.* 2012
10. Schreiber & Engelhorn 1998
11. Espanhol *et al.* 2007
12. Blank *et al.* 2008
13. Mateus *et al.* 2011
14. Mateus *et al.* 2013
15. Yamazaki & Goto 1998
16. Yamazaki *et al.* 2006
17. Okada *et al.* 2010
18. Yamazaki *et al.* 2011

CHAPTER 2. TIMING OF OVARIAN DIFFERENTIATION IN CHESTNUT AND NORTHERN BROOK LAMPREYS

2.1 Abstract

Lampreys are unlike most jawed fishes in that sex differentiation occurs up to several years after hatching, and ovarian and testicular differentiation are separated by several years. As adults, non-parasitic females have much smaller body size and therefore much lower fecundity than parasitic females. Previous studies have suggested that this fecundity difference is established in the larval stage through ovarian differentiation at a younger age (and therefore smaller body size) in non-parasitic females, leading to production of fewer oocytes. Earlier ovarian differentiation in non-parasitic species may be the earliest observable difference between closely related parasitic and non-parasitic species. This study examined whether this pattern is broadly applicable in lampreys. The timing of histological ovarian differentiation was determined in larval parasitic chestnut lamprey *Ichthyomyzon castaneus* and non-parasitic northern brook lamprey *I. fossor*. Ovarian differentiation occurred in age-classes 1 and 2 in both chestnut and northern brook lampreys, and there were no significant differences in the timing of ovarian differentiation between species. The variable timing of ovarian differentiation in these species suggests that: a) life history type is not yet determined at ovarian differentiation; or b) the timing of ovarian differentiation is not a reliable marker of life history type. As well, factors such as growth and body condition may determine whether an individual undergoes ovarian differentiation in age-class 1 or 2.

2.2 Introduction

In many fish species, **sex differentiation** (note that terms that are bolded on first use within each chapter are defined in the glossary in Appendix 1) occurs early in development. Teleosts may undergo sex differentiation within a few weeks of fertilization or hatching; examples of this are zebrafish *Danio rerio* (Wang *et al*. 2007) and masu salmon *Oncorhynchus masou* (Nakamura *et al*. 1974). Lampreys are unusual in that sex differentiation occurs much later, sometimes several years after hatching. Lamprey young hatch in late spring or early summer and develop as larvae for four to six years before metamorphosis (Hardisty 2006). Ovarian differentiation typically occurs in the spring of the first, second, or third year following hatch (Hardisty 1969a, 1970). Testicular differentiation is even more delayed, and does not occur until metamorphosis (Hardisty 1971). Although testicular differentiation occurs slightly later than ovarian differentiation in many teleost species (Patino & Takashima 1995; Meijide *et al.* 2005; Sandra & Norma 2010), the large separation in the timing of ovarian and testicular differentiation in lampreys is highly unusual. One of the only groups known to have a similar pattern is the anguillid eels (Davey & Jellyman 2005). In European eel *Anguilla anguilla* ovarian development occurs directly from the undifferentiated gonad at around two years of age, and testicular development occurs at around three years of age following an intersexual stage (Beullens, Eding, Gilson, *et al.* 1997; Beullens, Eding, Ollevier, *et al.* 1997). The timing of sex differentiation in non-teleost fish is less well studied; however, sturgeons appear to have relatively late sex differentiation at 16–18 months of age (in Siberian sturgeon *Acipenser baerii*; Berbejillo et al. 2012).

In addition to differences in the timing of ovarian and testicular differentiation within lamprey species, there are also differences in the timing of ovarian differentiation between species. In general, there appears to be a trend for later ovarian differentiation with increasing adult body size. Ovarian differentiation typically occurs in the first year following hatch in non-parasitic species (which, given that they do not feed following metamorphosis, are small at sexual maturity) and in the second year following hatch in parasitic species (Hardisty 1970). In the sea lamprey *Petromyzon marinus*, ovarian differentiation occurs at two years post-hatch in the landlocked form, but the larger anadromous form delays ovarian differentiation until three years post-hatch (Hardisty 1969a; Barker & Beamish 2000). As adults, the larger parasitic species have much greater fecundity than the smaller non-parasitic species (Vladykov 1951); this fecundity difference may be established in the larval stage through ovarian differentiation at a younger age and therefore smaller body size in non-parasitic species (Hardisty 1970).

Earlier ovarian differentiation in smaller non-parasitic species generally occurs even in the very closely related lamprey species pairs. Earlier ovarian differentiation in the non-parasitic member of a species pair has been observed in European river and brook lampreys (*Lampetra fluviatilis* and *L. planeri*; Hardisty 1970), chestnut lamprey and southern brook lamprey (*Ichthyomyzon castaneus* and *I. gagei*; Beamish & Thomas 1983), and Arctic lamprey and Far Eastern brook lamprey (*Lethenteron camtschaticum* and *Le. reissneri*; Fukayama & Takahashi 1982, 1983). Ovarian differentiation occurs several years prior to metamorphosis; at this time, many species pairs are still morphologically indistinguishable (Docker 2009). The earlier timing of ovarian

differentiation in non-parasitic species may be the earliest observable difference between paired species (although it is not an externally visible difference).

The difference in the timing of ovarian differentiation among lamprey species is an example of heterochrony. Heterochrony can be defined as "a change in the relative timing of developmental events and processes in evolution" (Smith 2003). Two types of heterochrony are sequence heterochrony (differences in the timing of an event, relative to other developmental events) and growth heterochrony (differences in the timing of an event, relative to body growth; Smith 2001). Lamprey ovarian differentiation could be considered an example of both types: ovarian differentiation occurs more quickly after hatch (sequence heterochrony) and at a smaller body size (growth heterochrony) in non-parasitic species. In lampreys, heterochrony appears to be an important factor in the evolution of non-parasitic species. Non-parasitic species appear to have evolved through a lengthening of the larval period and a more rapid **sexual maturation** after metamorphosis, relative to parasitic species (Hardisty 2006). The differences in timing of ovarian differentiation suggest that heterochronic differences between parasitic and non-parasitic species may be evident well before metamorphosis. This makes the ovarian differentiation period of particular interest for those studying **life history types** in lampreys. However, one difficulty in studying lampreys is that researchers tend to assume that similar trends (e.g., earlier ovarian differentiation in non-parasitic species) occur in all species pairs, regardless of differences in degree of divergence (Docker 2009). Thus, it is important to confirm whether the "rule" of earlier ovarian differentiation in non-parasitic species is broadly applicable across all lamprey taxa.

Heterochrony may be important in the evolution of alternate life history types in other taxa. In the mole salamander *Ambystoma talpoideum*, populations contain some individuals that undergo metamorphosis and some that do not (paedomorphic individuals); this is an example of alternate life history types achieved through heterochrony (Ryan & Semlitsch 1998). In salmonids, differential timing of the onset of salinity tolerance may be related to life history type (anadromous or freshwater resident) and timing of migration (McCormick 1994). Some marine invertebrates also display heterochrony in different life history types; larvae that eat nurse eggs develop juvenile traits more quickly than those than eat plankton (Gibson & Gibson 2004).

This study used histological examination of the gonads to identify the stages of gonadal development (specifically ovarian differentiation, which is discussed in more detail in Section 1.2.2) in larvae of parasitic chestnut lamprey and non-parasitic northern brook lamprey *Ichthyomyzon fossor*. In order to determine whether ovarian differentiation occurs earlier in non-parasitic species, each individual was classified by age (inferred from length-frequency curves; see Section 2.3.3) and stage of gonadal development, and the timing of ovarian differentiation was compared in chestnut and northern brook lampreys. Although chestnut and northern brook lampreys are not a species pair, they are in the same genus, and use of these two species allows the comparison of gonadal development in the two life history types. Ovarian differentiation may be the earliest time when heterochronic differences between parasitic and non-parasitic lamprey species become apparent. Thus, this period may yield clues as to how and when life history type is determined in lampreys.

2.3 Methods

2.3.1 Sample collection

Larval northern brook lamprey (*n* = 87, length range 32–150 mm) were collected from the Birch River (N 49.68003, W 95.78956), and larval chestnut lamprey (*n* = 115, length range 35–117 mm) were collected from the Rat River (N 49.32082, W 96.94621; Figure 2.1) in southeastern Manitoba. Larvae were collected using a backpack electroshocker (Smith-Root LR-24) 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). Collections occurred from April 2011 to July 2012, with most larvae collected from April to July in each year.

Of Manitoba's three lamprey species, only northern brook lamprey are known to occur in the Birch River (Stewart & Watkinson 2004). Because parasitic species such as the silver lamprey and chestnut lamprey are generally more visible, we can be reasonably confident that only northern brook lamprey are present in the Birch River. However, histological analysis of metamorphosing lampreys for a related project revealed that some females from the Rat River were becoming sexually mature upon completion of metamorphosis; this is a characteristic of non-parasitic species (Hardisty 1971), indicating that a non-parasitic species is also present in the Rat River. All samples from the Rat River were therefore genetically tested to determine species, and the sexually maturing females were identified as northern brook lamprey. Chestnut and northern brook lampreys were distinguished as described in Neave *et al*. (2007). In brief, a fragment of the mitochondrial *ND5* gene was amplified using PCR and digested using the

restriction enzyme *Bst*NI, producing one fragment in chestnut lamprey and two or three fragments in northern brook lamprey. In 2011, three of the larvae collected from the Rat River were identified as northern brook lamprey; all those collected from the Rat River in 2012 were chestnut lamprey. Because of the very small numbers of northern brook lamprey in the Rat River, these northern brook lamprey were not used in the analysis.

2.3.2 Histological preparation

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 (AHF) until sacrifice, in accordance with Animal Use Protocol F11-019. Only individuals sacrificed within one week of collection were included in the analysis, to minimize the possible effect of laboratory holding on growth and gonadal development. Larvae were euthanized using an overdose of MS-222 (> 400 mg/L) and decapitated to ensure death. A cross-section through the entire body was taken from the midsection of each individual, where the size of the gonad is greatest (Docker 1992). Cross-sections were fixed in 10% buffered formalin (pH 7.0) for a minimum of 48 hours before use. Samples were prepared for histological analysis following the procedure of Docker (1992), with a few modifications. Briefly, cross-sections were washed in four changes of water over a minimum of two hours to remove formalin, then dehydrated in increasingly pure (from 70% to 100%) ethanol. Samples were cleared using Slide-Brite, infiltrated with paraffin in a vacuum oven, and embedded in paraffin blocks. A rotary microtome was used to cut ten 8 µm sections from each sample. These sections were fixed to slides and stained using a

progressive hematoxylin and eosin staining procedure. For some samples, it was necessary to cut thicker sections (up to 30 µm) because of poor sample quality; however, it was still possible to examine the histological characteristics of most of these samples.

2.3.3 Histological and statistical analysis

Slides of each lamprey gonadal cross-section were examined using a compound microscope (Leica Galen III), and seven stages of gonadal development were identified following Hardisty (1965) (Table 2.1; Figure 2.1). Pictures of representative gonads of each stage were taken using an Olympus BX43 compound microscope with a QImaging MicroPublisher 3.3 RTV camera and Q-Capture Pro 7 software (QImaging).

Length-frequency distributions are often used to estimate age in lampreys. These distributions are usually prepared using data from approximately 100 to 300 individuals (e.g., Hardisty 1969b, Potter & Bailey 1972); however, interpretable distributions have been produced with as few as 27 individuals (Hardisty 1961b). Statolith banding patterns can also be used to age lamprey larvae (Beamish & Medland 1988); however, this method is much more time-consuming and expensive than constructing length-frequency diagrams and is generally not necessary for ageing the youngest few age classes. Lampreys experience rapid growth in the first two years of larval life, resulting in a length-frequency distribution with clear peaks corresponding to one- and two-year-old larvae (Hardisty 2006). As increases in lamprey length slow with lamprey age, it is often more difficult to determine precise age-classes for older larvae (Hardisty 2006); however, as ovarian differentiation in chestnut and northern brook lampreys was expected to occur in the first or second year following hatch, precise age classification was not required for

lampreys older than two years. Length-frequency diagrams were prepared separately for the Rat and Birch rivers (Figure 2.2), and approximate age classes were determined (Table 2.2) through visual examination of these diagrams. Larvae were considered to be age-class 0 from the time of hatch until December 31 of that year, age-class 1 in the following year, and so forth. All larvae larger than would be expected for age-class 2 were grouped together as age-class 3+.

The stage of gonadal development was determined for each individual. Although Hardisty (1971) suggested that many future males undergo a cystic stage and the cysts later degenerate (see Section 1.2.2), degenerating cysts were rarely observed in the present study (see Table 2.3). This suggests that most gonads in the cystic stage will progress to oocyte growth in chestnut and northern brook lampreys; thus, the present study considered ovarian differentiation to occur in the cystic and oocyte growth stages (stages 2 and 3). A "differentiating" individual would be in one of these two stages.

For each individual, the day of the year that it was sacrificed was determined (e.g., 30 April 2012 becomes day 121). The overall distribution of these data (including age-classes 1 and 2 for both chestnut and northern brook lampreys) was tested for normality using a Shapiro-Wilk W test in JMP 10 (SAS Institute Inc., Cary, NC), and this test showed that the data were not normally distributed ($p < 0.0001$). Therefore, differences in date of sacrifice between species within an age-class and between age-classes within a species were examined using a Kruskal-Wallis test with a Dunn's correction for multiple comparisons in GraphPad InStat 3 (GraphPad Software, San Diego, California). The Kruskal-Wallis test is a non-parametric alternative to ANOVA, and it can be used when data are not normally distributed and/or sample sizes are small. It tests for differences in

medians, rather than means (Moore & McCabe 2006). This test was performed to ensure that the interpretation of further results was not biased by differential time of sacrifice (e.g., if age-class 1 chestnut lamprey were sacrificed later in the year than age-class 1 northern brook lamprey).

The length distributions of age-classes 1 and 2 were tested for normality using Shapiro-Wilk W-tests in JMP, and these tests indicated that the data were not normally distributed (age-class 1: $p = 0.0053$, age-class 2: $p = 0.0065$). Therefore, median lengths of chestnut and northern brook lampreys in each age-class were compared to each other using a Mann-Whitney U-test (also known as a Wilcoxon rank sum test) in GraphPad InStat. This test is a non-parametric alternative to the student's t-test, and it compares medians instead of means. It is more suitable than the t-test in situations where the data are not normally distributed and sample sizes are small (Moore & McCabe 2006).

The proportion of presumptive males, differentiating individuals, and differentiated females was compared between species within an age-class and between age-classes within a species using Fisher's exact tests in R 2.15.2 (R Core Team 2012). Fisher's exact tests are more accurate than chi-square tests when sample sizes are small (McDonald 2009). Significance of differences was determined after Bonferroni correction for multiple comparisons (Bonferroni 1935).

2.4 Results

All stages of gonadal development shown in Table 2.1 and Figure 2.1 were observed in both northern brook and chestnut lampreys. At each gonadal stage, there were no observable differences in the histological characteristics of chestnut and northern brook lampreys. Due to varying thickness of histological sections, it was not possible to statistically compare oocyte numbers per section between chestnut and northern brook lampreys. The maximum number of oocytes observed in an 8 µm thick cross-section was 69 for chestnut lamprey and 43 for northern brook lamprey; however, oocyte counts were only possible in five chestnut lamprey and two northern brook lamprey.

Length-frequency distributions were similar for chestnut and northern brook lampreys (Table 2.2). In age-class 1, median length was significantly greater in chestnut lamprey than in northern brook lamprey (45 mm vs. 40.5 mm; U = 907.50, $p < 0.0001$). However, in age-class 2, median length was significantly greater in northern brook lamprey than in chestnut lamprey (63 mm vs. 56 mm; U = 135, $p = 0.0006$).

The median date of sacrifice was significantly later in the year for age-class 1 northern brook lamprey compared to age-class 1 chestnut lamprey (mean rank difference 31.761, $p < 0.001$). The median date of sacrifice was also significantly later for age-class 2 chestnut lamprey compared to age-class 1 chestnut lamprey (mean rank difference 29.902, $p < 0.05$). There were no significant differences in date of sacrifice between the age-classes for northern brook lamprey or between chestnut and northern brook lampreys in age-class 2.

Of the individuals sampled in age-class 1, 57.6% of chestnut lamprey and 60% of northern brook lamprey were undergoing ovarian differentiation (Table 2.3). Of those sampled in age-class 2, 33.3% of chestnut lamprey and 52.2% of northern brook lamprey were undergoing ovarian differentiation. Ovarian differentiation was observed in age-classes 1 and 2 in both species, but never in age-class 3+. Comparisons of the numbers of individuals in different stages of gonadal development showed very few significant differences between species or between age-classes. The number of differentiated females was significantly higher in age-class 2 than age-class 1 for chestnut lamprey (p = 0.001; Table 2.4); all other comparisons were non-significant after Bonferroni correction. Sample sizes at age 3+, after ovarian differentiation is expected to be complete in both species (and when ovarian differentiation was no longer observed in this study), were too small to allow any meaningful comparisons of population sex ratios.

The annual timing of ovarian differentiation appeared similar between species and between age classes. Since sample sizes were often small, age-classes 1 and 2 were considered together (Table 2.5). The prevalence of the cystic stage appeared to peak in early spring (April), and degenerating cysts were most often observed in May. Similarly, the oocyte growth stage was most common in June, and differentiated females and degenerating oocytes became more common in both species in July.

2.5 Discussion

Ovarian differentiation occurs in the spring and summer of the first and second years following hatch (i.e., age-classes 1 and 2) for both chestnut and northern brook lampreys in Manitoba, and is complete before the third year following hatch (i.e., age-class 3+) for both species. This study therefore indicates that previous studies suggesting that non-parasitic lampreys differentiate at least one year earlier than parasitic species (e.g., Hardisty 1970) oversimplify the ovarian differentiation process.

The present study found no evidence for differential timing of ovarian differentiation in chestnut and northern brook lampreys. The only significant difference was between age-classes in chestnut lamprey; there were more differentiated females in age-class 2 than in age-class 1. This may be because age-class 2 chestnut lamprey were collected significantly later in the year. The number of individuals differentiating was not significantly different between chestnut and northern brook lampreys in age-classes 1 or 2. Previous studies of paired species indicated that non-parasitic larvae differentiated in age-class 1, whereas parasitic larvae differentiated in age-class 2 (Hardisty 1960; Hardisty 1970; Fukayama & Takahashi 1982; Beamish & Thomas 1983; Fukayama & Takahashi 1983). This relationship was particularly clear in the examination of European river and brook lampreys (Hardisty 1970). Later studies also suggested this pattern; however, a closer examination reveals more nuance. Fukayama & Takahashi (1983) found that although most non-parasitic Far Eastern brook lamprey differentiated in age-class 1, differentiation could also occur later. Some individuals of parasitic Arctic lamprey began ovarian differentiation in age-class 1, although the majority differentiated

in age-class 2 (Fukayama & Takahashi 1982). In non-parasitic southern brook lamprey, ovarian differentiation was observed to occur in age-class 1, at 50–90 mm length (Beamish & Thomas 1983). In its parasitic counterpart chestnut lamprey, age-classes could not be determined; however, all individuals were differentiated by the time they reached a length of 88 mm (Beamish & Thomas 1983). Unfortunately, Beamish & Thomas (1983) did not indicate the size of the earliest-differentiating chestnut lamprey in their study; however, their findings do not rule out the possibility that some chestnut lamprey may undergo ovarian differentiation in age-class 1. In general, the timing of ovarian differentiation may be more variable than has been explicitly stated in previous studies.

The variable timing of ovarian differentiation (i.e., in age-class 1 or age-class 2) within these populations of chestnut and northern brook lampreys raises the question of how ovarian differentiation is triggered. Differentiating individuals were occasionally observed in late fall, although sample sizes were small. This could indicate that a small proportion of the population begin or finish differentiating in the late summer or fall, although previous studies have only documented differentiation in the spring and summer (e.g., Hardisty 1970). Differentiation may be triggered by external cues such as water temperature (like metamorphosis; Youson *et al.* 1993), and larvae may need to attain a certain body condition before differentiation is possible. If this body condition is not attained by the time the appropriate temperature cue occurs in the spring of the first year following hatch (i.e., age-class 1), sex differentiation could be delayed until late summer or the next spring. Sample sizes in this study were too small to test this hypothesis directly; however, lamprey metamorphosis requires the larvae to reach a certain

minimum length, body weight, and condition factor (a ratio of length to weight; Youson *et al.* 1993). In other species, different developmental processes are dependent upon size or body condition. For example, Chinook salmon *Oncorhynchus tshawytcha* males are more likely to undergo sexual maturation at an early age if they have a relatively high body weight (Shearer *et al.* 2006), and Japanese eel *Anguilla japonica* larvae must reach a size of 50−55 mm in order to undergo metamorphosis when prompted by external cues (Okamura *et al.* 2012). Thus, it seems reasonable that lamprey larvae might need to reach a certain size or body condition prior to ovarian differentiation. Future studies could collect many larvae at the same time of year and determine whether undifferentiated larvae are smaller than differentiating or differentiated larvae of a similar age. Growth could also be manipulated in a laboratory setting and the effects on timing of ovarian differentiation could be observed.

In general, the length ranges of age-classes were similar for chestnut and northern brook lampreys. However, the average length of chestnut lamprey was greater in age-class 1 (even though the median date of collection was later for northern brook lamprey). In age-class 2, the average length of northern brook lamprey was greater (and there were no significant differences in date of collection). Chestnut and northern brook lampreys were sampled from one river each; thus, it is difficult to determine whether any size differences are due to innate characteristics of the species or to environmental differences, which can have a profound effect on the growth of ectotherms (Angilletta *et al*. 2004). In least brook lamprey *Lampetra aepyptera*, differences in growth rate have been documented among populations in different rivers; this may be due to environmental conditions or differences in larval density (Docker & Beamish 1994).

Size differences suggest that early growth in parasitic and non-parasitic species, and the effect of environmental characteristics on larval growth, should be examined more closely. Ideally, this should be done in multiple rivers for each species, and also in rivers where parasitic and non-parasitic species co-occur. Both chestnut and northern brook lampreys were found in the Rat River; however, the prevalence of northern brook lamprey was so low that northern brook lamprey from the Rat River were excluded from this study. Future studies could attempt to locate more northern brook lamprey in the Rat River and examine growth of the two species where they co-occur.

Previous studies have suggested that differences in fecundity between parasitic and non-parasitic species were probably due primarily to the differences in timing of ovarian differentiation (Hardisty 1970). Parasitic species, considered older and therefore larger at the time of ovarian differentiation, were thought to be able to support more mitotic divisions of germ cells, resulting in greater fecundity (Hardisty 1970). Beamish & Thomas (1983) found that parasitic chestnut lamprey from Michigan had higher potential fecundity following ovarian differentiation than its non-parasitic counterpart southern brook lamprey from Alabama. In the present study, the maximum number of oocytes observed per cross-section was 69 for chestnut lamprey and 43 for northern brook lamprey, but sample sizes were small. Therefore, this study was unable to determine whether there are differences in potential fecundity between congeneric parasitic and non-parasitic lampreys at the time of ovarian differentiation, even when parasitic and non-parasitic species differentiate in the same age-class. It should be noted, however, that chestnut lamprey were larger than northern brook lamprey in age-class 1, which could contribute to greater fecundity of chestnut lamprey. Beamish and Thomas (1983)

found that, within an age-class, the largest individuals had the highest potential fecundity. Furthermore, body size alone may not be the only factor affecting oocyte production. Non-parasitic species could be the same size as parasitic species at ovarian differentiation and still produce fewer oocytes through some other mechanism (e.g., genetic differences). Differences in fecundity could also originate or become more pronounced later in life. For example, fecundity could be reduced in northern brook lamprey through oocyte atresia at metamorphosis, as has been documented in other lamprey species (e.g., European brook lamprey and Far Eastern brook lamprey; Hardisty 1971; Fukayama & Takahashi 1983; see Section 1.1.2.4).

The observation in the present study that differences between the life history types were not evident through histological examination of the gonads at ovarian differentiation could indicate that: a) life history type is determined after ovarian differentiation, and thus there are no differences between life history types at the time of ovarian differentiation; or b) life history type is determined prior to ovarian differentiation, but differences are not evident until later in larval life or at metamorphosis. Docker (2009) has suggested that different species pairs might represent different points on a speciation continuum. Although chestnut lamprey and northern brook lamprey are not a species pair, both are members of other species pairs. Chestnut lamprey cannot be genetically distinguished from its non-parasitic counterpart southern brook lamprey, and northern brook lamprey cannot be genetically distinguished from parasitic silver lamprey (Lang *et al.* 2009; April *et al.* 2011; Docker *et al.* 2012; see Table 1.1). This suggests that both chestnut and northern brook lampreys may be recently diverged from their paired species. It is possible that the timing of ovarian differentiation is more flexible in recently

diverged life history types. Future non-parasitic females that underwent ovarian differentiation earlier in larval life would "waste" fewer oocytes through atresia at metamorphosis, whereas future parasitic females that differentiated later in larval life would benefit from higher potential fecundity. Over evolutionary time, this would select for differential timing of ovarian differentiation; however, recently diverged species pairs might not yet have established this pattern. Differential timing of ovarian differentiation might be less common in more northerly species pairs, which may have diverged more recently post-glaciation.

One example of rapid evolution of earlier ovarian differentiation has occurred in the landlocked sea lamprey, which undergoes ovarian differentiation one year earlier than the anadromous sea lamprey (Hardisty 1969a). The freshwater environment results in smaller adult body size, leading to lower adult fecundity; this may select for reduced oocyte production through earlier ovarian differentiation. Some authors have suggested that freshwater parasitic species are an intermediate form in the evolution of non-parasitic species from anadromous parasitic species (Beamish 1985, Salewski 2003, Hardisty 2006). Thus, freshwater parasitic species such as the chestnut lamprey might be expected to show some characteristics that are more typical of brook lampreys than anadromous parasitic lampreys (e.g., earlier ovarian differentiation in some individuals, as in the present study). Although there is debate whether sea lamprey colonized Lake Ontario following the last glacial retreat (e.g., Bryan *et al.* 2005; Waldman *et al.* 2009) or within historical times through manmade canals (see Eshenroder 2009), it is generally agreed that they became freshwater resident sometime within the last 11 500 years (Bryan *et al.* 2005). Presumably, earlier ovarian differentiation evolved after this point. Northern

brook lamprey in Manitoba may have had a similar amount of time to evolve earlier ovarian differentiation (due to their relatively recent, postglacial origin; Docker *et al.* 2012); however, in the present study, the expected advancement in time of ovarian differentiation was not observed. It is possible that the population of northern brook lamprey in Manitoba is more recently derived than the landlocked sea lamprey. Alternatively, the selection pressures experienced by northern brook lamprey may be different from those experienced by landlocked sea lamprey. Because northern brook lamprey is non-parasitic and landlocked sea lamprey is parasitic, it is difficult to make direct comparisons between the two.

In summary, this study found no evidence for heterochrony in the timing of ovarian differentiation between chestnut and northern brook lampreys; this is in contrast to previous assumptions about ovarian differentiation in parasitic and non-parasitic species. These results suggest that gonadal differences between parasitic and non-parasitic lamprey species may not always be evident until later in larval life or at metamorphosis; however, this study could not rule out the possibility of differences in oocyte number at or soon after ovarian differentiation. Within each species, ovarian differentiation occurred in some individuals in age-class 1 and in others in age-class 2. Future research should more rigorously examine differences between these species at and after ovarian differentiation (including through metamorphosis) and examine factors (e.g., growth, environmental conditions) that may affect the timing of ovarian differentiation.

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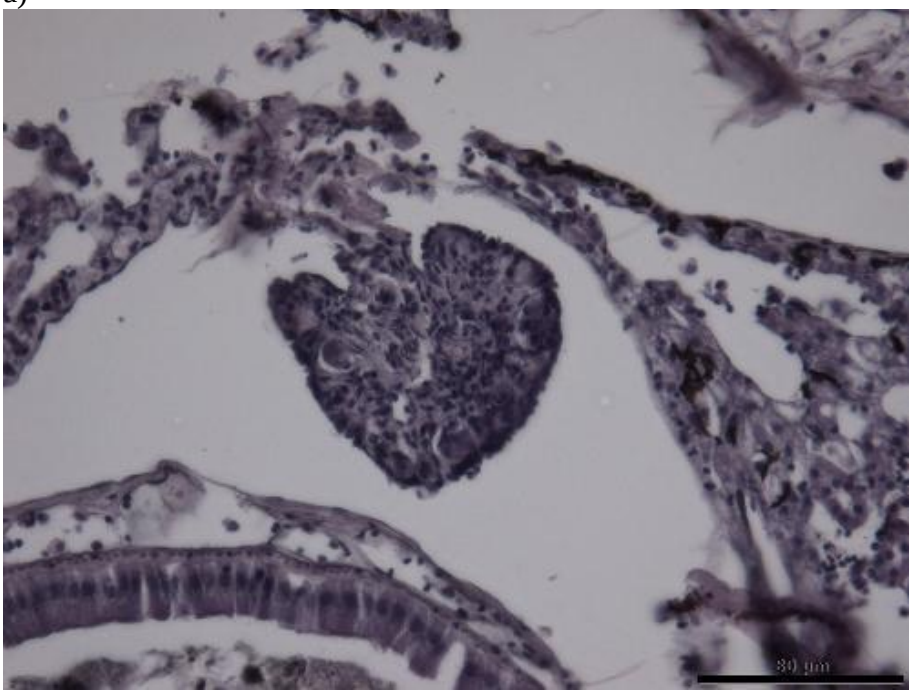
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2.7 Tables and Figures

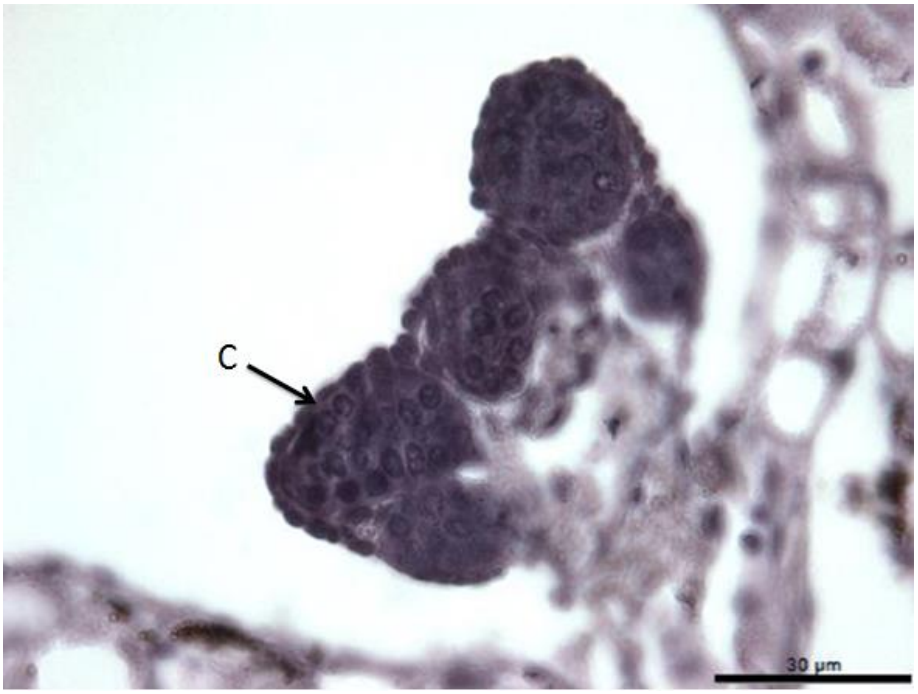
Table 2.1. Stages of gonadal development during the larval phase in chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor* larvae. Ovarian differentiation is considered to occur during stages 2 and 3. Stages were identified and described with reference to Hardisty (1965). See Figure 2.1.

Stage number	Stage name	Histological description
1	Presumptive male or undifferentiated	No germ cell cysts are visible; two or fewer oocytes are visible (e.g., Figure 2.1a)
2	Cystic	Contains multiple cysts of four or more germ cells each (e.g., Figure 2.1b)
3	Oocyte growth	Contains germ cells that have increased in size and are intermediate in basophilicity (i.e., degree of dark purple staining) between germ cells in the cystic and differentiated female stages; multiple stages of growth may be visible; some germ cell cysts may remain (e.g., Figure 2.1c)
4	Differentiated female	Gonad is composed primarily of oocytes; all oocytes are basophilic and of approximately uniform size; no germ cell cysts remain (e.g., Figure 2.1d)
5	Degenerating cysts	Germ cell cysts are present but there is no evidence of cytoplasmic growth. Cysts are characterized by dark patches and/or fuzzy borders of germ cells, indicating degeneration. Degeneration is present in more than half of the germ cell cysts in the gonad (e.g., Figure 2.1e)
6	Degenerating oocytes	The gonad is composed primarily of oocytes. More than half of the oocytes in the gonad are degenerating, characterized by patches in the cytoplasm that are much more or much less basophilic than the rest of the cytoplasm (e.g., Figure 2.1f)
7	Atypical	Gonad cannot be classified as any of the other stages (e.g., Figure 2.1g)

a)



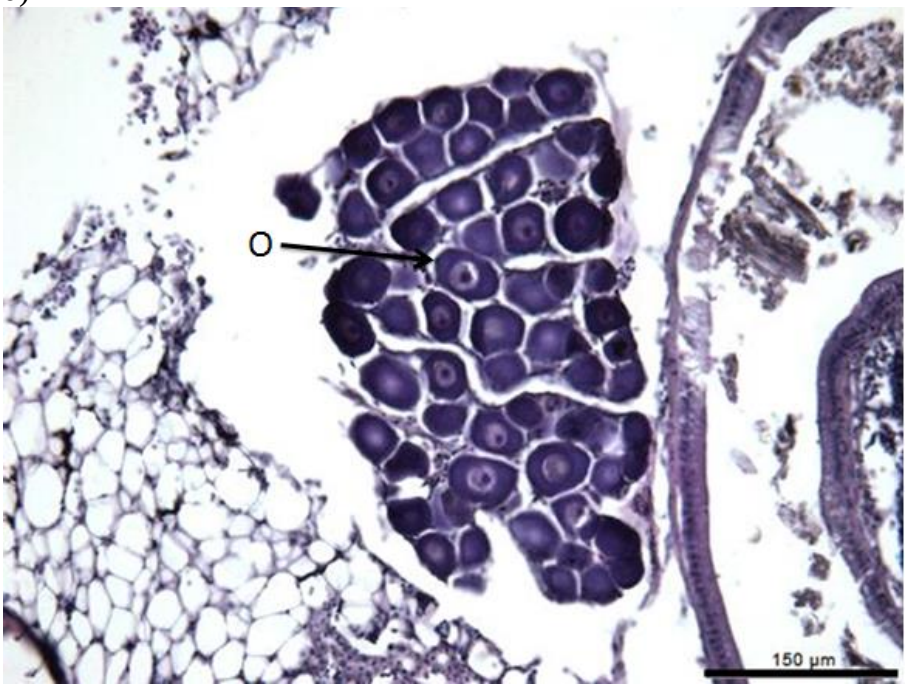
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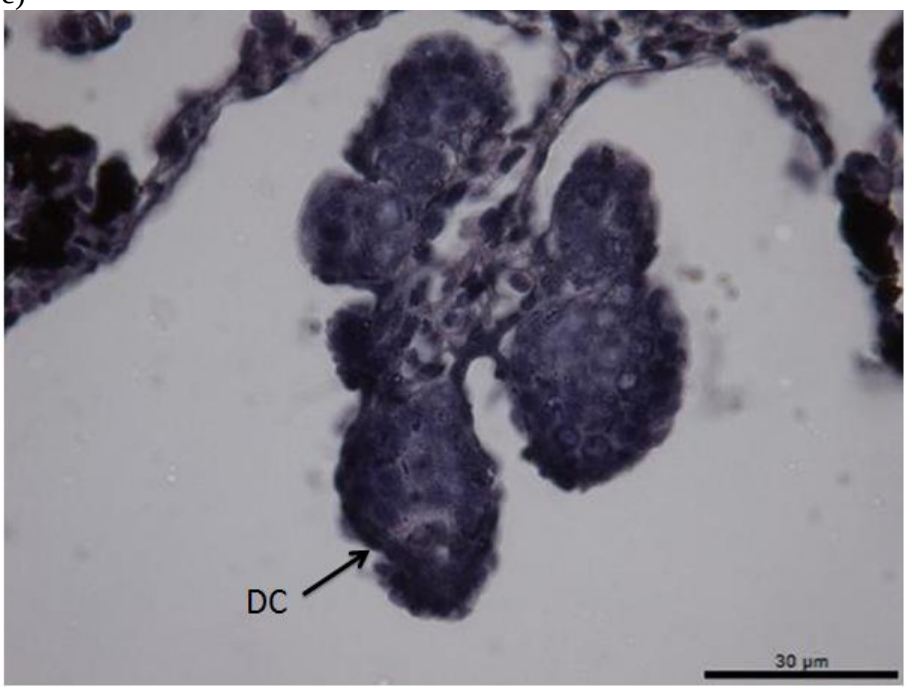
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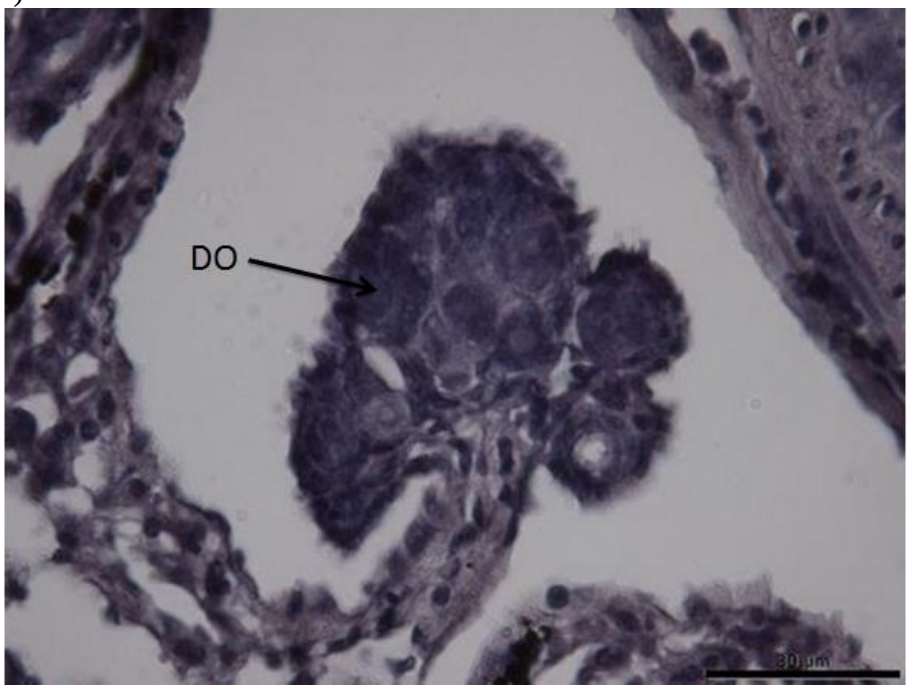
d)



e)



f)



g)

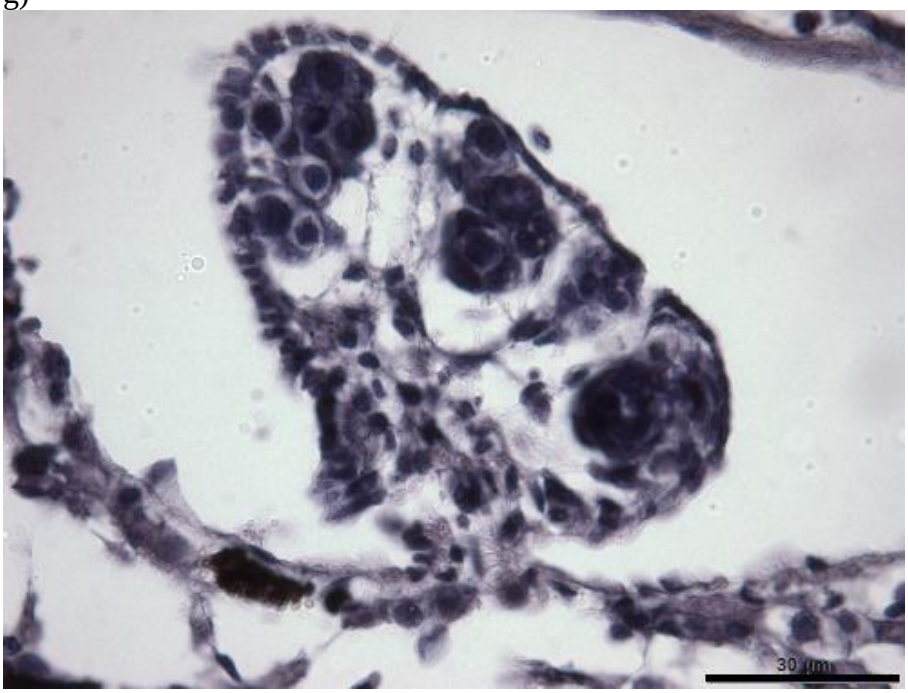


Figure 2.1. Stages of gonadal development in chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor* larvae. Stages are similar in the two species and correspond to those given in Table 2.1. Scale bars are present in the lower-right corner of each image.

a) Stage 1, undifferentiated or presumptive male. The gonad is composed primarily of somatic tissue, with a few undifferentiated germ cells around the edges. Taken from a 63 mm northern brook lamprey larva, age-class 2.

b) Stage 2, cystic. Several cysts of germ cells are clearly visible. Taken from a 46 mm chestnut lamprey larva, age-class 1. "C" indicates a germ cell cyst.

c) Stage 3, oocyte growth. Oocytes have begun to increase in size and become more basophilic. Remnants of cysts are still visible. Taken from a 51 mm chestnut lamprey larva, age-class 1. "G" indicates a growing oocyte.

d) Stage 4, differentiated female. Oocytes are clearly basophilic. Some degenerating oocytes are apparent, staining a darker shade of purple; however, this individual is still considered a differentiated female, because fewer than half of the oocytes show signs of degeneration. Taken from a 58 mm chestnut lamprey larva, age-class 2. "O" indicates a differentiated oocyte.

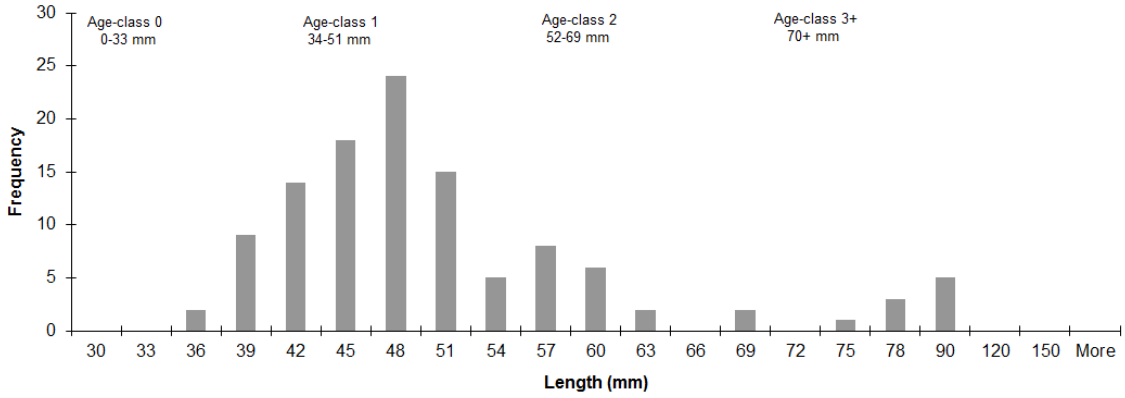
e) Stage 5, degenerating cysts. Several cysts of germ cells are clearly visible; however, degeneration is evident in the dark staining around the edges and the fuzzy borders of many of the germ cells. Taken from a 36 mm chestnut lamprey larva (presumably age-class 1; not used for this study because the individual was held in the laboratory for more

than one week). Degenerating cysts were also observed in some individuals not held in the laboratory (see Table 2.3). “DC” indicates a degenerating cyst.

f) Stage 6, degenerating oocytes. This individual was probably in the oocyte growth phase when the oocytes began to degenerate. Degeneration is evident in the fuzzy borders of the oocytes. Taken from a 49 mm chestnut lamprey larva (presumably age-class 1; not used for this study because the individual was held in the laboratory for more than one week). Degenerating oocytes were also observed in some individuals not held in the laboratory (see Table 2.3). “DO” indicates a degenerating oocyte.

g) Stage 7, atypical. The gonad has a large amount of empty space and contains some cells that may be degenerating oocytes. Taken from a 38 mm chestnut lamprey larva (presumably age-class 1; not used for this study because the individual was held in the laboratory for more than one week). Atypical gonads were also observed in some individuals not held in the laboratory (see Table 2.3).

a)



b)

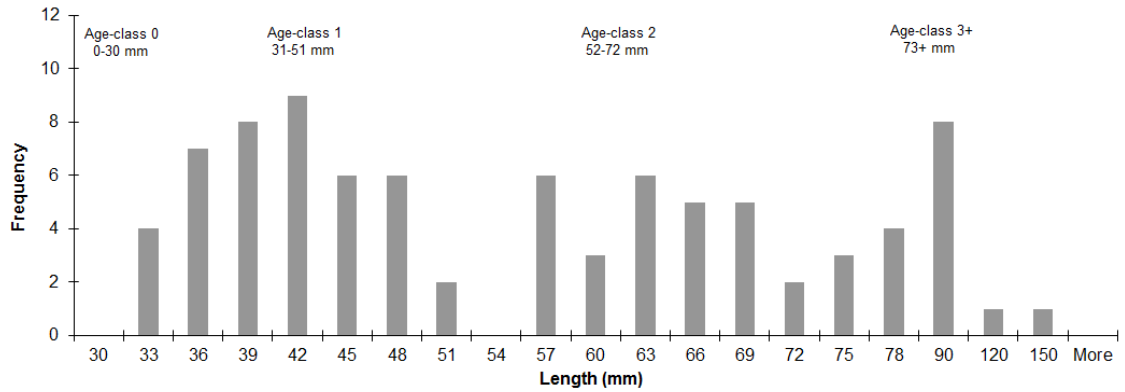


Figure 2.2. Length frequency diagrams for lampreys collected in Manitoba in 2011−2012. a) Chestnut lamprey *Ichthyomyzon castaneus* collected from the Rat River, $n = 115$; b) Northern brook lamprey *I. fossor* collected from the Birch River, $n = 87$. Ranges for each age class are indicated about the diagrams.

Table 2.2. Basic information on chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor* collected in 2011−2012. For each age-class, the length range, number collected, mean length, and number of individuals for which stage of gonadal development was determined are given.

	Rat River (*I. castaneus*)				Birch River (*I. fossor*)			
Age-class	Length range (mm)	*n*	Mean length (mm)	Number staged	Length range (mm)	*n*	Mean length (mm)	Number staged
0	0–33	0	–	–	0–30	0	–	–
1	34–51	82	44.6	66	31–51	42	40.4	35
2	52–69	23	57.5	18	52–72	27	62.6	23
3+	≥70	10	84.5	9	≥73	18	86.4	6

Table 2.3. Distribution of stages of gonadal development among age-classes of chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor* collected in 2011–2012. Stages correspond to those given in Table 2.1 and Figure 2.1.

			Percentage in stage						
Age-class		N	1	2	3	4	5	6	7
1	*I. castaneus*	66	21.2	27.3	30.3	6.1	6.1	6.1	3.0
	I. fossor	35	5.7	5.7	54.3	22.9	2.9	5.7	2.9
2	*I. castaneus*	18	11.1	11.1	22.2	38.9		16.7	
	I. fossor	23	34.8		52.2		8.7		
3+	*I. castaneus*	9	44.4			55.6			
	I. fossor	6	66.7			16.7		16.7	

Table 2.4. *p*-values of Fisher's exact tests for differences in the proportions of individuals in a particular stage of gonadal development. Comparisons were made between age-classes within a species and between species within an age-class, in chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor*. Stages 2 and 3 are grouped together because ovarian differentiation occurs during these two stages (see Table 2.1 and Figure 2.1). * indicates values that were significant after Bonferroni correction (Bonferroni corrected *p*-value = 0.0042).

Comparison		Stage		
Group 1	Group 2	1	2 and 3	4
I. castaneus age 1	*I. fossor* age 1	0.081	0.836	0.021
I. castaneus age 2	*I. fossor* age 2	0.142	1.000	0.028
I. castaneus age 1	*I. castaneus* age 2	0.503	0.109	0.001*
I. fossor age 1	*I. fossor* age 2	0.010	0.597	0.287

Table 2.5. Annual distribution of the stages of gonadal development in chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor* collected in Manitoba in 2011-2012. Stages correspond to those given in Table 2.1 and Figure 2.1.

			Stage						
Month	Species	*n*	1	2	3	4	5	6	7
April	*I. castaneus*	6		100.0					
	I. fossor	0							
May	*I. castaneus*	17	11.8	58.8	11.8		17.6		
	I. fossor	1			100.0				
June	*I. castaneus*	32	18.8	3.1	59.4	3.1	3.1	6.3	6.3
	I. fossor	31	12.9		83.9			3.2	
July	*I. castaneus*	22	22.7		9.1	45.5		22.7	
	I. fossor	22	18.2	4.5	9.1	54.5	4.5	4.5	4.5
November	*I. castaneus*	6	33.3	50.0	16.7				
	I. fossor	5	40.0		20.0	20.0			20.0

CHAPTER 3. EXPRESSION OF FIVE CANDIDATE GENES POTENTIALLY INVOLVED IN OVARIAN DIFFERENTIATION IN CHESTNUT AND NORTHERN BROOK LAMPREYS

3.1 Abstract

Although there is some information about the genetic basis of sex differentiation in model species of teleost fishes and tetrapods, nothing is known about genes involved in sex differentiation in lampreys. Lampreys are ancient vertebrates that diverged from jawed vertebrates approximately 500 million years ago. Relative to teleosts, lampreys have much later ovarian differentiation and a much longer time period between ovarian and testicular differentiation. The genetic basis of lamprey sex differentiation is therefore of particular interest both because of the phylogenetic importance of lampreys and because of their unusual pattern of sex differentiation. As well, differences between lamprey life history types (parasitic and non-parasitic) may first become evident at ovarian differentiation. 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 *I. fossor* in age-classes 1 and 2. Five target genes (17β-hydroxysteroid dehydrogenase, germ cell-less, estrogen receptor β, insulin-like growth factor 1 receptor, and daz-associated protein 1), chosen due to an apparent role in sex differentiation in other vertebrates, were examined. When both age-classes were combined, average expression of insulin-like growth factor 1 receptor was higher in chestnut lamprey ($p = 0.0807$), and average expression of daz-associated protein 1 was higher in northern brook lamprey ($p = 0.0501$). In age-class 1, there were no significant differences in gene expression between chestnut and northern

brook lamprey. However, at age 2, northern brook lamprey had significantly higher expression of estrogen receptor β than chestnut lamprey ($p < 0.01$). Germ cell-less was expressed at higher levels in age-class 2 versus age-class 1 northern brook lamprey ($p < 0.01$), and 17β-hydroxysteroid dehydrogenase was not differentially expressed between any groups. Gene Ontology terms for the differentially expressed genes suggest that parasitic and non-parasitic species may have differences in growth, fecundity, and apoptosis in the gonads during early larval life. This study is the first to identify genes that may be involved in ovarian differentiation in lampreys.

3.2 Introduction

Sex differentiation (note that terms bolded on first use in each chapter are defined in the glossary in Appendix 1) is a complex process involving the development of a previously undifferentiated gonad into a recognizable ovary or testis. Although some information is known about the genetic basis of sex differentiation in model fish species (reviewed in Piferrer & Guiguen 2008; Sandra & Norma 2010; Piferrer *et al.* 2012), nothing is known about the genetic factors involved in sex differentiation in lampreys, ancient jawless fishes.

Many previous studies have examined genes known to be involved in sex differentiation in other organisms (e.g., mammals) and attempted to determine whether these roles are conserved in fish (reviewed in Piferrer *et al.* 2012). In fish, there is a great deal of variability in genes involved in upstream processes such as **sex determination**

and downstream processes such as responses to steroid hormones; however, genes involved in sex differentiation are more conserved (Piferrer & Guiguen 2008). Several genes have been identified that are involved in sex differentiation in multiple fish species. For example, cytochrome p450 aromatase 19a (*cyp19a*), the gene for an enzyme that converts androgens to estrogens, is very important for ovarian differentiation because the balance between androgens and estrogens has a major effect on the differentiating gonad (Piferrer & Guiguen 2008). Hydroxysteroid dehydrogenase enzymes are also important in the production of steroid hormones. Androgen and estrogen receptors mediate the effects of the steroid hormones produced by these enzymes, and these receptors are important in male and female development, respectively (Piferrer & Guiguen 2008). There are also several transcription factors known to be important in sex differentiation. Doublesex and mab-3 related transcription factor 1 (*dmrt1*) is important in male sex differentiation in many fish species and other vertebrates (Piferrer & Guiguen 2008). Sex-determining region Y-related high mobility group containing box genes (*sox* genes) are involved in multiple developmental processes, including sex differentiation, in vertebrates. In mammals, *sox9* is involved in testis differentiation, but its role in fish is less well-studied (Piferrer & Guiguen 2008). Forkhead box L2 (*foxl2*) is involved in female sex differentiation in fish (Piferrer & Guiguen 2008). Some of these genes are expressed in both males and females during sex differentiation, but expression levels are different, producing different responses (Piferrer *et al.* 2012).

Although previous studies have identified conserved genes involved in sex differentiation in jawed fishes (primarily teleosts), there are reasons to suspect that not all of these genes will be conserved in lampreys (see Figure 1.1 re: the phylogenetic

relationships among these taxa). In teleosts, 17β-estradiol is the most important steroid hormone for ovarian differentiation and 11-ketotestosterone for testicular differentiation (Sandra & Norma 2010). Lampreys, in contrast, use primarily 15α-hydroxylated sex steroids (Bryan *et al.* 2008). Application of teleost forms of sex steroids does not appear to affect the sex ratio of larval lampreys (Docker 1992); however, the effect of 15α-hydroxylated steroids on sex differentiation in lampreys is still unknown. Lamprey sex differentiation is also very different from teleost sex differentiation. Teleosts typically differentiate 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 & Takashima 1995; Sandra & Norma 2010; see Section 1.2.2). In contrast, lamprey ovarian differentiation occurs in the spring of the first, second, or third year following hatch (Hardisty 1969a, 1970), and testicular differentiation does not occur until metamorphosis, several years later (Hardisty 1965). Although some non-teleost fishes do display delayed sex differentiation (e.g., Siberian sturgeon *Acipenser baerii*; Berbejillo *et al.* 2012), there are relatively few studies of the genetic factors that may be involved.

One area that is of particular interest is potential differences in gene expression during ovarian differentiation in parasitic and non-parasitic lamprey species. Ovarian differentiation typically occurs earlier in non-parasitic species (Hardisty 1970), although this is not always the case (see Chapter 2). Furthermore, parasitic species are much more fecund as adults (Vladykov 1951); this difference in fecundity likely begins in the larval stage, where parasitic species 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 Sections 1.1.1.2 and 1.1.2.3). These differences may contribute to the vastly different morphology and life history of lamprey adults.

Previous studies on other topics have used lampreys as a model for the ancestral vertebrate. For example, Sower *et al.* (2009) used lampreys to provide insight into the evolution of the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-thyroid axes in vertebrates. However, gonadal development is very different in lampreys than in many other vertebrates. In order to make effective comparisons between lampreys and other vertebrates, a fuller understanding of sex differentiation in lampreys is necessary.

This study used publically available lamprey genomic sequence and transcriptomic sequence available from previous studies in the Docker laboratory to identify and amplify candidate genes for ovarian differentiation in parasitic chestnut lamprey *Ichthyomyzon castaneus* and non-parasitic northern brook lamprey *I. fossor*. Seventeen genes involved in gonadal development, steroidogenesis, and other processes of interest in other vertebrates were identified in lampreys using these sequence resources. Ultimately, five of these genes (17β-hydroxysteroid dehydrogenase, germ cell-less, estrogen receptor β, insulin-like growth factor 1 receptor, and daz-associated protein 1) were amplified and chosen for further analysis. Expression of these genes was examined before, during, and after ovarian differentiation in age-class 1 and 2 lamprey larvae.

3.3 Methods

3.3.1 Sample collection and preparation

Chestnut lamprey (*n* = 19, size range 41–61 mm) were collected from the Rat River in St. Malo, Manitoba. The primary collection location for northern brook lamprey was the Birch River near Prawda, Manitoba (*n* = 10, 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 3.1. All larvae were collected using a backpack electroshocker (Smith-Root LR-24) between April and July in 2011 and 2012, 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).

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 (AHF) 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. The gonad was removed from each individual and flash-frozen in liquid nitrogen, then stored at –80 °C until use. For individuals from Canada

Creek, the gonad was removed and stored in RNA Later solution (Ambion) at –30 °C until use.

The age and stage of gonadal development of each individual were determined as described in Section 2.3.3. Briefly, age was determined using length-frequency curves. Age-class 1 corresponded to larvae 34–51 mm total length in chestnut lamprey and 31–51 mm in northern brook lamprey; age-class 2 corresponded to 52–69 mm in chestnut lamprey and 52–72 mm in northern brook lamprey. Gonadal stage was determined through histological examination. Stages 1 to 4 (Table 2.1, Figure 2.1) were used in this part of the study. Stage 1 gonads are undifferentiated or presumed male; stage 2 gonads contain mostly cysts of dividing germ cells that will presumably develop into oocytes in most individuals (see Section 2.3.3); stage 3 gonads contain mostly growing oocytes; and stage 4 gonads contain differentiated oocytes. Stages 2 and 3 are considered to be ovarian differentiation; however, when these stages were analyzed with stages 1 and 4, the combined group was referred to as differentiating for convenience. Up to three individuals of each stage were randomly selected from age-class 1 for both species; for age-class 2, up to two individuals of each stage were randomly selected for use. In some cases, only one or two individuals were available for a given stage; in those situations, all available samples were used. Sample sizes and information are given in Table 3.2.

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. Ten-fold dilutions of cDNA were prepared and stored at –30 °C until use.

3.3.2 Selection of target and reference genes

Seventeen potential target genes and three potential reference genes (see Tables 3.3, 3.4, 3.5) were identified. Two sources of sequence information were used: a) the sea lamprey *Petromyzon marinus* genome (Smith *et al.* 2013); and b) a preliminary northern brook lamprey **transcriptome** generated in a previous project in the Docker laboratory (Craig T. McFarlane, MSc thesis, in preparation). In brief, in the previous project, RNA sequencing was performed using RNA from the gonad of a differentiated female northern brook lamprey larva (gonadal stage 4, length 97 mm, date of sacrifice 25 May 2011) from McKinnon Creek. Initially, the short sequences ("**reads**") generated by RNA sequencing were aligned to **contigs** from the sea lamprey genome (i.e., source "a") for preliminary analysis. Later, these reads were assembled into northern brook lamprey contigs (i.e., source "b"; see Sections 4.3.2 and 4.3.3 for more details on transcriptome generation). Potential target and reference genes were identified from both sets of contigs (sea lamprey and northern brook lamprey) in two ways, as described in the next two paragraphs.

Both sea lamprey and northern brook lamprey contigs were identified using Blast2GO v. 2.6.4 (Conesa *et al.* 2005). Contigs were queried against the non-redundant (nr) database using a **blastx** method with an **e-value** threshold of 1.0×10^{-6}, and the top five results were retrieved. Each contig was identified as the gene corresponding to the

best match among these top five results. **Gene Ontology (GO) terms** and annotations for the identified contigs were retrieved using default settings. Eight contigs with GO terms involving sex differentiation, steroidogenesis, growth, and gonadal development were selected as potential target genes. Two contigs with GO terms involving structural and housekeeping functions were selected as potential reference genes.

Nine potential target genes involved in sex differentiation and other processes of interest in other vertebrates were identified from the literature, along with one potential reference gene (see Tables 3.3 and 3.5). Protein sequences for these genes in other vertebrates were downloaded from the NCBI database. These sequences were queried against the sea lamprey genome and/or northern brook lamprey transcriptome 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 homology to these sequences were selected for further analysis.

All 20 contigs identified through Blast2GO or genome/transcriptome searches were further analyzed in Geneious. Contigs were again compared to sequences within the nr database using a blastx method and an e-value threshold of 1.0×10^{-5}, and the top 20 BLAST hits were retrieved. A multiple sequence alignment of these BLAST results and the lamprey contig was performed using the Geneious alignment method with default settings. For each gene, a region with the highest sequence identity was identified, and the lamprey nucleotide sequence for this region was extracted. This region was translated to protein sequence and aligned to target gene sequences from other chordate species using the Geneious alignment method with default settings. These multiple sequence alignments are given in Appendix 2. For each gene, a neighbour-joining tree was

constructed using the Jukes-Cantor genetic distance model and 500 bootstrap replicates. These trees are shown in Appendix 2.

Primers were designed for these conserved regions in 20 genes using Beacon Designer 7.20 (Premier Biosoft). Initial testing of primers was performed with a final primer concentration of 1.33 µM per primer, using thermal cycler conditions of denaturation at 94 °C for 2 minutes; followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at a range of 48 to 64 °C for 30 seconds, and extension at 72 °C for 30 seconds; followed by a final extension at 72 °C for 5 minutes. The resulting PCR products were visualized on a 1.5% agarose gel.

Primer pairs for each of the three potential reference genes (alpha tubulin, beta tubulin, and glyceraldehyde-3-phosphate-dehydrogenase) produced a single product of the expected size. However, expression of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) has been shown to vary more among individuals and developmental stages than other potential reference genes (Bustin 2000); thus, it was discarded. Alpha tubulin was chosen as the reference gene because it amplified over a wider range of annealing temperatures than beta tubulin.

Primer pairs for nine target genes produced a single product of the expected size. Three primer pairs were discarded because the genes could not be reliably amplified. One primer pair (serine-threonine protein kinase chk1) amplified well but was not chosen for further analysis due to financial considerations.

Thus, five target genes and one reference gene were used for further analysis (Tables 3.3 and 3.4). Genes that were not used for further analysis (n = 14) are listed in Table 3.5.

3.3.3 Quantitative reverse-transcriptase PCR

Quantitative reverse-transcriptase PCR (qRT-PCR) was performed in a 15 µL reaction volume using SYBR Green PCR Mastermix (Life Technologies) and analyzed on a Bio-Rad iQ5 instrument. For each primer pair, a standard curve with multiple different concentrations of cDNA was used to calculate amplification efficiency (Pfaffl 2001). Standard curves are shown in Appendix 3.

The final reaction included 1 µL each of the forward and reverse primers, for a final concentration of 1.33 µM each, and 2 µL of the one-tenth solution of cDNA. Thermal cycler conditions were an initial denaturation at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C for 10 seconds and annealing at 57.8 °C for 30 seconds. In preliminary testing, an annealing temperature of 57.8 °C was found to produce the best efficiency for the reference gene (alpha tubulin). Melting curve analysis was performed for each amplification. For each individual, two technical replicates were performed.

3.3.4 Analysis

In qRT-PCR, fluorescence is produced from PCR product amplification. The expression of each gene is inferred from the number of PCR cycles required for this fluorescence to reach a certain threshold; this cycle number is referred to as the threshold cycle (C_T; Jozefczuk & Adjaye 2011). The average C_T value of the two technical replicates for each individual was considered to be the mean C_T. In some individuals, certain genes did not amplify within 40 PCR cycles. In this situation, an arbitrary mean C_T value of 41 cycles was assigned to facilitate the calculations, resulting in gene

expression values that were effectively zero, relative to the other genes' expression levels (see http://www.protocol-online.org/biology-forums-2/Real-time-PCR.html). Because an arbitrary value was assigned to some individuals, non-parametric statistics were used for all analyses.

An individual with low C_T values for most genes was identified, and all other samples were standardized to that individual using the Pfaffl method for calculation of relative gene expression (Pfaffl 2001).

GraphPad InStat 3.10 (GraphPad Software, San Diego, California) was used for all statistical comparisons of gene expression. All tests were done with $\alpha = 0.05$. Tests that were performed were: a) pairwise comparisons of all stages of gonadal development in both species and both age-classes, using a Kruskal-Wallis test with a Dunn's post-test for multiple comparisons; b) pairwise comparisons between age-classes within a species and between species within an age-class, using a Kruskal-Wallis test with a Dunn's post-test for multiple comparisons; and c) overall expression of each gene in the two species, using a Mann-Whitney U-test (also known as a Wilcoxon rank sum test). Originally, the intention of this project was to relate gene expression to gonadal stage (i.e., test "a"). However, differences in gene expression among gonadal stages could not be detected (see Section 3.4); thus, broader categories (i.e., tests "b" and "c") were also used.

3.4 Results

Raw C_T values are given in Appendix 4.

There were no significant differences in the expression of 17β-hydroxysteroid dehydrogenase, germ cell-less, estrogen receptor beta, insulin-like growth factor 1 receptor, or daz-associated protein 1 between individual stages within species or between species in a particular stage. Graphs of relative gene expression for individual stages are given in Appendix 4.

There were no significant differences in the overall expression of these five genes between all chestnut lamprey and all northern brook lamprey (Figure 3.1). However, two comparisons approached significance. Expression of insulin-like growth factor 1 receptor (*igf1r*) may be higher in chestnut lamprey ($p = 0.0807$), and expression of daz-associated protein 1 (*dazap1*) may be higher in northern brook lamprey ($p = 0.0501$).

Expression of estrogen receptor beta (*erβ*) was significantly lower in age-class two chestnut lamprey than in age-class 1 chestnut lamprey ($p < 0.05$, Figure 3.2a). Expression of germ cell-less (*gcl*) was significantly higher in age-class 2 than in age-class 1 for northern brook lamprey ($p < 0.01$, Figure 3.2b).

There were no significant differences in gene expression between chestnut and northern brook lamprey at age 1 (Figure 3.3a). At age 2, northern brook lamprey had significantly higher expression of *erβ* than chestnut lamprey ($p < 0.01$, Figure 3.3b).

3.5 Discussion

This is the first study to look at expression of target genes in lampreys prior to, during, and after ovarian differentiation. Target genes involved in hormone response,

early ovarian differentiation, growth, and apoptosis in other vertebrates were differentially expressed between age-classes or between species. Differential expression of these genes suggests that they may be involved in processes related to biological differences between parasitic and non-parasitic lampreys. Furthermore, the presence of differences in gene expression between **life history types** during ovarian differentiation suggests that life history type may already be determined at this stage.

Two of the genes examined in this study (*hsd17β* and *erβ*) are involved in hormone production and response. 17β hydroxysteroid dehydrogenase isoforms are involved in the production of 17-hydroxysteroids (Labrie *et al.* 1997), and estrogen receptors bind to estrogen (in most species, 17β-estradiol) and mediate its effects on the organism (Hall *et al.* 2001). Although lampreys do not appear to use 17-hydroxysteroids to any substantial extent (Bryan *et al.* 2008), *hsd17β* was identified in the sea lamprey genome during Blast2GO analysis. This suggests that lampreys may produce small amounts of 17-hydroxysteroids; this is consistent with the findings of Bryan *et al.* (2008). However, *hsd17β* was the only gene that had no significant differences in expression between species or age-classes. The function of 17-hydroxysteroids in lampreys is still unknown (Bryan *et al.* 2008); however, the lack of any significant differences in *hsd17β* expression suggests that these steroids are probably not involved in lamprey ovarian differentiation. In chestnut lamprey, there was a significant decrease in *erβ* expression between ages 1 and 2; this resulted in significantly higher expression in northern brook lamprey compared to chestnut lamprey at age 2. Research into estrogen receptors has focused on their roles in human disease (e.g., Deroo & Korach 2006) and response to chemical exposure (e.g., Wang *et al.* 2011); thus, the role of estrogen receptors in normal

fish gonads is poorly understood. Differences in *erβ* expression may be related to its potential roles in ovarian development, growth, and apoptosis (discussed below).

The genes *gcl* and *erβ* have been shown to be expressed at higher levels during early ovarian development (relative to other stages) in other fish species (e.g., Baron *et al.* 2005; Chen *et al.* 2011; Nagler *et al.* 2012). Between ages 1 and 2, *gcl* expression increased in northern brook lamprey and *erβ* expression decreased in chestnut lamprey (and *erβ* expression was significantly higher in northern brook lamprey than in chestnut lamprey at age 2). *erβ* has GO terms including "positive regulation of apoptotic process" and "negative regulation of cell growth"; thus, the relatively higher expression of this gene in northern brook lamprey may contribute to their lower fecundity. *gcl* is required for normal germ cell formation and fertility in the fruit fly *Drosophila* (Jongens *et al.* 1992) and spermatogenesis in mice *Mus musculus* (Maekawa *et al.* 2004). In zebrafish, *gcl* was expressed during primordial germ cell formation and during ovarian and testicular development (Li *et al.* 2006). The higher expression in older northern brook lamprey could possibly be related to the presence of more developing oocytes in individuals that differentiate later in life; however, there was no difference in expression between age-class 1 and 2 chestnut lamprey.

dazap1 is a member of the deleted in azoospermia family of genes; these genes are involved in male fertility in humans and are known to be required for germ cell formation, differentiation, and maturation in other species (Yen 2004). *dazap1* was expressed at higher levels in northern brook lamprey (although this difference was also not quite significant; $p = 0.0501$). Genes in this family are expressed in the gonads of both sexes in other fish species (Xu *et al.* 2007; Peng *et al.* 2009; Li *et al.* 2011);

however, their exact role in fish development is uncertain. Interestingly, expression of *dazap1* may increase between the first and second year of larval life in both chestnut and northern brook lampreys (Figure 3.2); however, this increase is not significant and must be confirmed with a larger sample size. If *dazap1* expression does increase throughout larval life, higher expression in northern brook lamprey may reflect greater ovarian maturity in some way that is not evident histologically.

Expression of *igf1r* was examined as a way to look at growth, specifically the effects of insulin-like growth factor 1 (IGF1), which stimulates growth in response to growth hormone (Moriyama *et al.* 2000). Studies suggest that IGF1 helps to regulate growth and reproduction by promoting cell division and differentiation while inhibiting apoptosis (Reinecke 2010). This gene may be expressed at a higher level in chestnut lamprey than northern brook lamprey, although this difference was not quite significant ($p = 0.0807$). Insulin-like growth factor 1 is associated with increased growth and fecundity but reduced lifespan, and it also stimulates the production of sex steroids (Dantzer & Swanson 2012). Increased expression of *igf1r* in chestnut lamprey may be related to their greater size and fecundity as adults. Because of the many effects of IGF1 on growth and life history traits, Dantzer & Swanson (2012) suggest that it may be an important gene in rapid population divergence and speciation; thus, this gene may be of interest for future research into lamprey life history types.

This study is a preliminary investigation of target gene expression in lampreys during ovarian differentiation. As such, it is limited by several factors.

First, it was not possible to identify any significant differences in gene expression between individual stages of gonadal development; this is probably due to small sample

size. The results of this study should be confirmed and expanded upon with larger sample sizes. Increasing the sample size may make it possible to identify genes that are important in each stage of gonadal development.

Second, many genes involved in sex differentiation in other vertebrate species were not identified or not successfully amplified in chestnut or northern brook lampreys (see Table 3.5). This does not necessarily mean that these genes are not present or not expressed in lampreys. Although lamprey genomic resources are increasing, especially with the sequencing of the sea lamprey genome (Smith *et al.* 2013), sequence data is still limited, especially in species other than the sea lamprey. With increased availability of genomic and transcriptomic data, it may be possible to design better primers for some of these genes in the future. Of particular interest would be the genes that are known to be conserved in other vertebrates but were not successfully amplified in lampreys (e.g., *dmrt1*, *foxl2*, *sf1*). Some of these genes (e.g., *dmrt1*, *sf1*; Sandra & Norma 2010) are primarily involved in male sex differentiation in teleosts. As ovarian and testicular differentiation are separated by several years in lampreys (Hardisty 1965, Hardisty 1971), genes involved in male and female sex differentiation may not be expressed at the same time. Some of the genes involved in male sex differentiation in teleosts may not be expressed at detectable levels in lampreys until testicular differentiation occurs (at metamorphosis; Hardisty 1965).

Finally, chestnut and northern brook lampreys were not collected in the same rivers; thus, it is difficult to determine whether differences in gene expression are due to species differences or to environmental factors. Chestnut and northern brook lampreys both occur in the Rat River (see Section 2.3.1), although northern brook lamprey appear

to be less common. Future studies could examine gene expression in sympatric chestnut and northern brook lamprey larvae and in laboratory stocks raised under identical conditions. As well, individuals could be collected from multiple allopatric populations to determine whether differences between species are consistently observed across multiple rivers. Examination of gene expression could also be extended to other parasitic and non-parasitic species.

Differences in gene expression between chestnut and northern brook lampreys at this early stage of the life cycle suggest that some differences between parasitic and non-parasitic species may be evident during ovarian differentiation. In particular, differential expression of genes such as *igf1r*, *gcl*, and *erβ* suggests that there may be important differences in growth, fecundity, and apoptosis in the gonads during early larval life. Genes involved in these processes are potential areas for further investigation into the genetic basis of life history type.

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3.7 Tables and Figures

Table 3.1. Sampling locations for chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor* used in this project. "Coordinates" refers to the geographic coordinates of the location where each individual was collected.

Name	Province/State	Species collected	Coordinates	*n*
Rat River	Manitoba	*I. castaneus*	49.321, 96.946	19
Birch River	Manitoba	*I. fossor*	49.680, 95.790	10
McKinnon Creek	Ontario	*I. fossor*	46.3, 83.6	3
Canada Creek	Michigan	*I. fossor*	45.2, 84.2	5

Table 3.2. Information on individuals of chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor* used in this study. "Stage" refers to stage of gonadal development; see Table 2.1.

Species	Age-class	Stage	*n*	Length (mm)	Date sacrificed	Collection location
I. castaneus	1	1	3	42	07-Jun-12	Rat River
				43	07-Jun-12	Rat River
				50	25-Jul-12	Rat River
		2	3	47	30-Apr-12	Rat River
				46	30-Apr-12	Rat River
				41	30-May-12	Rat River
		3	3	51	08-Jun-12	Rat River
				45	22-Jun-12	Rat River
				49	22-Jun-12	Rat River
		4	3	49	25-Jul-12	Rat River
				51	25-Jul-12	Rat River
				43	25-Jul-12	Rat River
I. castaneus	2	1	1	55	07-Jun-12	Rat River
		2	2	59	17-May-12	Rat River
				54	24-May-12	Rat River
		3	2	61	22-Jun-12	Rat River
				56	22-Jun-12	Rat River
		4	2	58	25-Jul-12	Rat River
				58	25-Jul-12	Rat River
I. fossor	1	1	3	48	Jun-11	Canada Creek
				48.5	Jun-11	Canada Creek
				52	Jun-11	Canada Creek
		2	1	54	25-May-11	McKinnon Creek
		3	3	45	13-Jun-12	Birch River
				42	13-Jun-12	Birch River
				41	28-Jun-12	Birch River
		4	3	50	29-Jul-11	Birch River
				47	29-Jul-11	Birch River
				42	20-Jul-12	Birch River
I. fossor	2	1	2	64.5	Jun-11	Canada Creek
				68	20-Jul-12	Birch River
		2	2	65	25-May-11	McKinnon Creek
				66	25-May-11	McKinnon Creek
		3	2	66	24-May-12	Birch River
				62	13-Jun-12	Birch River
		4	2	63	Jun-11	Canada Creek
				55	29-Jul-11	Birch River

Table 3.3. Information on target and reference genes used in this study. "Source of sequence data" refers to the sequence that was used to identify the gene and design primers (see Table 3.4); "Selected GO terms" refers to Gene Ontology terms that are potentially relevant. Gene Ontology terms were identified using Blast2GO (Conesa *et al.* 2005). For Gene Ontology terms, C = cellular component, F = molecular function, P = metabolic process. * denotes genes that were identified from the literature and searched for in genomic and transcriptomic sequence; other genes were identified through Blast2GO analysis.

Gene	Abbreviation	Type	Potential roles	Source of sequence data	Selected GO terms
Alpha tubulin*	*tubα*	Reference	Housekeeping gene involved in structure of cytoskeleton[1]	Sea lamprey genome	P:protein polymerization; P:microtubule-based movement; F:structural molecule activity
17-beta hydroxysteroid dehydrogenase	*hsd17β*	Target	Production of sex steroids[2], specifically androsterone and androstenedione[3]	Sea lamprey genome	P:metabolic process; F:oxidoreductase activity
Germ cell-less	*gcl*	Target	Spermatogenesis[4], germ cell formation and development[5]	Sea lamprey genome	P:regulation of transcription, DNA-dependent; P:multicellular organismal process; P:developmental process
Estrogen receptor beta	*erβ*	Target	Regulates estrogen signalling in ovary[6], early ovarian development[7]	Northern brook lamprey transcriptome	P:ovarian follicle development; F:estrogen receptor activity; P:positive regulation of apoptotic process; F:estrogen response element binding; P:negative regulation of cell growth; P:intracellular estrogen receptor signaling pathway; P:negative regulation of androgen receptor signaling pathway
Insulin-like growth factor 1 receptor*	*igf1r*	Target	Receptor for insulin-like growth factor 1, which is involved in oocyte growth[8] and body growth[9]	Northern brook lamprey transcriptome	P:positive regulation of cell proliferation; F:insulin-like growth factor I binding; P:positive regulation of developmental growth; P:insulin-like growth factor receptor signaling pathway; P:positive regulation of anti-apoptosis; P:male sex determination; P:cellular response to growth factor stimulus; P:positive regulation of mitosis
Daz-associated protein 1	*dazap1*	Target	Growth[10], spermatogenesis[10]	Northern brook lamprey transcriptome	P:spermatogenesis; P:cell differentiation; P:multicellular organismal development; P:oogenesis; F:nucleotide binding

[1] Thellin *et al.* 1999
[2] Labrie *et al.* 1997
[3] Zhai *et al.* 2012
[4] Maekawa *et al.* 2004
[5] Li *et al.* 2006
[6] Harris 2007
[7] Chen *et al.* 2011
[8] Campbell *et al.* 2006
[9] Moriyama *et al.* 2000
[10] Hsu *et al.* 2008

Table 3.4. Sequences of primers used in quantitative real-time reverse-transcriptase PCR. More information on these genes is given in Table 3.3.

Gene	Type	Forward primer	Reverse primer
Alpha tubulin	Reference	AAGGTGGGCATCAACTAC	CTTGGCGTACATGAGGTC
17-β hydroxysteroid dehydrogenase	Target	CAGAAGTGAGCCAGGATG	CGGAACAGGTGAAGAAGG
Germ cell-less	Target	TCCACCACAACTTGATGAC	AACAGCCACTTCTTGAGC
Estrogen receptor beta	Target	TTCCAGCCAGCAAGACTC	TCCAAGCAGCCATTCTCC
Insulin-like growth factor 1 receptor	Target	AGTTGTTTCACAGGCTCAG	ATCCCATCGGCAATTTCG
Daz-associated protein 1	Target	GGGAAACTATTTGTTGGCGGTCTG	CGAGGCAGTGAGGTCTGTTGG

Table 3.5. Information on target and reference genes that were tested but not pursued further in this study. "Source of sequence data" refers to the sequence that was used to identify the gene and design primers. "Selected GO terms" refers to Gene Ontology terms that were potentially relevant. Gene Ontology terms were identified using Blast2GO (Conesa *et al.* 2005). For Gene Ontology terms, C = cellular component, F = molecular function, P = metabolic process. * denotes genes that were identified from the literature and searched for in genomic and transcriptomic sequence; other genes were identified through Blast2GO analysis.

Gene	Type	Source of sequence data	Status	Selected GO terms
Beta tubulin	Reference	Sea lamprey genome	Not chosen as a reference gene because of marginal amplification	C:cytoskeleton; P:protein polymerization; P:microtubule-based process; F:structural component of cytoskeleton
Glyceraldehyde-3-phosphate-dehydrogenase	Reference	Sea lamprey genome	Not chosen as a reference gene because of variability in expression (Bustin 2000)	P:glucose metabolic process; P:glycolysis
Doublesex and mab-3 related transcription factor 1*	Target	Sea lamprey genome	Failed to amplify	P:cell differentiation; P:sex differentiation
Steroidogenic factor 1*	Target	Sea lamprey genome	Failed to amplify	P:cell differentiation; P:hormone metabolic process; P:male gonad development; P:primary sex determination
Doublesex and mab-3 related transcription factor a2	Target	Sea lamprey genome	Non-specific amplification	P:sex differentiation
Gonadotropin releasing hormone receptor 1*	Target	Sea lamprey genome	Non-specific amplification	P:cellular response to gonadotropin-releasing hormone
Gonadotropin releasing hormone receptor 2*	Target	Northern brook lamprey transcriptome	Failed to amplify	P:cellular response to gonadotropin-releasing hormone
Progestin receptor 1*	Target	Northern brook lamprey transcriptome	Failed to amplify	P:oogenesis; F:steroid binding
Forkhead box L2*	Target	Northern brook lamprey transcriptome	Failed to amplify	P:female somatic sex determination; P:ovarian follicle development; F:estrogen receptor binding
SRY-related homeobox 9*	Target	Northern brook lamprey transcriptome	Non-specific amplification	P:male germ-line sex determination; P:male gonad development
Proliferating cell nuclear antigen*	Target	Northern brook lamprey transcriptome	Amplified poorly	P:cell proliferation; P:DNA replication
Meiotic recombination protein DMC1	Target	Northern brook lamprey transcriptome	Amplified poorly	P:reciprocal meiotic recombination
3-beta hydroxysteroid dehydrogenase	Target	Northern brook lamprey transcriptome	Amplified poorly	P:progesterone metabolic process; P:steroid biosynthetic process
Serine-threonine protein kinase Chk1	Target	Sea lamprey genome	Not chosen as a target gene due to financial considerations	P:cell cycle checkpoint; P:negative regulation of mitosis

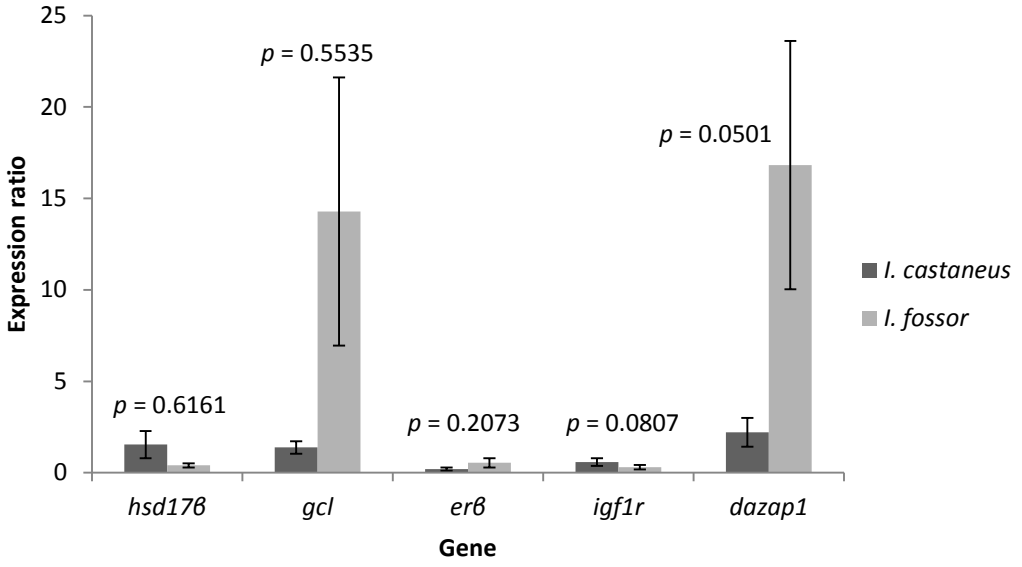
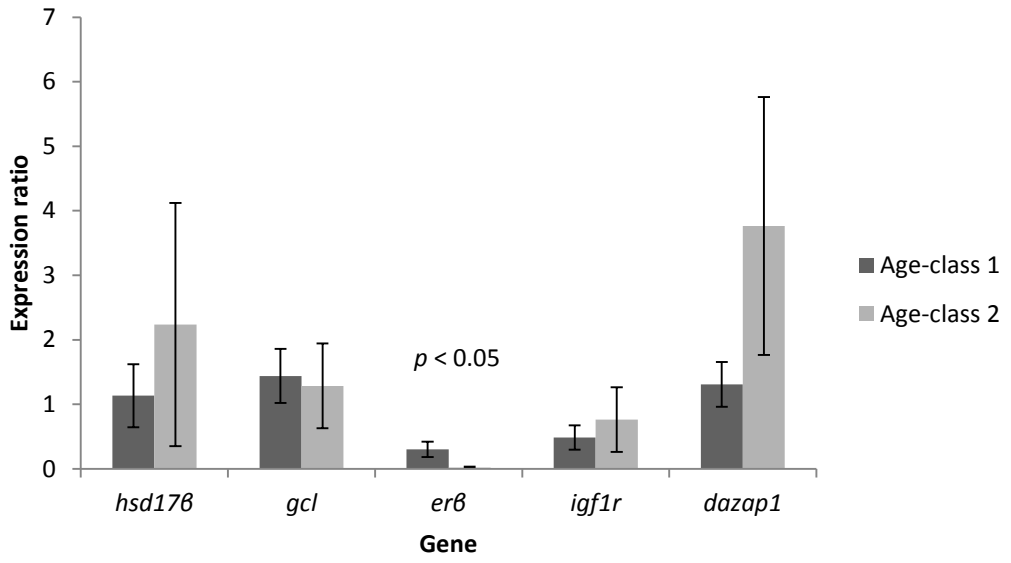


Figure 3.1. Means and standard errors of gonadal gene expression in all individuals of chestnut lamprey *Ichthyomyzon castaneus* (*n* = 19) and northern brook lamprey *I. fossor* (*n* = 18). Significance of pairwise comparisons between species was determined using a Mann-Whitney test in GraphPad InStat 3; all *p*-values are shown. Expression ratio refers to relative gene expression, standardized to one individual using the Pfaffl method (Pfaffl 2001). Abbreviations are defined in Table 3.3.

a)



b)

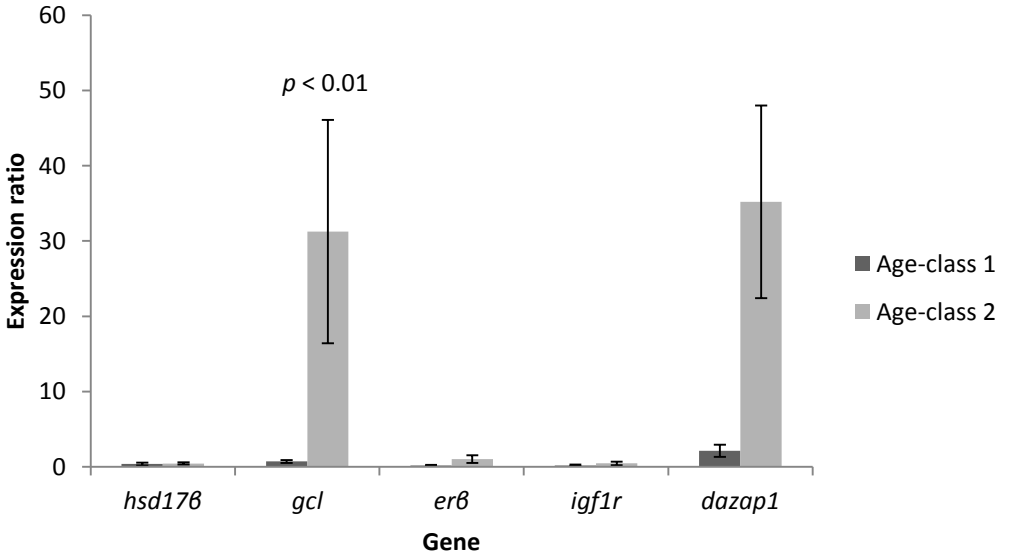
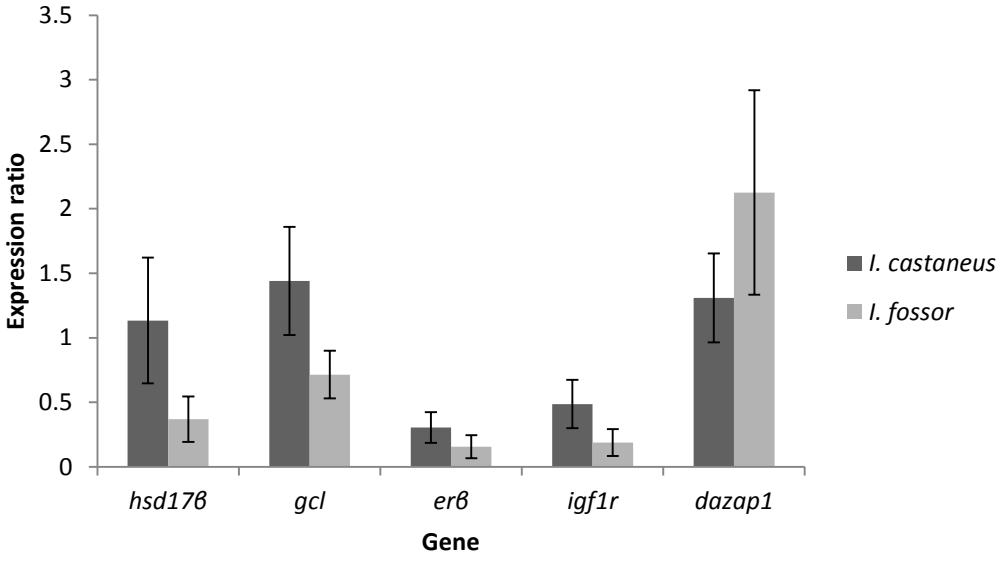


Figure 3.2. Means and standard errors of gonadal gene expression in a) chestnut lamprey *Ichthyomyzon castaneus* (age-class 1, *n* = 12; age-class 2, *n* = 7) and b) northern brook lamprey *I. fossor* (age-class 1, *n* =10; age-class 2, *n* = 8), in age-classes 1 and 2. Significance of pairwise comparisons was determined using a Kruskal-Wallis test with a Dunn's post-test in GraphPad InStat 3; *p*-values less than 0.05 are shown. Expression ratio refers to relative gene expression, standardized to one individual using the Pfaffl method (Pfaffl 2001). Abbreviations are defined in Table 3.3.

a)



b)

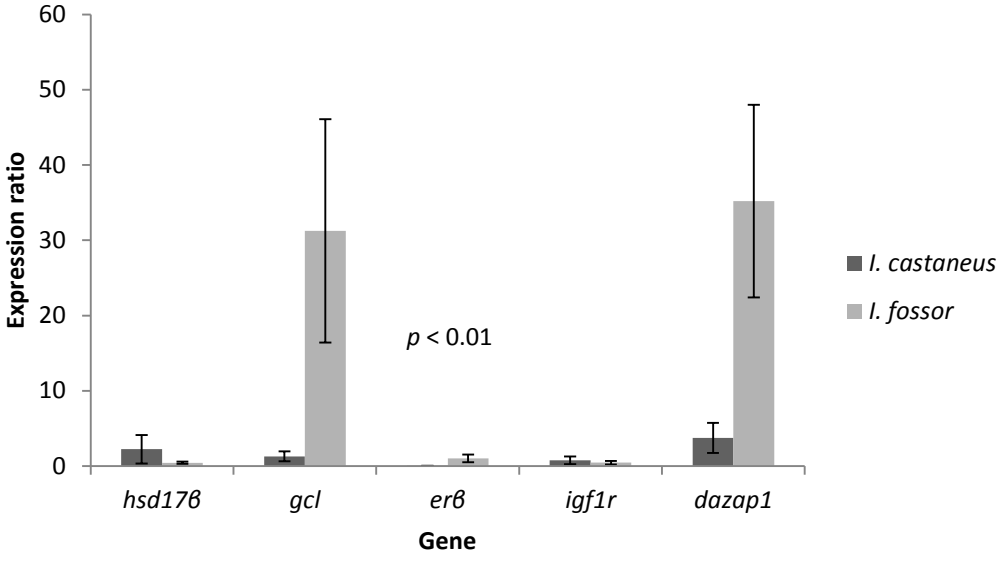


Figure 3.3. Means and standard errors of gonadal gene expression in chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor* in a) age-class 1 (*I. castaneus*, *n* = 12; *I. fossor*, *n* = 10) and b) age-class 2 (*I. castaneus*, *n* = 7; *I. fossor*, *n* = 8). Significance of pairwise comparisons was determined using a Kruskal-Wallis test with a Dunn's post-test in GraphPad InStat 3; *p*-values less than 0.05 are shown. Expression ratio refers to relative gene expression, standardized to one individual using the Pfaffl method (Pfaffl 2001). Abbreviations are defined in Table 3.3.

CHAPTER 4. COMPARISON OF GLOBAL GENE EXPRESSION DURING OVARIAN DIFFERENTIATION IN CHESTNUT AND NORTHERN BROOK LAMPREYS

4.1 Abstract

In lampreys, there are some closely related parasitic and non-parasitic species for which no differences in gene sequence or allele frequency are known. This suggests that differences in gene expression, rather than differences in gene sequence, may be responsible for the large morphological differences between adults. As adults, non-parasitic females have much lower fecundity than parasitic females; this difference may begin during the larval stage, at the time of initial oocyte production. This study used RNA sequencing (RNA-Seq) to identify differences in global gene expression in the ovaries of parasitic chestnut lamprey *Ichthyomyzon castaneus* and non-parasitic northern brook lamprey *Ichthyomyzon fossor* during ovarian differentiation. Two individuals (one in each of the two stages of ovarian differentiation) from each species were examined, along with one differentiated northern brook lamprey. Genes overexpressed during each stage and in each species were identified and assigned Gene Ontology terms. The results show that chestnut lamprey have higher expression of some genes (e.g., cytochrome c oxidase) related to energy metabolism, suggesting greater activity in the gonad during ovarian differentiation. There also appear to be some differences in lipid metabolism between chestnut and northern brook lampreys. In particular, northern brook lamprey have greatly increased (270-fold) expression of 7-dehydrocholesterol reductase during ovarian differentiation. Genes potentially involved in ovarian differentiation (e.g.,

Wilms' tumour suppressor protein 1, leucine-rich repeat-containing protein 17, and meiotic recombination protein DMC1) were identified, as were genes that appeared to be important in the differentiated ovary (e.g., zona pellucida sperm binding protein 4). The results of this study will inform future investigations into the genetic basis of ovarian differentiation and life history type in this ancient vertebrate.

4.2 Introduction

Alternate **life history types** (note that terms bolded on first use within each chapter are defined in the glossary in Appendix 1) are common in freshwater fishes (see Section 1.1). Well studied examples include the benthic and limnetic forms of the threespine stickleback *Gasterosteus aculeatus* (reviewed in McKinnon & Rundle 2002) and the dwarf and normal forms of the lake whitefish *Coregonus clupeaformis* (reviewed in Bernatchez *et al.* 1999, 2010). Even in model systems of alternate life history types, it is sometimes uncertain whether alternate life history types are members of the same species or different species and whether morphological differences are caused by genetic or environmental factors (Wimberger 1994).

Lamprey parasitic and non-parasitic species pairs are a system in which the genetic and/or environmental factors contributing to life history type are completely unknown (Docker 2009). Searches for the genetic basis of life history type have primarily focused on sequence divergence and population differentiation using neutral genetic markers. Early studies examined species pairs using low-resolution markers such

as allozymes (e.g., Beamish & Withler 1986), and many have looked for species-specific differences at the "DNA barcoding" or other mitochondrial genes (e.g., April *et al.* 2011; Docker *et al.* 2012); later studies looked at higher-resolution markers typically used for population structure, such as microsatellites (e.g., McFarlane 2009; Docker *et al.* 2012). Even with the greater resolution provided by new genetic markers, the majority of these studies have not found genetic differences between paired species (see Table 1.1). This suggests that many lamprey species pairs are very recently diverged or have ongoing gene flow (Docker 2009; Docker *et al.* 2012); thus, there has been little time for the development of fixed sequence differences. Instead, the repeated rapid evolution of non-parasitism in lampreys suggests that alteration of gene expression patterns may have occurred. Similar rapid changes in life history have been accompanied by gene expression changes in anadromous and landlocked forms of rainbow trout *Oncorhynchus mykiss* (Aykanat *et al.* 2011) and in dwarf and normal forms of lake whitefish (Jeukens *et al.* 2009).

In the past, studies of gene expression have focused on a few specific candidate genes (e.g., Baron *et al.* 2005) or on larger numbers of candidate genes using microarrays (e.g., Cavileer *et al.* 2009); see Section 1.4.2 for more details on these methods. However, both of these approaches require prior sequence information, sometimes in large quantities; these types of studies are also limited because they focus on already-identified genes (Shiu & Borevitz 2008; Nygaard & Hovig 2009). A microarray has been developed for sea lamprey *Petromyzon marinus*, but it is unpublished to date and limited by the minimal sequence information that was available at the time of construction (http://www.glfc.org/research/reports/Li_microarray.html). However, many next

generation sequencing (NGS) techniques, such as RNA sequencing (RNA-Seq), do not require prior sequence information, making them more suitable for use in non-model species (Ekblom & Galindo 2011). Studies of global gene expression look at all of the genes expressed in the **transcriptome** of an organism (in a certain tissue, at a certain life stage, under certain conditions), and they are an increasingly popular method of comparing gene expression patterns in alternate life history types. RNA-Seq produces thousands or millions of short sequence **reads** that can be assembled into a transcriptome; this provides both sequence information and quantification of gene expression (Ekblom & Galindo 2011).

In this study, the primary goal was to detect genes or groups of genes that may be involved in lamprey life history type (i.e., that are differentially expressed in parasitic and non-parasitic lampreys); RNA-Seq is a suitable technology because it does not require prior knowledge of the genes involved or their sequence. Several studies of life history types in other fishes have used RNA-Seq. RNA-Seq studies tend to identify a large number of differentially expressed genes, rather than one or a few genes that are responsible for life history differences. In these studies, it is common to identify categories of genes that may be involved in life history differences. **Gene Ontology (GO) terms** are of particular use here; these terms identify multiple biological processes, molecular functions, and cellular components that may be associated with a gene sequence (Ashburner *et al.* 2000). In lake whitefish, RNA-Seq revealed differential expression of 116 genes related to protein synthesis and 51 genes related to energy metabolism in the livers of dwarf and normal life history types (Jeukens *et al.* 2010). Goetz *et al.* (2010) used RNA-Seq to examine differences in liver gene expression

between lean and siscowet forms of lake trout *Salvelinus namaycush* and found differences in groups of genes involved with immunity, lipid synthesis, metabolism, and transport. In the Midas cichlid *Amphilophus citrinellus*, thick- and thin-lipped forms have evolved repeatedly in lakes in Nicaragua. RNA sequencing was used to examine gene expression in the lips of these forms in four lakes, and revealed parallel differences in gene expression between forms, with greater divergence in gene expression in forms from older lakes (Manousaki *et al.* 2013).

In lampreys, most morphological divergence of life history types occurs after metamorphosis (see Section 1.1.2.2). An important area of divergence is in fecundity: non-parasitic females, because of their smaller adult body size, have greatly reduced adult fecundity compared to parasitic females (Vladykov 1951). However, the origins of this difference may occur in the larval period. Previous studies suggested that non-parasitic species undergo ovarian differentiation earlier (and thus at smaller sizes) than parasitic species, contributing to the lower fecundity of non-parasitic females (Hardisty 1970). Although this may not be true in all cases (see Chapter 2), at least some non-parasitic species appear to have lower maximum potential fecundity as larvae (e.g., northern brook lamprey *Ichthyomyzon fossor*; Neave *et al.* 2007). This suggests that there may be detectable differences in gonadal gene expression between life history types at the time of ovarian differentiation.

This study therefore used RNA-Seq to attempt to identify differences in global gene expression between the parasitic chestnut lamprey *Ichthyomyzon castaneus* and non-parasitic northern brook lamprey during ovarian differentiation. Although these two species are not a species pair (see Section 1.5), differences in the timing or extent of gene

expression may still provide insight into differences between parasitic and non-parasitic life history types (although sequence differences will be less informative). Comparisons were also made between the stages of ovarian differentiation and between ovaries undergoing differentiation and those in which differentiation was complete, in order to identify genes that might be important in ovarian differentiation in this ancient group of vertebrates. Because of the high cost of RNA-Seq, only one individual from each species and stage were sequenced, but this study provides preliminary information about global gene expression that will inform future targeted studies of gene expression. In addition to identifying genes that may be differentially expressed in the two lamprey life history types during ovarian differentiation, this study will contribute to the knowledge about how conserved these genes are across vertebrates (see Chapter 5).

4.3 Methods

4.3.1 Sample collection and preparation

Lampreys were collected using a backpack electroshocker (Smith-Root LR-24) 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). The individuals used for RNA-Seq were a subset of those sampled for the entire thesis, chosen based on stage of gonadal development. All chestnut lamprey were collected from the Rat River in St. Malo, Manitoba. One northern brook lamprey was collected from the Birch River near

Prawda, Manitoba, and two were collected from McKinnon Creek near Sault Ste. Marie, Ontario. Details on samples are given in Table 4.1.

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 (AHF) 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. The gonad was removed from each individual and flash-frozen in liquid nitrogen, then stored at –80 °C until use.

The age of each individual was determined using length-frequency curves for the population, as described in Section 2.3.3. The stage of gonadal development was determined through histological analysis, as described in Section 2.3.2.

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.

4.3.2 RNA sequencing

In brief, RNA-Seq involves reverse-transcribing messenger RNA (mRNA) to complementary DNA (cDNA) fragments, attaching adapters to one or both ends of these

fragments, and sequencing the cDNA from one or both ends using high-throughput sequencing technology (Hudson 2008; Wang *et al.* 2009; Tucker *et al.* 2009; Ekblom & Galindo 2011). Sample preparation and RNA-Seq were performed at the Oklahoma Medical Research Foundation (Oklahoma City, Oklahoma) for sample numbers IC3, M01, and N1-10, and at the Hussman Institute for Human Genomics (Miami, Florida) for sample numbers C13-3, C20-3, and N17-1 (because the Oklahoma Medical Research Foundation no longer offered this service to external researchers).

For sample numbers IC3, M01, and N1-10, messenger RNA (mRNA) was isolated from ribosomal RNA and small modulating RNA using a poly-T bead step. **Non-normalized** libraries were prepared for each individual following Illumina (San Diego, CA) manufacturer guidelines, and each library was barcoded so that multiple samples could be sequenced in one lane. Sequencing was performed using 75 base pair paired-end sequences on an Illumina Hi-Seq 2000. For sample IC3, sequencing was performed with greater depth (i.e., a higher number of reads were sequenced for this sample), because this sample was intended to be used for transcriptome construction in another project being conducted in the Docker laboratory (Craig T. McFarlane, MSc thesis, in preparation).

For sample numbers C13-3, C20-3, and N17-1, mRNA was isolated using a poly-A step and non-normalized libraries were prepared using the Illumina TruSeq DNA Kit and Epicentre ScriptSeq Kit. Each library was barcoded so that multiple samples could be sequenced in one lane. Sequencing was performed using 100 base pair paired-end sequences on an Illumina Hi-Seq 2000.

4.3.4 Transcriptome assembly

The short sequences that result from RNA-Seq are called reads, and these reads can be aligned to a reference genome or transcriptome or assembled *de novo* into longer sequences called **contigs**. Gene expression can then be compared among samples, based on the number of reads that aligned to each contig in each sample, and contigs can be identified as particular genes based on alignment to sequences in databases (Hudson 2008; Wang *et al.* 2009; Tucker *et al.* 2009; Ekblom & Galindo 2011).

Transcriptome construction for sample IC3, as mentioned above, was performed as part of a previous project; sequencing for IC3 was performed with much greater depth but this sample was from a metamorphosing female. Thus, larval transcriptomes were also constructed for samples C20-3 and N17-1 (one chestnut lamprey and one northern brook lamprey) in the same manner, which is described immediately below. This provided three transcriptomes that could potentially be used for alignment of reads from all five larval samples.

For construction of the three transcriptomes, reads were assembled into contigs using SOAPdenovo (Luo *et al.* 2012). In this procedure, reads from RNA-Seq were broken into smaller sequences of length k (k-mers); k-mers with similar sequence were assembled together, and the paired-end reads were used to guide assembly into longer sequences. Three rounds of assemblies were performed at multiple k values, which should improve the accuracy of the final assembly (Crawford *et al.* 2010). In the first round, six k values were used (23, 33, 39, 43, 49, 59) and these assemblies were then combined into a single data set. In the second round, three k values were used (29, 35,

53) and the assemblies were again combined. In the final round, one assembly was performed with a k value of 39 (after Crawford *et al.* 2010).

For each transcriptome, contigs were validated (i.e., it was confirmed that each contig represented an actual gene) by aligning them to the Swiss-Prot and NCBI non-redundant (nr) databases using **blastx** with a maximum **e-value** of 10^{-5}. Contigs matching viral, bacterial, and fungal sequences were removed from the dataset (see Table 4.2 and Section 4.4). This may have removed some highly conserved lamprey sequences that matched most closely to a viral, bacterial, or fungal sequence; however, it was impossible to determine whether these contigs originated from lamprey RNA or from contaminating organisms or pathogens, and removing them was the most cautious approach.

In order to remove duplicate contigs and join fragmented contigs, Minimo (part of the Amos package; Treangen *et al.* 2011) was used to join overlapping reads with a 90% match over at least ten base pairs. This contig set was again aligned to Swiss-Prot and nr using blastx, and any contigs without a blastx match were removed from the dataset.

4.3.5 Transcriptome comparison

Because sample IC3 was sequenced with greater depth of coverage, the transcriptome constructed from this sample was expected to be of higher quality than transcriptomes constructed for larval samples C20-3 and N17-1. However, the IC3 transcriptome may not have included genes expressed at higher levels in larval stages. Therefore, to determine which of the three was the best transcriptome to use for further analysis, reads from each of the five larval samples were aligned to each of the three

transcriptomes (as described below in Section 4.3.6). The percentages of reads aligned to each transcriptome, and other transcriptome characteristics, are compared in Table 4.2. The IC3 transcriptome was superior in terms of the total number of nucleotides sequenced, the average length of contigs (as indicated by the N50 and N90 values), and the maximum contig length. As well, the percentage of reads from the five larval samples that aligned to a transcriptome was consistently higher for IC3 than for either of the other two transcriptomes. The percentage of reads aligned to IC3 ranged from 20.85 to 31.78%, versus 7.86 to 21.80% for C20-3 and 7.81 to 21.55% for N17-1. The better alignment of reads to the IC3 transcriptome for all samples indicated that it was the best choice for further analysis, even though it is not from a larval sample. Therefore, the IC3 transcriptome was used for alignment of reads, analysis of differential expression, and contig identification. However, no comparisons of gene expression were made between sample IC3 and the other samples in this project, because IC3 is from a metamorphosing female chestnut lamprey and this project was limited to analysis of larval gene expression.

4.3.6 Differential gene expression

Reads from each of the five individual samples were aligned to the IC3 transcriptome using STAR v 2.14 (Dobin *et al.* 2013) with default settings. This program aligns paired-end reads as single sequences, allowing for a possible gap or overlap between the two ends of a paired-end read (Dobin *et al.* 2013). Although there is little information available about how using paired-end reads of different lengths (i.e., 75 bp and 100 bp) might affect analysis, this approach should minimize the bias caused by

comparing samples with different read lengths. The number of sequences that aligned to each contig were tabulated in R 2.15.2 (R Core Team 2012). More reads from RNA-Seq will align to longer contigs, making differential expression of these contigs easier to detect (Bullard *et al.* 2010); however, this bias will affect all samples similarly, so it can often be ignored (Oshlack *et al.* 2010; Soneson & Delorenzi 2013). For example, length bias would be of concern if comparing expression of gene X and gene Y in the same sample; however, it is not a major concern if comparing expression of gene X in two different samples (as in this study), although it may be more difficult to detect differential expression of shorter contigs.

To analyze differential gene expression, the R Bioconductor package BaySeq (Hardcastle & Kelly 2010) was used. This program uses a Bayesian approach to compare counts of reads aligning to each contig among samples, while correcting for the different number of reads in each library. It assumes a negative binomial distribution of the data, which is robust even when the data are not distributed that way. It is superior to many previous programs in that it can compare groups of samples, rather than single pairs (Hardcastle & Kelly 2010).

Eight major comparisons of gene expression were made (Table 4.3). These pairwise comparisons were chosen in order to identify potential differences in gene expression between differentiating (stage 2 and 3) chestnut and northern brook lampreys, between the two stages of ovarian differentiation (stage 2 versus stage 3), and between differentiating (stage 2 and 3) and differentiated (stage 4) ovaries. Unfortunately, a differentiated chestnut lamprey was not available at the time RNA-Seq was performed, and the metamorphosing chestnut lamprey IC3 was not comparable to larval samples;

thus, only the differentiated northern brook lamprey sample (M01) was used to represent differentiated lampreys.

The false discovery rate (FDR) is the proportion of false positive results expected among all comparisons (Benjamini & Hochberg 1995). For comparisons 1–6 and 8, the FDR was set at 0.10 because there were few differences in gene expression with a lower FDR for most comparisons; this allowed identification of some genes that are potentially differentially expressed. However, caution should be used when interpreting these results, as the relatively high FDR may lead to false positive results. When comparing differentiating chestnut lamprey to differentiated northern brook lamprey (comparison 7), many more genes were differentially expressed; thus, an FDR of 0.01 was used.

For each comparison in Table 4.3, the contigs overexpressed by each group were extracted from the transcriptome using Biostrings (Pages *et al.* 2013) and further analyzed in Blast2GO (Conesa *et al.* 2005). Although contigs had been previously validated through alignment to the Swiss-Prot and nr databases (see Section 4.3.4), the sequence data extracted by Biostrings did not include the contig identification. Thus, contigs were re-identified in Blast2GO through a blastx search of the nr database with a maximum e-value of 10^{-6}.

4.3.7 Gene Ontology analysis

After contig identification, Gene Ontology (GO) terms were assigned to each contig using default settings in Blast2GO.

To test for differences in the GO terms associated with contigs overexpressed by each group in a comparison, a two-tailed Fisher's exact test with an FDR of 0.10 was performed for each comparison.

To look for qualitative differences in GO term representation, pie charts comparing the GO terms associated with genes overexpressed by each group in each comparison were constructed using Level 2 GO categories (i.e., Level 2 graph nodes; see Ashburner *et al.* 2000; Conesa *et al.* 2005). A node is a category of gene function (Ashburner *et al.* 2000), and higher levels give more specific information about the function of a particular gene. For example, the three major categories of GO terms (biological process, cellular component, and molecular function) are Level 1 nodes; Level 2 nodes give more specific information about gene function but still allow classification of genes into broad categories. For pie chart construction, only GO terms in the biological process category were used, because the limitations of Blast2GO did not allow all three major categories to be analyzed at the same time and biological process terms were of the most interest.

4.4 Results

The transcriptome constructed from sample IC3 had 560 204 contigs in the initial assembly, 42 154 validated contigs after blastx search, and 33 813 contigs remaining after fungal, viral, and bacterial sequences were removed (Table 4.2). Percentages of

reads successfully aligned to this transcriptome ranged from 20.85% (for sample C20-3) to 31.78% (for sample N1-10) and averaged 27.26%.

For each comparison, differentially expressed genes of interest were identified and highlighted in Tables 4.4 to 4.7 (see immediately below). These genes were chosen because they were clearly identified (i.e., not just identified as a "hypothetical protein") and had GO terms involved in gonadal development, cell proliferation, steroidogenesis, cell differentiation, energy metabolism, and other areas of interest.

Comparisons 1–3 examined potential differences in gene expression between chestnut and northern brook lampreys at equivalent stages of gonadal development. In comparison 1, differentiating (stages 2 and 3) chestnut lamprey expressed 20 genes at a higher level than differentiating northern brook lamprey; in comparison, only one gene was overexpressed in northern brook lamprey (Table 4.4, Figure 4.1). There were no statistically significant differences in the GO terms associated with contigs overexpressed by each group. However, there were some qualitative differences in the GO process terms associated with each group. Differentiating chestnut lamprey had GO process terms mostly involved in metabolic and cellular processes, whereas differentiating northern brook lamprey (with only one overexpressed gene) had an even distribution of GO process terms over several categories. In comparison 2, stage 2 chestnut lamprey overexpressed 41 genes relative to stage 2 northern brook lamprey, whereas stage 2 northern brook lamprey overexpressed 189 genes (Table 4.4, Figure 4.2). There were no statistically significant differences in GO term representation and no qualitative differences in GO biological process term representation. In comparison 3, no genes were found to be differentially expressed between stage 3 chestnut and northern brook

lampreys. Genes of interest in comparisons 1–3 are highlighted in Table 4.4. Differences in expression levels ranged from 1.54-fold (cytochrome c oxidase subunit 2) to 270-fold (7-dehydrocholesterol reductase).

Comparisons 4 and 5 examined potential differences between the two stages of ovarian differentiation (stages 2 and 3) within each species. There were no differences in gene expression between stage 2 and 3 chestnut lamprey (comparison 4). In comparison 5, stage 2 northern brook lamprey overexpressed 269 genes relative to stage 3 northern brook lamprey (Table 4.5, Figure 4.3), but stage 3 did not overexpress any genes relative to stage 2. Genes of interest in comparisons 4 and 5 are highlighted in Table 4.5. Differences in expression levels ranged from 2.5-fold (transcription factor sox6) to 18.5-fold (meiotic recombination protein DMC1).

Comparisons 6–8 examined differences between differentiating and differentiated ovaries (Tables 4.6 and 4.7). In comparison 6, differentiating (stages 2 and 3) northern brook lamprey overexpressed 41 genes relative to differentiated (stage 4) northern brook lamprey; stage 4 overexpressed 106 genes relative to stages 2 and 3. There were no significant differences in overall GO term representation and no clearly visible qualitative differences in GO biological process term representation (Figure 4.4). In comparison 7, differentiating (stages 2 and 3) chestnut lamprey overexpressed 255 genes relative to differentiated (stage 4) northern brook lamprey; stage 4 northern brook lamprey in turn overexpressed 767 genes relative to stages 2 and 3 chestnut lamprey (note that this is with an FDR of 0.01). This is the only comparison for which there were statistically significant differences in GO term representation. A total of 263 GO terms were over-represented in genes overexpressed by differentiating chestnut lamprey, whereas 29 GO

terms were over-represented in genes overexpressed by differentiated northern brook lamprey. The top ten GO terms that were significantly over-represented in each group are given in Table 4.8. In general, differentiating chestnut lamprey appear to have more GO terms involved in cellular components, whereas northern brook lamprey with an already-differentiated ovary have more GO terms involved in metabolic processes and molecular functions. This is in agreement with the qualitative differences seen in Figure 4.5. When differentiating (stages 2 and 3) chestnut and northern brook lampreys were combined (comparison 8), they overexpressed 9 genes relative to differentiated (stage 4) northern brook lamprey; stage 4 northern brook lamprey in turn overexpressed 70 genes. There were no significant differences in GO term representation; however, in the qualitative comparison, differentiating lampreys appeared to have more GO process terms involved in cellular component organization and biogenesis, whereas differentiated northern brook lamprey had more GO terms involved in cellular and metabolic processes (Figure 4.6). Genes of interest in comparisons 6–8 are highlighted in Tables 4.6 and 4.7. Differences in expression levels ranged from 7.81-fold (testis-expressed sequence 10 protein homolog) to 106.03-fold (zona pellucida sperm binding protein 4).

4.5 Discussion

This is the first study to examine global gene expression in lampreys during ovarian differentiation. Previous studies have used NGS technology to sequence mRNA from sea lamprey brain, intestine, liver, kidney, olfactory tissue and embryonic tissue (Smith *et al.* 2013), as well as sea lamprey dorsal rope, gill, and muscle tissue (Chung-

Davidson *et al.* 2013). The present study developed transcriptomic resources in a new tissue (gonadal tissue), during a new developmental period (ovarian differentiation), for two species other than the sea lamprey (chestnut and northern brook lampreys), with a focus on ovarian differentiation and potential differences between parasitic and non-parasitic lampreys. Genes differentially expressed between chestnut and northern brook lampreys during ovarian differentiation were identified. In particular, there may be differences in energy metabolism and lipid metabolism between the two species. As well, this study identified genes of interest in differentiating and already-differentiated ovaries. The results of this study will inform future investigations into the genetic basis of lamprey life history type and provide additional transcriptomic resources for these ancient vertebrates.

There may be higher energy demands in the developing ovary of the chestnut lamprey during ovarian differentiation. The top two genes overexpressed in differentiating chestnut lamprey compared to differentiating northern brook lamprey were cytochrome c oxidase subunits II and III. Cytochrome c oxidase is an enzyme in the electron transport chain of the mitochondrion, and it plays a role in the production of ATP (Hill *et al.* 2008). Chestnut lamprey may require more ATP for the production of a higher number of oocytes. Chestnut lamprey also overexpressed a larger number of genes than northern brook lamprey (20 versus one); this may suggest that the gonadal tissue is more metabolically active in chestnut lamprey at this stage. If the greater fecundity of chestnut lamprey begins at ovarian differentiation (as has been suggested for parasitic species; Hardisty 1970), greater energy requirements in the differentiating ovary would be expected.

Differences in energy metabolism between life history types have also been documented in the lake whitefish. Dwarf forms are smaller, mature earlier, and have a shorter lifespan than normal forms (Chouinard *et al.* 1996; Bernatchez *et al.* 1999), and the two forms can be readily distinguished based on size at maturity (Bernatchez *et al.* 2010). An early microarray experiment, using a 3557 gene microarray developed for Atlantic salmon *Salmo salar*, showed that ATP synthase is expressed at a higher level in the muscle tissue of adults of the dwarf form compared to the normal form (Derome *et al.* 2006). In the liver, higher expression of genes associated with energy metabolism in the adults of the dwarf form was shown with a larger (16 006 gene) Atlantic salmon microarray (St-Cyr *et al.* 2008) and with RNA-Seq (Jeukens *et al.* 2010). Renaut *et al.* (2010) found that single nucleotide polymorphisms (SNPs) that had differences in allele frequency between the two forms of the lake whitefish (in muscle, brain, and liver tissue of adults) were often associated with genes involved in energy metabolism (including forms of cytochrome c oxidase). They suggested that mitochondrial and nuclear genes involved in energy metabolism may be very important in the divergence of dwarf and normal lake whitefish. Higher expression of genes involved in energy metabolism in the dwarf whitefish may be associated with their higher metabolic rate and greater activity levels (Bernatchez *et al.* 2010). In lampreys, higher expression of cytochrome c oxidase subunits in chestnut lamprey may likewise be an indication of higher metabolic rate in parasitic species (at least in the developing ovary). Future studies of life history type in lampreys should examine energy metabolism in more detail.

There may also be differences in lipid metabolism between chestnut and northern brook lampreys. The one gene overexpressed in differentiating northern brook lamprey

compared to differentiating chestnut lamprey was 7-dehydrocholesterol reductase (7-DHCR); this gene was overexpressed by a large amount (270-fold) in differentiating northern brook lamprey. This is an enzyme that catalyzes the final reaction for biosynthesis of cholesterol (Moebius *et al.* 1998); thus, it is important in all downstream reactions of steroidogenesis. In mice, 7-DHCR is involved in Leydig cell differentiation and testosterone production in the testis (Anbalagan *et al.* 2004). Low-density lipoprotein (LDL) receptor-related protein 2 was overexpressed in stage 2 chestnut lamprey relative to stage 2 northern brook lamprey. Low-density lipoprotein receptors are involved in endocytosis of LDL, a form of cholesterol (Goldstein *et al.* 1983).

Lipid metabolism is important in life history types in other fish. In lake whitefish, a microarray study showed upregulation of lipid metabolism genes in the liver of adults of the dwarf form compared to the normal form (St-Cyr *et al.* 2008). Using RNA-Seq to examine liver gene expression in adults, lipid metabolism genes such as apolipoproteins and vitellogenins were likewise identified as candidate genes for differences between life history types in lake whitefish (Jeukens *et al.* 2010). Higher expression of lipid metabolism genes in the dwarf form is probably related to their higher metabolic rate and activity levels (Bernatchez *et al.* 2010). Possible parallels between whitefish and lampreys are less clear in lipid metabolism than in energy metabolism, and it is difficult to make direct comparisons between the liver and the ovary. Differences in lipid metabolism may be related to timing of development and maturation, which may differ between parasitic and non-parasitic species (see Chapter 2). In Chinook salmon *Oncorhynchus tshawytscha*, males can sometimes undergo **sexual maturation** one to four years earlier than females in the same age-class (Taylor 1989; Healey 1991). When

hatchery fish were fed a high-lipid diet (which caused increased whole-body lipid levels), the percentage of early-maturing males increased (Shearer & Swanson 2000). Combined with the suggestion that increased growth in the larval stage in lampreys could lead to a higher incidence of non-parasitism (Kucheryavyi, Savvaitova, Pavlov, *et al.* 2007), these studies suggest that lipid metabolism is an important area for further research into lamprey life history types.

To attempt to identify genes important in ovarian differentiation, northern brook lamprey with already-differentiated ovaries were compared to chestnut and northern brook lampreys still undergoing differentiation. In general, genes overexpressed in differentiating lampreys of both species had more GO terms involved in cellular components; this may be because the rapid cell division occurring during ovarian differentiation requires the production of a large number of cellular components. Genes overexpressed in differentiated northern brook lamprey were more often involved in molecular functions and biological processes. This suggests that differentiated oocytes may be more metabolically active than undifferentiated cells, despite the changes occurring in the gonads during ovarian differentiation.

Some genes that may be involved in ovarian differentiation are Wilms' tumour suppressor protein 1 (*wt1*; overexpressed in differentiating northern brook lamprey) and leucine-rich repeat containing protein 17 (*lrrc17*; overexpressed in differentiating individuals of both species). *wt1* is essential for gonad development in mammals and in other fish species (Scharnhorst *et al.* 2001; Sandra & Norma 2010); it is also required for expression of *sox9* (Gao *et al.* 2006), which is important in gonadal development in many species (see Chapter 3). *lrrc17* has been identified as a gene expressed during oocyte

development in mice (Malcuit *et al.* 2009), although little is known about its function. Two genes associated with meiosis were also identified: meiotic inhibitor protein 1 and meiotic recombination protein DMC1, both of which are required for synapsis of homologous chromosomes during meiosis (Yoshida *et al.* 1998; Libby *et al.* 2002). In the differentiated northern brook lamprey, zona pellucida sperm binding protein 4 was overexpressed relative to differentiating chestnut and northern brook lampreys. The zona pellucida surrounds the oocyte and is especially important for sperm binding and fertilization (Sinowatz *et al.* 2001); this gene is probably overexpressed in the differentiated ovary because of the larger number of differentiated oocytes that are present. Genes identified in this study may be useful in future studies of candidate gene expression and sequence.

Due to the high cost of RNA sequencing, only a single individual from each species and stage was sequenced. Thus, the results of this study may be affected by individual variation, handling conditions, and environmental conditions, and should be treated as preliminary. Next generation sequencing results are frequently confirmed with other techniques such as quantitative real-time reverse transcriptase PCR (qRT-PCR; see Section 1.4.2). These techniques, which are less expensive, allow for more detailed examination of gene expression (for specific genes of interest) in a larger number of samples (Nygaard & Hovig 2009; Huestis & Marshall 2009). RNA-Seq provides both gene expression and gene sequence data (Ekblom & Galindo 2011); thus, the results of RNA-Seq can be used both to identify differentially expressed genes (as in this chapter) and to design primers for further analysis of these genes (as in Chapter 3). Multiple genes of interest (e.g., 5–20, as in Chapter 3) could be identified and their expression

analyzed through qRT-PCR in many more individuals and developmental stages, at a much lower cost than performing further RNA sequencing. Genes of particular interest for qRT-PCR would be 7-dehydrocholesterol reductase and cytochrome c oxidase (which may contribute to species differences), as well as Wilms' tumor suppressor protein 1 and zona pellucida sperm binding protein 4 (which may be involved in ovarian differentiation and the differentiated ovary, respectively). If any of these contigs were chosen for further investigation, a procedure similar to that described in Section 3.3.2 could be implemented to further verify gene identity. However, there are no major concerns about misidentification of contigs in this study. E-values below 1.0×10^{-5} are generally considered to be reliable (Claverie & Notredame 2006), and e-values for genes of interest in the present study ranged from 1.72×10^{-8} (zona pellucida sperm binding protein 4) to effectively zero (cytochrome p450).

The sequence generated by RNA-Seq can be used for other purposes than designing primers for target genes. Sequence data could be mined to identify fixed sequence differences between chestnut and northern brook lampreys that have not yet been identified. There are already genetic methods to distinguish chestnut and northern brook lampreys (Neave *et al.* 2007), so it would be of greater value to collect and analyze silver lamprey (which cannot yet be genetically distinguished from northern brook lamprey; Docker *et al.* 2012) in order to identify sequence differences. Sequence data can also be used to identify nuclear genes for phylogenetic analysis. Many studies of lamprey phylogeny rely on mitochondrial DNA (e.g., Docker *et al.* 1999; Lang *et al.* 2009), which is maternally inherited and thus unable to detect events such as hybridization between species (Sunnucks 2000). In other species, RNA-Seq data have

been used to generate a set of single nucleotide polymorphism markers (SNPs), which are useful for studies of species divergence and population structure (e.g., Renaut *et al.* 2010). With other recent studies of the sea lamprey genome and transcriptome (Smith *et al.* 2013; Chung-Davidson *et al.* 2013), sequence from this study will contribute greatly to lamprey genetic resources. These resources may be used to answer questions about lamprey phylogeny and vertebrate evolution.

Development of quality transcriptomic resources in non-model species can often be difficult. When IC3 contigs were validated through alignment to the nr and Swiss-Prot databases, only about 7.5% of contigs were retained for further analysis. In recent studies using similar methods for transcriptome assembly in non-model species, the percentage of contigs retained has generally been higher. Values ranged from about 30% (in the mosquito *Anopheles funestus* and Chinese sika deer *Cervus nippon*; Crawford *et al.* 2010 and Yao *et al.* 2012, respectively) to about 50% (in salt marsh minnow *Fundulus grandis* and soft-shelled turtle *Pelodiscus sinensis*; Garcia *et al.* 2012 and Wang *et al.* 2013, respectively). However, the total number of contigs in this project (33 813) is similar to the total number reported by Smith *et al.* (2013) for sea lamprey (26 046). The long divergence time of lampreys from model species (lampreys and jawed fishes have been diverged for about 510 million years; Donoghue *et al.* 2000; Near 2009) suggests that lamprey sequences may be quite divergent from model species sequences; this is reflected in the low percentage of contigs that were validated by a blastx search in the present study. The percentage of reads that aligned to the transcriptome is less frequently reported in RNA-Seq studies; however, the relatively low percentage of reads aligned in the present study may be due to the high proportion of contigs that were discarded. With

the increasing availability of lamprey genomic and transcriptomic resources (e.g. Smith *et al.* 2013; Chung-Davidson *et al.* 2013), it may be possible to create a higher-quality transcriptome from this project's data in the near future.

In summary, this study found differences in global gene expression between chestnut and northern brook lampreys during ovarian differentiation. Areas of particular interest are energy metabolism and lipid metabolism, which have been implicated in the divergence of alternate life history types in other fishes. These results provide many potential directions for future research into lamprey life history types. In addition, this project identified genes that may be involved in ovarian differentiation in lampreys and contributed to the recent expansion in lamprey genomic and transcriptomic resources.

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4.7 Tables and Figures

Table 4.1. Details on individuals of chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor* used in this project. "Coordinates" refers to the geographic coordinates of the location where each individual was collected. "Gonadal stage" refers to the histological characteristics of the gonad (see Table 2.1 and Figure 2.1); ovarian differentiation occurs in stages 2 and 3, and the differentiated ovary occurs in stage 4. Sample number IC3 was included because it was used for transcriptome assembly; however, no comparisons of gene expression were made with IC3, because this project examined only the larval stage.

Sample number	Species	Location collected	Coordinates	Date sacrificed	Life stage	Age-class	Gonadal stage	Length (mm)
IC3	*I. castaneus*	Rat River	49.321, 96.946	27 July 2011	Transformer	3+	4	133
C13-3	*I. castaneus*	Rat River	49.321, 96.946	30 April 2012	Larva	1	2	41
C20-3	*I. castaneus*	Rat River	49.321, 96.946	07 June 2012	Larva	2	3	57
M01	*I. fossor*	McKinnon Creek	46.3, 83.6	25 May 2011	Larva	3+	4	97
N1-10	*I. fossor*	McKinnon Creek	46.3, 83.6	25 May 2011	Larva	2	2	59
N17-1	*I. fossor*	Birch River	49.680, 95.790	13 June 2012	Larva	2	3	67

Table 4.2. Characteristics of the three transcriptomes used in this project. "Transcriptome source" indicates the individual from which the transcriptome was constructed. Preliminary contigs = contigs constructed in the initial assembly; validated contigs = contigs that were matched to a sequence in the nr or Swiss-Prot database using blastx; final contigs = contigs remaining after those matching fungal, viral, and bacterial sequences were removed. N50 = the contig length above which the assembly contains half the total number of bases; N90 = the contig length above which the assembly contains 90% of the total number of bases. Higher values for N50 and N90 indicate an assembly with a longer average contig length. The percentages of RNA-Seq reads that aligned to each transcriptome are shown for all larval samples.

	Transcriptome source		
	IC3	C20-3	N17-1
Number of preliminary contigs	560 204	241 667	284 396
Number of validated contigs	42 154	30 044	31 079
Number of final contigs	33 813	29 393	30 595
Total nucleotides	54 297 638	15 451 819	23 818 944
Maximum contig length (bp)	262 216	7 884	14 349
N50	4 321	849	1 301
N90	747	212	335
Contigs longer than 8000 bp (%)	3.25	0.00	0.03
Reads aligned per sample (%)			
C13-3	25.03	8.73	12.36
C20-3	20.85	7.86	9.41
N1-10	31.78	13.67	12.16
N17-1	29.42	17.22	14.71
M01	29.23	21.80	21.55
Average percentage of reads aligned	27.26	13.86	14.04

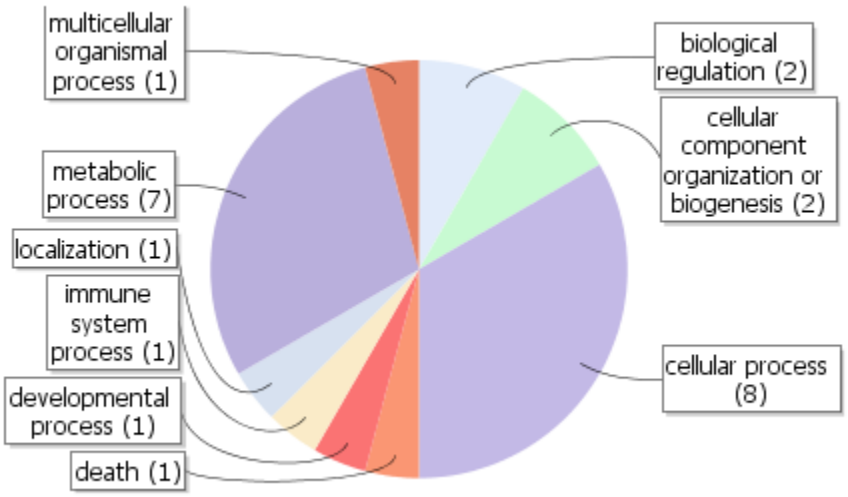
Table 4.3. Pairwise comparisons (i.e., Group 1 versus Group 2) of gene expression made between individuals or groups of chestnut lamprey *Ichthyomyzon castaneus* and/or northern brook lamprey *I. fossor*. "Gonadal stage" refers to the histological characteristics of the gonad (see Table 2.1 and Figure 2.1); ovarian differentiation occurs in stages 2 and 3, and the differentiated ovary occurs in stage 4. "Tables" and "Figures" refer to the tables and figures in this chapter where the results of each comparison are shown.

		Group 1			Group 2					
Comparison number	Purpose of comparison	Species	Gonadal stage	Samples	Species	Gonadal stage	Samples	Differential expression?	Tables	Figures
1	Detect species differences during ovarian differentiation	*I. castaneus*	2 and 3	C13-3, C20-3	*I. fossor*	2 and 3	N1-10, N17-1	Yes	4.4	4.1
2	Detect species differences during ovarian differentiation	*I. castaneus*	2	C13-3	*I. fossor*	2	N1-10	Yes	4.4	4.2
3	Detect species differences during ovarian differentiation	*I. castaneus*	3	C20-3	*I. fossor*	3	N17-1	No	-	-
4	Detect differences between stages of ovarian differentiation	*I. castaneus*	2	C13-3	*I. castaneus*	3	C20-3	No	-	-
5	Detect differences between stages of ovarian differentiation	*I. fossor*	2	N1-10	*I. fossor*	3	N17-1	Yes	4.5	4.3
6	Detect differences between differentiating and differentiated females	*I. fossor*	2 and 3	N1-10, N17-1	*I. fossor*	4	M01	Yes	4.6, 4.7	4.4
7	Detect differences between differentiating and differentiated females	*I. castaneus*	2 and 3	C13-3, C20-3	*I. fossor*	4	M01	Yes	4.6,4.7, 4.8	4.5
8	Detect differences between differentiating and differentiated females	*I. castaneus*, *I. fossor*	2 and 3	C13-3, C20-3, N1-10, N17-1	*I. fossor*	4	M01	Yes	4.6, 4.7	4.6

Table 4.4. Potential genes of interest for species differences between chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor*. These are a subset of genes differentially expressed between stage 2 and/or 3 *I. castaneus* and *I. fossor*; these genes were selected because they were clearly identified and had Gene Ontology terms of interest. FDR = false discovery rate. Similarity = mean similarity to the top ten gene matches identified by blastx in Blast2GO (Conesa *et al.* 2005). For Gene Ontology terms, C = cellular component, F = molecular function, P = metabolic process. "Comparison" refers to the comparisons of gene expression indicated in Table 4.3. "Stage" refers to the histological characteristics of the gonad (see Table 2.1 and Figure 2.1); ovarian differentiation occurs in stages 2 and 3, and the differentiated ovary occurs in stage 4. No genes were differentially expressed between stage 3 *I. castaneus* and stage 3 *I. fossor*.

Name	FDR	Contig length (bp)	E-value	Similarity	Selected Gene Ontology terms	Comparison	Overexpressed in	Underexpressed in	Fold difference
cytochrome c oxidase subunit iii	0.000	1847	2.39E-136	86.50%	P:aerobic electron transport chain; C:mitochondrial inner membrane;:cytochrome-c oxidase activity	1	Stage 2 and 3 *I. castaneus*	Stage 2 and 3 *I. fossor*	2.2
cytochrome oxidase subunit 2	0.000	1142	5.48E-70	93.30%	C:mitochondrial inner membrane; C:respiratory chain; F:electron carrier activity; P:respiratory electron transport chain; F:cytochrome-c oxidase activity	1	Stage 2 and 3 *I. castaneus*	Stage 2 and 3 *I. fossor*	1.54
7-dehydrocholesterol reductase	0.098	292	1.46E-44	84.50%	F:7-dehydrocholesterol reductase activity; P:multicellular organism growth; P:cell differentiation; P:regulation of cell proliferation; P:cholesterol biosynthetic process	1	Stage 2 and 3 *I. fossor*	Stage 2 and 3 *I. castaneus*	270
7-dehydrocholesterol reductase	0.058	292	1.48E-44	84.50%	F:7-dehydrocholesterol reductase activity; P:multicellular organism growth; P:cell differentiation; P:regulation of cell proliferation; P:cholesterol biosynthetic process	2	Stage 2 *I. fossor*	Stage 2 *I. castaneus*	78
low-density lipoprotein receptor-related protein 2	0.093	209	4.07E-23	75.00%	C:lysosome; P:organ development; P:protein glycosylation; P:endocytosis; P:vitamin D metabolic process	2	Stage 2 *I. castaneus*	Stage 2 *I. fossor*	3

a)



b)

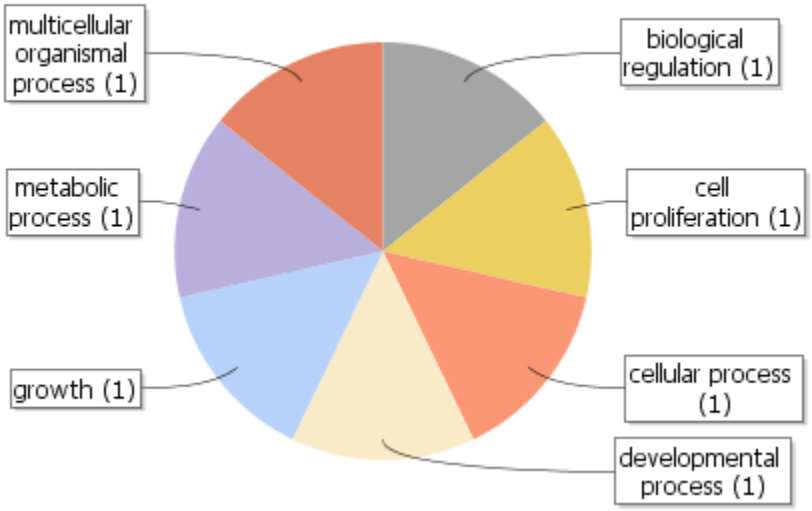
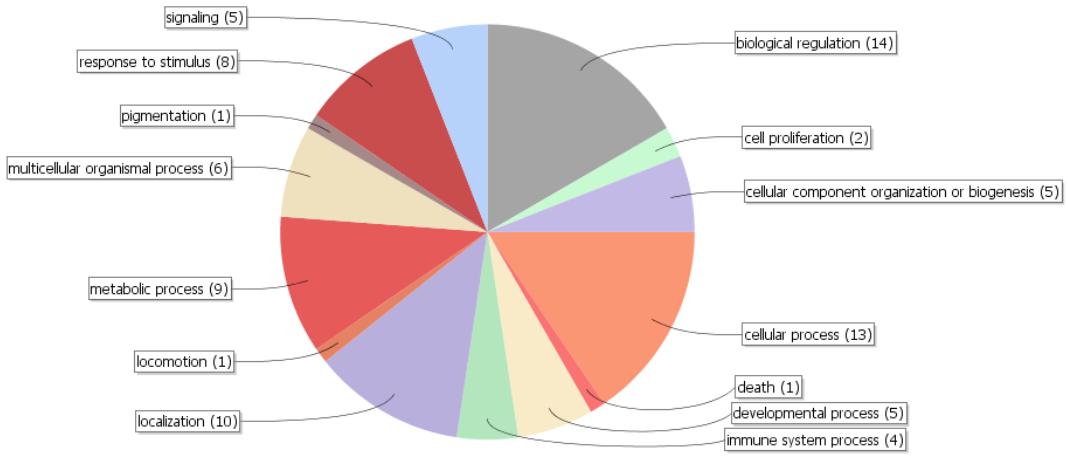


Figure 4.1. Level 2 categories of Gene Ontology biological process terms (cellular component and molecular function terms are not included) for genes differentially expressed in stage 2 and 3 chestnut lamprey *Ichthyomyzon castaneus* versus stage 2 and 3 northern brook lamprey *I. fossor* (comparison 1). a) Genes (n = 20) overexpressed in stage 2 and 3 *I. castaneus*; b) Genes (n =1) overexpressed in stage 2 and 3 *I. fossor*. "Stage" refers to the histological characteristics of the gonad (see Table 2.1 and Figure 2.1); ovarian differentiation occurs in stages 2 and 3, and the differentiated ovary occurs in stage 4.

a)



b)

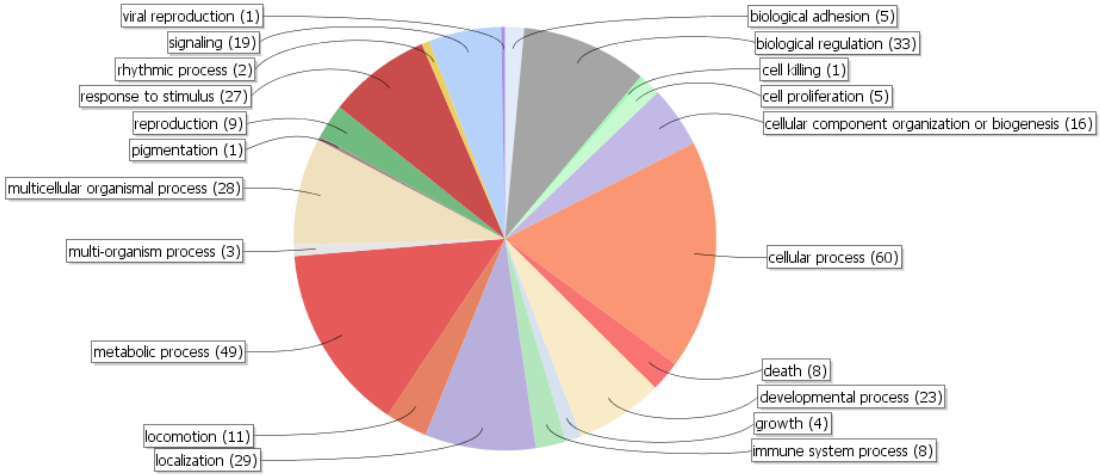


Figure 4.2. Level 2 categories of Gene Ontology biological process terms (cellular component and molecular function terms are not included) for genes differentially expressed between stage 2 chestnut lamprey *Ichthyomyzon castaneus* and stage 2 northern brook lamprey *I. fossor* (comparison 2). a) Genes (n = 41) overexpressed in stage 2 *I. castaneus*; b) Genes (n = 189) overexpressed in stage 2 *I. fossor*. "Stage" refers to the histological characteristics of the gonad (see Table 2.1 and Figure 2.1); ovarian differentiation occurs in stages 2 and 3, and the differentiated ovary occurs in stage 4.

Table 4.5. Genes that may be involved in stage 2 of ovarian differentiation in northern brook lamprey *Ichthyomyzon fossor*. These are a subset of genes overexpressed in stage 2 relative to stage 3 northern brook lamprey; these genes were selected because they were clearly identified and had Gene Ontology terms of interest. FDR = false discovery rate. Similarity = mean similarity to the top ten gene matches identified by blastx in Blast2GO (Conesa *et al.* 2005). For Gene Ontology terms, C = cellular component, F = molecular function, P = metabolic process. "Comparison" refers to the comparisons of gene expression indicated in Table 4.3. "Stage" refers to the histological characteristics of the gonad (see Table 2.1 and Figure 2.1); ovarian differentiation occurs in stages 2 and 3, and the differentiated ovary occurs in stage 4.

Name	FDR	Length (bp)	E-value	Similarity	Selected Gene Ontology terms	Comparison	Overexpressed in	Underexpressed in	Fold difference
transcription factor sox6	0.095	297	1.47E-31	85.40%	F:DNA binding; P:multicellular organismal development; P:regulation of transcription, DNA-dependent	5	Stage 2 *I. fossor*	Stage 3 *I. fossor*	2.5
meiotic recombination protein dmc1	0.046	308	3.77E-10	83.83%	F:DNA binding; P:reciprocal meiotic recombination; F:ATP binding; P:DNA repair	5	Stage 2 *I. fossor*	Stage 3 *I. fossor*	18.5
meiosis inhibitor protein 1	0.043	664	1.03E-22	53.70%	P:spermatid development; P:male meiosis I; P:gamete generation; P:meiosis	5	Stage 2 *I. fossor*	Stage 3 *I. fossor*	12
retinoic acid receptor 1	0.068	788	6.61E-15	97.80%	F:steroid hormone receptor activity; P:retinoic acid receptor signaling pathway; P:negative regulation of cell proliferation; P:steroid hormone mediated signaling pathway; P:positive regulation of apoptotic process; P:multicellular organism growth	5	Stage 2 *I. fossor*	Stage 3 *I. fossor*	3
insulin gene enhancer protein isl-1	0.097	803	3.94E-72	71.70%	P:positive regulation of insulin secretion; P:negative regulation of intracellular estrogen receptor signaling pathway; P:positive regulation of cell proliferation; P:cellular response to glucocorticoid stimulus; P:negative regulation of canonical Wnt receptor signaling pathway; F:estrogen receptor binding	5	Stage 2 *I. fossor*	Stage 3 *I. fossor*	2.5

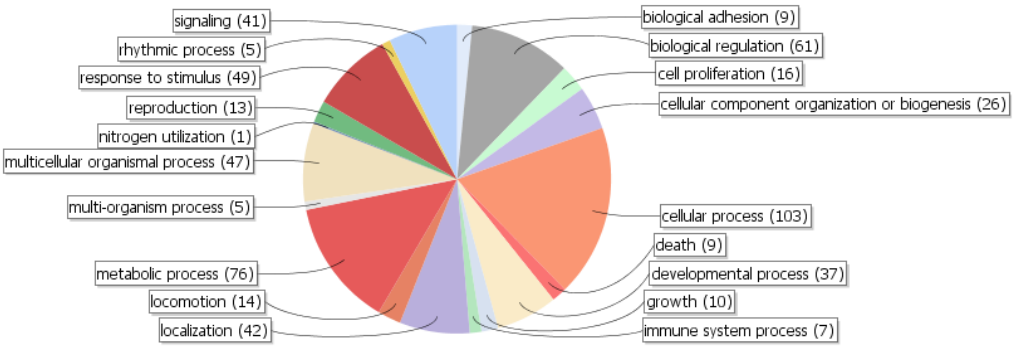


Figure 4.3. Level 2 categories of Gene Ontology biological process terms (cellular component and molecular function terms are not included) for genes (n = 269) overexpressed in stage 2 northern brook lamprey *Ichythyomyzon fossor* relative to stage 3 *I. fossor* (comparison 5). No genes were overexpressed in stage 3 *I. fossor* relative to stage 2. "Stage" refers to the histological characteristics of the gonad (see Table 2.1 and Figure 2.1); ovarian differentiation occurs in stages 2 and 3, and the differentiated ovary occurs in stage 4.

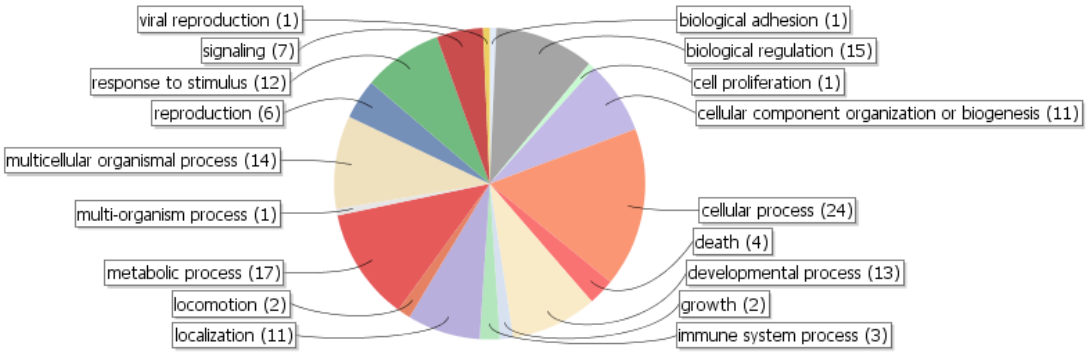
Table 4.6. Genes that may be involved in ovarian differentiation in chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor*. These are a subset of genes overexpressed in stage 2 and 3 individuals of either or both species, relative to stage 4 northern brook lamprey; these genes were selected because they were clearly identified and had Gene Ontology terms of interest. FDR = false discovery rate. Similarity = mean similarity to the top ten gene matches identified by blastx in Blast2GO (Conesa *et al.* 2005). For Gene Ontology terms, C = cellular component, F = molecular function, P = metabolic process. "Comparison" refers to the comparisons of gene expression indicated in Table 4.3. "Stage" refers to the histological characteristics of the gonad (see Table 2.1 and Figure 2.1); ovarian differentiation occurs in stages 2 and 3, and the differentiated ovary occurs in stage 4.

Name	FDR	Length (bp)	E-value	Similarity	Selected Gene Ontology terms	Comparison	Overexpressed in	Underexpressed in	Fold difference
leucine-rich repeat-containing protein 17-like	0.058	3140	7.14E-22	62.50%	P:cell differentiation	6	Stage 2 and 3 *I. fossor*	Stage 4 *I. fossor*	53.93
leucine-rich repeat-containing protein 17-like	0.002	3140	7.10E-22	62.30%	P:cell differentiation	7	Stage 2 and 3 *I. castaneus*	Stage 4 *I. fossor*	46.14
wilms tumor 1 protein	0.067	157	7.53E-20	95.00%	P:negative regulation of female gonad development; P:germ cell development; P:negative regulation of apoptotic process; P:negative regulation of cell growth; P:positive regulation of male gonad development; P:induction of apoptosis	6	Stage 2 and 3 *I. fossor*	Stage 4 *I. fossor*	65.5
cytochrome c oxidase polypeptide vic-2	0.001	219	1.09E-15	82.20%	C:mitochondrial inner membrane; F:cytochrome-c oxidase activity	7	Stage 2 and 3 *I. castaneus*	Stage 4 *I. fossor*	24.56
cytochrome c oxidase subunit vib isoform 1	0.002	449	5.13E-45	86.90%	P:ion transmembrane transport; P:oxidation-reduction process; F:cytochrome-c oxidase activity	7	Stage 2 and 3 *I. castaneus*	Stage 4 *I. fossor*	10.21
nadh dehydrogenase	0.004	517	7.98E-19	71.90%	P:mitochondrial electron transport, NADH to ubiquinone; C:mitochondrial respiratory chain complex I	7	Stage 2 and 3 *I. castaneus*	Stage 4 *I. fossor*	9.85

Table 4.7. Genes that may be important in the differentiated ovary in chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor*. These are a subset of genes overexpressed in stage 4 northern brook lamprey relative to stage 2 and 3 individuals of either or both species; these genes were selected because they were clearly identified and had Gene Ontology terms of interest. FDR = false discovery rate. Similarity = mean similarity to the top ten gene matches identified by blastx in Blast2GO (Conesa *et al.* 2005). For Gene Ontology terms, C = cellular component, F = molecular function, P = metabolic process. "Comparison" refers to the comparisons of gene expression indicated in Table 4.3. "Stage" refers to the histological characteristics of the gonad (see Table 2.1 and Figure 2.1); ovarian differentiation occurs in stages 2 and 3, and the differentiated ovary occurs in stage 4.

Name	FDR	Length (bp)	E-value	Similarity	Selected Gene Ontology terms	Comparison	Overexpressed in	Underexpressed in	Fold difference
zona pellucida sperm-binding protein 4	0.005	429	1.72E-08	46.40%	P:single fertilization; C:cell part; P:regulation of cellular process; P:cellular process involved in reproduction	6	Stage 4 *I. fossor*	Stage 2 and 3 *I. fossor*	106.03
testis-expressed sequence 10 protein homolog	0.053	831	5.17E-31	55.00%	C:nuclear part	6	Stage 4 *I. fossor*	Stage 2 and 3 *I. fossor*	7.81
testis-expressed sequence 10 protein homolog	0.003	831	5.13E-31	55.00%	C:nuclear part	8	Stage 4 *I. fossor*	Stage 2 and 3 *I. castaneus* and *I. fossor*	10.63
cytochrome p450	0.002	3206	0	88.60%	P:two-component signal transduction system (phosphorelay); P:oxidation-reduction process; P:regulation of transcription, DNA-dependent; F:oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen; F:electron carrier activity; F:monooxygenase activity; P:signal transduction by phosphorylation; F:heme binding; F:two-component sensor activity; C:membrane	7	Stage 4 *I. fossor*	Stage 2 and 3 *I. castaneus*	not expressed in stage 2 and 3 *I. castaneus*

a)



b)

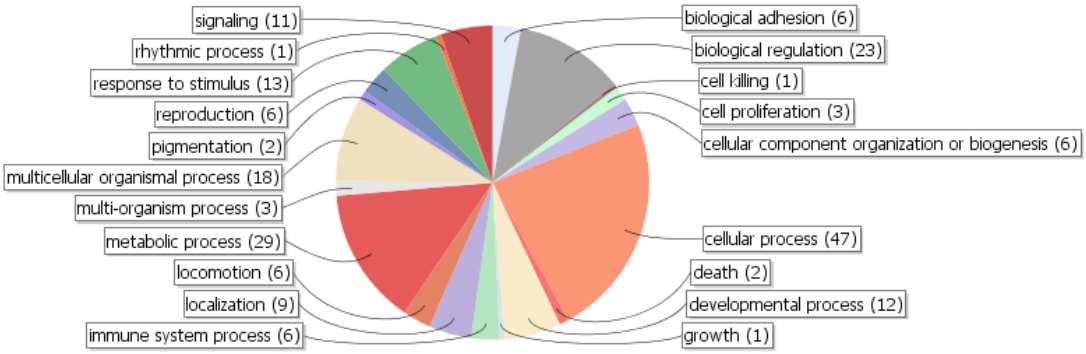
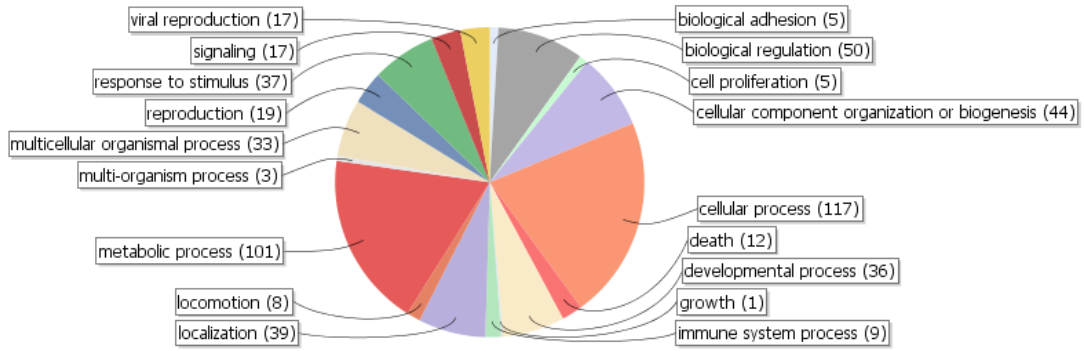


Figure 4.4. Level 2 categories of Gene Ontology biological process terms (cellular component and molecular function terms are not included) for genes differentially expressed between stage 2 and 3 northern brook lamprey *Ichthyomyzon fossor* and stage 4 *I. fossor*. a) Genes (n = 41) overexpressed in stage 2 and 3 *I. fossor*; b) Genes (n = 106) overexpressed in stage 4 *I. fossor*. "Stage" refers to the histological characteristics of the gonad (see Table 2.1 and Figure 2.1); ovarian differentiation occurs in stages 2 and 3, and the differentiated ovary occurs in stage 4.

a)



b)

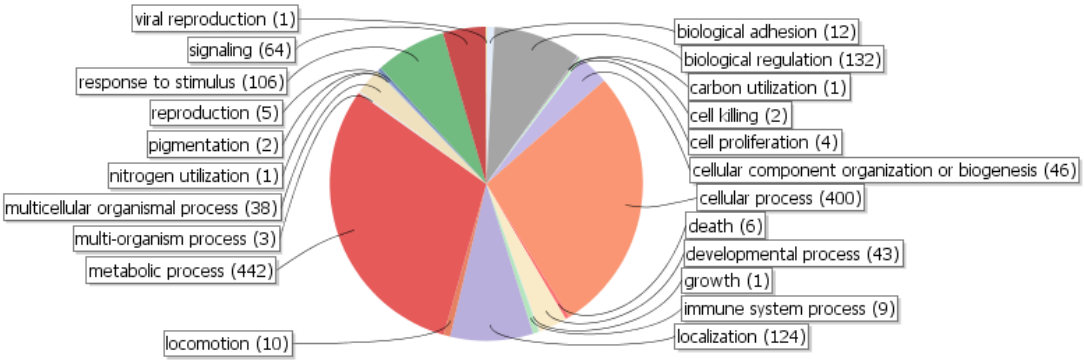
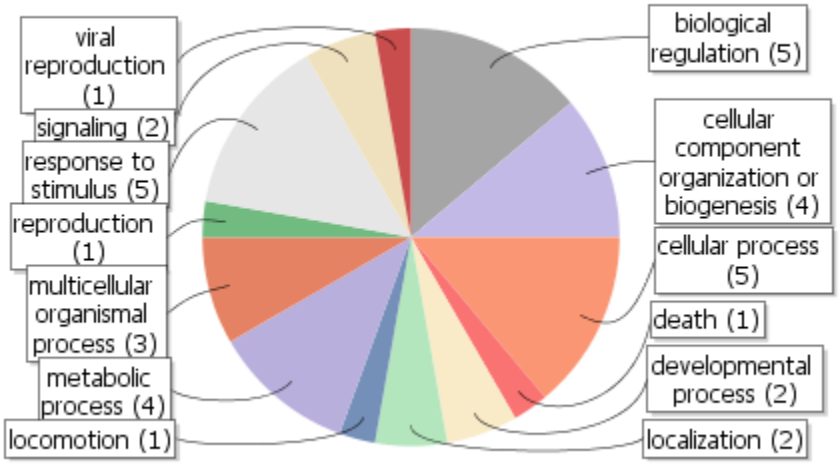


Figure 4.5. Level 2 categories of Gene Ontology biological process terms (cellular component and molecular function terms are not included) for genes differentially expressed between stage 2 and 3 chestnut lamprey *Ichthyomyzon castaneus* and stage 4 northern brook lamprey *I. fossor* (comparison 7). a) Genes (n = 255) overexpressed in stage 2 and 3 *I. castaneus*; b) Genes (n = 767) overexpressed in stage 4 *I. fossor*. "Stage" refers to the histological characteristics of the gonad (see Table 2.1 and Figure 2.1); ovarian differentiation occurs in stages 2 and 3, and the differentiated ovary occurs in stage 4.

Table 4.8. Top ten Gene Ontology terms over-represented in stage 2 and 3 chestnut lamprey *Ichthyomyzon castaneus* relative to stage 4 northern brook lamprey *I. fossor* and *vice versa* (comparison 7), using a Fisher's exact test in Blast2GO (Conesa *et al.* 2005). This is the only comparison for which there were statistically significant differences in GO term representation. In total, 263 GO terms were over-represented in genes overexpressed by stage 2 and 3 chestnut lamprey, whereas 29 GO terms were over-represented in genes overexpressed by stage 4 northern brook lamprey. FDR = false discovery rate. For Gene Ontology terms, C = cellular component, F = molecular function, P = metabolic process. "Stage" refers to the histological characteristics of the gonad (see Table 2.1 and Figure 2.1); ovarian differentiation occurs in stages 2 and 3, and the differentiated ovary occurs in stage 4.

Term	Category	FDR	Over-represented in
intracellular organelle	C	2.10E-26	Stage 2 and 3 *I. castaneus*
organelle	C	2.10E-26	Stage 2 and 3 *I. castaneus*
structural molecule activity	F	2.85E-25	Stage 2 and 3 *I. castaneus*
ribosome	C	9.91E-25	Stage 2 and 3 *I. castaneus*
structural constituent of ribosome	F	2.11E-23	Stage 2 and 3 *I. castaneus*
cytoplasmic part	C	1.09E-22	Stage 2 and 3 *I. castaneus*
intracellular non-membrane-bounded organelle	C	2.28E-22	Stage 2 and 3 *I. castaneus*
non-membrane-bounded organelle	C	2.28E-22	Stage 2 and 3 *I. castaneus*
ribonucleoprotein complex	C	2.53E-22	Stage 2 and 3 *I. castaneus*
macromolecular complex	C	2.80E-20	Stage 2 and 3 *I. castaneus*
catalytic activity	F	7.80E-15	Stage 4 *I. fossor*
cellular ketone metabolic process	P	3.35E-06	Stage 4 *I. fossor*
organic acid metabolic process	P	3.40E-06	Stage 4 *I. fossor*
oxoacid metabolic process	P	5.30E-06	Stage 4 *I. fossor*
carboxylic acid metabolic process	P	5.30E-06	Stage 4 *I. fossor*
small molecule binding	F	2.11E-05	Stage 4 *I. fossor*
cofactor binding	F	4.03E-05	Stage 4 *I. fossor*
lyase activity	F	1.67E-04	Stage 4 *I. fossor*
nucleotide binding	F	1.95E-04	Stage 4 *I. fossor*
transferase activity	F	2.55E-04	Stage 4 *I. fossor*

a)



b)

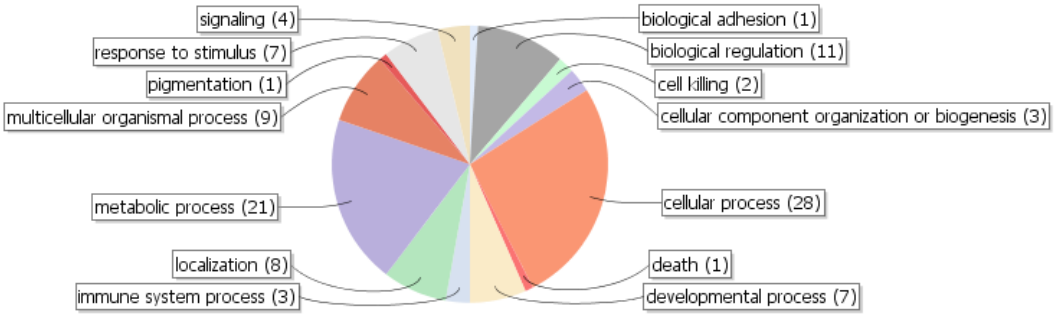


Figure 4.6. Level 2 categories of Gene Ontology biological process terms (cellular component and molecular function terms are not included) for genes differentially expressed in stage 2 and 3 chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor* versus stage 4 *I. fossor* (comparison 8). a) Genes (n = 9) overexpressed in stage 2 and 3 *I. castaneus* and *I. fossor*; b) Genes (n = 70) overexpressed in stage 4 *I. fossor*. "Stage" refers to the histological characteristics of the gonad (see Table 2.1 and Figure 2.1); ovarian differentiation occurs in stages 2 and 3, and the differentiated ovary occurs in stage 4.

CHAPTER 5. GENERAL DISCUSSION

The genetic basis of life history differences is an area of interest in evolutionary biology and particularly in biology of freshwater fishes (e.g., Peichel *et al.* 2001; Bernatchez *et al.* 2010). In lampreys, the genetic and/or environmental factors that determine **life history type** (note that terms bolded on first use within each chapter are defined in the glossary in Appendix 1) are still unknown. This thesis explored potential differences between parasitic chestnut lamprey *Ichthyomyzon castaneus* and non-parasitic northern brook lamprey *Ichthyomyzon fossor* during ovarian differentiation, which is the earliest period when differences between life history types have been suggested to occur.

5.1 Implications for lamprey life history types

5.1.1 When do life history types diverge?

One of the major questions about lamprey life history types is: at what point in the life cycle is life history type determined? This thesis examined potential differences between life history types in three areas: timing of ovarian differentiation; expression of individual genes before, during, and after ovarian differentiation; and global gene expression during ovarian differentiation. Clear differences between parasitic and non-parasitic species at ovarian differentiation would suggest that life history type is determined during or prior to differentiation. Absence of differences would suggest either that: a) life history type is determined after ovarian differentiation; or b) life history

type is determined prior to ovarian differentiation but differences are not apparent until later in the life cycle.

From previous studies of ovarian differentiation in parasitic and non-parasitic lamprey species (e.g., Hardisty 1960; Hardisty 1970; Fukayama & Takahashi 1982; Beamish & Thomas 1983; Fukayama & Takahashi 1983), it was expected that non-parasitic species would undergo ovarian differentiation one year earlier than parasitic species. However, the present study found that parasitic chestnut lamprey and non-parasitic northern brook lamprey in Manitoba had similar timing of ovarian differentiation (Chapter 2). Thus, the timing of ovarian differentiation does not appear to be a reliable marker of life history type; this may be because life history type is not yet determined by this time, or because differences do not become evident until sometime later in the life cycle. Chestnut lamprey and northern brook lamprey are each genetically indistinguishable from their paired species (southern brook lamprey *Ichthyomyzon gagei* and silver lamprey *I. unicuspis*, respectively; see Table 1.1), suggesting that members of these species pairs are recently diverged. As well, lamprey populations at northern latitudes (such as Manitoba) will have been more recently affected by glaciation than southern populations, allowing less time for the divergence of life history types. Differences in timing of ovarian differentiation may be less common in recently diverged life history types (see Section 2.5).

In contrast to the lack of differences in the timing of ovarian differentiation, analysis of gene expression in chestnut and northern brook lampreys during ovarian differentiation suggested that there may be differences between the life history types at this stage. In particular, differential expression of genes such as *igf1r* and *erβ* (Chapter 3)

suggests that there may be differences in growth and apoptosis in the gonad at this stage; these are possible contributors to later differences in fecundity. RNA sequencing (RNA-Seq; Chapter 4) indicated that differentiating chestnut lamprey may have higher expression of genes involved in energy metabolism (such as cytochrome c oxidase), suggesting that the gonads are more active in differentiating parasitic species. Differentiating northern brook lamprey had much higher expression of 7-dehydrocholesterol reductase, which catalyzes the biosynthesis of cholesterol (Moebius *et al.* 1998) and is therefore important in all downstream reactions that produce steroid hormones. Thus, there may be differences in lipid metabolism and energy metabolism between parasitic and non-parasitic species during ovarian differentiation. However, differences in gene expression during ovarian differentiation do not necessarily mean that feeding type has been determined by that point. With the single individual used for each stage in the RNA-Seq study, some of the differences detected could be due to individual variation or related to environmental conditions or other factors unrelated to life history type (see Section 5.3).

This study did not address the question of when life history types diverge in future male lampreys. Unlike ovarian differentiation, testicular differentiation occurs at the same time (metamorphosis; Hardisty 1965) in all species; thus, testicular differentiation appears less likely to be important in determining life history type. No differences in sperm count between life history types have been documented; the smaller adult body size of non-parasitic species probably has a much greater effect on oocyte numbers (e.g., Vladykov 1951), because eggs are much larger and more energetically demanding than sperm. Examination of gene expression during testicular differentiation would be

valuable primarily because some genes involved in **sex differentiation** in other vertebrates were not identified during ovarian differentiation in the present study (see Section 5.2 and Table 5.3). Genes such as *dmrt1* and *sf1*, which are involved in male sex differentiation in other vertebrates (Sandra & Norma 2010), might be successfully identified in differentiating male lampreys.

This study provided no definitive answers about when life history type is determined in lampreys. There were no observable differences in gonadal morphology or the timing of ovarian differentiation; however, differences in gene expression suggest that more subtle underlying differences may be present. Several potential avenues for future research into gene expression in lampreys have been identified. Future studies may be able to determine whether there are consistent differences in gene expression between life history types during ovarian differentiation or whether gene expression can be manipulated by changing environmental conditions in the laboratory.

5.1.2 What causes lower fecundity in non-parasitic species?

One of the major gonadal differences between parasitic and non-parasitic lamprey species is in adult female fecundity: non-parasitic females have much lower fecundity than parasitic females (Vladykov 1951). This reduced fecundity in non-parasitic species may originate from ovarian differentiation at an earlier age and smaller size, leading to production of fewer oocytes (e.g., southern brook lamprey; Beamish & Thomas 1983), from oocyte atresia at metamorphosis (e.g., European brook lamprey *Lampetra planeri*; Hardisty 1971), or from a combination of these factors. Chapter 2 of this thesis examined the "rule" of earlier ovarian differentiation in non-parasitic species (Hardisty 1970) and

found that it is not universally applicable: parasitic chestnut lamprey and non-parasitic northern brook lamprey have similar timing of ovarian differentiation. (As well, there may be some more subtle overlap in the timing of ovarian differentiation between Arctic lamprey *Lethenteron camtschaticum* and Far Eastern brook lamprey *Le. reissneri*; see Section 2.5.) Although it was not possible to quantify potential fecundity at the time of ovarian differentiation in this study, the lack of differential timing suggests that the lower fecundity of northern brook lamprey does not arise from ovarian differentiation at an earlier age and smaller size. Instead, other factors must contribute to the lower adult fecundity of northern brook lamprey. In recently derived non-parasitic species, there may not have been enough time for the evolution of earlier ovarian differentiation, and oocyte atresia at metamorphosis may be account for the reduction in fecundity (see Sections 2.5 and 5.1.2). Unfortunately, examination of oocyte atresia at metamorphosis was beyond the scope of this study. However, differences in gonadal gene expression around the time of ovarian differentiation may contribute to lower fecundity in northern brook lamprey. In particular, chestnut lamprey may have higher expression of insulin-like growth factor 1 receptor (*igf1r*; although this was only significant with $\alpha = 0.10$). This may indicate that more growth is occurring in the gonads in chestnut lamprey, which could contribute to their greater fecundity. Age-class 2 northern brook lamprey had significantly higher expression of estrogen receptor beta (*erβ*) than age-class 2 chestnut lamprey. This gene has Gene Ontology (GO) terms such as "positive regulation of apoptotic process" and "negative regulation of cell growth" (see Table 3.3). Higher expression of *erβ* in northern brook lamprey may contribute to their reduced fecundity. Furthermore, RNA-Seq indicated that chestnut lamprey have higher expression of some genes related to

energy metabolism during ovarian differentiation (e.g., cytochrome c oxidase). If chestnut lamprey produce more oocytes during ovarian differentiation, this could account for higher energy demands. Thus, although differential timing of ovarian differentiation does not appear to be the explanation for lower fecundity in northern brook lamprey, this study identified several genes of interest for further investigation.

5.2 Implications for vertebrate sex differentiation

No previous studies have examined the genetic basis of lamprey sex differentiation. Although there are many studies of genetic factors in teleost sex differentiation (reviewed in Piferrer & Guiguen 2008; Sandra & Norma 2010; Piferrer *et al.* 2012) and a few studies of genetic factors in non-teleost fishes (e.g., Berbejillo *et al.* 2012), lampreys have been diverged from jawed fishes for approximately 510 million years (Donoghue *et al.* 2000; Near 2009; see Figure 1.1). Thus, genetic factors involved in lamprey sex differentiation may be substantially different. This study identified several genes expressed during lamprey ovarian differentiation. Understanding the roles of these genes in lamprey ovarian differentiation contributes to knowledge about the evolution of these genes and their functions across taxa.

Five genes known to be involved in sex differentiation in other taxa were amplified in chestnut and northern brook lampreys using quantitative reverse-transcriptase PCR (qRT-PCR; Table 5.1). This indicates that these genes are present in lampreys and expressed in the gonad during ovarian differentiation, although their roles

are still unclear. Of these genes, all but daz-associated protein 1 (*dazap1*) are present or have a known function in some invertebrates, suggesting that they evolved prior to the divergence of vertebrates and invertebrates. Daz-associated protein 1 or closely related genes (e.g., daz-like in medaka *Oryzias latipes*; Xu *et al.* 2007) have known functions in sex differentiation in some teleosts and tetrapods. The expression of *dazap1* in lampreys extends the phylogenetic history of the daz-like gene family into agnathans.

RNA-Seq also identified multiple genes that may be involved in lamprey ovarian differentiation and that may be important in the differentiated ovary. Several genes of interest were discussed in more detail in Chapter 4, and the presence of these genes in other taxa is summarized in Table 5.2. Only Wilms' tumour suppressor protein 1 (*wt1*) is known to be present in invertebrates; none of the other genes have yet been identified in groups more ancient than teleosts or tetrapods. The presence of these genes in lampreys suggests that they may also be present but not yet characterized in non-teleost fishes. Identifying these genes as having a potential role in ovarian differentiation in lampreys is a substantial increase in understanding of the evolution of these genes.

Several genes that are known to have conserved roles in sex determination and differentiation in other vertebrates were not successfully amplified in lampreys through qRT-PCR nor identified as genes overexpressed during ovarian differentiation through RNA-Seq (Table 5.3). Four of these five genes were identified in other vertebrates and even invertebrates; thus, these genes are probably present in lampreys in some form, although they may not all be involved in sex differentiation. The increasing availability of genomic and transcriptomic sequence for lampreys may allow these genes to be amplified in the future. One gene, cytochrome p450 aromatase 19a (*cyp19a*), was not

identified in the available transcriptomic sequence and is not known to be present in invertebrates or other basal vertebrates such as the hagfish. Thus, *cyp19a* may not be present in lampreys; this would not be surprising, because *cyp19a* converts androgens to estrogens in other vertebrates (Piferrer & Guiguen 2008), and lampreys use different steroid hormones from many other vertebrates (Bryan *et al.* 2008). Lampreys may use a different form of aromatase for this purpose.

5.3 Technical difficulties and limitations of this study

The original intention of this study was to use the results of RNA-Seq differential expression analysis (i.e., Chapter 4) to inform the selection of target genes for qRT-PCR (i.e., Chapter 3). However, due to the lengthy sample processing and data analysis times required for RNA-Seq, this was not possible. Instead, preliminary **transcriptome** assemblies were available to supply sequence for potential target genes, but whether these genes were differentially expressed was not known. In the end, none of the five target genes studied using qRT-PCR were identified as differentially expressed between any species or life stages using RNA-Seq. This is not particularly surprising; target gene selection was a "needle in a haystack" process. In addition, qPCR is more sensitive to small differences in target gene expression than RNA-Seq (Nygaard & Hovig 2009; Huestis & Marshall 2009); thus, finding differential expression of these genes with qPCR but not with RNA-Seq is not unusual. One gene that was considered as a target gene (meiotic recombination protein DMC1) but rejected because of poor amplification was

discovered to be overexpressed in stage 2 vs. stage 3 northern brook lamprey; this would be expected because meiosis occurs in stage 2 (Hardisty 1971; Hardisty *et al.* 1986). It is unfortunate that the results of RNA-Seq could not be used to inform target gene selection for this thesis. Genes of particular interest would have been 7-dehydrocholesterol reductase (for its large fold difference in expression levels between differentiating chestnut and northern brook lampreys) and cytochrome c oxidase (for its possible use as a marker of general metabolism level, as well as for the differences in expression between chestnut and northern brook lampreys). RNA-Seq also identified genes that may have a role in ovarian differentiation (*wt1*, leucine-rich repeat-containing protein 17) and the differentiated ovary (zona pellucida sperm binding protein 4). Fortunately, the sequence data generated by RNA-Seq can be used to design primers for qRT-PCR analysis of these genes in the future.

All parts of this thesis used lampreys from a limited number of rivers; northern brook lamprey were collected from three rivers and chestnut lamprey from only one. Thus, all results may be biased by environmental variation. Chapter 2 documents the potential for ovarian differentiation in age-class 1 or age-class 2 in both species; this potential remains despite any influence of environmental variation on the proportions of individuals differentiating in each year. However, gene expression differences identified through qRT-PCR and RNA-Seq may be the result of environmental differences. Future studies could examine the expression of genes of interest in parasitic and non-parasitic lamprey larvae kept under similar conditions, to determine whether these differences are innate or caused by environmental factors. Sample sizes were small for both qRT-PCR and RNA-Seq; thus, gene expression results must be considered preliminary. For qRT-

PCR, it was necessary to combine samples into age-classes in order to detect any statistically significant differences; thus, it is difficult to be sure that these differences reflect what was occurring histologically. However, it may be possible to increase sample sizes in the future, so that comparisons among gonadal stages are more statistically meaningful. For RNA-Seq, a single individual was used for each species and stage. Thus, these results are particularly vulnerable to individual variation, environmental variation, and differences in sample preparation. However, the results of RNA-Seq can be used to guide future studies using less expensive techniques with larger sample sizes (e.g., qRT-PCR; Nygaard & Hovig 2009; Huestis & Marshall 2009).

5.4 Directions for future research

The original intention of this study was to compare ovarian differentiation in the closely related species pair silver lamprey and northern brook lamprey; however, it was not possible to collect silver lamprey larvae (see Section 1.5). Although the findings of this study can provide clues as to differences between life history types, the results cannot be directly applied to paired species. A priority for future research would be to discover reliable collection locations for silver lamprey larvae and repeat this research using silver lamprey as the parasitic species. Performing RNA-Seq on silver lamprey larvae (or silver lamprey of any stage) would be particularly useful. Previous studies have used results from next generation sequencing to generate a set of single nucleotide polymorphism markers (SNPs), which can be used to study species differences and population

divergence (e.g., Renaut *et al.* 2010; Mateus *et al.* 2013); silver lamprey of any stage could be used for identification of SNPs. Using SNPs in silver and northern brook lampreys might help to identify genes that are divergent between life history types or even identify fixed sequence differences. RNA-Seq data can also be mined to discover microsatellite loci (Ekblom & Galindo 2011) and alternative splicing (Costa *et al.* 2010).

It would also be valuable to perform RNA-Seq in other life stages and tissues for whatever lamprey species are available, in order to sequence genes that may not be expressed in the gonads of larval and metamorphosing individuals. One of the uses of RNA-Seq data is the discovery of candidate genes that may be involved in processes of interest (Ekblom & Galindo 2011). This study has identified several candidate genes expressed in the gonad that may be involved in species differences and ovarian differentiation; however, other tissues may provide additional information. Lampreys, like other vertebrates, have a hypothalamic-pituitary-gonadal axis (Sower *et al.* 2009), meaning that gene expression in the gonad may be controlled by gene expression in the brain. Examining gene expression in the brain prior to and during ovarian differentiation might identify other genes that control ovarian differentiation in lampreys. As well, RNA sequencing of other tissues and life stages may identify candidate genes for other processes.

Many studies of paired lamprey species have focused on the extent to which the two life history types diverge at metamorphosis (e.g., Yamazaki *et al.* 2001; Kucheryavyi *et al.* 2007); this study examined whether the alternate pathways diverge years in advance of metamorphosis. Although the results of this study are preliminary, future studies may provide practical research tools for further studying the environmental and genetic basis

of lamprey life history type. A gene expression profile can be considered a "molecular phenotype," because gene expression is affected by both genes and the environment (Pavey *et al.* 2010). If any of the genes of interest identified in this study are later found to be reliable markers of life history type, researchers might be able to examine the gene expression profile of an individual during ovarian differentiation and determine its future life history type then, rather than waiting several more years until metamorphosis. This would facilitate experiments examining whether life history type is heritable or determined by environmental factors such as food availability (e.g., Kucheryavyi *et al.* 2007), or whether both genetic and environmental factors play a role in determining life history type. Being able to determine the future life history type of an individual several years before metamorphosis would be particularly valuable for studies such as common garden experiments or reciprocal transplant experiments.

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5.6 Tables

Table 5.1. Phylogenetic distribution of candidate genes examined through quantitative reverse-transcriptase PCR in chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor* during ovarian differentiation. "Known role" indicates genes that are known to be involved in sex differentiation or the gonad in a taxon; "Present" indicates genes that have been identified in a taxon but are not known to be involved in sex differentiation or the gonad. * indicates that the identified gene was not exactly the same gene used in this study, but instead a closely related gene.

	Gene				
Group	*hsd17β*	*gcl*	*erβ*	*igf1r*	*dazap1*
Invertebrates	Known role[1]	Known role[2]	Known role[3*]	Present[4*]	
Hagfishes			Present[3*]	Present[5]	
Cartilaginous fishes			Present[6*]	Present[5]	
Sturgeons			Present[7]		
Gars			Present[7]		
Teleosts	Known role[8]	Known role[9]	Known role[10]	Known role[11]	Known role[12*]
Tetrapods	Known role[13]	Known role[14]	Known role[15]	Known role[16]	Known role[17]

1. Zhai *et al.* 2012
2. Jongens *et al.* 1992
3. Eick & Thornton 2011
4. Lin *et al.* 2001
5. Drakenberg *et al.* 1993
6. Katsu *et al.* 2010
7. Katsu *et al.* 2008
8. Devlin & Nagahama 2002
9. Li *et al.* 2006
10. Chakraborty *et al.* 2011
11. Campbell *et al.* 2006
12. Xu *et al.* 2007
13. Labrie *et al.* 1997
14. Maekawa *et al.* 2004
15. Deroo & Korach 2006
16. Hillier 2001
17. Hsu *et al.* 2008

Table 5.2. Phylogenetic distribution of genes that may be involved in ovarian differentiation or the differentiated ovary in chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor*, identified through RNA sequencing. "Known role" indicates genes that are known to be involved in sex differentiation or the gonad in a taxon; "Present" indicates genes that have been identified in a taxon but are not known to be involved in sex differentiation or the gonad. * indicates that the identified gene was not exactly the same gene used in this study, but instead a closely related gene.

	Gene				
Group	*wt1*	*lrrc17*	*zp4*	*dmc1*	*mei1*
Invertebrates	Present[1*]				
Hagfishes					
Cartilaginous fishes					
Sturgeons					
Gars					
Teleosts	Known role[2]		Present[3]	Known role[4]	Present[5]
Tetrapods	Known role[6]	Known role[7]	Known role[8]	Known role[9]	Known role[10]

1. Shimeld 2008
2. Sandra & Norma 2010
3. Goudet *et al.* 2008
4. Kajiura-Kobayashi *et al.* 2005
5. Libby *et al.* 2003
6. Scharnhorst *et al.* 2001
7. Malcuit *et al.* 2009
8. Sinowatz *et al.* 2001
9. Yoshida *et al.* 1998
10. Libby *et al.* 2002

Table 5.3. Genes that are known to have a conserved role in sex differentiation in vertebrates but were not successfully amplified in chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor* using transcriptome sequence. "Known role" indicates genes that are known to be involved in sex differentiation or the gonad in a taxon; "Present" indicates genes that have been identified in a taxon but are not known to be involved in sex differentiation or the gonad. * indicates that the identified gene was not exactly the same gene used in this study, but instead a closely related gene. For more details on these genes, see Table 3.5.

	Gene				
Group	*cyp19a*	*dmrt1*	*foxl2*	*sf1*	*sox9*
Invertebrates		Known role[1]	Known role[1]	Known role[2*]	Known role[3*]
Hagfishes					Present[4]
Cartilaginous fishes	Known role[5]				
Sturgeons		Known role[6]	Known role[7]		Known role[6]
Gars					Present[8]
Teleosts	Known role[9]	Known role[9]	Known role[9]	Known role[9]	Known role[9]
Tetrapods	Known role[10]	Known role[11]	Known role[11]	Known role[11]	Known role[11]
In this study	Sequence not found	Failed to amplify	Failed to amplify	Failed to amplify	Non-specific amplification

1. Ma *et al.* 2012
2. Asahina *et al.* 2000
3. DeFalco *et al.* 2003
4. Ota *et al.* 2007
5. Ijiri *et al.* 2000
6. Berbejillo *et al.* 2012
7. Amberg *et al.* 2010
8. Eames *et al.* 2012
9. Sandra & Norma 2010
10. Bulun *et al.* 2003
11. Wilhelm *et al.* 2007

APPENDIX 1: GLOSSARY

blastx: a search method using the Basic Local Alignment Search Tool (BLAST). In this method, a nucleotide sequence is translated and compared to a protein sequence database (Claverie & Notredame 2006).

Contig: a contiguous nucleotide sequence generated from overlapping shorter sequences (Ekblom & Galindo 2011)

E-value: in BLAST, a measure of the probability of finding a sequence alignment by chance; lower e-values indicate a better alignment (Claverie & Notredame 2006)

Gene Ontology (GO) term: a term that indicates a particular cellular component, molecular function, or biological process that may be associated with a gene product (Ashburner *et al.* 2000). Multiple GO terms are typically assigned to a single gene (e.g., as in Table 3.3).

Life history types: Members of the same or related species that have very different life history characteristics (e.g., morphology, feeding, reproduction, growth, age at maturity, habitat use). These different life history characteristics may have a genetic basis, an environmental basis, or some combination of the two. In lampreys, the major groupings of life history types are parasitic versus non-parasitic and migratory versus non-migratory.

Non-normalized sequence library: a sequence library generated for next generation sequencing without the use of procedures to equalize the abundance of different transcripts. Non-normalized libraries can be used to compare relative abundance of different transcripts, whereas normalized libraries cannot (Ekblom & Galindo 2011).

Read: a short nucleotide sequence generated through next generation sequencing (Ekblom & Galindo 2011)

Sex determination: the event that "predisposes a bipotential gonad to develop as an ovary or a testicle" (Sandra & Norma 2010)

Sex differentiation: the process by which the phenotypic sex of an individual is produced (Piferrer & Guiguen 2008); may be subdivided into ovarian differentiation and testicular differentiation

Sexual maturation: the final changes in a gonad prior to reproduction (Patiño & Sullivan 2002)

Transcriptome: all RNA sequences produced in a particular tissue, at a particular developmental stage, in a particular organism (Ekblom & Galindo 2011)

Ashburner M, Ball C, Blake J, Botstein D (2000) Gene Ontology: tool for the unification of biology. *Nature Genetics*, **25**, 25–29.

Claverie J-M, Notredame C (2006) *Bioinformatics for Dummies 2nd Edition*. Wiley Publishing, Inc., Hoboken, New Jersey.

Ekblom R, Galindo J (2011) Applications of next generation sequencing in molecular ecology of non-model organisms. *Heredity*, **107**, 1–15.

Patiño R, Sullivan C (2002) Ovarian follicle growth, maturation, and ovulation in teleost fish. *Fish Physiology and Biochemistry*, **26**, 57–70.

Piferrer F, Guiguen Y (2008) Fish gonadogenesis. Part II: Molecular biology and genomics of sex differentiation. *Reviews in Fisheries Science*, **16**, 35–55.

Sandra G-E, Norma M-M (2010) Sexual determination and differentiation in teleost fish. *Reviews in Fish Biology and Fisheries*, **20**, 101–121.

APPENDIX 2. AMINO ACID SEQUENCE ALIGNMENTS AND NEIGHBOUR-JOINING TREES FOR TARGET AND REFERENCE GENES USED FOR QUANTITATIVE REVERSE-TRANSCRIPTASE PCR

Table A2.1. List of amino acid sequences used to construct the alignment (Figure A2.1) and neighbour-joining tree (Figure A2.2) for alpha tubulin (alpha-tub).

Species	Common name	GenBank accession number	Gene name
Callorhinchus milii	Elephant shark	gi|392884398|gb|AFM91031.1|:174-430	Tubulin alpha chain
Canis lupus familiaris	Domestic dog	XP_534765	PREDICTED: tubulin alpha-3 chain-like
Chelonia mydas	Green sea turtle	EMP38011	Tubulin alpha-3 chain
Ciona intestinalis	Sea squirt	XP_002129440	PREDICTED: tubulin alpha-1A chain isoform 1
Columba livia	Rock dove	EMC81649	Tubulin alpha-3 chain, partial
Danio rerio	Zebrafish	gi|300244539|ref|NP_001177911.1|:174-430	tubulin, alpha 1, like 2
Dicentrarchus labrax	European seabass	gi|32895272|gb|AAP89017.1|:108-364	alpha-tubulin
Esox lucius	Northern pike	gi|225716392|gb|ACO14042.1|:174-430	Tubulin alpha chain
Homo sapiens	Human	AAC39578	alpha tubulin
Mus musculus	House mouse	AAH04790	TubA2b protein
Myxine glutinosa	Atlantic hagfish	gi|21667239|gb|AAM73997.1|:98-336	alpha-tubulin 1
Oryzias latipes	Medaka	gi|432932029|ref|XP_004081750.1|:174-430	PREDICTED: tubulin alpha chain-like
Osmerus mordax	Rainbow smelt	gi|225707296|gb|ACO09494.1|:174-430	Tubulin alpha-1 chain
Salmo salar	Atlantic salmon	gi|197632605|gb|ACH71026.1|:174-430	tubulin, alpha 8 like 3-2
Xenopus tropicalis	Western clawed frog	XP_002934010	PREDICTED: tubulin alpha-1D chain-like

```
Callorhinchus milii alpha-tub   APQASTAVVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIDRPTYTNLNRLISQ
        Canis lupus alpha-tub   APQVSTAVVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLIGQ
     Chelonia mydas alpha-tub   APQVSTAVVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLIGQ
  Ciona intestinalis alpha-tub   APQVSTAVVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLMGQ
       Columba livia alpha-tub   APQVSTAVVEPYNTILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLIGQ
         Danio rerio alpha-tub   APQVSTAVVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLIGQ
Dicentrarchus labrax alpha-tub   APQVSTAVIEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPSYTNLNRLISQ
         Esox lucius alpha-tub   APQVSTAVVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPSYTNLNRLIGQ
        Homo sapiens alpha-tub   APQVSTAVVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLIGQ
        Mus musculus alpha-tub   APQVSTAVVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLISQ
    Myxine glutinosa alpha-tub   APQVSTAVVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLIGQ
     Oryzias latipes alpha-tub   APQVSTAVVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLIGQ
      Osmerus mordax alpha-tub   APQVSTAVVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLIGQ
         Salmo salar alpha-tub   APQVSTAVVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLIGQ
  Xenopus tropicalis alpha-tub   APQISTAVVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLIGQ
  Petromyzon marinus alpha-tub   APQVSTAVVEPYN?ILTTHTTLEHSDCAFMVDNEAIYDI?RRNLDIERPTYTNLNRLIGQ

Callorhinchus milii alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVSEITN
        Canis lupus alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVAEITN
     Chelonia mydas alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVAEITN
  Ciona intestinalis alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVAEITN
       Columba livia alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLVTYAPVISAEKAYHEQLSVAEITN
         Danio rerio alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVAEITN
Dicentrarchus labrax alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAERAYHEQLSVAEITN
         Esox lucius alpha-tub   IVSSITASLRFDGVLNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVADITN
        Homo sapiens alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVAEITN
        Mus musculus alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVAEITN
    Myxine glutinosa alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVAEITN
     Oryzias latipes alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVADITN
      Osmerus mordax alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVADITN
         Salmo salar alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVADITN
  Xenopus tropicalis alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVSEITN
  Petromyzon marinus alpha-tub   IVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSV?EITN

Callorhinchus milii alpha-tub   ACFEPANQMVKCDPRHGKYMACCLLYRGDVVPKDVNAAIATIKTKRSIQFVDWCPTGFKV
        Canis lupus alpha-tub   ACFEPANQMVKCDPRHGKYMACCMLYRGDVVPKDVNAAIATIKTKRTIQFVDWCPTGFKV
     Chelonia mydas alpha-tub   ACFEPANQMVKCDPRHGKYMACCMLYRGDVVPKDVNAAIATIKTKRTIQFVDWCPTGFKV
  Ciona intestinalis alpha-tub   ACFEPANQMVKCDPRHGKYMACCMLYRGDVVPKDVNAAIATIKTKRTIQFVDWCPTGFKV
       Columba livia alpha-tub   ACFEPANQMVKCDPRHGKYMACCMLYRGDVVPKDVNAAIATIKTKRTIQFVDWCPTGFKV
         Danio rerio alpha-tub   ACFEPANQMVKCDPRHGKYMACCLLYRGDVVPKDVNAAIATIKTKRTIQFVDWCPTGFKV
Dicentrarchus labrax alpha-tub   SCFEPANQMVKCDPRHGKYMACCLLYRGDVVPKDVNVAIAAIKTKRSIQFVDWCPTGFKV
         Esox lucius alpha-tub   ACFEPANQMVKCDPRHGKYMACCLLYRGDVVPKDVNSAIAAIKTIRSIQFVDWCPTGFKV
        Homo sapiens alpha-tub   ACFEPANQMVKCDPRHGKYMACCMLYRGDVVPKDVNAAIATIKTKRTIQFVDWCPTGFKV
        Mus musculus alpha-tub   ACFEPANQMVKCDPRHGKYMACCLLYRGDVVPKDVNAAIATIKTKRSIQFVDWCPTGFKV
    Myxine glutinosa alpha-tub   ACFEPANQMVKCDPRHGKYMACCMLYRGDVVPKDVNAAIATIKTKRTIQFVDWCPTGFKV
     Oryzias latipes alpha-tub   ACFEPANQMVKCDPRHGKYMACCLLYRGDVVPKDVNSAIAAIKTKRTIQFVDWCPTGFKV
      Osmerus mordax alpha-tub   ACFEPANQMVKCDPRHGKYMACCLLYRGDVVPKDVNSAIATIKTKRTIQFVDWCPTGFKV
         Salmo salar alpha-tub   ACFEPANQMVKCDPRHGKYMACCLLYRGDVVPKDVNSAIATIKTKRTIQFVDWCPTGFKV
  Xenopus tropicalis alpha-tub   ACFEPANQMVKCDPRHGKYMACCMLYRGDVVPKDVNAAIATIKTKRTIQFVDWCPTGFKV
  Petromyzon marinus alpha-tub   ACFEPANQMVKCDPRHGKYMACCMLYRGDVVPKDVNAAIA?IKTKR?IQFVDWCPTGFKV

Callorhinchus milii alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM
        Canis lupus alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM
     Chelonia mydas alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM
  Ciona intestinalis alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM
       Columba livia alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM
         Danio rerio alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM
Dicentrarchus labrax alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM
         Esox lucius alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM
        Homo sapiens alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM
        Mus musculus alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM
    Myxine glutinosa alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEG-
     Oryzias latipes alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM
      Osmerus mordax alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM
         Salmo salar alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM
  Xenopus tropicalis alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM
  Petromyzon marinus alpha-tub   GINYQPPTVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGM

Callorhinchus milii alpha-tub   EEGEFSEAREDMAALEK
        Canis lupus alpha-tub   EEGEFSEAREDLAALEK
     Chelonia mydas alpha-tub   EEGEFSEAREDLAALEK
  Ciona intestinalis alpha-tub   EEGEFSEAREDLAALEK
       Columba livia alpha-tub   EEGEFSEAREDLAALEK
         Danio rerio alpha-tub   EEGEFSEAREDMAALEK
Dicentrarchus labrax alpha-tub   EEGEFSEAREDMAALEK
         Esox lucius alpha-tub   EEGEFSEAREDMAALEK
        Homo sapiens alpha-tub   EEGEFSEAREDLAALEK
        Mus musculus alpha-tub   EEGEFSEAREDMAALEK
    Myxine glutinosa alpha-tub   ----------------
     Oryzias latipes alpha-tub   EEGEFSEAREDMAALEK
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     Osmerus mordax alpha-tub   EEGEFSEAREDMAALEK
        Salmo salar alpha-tub   EEGEFSEAREDMAALEK
 Xenopus tropicalis alpha-tub   EEGEFSEAREDMAALEK
 Petromyzon marinus alpha-tub   EEGEFSEAREDLAALEK
```

Figure A2.1. Alignment of translated amino acid sequence for *Petromyzon marinus* alpha tubulin (alpha-tub) to alpha-tub amino acid sequences from other chordate species. Sequences are in alphabetical order by scientific name, except for the lamprey sequence, which is given at the bottom of each section. Locations of primers are highlighted in light gray (forward) and dark gray (reverse). GenBank accession numbers and common names are given in Table A2.1.

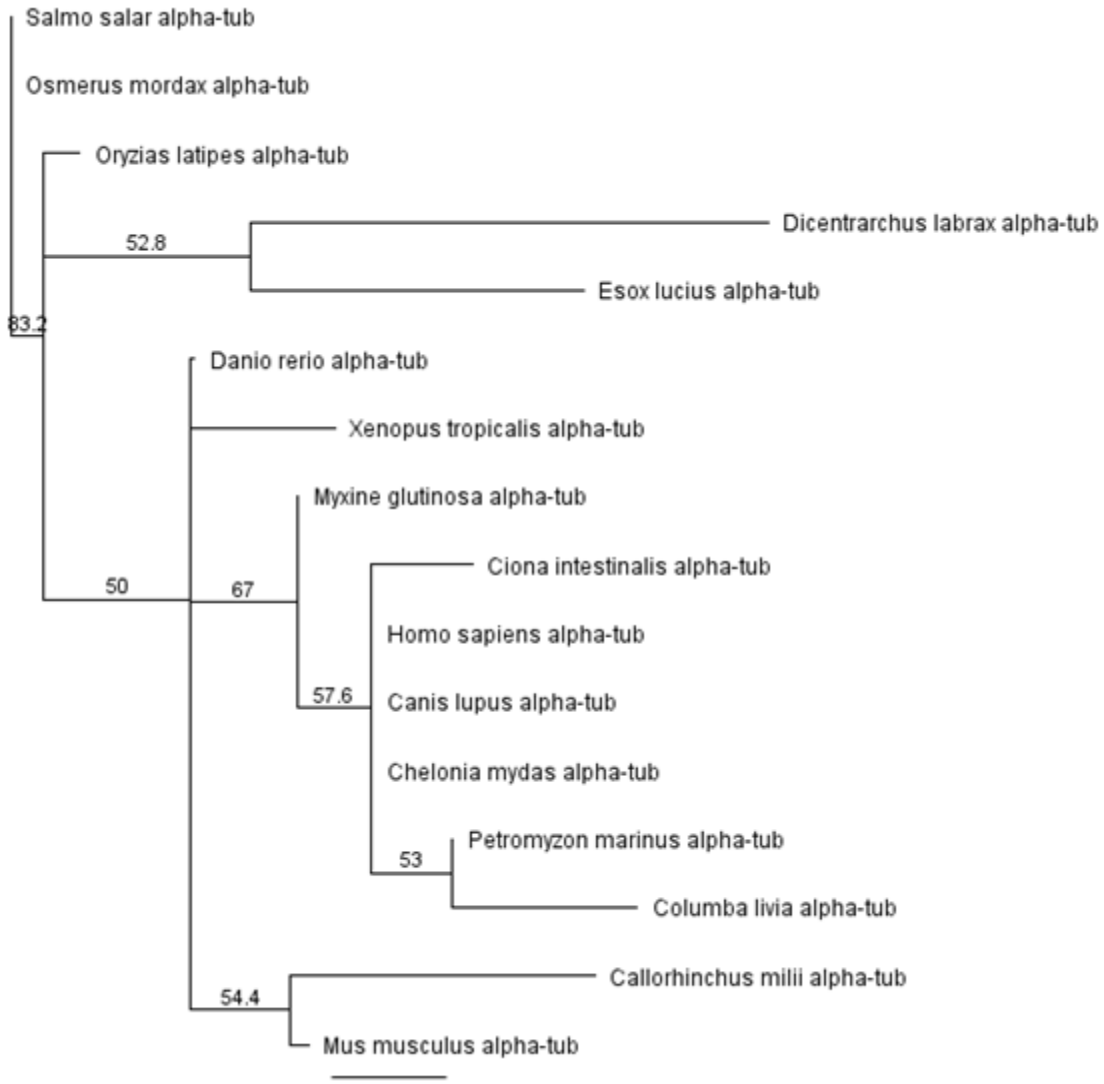


Figure A2.2. Neighbour-joining tree for alpha tubulin (alpha-tub) amino acid sequences. This tree was constructed in Geneious using the Jukes-Cantor genetic distance model and 500 bootstrap replicates. GenBank accession numbers and common names are given in Table A2.1.

Table A2.2. List of amino acid sequences used to construct the alignment (Figure A2.3) and neighbour-joining tree (Figure A2.4) for 17-beta hydroxysteroid dehydrogenase (17betaHSD).

Species	Common name	GenBank accession number	Gene name
Anguilla japonica	Japanese eel	gi|40647365|gb|AAR88433.1|:62-194	17b-hydroxysteroid dehydrogenase type I
Callorhinchus milii	Elephant shark	gi|387915544|gb|AFK11381.1|:67-274	hydroxysteroid dehydrogenase like 1
Canis lupus familiaris	Domestic dog	XP_544970	PREDICTED: estradiol 17-beta-dehydrogenase 11
Cavia porcellus	Guinea pig	XP_003469425	PREDICTED: estradiol 17-beta-dehydrogenase 11-like
Columba livia	Rock dove	EMC85030	Estradiol 17-beta-dehydrogenase 11
Felis catus	Domestic cat	XP_003985243	PREDICTED: estradiol 17-beta-dehydrogenase 11 isoform 1
Gallus gallus	Domestic chicken	XP_420547	PREDICTED: estradiol 17-beta-dehydrogenase 11 isoform 2
Loxodonta africana	African elephant	XP_003414237	PREDICTED: estradiol 17-beta-dehydrogenase 11-like
Oryzias dancena	Indian ricefish	gi|224473810|gb|ACN49151.1|:11-219	estradiol 17 beta-dehydrogenase 8
Ovis aries	Domestic sheep	XP_004010030	PREDICTED: estradiol 17-beta-dehydrogenase 11
Xenopus tropicalis	Western clawed frog	NP_001011304	hydroxysteroid (17-beta) dehydrogenase 11



Figure A2.3. Alignment of translated amino acid sequence for *Petromyzon marinus* 17-beta hydroxysteroid dehydrogenase (17betaHSD) to 17betaHSD amino acid sequences from other species. Sequences are in alphabetical order by scientific name, except for the lamprey sequence, which is given at the bottom of each section. Locations of primers are highlighted in light gray (forward) and dark gray (reverse). GenBank accession numbers and common names are given in Table A2.2.

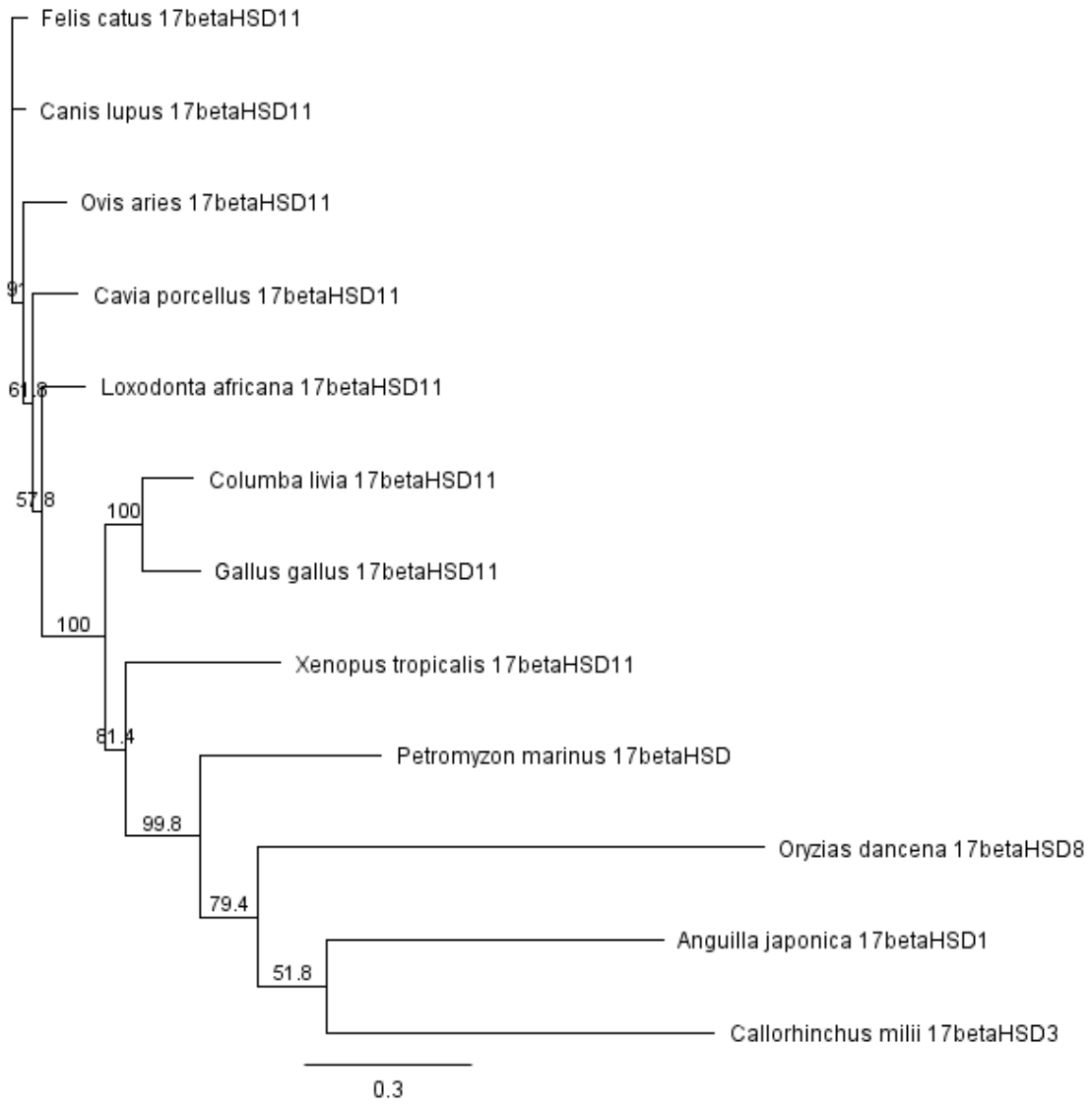


Figure A2.4. Neighbour-joining tree for 17-beta hydroxysteroid dehydrogenase (17betaHSD) amino acid sequences. This tree was constructed in Geneious using the Jukes-Cantor genetic distance model and 500 bootstrap replicates. GenBank accession numbers and common names are given in Table A2.2.

Table A2.3. List of amino acid sequences used to construct the alignment (Figure A2.5) and neighbour-joining tree (Figure A2.6) for germ cell-less 1 (GCless1).

Species	Common name	Gen Bank accession number	Gene name
Canis lupus familiaris	Domestic dog	XP_538534	PREDICTED: germ cell-less protein-like 1 isoform 1
Cavia porcellus	Guinea pig	XP_003468977	PREDICTED: germ cell-less protein-like 1-like
Ciona intestinalis	Sea squirt	XP_002125481	PREDICTED: germ cell-less protein-like 1-like
Danio rerio	Zebrafish	NP_996933	germ cell-less protein-like 1
Felis catus	Domestic cat	XP_003984137	PREDICTED: germ cell-less protein-like 1
Gallus gallus	Domestic chicken	XP_423987	PREDICTED: germ cell-less protein-like 1, partial
Oreochromis niloticus	Nile tilapia	XP_003444365	PREDICTED: germ cell-less protein-like 1
Sus scrofa	Domestic pig	XP_003125107	PREDICTED: germ cell-less protein-like 1-like
Taeniopygia guttata	Zebra finch	XP_002192180	PREDICTED: germ cell-less protein-like 1
Takifugu rubripes	Pufferfish	XP_003965640	PREDICTED: germ cell-less protein-like 1-like

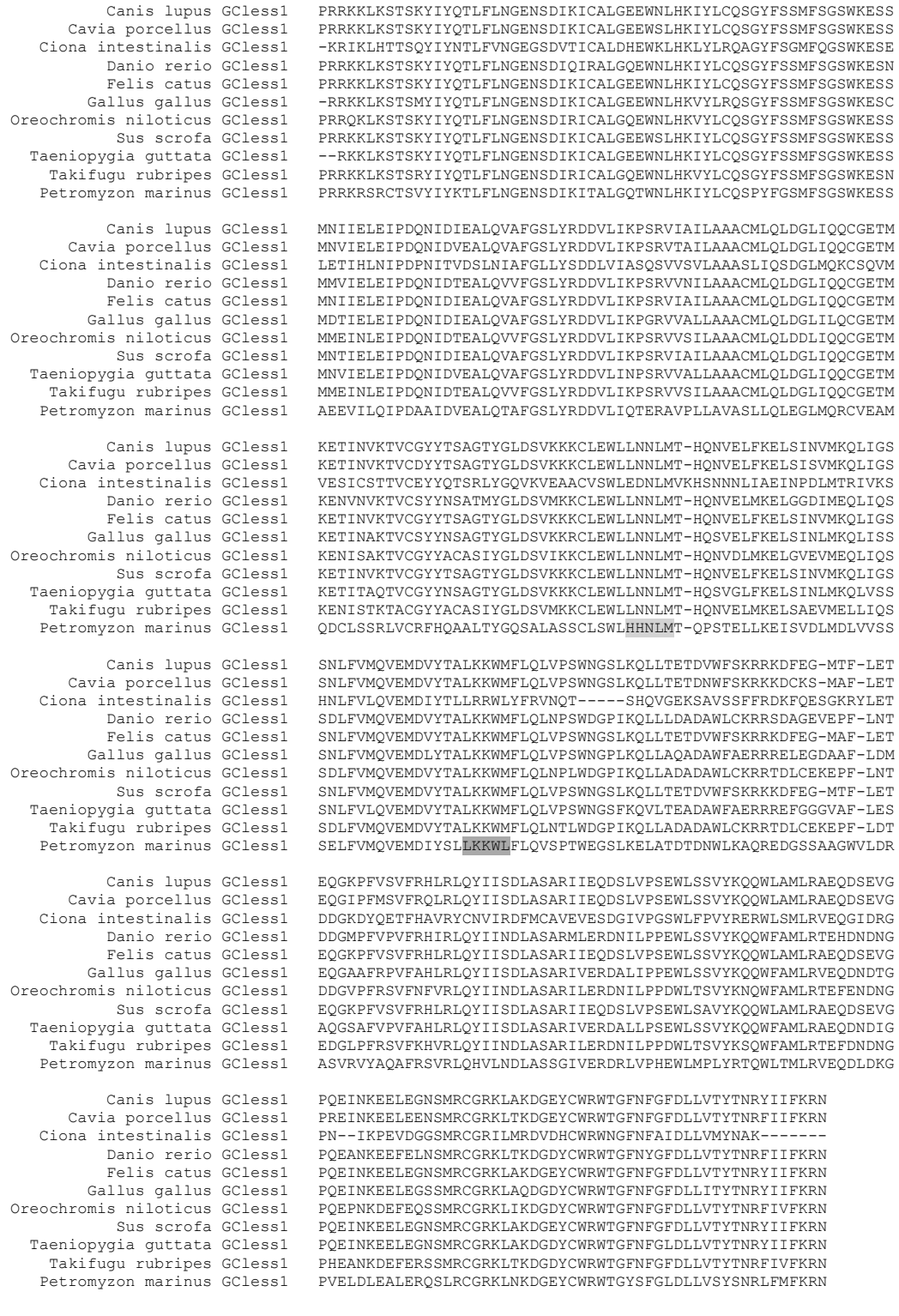


Figure A2.5. Alignment of translated amino acid sequence for *Petromyzon marinus* germ cell-less 1 (GCless1) to GCless1 amino acid sequences from other species. Sequences are in alphabetical order by scientific name, except for the lamprey sequence, which is

given at the bottom of each section. Locations of primers are highlighted in light gray (forward) and dark gray (reverse). GenBank accession numbers and common names are given in Table A2.3.

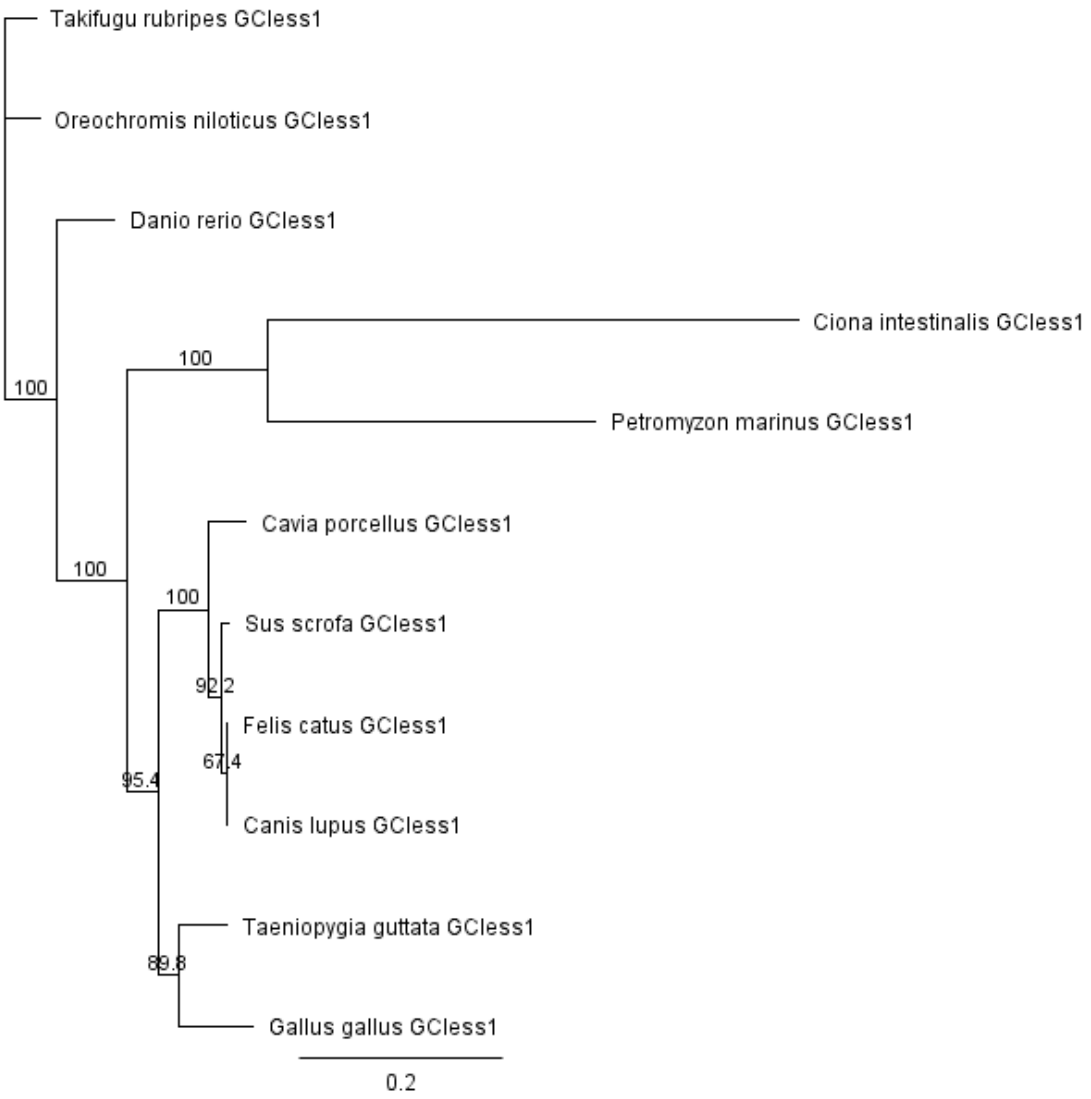


Figure A2.6. Neighbour-joining tree for germ cell-less 1 (GCless1) amino acid sequences. This tree was constructed in Geneious using the Jukes-Cantor genetic distance model and 500 bootstrap replicates. GenBank accession numbers and common names are given in Table A2.3.

Table A2.4. List of amino acid sequences used to construct the alignment (Figure A2.7) and neighbour-joining tree (Figure A2.8) for estrogen receptor beta (ERbeta). Some estrogen receptor alpha (ERalpha) sequences were also used.

Species	Common name	GenBank accession number	Gene name
Atractosteus tropicus	Tropical gar	BAG82654	estrogen receptor beta
Capra hircus	Goat	ACF24870	estrogen receptor beta
Carassius auratus	Goldfish	AAL12298	estrogen receptor alpha
Danio rerio	Zebrafish	CAB77022	estrogen receptor alpha
Gorilla gorilla	Gorilla	ABY64728	estrogen receptor beta
Homo sapiens	Human	BAD92857	estrogen receptor 2 (ER beta) variant
Latimeria menadoensis	Indonesian coelacanth	CCP19149	estrogen receptor-beta
Loxodonta africana	African elephant	XP_003408751	PREDICTED: estrogen receptor beta-like
Meleagris gallopavo	Indian peafowl	XP_003206826	PREDICTED: estrogen receptor beta-like
Mus musculus	House mouse	AAF22561	estrogen receptor alpha
Ovis aries	Domestic sheep	NP_001009737	estrogen receptor beta
Pan paniscus	Bonobo	ABY64727	estrogen receptor beta
Scyliorhinus torazame	Cloudy catshark	BAJ15288	estrogen receptor beta
Squalus acanthias	Spiny dogfish	AAK57823	estrogen receptor beta
Sus scrofa	Domestic pig	NP_001001533	estrogen receptor beta
Taeniopygia guttata	Zebra finch	XP_002200631	PREDICTED: estrogen receptor beta

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 Atracosteus tropicus ERbeta   AEPPDVYLMDYPKKPFTEATMMMSLTNLADKELVHMISWAKKIPGFMEFSLYDQVRLLEC
         Capra hircus ERbeta   AEPPHV-LMSRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLYDQVRLLES
    Carassius auratus ERalpha  AEPPAVCSRQKHSRPYTEITMMSLLTNMADKELVHMIAWAKKVPGFQDLSLHDKVQLLES
      Cyprinus carpio ERalpha  AEPPAVCSRQKHSRPYTEITMMSLLTNMADKELVHMIAWAKKVPGFQDLSLHDQVQLLES
          Danio rerio ERalpha  AEPPAVCSRQKHSRPYTEITMMSLLTNMADKELVHMIAWAKKVPGFQDLSLHDQVQLLES
       Gorilla gorilla ERbeta   AEPPHV-LISRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLFDQVRLLES
          Homo sapiens ERbeta   AEPPHV-LISRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLFDQVRLLES
 Latimeria menadoensis ERbeta   AEPPEVLLLVHPKKPFTEASMMMSLTNLADKELVHMISWAKKIPGFIELNLYDQVRLLES
    Loxodonta africana ERbeta   AEPPNV-LVSRPTSPFTEASMMMSLTKLADKELVHMIGWAKKIPGFVELSLYDQVRLLES
   Meleagris gallopavo ERbeta   AEPPNV-LVSRPSKPFTEASMMMSLTKLADKELVHMIGWAKKIPGFIDLSLYDQVRLLES
         Mus musculus ERalpha   AEPPMIYSEYDPSRPFSEASMMGLLTNLADRELVHMINWAKRVPGFGDLNLHDQVHLLEC
            Ovis aries ERbeta   AEPPHV-LMSRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLYDQVRLLES
          Pan paniscus ERbeta   AEPPHV-LISRPSAPFTEASMMMSLTKLADKELVHMISWAKKIPGFVELSLFDQVRLLES
 Scyliorhinus torazame ERbeta   AEPPNVYSLKHPNKPYTEASMMMSLTNLADRELVHMIAWAKKVPGFLELDLHEQVQLLEC
     Squalus acanthias ERbeta   AEPPNVYSLNHPNKPYTEVSMMMSLTNLADRELVHMIAWAKKVPGFVELDLHDQVQLLEC
           Sus scrofa ERbeta   AEPPHV-LVSRPSTPFTEASMMMSLTKLADKELVHMISWAKKIPGFMELSLYDQVRLLES
   Taeniopygia guttata ERbeta   AEPPHV-LVSRPSKPFTEASMMMSLTKLADKELVHMIGWAKKIPGFIDLSLYDQVRLLES
   Ichthyomyzon fossor ERbeta   AEPPKVLSLQESKQPFSEASLMNVLTNLADRELVHMIAWAKKIPGFMDINLEDQVQLLES

 Atracosteus tropicus ERbeta   CWLEVLMIGLMWRSVDHPGKLIFSRDLTLNRDEGNCVEGIVEIFDMLLAATSRFRELKLQ
         Capra hircus ERbeta   CWLEVLMVGLMWRSIDHPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLATTSRFRELKLQ
    Carassius auratus ERalpha  TWLEVLMIGLIWRSIHSPGKLIFAQDLILDRNEGECVEGMAEIFDMLLATVTRFRSLKLK
      Cyprinus carpio ERalpha  TWLEVLMIGLIWRSIHSPGKLIFAQDLILDRSEGECVEGMAEIFDMLLATVTRFRNLKLK
          Danio rerio ERalpha  SWLEVLMIGLIWRSIHSPGKLIFAQDLILDRSEGECVEGMAEIFDMLLATVARFRSLKLK
       Gorilla gorilla ERbeta   CWMEVLMVGLMWRSIDHPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLATTSRFRELKLQ
          Homo sapiens ERbeta   CWMEVLMVGLMWRSIDHPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLATTSRFRELKLQ
 Latimeria menadoensis ERbeta   SWLEILMVGLMWRSIDYPGKLIFAPDLILDRDEGTCVEGILEIFDLLLVTTARFRELKLQ
    Loxodonta africana ERbeta   CWMEVLMVGLMWRSIDHPGKLIFAPDLILDRDEGKCVEGILEIFDMLLATTSRFRELKLQ
   Meleagris gallopavo ERbeta   CWMEVLMIGLMWRSIDHPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLAMTSRFRELKLQ
         Mus musculus ERalpha   AWLEILMIGLVWRSMEHPGKLLFAPNLLLDRNQGKCVEGMVEIFDMLLATSSRFRMMNLQ
            Ovis aries ERbeta   CWLEVLMVGLMWRSIDHPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLATTSRFRELKLQ
          Pan paniscus ERbeta   CWMEVLMVGLMWRSIDHPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLATTSRFRELKLQ
 Scyliorhinus torazame ERbeta   CWLEVLMVGLMWRSIEYPGKLLFAPDLILDRDEGQCVEGILEIFDMLLAATSRFRELKLQ
     Squalus acanthias ERbeta   CWLEVLMVGLMWRSIEYPGKLLFAPDLILDRDEGQCVEGILEIFDMLLAATSRFRDLKLQ
           Sus scrofa ERbeta   CWLEVLMVGLMWRSIDHPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLATTSRFRELKLQ
   Taeniopygia guttata ERbeta   CWMEVLMIGLMWRSIDHPGKLIFAPDLVLDRDEGKCVEGILEIFDMLLAMTSRFRELKLQ
   Ichthyomyzon fossor ERbeta   CWLEVLMVGLIWRSMTHPGKLVFAADLVLERDDGCCVEGIVDIFDLLLRTTSQFIELSVT

 Atracosteus tropicus ERbeta   REEYLCLKAMILLNS
         Capra hircus ERbeta   HKEYLCVKAMILLNS
    Carassius auratus ERalpha  LEEFVCLKALILLNS
      Cyprinus carpio ERalpha  LEEFVCLKAIILLNS
          Danio rerio ERalpha  LEEFVCLKAIILINS
       Gorilla gorilla ERbeta   HKEYLCVKAMILLNS
          Homo sapiens ERbeta   HKEYLCVKAMILLNS
 Latimeria menadoensis ERbeta   QQEYLCLKAMVLLNS
    Loxodonta africana ERbeta   HKEYLCVKAMILLNS
   Meleagris gallopavo ERbeta   HKEYLCVKAMILLNS
         Mus musculus ERalpha   GEEFVCLKSIILLNS
            Ovis aries ERbeta   HKEYLCVKAMILLNS
          Pan paniscus ERbeta   HKEYLCVKAMILLNS
 Scyliorhinus torazame ERbeta   HEEYLCLKAMVLLNS
     Squalus acanthias ERbeta   HEEYLCLKAMVLLNS
           Sus scrofa ERbeta   HKEYLCVKAMILLNS
   Taeniopygia guttata ERbeta   HKEYLCVKAMILLNS
   Ichthyomyzon fossor ERbeta   LEEFICLKAMVLLNS
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Figure A2.7. Alignment of translated amino acid sequence for *Ichthyomyzon fossor* estrogen receptor beta (ERbeta) to ERbeta and estrogen receptor alpha (ERalpha) sequences from other species. Sequences are in alphabetical order by scientific name, except for the lamprey sequence, which is given at the bottom of each section. Locations of primers are highlighted in light gray (forward) and dark gray (reverse). GenBank accession numbers and common names are given in Table A2.4.

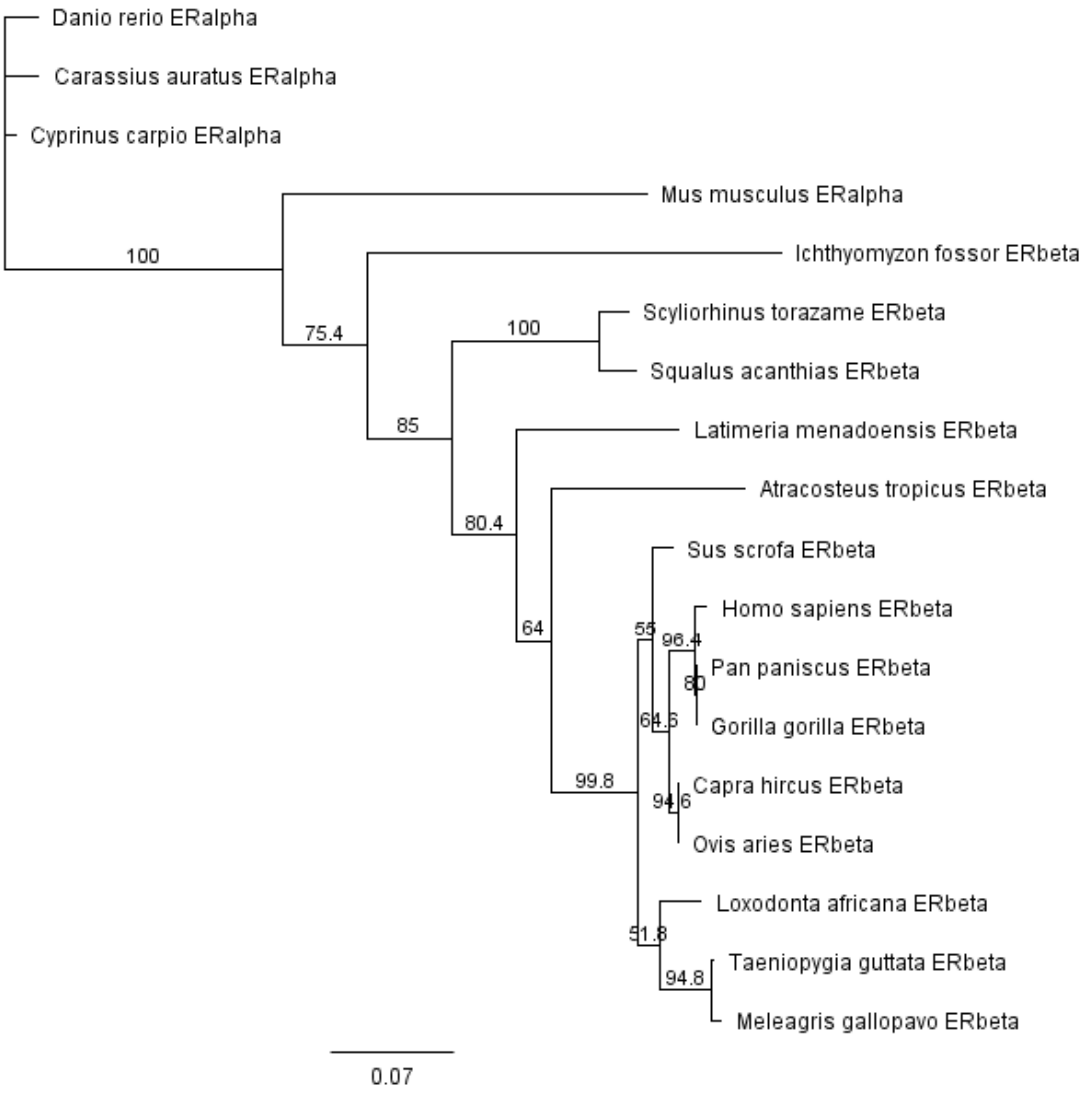


Figure A2.8. Neighbour-joining tree for estrogen receptor beta (ERbeta) and estrogen receptor alpha (ERalpha) amino acid sequences. This tree was constructed in Geneious using the Jukes-Cantor genetic distance model and 500 bootstrap replicates. GenBank accession numbers and common names are given in Table A2.4.

Table A2.5. List of amino acid sequences used to construct the alignment (Figure A2.9) and neighbour-joining tree (Figure A2.10) for insulin-like growth factor 1 receptor (IGF1R).

Species	Common name	Gen Bank accession number	Gene name
Acipenser ruthenus	Sterlet	ABC69707	insulin-like growth factor 1 receptor
Bos taurus	Domestic cattle	AEX15867	insulin-like growth factor 1 receptor, partial
Capra hircus	Goat	AEK98517	insulin-like growth factor-I receptor
Chelonia mydas	Green sea turtle	EMP41689	Insulin-like growth factor 1 receptor, partial
Columba livia	Rock dove	EMC77329	Insulin-like growth factor 1 receptor
Coturnix japonica	Japanese quail	BAF73400	insulin-like growth factor 1 receptor
Ctenopharyngodon idella	Grass carp	ACF33512	insulin-like growth factor 1 receptor type b
Epinephelus coioides	Orange-spotted grouper	AAX09649	insulin-like growth factor I receptor form B
Felis catus	Domestic cat	XP_003986920	PREDICTED: insulin-like growth factor 1 receptor
Gallus gallus	Domestic chicken	AGG38009	insulin-like growth factor I receptor
Heterocephalus glaber	Naked mole rat	EHA99789	Insulin-like growth factor 1 receptor
Morone chrysops	White bass	AAO73860	insulin-like growth factor I receptor
Oreochromis mossambicus	Mozambique tilapia	AAN77006	insulin-like growth factor-I receptor
Ornithorhynchus anatinus	Duck-billed platypus	XP_001508767	PREDICTED: insulin-like growth factor 1 receptor-like
Pimephales promelas	Fathead minnow	AAT45196	insulin-like growth factor-I receptor
Pongo abelii	Sumatran orangutan	NP_001125259	insulin-like growth factor 1 receptor
Xenopus laevis	African clawed frog	CAA90517	insulin-like growth factor I receptor
Xenopus tropicalis	Western clawed frog	XP_002933351	PREDICTED: insulin-like growth factor 1 receptor

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      Acipenser ruthenus IGF1R   IAKGVVKDEPETRVAIKTVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
              Bos taurus IGF1R   VAKGVVKDEPETRVAIKTVNEAASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
            Capra hircus IGF1R   VAKGVVKDEPETRVAIKTVNEAASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
          Chelonia mydas IGF1R   IAKGVVKDEPETRVAIKTVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
           Columba livia IGF1R   IAKGVVKDEPETRVAIKTVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
       Coturnix japonica IGF1R   IAKGVVKDEPETRVAIKTVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
 Ctenopharyngodon idella IGF1R   -AKGVVKDEPETRVAIKTVNESASLHERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
    Epinephelus coioides IGF1R   -AKGVVKDEPETRVAIKTVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
             Felis catus IGF1R   VAKGVVKDEPETRVAIKTVNEAASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
           Gallus gallus IGF1R   IAKGVVKDEPETRVAIKTVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
   Heterocephalus glaber IGF1R   VAKGVVKDEPETRVAIKTVNEAASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
         Morone chysops IGF1R   LAKGVVKDEPETRVAIKTVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
 Oreochromis mossambicus IGF1R   IAKGVVKDEPETRVAIKTVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
Ornithorhynchus anatinus IGF1R   IAKGVVKDEPETRVAIKTVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
     Pimephales promelas IGF1R   --KGVVKDEPETRVAIKTVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
           Pongo abelii IGF1R   VAKGVVKDEPETRVAIKTVNEAASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
         Xenopus laevis IGF1R   IAKGVVKDEAETKVAIKTVNEAASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
       Xenopus tropicalis IGF1R   IAKGVVKDEAETRVAIKTVNESASMRERIEFLNEASVMKEFNCHHVVRLLGVVSQGQPTL
       Icthyomyzon fossor IGF1R   MARGIVKGEDETRVAIKTVNETATMRERYEFLNEASVMKSFNCHHVVRLLGVVSQAQPIL

      Acipenser ruthenus IGF1R   VIMELMTRGDLKSYLRSLRSEAEKNTGQPLPP-LKKMIQMTGEIADGMSYLNANKFVHRD
              Bos taurus IGF1R   VIMELMTRGDLKSYLRSLRPEME-NNPVLAPPSLSKMIQMAGEIADGMAYLNANKFVHRD
            Capra hircus IGF1R   VIMELMTRGDLKSYLRSLRPEME-NNPVLAPPSLSKMIQMAGEIADGMAYLNANKFVHRD
          Chelonia mydas IGF1R   VIMELMTRGDLKSYLRSLRPDTE-NNPGQSPPTLKKMIQMAGEIADGMAYLNANKFVHRD
           Columba livia IGF1R   VIMELMTRGDLKSYLRSLRPDTE-NNPGQAPPTLKKMIQMAGEIADGMAYLNANKFVHRD
       Coturnix japonica IGF1R   VIMELMTRGDLKSYLRSLRPDTE-SNPGQAPPTLKKMIQMAGEIADGMAYLNANKFVHRD
 Ctenopharyngodon idella IGF1R   VIMELMTRGDLKSYLRSLRS-AENTSSLPLPP-LKKMIQMAGEIADGMAYLNANKFVHRD
    Epinephelus coioides IGF1R   VIMELMTRGDLKSYLRSLRPKEQQWSSLSLPP-LKKMLQMAGQIADGMAYLNANKFVHRD
             Felis catus IGF1R   VIMELMTRGDLKSYLRSLRPEIE-NNPVLAPPSLSKMIQMAGEIADGMAYLNANKFVHRD
           Gallus gallus IGF1R   VIMELMTRGDLKSYLRSLRPDTE-SNPGQAPPTLKKMIQMAGEIADGMAYLNANKFVHRD
   Heterocephalus glaber IGF1R   VIMELMTRGDLKSYLRSLRPEME-NNPVLAPPSLSKMIQMAGEIADGMAYLNANKFVHRD
         Morone chysops IGF1R   VIMELMTRGDLRSYLRSLRPKEQQWSSLSLPP-LKKMLQMAGQIADGMAYLNANKFVHRD
 Oreochromis mossambicus IGF1R   VIMELMTRGDLKSHLRSMRSQ-ENSSCQSLPP-LKKMIQMAGEIADGMAYLNANKFVHRD
Ornithorhynchus anatinus IGF1R   VIMELMTRGDLKSYLRSLRPDTEQNNPGQAPPTLKKMIQMAGEIADGMAYLNANKFVHRD
     Pimephales promelas IGF1R   VIMELMTRGDLKSYLRSLRSKEQGSSSQSLPP-LKKMIQMAGEIADGMAYLNANKFVHRD
           Pongo abelii IGF1R   VIMELMTRGDLKSYLRSLRPEME-NNPVLAPPSLSKMIQMAGEIADGMAYLNANKFVHRD
         Xenopus laevis IGF1R   VIMELMTRGDLKSYLRSLRPDTE-SNSGQSPPSLKKMIQMAGEIADGMSYLNANKFVHRD
       Xenopus tropicalis IGF1R   VIMELMTRGDLKSYLRSLRPDTE-SNSGQPPPSLKKMIQMAGEIADGMAYLNANKFVHRD
       Icthyomyzon fossor IGF1R   VIMELMTRGDLKSYLRSLRPDAE-KKPTRPPSSLPEMLQMAGEIADGMAYLNAKKFVHRD

      Acipenser ruthenus IGF1R   LAARNCMVA-----
              Bos taurus IGF1R   LAARNCMVAEDFTV
            Capra hircus IGF1R   LAARNCMVAEDFTV
          Chelonia mydas IGF1R   LAARNCMVAEDFTV
           Columba livia IGF1R   LAARNCMVAEDFTV
       Coturnix japonica IGF1R   LAARNCMVAEDFTV
 Ctenopharyngodon idella IGF1R   LAARNCMVAEDFTV
    Epinephelus coioides IGF1R   LAARNCMVAEDFTV
             Felis catus IGF1R   LAARNCMVAEDFTV
           Gallus gallus IGF1R   LAARNCMVAEDFTV
   Heterocephalus glaber IGF1R   LAARNCMVAEDFTV
         Morone chysops IGF1R   LAARNCMVAEDFTV
 Oreochromis mossambicus IGF1R   LAARNCMVAEDFTV
Ornithorhynchus anatinus IGF1R   LAARNCMVAEDFTV
     Pimephales promelas IGF1R   LAARNCMVAEDFTV
           Pongo abelii IGF1R   LAARNCMVAEDFTV
         Xenopus laevis IGF1R   LAARNCMVTEDFTV
       Xenopus tropicalis IGF1R   LAARNCMVAEDFTV
       Icthyomyzon fossor IGF1R   LAARNCMVAEDFTV
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Figure A2.9. Alignment of translated amino acid sequence for *Ichthyomyzon fossor* insulin-like growth factor 1 receptor (IGF1R) to IGF1R amino acid sequences from other species. Sequences are in alphabetical order by scientific name, except for the lamprey sequence, which is given at the bottom of each section. Locations of primers are highlighted in light gray (forward) and dark gray (reverse). GenBank accession numbers and common names are given in Table A2.5.

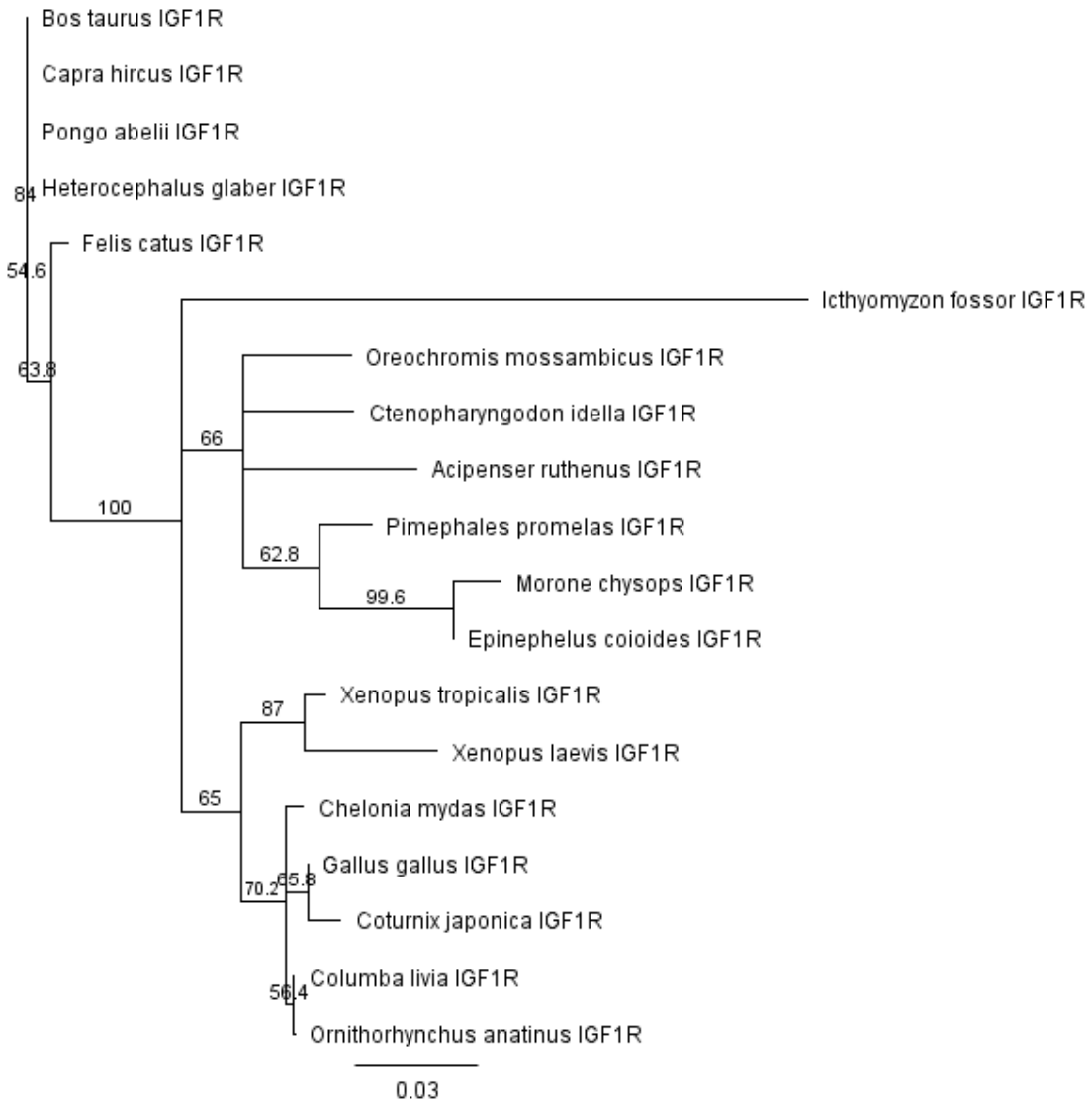


Figure A2.10. Neighbour-joining tree for insulin-like growth factor 1 receptor (IGF1R) amino acid sequences. This tree was constructed in Geneious using the Jukes-Cantor genetic distance model and 500 bootstrap replicates. GenBank accession numbers and common names are given in Table A2.5.

Table A2.6. List of amino acid sequences used to construct the alignment (Figure A2.11) and neighbour-joining tree (Figure A2.12) for daz-associated protein 1 (DAZAP1).

Species	Common name	Gen Bank accession number	Gene name
Bos taurus	Domestic cattle	NP_001179960	DAZ-associated protein 1
Canis lupus familiaris	Domestic dog	XP_003432858	PREDICTED: DAZ-associated protein 1
Danio rerio	Zebrafish	NP_001166975	DAZ associated protein 1
Gallus gallus	Domestic chicken	NP_001026599	DAZ-associated protein 1
Homo sapiens	Human	AAF78364	DAZ associated protein 1
Mus musculus	House mouse	AAF81071	DAZ-associated protein 1
Oreochromis niloticus	Nile tilapia	XP_003449071	PREDICTED: DAZ-associated protein 1-like
Oryzias latipes	Medaka	XP_004067962	PREDICTED: DAZ-associated protein 1-like isoform 1
Salmo salar	Atlantic salmon	ACI34285	DAZ-associated protein 1
Takifugu rubripes	Pufferfish	XP_003974355	PREDICTED: DAZ-associated protein 1-like
Xenopus tropicalis	Western clawed frog	CAJ83458	DAZ associated protein 1

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          Bos taurus DAZAP1   GADEIGKLFVGGLDWSTTQETLRSYFSQYGEVVDCVIMKDKTTNQSRGFGFVKFKDPNCV
         Canis lupus DAZAP1   GADEIGKLFVGGLDWSTTQETLRSYFSQYGEVVDCVIMKDKTTNQSRGFGFVKFKDPNCV
         Danio rerio DAZAP1   AGDEIGKLFVGGLDWSTTQETLRNYFSQYGEVVDCVIMKDKSTNQSRGFGFVKFKDPNCV
       Gallus gallus DAZAP1   GADEIGKLFVGGLDWSTTQETLRSYFSQYGEVVDCVIMKDKTTNQSRGFGFVKFKDPNCV
        Homo sapiens DAZAP1   GADEIGKLFVGGLDWSTTQETLRSYFSQYGEVVDCVIMKDKTTNQSRGFGFVKFKDPNCV
        Mus musculus DAZAP1   GADEIGKLFVGGLDWSTTQETLRSYFSQYGEVVDCVIMKDKTTNQSRGFGFVKFKDPNCV
Oreochromis niloticus DAZAP1   -GDEVGKLFVGGLDWSTTQETLRNYFSQYGEVVDCVIMKDKTTNQSRGFGFVKFKDPNCV
      Oryzias latipes DAZAP1   -GDEVGKLFVGGLDWSTTQETLRNYFSQYGEVVDCVIMKDKTTNQSRGFGFVKFKDPNCV
          Salmo salar DAZAP1   AGDEVGKLFVGGLDWSTTQETLRNYFSQYGEVVDCVIMKDKTTNQSRGFGFVKFKDPNCV
    Takifugu rubripes DAZAP1   -GDEVGKLFVGGLDWSTTQETLRNYFSQYGEVVDCVIMKDKTTNQSRGFGFVKFKDPNCV
   Xenopus tropicalis DAZAP1   GGDEIGKLFVGGLDWSTTQETLRSYFSQYGEVVDCVIMKDKTTNQSRGFGFVKFKDPNCV
  Ichthyomyzon fossor DAZAP1   AGDEIGKLFVGGLDWNTSQEGLQMYFSQFGEVIDCVIMKDKETNQSRGFGFVKFKDPNCI

          Bos taurus DAZAP1   GTVLASRPHTLDGRNIDPKPCTPRGMQPERTRPKEGWQ-KGPRSDNSKSNKIFVGGIPHN
         Canis lupus DAZAP1   GTVLASRPHTLDGRNIDPKPCTPRGMQPERTRPKEGWQ-KGPRSDNSKSNKIFVGGIPHN
         Danio rerio DAZAP1   RTVLDTKPHNLDGRNIDPKPCTPRGMQPEKTRTKDGW--KGSKSDSNKSKKIFVGGIPHN
       Gallus gallus DAZAP1   GTVLASRPHTLDGRNIDPKPCTPRGMQPERTRPKEGWQ-KGSRSDNNKSNKIFVGGIPHN
        Homo sapiens DAZAP1   GTVLASRPHTLDGRNIDPKPCTPRGMQPERTRPKEGWQ-KGPRSDYSKSNKIFVGGIPHN
        Mus musculus DAZAP1   GTVLASRPHTLDGRNIDPKPCTPRGMQPERTRPKEGW--KGPRSDSSKSNKIFVGGIPHN
Oreochromis niloticus DAZAP1   RTVLETKPHNLDGRNIDPKPCTPRGMQPEKSRTKEGW--KGSKADSNKSKKIFVGGIPHN
      Oryzias latipes DAZAP1   RTVLETKPHNLDGRNIDPKPCTPRGMQPEKSRTKEGW--KGSKADSNKSKKIFVGGIPHN
          Salmo salar DAZAP1   RTVLETKPHNLDGRNIDPKPCTPRGMQPEKVRTKDGW--KGSKADSNKSKKIFVGGIPHN
    Takifugu rubripes DAZAP1   RTVLETKPHNLDGRNIDPKPCTPRGMQPEKSRTKEGW--KGSKADSNKSKKIFVGGIPHN
   Xenopus tropicalis DAZAP1   GTVLASRPHTLDGRNIDPKPCTPRGMQPERTRPREGWQQKGPRTENSRSNKIFVGGIPHN
  Ichthyomyzon fossor DAZAP1   GMVLANRPHCLDGRTIDPKPCTPRSMQTPKQNHQERP--PPVNQNVIRCKKIFVGGIPHN

          Bos taurus DAZAP1   CGETELREYFKKFGVVTEVVMIYDAEKQRPRGFGFITFEDEQSVDQAVNMHFHDIMGKKV
         Canis lupus DAZAP1   CGETELREYFKKFGVVTEVVMIYDAEKQRPRGFGFITFEDEQSVDQAVNMHFHDIMGKKV
         Danio rerio DAZAP1   CGEAELRDYFNRFGVVTEVVMIYDAEKQRPRGFGFITFEAEQSVDQAVNMHFHDIMGKKV
       Gallus gallus DAZAP1   CGETELREYFKKFGVVTEVVMIYDAEKQRPRGFGFITFEDEQSVDQAVNMHFHDIMGKKV
        Homo sapiens DAZAP1   CGETELREYFKKFGVVTEVVMIYDAEKQRPRGFGFITFEDEQSVDQAVNMHFHDIMGKKV
        Mus musculus DAZAP1   CGETELREYFKKFGVVTEVVMIYDAEKQRPRGFGFITFEDEQSVDQAVNMHFHDIMGKKV
Oreochromis niloticus DAZAP1   CGEPELRDYFNRFGVVTEVVMIYDAEKQRPRGFGFITFEAEQSVDQAVNMHFHDIMGKKV
      Oryzias latipes DAZAP1   CGEPELRDYFNRFGVVTEVVMIYDAEKQRPRGFGFITFEAEQSVDQAVNMHFHDIMGKKV
          Salmo salar DAZAP1   CGEPELREYFNRFGVVTEVVMIYDAEKQRPRGFGFITFEAEQSVDQAVNMHFHDIMGKKV
    Takifugu rubripes DAZAP1   CGEPELRDYFNRFGAVTEVVMIYDAEKQRPRGFGFITFEAEQSVDQAVNMHFHDIMGKKV
   Xenopus tropicalis DAZAP1   CGETELREYFKRFGVVTEVVMIYDAEKQRPRGFGFITFEDEQSVDQAVNMHFHDIMGKKV
  Ichthyomyzon fossor DAZAP1   CGDMELREYFKRFGMVTEVVMIYDQLKQRPRGFGFISFEDEQSVDQAVNLHFHDIMGKKV

          Bos taurus DAZAP1   EVKRAEPRDSK
         Canis lupus DAZAP1   EVKRAEPRDSK
         Danio rerio DAZAP1   EVKKAEPRDSK
       Gallus gallus DAZAP1   EVKRAEPRDSK
        Homo sapiens DAZAP1   EVKRAEPRDSK
        Mus musculus DAZAP1   EVKRAEPRDSK
Oreochromis niloticus DAZAP1   EVKKAEPRDSK
      Oryzias latipes DAZAP1   EVKKAEPRDSK
          Salmo salar DAZAP1   EVKKAEPRD--
    Takifugu rubripes DAZAP1   EVKKAEPRDSK
   Xenopus tropicalis DAZAP1   EVKRAEPRDSK
  Ichthyomyzon fossor DAZAP1   EVKRAEPRDGK
```

Figure A2.11. Alignment of translated amino acid sequence for *Ichthyomyzon fossor* daz-associated protein 1 (DAZAP1) to DAZAP1 amino acid sequences from other species. Sequences are in alphabetical order by scientific name, except for the lamprey sequence, which is given at the bottom of each section. Locations of primers are highlighted in light gray (forward) and dark gray (reverse). GenBank accession numbers and common names are given in Table A2.6.

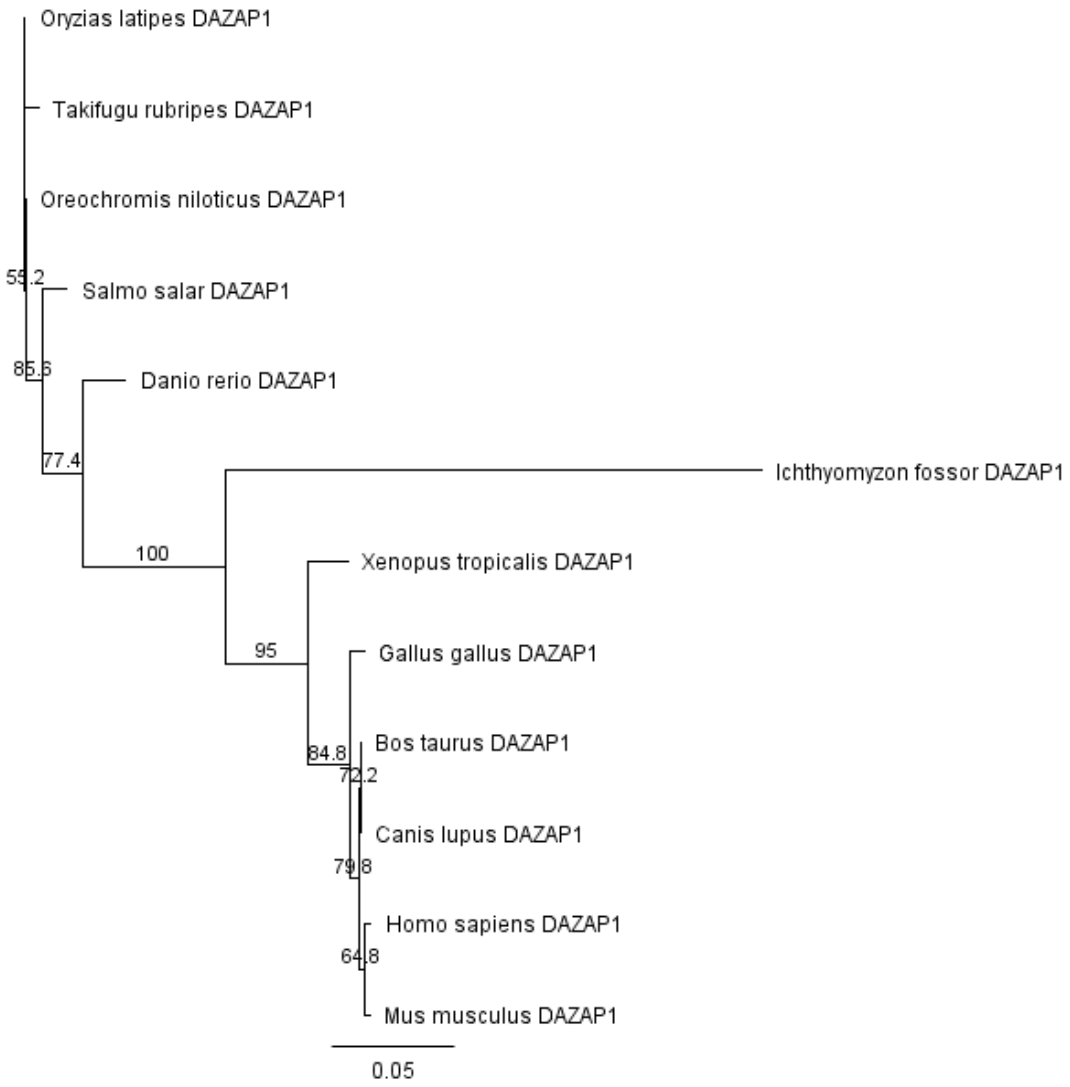


Figure A2.12. Neighbour-joining tree for daz-associated protein 1 (DAZAP1) amino acid sequences. This tree was constructed in Geneious using the Jukes-Cantor genetic distance model and 500 bootstrap replicates. GenBank accession numbers and common names are given in Table A2.6.

APPENDIX 3. CALIBRATION CURVES FOR TARGET AND REFERENCE GENES FOR QUANTITATIVE REVERSE-TRANSCRIPTASE PCR

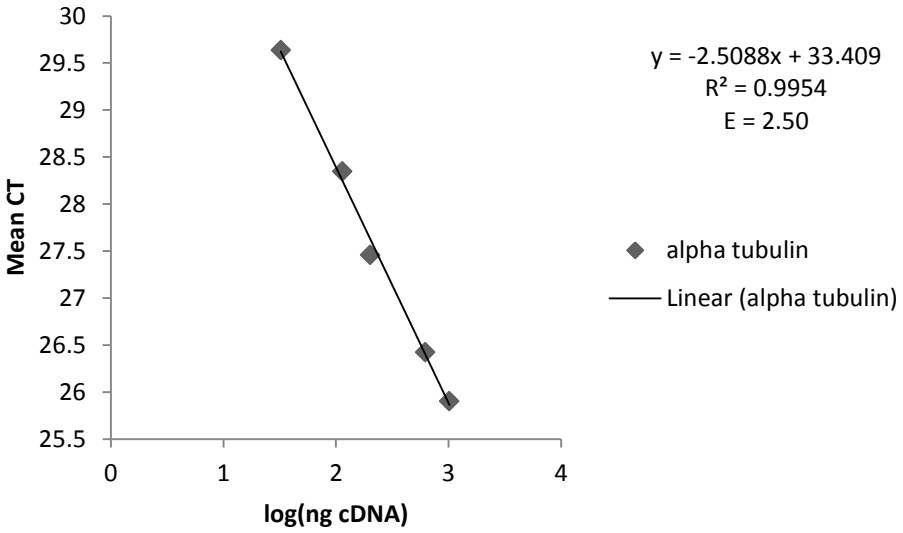


Figure A3.1. Calibration curve for alpha tubulin. E = calculated efficiency according to the equation $E = 10^{[-1/slope]}$.

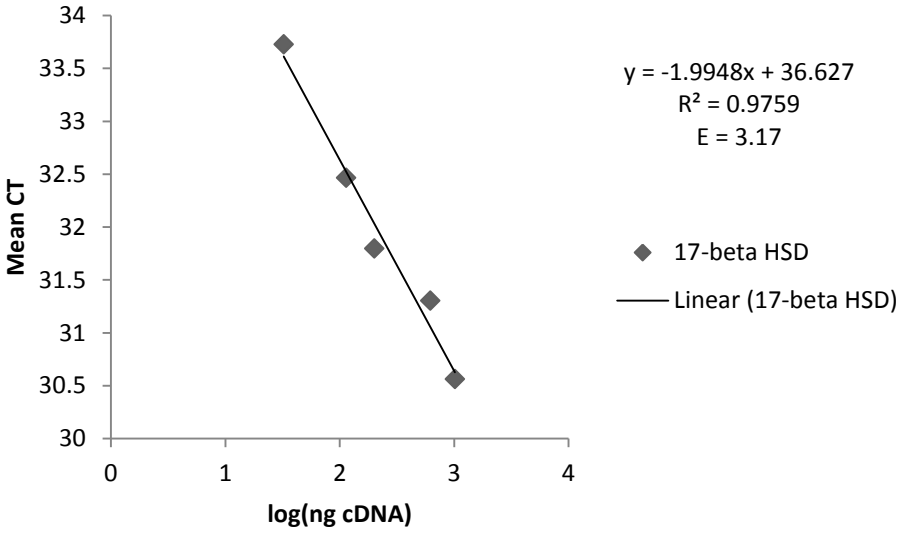


Figure A3.2. Calibration curve for 17-beta hydroxysteroid dehydrogenase (17-beta HSD). E = calculated efficiency according to the equation $E = 10^{[-1/slope]}$.

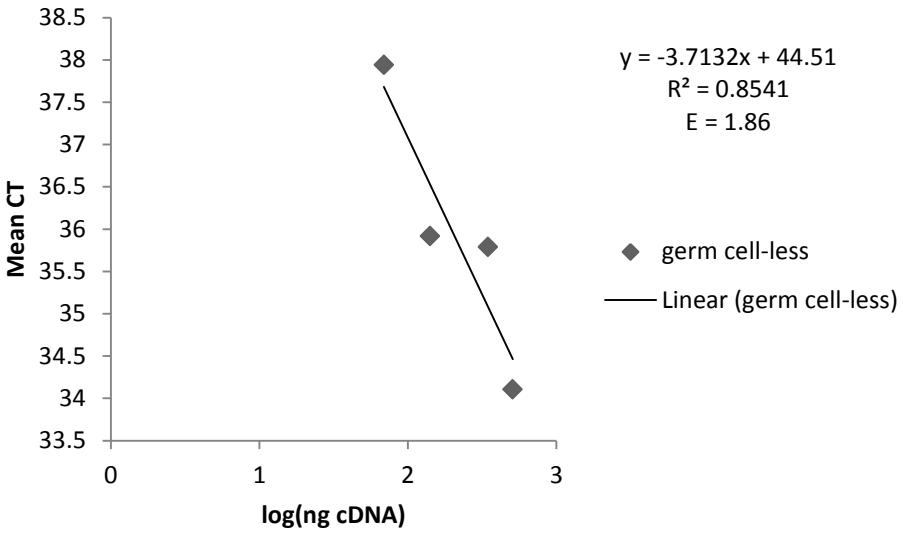


Figure A3.3. Calibration curve for germ cell-less. E = calculated efficiency according to the equation $E = 10^{[-1/slope]}$.

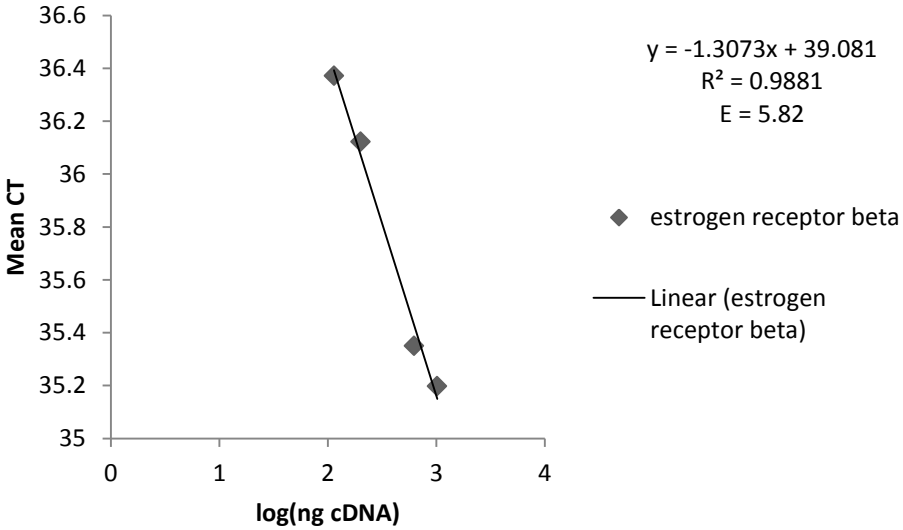


Figure A3.4. Calibration curve for estrogen receptor beta. E = calculated efficiency according to the equation $E = 10^{[-1/slope]}$.

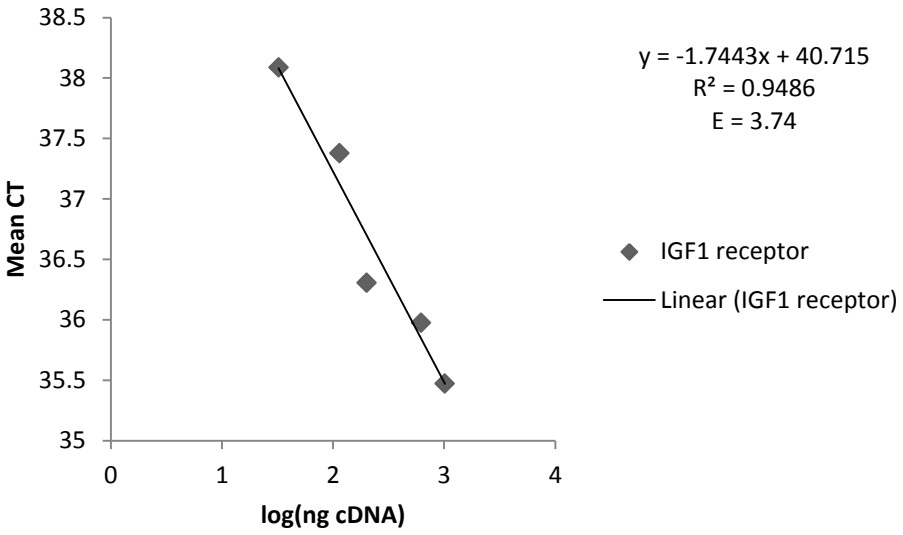


Figure A3.5. Calibration curve for insulin-like growth factor 1 receptor (IGF1 receptor). E = calculated efficiency according to the equation $E = 10^{[-1/slope]}$.

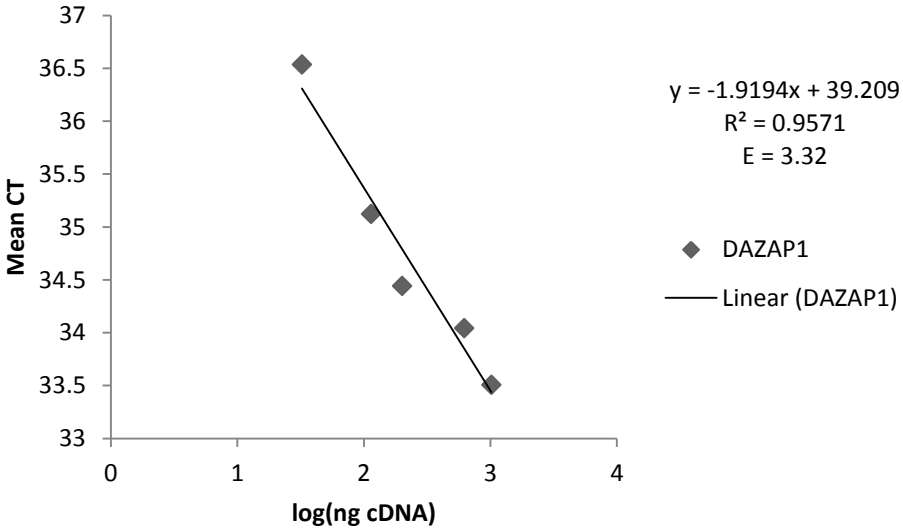


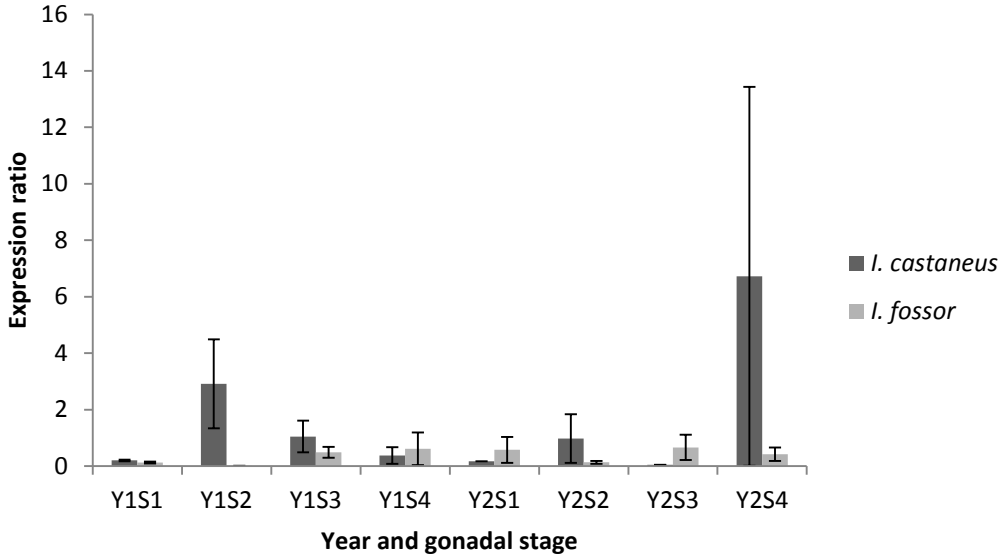
Figure A3.6. Calibration curve for daz-associated protein 1 (DAZAP1). E = calculated efficiency according to the equation $E = 10^{[-1/slope]}$.

APPENDIX 4. RAW DATA FROM QUANTITATIVE REVERSE-TRANSCRIPTASE PCR AND COMPARISONS OF GENE EXPRESSION IN GONADAL STAGES 1–4

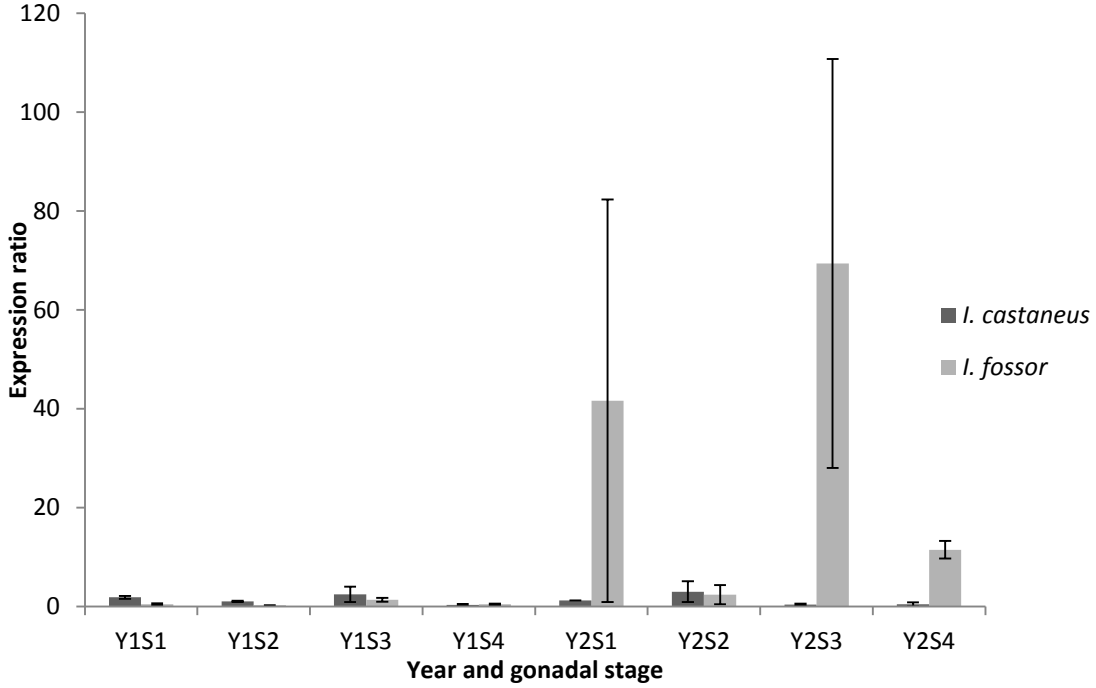
Table A4.1. Mean C_T values (from two replicates) for one reference gene (alpha tubulin) and five target genes for chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor*. Stage indicates stage of gonadal development (corresponding to those in Table 2.1 and Figure 2.1); year indicates age-class. 17-beta HSD = 17-beta hydroxysteroid dehydrogenase; GC-less = germ cell-less; ER beta = estrogen receptor beta; IGF1R = insulin-like growth factor 1 receptor; DAZAP1 = daz-associated protein 1. All values of 41.00 are arbitrary values assigned to individuals that failed to amplify a particular gene to threshold.

		I. castaneus						*I. fossor*					
		alpha tubulin	17-beta HSD	GC-less	ER beta	IGF1R	DAZAP1	alpha tubulin	17-beta HSD	GC-less	ER beta	IGF1R	DAZAP1
Stage 1	Year 1	32.43	35.72	36.67	36.62	38.10	37.54	34.12	37.03	41.00	41.00	38.25	36.74
		31.91	35.10	36.86	36.69	37.33	35.02	31.90	36.15	41.00	38.54	41.00	36.20
		32.71	35.53	37.30	37.99	41.00	35.29	31.56	34.96	37.59	38.29	41.00	33.57
	Year 2	32.53	35.70	37.77	41.00	38.26	36.22	32.09	35.68	37.64	38.31	41.00	35.46
								37.44	38.06	38.33	38.22	41.00	36.53
Stage 2	Year 1	31.26	31.70	36.59	37.32	36.74	34.44	30.14	35.93	36.85	36.83	37.64	33.94
		31.32	32.23	36.19	37.18	36.30	35.24						
		30.53	34.84	34.89	36.49	36.34	35.33						
	Year 2	34.17	34.96	37.96	41.00	37.14	35.14	33.96	36.78	37.92	37.65	41.00	36.09
		31.71	35.44	37.10	38.41	36.93	33.92	30.78	35.02	36.75	36.49	38.46	34.57
Stage 3	Year 1	29.74	34.00	34.21	36.11	36.78	34.23	30.87	33.22	34.90	35.64	36.04	34.34
		31.79	32.97	34.30	35.89	35.85	34.51	31.00	33.27	34.92	35.93	37.14	34.15
		28.82	31.24	32.68	34.45	34.42	33.29	31.11	35.08	36.80	37.59	41.00	34.30
	Year 2	28.50	33.84	34.86	37.18	37.46	32.95	36.92	37.58	37.07	38.00	41.00	36.12
		29.53	34.40	34.50	36.13	36.24	33.50	35.50	37.85	37.19	38.47	41.00	34.81
Stage 4	Year 1	31.02	33.01	36.46	37.72	37.54	35.16	31.67	33.00	37.36	37.76	41.00	35.84
		27.05	32.14	34.92	36.06	35.75	33.03	30.41	37.04	37.07	37.71	38.09	34.91
		29.68	34.08	36.28	37.62	37.01	35.78	31.26	35.59	38.12	37.48	41.00	36.68
	Year 2	28.50	34.67	38.08	38.45	37.24	36.76	34.27	37.03	37.07	38.00	37.71	33.75
		29.53	29.54	33.93	36.83	35.13	33.50	34.69	36.26	37.19	38.47	38.56	36.79

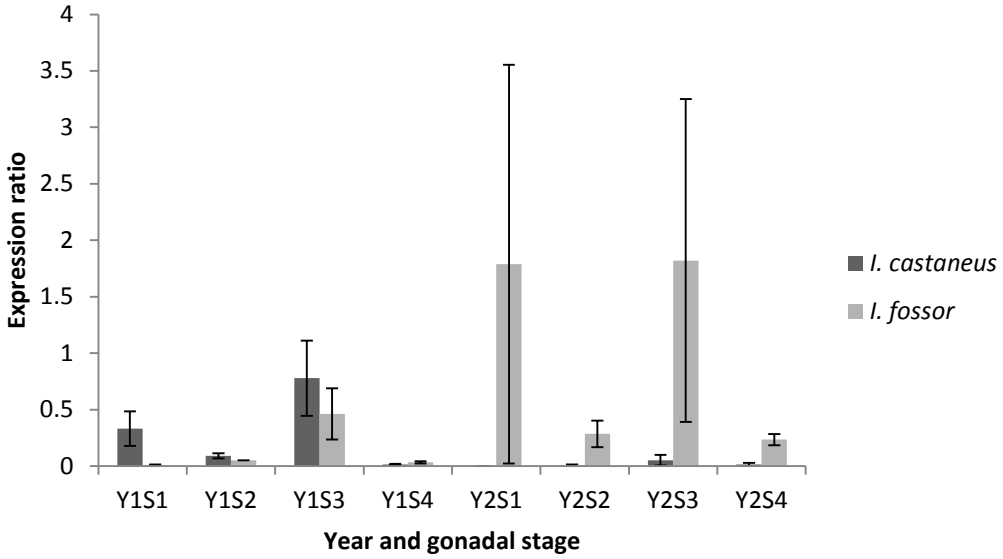
a)


16
14
12
10
8
6
4
2
0
Expression ratio
Y1S1
Y1S2
Y1S3
Y1S4
Y2S1
Y2S2
Y2S3
Y2S4
Year and gonadal stage
I. castaneus
I. fossor

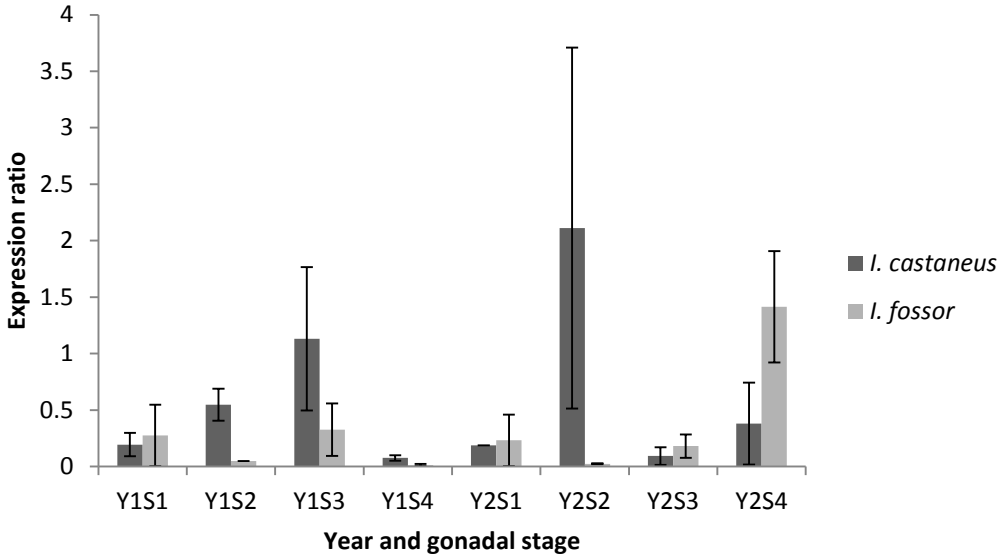
b)


120
100
80
60
40
20
0
Expression ratio
Y1S1
Y1S2
Y1S3
Y1S4
Y2S1
Y2S2
Y2S3
Y2S4
Year and gonadal stage
I. castaneus
I. fossor

c)


Expression ratio
4
3.5
3
2.5
2
1.5
1
0.5
0
Y1S1
Y1S2
Y1S3
Y1S4
Y2S1
Y2S2
Y2S3
Y2S4
Year and gonadal stage
I. castaneus
I. fossor

d)


Expression ratio
4
3.5
3
2.5
2
1.5
1
0.5
0
Y1S1
Y1S2
Y1S3
Y1S4
Y2S1
Y2S2
Y2S3
Y2S4
Year and gonadal stage
I. castaneus
I. fossor

e)

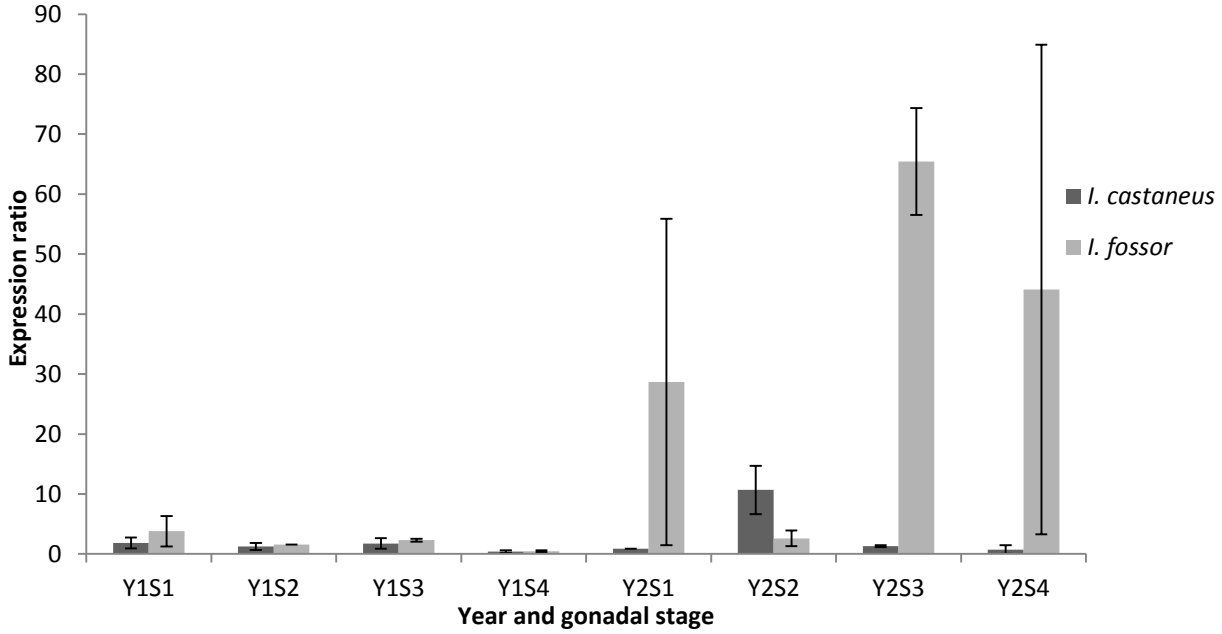


Figure A4.1. Means and standard errors of gonadal gene expression in chestnut lamprey *Ichthyomyzon castaneus* and northern brook lamprey *I. fossor*. Age-class is indicated by Y; stage of gonadal development is indicated by S (e.g., Y1S1 indicates age-class 1, stage 1). Stages correspond to those given in Table 2.1 and Figure 2.1. a) 17-beta hydroxysteroid dehydrogenase; b) germ cell-less; c) estrogen receptor beta; d) insulin-like growth factor 1 receptor; e) daz-associated protein 1.