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UNUSUAL SEX STEROIDS IN LAMPREYS

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UNUSUAL SEX STEROIDS IN LAMPREYS

By

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ABSTRACT
UNUSUAL SEX STEROIDS IN LAMPREYS

By

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The lamprey is one of the earliest evolving extant vertebrates for which the hypothalamus-pituitary-gonadal (HPG) axis has been shown to control reproduction. The hypothalamic hormones, particularly gonadotropin-releasing hormone (GnRH), have been well-characterized, but the identity and function of the gonadal steroids remain elusive. Further knowledge regarding the proximate endocrine controls over reproduction in the lamprey is desirable because of the lamprey's unique place in the phylogenic tree and the economic importance of the species. The objective of this dissertation research was to identify possible functional sex steroids in lampreys, develop methods with which to measure the sex steroids, and compare sex steroid concentrations in response to stimulation with lamprey GnRH.

This dissertation research has shown that sea lamprey testes rapidly metabolize classical steroids into 15α-hydroxylated derivatives *in vitro,* producing 15α-hydroxyprogesterone (15α-P) and 15α-hydroxytestosterone (15α-T). It was further demonstrated, using radioimmunoassays (RIAs) developed as part of this dissertation research, that these steroids are produced *in vivo* and circulated in the plasma. Plasma concentrations of 15α-T are < 1 ng/ml in prespermiating male sea lampreys, but rise 2-5 times in response to injections of GnRH, and do not show differing responses to the two types of endogenous lamprey GnRH. Plasma concentrations of 15α-P are also < 1 ng/ml in prespermiating

male sea lampreys, but rise to average concentrations of 36 ng/ml in response to injections of GnRH. 15α-P concentrations rise higher in response to GnRH III than to GnRH I, are higher at 8 h than 24 h post-injection, and higher doses of either type of GnRH elicit higher plasma levels of 15α-P. *In vitro* and *in vivo* production of 15α-T and 15α-P was investigated in male silver lampreys (*Ichthyomyzon unicuspis*), chestnut lampreys (*I. casteneus*), American brook lampreys (*Lethenteron appendix*), Pacific lampreys (*Entosphenus tridentatus*), and European river lampreys (*Lampetra fluviatilis*). While 15α-hydroxylated steroids were produced *in vitro* and *in vivo* in all species examined, the response in plasma levels of 15α-hydroxylated steroids to injections of GnRH varied among species.

This research provides a first step toward understanding the proximate controls over reproduction in lamprey species. In sea lampreys, 15α-T is the first androgen that has been shown to respond to hypothalamic hormones. Levels of 15α-P also increase dramatically in response to injections of GnRH to levels far higher than those measured previously for any steroid. It appears that 15α-hydroxylation is a common pathway in the testes of lamprey species, but differences exist in the controls over steroidogenesis among lamprey species.

This dissertation is dedicated to my husband, Andrew, without whose support I would have never been able to accomplish so much.

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CHAPTER ONE

Classical and 15α-hydroxylated steroids in lamprey species

Importance of knowledge regarding lamprey steroids

Information regarding lamprey steroids is vital from both basic and applied science perspectives. Lampreys (class Cephalaspidomorphi) are one of the earliest-evolving extant vertebrates (Hardisty and Potter 1971), and with hagfish (class Myxini) form a monophyletic group, superclass Agnatha (Kuraku et al. 1999). Lampreys are the earliest-evolving animals in which research has demonstrated that the hypothalamal-pituitary-gonadal (HPG) axis controls reproduction, as it is known to do in all higher vertebrates (reviews: Sower 1990, 1998, 2003; Sower and Kawauchi 2001). The hypothalamic hormones in lampreys have been well-characterized (Sower 1990, 2003; Sower and Kawauchi 2001), with two morphs of endogenous gonadotropin-releasing hormone (GnRH) identified (Sherwood et al. 1986; Sower et al. 1993). However, research concerning the gonadal steroids in lampreys has yielded enigmatic, and sometimes conflicting, results. Sex steroids are hypothesized to be important to vertebrate evolution and biocomplexity (Baker 1997, 2003, 2004), and the sex steroid-receptor system found in lampreys may be the best living proxy for those found in ancestral vertebrates (Neidert et al. 2001).

Besides being an important group for physiological studies based on its phylogeny, studies of lamprey steroids have practical applications as well. Lamprey species are in decline over much of their range (Renaud 1997). Sea lampreys (*Petromyzon marinus*) are considered delicacies in Europe (Maitland 1980) and suffer from over-fishing and habitat destruction (Almeida et al. 2000). In the United States, the Pacific lamprey (*Entosphenus tridentatus*) is a culturally important food source for Native Americans, but

populations are dwindling due to habitat loss and prevention of migration due to hydroelectric dams (Close et al. 2002). However, in the Great Lakes, the sea lamprey is an invasive pest that has significantly altered the food web and is subject to an expensive international control program (Christie and Goddard 2003). Knowledge regarding lamprey reproductive steroids may aid both conservation and control efforts (Docker et al. 2003). Without first grasping how reproduction is controlled under natural conditions in lampreys, it cannot be understood how adverse conditions or control techniques affect reproduction. It also cannot be determined how a lamprey's reproductive physiology responds to or copes with control techniques. In addition, there has been an increased emphasis in using and developing alternative sea lamprey control techniques, many of which focus on the reproductive phase, in order to reduce reliance on TFM, a chemical lamprey larvacide (Christie and Goddard 2003).

The timing and order of the evolution of steroid hormones and their receptors is a subject of great interest and debate (e.g. Baker 2004). Studies based on nuclear receptor sequences have led to the hypotheses that agnathans diverged from gnathostomes prior to the evolution of a nuclear androgen receptor, and that the nuclear estrogen receptor was the first to evolve (Thornton 2001); studies based on classical steroids (i.e., those found in higher vertebrates; Sower 1990) in lampreys have been used to support these hypotheses. However, if the functional steroid hormones in lampreys differ structurally from those of other vertebrates, the results from previous studies and hypothesis based upon them may need to be questioned.

Lamprey Life Cycle

Lamprey species can be anadromous or stay entirely in riverine habitats, and anadromous species can give rise to landlocked forms (Hardisty and Potter 1971). In some species the adults do not feed parasitically, and these species are always restricted to riverine habitats. All lamprey species are semelparous, meaning that they spawn only once before dying. The eggs are laid in gravel nests in rivers, and hatched larvae burrow into silt after being carried down stream (Applegate 1950). The larvae, or ammocoetes, are filter feeders and remain in the river bottom for 5-8 years (Potter 1980) after which the species which undergo a parasitic stage metamorphose into adults and migrate downstream to oceans or lakes. The lampreys feed by attaching to large fish with their sucker-like mouths and perforating the flesh, and different species of lampreys have different preferred attachment points depending on whether host body fluids or muscle are the preferred food (Potter and Hillard 1987). The parasitic phase has a duration of approximately eighteen months (Farmer 1980). Sexually mature lampreys migrate upstream and then reproduce in streams in the spring. The upstream migration occurs in early spring for lampreys of the genera *Ichthyomyzon* and *Petromyzon*, but occurs in autumn for lampreys of the genera *Entosphenus* and *Lampetra* (Beamish 1980, Manion and Hanson 1980).

Classical steroids in lampreys

Steroid levels in lampreys have often been investigated using immunoassays for classical steroids that are commercially available (Table 1). Some of the immunoassays have been validated by confirming the identity of the steroids chromatographically (e.g., Botticelli

1963; Kime and Larsen 1987) so that it is likely that classical steroids are produced and circulated in lamprey plasma. Additionally, levels of classical steroids fall after hypophysectomy (Kime and Larsen 1987; Sower and Larsen 1991), which indicates that levels are regulated by neuroendocrine hormones, and large-dose implants of some steroids can lead to development of secondary sex characteristics (Larsen 1974). However, implants of classical steroids do not accelerate gonadal sexual maturation or slow it through negative feedback loops (Larsen 1974), and exposure to exogenous classical steroids does not affect sex determination in larvae (Docker 1992).

Progestagens. Progesterone (P) has been detected in sea lamprey plasma, and differences in P concentrations in plasma have been detected between sexes (Linville et al., 1987), between reproductive stages (Bolduc and Sower 1992), and in response to GnRH (Sower et al. 1987; Sower 1989; Sower et al. 1993; Derragon and Sower 1994; Gazourian et al. 2000). The concentrations of immunoreactive (ir)-P in most sea lampreys are normally less than 1 ng/ml but exhibit a 2- to 5-fold increase in response to GnRH injection (Gazourian et al. 2000). Because P can be detected by RIA in lamprey plasma and its plasma concentrations differ in response to stimulation, it has been suggested that P is a functional hormone in lampreys (Sower 1990).

Androgens. There is little evidence for functional classical androgens in lampreys. In most cases, the amounts of testosterone (T) in sea lampreys were either undetectable or very low (<1 ng/ml). Although one study did find sexual dimorphism and correlations between maturational stage and steroid levels (Linville et al. 1987), other studies did not

support these findings (Sower et al. 1985a), and showed that T concentrations did not change in response to GnRH injection (Sower et al. 1985b). In other species examined, T appears to be present, but at low concentrations that bear no relationship to stage of maturation, gender, or treatment (Fukayama and Takahashi 1985; Kime and Larsen 1987). The only exception is the brook lamprey, *Lampetra planeri* Bloch, in the blood of which Seiler et al. (1985) recorded concentrations of T of up to 15 ng/ml.

Estrogens. Estradiol (E_2) has been measured using immunoassays in sea lamprey plasma of both sexes on numerous occasions, and research regarding E_2 provides the strongest evidence of any classical steroid of it being a functional hormone (Sower 1990). Differences in E_2 concentrations in plasma have been detected between sexes (Sower et al. 1985a; Linville et al. 1987), between reproductive stages (Sower et al. 1985a; Linville et al. 1987; Bolduc and Sower 1992) and in response to heterologous and lamprey GnRH (Sower 1989; Sower et al. 1983, 1985b, 1987, 1993; Derragon and Sower 1994; Gazourian et al. 1997, 2000). Levels in GnRH-stimulated lampreys have been found to rise as high as 12 ng/ml (Sower et al. 1983). E_2 has also been measured on multiple occasions in other lamprey species. In European river lampreys (*Lampetra fluviatilis*) and Japanese river lampreys (*Lampetra japonica)*, plasma E_2 levels have been associated with vitellogenesis in females and also shown to increase at spawning in males (Barranikova et al. 1995; Mewes et al. 2002; Fukayama and Takahashi 1985). The gonadal origin of E_2 is in doubt, as Kime and Larsen (1987) found that both E_2 and T plasma concentrations rose in European river lampreys after gonadectomy, and the authors suggested that these steroids may be inactive precursors synthesized in extra-

gonadal endocrine tissues, such as the interrenal glands (and then possibly converted to active sex hormones in the gonads).

Receptors for classicals steroids. Thus far, the only steroid in lampreys for which there is evidence of tissue binding is E_2 (Ho et al. 1987). In this study, nuclear receptors with an affinity to E_2 were found in the nuclear and cytosolic fractions of testicular tissue in sea lampreys. This study also found that nuclear receptors for classical androgens were absent in the testes. Recently, it has been determined that membrane-bound steroid receptors are found in vertebrate animals (Zhu et al. 2003a, 2003b). This mechanism of steroid action has not been investigated in lampreys.

Sequences for partial putative nuclear receptors have been amplified using PCR-based experiments with degenerate primers (Thornton 2001). Sequences homologous to nuclear estrogen, progestagen, and corticoid receptors were cloned, but no androgen receptor could be amplified. Thornton (2001) used the lack of a nuclear androgen receptor, combined with the poor results garnered from experiments using immunoassays for T, to hypothesize that the androgen receptor evolved through gene duplications (e.g., Suga et al. 1999; Escriva et al. 2002) after gnathostomes diverged from agnathans, and therefore lampreys lack functional androgens. The binding affinities of the amplified receptors have not yet been investigated.

Steroid metabolism in lampreys

Using radiolabeled precursors either *in vitro* or *in vivo,* classical steroids have rarely been shown to be synthesized or metabolized to recognizable steroids as major products in lampreys (Table 2). Weisbart and Youson (1975) incubated testicular tissue from parasitic phase lampreys with ^{14}C-P, and could only identify one product, which was a very small amount of 11-deoxycorticosterone. In the same study, presumptive adrenocortical tissue from larval and parasitic phase lampreys was incubated with ^{14}C-P and small amounts of 17-hydroxyprogesterone, androstenedione, and 11-deoxycortisol were formed. With both the testes and presumptive adrenocortical tissues, most of the radiolabel was converted to unknown steroids. Weisbart et al. (1977) investigated the conversion of ^{3}H-P *in vivo* in parasitic phase sea lampreys, and 11-deoxycorticosterone was the only one of the several products that could be identified. Again, most of the radioactivity was converted to unknown compounds. When ^{3}H-cholesterol was used as a substrate for *in vitro* incubations with adult sea lamprey testicular, ovarian, and presumptive adrenocortical tissues, none of the steroid metabolites could be identified (Weisbart et al. 1978). Weisbart et al. (1978) also found that testicular 3β-hydroxysteroid dehydrogenase activity (an enzyme required for the conversion of Δ-5 steroids such as pregnenolone to Δ-4 steroids such as P and T) was very low in comparison to other vertebrates. Additionally, Weisbart and Idler (1970) found no evidence for the presence of Δ-4 steroids in an extract of 300 ml of plasma from prespermiating males. Callard et al. (1980) demonstrated the presence of aromatase and 5α-reductase in the gonads by using ^{3}H-androstenedione as a precursor, and were able to identify small amounts of 5α-

androstenedione, estrone, and estradiol, but once again, the majority of the radioactivity was converted to unknown products.

One explanation for the lack of identifiable products in the above studies is that lamprey may use atypical enzymes for steroidogenic pathways. Several studies have shown that lamprey gonads contain an unusual 15α-steroid hydroxylase. This enzyme (or family of enzymes) converts classical steroids and steroid precursors to more polar 15α-hydroxylated derivatives (Fig. 1), which have so far not been identified as metabolic products of the gonads in any other vertebrate species. The first study was by Kime and Rafter (1981), who demonstrated that the gonads of river lamprey contain a 15-hydroxylase that acts on P and T, although it was unclear whether T was hydroxylated in the 15α or 15β position. Kime and Callard (1982) demonstrated that the testes of the sea lamprey also contained enzymes that synthesized 15α-hydroxytestosterone (15α-T) and 15α-hydroxyandrostenedione from androstenedione. Kime and Larsen (1987) later hypothesized that the 15α-hydroxylated steroids were the functional steroids in lampreys, and that the classical steroids found in plasma were from extra-gonadal sources and not active hormones - and were metabolized to their active form in the gonads.

More recent studies have been carried out on 15α-hydroxylated steroids in lampreys. Lowartz et al. (2003) showed that steroids produced *in vitro* and *in vivo* in adult lampreys coeluted with 15α-hydroxylated steroids on high performance liquid chromatography (HPLC), and later demonstrated that the same phenomenon occurs in large ammocoetes and parasitic phase lampreys (Lowartz et al. 2004). In the course of this dissertation,

Bryan et al. (2003, 2004) confirmed the structures of 15α-T and 15α-hydroxyprogesterone (15α-P) as the major products of T and P in lamprey testes *in vitro* using chromatographic, microchemical, and immunological techniques. 15α-T was shown to be the only product of T, but P was converted into four different products, the relative amounts of which varied with reproductive season. 15α-P was the only product of P that could be identified using reference steroids.

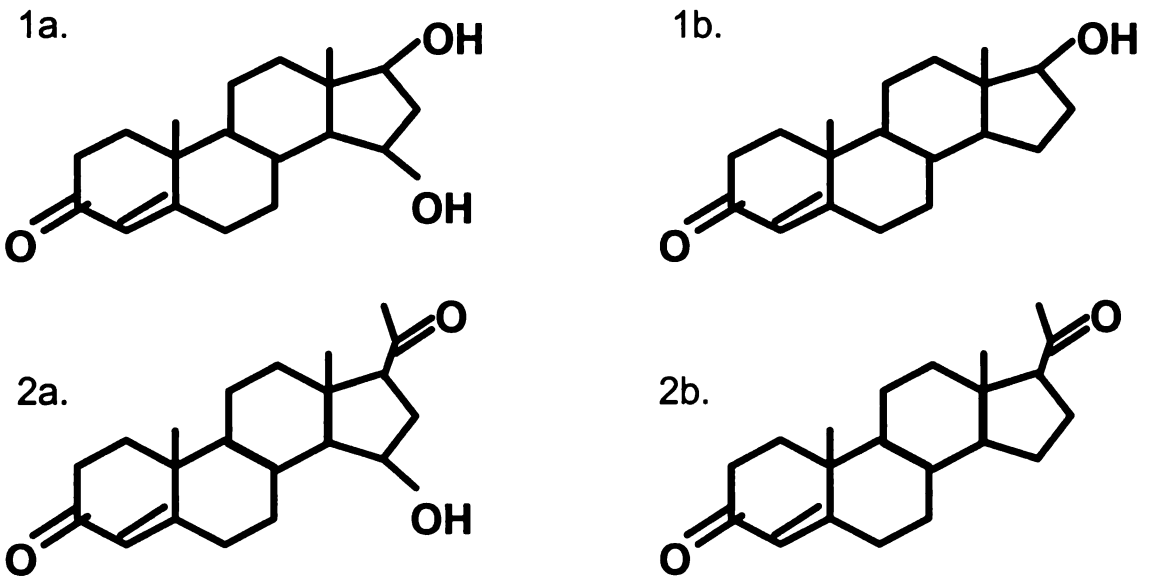

Figure 1: Structures of hypothesized lamprey and classical steroids. Hypothesized lamprey steroids shown are 15α-hydroxytestosterone (15α-T, 1a.) and 15α-hydroxyprogesterone (15α-P4, 2a.). For comparison, the structures of the classical hormones testosterone (1b.) and progesterone (2b.) are provided.

Plasma levels of 15α-hydroxylated steroids in sea lampreys

Although there was prior evidence for *in vitro* production of 15α-hydroxylated steroids, *in vivo* production had not yet been investigated. The research performed for this dissertation included developing a method to measure plasma concentrations of 15α-T

and 15α-P. Radioimmunoassays (RIAs) for 15α-hydroxylated steroids have been developed and used to show that 15α-hydroxylated steroids are produced *in vivo* (Table 1; Bryan et al. 2003, 2004). The radiolabel for the RIAs was produced using a large amount of commercial tritiated T or P, and incubating it with lamprey testes so that the endogenous 15α-hydroxylase would produce tritiated 15α-P and 15α-T.

15α-Hydroxyprogesterone. Bryan et al. (2004) used the RIA to measure the plasma concentrations of ir-15α-P in prespermiating males that had been given two serial injections, 24 h apart, of either lamprey GnRH I (50, 100, or 200 μg/kg), GnRH III (50, 100, or 200 μg/kg), or saline control, with plasma being sampled 8 and 24 h after the second injection. Plasma concentrations of ir-15α-P rose from less than 1 ng/ml to 36 ng/ml (mean of all treatments) at 8 h post-injection and declined at 24 h after injection, but not to base levels. At 8 h after the second injection, both types of GnRH had similar effects, but GnRH III elicited higher plasma levels at 24 h. The levels of 15α-P detected in the treatment groups are nearly an order of magnitude greater than those measured for any other steroid in lampreys. Levels in females were found to be less than 1 ng/ml, but ovulating females still had significantly higher levels than preovulating females. Spermiating males had average plasma concentrations of 2.48 ng/ml.

15α-Hydroxytestosterone. Bryan et al. (2003) used the RIA to measure 15α-T plasma levels in adult lampreys and found that concentrations were all less than 1 ng/ml, but differed significantly between sexes and between immature and mature lampreys. Young et al. (2004a) used the RIA to measure the plasma concentrations of 15α-T in

prespermiating male lampreys treated with GnRH as above, and it was found that 15α-T plasma levels rose 2-5 times in treated fish at 8 h and remained elevated at 24 h. Both types of GnRH elicited the same response. A second study (Young et al. 2004b) used low-dose time-release pellets of D-Arg(6)-GnRH analogs in prespermiating male lampreys. By 6 h after the implantation of the pellets, 15α-T level were elevated 1.6-2.4 times, and remained elevated for 48 h, and after 12 h, GnRH I elicited higher responses. Levels of 15α-T did not differ between non-treated males and males treated with bizasir (*P*,*P*-bis(1-aziridinyl)-*N*-methylphosphinothioic amide). Bizasir is used as a chemosterilant in a sterile male release program used to reduce the numbers of viable eggs in areas which cannot be treated with lamprey larvacides (Hanson and Manion 1980, Twohey et al. 2003).

15α-Hydroxylated steroids in other lamprey species

Once it was determined that 15α-T and 15α-P are produced in the testes and circulated in the plasma of sea lampreys, a study was then conducted to examine whether these unusual steroids and steroidogenic enzymes were a unique feature of sea lampreys, or whether they are common among lamprey species (Figure 2). The dissertation research established that other lamprey species that evolved both before and after sea lampreys produce 15α-P and 15α-T *in vitro* and *in vivo* (Table 1, Table 2), but how 15α-hydroxylated steroids function within the HPG axis appears to differ among the species (Bryan et al. submitted, in preparation). All species examined thus far, including members of the most ancestral genus, *Ichthyomyzon*, and the most derived genus,

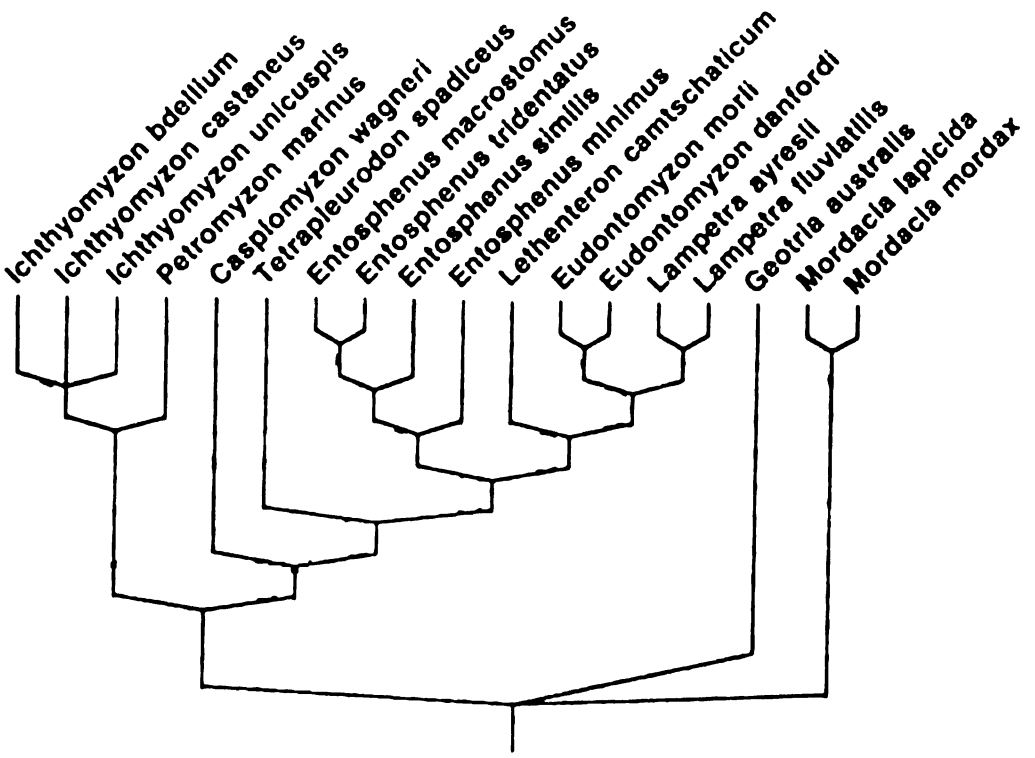

Figure 2. Lamprey phylogeny excerpted from Gill et al. 2003. The phylogeny is based on analysis of 32 morphological, anatomical, and karyological characters to produce a consensus tree.

Lampetra, appear to have 15α-steroid hydroxylase in adult testes (Gill et al. 2003; Kime and Rafter 1981; Bryan et al. submitted, in preparation), and have ir-15α-hydroxylated steroids that coelute with standards on HPLC. However, neither 15α-P nor 15α-T plasma concentrations change in response to either type of GnRH in male silver lampreys (*I. unicuspis*), and 15α-T plasma concentrations are unaffected by either type of GnRH in male river lampreys. Both types of GnRH cause higher plasma concentrations of 15α-P in male river lampreys, and GnRH III, but not GnRH I, causes higher plasma concentrations of both 15α-P and 15α-T in pacific lampreys (*Entosphenus tridentatus*). However, these increases, while statistically significant, are still biologically small.

Although this difference may be explained by differences in life history, feeding ecology, maturational stage at which experiments were conducted, and differences in experimental protocol, these experiments are beginning to suggest that sea lamprey reproductive physiology has diverged from that of other lamprey species.

Non-classical steroids in other aquatic vertebrates

Hagfish are the closest extant relative to lampreys, with which they form a monophyletic group (Kuraku et al. 1999). Lamprey-like and hagfish-like species evolved in the early Cambrian period (Shu et al. 1999) more than 500 million years ago, which supports a molecular clock-based estimate that agnathans diverged from gnathostomes approximately 564 million years ago (Kumar and Hedges 1998). Hagfish steroids have not received the same attention that sea lamprey steroids have. However, Kime and Hews (1980) and Kime et al. (1980) demonstrated that hagfish ovaries and testes possess 5α-reductase and 6β- and 7α-hydroxylases, along with other enzymes that convert P and T into unidentified metabolites. The unusual steroids found in lampreys and hagfish may be evolutionary artifacts.

However, many 'non-classical' gonadal steroids other than P, T, and E_2 have been definitively identified in teleosts (Kime 1993) though functions have only been ascribed to a very few of them. One of the best known is 17,20β-dihydroxypregn-4-en-3-one (17,20β-P) that is used as a maturation-inducing hormone in many teleost species and also as a pheromone in fish of the carp family (Stacey et al. 1989). There is no evidence that lampreys make this steroid, or any of the non-classical steroids that have been

studied in detail in teleost fishes (Table 2). There appears to be some variation in steroid structure in fishes (Kime 1993) compared to other vertebrate groups, but the 15α-hydroxylated steroids have not been found to be metabolic products of the gonads in any other vertebrate group.

Implications, conclusions, and future work

Steroids have been linked to maturation (Nagahama 1994), courtship behavior (Stacey and Kobayashi 1996), aggressive behavior (Elofsson et al. 2000), and migratory movements (Munakata et al. 2001a, 2001b) in teleosts. In all vertebrates studied to date, a common function of sex steroids is regulation of gonadal development. The gonads of the lamprey are morphologically similar to those of teleost fish and undergo the same developmental processes, including vitellogenesis and oocyte final maturation in females, and spermatogenesis and spermiation in males. In all teleost species studied thus far, these processes are known to be under the control of sex steroids produced by the gonads: e.g. estradiol stimulates vitellogenesis in females and spermatogonial proliferation in males; testosterone stimulates gonadotropin production by the pituitary gland of both sexes; 11-ketotestosterone stimulates spermatogenesis; 17,20β-P stimulates oocyte final maturation and spermiation (review: Miura and Miura 2001). It is generally accepted that steroids control these same processes in lampreys since the HPG axis is known to control reproduction in lampreys.

However, further research is needed to elucidate the functions of each of the 15α-hydroxylated steroids. First, binding studies must be conducted to determine the

locations and types of receptors present for 15α-P and 15α-T. Once the target tissues and mechanism of action of each of the 15α-hydroxylated steroids are known, it will become easier to determine the functions of these steroids.

In teleost fish, progestagens, particularly 17α,20β-P and 17α, 20β,21-trihydroxyprogesterone, are closely linked to final maturation (Nagahama 1994). In teleosts, it has been shown not only that injection with gonadotropins or GnRH-agonists result in spermiation and a rise in progestagens (Ueda et al. 1985; Mylonas et al. 1997; Vermeirssen et al. 2000), but that injection with progestagens alone results in spermiation (Ueda et al. 1985; Yueh and Chang 1997). Injections with GnRH are known to cause an increase in 15α-P plasma concentrations (Bryan et al. 2004) and decrease the time to final maturation (Sower 1990), but more research is needed to determine if final maturation caused by GnRH is mediated by 15α-P. Additionally, since male lampreys release a potent sex pheromone concurrent with the onset of spermiation (Li et al. 2002), it is likely that the same steroidal cues are responsible for both final maturation and pheromone release. 15α-P may be responsible for stimulating either pheromone synthesis, the development of the secretory cells in the gills (Siefkes et al. 2003), or pheromone release, in addition to the possibility that it stimulates final maturation and spermiation.

Androgens have been implicated in spermatogenesis and development of secondary sexual characteristics (Borg 1994) and aggressive behavior (Elofsson et al. 2000) in teleosts. An additional function deserving further research in lampreys is the possible

involvement in migration, as androgens are known to be a factor in upstream migration in salmon (Munataka et al. 2001a; 2001b). No classical androgens have any characteristics of functional hormones in lampreys, and the prevailing theory is that lampreys do not use androgens (Sower 1990; Thornton 2001). However, though its circulating levels are relatively low, 15α-T may have androgenic functions in lampreys.

The research performed for this dissertation has furthered our knowledge of steroid structure, functionality, and evolution in lampreys. The synthesis of 15α-P and 15α-T by the testes has been confirmed. *In vivo* production and changes in circulatory levels in response to GnRH has been demonstrated, indicating that these two steroids are likely part of the HPG axis in sea lampreys. The enzyme pathway that produces 15α-T and 15α-P in the testes appears to be present in all other lamprey species examined, indicating that the 15α-hydroxylase enzyme is an ancestral trait within the Petromyzontidae family. However, the effects of neuroendocrine hormones on the steroidogenic pathways that produce 15α-T and 15α-P appear to vary widely among species, which demonstrates that lamprey species have diverged in their controls over steroidogenesis and possibly in the functionality of 15α-hydroxylated steroids.

Much more research regarding lamprey steroids is needed to understand the significance of the 15α-hydroxylated steroids in lampreys. We do not yet know which classical or 15α-hydroxylated steroids are functional hormones, the physiological roles of any of the steroids, or which steroids have nuclear and/or membrane receptors. It is important to answer these research questions, as they will further our understanding of the evolution of

the enzymes involved in steroidogenesis (Baker 2004), the evolution of nuclear steroid receptors (Thornton 2001), and the evolution of ligand-receptor based methods of regulation (Baker 2003).

Table 1: Studies using steroid immunoassays in lampreys.

Reference	Stage or sex	Steroid	Range/ mean in plasma (ng/ml)	Difference between sexes?	Difference between reproductive stages?	Levels in response to GnRH (ng/ml)?
Sea lampreys (*P. marinus*)						
Sower et al. 1983	POF, OF	E_2	3-5	-	Yes	Up to 12
Weisbart et al. 1980	Early migrating POF and PSM	17α,20β-P	1.6 – 3.1	-	-	-
		T	4.1	-	-	-
Katz et al. 1982	SM, OF	P	0-1.25	-	Rose after stress	-
		DHT	0	-	-	-
		T	0	-	-	-
		AD	1.05-5.58	-	Rose after stress	-
		E	0.74-7.77		-	-
		E_2	0.51-3.14	-	-	-
Sower et al. 1985	POF, OF, PSM, SM	T	0.1 – 0.2	No	No	-
		E_2	0.5 – 3.0	Only in mature lampreys	At spawning, rises in SM and drops in OF	-
Sower et al. 1985b	POF, OF, PSM, SM	T	0.10-0.18	-	-	No effect
		E_2	2	-	More mature F have lower levels	Up to 6.5
Linville et al. 1987	POF, OF, PSM, SM	T	0.005-0.170	M higher than F	No change with spawning behavior	-
		P	0.1 – 2.8	M higher than F		-
		E_2	0.6 – 2.3	M higher than F	During spawning no change in M; drop in F	
Sower et al. 1987	POF, OF	P	<1	-	-	Increased
		E_2	1.5	-	-	Up to 5.5
Sower 1989	PSM	P	0.25	-	-	Up to 3
		E_2	1.5	-	-	Up to 3
Sower et al. 1993	POF	P	0.52	-	-	Up to 0.71
		E_2	0.64	-	-	Up to 2.06
Sower and Larsen 1991	POF	P	0.3	-	Decreases after hypophysectomy	-

Reference	Stage or sex	Steroid	Range/ mean in plasma (ng/ml)	Difference between sexes?	Difference between reproductive stages?	Levels in response to GnRH (ng/ml)?
Sower and Larsen 1991 (cont'd)		E_2	1.91	-	Decreases after hypophysectomy	-
Bolduc and Sower 1992	POF, OF	P	0.1-0.6	-	Fluctuates or slowly increases	-
		E_2	0.25-3	-	Increases through spawning season, drops suddenly at end	-
Deragon and Sower 1994	PSM	P	0.2	-	-	Up to 2
		E_2	1.2	-	-	Up to 2.4
Gazourian et al. 1997	POF	P	2			Up to 12
		E_2	0.2			Up to 0.5
Gazourian et al. 2000	PSM	E_2	2	-	-	Up to 8
		P	0.2			Up to 2.6
Rinchard et al. 2000	SM	T	0.03-0.15	-	-	-
		E_2	1-2	-	-	-
		P	0.4-1.2	-	-	-
Bryan et al. 2003	POF, OF, PSM, SM	15α-T	<1	Yes	Yes	
Bryan et al. 2004	POF, OF, PSM, SM	15α-P	<1 – 2.48	Yes	Yes	PSM only, mean of 36
Young et al. 2004a	PSM	15α-T	<0.5	-	-	Up to 3
Young et al. 2004b	PSM	15α-T	0.3			Up to 0.6
		E_2	1			Up to 3.5
River lampreys (*L. fluviatilis*)						
Kime and Larsen 1987	POF, PSM	T	0.1	None	Increases after gonadectomy to 1.2	-
		E_2	1	None	Increases after gonadectomy to 2	
Barranikova et al. 1995	POF, OF, PSM, SM	P	1-10	-	-	-

Reference	Stage or sex	Steroid	Range/ mean in plasma (ng/ml)	Difference between sexes?	Difference between reproductive stages?	Levels in response to GnRH (ng/ml)?
Barranikova et al. 1995 (cont'd)		E_2	0.5 - 3.5	-	Decreases near ovulation	-
Mewes et al. 2002	POF, PSM, OF, SM	E_2	0.01 – 3.2	Higher in M than F	-	-
Other species						
Fukayama and Takahashi	*L. japonica* adults	T	0	-	-	-
		E_2	0.44 – 4.52	Higher in M than F near spawning	F levels highest near migration, M increase with maturation	-
Seiler et al. 1985	*L. planeri* adults	P_5	2 - 3	-	None	-
		AD	0 - 2.5	-	None	-
		T	2.5-17	-	Increased with maturity	-

POF: pre-ovulating females; OF: ovulating females; PSM: prespermiating males; SM: spermiating males: P: progesterone; P_5: pregnenolone; 17α,20β-P: 17α,20β-dihydroxyprogesterone; T: testosterone; AD: androstenedione; DHT: 5α-dihydrotestosterone; E: estrone; E_2: estradiol; 15α-T: 15α-hydroxytestosterone; 15α-P: 15α-hydroxyprogesterone

Table 2: Metabolic Studies

Reference	Species	Tissue	Stage	Precursor	Products	Not produced
Weisbart and Youson 1975	*P. marinus*	PAT	larvae, parasites	P	S, 17-P, AD, UCs	F, E, B, T, DOC
		testes	parasites	P	DOC, UCs	F, E, B, S, T, 17-P, AD
Weisbart and Youson 1977	*P. marinus*	intracardiac injection	parasites	P	DOC, UCs	F, E, B, S, T, 17-P, AD
Weisbart et al. 1978	*P. marinus*	testes, ovaries, PAT	adults	cholesterol	UCs	T, AD, P, P_5, 17α-P
Callard et al. 1980	*P. marinus*	ovary, kidney	adults	AD	E_1, 5α-AD, UCs	DHT
		testes	adults	AD	E_2, E, 5α-AD, UCs	DHT
Kime and Rafter 1981	*L. fluviatilis*	ovaries	adults	P, T	15α-P, AD, 15β-T	T, 17-P, E_2
		testes	adults	P, T	15α-P, AD, 15β-T	T, 17-P, E_2
Kime and Callard 1982	*P. marinus*	testes	adults	AD	15α-T, 15α-AD	15β-T
		brain, liver, kidney, ovary	adults	AD	15α-AD	
Bryan et al. 2003	*P. marinus*	testes	adults	T	15α-T	15β-T
Lowartz et al. 2003	*P. marinus*	testes	adults	P_5, 17-P, AD	15α-T, 15α-P, small amount of E_2	T, P
		ovaries		P_5, 17-P, AD	15α-estrogens, small amount of E_2	T, P
Bryan et al. 2004	*P. marinus*	testes	adults	P	15α-P, UCs	
Lowartz et al. 2004	*P. marinus*	ovaries, testes	Larvae, transformers, parasites	P_5, P, AD	7α-P_5, 15α-P, 15α-AD, 15α-T, 15α-E_2, small amount of E_2, UCs	T, 15β-steroids
Bryan et al. submitted	*I. unicuspis* *I. castaneus* *L. appendix*	ovaries, testes	adults	P, T	15α-P, 15α-T, UCs	
Bryan et al. in preparation	*L. fluviatilis*	testes	adults	P, T	15α-P, 15α-T, UCs	

PAT: presumptive adrenocorticol tissue; P: progesterone; P_5: pregnenolone; 7α-P_5: 7α-hydroxypregnenolone; 17-P: 17α-hydroxyprogesterone; AD: androstenedione: 5α-AD: 5α-reduced androstenedione;15α-AD: 15α-hydroxyandrostenedione; T: testosterone: S: 11-deoxycortisol: DOC: 11-deoxycorticosterone; F: cortisol; E: cortisone; B: corticosterone; E_1: estrone; 15α-T: 15α-hydroxytestosterone; 15β-T: 15β-hydroxytestosterone; 15α-AD: 15α-hydroxyandrostenedione; 15α-P: 15α-hydroxyprogesterone; UCs: unidentified compounds

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CHAPTER TWO

Bryan M.B, Scott A.P, Černý I., Yun S.S, Li W. 2003. 15α-Hydroxytestosterone produced *in vitro* and *in vivo* in the sea lamprey, *Petromyzon marinus*. General and Comparative Endocrinology 132, 418-426.

15α-Hydroxytestosterone produced *in vitro* and *in vivo* in the sea lamprey, *Petromyzon marinus*

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Abstract

Prior research has shown that the testes of lampreys are able to synthesize 15-hydroxylated steroid hormones *in vitro*. Here we show that testes of the sea lamprey *Petromyzon marinus* L. are able to convert tritiated testosterone into tritiated 15α-hydroxytestosterone (15α-T) in high yield. The identity of the tritiated 15α -T has been confirmed by: co-elution with standard 15α-T on high performance liquid chromatography (HPLC); co-elution on thin layer chromatography (TLC); co-elution of acetylated tritiated and standard 15α-T on TLC; and strong binding to an antiserum developed against 15α-T. The strong reaction between the tritiated 15α-T and the antiserum has been used to develop a radioimmunoassay (RIA). The RIA operates over the range of 500 pg to 2 pg per tube; and can be applied directly to plasma samples. This assay has been used to demonstrate that 15α-T is present in blood plasma of the sea lampreys. The concentrations of 15α-T in captive lampreys were found to be as follows (pg/ml; mean ± sem, n): parasitic stage (reproductively immature), < 20 pg/ml, n = 7; pre-ovulatory females, 156 ± 30 pg/ml, n = 8; ovulated females, 62 ± 9, n = 5; pre-spermiating males, 275 ± 19, n = 8; spermiating males, 216 ± 48, n = 8. When spermiating male plasma was fractionated on HPLC, immunoreactivity was found exclusively in the expected elution position of 15α-T. The biological significance of this steroid has yet to be established.

Key words: sea lamprey, Petromyzon, endocrinology, steroid, androgen

Introduction

The sea lamprey, *Petromyzon marinus* L., is a useful model species in studies of comparative and evolutionary endocrinology. As a member of superclass Agnatha, the lamprey is one of the earliest evolving vertebrates still alive today. It has an anadromous life cycle, with filter-feeding ammocoetes living in stream bottoms, parasitic phase immature lamprey feeding in large bodies of water, and reproductive adults migrating to tributary streams to spawn (Hardisty and Potter 1971). Additionally, information concerning basic lamprey biology may be useful in a conservation context due to the effort spent controlling the invasive sea lamprey population in the Laurentian Great Lakes and conserving dwindling populations in Europe (Maitland 1980).

Previous research on lampreys has established that, similar to teleosts and higher vertebrates, the hypothalamus-pituitary-gonadal axis, which includes two forms of GnRH, regulates reproduction (reviews: Sower 1998; Sower and Kawauchi 2001). However, the identity of the gonadal steroids in lampreys and their role in reproduction is still not clear, especially with regard to the existence of androgens. There have been several studies in which measurements have been made of plasma concentrations of testosterone in the sea lamprey (Sower et al. 1985a, 1985b; Linville et al. 1987; Katz et al. 1982; Weisbart et al. 1980). In most cases, the amounts of testosterone were either undetectable or very low (<1 ng/ml) – the exception being the paper by Weisbart et al. (1980) in which a single value of 4.2 ng/ml was reported. This was the only study that did not use radioimmunoassay (RIA) to measure the steroid. Despite the low concentrations, Linville et al. (1987) found significant differences in plasma testosterone

concentrations between male and female lampreys and an association between testosterone concentrations and stage of maturation. However, this finding was in contrast to that of Sower et al. (1985a), which found no differences between the sexes and no relationship to the stage of reproduction. Sower et al. (1985b) also showed that testosterone concentrations did not change in response to GnRH injection. The story with other species of lamprey is very similar. Testosterone (or at least a substance or substances cross-reacting with testosterone antibodies) is present, but at low concentrations that bear no relationship to stage of maturation, gender, or treatment (Fukayama and Takahashi 1985; Kime and Larsen 1987; Rinchard et al. 2000). The only exception is the brook lamprey, *Lampetra planeri* Bloch, in the blood of which Seiler et al. (1985) recorded concentrations of testosterone of up to 15 ng/ml.

Attempts have also been made to demonstrate the *in vivo* (Weisbart et al. 1977) and *in vitro* biosynthesis of testosterone by the gonads of immature (Weisbart and Youson 1975) and mature sea lamprey (Weisbart et al. 1978). In both cases, no testosterone was found. In the latter study, it was also reported that testicular 3β-hydroxysteroid dehydrogenase activity (that is required for the production of 4-ene steroids such as testosterone) was relatively low in comparison to other vertebrates.

The most likely reason for the apparent absence of testosterone in lamprey comes from a study by Kime and Rafter (1981). These authors incubated the testes of the river lamprey, *Lampetra fluviatilis* L., *in vitro* with either radio-labeled testosterone or radio-labeled progesterone. In both cases, the steroids were rapidly transformed into,

respectively, 15β-hydroxytestosterone and 15α-hydroxyprogesterone. When Kime and Callard (1982) incubated testes of the sea lamprey with radio-labeled androstenedione they identified both 15α-hydroxyandrostenedione and 15α-hydroxytestosterone (15α-T). These findings prompted the authors to predict that the functional steroids in lampreys (whether androgens, estrogens, or progestagens) differed substantially from those of most other vertebrates in possessing a 15-hydroxyl group. Recently there has been confirmation that steroids produced *in vivo* and *in vitro* in sea lamprey have elution times corresponding to 15-hydroxylated steroids (Lowartz et al. 2003). The aim of the present study was to establish chemical evidence determining whether or not 15α-T is present in the blood plasma of reproductively mature sea lamprey.

Our first objective was to establish whether the incubation of sea lamprey testes with tritiated testosterone would yield 15α-T. Our second objective was to develop an immunoassay – whether by enzyme-linked immunoassay (ELISA) or RIA – in order to determine whether 15α-T is present in sea lamprey plasma. Our third objective was to determine whether there were any differences between the sexes and between stages of maturity in plasma concentrations of 15α-T.

Methods

All chemicals were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Sea lamprey were collected in streams by U.S. Fish and Wildlife Service employees, and transported to either Michigan State University (East Lansing, MI) for incubation experiments or Hammond Bay Biological Station (Millersburg, MI) for plasma sampling

where they were held at 10° C. Blood was obtained from the caudal vein using heparinized syringes, held at 4° C for 20 min, and centrifuged at 2500 rpm for 20 min. The plasma was removed and stored at -80° C.

Synthesis of 15-hydroxylated standards

15α-T was prepared by a fifteen-step synthesis beginning with DHEA. The 15α-hydroxy group was introduced using a modification of the procedure described by Hosoda et al. (1976). A 15-hydroxyandrostane derivative was obtained and, after change of protection, was transformed into the testosterone series using the method described by Raggio and Watt (1976). The final product had a melting point and optical rotation that was identical to 15α-T obtained through microbial transformation (Tamm et al. 1963). The fully resolved 1H NMR spectrum corresponded to established data (Kirk et al. 1990); selected couplings in Hz: J(14α,15β) 9.1, J(15β,16α) 9.7, J(15β,16β) 3.4, J(16α,16β) 14.4, J(16α,17α) 8.7, J(16β,17α) 9.1. 15β-T was prepared as in Cerny et al. (1996).

In vitro biosynthesis of tritiated 15α-T

To confirm the biosynthesis of 15α-T, testicular tissue from two freshly killed non-spermiating adult male lampreys was finely diced with a scalpel. Replicate 500 mg portions of diced tissue from each lamprey were placed in 50 ml polypropylene tubes containing 10 ml L15 medium (at 6° C) and 25 μCi [1,2,6,7-^{3}H]-testosterone. The tube was then shaken for 4 h at 17° C. At the end of this period, the medium was removed from each tube, filtered, and passed through an activated Sep-Pak C18 extraction

cartridge (Waters, Milford, MA). Each cartridge was washed with 5 ml distilled water and eluted with 5 ml methanol. The solutions were stored at –20° C.

In order to characterize the products of the reaction, 50 µl of methanol extract was mixed with 10 µg each of T, 15α-T, and 15β-T (dissolved in 20 µl ethanol), dried down under a stream of nitrogen at 45° C, redissolved in 500 µl acetonitrile/water/TFA (28/72/0.01, v/v/v) and loaded onto a reverse-phase HPLC column (Waters, Milford, MA; 5 µm octadecylsilane; 4.6 mm x 250 mm; fitted with a guard module). Two solvents were used to deliver a gradient to the column. Solvent A was 0.01% TFA in distilled water and solvent B was 70% acetonitrile and 0.01% TFA in distilled water. The pattern of development was as follows: 0 → 10 min, 28% B; 10 → 60 min, 28% → 100% B; 60 → 80 min, 100% B. The eluate was monitored for UV absorption with a photodiode array detector (Waters). Fractions were collected every 1 min between 20 and 60 min into scintillation vials. After addition of 3 ml ScintSafe Econo 1 scintillation fluid (Fisher Scientific, Pittsburgh, PA) the vials were counted on an LS 6500 Scintillation Counter (Beckman Coulter).

Further characterization of radioactive 15α-T was carried out by thin layer chromatography (TLC). A 1.5 ml aliquot of methanol extract from one of the incubations was mixed with 20 µg 15α-T and testosterone, dried down, and fractionated on HPLC as described above. Part of the fraction (30 µl containing 50,000 dpm) corresponding to the elution position of 15α-T was placed in a glass tube containing 10 µg 15α-T. The solvents were removed, replaced by 100 µl pyridine and 100 µl acetic anhydride, and left

overnight at room temperature. A further 30 µl of the same fraction was mixed with 10 µg of 15α-T and 15β-T in a separate glass tube. The solvents in both tubes were evaporated and replaced with 40 µl ethyl acetate. These were loaded onto separate lanes of a TLC plate, which was developed with chloroform/ethanol (50/2; v/v). The positions of the standards were noted by placing the plate under a UV source. The lanes were then divided into 4 mm fractions, scraped off, placed in scintillation vials, mixed with 3 ml scintillation fluid and counted.

Further confirmation of the identity of radioactive 15α-T was carried out by testing its ability to bind to different dilutions of antisera developed to 15α-T-3-CMO (as described below), 15β-T-3-CMO and 15α-P-3-CMO (and to a control serum). This required a further 1.5 ml of the methanol extract to be fractionated on HPLC without being mixed with any standard. The antibody dilutions were made up in 100 µl assay buffer in glass tubes (as described below) and the radioactive 15α-T was added in a further 100 µl buffer at the rate of 5,000 dpm per tube. After overnight incubation at 4° C, 500 µl of ice-cold charcoal solution (50 mM sodium phosphate pH 7.4, 0.1% gelatin, 1.0% dextran-coated charcoal) was added to each tube. The tubes were kept in ice for 20 min, and then centrifuged at 1000 g for 12 min. The supernatants were poured into 8 ml scintillation vials, mixed with 6 ml scintillation fluid and counted.

Preparation of 15α-T radiolabel for use in radioimmunoassay

Approximately 1 g of minced testicular tissue was added to 12 ml L-15 medium containing 150 µCi tritiated testosterone and 7.3 mg NAD. After incubation at 10° C for 4 h, the medium was filtered and loaded onto an extraction cartridge as described above.

The 15α-T label was preferentially eluted by passing through the cartridge 10 ml of 23% acetonitrile and 0.01% TFA in distilled water (v/v/v). This was then diluted with 20 ml distilled water and passed through a fresh extraction cartridge, washed with 5ml distilled water and eluted with 5 ml ethanol. The extract was stored at -20° C.

Development of antiserum

In order to develop an antiserum, a mixture of 15 mg 15α-T, 17 mg carboxymethyloxime hydrochloride, and 25 mg sodium acetate was dissolved in 1.5 ml methanol and left overnight at 4° C. The methanol was then dried down under a stream of nitrogen at 45° C and redissolved in 400 µl methanol, followed by 1 ml of water which had been adjusted to pH 2.0 with acetic acid. The product from the reaction, 15α-T-CMO, was extracted from this solution by shaking it twice with 4 ml ethyl acetate. The ethyl acetate was evaporated and the extract redissolved with a small amount of methanol and then precipitated by addition of diethyl ether. The powder was dried under vacuum.

Five mg 15α-T-CMO was dissolved in 1.5 ml of dimethylformamide (DMF) in a 20 ml glass scintillation vial. The vial was placed in crushed ice within a polystyrene container that was placed on top of a magnetic stirrer. A small magnetic flea was added to the beaker. The ice was prevented from thawing by the occasional addition of small amounts of liquid nitrogen to the container. With constant stirring, 3.5 µl tri-butylamine and 2.5 µl isochloroformate were added to the vial and the reaction was allowed to proceed for 40 min. In the meantime, 20 mg BSA was dissolved in 1 ml distilled water and then diluted with 1 ml DMF and 1 drop of 2 N sodium hydroxide. This mixture, after being chilled on

ice, was added to the vial and left to stir for a further 3 h. After this time, the mixture (which was slightly opaque) was centrifuged for 10 min at 1000 g. The clear supernatant was made up to 2.5 ml with distilled water and then desalted on a PD-10 column (Nash et al., 2000), using 3.5 ml distilled water to elute the protein fraction. The eluate was frozen and freeze-dried.

Approximately 2 mg 15α-T-CMO-BSA was dissolved in 1 ml saline and 1 ml Freund's Complete Adjuvant and each injected intradermally into two rabbits. Booster injections using the same amount of powder, but suspended in Freund's Incomplete Adjuvant, were given at four, six, and eight weeks following the first injection. Blood (20 ml) was obtained eight weeks after the first injection and allowed to clot before being centrifuged at 2500 rpm for 15 min. The serum was removed and frozen in 0.5 ml aliquots at -80° C.

Radioimmunoassay (RIA) procedure

RIAs were conducted in glass culture tubes (10 mm x 75 mm, Fisher Scientific, Pittsburgh, PA) according to Scott et al. (1980). The assay buffer consisted of 50 mM sodium phosphate pH 7.4, 0.2% BSA, 137 mM NaCl, 0.40 mM EDTA, 0.77 mM sodium azide. Nine standards were made up in duplicate over the range 500 to 1.95 pg/ 100µl/tube. The tubes containing unknowns also had a volume of 100 µl. Binding reagent was made up by adding radiolabel and antiserum to 20 ml of assay buffer in amounts such that, when 100 µl was dispensed to all tubes, each tube contained 7,000 dpm and, in the absence of any standard, 50% of the radiolabel was bound to the antiserum. Blank tubes, to which no antibody was added, and tubes necessary to

determine the total and maximum dpm counts were also included in the assay. All tubes were incubated overnight at 4 °C, and then placed on ice and separated with charcoal as described above.

The specificity of the antiserum was tested by making, in 100 µl buffer, six five-fold serial dilutions of 15α-T over a range of 10,000 to 0.64 pg/tube; of 15β-T and T over a range of 10,000 to 3.2 pg/ tube; and 11keto-testosterone, androstenedione, estradiol, cholic acid, 15α-hydroxyprogesterone, 3keto-petromyzonol sulfate, 3keto-allochoic acid, petromyzonol sulfate, and allochoic acid over a range of 10,000 to 400 pg/tube. A further 100 µl of buffer containing 7,000 dpm radiolabel and the 15α-T antiserum at a dilution of 1:100,000 (v/v) was added to each tube. After overnight incubation at 4° C they were separated with dextran-coated charcoal as described above.

Some cross-reaction was found with 15β-T (see Results). An experiment was thus performed to determine whether this was due to: genuine cross-reaction; possible contamination of the standard with 15α-T; or a mixture of both. Small amounts of standard 15α-T (200 ng) and 15β-T (2 µg) were loaded on to separate lanes of a TLC plate (type LK6DF; Whatman International). The plate was then developed with chloroform/ethanol (50/3; v/v) and the positions of the steroids detected by placing the plates under a UV light source. Both lanes were divided into 5 mm sections that were scraped into glass tubes. Assay buffer (1 ml) was added to each tube, held overnight at 4 °C, vortexed, and centrifuged briefly. Replicate 100 µl aliquots from each tube were assayed for 15α-T.

Preliminary experiments with several plasma extraction procedures (involving diethyl ether, ethyl acetate or dichloromethane) indicated that there appeared to be little or no interference with the assay if plasma was added directly to the tubes. An experiment was conducted to determine whether plasma proteins that bind 15α-T are present. Pooled plasma from parasitic phase lampreys, pre-ovulating females, ovulating females, pre-spermiating males, and spermiating males was diluted 1:2, 1:4, 1:8, and 1:16 in assay buffer (100 µl/tube). Radiolabel was added to all tubes (100 µl containing 7,000 dpm). The tubes were then incubated and separated as described above for the RIA. In a second experiment, 2 ml male plasma (pooled from several males) was mixed with 20 ng 15α-T. The plasma was then diluted four times with the same plasma to yield a range of dilutions of 10 to 1.25 ng/ ml. Each of these dilutions was then assayed using aliquots of 25, 50 and 100 µl aliquots. The two lower aliquots were made up to 100 µl with assay buffer.

Inter-assay variation was determined by measuring the amount of 15α-T in the same plasma sample (*c.* 2 ng/ml) in six separate assays. Intra-assay variation was determined by measuring 15α-T in the same sample six times in the same assay.

Concentrations of 15α-T in lamprey plasma

Plasma was collected as described above from seven parasitic lamprey, five ovulating females and eight each of pre-spermiating males, pre-ovulatory females and spermiating males. Parasitic phase lampreys were held for at least one week without feeding. For each sample, 50 µl of plasma was assayed in duplicate.

To confirm that cross-reacting material in plasma had the same chromatographic properties as 15α-T, 5 ml pooled male sea lamprey plasma was passed through an extraction cartridge. The steroids were eluted with 7 ml methanol and then dried with a rotary evaporator (RE 200, Yamato, Orangesburg, NJ). The residue was subjected to HPLC separation as described above. All fractions were assayed for 15α-T using 20 µl/ fraction.

Results

Incubation of lamprey testes with tritiated testosterone resulted in two major peaks on HPLC (Fig. 3). The first and largest of these peaks corresponded to the elution position of 15α-T and the second to the elution position of testosterone. The conversion rates of ^{3}H-testosterone to 15α-T in the two testes were 85.6 % and 64.5 %.

Further confirmation of the identity of the radioactive 15α-T was obtained by running some of the HPLC fraction on TLC. The bulk of the radioactivity eluted in the same position as standard 15α-T (Fig. 4). Very little was associated with 15β-T. Furthermore, when the radioactive peak was mixed with standard 15α-T and acetylated, both radioactivity and UV absorption co-migrated.

Incubation of 1 g lamprey testis with 150 µCi tritiated T yielded approximately 75 µCi 15α-T. The dilution of antiserum that was required to bind only 50% of this radiolabel (at 7000 dpm/ 200 µl) was 1:100,000 (v/v). The radioactive 15α-T bound strongly to the

15α-T antiserum, slightly to the 15β-T antiserum and not at all to the 15α-P antiserum or control serum (Fig. 5).

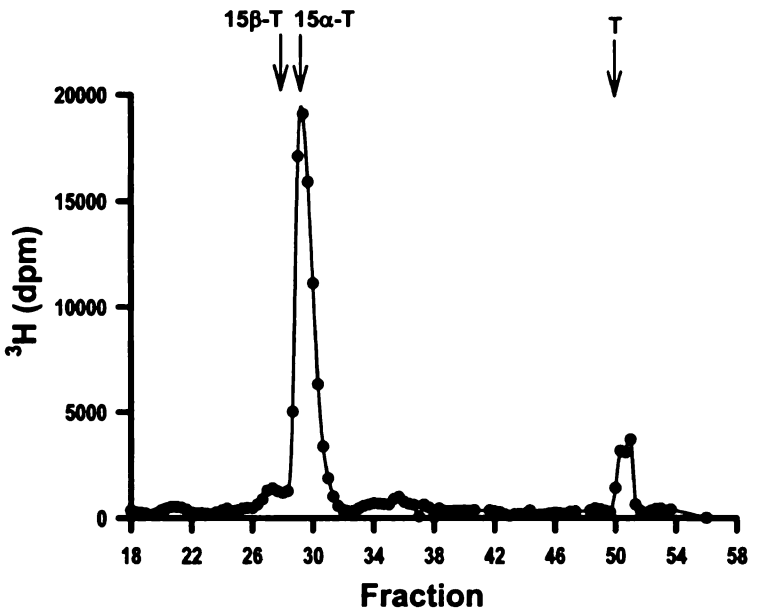

Figure 3: ^{3}H counts (dpm) in fractions following HPLC analysis of 20 µl of media from incubation of testicular tissue with ^{3}H testosterone. Arrows show where 15α-T (15α-hydroxytestosterone), 15β-T (15β-hydroxytestosterone), and T (testosterone) standards elute.

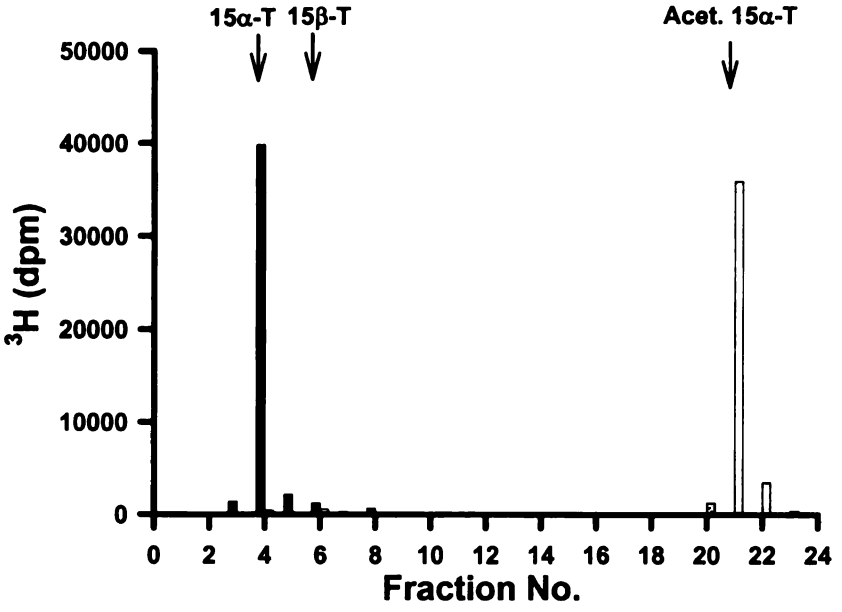

Figure 4: ^{3}H counts (dpm) from TLC fractionation of 30 µl of incubation media after purification with HPLC. Black bars represent the purified product of incubation, and grey bars represent the acetylated product. Arrows show the elution points of 15α-T (15α-hydroxytestosterone) standard, acetylated 15α-T, and 15β-T (15β-hydroxytestosterone) standard.

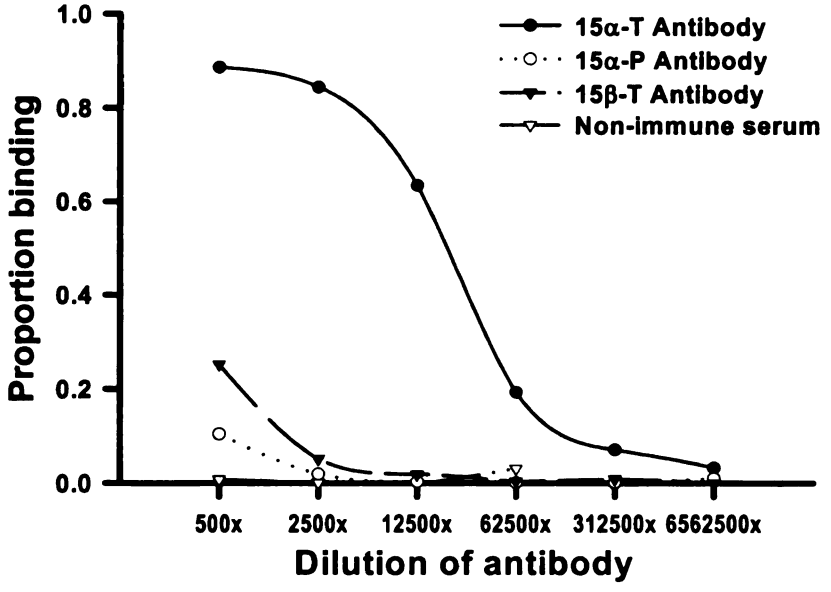

Figure 5: Ability of antibodies raised against different steroids to bind purified 15α-T produced *in vitro*. 15α-T is 15α-hydroxytestosterone, 15α-P is 15α-hydroxyprogesterone, and 15β-T is 15β-hydroxytestosterone.

There was negligible cross-reaction between the 15α-T antiserum and most of the tested steroids (Fig. 6). However, there was substantial, but non-parallel, cross-reaction of the 15β-T (36% towards the top of the standard curve and 6.4% towards the bottom). By running the 15β-T on TLC, it was established that a large part of the cross-reaction was due to probable contamination of the 15β-T standard with 15α-T (Fig. 7). Some cross-reaction was still associated, however, with the elution position of 15β-T.

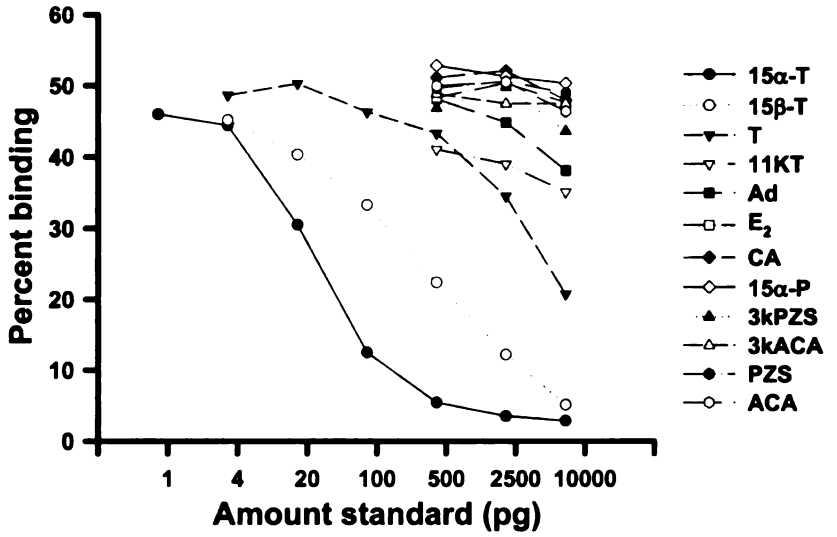

Figure 6: Ability of various common steroids and bile acids to displace radio-labeled 15α-T (produced *in vitro*) from the antibody raised against 15α-T. 15α-T is 15α-hydroxytestosterone, 15β-T is 15β-hydroxytestosterone, T is testosterone, 11KT is 11keto-testosterone, Ad is androstenedione, E_2 is estradiol, CA is cholic acid, 15α-P is 15α-hydroxyprogesterone, 3kPZS is 3keto-petromyzonol sulfate, 3kACA is 3keto-allochoic acid, PZS is petromyzonol sulfate, and ACA is allochoic acid.

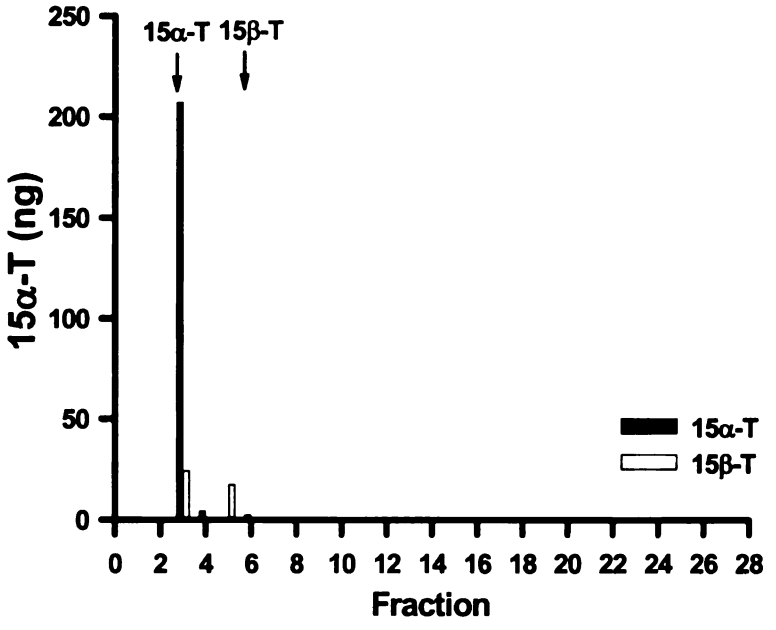

Figure 7: Assay for 15α-T (15α-hydroxytestosterone) of TLC fractions from 2 μg of 15β-T (15β-hydroxytestosterone) standard (filled bars) and 200 ng of 15α-T standard (open bars). Arrows indicate the elution points of 15α-T and 15β-T. There is only a small positive result in the fraction containing 15β-T, indicating that actual cross-reactivity is small. However, there are positive results in other fractions in the 15β-T lane, indicating that the standard is contaminated.

Intra-assay variation for 15α-T was *c.* 5% over the middle of the standard curve. Inter-assay variation was *c.* 14%.

Lamprey plasma from any sex or reproductive stage did not bind significantly to tritiated 15α-T even at a dilution of 1:2 (v/v). Plasma dilutions yielded values near expected when assayed in 25 μl or 50 μl volumes (Fig. 8). Assay of plasma in 100 μl volumes yielded values slightly lower than expected.

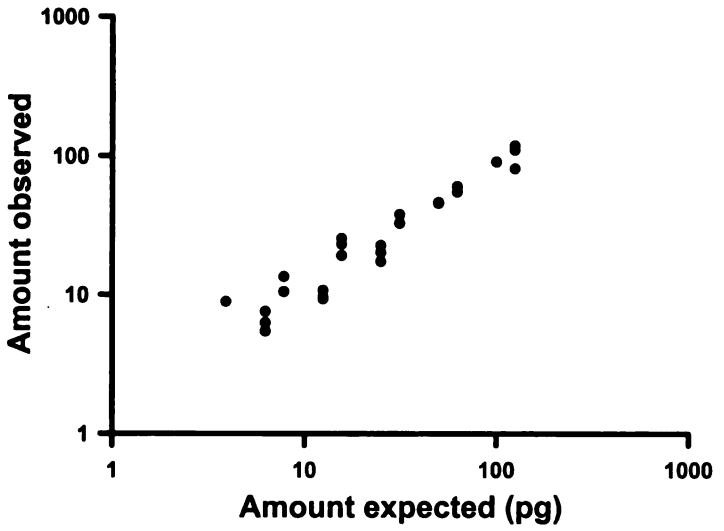

Figure 8: Expected amounts of 15α-T plotted against observed amounts in the recovery experiment. Known amounts of 15α-T were added to plasma, which was assayed in different quantities and dilutions.

No 15α-T (< 20 pg/ml) was detected in plasma obtained from parasitic phase lamprey. The concentrations of 15α-T in captive lamprey were found to be as follows (pg/ml; mean ± sem): parasitic stage, < 20; pre-ovulatory females, 156 ± 30; ovulated females, 62 ± 9; pre-spermiating males, 275 ± 19; spermiating males, 216 ± 48. Since 15α-T was not detected in plasma obtained from parasitic phase lamprey, they were not included in statistical tests. Significant differences existed in plasma 15α-T concentrations among other groups (ANOVA, $F_{3, 25} = 6.53$, $P = 0.002$, Fig. 9). In pairwise comparisons (with a Bonferroni correction) ovulating females were found to have significantly lower concentrations than pre-spermiating males ($P = 0.002$) and spermiating males ($P = 0.028$). After HPLC fractionation of male plasma, only one fraction contained cross-reactive material and that was in the expected elution position of 15α-T (Fig. 10).

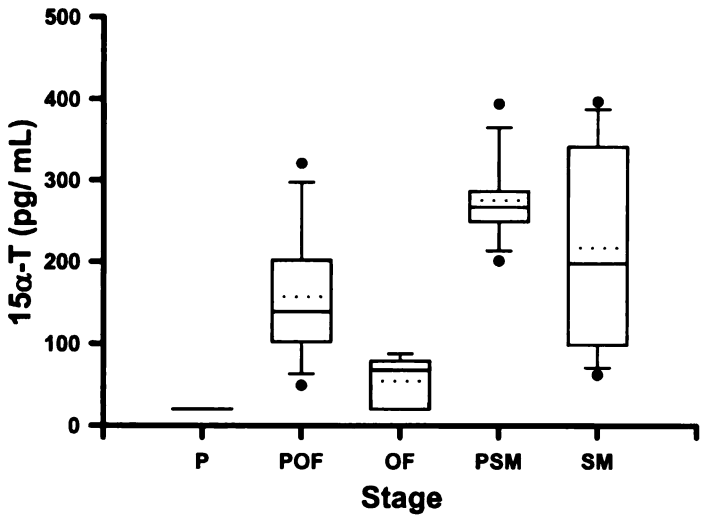

Figure 9: Differences in 15α-T concentrations among sexes and stages of maturity. P is parasitic phase lamprey (n = 7), OF is ovulated females (n = 5), POF is pre-ovulatory females (n = 8), PSM is pre-spermiating males (n = 8), and SM is spermiated males (n = 8). Dotted lines are means, solid center lines are medians, boxes cover the 25-75% range, whiskers show the 10% and 90% percentiles, and dots are values either below 10% or above 90% percentile.

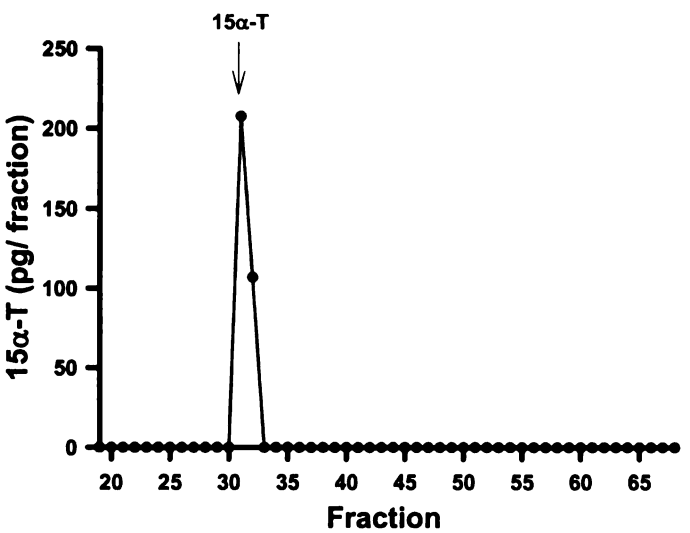

Figure 10: Amount of 15α-T found in HPLC fractions from 5 ml pooled male sea lamprey plasma based on RIA of 20 μL of each fraction. An arrow shows where 15α-T elutes.

Discussion

This study provides conclusive evidence that the testis of the sea lamprey is able to produce 15α-T both *in vitro* from exogenous testosterone and *in vivo*. *In vitro* production of 15α-T using testosterone as a precursor has so far only been demonstrated in the river lamprey (Kime and Rafter 1981; Golla et al. 2000), though a recent study found that the *in vitro* product of testosterone co-eluted with 15α-T on HPLC (Lowartz et al. 2003). Kime and Callard (1982) found 15α-T as one of the products of incubation of sea lamprey testes with radioactive androstenedione. Kime and Rafter (1981) obtained high yields of 15β-T from testes of the river lamprey. However, in both the present study and that by Kime and Callard (1982) this isomer was not formed – suggesting that the sea lamprey lacks a 15β-hydroxylase.

The 15α-hydroxylating activity in the sea lamprey testis is so strong and specific that it has enabled us to use lamprey testicular tissue to produce tritiated 15α-T suitable for use in RIA. This proved to be beneficial as we had originally set out to develop an ELISA for 15α-T. However, we experienced persistent problems with replication, specificity and generation of 'false positives' with the prototype ELISA. These problems were ameliorated when we developed the RIA.

The amounts of the steroid that we have found in plasma are no higher than those of testosterone that have previously been measured in this species. However, as opposed to the situation with testosterone (with the exception of one study – see Introduction) there

are very clear differences in 15α-T concentrations between the sexes and stages of maturation. Additionally, we have HPLC evidence that what we are measuring is in fact 15α-T and not some other cross-reacting compound(s). There are no published data demonstrating similar validation for RIAs of other androgens in lamprey. We have also established (unpublished studies) that 15α-T concentrations of between 2 and 4 ng/ml are found in the plasma of individual GnRH-injected males.

One possibility for the relatively low amounts found in the present study may be due to the fact that the fish had been held in tanks for some weeks before they were sampled. It is known that captivity causes a substantial reduction in sex steroid concentrations in teleost flatfish (Vermeirssen et al. 1998). However, this possibility remains to be established in the lamprey. Another possible reason for low amounts of 15α-T is that it is cleared very rapidly from the plasma. The fact that there is no steroid binding activity in the plasma would certainly be a factor that favors its rapid excretion (see discussion by Weisbart et al. 1980).

The RIA for 15α-T has the ability to measure 15α-T over a wide range, and has low cross-reactivity with all hormones tested, with the exception of 15β-T. However, TLC fractionation of 15β-T with subsequent quantification of the amount of 15α-T in the fractions via RIA revealed that the 15β-T standard used in these analyses was heavily contaminated and that the actual detection rate of 15β-T is very low (0.87%). Additionally, since 15β-T does not appear to be synthesized by the testes of sea lamprey,

cross-reactivity is not a concern. The use of plasma, rather than a plasma extract, in the assay did not appear to be a problem.

Although we have demonstrated that 15α-T is produced by lamprey gonads and is present in the blood, we still cannot state whether it has a function. For this, we would need to demonstrate the presence of receptors in one or more putative target organs – and then to demonstrate that injection of 15α-T causes a specific biochemical, physiological or behavioral change. Larsen (1974) has already shown that treatment of river lamprey with exogenous testosterone causes the development of male secondary characters in the river lamprey. This does not exclude the possibility that the testosterone exerted its activity through conversion to 15α-T by endogenous enzyme (see discussion by Kime and Larsen 1987).

An attempt has already been made to identify DNA sequences that are common to vertebrate androgen receptors by searching sea lamprey liver mRNA with PCR primers (Thornton 2001). The results of this study were negative, suggesting that androgen receptors evolved after the divergence between cyclostomes and gnathostomes, and that lamprey therefore may not utilize androgens at all and instead use estrogens as the main steroids in both males and females. This hypothesis is supported by findings that 17β-estradiol is present in the blood of male sea lampreys at up to 3 ng/ml (e.g. Sower et al. 1985a) and that estrogen-binding activities were detected in both the cytosolic and nuclear extracts of sea lamprey testes (Ho et al. 1987). However, since the search for lamprey androgen receptors was performed using PCR primers for known (gnathostome)

androgen receptors, the presence of unique receptors with unique DNA sequences for '15α-hydroxylated androgens' cannot be excluded.

If 15α-T is a functional hormone, there are several possible reasons why the lamprey may utilize it rather than testosterone. As a parasite feeding on other vertebrates, the sea lamprey would possibly need a mechanism to distinguish endogenous from exogenous ingested hormones. Using a unique set of hormones, such as 15-hydroxylated steroids, would allow lampreys an easy way to distinguish between the two. It is also possible that the 15-hydroxylated steroids are not an evolved response to parasitism, but are simply a primitive form of steroid hormones.

Studies of the endocrine systems of other parasites would be useful in distinguishing between the above two hypotheses. Unfortunately, there has been very little research performed on the steroids of other parasites, although one study did find that ticks inactivate ingested ecdysteroids by conjugating them with fatty acid chains (Connat et al. 1986). Most studies have instead focused on the effect of parasites on host steroids. However, based on studies of this type, it seems likely that host hormones affect parasites feeding upon them. Lawrence (1991) found that various host steroids were found to promote growth and/or reproductive activities in a variety of invertebrate parasites. In certain cases, such as that of the coccidian, the parasite's endocrine system becomes strictly correlated with the host's in order to synchronize reproductive activities (Porchet-Hennere and Dugimon 1992). In situations such as that of the lamprey where the parasite and host are very similar, having a system to distinguish endogenous from exogenous hormones would perhaps be essential in order for the lamprey to prevent its own body

from responding to the host's hormones especially since the sea lamprey feeds upon several species with differing reproductive seasons. Further research on sea lamprey endocrinology may help illustrate how parasites in general cope with host hormones to prevent endocrine disruption.

In contrast, examining the endocrine systems of animals related to lamprey may help to distinguish if 15-hydroxylated steroids are an incidental event in evolution. Hagfish are the only other group of agnathans still alive today, though hagfish are scavengers and not parasites, so they do not need a mechanism to cope with exogenous hormones. However, it has been demonstrated that the hagfish produce some unusual steroids, including 5, 6, and 7-hydroxylated hormones (Kime et al. 1980; Kime and Hews 1980). Based on hagfish endocrinology, it appears possible that unusual steroids are due to incidental or ancestral events in evolution.

Lampreys are not the only animals that produce steroids with a 15-hydroxyl group, although steroids of this type are rare, and the reasons for the 15-hydroxyl group are seldom explored. Estrogens with a 15-hydroxyl group have been found *in vivo* in the peripheral plasma of the laying turkey (Brown et al. 1979) and *in vitro* in bovine adrenal glands (Levy et al. 1965). Bullfrog liver slices were shown to produce 15α- and 15β-hydroxydeoxycorticosterone *in vitro* (Schneider 1965). Additionally, 15-hydroxylated steroids have been isolated from human pregnancy urine (Giannopoulos and Solomon 1967, 1970; Giannopoulos et al. 1970). Hepatic microsomes from mice have been shown to metabolize testosterone by hydroxylation, some of which occur at the 15α or 15β positions (Ford et al. 1975). A final instance of 15-hydroxylation has a definitive reason;

it was determined that in rat liver microsomes cytochrome p450 hydroxylates steroids at several different points including the 15-C, which may make the steroids more water-soluble in order to aid in excretion (Hrycay et al. 1976; Gustafsson 1970).

Further research is needed to elucidate if 15α-T has a function and determine the reason for the presence of this unique hormone. It is clear that knowledge of sea lamprey hormones can provide useful information for comparative endocrinology and the evolution of steroid hormones.

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CHAPTER THREE

Bryan M.B., Scott A.P., Young B.A., Černý I., Li W. 2004. 15α-Hydroxyprogesterone in male sea lampreys, *Petromyzon marinus L.* Steroids 69, 273-281.

15α-Hydroxyprogesterone in male sea lampreys, *Petromyzon marinus L.*

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Abstract

There is growing evidence that sea lampreys, *Petromyzon marinus* L., produce gonadal steroids differing from those of other vertebrates by possessing an additional hydroxyl group at the C15 position. Here we demonstrate that sea lamprey testes produce 15α-hydroxyprogesterone (15α-P) *in vitro* when incubated with tritiated progesterone, that 15α-P is present in the plasma of sea lampreys, and that plasma concentrations of immunoreactive (ir) 15α-P rise dramatically in response to injections of gonadotropin-releasing hormone (GnRH). The identity of the tritiated 15α-P produced *in vitro* was confirmed by co-elution with standard 15α-P on high performance liquid chromatography, co-elution with standard and acetylated 15α-P on thin layer chromatography, and specific binding to antibodies raised against standard 15α-P. The *in vitro* conversion was used to produce tritiated 15α-P label for a radioimmunoassay (RIA), which is able to detect 15α-P in amounts as low as 2 pg per tube. The RIA has been used to measure the plasma concentrations of 15α-P in males given two serial injections, 24 h apart, of either lamprey GnRH I or GnRH III (50, 100, or 200 μg/kg) or saline control, with plasma being sampled 8 and 24 h after the second injection. Plasma concentrations of ir-15α-P rose from < 1 ng/ml to 36 ng/ml (mean of all treatments) 8 h after injection and declined within 24 h. This is the first time an RIA has detected such high steroid concentrations in lampreys. This finding is suggestive of a role for 15α-P in control of reproduction in the sea lamprey.

Keywords: sea lamprey, 15-hydroxylation, Petromyzon, GnRH, steroid, 15α-hydroxyprogesterone

Introduction

As a member of superclass Agnatha, the lamprey is one of the earliest evolved extant vertebrates (Hardisty and Potter 1971). Because of the unique position of the lamprey in the phylogenetic tree, understanding of the mechanisms and enzymes involved in steroidogenesis in lampreys may have important implications for our understanding of the evolution of steroids in vertebrate species. Additionally, further knowledge regarding lamprey reproductive processes is desirable because of the economic significance of sea lampreys both as an invasive species targeted for control in the Great Lakes and as a commercially important species in Europe (Maitland 1980; Almeida et al. 2000).

There is strong evidence that sea lamprey (*Petromyzon marinus*) gonadal steroids differ structurally from classical steroids (i.e. those found in higher vertebrates). Investigations into androgen production in sea lampreys have revealed that testosterone is rapidly metabolized into 15α,17α-dihydroxy-androst-4-en-3-one (15α-hydroxytestosterone; 15α-T) *in vitro* (Kime and Callard 1982; Bryan et al. 2003; Lowartz et al. 2003) and that 15α-T is produced *in vivo* and circulates in the plasma (Bryan et al. 2003; Lowartz et al. 2003), although at relatively low concentrations (< 1 ng/ml). When prespermiating male lampreys are injected with exogenous gonadotropin-releasing hormone (GnRH) there is a 2- to 5- fold increase in the plasma concentrations of 15α-T (Young et al. 2004).

With respect to C_{21} steroids, progesterone (P) has been detected in sea lamprey plasma using immunoassays, and differences in immunoreactive P concentrations in plasma have been detected between sexes (Linville et al. 1987), between reproductive stages (Bolduc

and Sower 1992) and in response to GnRH (Sower et al. 1987; Deragon and Sower 1994; Gazourian et al. 2000). Very similar to the plasma concentrations of 15α-T, the concentrations of immunoreactive P in most sea lampreys are normally less than 0.5 ng/ml but exhibit a 2- to 5-fold increase in response to GnRH injection (Gazourian et al. 2000). Because P is a hormone in mammals, can be detected by RIA in lamprey plasma, and its plasma concentrations change in response to stimulation, it has been suggested that P is a functional hormone in lampreys (Sower 1990).

Despite being detected by immunoassay, P has not been found to be produced in any studies using radiolabeled precursors either *in vitro* or *in vivo*. In fact, when adult sea lamprey testicular, ovarian, and presumptive adrenocortical tissues were incubated *in vitro* with ^{3}H-cholesterol, none of the steroid metabolites could be identified (Weisbart et al. 1978). However, if P is added to gonads *in vitro*, it does appear to be readily metabolized. When testicular tissue from parasitic phase lampreys was incubated with ^{14}C-P, several products were formed of which the only one that was identified was 11-deoxycorticosterone (Weisbart and Youson 1975). In the same study, presumptive adrenocortical tissue from larval and parasitic phase lampreys was incubated with ^{14}C-P and small amounts of 17-hydroxyprogesterone, androstenedione, and 11-deoxycortisol were formed. However, most of the radiolabel was converted to unknown steroids. The conversion of ^{3}H-P was investigated *in vivo* in parasitic phase sea lampreys, and 11-deoxycorticosterone was the only identifiable product (Weisbart and Youson 1977). Again, much of the radioactivity was converted to unknown compounds.

The lack of evidence for *in vitro* synthesis of P and its relatively low physiological concentrations led Kime and Rafter (1981) to hypothesize that lampreys may use a steroid other than P as a hormone during the final stages of gonadal maturation. These authors incubated ovaries and testes of the river lamprey (*Lampetra fluviatilis*) with ^{3}H-P and found that the major product was 15α-hydroxy-pregn-4-ene-3,20-dione (15α-hydroxyprogesterone; 15α-P). Recently, it has been shown that the elution time of a steroid produced *in vitro* in male sea lampreys corresponded on high performance liquid chromatography (HPLC) to the elution time of 15α-P (Lowartz et al. 2003). However, the authors of this latter study did not definitively identify this steroid in the incubations, nor provide conclusive evidence for its presence in plasma.

In order to confirm that it is 15α-P that is being made by the testis of the sea lamprey and, more importantly, to demonstrate that it is produced *in vivo*, we have set out to: 1) incubate the testis of the sea lamprey with tritiated P and then tentatively identify 15α-P by co-migration on HPLC and on thin layer chromatography (TLC) before and after acetylation; 2) develop a radioimmunoassay (RIA) for 15α-P that can be applied to sea lamprey plasma, and 3) determine whether injection of GnRH increases plasma concentrations of 15α-P in adult male lampreys.

Methods

All chemicals were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Lamprey GnRH I and GnRH III were synthesized by Bachem Peptide Company (King of Prussia, PA). Sea lampreys for incubation experiments (and female sea lampreys) were

collected during their upstream spawning migration by U.S. Fish and Wildlife Service (USFWS) employees from northern Lake Huron tributaries. Sea lampreys for the GnRH-injection experiment were collected during their upstream spawning migration, between May 16 and May 23, 2003, by USFWS employees using traps at the mouth of the Cheboygan River in northern Lake Huron. The length and mass of each male for the GnRH experiment was recorded for subsequent dosage calculations, with the average male length (± SEM) being 490 ± 3.8 mm and average male mass being 245.8 ± 5.3 g. Lampreys were transported to either Michigan State University (East Lansing, MI) for incubation experiments or to the Hammond Bay Biological Station (USGS-BRD, Millersville, MI) for plasma sampling. At Michigan State University, lampreys were held in tanks containing 160 L of continuous-flow well water at approximately 12° C. At Hammond Bay Biological Station, lampreys were held in tanks containing 160 L of continuous-flow water from Lake Huron for at least one week prior to sampling. Temperature during acclimation and experimental periods at Hammond Bay was maintained at about 16 °C (±1 °C). All lampreys were anesthetized with 1 : 5000 MS222 before handling. Blood was obtained from the caudal vein using heparinized syringes, held at 4° C for 20 min, and centrifuged at 1000 x *g* for 20 min. The plasma was removed and stored at -80° C.

Chemical synthesis of 15α-P

The synthesis was performed according to standard methods starting with 3β-acetoxypregna-5,14-dien-20-one (Yoshii et al. 1977). The starting compound was protected at position 20 in form of ethylene glycol ketal (Liu et al. 1988) and transformed

(Suginome et al 1992) into a 3α,5α-cyclo-6β-methoxy derivative. This protected derivative was subjected to hydroboration and oxidation reactions to introduce a 15α-hydroxy group (Hosoda et al. 1977). The product was purified, acetylated and deprotected in acidic medium to yield 15α-acetoxy-3β-hydroxypregn-5-en-20-one. Oppenauer oxidation (Raggio and Watt 1977) and deacetylation yielded 15α-P.

The physico-chemical properties of the synthetic 15α-P included a melting point of 229-231°C (methanol), $[\alpha]_D$ +208 (c 0.8, chloroform). For $C_{21}H_{30}O_3$ (330.5) calculated: 76.33 %C, 9.15 %H; found: 76.24 %C, 9.08 %H. The Schubert method (Schubert et al. 1962) gave a melting point of 232°C, $[\alpha]_D$ +218 (chloroform). The Tamm method (Tamm et al. 1963) gave a melting point of 220-228°C, $[\alpha]_D$ +220 (c 1.183, chloroform). The ^1H NMR spectrum (500 MHz, $CDCl_3$) was in good agreement with previously published data (Kirk et al. 1990) at: 1.73 (H-1α), 2.04 (H-1β), 2.31 (H-2α), 2.43 (H-2β), 5.74 (H-4), 2.35 (H-6α), 2.41 (H-6β), 1.26 (H-7α), 2.18 (H-7β), 1.78 (H-8β), 1.03 (H-9α), 1.65 (H-11α), 1.45 (H-11β), 1.54 (H-12α), 2.02 (H-12β), 1.20 (H-14α), 4.11 (H-15β), 1.56 (H-16α), 2.78 (H-16β), 2.81 (H-17α), 0.701 (H-18), 1.204 (H-19), 2.134 (H-21). The ^{13}C NMR spectrum (125.7 MHz, $CDCl_3$) was: 35.74 (C-1), 33.93 (C-2), 199.31 (C-3), 123.89 (C-4), 170.54 (C-5), 32.69 (C-6), 31.99 (C-7), 35.20 (C-8), 53.70 (C-9), 38.57 (C-10), 20.88 (C-11), 38.87 (C-12), 44.55 (C-13), 62.84 (C-14), 73.39 (C-15), 35.35 (C-16), 60.86 (C-17), 14.61 (C-18), 17.48 (C-19), 208.20 (C-20), 31.53 (C-21); chemical shifts correspond to computed values from the Stothers survey (Blunt and Stothers 1977; computed data for most affected carbons: 63.1 (C-14), 74.4 (C-15), 35.2 (C-16), 61.1 (C-17)). Electron Impact Mass Spectrometry spectrum contained a molecular ion at m/z 330 (100%).

In vitro biosynthesis of tritiated 15α-P

Testicular tissue from a freshly killed adult male lamprey was finely diced in L15 medium. Fresh L15 medium (10 ml) and 0.5 g tissue was added to a 50 ml polypropylene tube containing 25 μCi [1,2,6,7-^{3}H]-progesterone. The tube was then shaken for 4 h at approximately 17° C. At the end of this period, the medium was removed, filtered, and passed through an activated Sep-Pak C18 extraction cartridge (Waters, Milford, MA). The Sep-Pak was washed with 5 ml distilled water and eluted with 5 ml methanol. The methanol eluate was stored at -20° C.

In order to characterize the products of the reaction, 20 μl of methanol extract was mixed with 10 μg each of P and 15α-P (dissolved in 20 μl ethanol), dried down under a stream of nitrogen at 45° C, fractionated using HPLC, and counted for disintegrations per minute (dpm) (Bryan et al. 2003).

Further characterization of radioactive 15α-P was carried out by thin layer chromatography (TLC). The remaining methanol eluate from the incubation was fractionated using HPLC as described above. From the fraction corresponding to the elution position of 15α-P, 50,000 dpm of the fraction was removed to each of two microcentrifuge tubes. To both tubes, 10 μg of standard 15α-P was added and the solvents were evaporated under nitrogen. In the first tube, 100 μl pyridine and 100 μl acetic anhydride were added and left overnight at room temperature, after which the solvents were again evaporated under nitrogen. The contents of both tubes were loaded

and developed on a TLC plate (Bryan et al. 2003). The positions of the standards were noted by placing the plate under a UV source. The lanes were then divided into 4 mm fractions, scraped off, placed in scintillation vials, mixed with 4 ml scintillation cocktail and counted.

Further confirmation of the identity of radioactive 15α-P was carried out by testing its ability to bind to different dilutions of antisera raised against haptens for 15α-P (see below), 15α-T (Bryan et al. 2003) and P (provided by Dr. G. Niswender, Colorado State University, Fort Collins, CO). The procedure was the same as that described by Bryan et al. (2003).

Preparation of 15α-P radiolabel for use in radioimmunoassay

Radiolabel was prepared on five separate occasions. For all incubations, approximately 1 g of minced testicular tissue from a single male was added to 20 ml L15 medium containing 150 to 200 μCi [1,2,6,7-^{3}H]-progesterone, and the mixture was incubated for 5 h at 12° C. After all incubations, the medium was filtered and loaded onto a Sep-pak extraction cartridge as described above. The radiolabel was further purified using HPLC as described above, and the fractions corresponding to the elution point of 15α-P were mixed 1 : 1 (v/v) with ethanol and stored at -20° C.

Development of antiserum

In order to develop an antiserum, 15α-P was first linked to a carboxymethyloxime (CMO) group to make 15α-P-3-CMO. To prevent the formation of 20-(O-

carboxymethyl)oxime, an intermediate 3-enamine derivative was first formed (David Kime, personal communication). To a glass tube was added 20 mg of 15α-P, 1.5 ml of methanol and 30 ml pyrrolidine. After 5 minutes, a precipitate was formed. With continued shaking, another 30 ml of pyrrolidine was added followed by 13 mg of carboxymethoxylamine hemihydrochloride. The mixture was heated to 55 °C for 5 min (during which the precipitate disappeared). The mixture was taken to dryness under a stream of nitrogen at 45 °C, redissolved in 1 ml water and acidified with 50 ml of concentrated HCl. The conjugate was extracted with ethyl acetate, taken to dryness, redissolved in two drops of methanol and crystallized with diethyl ether. The total yield was 4 mg. This was dissolved in 1.5 ml dimethyl formamide (DMF) and conjugated to BSA as described previously (Bryan et al. 2003).

Approximately 2 mg 15α-P-3-CMO-BSA was dissolved in 1 ml saline and 1 ml Freund's Complete Adjuvant and injected intradermally into two rabbits. Booster injections using the same amount of powder, but suspended in Freund's Incomplete Adjuvant, were given at ten weeks following the first injection. Blood (20 ml) was obtained twelve weeks after the first injection and allowed to clot before being centrifuged at 2500 rpm for 15 min. The serum was removed and frozen in 0.5 ml aliquots at -80° C.

RIA procedure

RIAs were conducted in glass culture tubes (10 mm x 75 mm, Fisher Scientific, Pittsburgh, PA) as described by Bryan et al. (2003). The assay buffer consisted of 50

mM sodium phosphate pH 7.4, 0.2% (w/v) BSA, 137 mM NaCl, 0.40 mM EDTA, 0.77 mM sodium azide.

The specificity of the antiserum was tested by making, in 100 µl buffer, six five-fold serial dilutions of 15α-P, P, and 17-hydroxyprogesterone over a range of 10,000 to 0.64 pg/tube; and 11-ketotestosterone, androstenedione, testosterone, 15α-T, 17β-estradiol, cholic acid, deoxycorticosterone, 11-deoxycortisol, 3-oxo-petromyzonol sulfate, 3-oxo-allocholic acid, petromyzonol sulfate, and allocholic acid over a range of 10,000 to 400 pg/tube. An additional 100 µl of buffer containing 5,000 dpm radiolabel and the 15α-P antiserum at a dilution of 1 : 12,500 (v/v) was added to each tube.

Inter-assay variation was determined by measuring the amount of 15α-P in the same plasma sample (*c.* 2 ng/ml) in six separate assays. Intra-assay variation was determined by measuring 15α-P in the same sample six times in the same assay.

Blood plasma concentrations of 15α-P

The reliability of the 15α-P RIA, when applied to raw (unextracted) lamprey plasma was tested in three ways. First, to determine specificity, 5 ml of pooled plasma from both control and GnRH-injected fish were extracted using a Sep-pak extraction cartridge and eluted with methanol. The eluate was evaporated with a rotary evaporator (Yamato RE200), fractionated using HPLC as described above, and 20 µl of each fraction was then assayed for 15α-P using RIA. Second, to determine whether there might be any proteins in plasma that might bind to 15α-P and hence interfere with the assay, dilutions

of pooled plasma from both control and GnRH-treated lampreys were combined with labeled 15α-P in the absence of antibody. Third, to establish parallelism within the assay, standard 15α-P at a range of dilutions from 156 pg/ml to 10 ng/ml was added to plasma from control lampreys and assayed at volumes of 25 µl, 50 µl, and 100 µl (total volume brought to 100 µl with assay buffer). Plasma from GnRH-treated lampreys (8 separate pools), pre-diluted 1 : 10 (v/v) with assay buffer, was tested the same way.

The experimental design comprised seven treatments, twelve lampreys per treatment, with two timed injections, and two timed bleedings following a previously published procedure (Sower 1989). Each group of 12 lampreys was injected with either 0.9% saline (control), lamprey GnRH I (Sherwood et al. 1986; 50, 100, or 200 µg/kg body weight) or lamprey GnRH III (Sower et al. 1993; 50, 100, or 200 µg/kg body weight). Peptides were dissolved in 0.9% saline less than 30 min prior to administration and injected intraperitoneally. Blood samples were collected 8 h and 24 h after the second set of injections. Blood samples of 0.5 to 1.0 ml were collected through the caudal vein using heparinized syringes. After centrifugation of blood samples, plasma was collected and stored at -80 °C. For RIA analysis, plasma from control lampreys was assayed using 20 µl plasma per replicate. Plasma from treatment lampreys was assayed using 20 µl of plasma diluted 1 : 10 (v/v) with assay buffer.

Statistical analyses were performed using the SAS system for windows, version 8. 15α-P plasma concentrations were natural log-transformed to attain normality for all statistical analyses. A 3-way (4x3x2) ANOVA was performed on all data investigating the main

effects of GnRH dosage (50, 100, or 200 μg/kg or saline), GnRH type (GnRH I or GnRH III), and time interval (8 h or 24 h) and the potential existence of interactions. If no meaningful interactions existed for main effects, Dunnett's multiple comparison tests were used to identify treatments that exceeded controls, while LSD multiple comparison tests were used to locate differences among treatment means. If interactions between factors were significant, the significance of differences among simple effects was calculated.

Females and males were allowed to reach maturity in tanks containing ambient water from Lake Huron. To determine the maturational stage of lampreys, gentle pressure was applied to the abdomen of males or females. If this action induced emissions of milt or eggs, the animals were classified as spermiating or ovulated. Blood samples were obtained and processed for plasma as above for 7 each of preovulatory females, ovulated females, and spermiating males. A t-test was used to compare 15α-P plasma concentrations between preovulatory and ovulated females.

Results

Incubation of P with sea lamprey testes yielded up to four peaks when the media were separated using HPLC (Fig. 11). The rate of conversion from P to any of the four peaks differed seasonally. The average rate of conversion from P to 15α-P was 51% with SEM of 4% during the natural spawning season (April-July) and was 14.6 ± 1.4% in animals that were kept alive artificially in cold water past the natural spawning season (August-September).

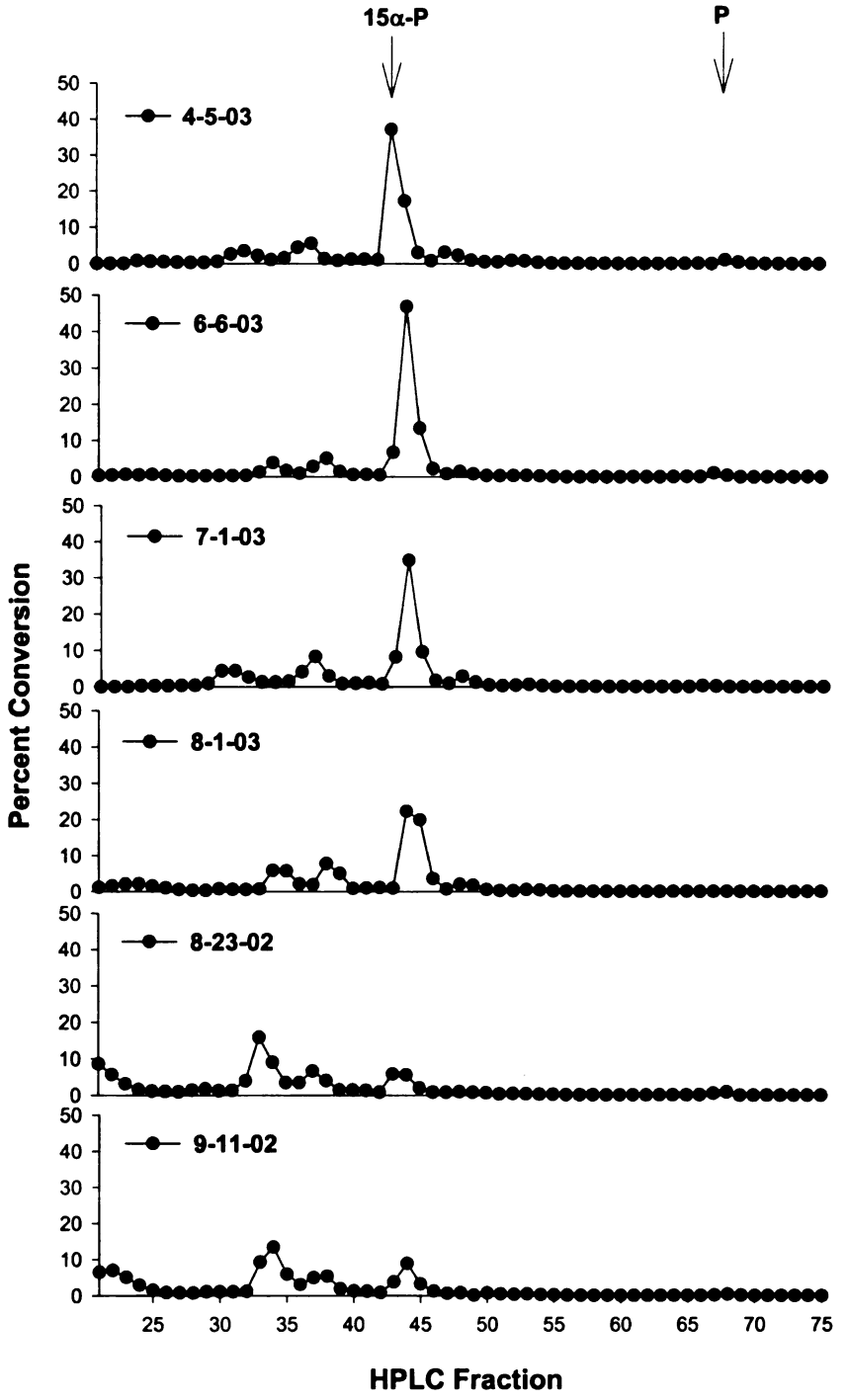

Figure 11: Products from *in vitro* metabolism of [^{3}H]-P by sea lamprey testes. In the legend, the labels stand for month-day-year. The relative amounts of each product appear to change with time. The data shown from April through August 1 represent the natural spawning season of lampreys, while the data from August 23 and September represent males kept in cold water to artificially keep them alive. Arrows show the elution positions of standard 15α-hydroxyprogesterone (15α-P) and progesterone (P).

Confirmation of the identity of the radioactive 15α-P was obtained by running some of the putative HPLC-purified 15α-P on TLC. The radioactivity co-migrated with standard 15α-P both before and after acetylation (Fig. 12).

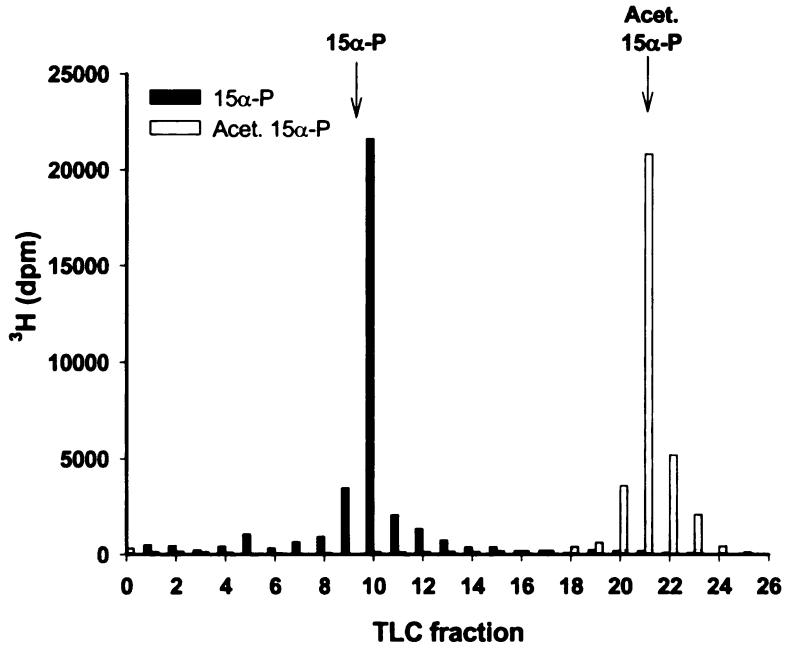

Figure 12: ^{3}H counts (dpm) from TLC fractionation of incubation media after purification with HPLC. Black bars represent the purified product of incubation, and grey bars represent the acetylated product. Arrows show the elution positions of 15α-hydroxyprogesterone (15α-P) and progesterone (P).

The dilution of antiserum that was required to bind 50% of 15α-P radiolabel (at 5000 dpm/ 200 μl) differed between batches of radiolabel, and ranged from 1 : 12,500 to 1 : 33,333 (v/v). The radioactive 15α-P bound strongly to the antisera raised against standard 15α-P, and did not display any affinity to P, 15α-T or control antisera (Fig. 13).

There was negligible cross-reaction between the 15α-P antiserum and most of the tested steroids. However, there was substantial, but non-parallel, cross-reaction with P (15.5% for 10 ng of P and 110% for 0.8 pg of P).

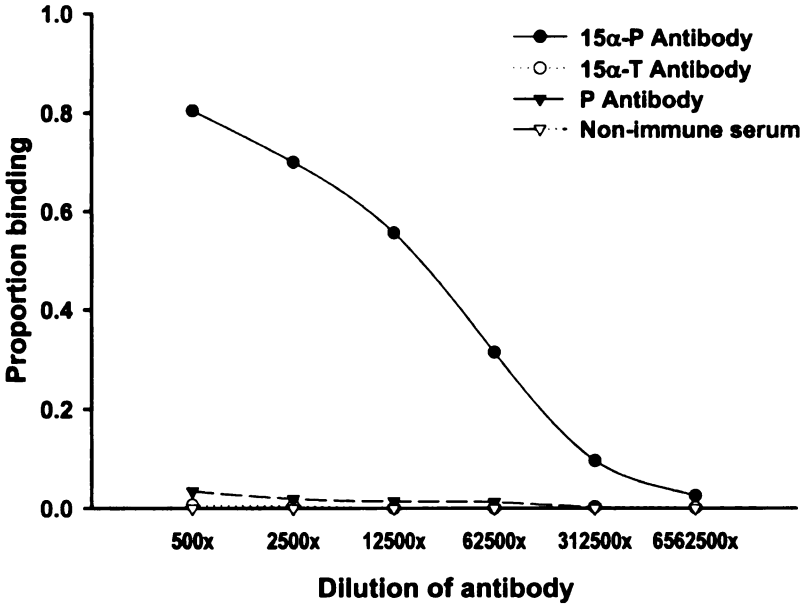

Figure 13: Ability of antibodies raised against different steroids to bind purified ^{3}H-15α-P produced *in vitro*. 15α-P is 15α-hydroxyprogesterone, 15α-T is 15α-hydroxytestosterone, and P is progesterone.

Intra-assay variation of 15α-P concentrations was 6.1%. Inter-assay variation was 13.2%.

On HPLC, there were several peaks of immunoreactive (ir)-15α-P in plasma from both control and GnRH-treated animals (Fig. 14). The highest peak (and the one that showed the largest difference following GnRH-treatment) corresponded to the elution position of 15α-P. There was no evidence for plasma protein binding of tritiated 15α-P in either control or treatment plasma as no radiolabel remained in the supernatant after addition of

charcoal and centrifugation. The recovery experiment performed on pooled plasma returned a line of expected versus observed values matching the equation of $y = 1.10x + 3.00$ with an $r^2 = 0.96$ for plasma from the control group and $y = 0.96x + 3.88$ with an $r^2 = 0.93$ for plasma from the treatment groups.

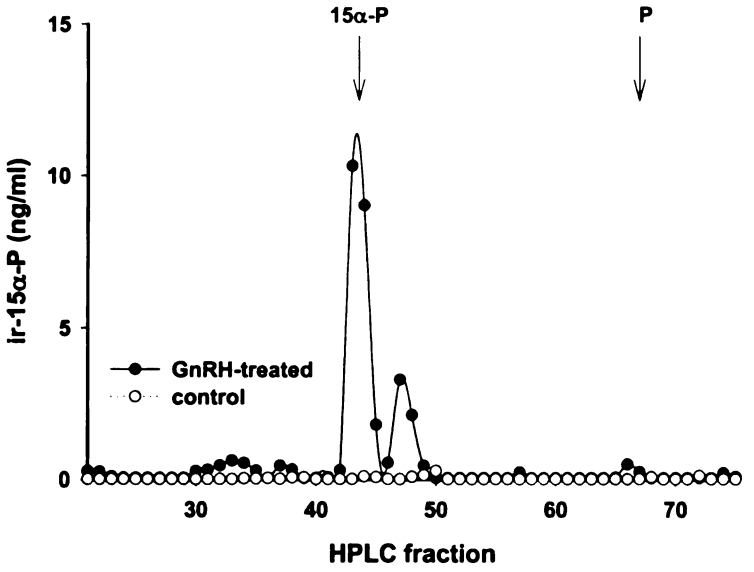

Figure 14: Immunoreactivity to 15α-hydroxyprogesterone (15α-P) in HPLC fractions of 1 ml pooled plasma extract from control and GnRH-treated males. The elution positions of 15α-P and progesterone (P) are indicated with arrows.

In the injection experiment, plasma concentrations of 15α-P were higher in all GnRH-treated animals compared to control animals at both 8 and 24 h after the second injection (Fig. 15; $P < 0.0001$). All factors (dosage, injection type, and sampling time) had significant effects. However, there was a significant interaction between the type of GnRH injected and the sampling time, so only simple effects could be investigated further for these two factors. Average plasma concentrations (average ng/ml ± SEM) of

15α-P were 36.3 ± 3 at 8 h and 5.8 ± 0.9 at 24 h for all GnRH-treated lampreys, compared to 0.6 ± 0.08 at 8 h and 0.7 ± 0.06 at 24 h for the control groups.

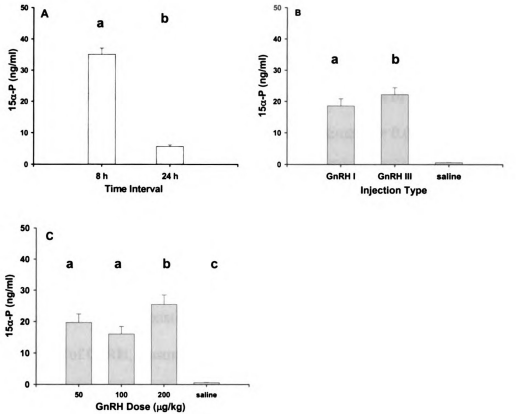

Figure 15: Responses of 15α-P plasma concentrations of prespermiating male lampreys to exogenous GnRH. Graph A shows the time at which sampling was done (levels of control animals were not included), graph B shows the type of injection the lampreys received (levels of control animals were not included in statistical analyses, but are in the graph for comparison), and Graph C shows the dose of the injection. Column heights represent group means of 15α-P plasma concentrations, and error bars represent the standard error of the mean. Lowercase letters indicate which treatment groups were significantly different from one another ($P < 0.05$) where main effects were tested for dosage and simple effects were tested for GnRH type and time interval.

Dunnett's comparisons to controls revealed that all doses of GnRH (50, 100, or 200 ug/kg) elicited plasma concentrations significantly higher ($P < 0.0001$) than the saline dose. Lampreys injected with 200 μg/kg of GnRH had significantly higher plasma

concentrations of 15α-P than lampreys injected with 50 or 100 μg/kg of GnRH ($P <$ 0.05). Plasma concentrations of 15α-P did not differ between lampreys injected with 50 or 100 μg/kg of GnRH ($P > 0.05$).

When examining the simple effects of the type of GnRH injected, differences in 15α-P plasma concentrations were found at both sampling times. Lampreys receiving injections of GnRH III showed a trend of higher plasma concentrations of 15α-P than lampreys receiving injections of GnRH I at the 8 h sampling time ($P = 0.0560$). At the 24 h sampling time, lampreys receiving injections of GnRH III had significantly higher plasma concentrations of 15α-P than lamprey receiving injections of GnRH I ($P < 0.0001$).

When examining the simple effects of the sampling time, significant differences in 15α-P plasma concentrations existed between the sampling times for both types of GnRH. For either type of GnRH, plasma levels of 15α-P were significantly higher at 8 h than at 24 h after the second injection ($P < 0.0001$).

Plasma concentrations (ng/ml; mean ± SEM, n = 7) of 15α-P in captive animals were: preovulatory females, 0.08 ± 0.03; ovulated females, 0.69 ± 0.10, spermiating males, 2.48 ± 0.89. The plasma concentrations of 15α-P in ovulated females were significantly higher than those found in preovulatory females ($P < 0.0001$).

Discussion

Our results lend support to the hypothesis (Kime and Rafter 1981) that lampreys use 15α-P as a reproductive hormone. 15α-P is readily produced both *in vitro* and *in vivo*. In response to GnRH, the concentrations of ir-15α-P rise far above those of any other steroid than has previously been measured in sea lampreys. The plasma concentrations of ir-15α-P rose very significantly within 8 h of the second GnRH injection, and decreased significantly, but did not fall to baseline levels, within 24 h of the second injection. The HPLC data indicated that it was mainly 15α-P itself, and not any of the other cross-reacting compounds, that was responsible for the large rise in ir-15α-P concentrations in plasma in response to GnRH injection.

First, we confirmed that sea lamprey produce 15α-P *in vitro*. Metabolism of ^{3}H-P changed seasonally, producing progressively more of the compounds that eluted early from the HPLC and less in the position of 15α-P. There is not enough evidence to indicate whether this progression is a natural phenomenon. This is because the final two incubations in the series were carried out on testes from fish that had had their spawning season artificially extended by keeping them in cold water. The only attempt that we have made to identify any of these other peaks has been a single test of their ability to bind to the 15α-P antiserum. The only peak to do so (unpublished results) was the one eluting at 38 and 39 min – suggesting, though not proving – that this metabolite of progesterone might also be 15α-hydroxylated.

The RIA for 15α-P revealed several peaks of immunoreactivity on the HPLC, of which the largest in GnRH-injected fish was 15α-P itself. One of the peaks corresponded to the elution position of P. This is consistent with the facts that the RIA cross-reacts with P (albeit with poor parallelism) and that P has previously been shown to be present in the plasma of male lampreys (see Introduction). The cross-reaction of the RIA with P is unlikely to be a problem for physiological studies on lampreys. P concentrations, even in GnRH-injected males, are very small relative to 15α-P plasma concentrations; baseline levels of ir-P have been measured at 0.2 ng/ml and levels rise to 2 to 2.6 ng/ml after GnRH administration (Deragon and Sower 1994; Gazourian et al. 2000). HPLC of pooled plasma samples showed that ir-15α-P in the elution position of P did not differ greatly between treatment (0.75 ng/ml) and control plasma (0.10 ng/ml) relative to the changes in 15α-P itself.

Two of the other immunoreactive peaks that eluted at 32-34 min and 38-39 min (cf. elution position of one of the metabolites of tritiated P) increased a small amount in response to GnRH. There was another immunoreactive peak at 47-48 min that showed a more marked increase in response to GnRH injection. The presence of these other immunoreactive peaks shows that there are at least three other, yet to be unidentified, steroids in the plasma of the male sea lamprey. Although they appear to be present in smaller amounts than 15α-P itself, this may be because they only exhibit partial cross-reaction with the antibody. This is a likely scenario, but can not be established until these steroids have been identified and synthesized. It is also not possible to determine at this

stage whether the immunoreactive steroids are simply metabolites of 15α-P or are any more or less likely to be 'hormones' (see below).

In order to measure only 15α-P in plasma, it will be necessary to either devise a steroid separation step or to raise a new antiserum that would hopefully have better specificity. At this stage in research regarding lamprey sex steroids, however, we would argue that this is not immediately necessary. Despite the interference from other steroids in the plasma, much of the increase in immunoreactivity in GnRH-injected animals was due to 15α-P itself. Also, standard 15α-P and plasma from these animals diluted parallel in the RIA. Since the scale of the increase in ir-15α-P in response to GnRH was far higher than that recorded for any other steroid in lampreys, we believe that the RIA described in the present paper has great value for physiological experiments in lampreys without the need for further modification.

The change in plasma concentrations of 15α-P in response to GnRH was rapid and significant. For both types of GnRH at all doses, 15α-P concentrations were at their highest at 8 h after the second injection, and had declined significantly, but not returned to basal levels, at 24 h after the second injection. A similar drop in E_2 (Gazourian et al. 1997, 2000) and P (Gazourian et al. 1997) concentrations occurred between 4 h and 24 h. However, mean concentrations of P in the latter study, even at 4 h, did not exceed 0.5 ng/ml.

The relatively poor dose-dependency for both peptides, at both sampling times, is almost certainly due to the fact that even the lowest doses (50 μg/kg) were sufficient to induce near maximum ir-15α-P responses. Doses of 100 μg/kg and 200 μg/kg for both GnRH I and GnRH III did not give a differential response in E_2 plasma concentration (Gazourian et al. 1997), though in this study doses of 200 μg/kg did elicit higher 15α-P levels than doses of 100 μg/kg. The present study indicates that GnRH III is probably more potent than GnRH I as ir-15α-P concentrations tended to be higher at 8 h and were significantly higher at 24 h in the animals that had been injected with GnRH III. This is in agreement with the observation that GnRH III is more potent than GnRH I in stimulating spermiation in male lampreys (Deragon and Sower 1994).

In a study by Young et al. (2004), using the same injection procedure as in the present paper, it was found that, although plasma concentrations of 15α-T increased in response to administration of exogenous GnRH (median level rose from 0.29 ng/ml to 1.19 ng/ml), there were no differences between either the type of GnRH, the dose or sampling time.

In many (but not all) teleosts, as spawning time approaches, there is a switch from the synthesis of C_{19} steroids (androgens) to C_{21} steroids (Scott et al. 1983; Barry et al. 1990). One C_{21} steroid in particular, 17,20β-dihydroxy-pregn-4-en-3-one, has an important role as an inducer of oocyte final maturation in females (Nagahama and Adachi 1985) and sperm activation in males (Miura et al. 1992; Miura and Miura 2001). If a similar switch in steroidogenesis takes place in lampreys, it could explain the differences seen in the differential response of 15α-T and ir-15α-P to GnRH administration. Our experiments

have mainly been carried out on adult prespermiating males very close to the onset of spermiation, at which stage enzyme biosynthesis might naturally favor 15α-P (C_{21}) over 15α-T (C_{19}) production.

Our results appear to be at variance with previous studies that indicate that lampreys have a poor ability to produce Δ-4 steroids (of which 15α-P is one). Weisbart et al. (1978), for example, found very low 3β-hydroxysteroid dehydrogenase (3β-HSD) activity in the testes of sexually mature sea lampreys. This enzyme is necessary for the conversion of Δ-5 (pregnenolone-based) to Δ-4 (progesterone-based) steroids. Additionally, there was no evidence for the presence of Δ-4 steroids in an extract of 300 ml of plasma from prespermiating males (Weisbart and Idler 1970). However, neither of those studies were carried out on GnRH-injected males. It is feasible that GnRH up-regulates 3β-HSD activity so that 15α-P can be formed. Additionally, non-stimulated prespermiating males have concentrations of 15α-P that were almost certainly too low (<1 ng/ml) to have displayed a typical Δ-4 steroid UV absorption signal in an extract of 300 ml plasma.

Further research will be needed to confirm if 15α-P or any of the cross-reacting steroids are hormones. One possible theory for the existence of these steroids in the lamprey is that P is the functional hormone but that it is rapidly metabolized into the forms that we detect in the present study. In support of this theory is the fact that presumptive inactive steroid metabolites are known to circulate in the plasma of teleosts at high concentrations (Inbaraj et al. 1997; Tveiten et al. 2000). Furthermore, these metabolites can be formed in the gonads and not in the peripheral circulation (Kime 1993). However, in most cases

presumptive inactive metabolites in teleosts are conjugated steroids; the conjugation is believed to facilitate the excretion and/or regulation of local concentrations of the steroids (Kime 1993). It is possible that this is the reason for 15α-hydroxylation of steroids in the lamprey testis. However, we have been careful to use the word 'presumptive' in referring to the steroid metabolites of the teleosts. It is only 'presumed' that they are metabolites in teleosts because no work has been done to determine whether they might also be hormones.

The critical step in establishing whether 15α-P is a hormone will be to demonstrate that there is a receptor for this steroid in one or more tissues of the lamprey. A partial sequence homologous to nuclear progesterone receptors has been identified in sea lampreys (Thornton 2001), but the binding properties of the putative receptor have not yet been examined. Even if 15α-P does not bind to this receptor, it might bind to an as yet unidentified nuclear receptor or even to a membrane receptor such as those recently found in a wide variety of vertebrates (Zhu et al. 2003a, 2003b); this mechanism of hormone action has yet to be examined in lampreys.

In conclusion, we have demonstrated the production of 15α-P by the testis of the sea lamprey; developed an RIA for this steroid; shown the presence of 15α-P and several immunoreactive steroids in plasma; shown that the concentrations of ir-15α-P are markedly elevated in response to GnRH injection; and provided a powerful new tool for studying the endocrine control of reproduction in lampreys.

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CHAPTER FOUR

Bryan M.B., Young B.A., Close D.A., Semeyn J., Robinson T.C., Bayer J., Li W. Comparison of synthesis of 15α-hydroxylated steroids in males of four North American lamprey species. General and Comparative Endocrinology, submitted.

Comparison of synthesis of 15α-hydroxylated steroids in males of four North American lamprey species

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Abstract

Recent studies have provided evidence that 15α-hydroxytestosterone (15α-T) and 15α-hydroxyprogesterone (15α-P) are produced *in vitro* and *in vivo* in adult male sea lampreys (*Petromyzon marinus*), and that circulatory levels increase in response to injections with gonadotropin-releasing hormone (GnRH). We examined four species from the Petromyzontidae family including silver lampreys (*Ichthyomyzon unicuspis*), chestnut lampreys (*I. castaneus*), American brook lampreys (*Lethenteron appendix*), and Pacific lampreys (*Entosphenus tridentata*) to determine if these unusual steroids were unique to sea lampreys or a common feature in lamprey species. *In vitro* production was examined through incubations of testis with tritiated precursors, and 15α-T and 15α-P production was confirmed in all species through co-elution with standards on both high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). *In vivo* production was proven by demonstrating that HPLC-fractionated plasma had peaks of immunoreactive 15α-T and 15α-P that coelute with standards through using previously developed radioimmunoassays (RIAs) for 15α-T and 15α-P. The possible functionality of 15α-T and 15α-P were further examined in silver and Pacific lampreys by investigating the effect of injection of either type of lamprey GnRH on plasma concentrations of 15α-T and 15α-P. Injections with exogenous GnRH did not affect circulatory levels of either steroid in silver lampreys, and only GnRH III elicited higher levels of both steroids in Pacific lampreys. The 15α-hydroxylase enzyme(s) for steroids appears to present in adult males of all species examined, but the question of whether 15α-hydroxylated steroids are functional in these lamprey species, and the significance of the 15-hydroxyl group, requires further research.

Keywords: lamprey, steroid, Petromyzontidae, 15α-hydroxylated

Introduction

There has been recent evidence to support the production of 15α-hydroxylated steroids *in vitro* and *in vivo* in male sea lampreys, *Petromyzon marinus* (Kime and Callard 1982; Bryan et al. 2003, 2004; Lowartz et al. 2003, 2004). Radioimmunoassays (RIAs) have been developed for 15α-hydroxytestosterone (15α-T; Bryan et al. 2003) and 15α-hydroxyprogesterone (15α-P; Bryan et al. 2004) and have been used to show that plasma concentrations of these steroids increase in response to both types of endogenous lamprey gonadotropin-releasing hormone (GnRH; Young et al. 2004a, 2004b; Bryan et al. 2004), and therefore may be functional hormones. Additionally, there is also research indicating that the gonads of the European river lamprey, *Lampetra fluviatilis*, produce 15-hydroxylated steroids *in vitro* using progesterone and testosterone as precursors, although there is some question as to whether testosterone was hydroxylated at the α or β position (Kime and Rafter 1981; Kime and Callard 1982).

In order to properly interpret the significance of 15α-hydroxylated steroids in an evolutionary context, it must first be established when this unique pathway evolved: is it a derived trait found only in certain lamprey lineages or is it common across lamprey genera and therefore an ancestral trait among lamprey species? If 15α-hydroxylated steroids are only found in *Petromyzon* and *Lampetra* species, these steroids may represent a derived trait that evolved one or more times within the lamprey lineage. However, if 15α-hydroxylated steroids are common to all lamprey species, it provides evidence that the ancestral vertebrate steroids were structurally different than those present in teleosts and higher vertebrates. There are major physiological differences,

including life cycles, feeding ecology, and morphology, among lamprey species (Potter and Gill 2003), which evolved millions of years ago (Hardisty and Potter 1971). Investigations into the presence of 15α-hydroxylated steroids across lamprey genera must be put in the context of lamprey phylogeny (Fig. 16). However, physiological studies of lampreys from genera other than *Petromyzon* are challenging because many lamprey species are in decline (Renaud 1997), making it difficult to obtain high numbers of individuals on which to perform extensive experiments.

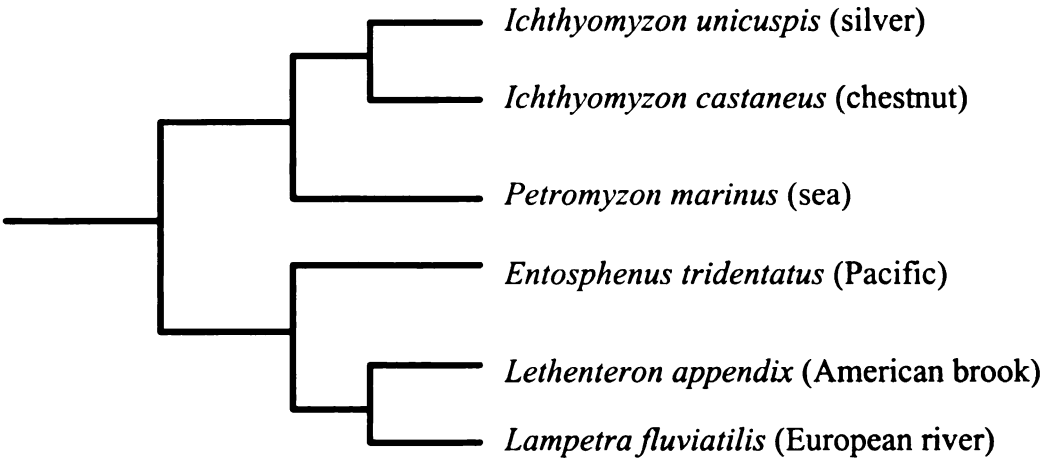

Figure 16. Phylogeny of lamprey species examined in Chapter 4. The phylogeny is based on that described by Gill et al. 2003.

Our hypothesis is that 15α-hydroxylated steroids are produced in the testes and circulated in the plasma of all lamprey species. We focused our research on genera found in North America to make comparisons with *Petromyzon*. Our objectives were 1) to determine if the 15α-hydroxylated derivatives of testosterone and progesterone are produced *in vitro* in lamprey species other than *P. marinus*, 2) to determine if 15α-P and 15α-T are produced *in vivo* and present in the plasma of lamprey species other than *P. marinus*, and

3) to determine (when possible) if 15α-P and 15α-T levels in plasma change in response to injection of GnRH I or GnRH III lamprey species.

Methods

All chemicals were obtained from Sigma unless otherwise noted. Taxonomic names are as designated by Gill et al. (2003).

Animals. For *in vitro* experiments and blood drawing, silver (*Ichthyomyzon unicuspis*), chestnut (*I. castaneus*), and American brook lampreys (*Lethenteron appendix*) were caught in sea lamprey traps by US Fish and Wildlife Service personnel and transported to Michigan State University (East Lansing, MI) in the spring of 2004. Lampreys were held at 12 ± 1 °C in flow-through tanks.

For GnRH-injection experiments, silver lampreys were caught in sea lamprey traps during the upstream spawning migration in May near the mouth of the St. Joseph's River by US Fish and Wildlife Service personnel and transported to Hammond Bay Biological Station (US Geological Survey; Millersburg, MI). The silver lampreys had an average weight ± standard error of 274.7 ± 5.30 g. Pacific lampreys (*Entosphenus tridentatus*) were collected by US Geological Survey personnel during upstream migration in November from the John Day Dam fish ladder on the Columbia River in Oregon. Pacific lampreys were transported to the Columbia River Research Laboratory and held under ambient conditions, and had an average weight of 324.9 ± 15.13 g.

All lampreys were anesthetized in 1:5000 MS222 prior to handling. Lampreys were classified as spermiating if gentle pressure on the abdomen resulted in the release of milt, and prespermiating if no milt was released (Siefkes et al. 2003)

In vitro incubations. This experiment used spermiating male silver lampreys (n = 2), chestnut lampreys (n = 2), and American brook lampreys (n = 6). For silver and chestnut lampreys 0.5 g of pooled gonadal tissue was used per incubation, but, because of the small gonad size, only 0.2 g of pooled gonadal tissue was used for American brook lampreys. The tissue was finely chopped in L-15 media added to a 50 ml conical tube containing 5 μCi of either ^{3}H-P or ^{3}H-T in 10 ml L-15. One incubation was performed per steroid per species. The tubes were shaken at 12 °C for 4 h. The tubes were then centrifuged at 1000 g for 20 min, the media was loaded onto activated Sep-paks (Waters), which were washed with 5 ml water and eluted with 5 ml methanol.

To identify the *in vitro* products, 2 ml of the methanol eluate was evaporated under nitrogen and resuspended in high performance liquid chromotagraphy (HPLC) buffer and fractionated using HPLC as by Bryan et al. (2003). A 20-μl aliquot was removed from each fraction and placed in a scintillation vial with 4 ml Safety-solve counting cocktail (Research Products International, Mount Prospect, IL) to count disintegrations per minute (dpm). The radioactivity in the fractions was compared to know elution points of 15α-hydroxylated and precursor standards. Percent conversion to a given product was calculated by dividing the dpm found to coelute with the product, including more than one fraction if necessary, by the total dpm found in all fractions.

Further characterization of radioactive products was carried out by thin layer chromatography (TLC). Based on coelution with 15α-P and 15α-T on HPLC, fractions were tentatively identified. From these fractions, an amount equivalent to 5,000 dpm was removed and added to microcentrifuge tubes along with 10 μg of standard 15α-T or 15α-P and evaporated under nitrogen. The contents of the tubes were loaded onto separate lanes of a TLC plate and developed (Bryan et al. 2003). The positions of the standards were noted by placing the plate under a UV source. The lanes were then divided into 5 mm fractions, scraped off, placed in scintillation vials, mixed with 4 ml scintillation cocktail and counted.

15α-hydroxylated steroid immunoreactivity in plasma. This experiment used spermiating male silver, chestnut, American brook, and Pacific lampreys (*Entosphenus tridentatus*). Blood was obtained through the caudal vein for (n, total plasma volume) silver (2, 1.25 ml), chestnut (2, 1.25 ml), and Pacific lampreys (8, 1.5 ml), and through cardiac puncture for American brook lampreys (6, 0.4 ml) using heparinized syringes. The blood was held at 4 °C for 20 min, centrifuged for 20 min at 1000 g, and the plasma removed and pooled for each species.

The pooled plasma was diluted 1:1 with 0.9% saline, passed through a 40 μM filter (Millipore), loaded onto an activated Sep-pak, and eluted as above. The methanol eluate was evaporated under a vacuum (CentriVap Concentrator, Labconco, Kansas City, MO)

and fractionated using HPLC as described previously (Bryan et al. 2003). Fractions 21-70 were assayed for 15α-P and 15α-T using RIA as in Bryan et al. (2003, 2004).

GnRH experiments. For silver lampreys, the experiment was carried out in May 2003 at the Hammond Bay Biological Station. For Pacific lampreys, the experiment was carried out in February 2003 at the USGS-Columbia River Research Laboratory in Cook, WA.

There were three treatment groups with 16 lampreys per group. An initial blood sample was taken to establish baseline steroid levels (for Pacific lampreys only). The lampreys were given one injection with either GnRH I (Sherwood et al. 1986) or GnRH III (Sower et al. 1993; both steroids synthesized by Bachem Peptide Company, King of Prussia, PA) in a dose of 100 μg/kg or 0.9% saline for control animals. Blood was sampled 24 h after the injection, and the lampreys were sacrificed so that sex could be determined. For silver lampreys, an ANOVA was used to compare plasma steroid levels from the three treatment groups (control, GnRH I, and GnRH III). For Pacific lampreys, t-tests were used to compare plasma levels before and after injection for each treatment group.

Results

In vitro incubations. See Table 3 for conversion rates. All species synthesized 15α-P and 15α-T from ^{3}H-P and ^{3}H-T, as determined by coelution on both HPLC (Fig. 17 and 18) and TLC. However, all species examined also converted ^{3}H-P into an unknown product that eluted at 32 minutes on HPLC, and American brook lampreys converted ^{3}H-T into an additional unknown product as well. Silver and chestnut lampreys had similar rates of

conversion for both ^{3}H-P and ^{3}H-T, but American brook lampreys had lower rates of conversion.

Male silver lampreys primarily metabolized P into 15α-P, but also produced steroids that eluted at 32 min (12.3% conversion) and 36 min (5.5% conversion) on HPLC. The sole product of T metabolism was 15α-T.

Table 3: Summary of results investigating *in vitro* and *in vivo* production of 15α-hydroxylated steroids.

	In vitro		*In vivo*		
	% converted (HPLC)[A]	Co-elutes on TLC[B]	ir-15α-OH in HPLC fraction[C]	GnRH I[D]	GnRH III[E]
Silver					
15α-P	46.1	√	√	NS	NS
15α-T	58.9	√	√	NS	NS
Chestnut					
15α-P	47.6	√	√	---	---
15α-T	69.3	√	√	---	---
American brook					
15α-P	30.4	√	√	---	---
15α-T	34.9	√	√	---	---
Pacific					
15α-P	---	---	√	NS	P = 0.07
15α-T	---	---	√	NS	√

The "√" symbol indicates a positive result, "NS" indicates a non-statistically significant result, and "---" indicates that no experiment was performed.

[A]The percent of the total radioactivity in the media after incubation that coelutes with 15α-P or 15α-T on high performance liquid chromatography (HPLC).
[B]Whether the *in vitro* product that coelutes with standard on HPLC also coelutes with standard on thin layer chromatography (TLC).
[C]Whether there is a peak of immunoreactivity in plasma extract that coelutes with standard 15α-P or 15α-T on HPLC.
[D]Whether an injection of GnRH I (100 μg/kg) causes an increase in circulatory levels of 15α-P or 15α-T at 24 h after injection.
[E]Whether an injection of GnRH III (100 μg/kg) causes an increase in circulatory levels of 15α-P or 15α-T at 24 h after injection.

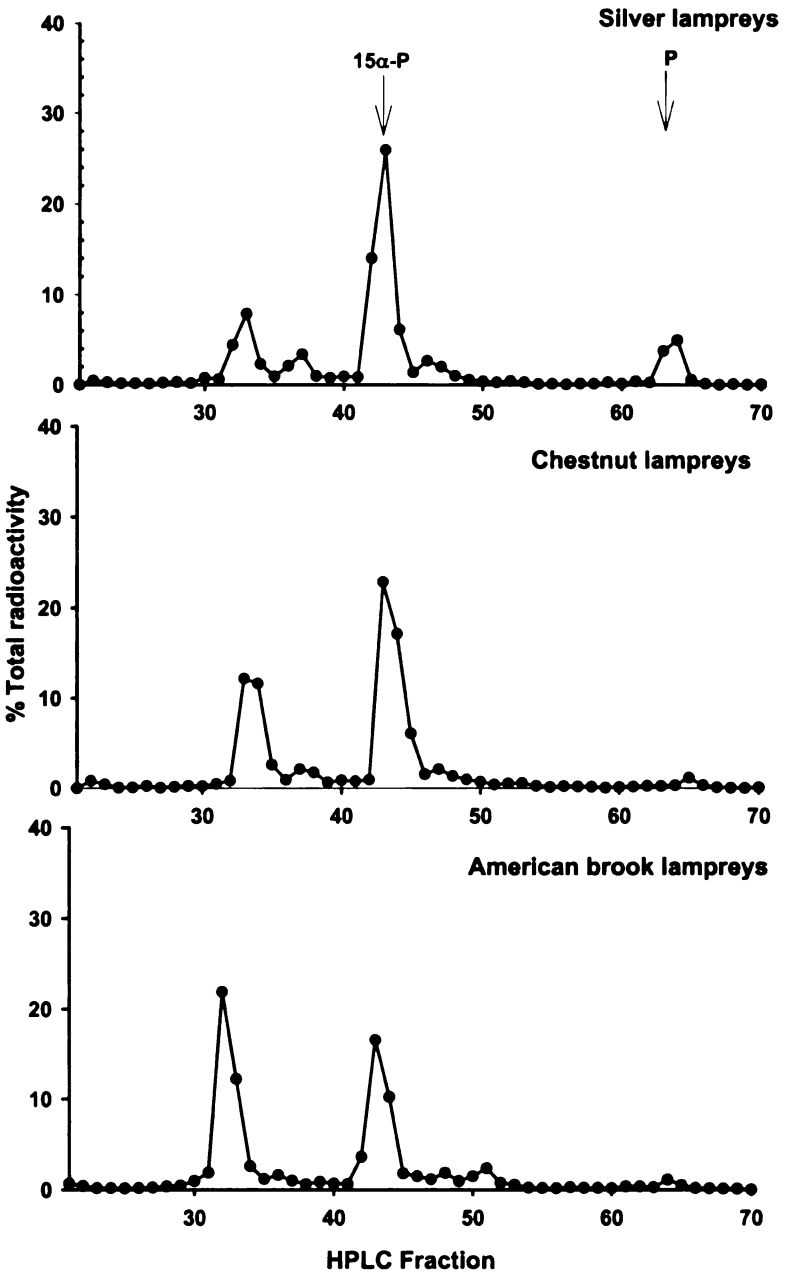

Figure 17: *In vitro* products of metabolism of tritiated progesterone (P) by testicular tissue of silver lampreys, chestnut lampreys, and American brook lampreys. The x-axis is HPLC fraction and the y-axis is percent of total radioactivity. Arrows indicate the elution points of 15α-hydroxyprogesterone (15α-P) and P.

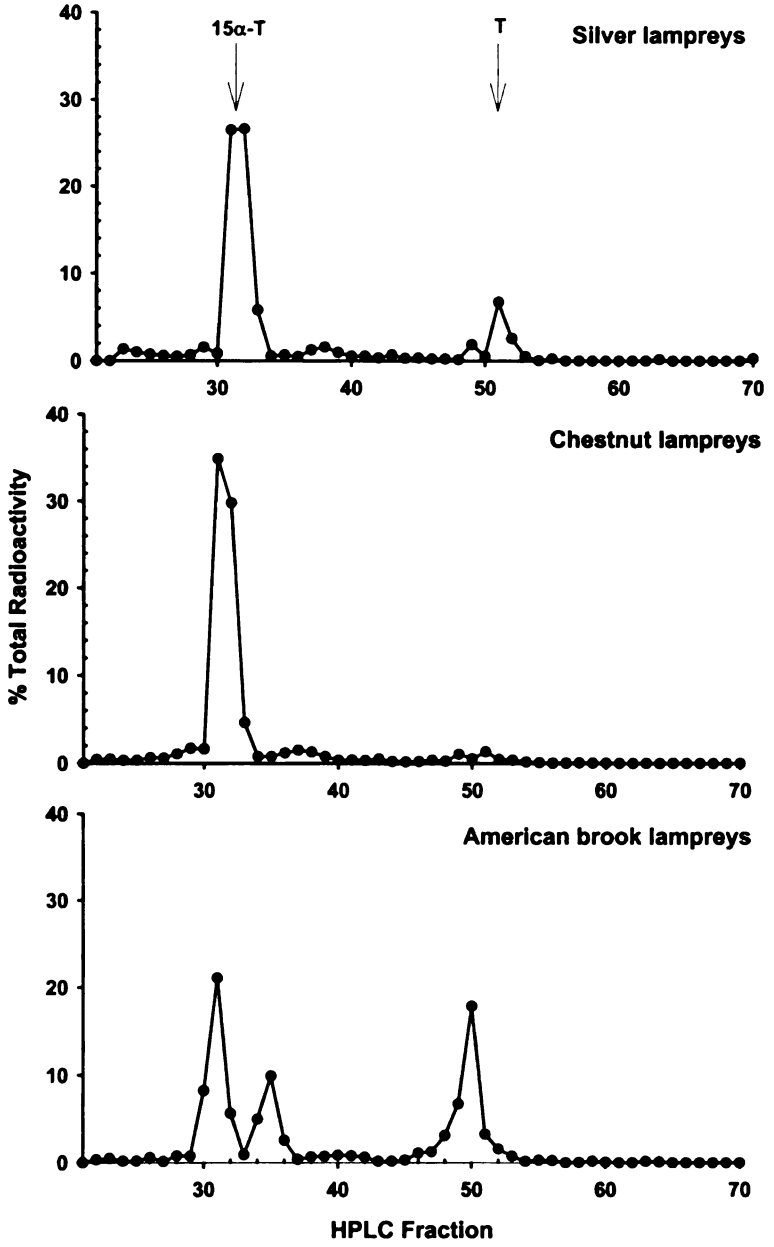

Figure 18: *In vitro* products of metabolism of tritiated testosterone (T) by testicular tissue of silver lampreys, chestnut lampreys, and American brook lampreys. The x-axis is HPLC fraction and the y-axis is percent of total radioactivity. Arrows indicate the elution points of 15α-hydroxytestosterone (15α-T) and T.

Male chestnut lampreys converted P into 15α-P, which was the major product, and a product that eluted at 32-33 min (28.3% conversion) on HPLC. The sole product of T metabolism was 15α-T.

Male American brook lampreys converted P to 15α-P, but there was more conversion (34.1%) to a product that eluted at 32-33 min on HPLC. T was converted to 15α-T, and was also converted (17.4%) to a product that eluted at 34 min on HPLC.

15α-hydroxylated immunoreactivity in plasma. Pooled, fractionated plasma from spermiating male silver, chestnut, American brook, and Pacific lampreys all had peaks of immunoreactivity that co-eluted with 15α-P and 15α-T (Fig.19).

For silver lampreys, immunoreactivity in the elution position of 15α-P was 1.98 ng/ml, or 31.6% of the total immunoreactivity. Immunoreactivity in the elution position of 15α-T was 1.09 ng/ml, or 66.9% of the total immunoreactivity.

For male chestnut lamprey plasma, the total immunoreactivity to 15α-P found in all HPLC fractions 6.04 ng/ml, with the largest amount of immunoreactivity coeluting with 15α-P. Immunoreactivity in the elution position of 15α-P was 2.06 ng/ml, or 34.1% of the total immunoreactivity. Immunoreactivity in the elution position of 15α-T was 2.57 ng/ml, or 96.3% of the total ir-15α-T, with the only peak of immunoreactivity corresponding to the elution time of 15α-T.

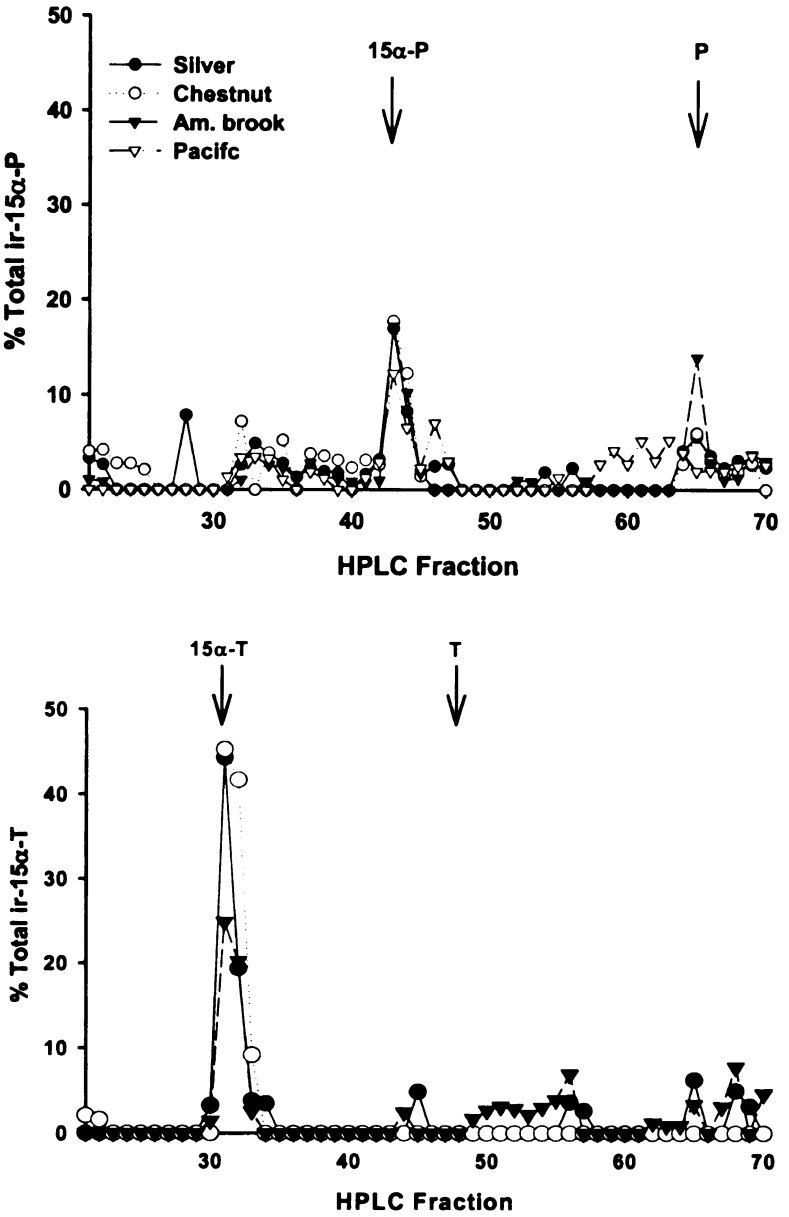

Figure 19: Immunoreactivity (ir) detected in HPLC fractions of extracted pooled plasma. The x-axis is HPLC fraction and the y-axis is percent of total immunoreactivity. The upper graph shows ir-15α-hydroxyprogesterone (15α-P) in pooled plasma obtained from male silver, chestnut, and American brook, and Pacific lampreys. The lower graph shows ir-15α-hydroxytestosterone (15α-T) to the same fractions (Pacific not shown). Arrows indicate the elution points of 15α-P, progesterone (P), 15α-T, and testosterone (T).

For male American brook lampreys, immunoreactivity in the elution position of 15α-P was 5.6 ng/ml, or 36.9% of the total immunoreactivity. A second large peak of immunoreactivity co-eluted with progesterone, and measured 3.82 ng/ml. Immunoreactivity in the elution position of 15α-T was 8.52 ng/ml, or 49.1% of the total ir-15α-T, which was the highest peak of immunoreactivity.

For male Pacific lampreys, immunoreactivity in the elution position of 15α-P was 0.75 ng/ml, or 21.7% of the total immunoreactivity. Immunoreactivity in the elution position of 15α-T was 0.25 ng/ml, or 53% of the total ir-15α-T.

GnRH experiments. For male silver lampreys, injections of neither type of GnRH caused circulatory levels of 15α-P or 15α-T to rise significantly higher than those in control animals injected with saline.

For male Pacific lampreys, there was no evidence that injections of GnRH I had an effect on circulatory levels of either 15α-P or 15α-T. However, lampreys injected with GnRH III showed a trend of higher plasma levels of 15α-P ($P = 0.07$) and significantly higher plasma levels of 15α-T ($P < 0.05$) after injection (Fig. 20).

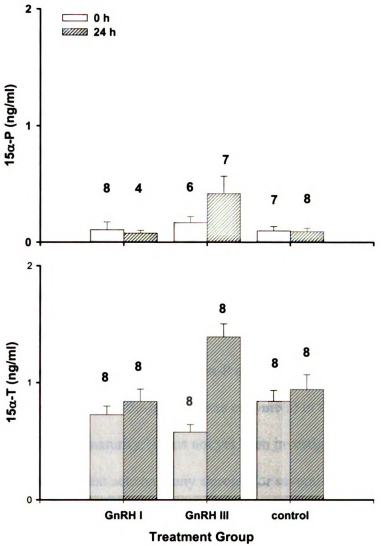

Figure 20: Results of GnRH-injection experiment using male Pacific lampreys. Pacific lampreys were injected with GnRH I or GnRH III (100 μg/kg) or saline. Blood was sampled prior to and 24 h after injection, and was assayed for 15α-P (upper graph) and 15α-T (lower graph), with the number of animals (n) indicated above each bar. Injection with GnRH III resulted in a trend toward higher plasma levels of 15α-P ($P = 0.07$) and significantly higher 15α-T plasma levels ($P < 0.05$). Injection with GnRH I did not cause a significant change in plasma levels of either steroid.

Discussion

15α-Hydroxylated steroids are produced by all lamprey species included in this study. Since this study included species from the oldest extant genus of lampreys, *Ichthyomyzon*, it is likely that the common ancestor of the species examined in this study also produced 15α-hydroxylated steroids. However, differences in production and circulation of 15α-hydroxylated steroids are apparent among species, which may be indicative of functional endocrine differences among lamprey species. It is important to note that 15α-hydroxylation has not been found to be a major steroidogenic pathway in the gonads of any other vertebrate.

All species examined formed 15α-P and 15α-T *in vitro*, and therefore all species examined express 15α-hydroxylase enzyme(s) in the gonads when nearing or at reproductive maturity. It has not yet been investigated whether there is one 15α-hydroxylase that acts on many steroids, or several different 15α-hydroxylases which are specific to androgens, progestagens, or estrogens. The lower rates of conversion found for both steroids by American brook lampreys may be a result of a smaller amount of tissue used in the incubations. In silver and chestnut lampreys, 15α-T was the sole product of testosterone metabolism, which is consistent with observations made of male sea lampreys (Bryan et al. 2003). In American brook lampreys, an additional unidentified product was formed, but 15α-T was still the major product.

Progesterone metabolism resulted in multiple products, some of which co-elute on HPLC with unidentified products observed in sea lamprey (Bryan et al. 2004). The relative

amounts of each metabolite varied greatly among species. In sea lampreys, it was found that the relative amounts of products changed as the reproductive season progressed, and the differences in steroid synthesis found among species using progesterone as a precursor may be reflective of this phenomenon. However, since these peaks are seen in several species, it is likely that there are more unusual progestagens in lampreys that have yet to be identified.

In addition to the species examined in this study, the *in vitro* conversion of progesterone and testosterone to their 15-hydroxylated derivatives has previously been demonstrated in immature adult male European river lampreys (Kime and Rafter 1981). In this study, the major product of testosterone metabolism was reported as 15β-T, but was later identified as possibly being 15α-T (Kime and Callard 1982). *In vivo* production of 15α-hydroxylated steroids has not been investigated in this species, which is a member of the latest genus of lamprey to evolve. Combined with the data presented here from *Ichthyomyzon* species, it lends greater support to the hypothesis that 15α-hydroxylase enzymes are pervasive in the testes of all species in the Petromyzontidae family.

All species examined in this study also had putative 15α-hydroxylated steroids produced *in vivo* and circulated in the plasma, as determined by HPLC elution time and immunoreactivity. The only major peak of ir-15α-T co-eluted with standard 15α-T for all species, but ir-15α-P was found in several fractions. Of particular interest may be the peak that elutes at 32 min on HPLC, as all species examined thus far also convert ^{3}H-P to

a product that elutes at this time. It is important to note that this peak does not coelute with 15α-T, which elutes at 30 min.

There are several possible reasons for the different responses of different species of lamprey to injection with GnRH. First, the injection and sampling scheme used for silver and Pacific lamprey was different than that used on sea lamprey (Young et al. 2004a; Bryan et al. 2004). Sea lampreys were given two serial injections, 24 h apart, and blood was sampled 6 h and 24 h after the second injection. While 15α-T circulatory levels were elevated at both the 6 h and 24 h sampling times (Young et al. 2004a), 15α-P circulatory levels were elevated at 6 h and declined significantly at 24 h, but were still above baseline levels (Bryan et al. 2004). It is possible that a single injection of GnRH did not stimulate steroidogenesis to the same extent that two serial injections do, or that levels of 15α-P were high at 6 h and declined by the time plasma was sampled at 24 h.

There is also a difference in the reproductive state of the animals used in these experiments. The experiments on sea lampreys used prespermiating adult males. The experiments on silver lampreys used adult males that were close to or already spermiated. The Pacific lamprey experiment used very immature adult males. It is very likely that GnRH affects steroidogenesis differently at each of these reproductive states.

Finally, it is possible that there are species-specific differences in endocrine function or response. Silver lampreys have a life cycle similar to that of sea lampreys in which the upstream spawning migration occurs in the spring directly prior to spawning (Manion and

Hanson 1980), and genus *Ichthyomyzon* is more closely related to genus *Petromyzon* than is *Entosphenus* (Gill et al. 2003). Pacific lampreys cease feeding and begin spawning migration between six months and a year prior to spawning, and overwinter in a fasting state in streams (Beamish 1980; Close 2002). This change in life cycle likely affects the timing of physiological events related to reproduction, and may necessitate different patterns in expression of hormone receptors or steroidogenesis.

The two endogenous forms of sea lamprey GnRH appear to be present in all three extant families of lamprey, including the Petromyzontidae family discussed in this paper, and the Geotriidae and Mordaciidea found in the southern hemisphere (Sower et al. 2000), and therefore likely evolved in an ancestral vertebrate before the families diverged. The two types of GnRH have been shown to be equipotent in inducing final maturation and steroidogenesis in sea lampreys, although larval sea lampreys have mainly GnRH III present in their brains (Sower et al. 2003). In sea lampreys, GnRH III appears to cause higher plasma levels of 15α-P in male sea lampreys (Bryan et al. 2004), but both types of GnRH cause similar increases in 15α-T circulatory levels (Young et al. 2004a). The results of the GnRH experiments on silver and Pacific lampreys may also point to differences in the effects and functions of these two types of GnRH. Determination of production of 15α-hydroxylated steroids in southern hemisphere lampreys would provide definitive evidence of the ancestry of 15α-hydroxylase, as these families likely diverged from Petromyzontidae in pre-Tertiary times (Gill et al. 2003).

The results of this study are best interpreted in the context of lamprey phylogeny. As determined by recently discovered fossils, lamprey-like and hagfish-like species evolved in the early Cambrian period (Shu et al. 1999) more than 500 million years ago, which supports a molecular clock-based estimate that agnathans diverged from gnathostomes approximately 564 million years ago (Kumar and Hedges 1998). However, times of divergence between extant lamprey species have not been calculated. It has been suggested that some groups of paired species are in the process of undergoing sympatric speciation (Salewski 2003), and have not fully diverged into separate biological species. For non-paired species and lampreys from different genera, it is difficult to make conclusions regarding differences in physiology and phylogenetic distances.

Some of the difficulties associated with making conclusions regarding differences in physiology and phylogenetic distances are due to controversies surrounding the phylogeny and nomenclature of species within the Petromyzontidae family (Bailey 1980). Most of the phylogenetic relationships among lamprey species have been determined through examination of their dentition (Hubbs and Potter 1971; Potter 1980; Potter and Hillard 1987), but a recent study using 32 morphological characteristics and rigorous statistical tests generated a new phylogenic tree (Gill et al. 2003). Most studies agree that *Ichthyomyzon* is the ancestral genus of all holarctic lampreys, and *Ichthyomyzon unicuspis* (silver lamprey) is the extant ancestral species. The genus *Petromyzon*, which contains only one species, *P. marinus*, is derived from *Ichthyomyzon*, whose two genera are monophyletic in origin (Gill et al. 2003). For the genus *Lampetra*, phylogenies based on dentition (Hubbs 1971; Potter 1980) divided it into three sub-

genera, *Entosphenus*, *Lethenteron*, and *Lampetra*, though a study using two mitochondrial genes found evidence for *Entosphenus* to be a separate taxon, and that the division between *Lethenteron* and *Lampetra* does not exist (Docker et al. 1999). However, analysis of morphological characteristics has led each of these sub-genera to be established as a separate genus (Gill et al. 2003). Of these three groups, *Entosphenus* is the most primitive, *Lampetra* is the most derived, and *Lethenteron* is intermediate. *L. fluviatilis* is known to produce 15-hydroxylated steroids *in vitro* (Kime and Rafter 1981), and the results of the current study support the hypothesis that the 15-hydroxylase is a common feature in lampreys, since this enzyme is found in representatives of the most ancestral, most derived, and intermediate genera.

Together with hagfish, lampreys form a monophyletic group (Kuraku et al. 1999), superclass Agnatha. It has been shown that hagfish gonads hydroxylate classical steroids at the C6 and C 7 positions *in vitro* (Kime and Hews 1980; Kime et al. 1980), although *in vivo* production has not been investigated. The combined evidence from lampreys and hagfish make it appear likely that ancestral vertebrates possessed steroid hydroxylases that are no longer found in the gonads of modern vertebrates. More research is needed to discern the reasons for both the existence of hydroxylated steroids in early vertebrates, and for their disappearance in higher vertebrates.

There have been few studies of the reproductive physiology of lamprey species in North American other than sea lamprey, although recorded observances of spawning behavior are often very similar among species (Case 1970; Manion and Hanson 1980; Cochran and

Lyons 2003). The emphasis on sea lampreys is likely due to both its abundance in freshwater lakes in northeastern North America and because of its economic and ecological impacts as an invasive species. Many lamprey species around the world are in decline (Renaud 1997) due to overfishing and habitat degradation, and obtaining sufficient numbers of a given lamprey species for physiological experiments can be difficult. Despite their importance as a food source to Native Americans (Close et al. 2002) and Europeans (Maitland et al. 1980; Almeida et al. 2000) native lamprey species have received little attention in North America. While the sea lamprey is often targeted as species for which hypotheses regarding early vertebrate evolution can be tested (e.g. Thornton 2001; Baker 2004), it is important to determine whether the physiological traits being investigated are common to all lamprey species, or are specific to sea lampreys.

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CHAPTER FIVE

Bryan M.B., Scott A.P., Lucas M.C., Li W. *In vitro* and *in vivo* production of 15α-hydroxylated steroids in adult male European river lampreys, *Lampetra fluviatilis* L.

***In vitro* and *in vivo* production of 15α-hydroxylated steroids in adult male European river lampreys, *Lampetra fluviatilis* L.**

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Running title: 15-OH Steroids in European River Lampreys

Abstract

The European river lamprey, *Lampetra fluviatilis*, is a member of the latest lamprey genus to evolve, and therefore represents a key group for examining the evolution of features within the Petromyzontidae family. Like the males of several North American species of lampreys, mature river lamprey testes produce15α-hydroxytestosterone (15α-T) and 15α-hydroxyprogestone (15α-P) *in vitro*. Males circulate immunoreactive (ir) 15α-T and 15α-P in the plasma. Plasma concentrations of 15α-P increase as the reproductive season approaches, and levels of 15α-T do not change. Plasma concentrations of 15α-P increase in response to injections of GnRH I or GnRH III, and plasma concentrations of 15α-T do not change in response to either type of GnRH. This finding provides further evidence that 15α-steroid hydroxylase in the testes is a common feature of holarctic lampreys, but raises questions regarding differences in potential functionality of 15α-hydroxylated steroids and the controls over steroidogenesis among lamprey species.

Keywords: river lamprey; *Lampetra*; 15α-hydroxylated; Petromyzontidae.

Introduction

The European river lamprey (*Lampetra fluviatilis*) is considered to belong to the latest genus of lampreys to evolve (Gill et al. 2003), and was the focus of several early studies investigating steroids in early vertebrates (Kime and Rafter 1981; Larsen 1987, 1990; Kime and Larsen 1987). One study found that immature adult river lamprey gonads of either sex metabolized progesterone and testosterone into 15-hydroxylated derivatives *in vitro* (Kime and Rafter 1981), although there were questions regarding whether the hydroxylation occurred at the α or β position (Kime and Callard 1982). Recent research has shown that 15α-hydroxytestosterone (15α-T) and 15α-hydroxyprogesterone (15α-P) are the major products of sea lamprey (*Petromyzon marinus*) testis *in vitro*, are circulated in the plasma, and plasma concentrations increase in response to injections with gonadotropin-releasing hormone (GnRH) in prespermiating adult males (Bryan et al. 2003, 2004; Young et al. 2004). Additionally, it has recently been shown that 15α-hydroxylated steroids are produced *in vitro* and *in vivo* in the males of four other North American lamprey species, including the silver lamprey (*Ichthyomyzon unicuspis*), chestnut lamprey (*I. castaneus*), American brook lamprey (*Lethenteron appendix*), and Pacific lamprey (*Entosphenus tridentatus*) (Bryan et al. submitted).

Lampreys have been considered the best living proxy for a vertebrate ancestor (Neidert et al. 2001), and constitute a key group for testing hypotheses regarding the timing and evolution of the steroid-receptor system (e.g., Thornton 2001; Baker 2004). To properly interpret the significance of 15α-hydroxylated steroids in the context of the evolution of steroids in vertebrates, the evolution of these steroids must be investigated in the context

of lamprey phylogenetics. It has already been demonstrated that 15α-T and 15α-P are present in the ancestral lamprey genus, *Ichthyomyzon* (Potter 1980; Bryan et al. submitted). The river lamprey belongs to the latest extant genus to evolve, *Lampetra* (Potter 1980; Gill et al. 2003), making it particularly important when addressing questions regarding the commonality of a particular trait in lamprey phylogenetics. The question as to whether 15α- or 15β-T, or both, are produced in river lampreys has never been resolved, and *in vivo* production of 15α-hydroxylated steroids has not previously been investigated. Further evidence regarding the status of these unusual steroids in *Lampetra* species would provide support for the theory that 15α-hydroxylated steroids are a common feature among species within the Petromyzontidae family (holarctic lampreys).

In this study, the objectives were to 1) Confirm *in vitro* production of 15α-hydroxylated steroids by mature adult river lamprey testis, 2) Determine if the *in vivo* production and circulation of 15α-hydroxylated steroids changes as natural maturation occurs in male lampreys, and 3) Determine if injections of either lamprey GnRH I or III (Sherwood et al. 1986; Sower et al. 1993) cause an increase in plasma concentrations of 15α-hydroxylated steroids in male river lampreys.

Methods

River lampreys were captured from River Ouse near Acaster Malbis in early December 2003 and transported to the University of Durham, where they were held in flow-through tanks at ambient temperatures and light conditions. Additionally, lampreys were captured while nesting on the River Ure near Ripon on April 20, 2004 which were also

transported to the University of Durham and held at ambient temperatures and light conditions for one day prior to sampling. Animal were anesthetized in 1:5000 MS-222 prior to handling for experiments. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise noted. Lamprey GnRHs were synthesized by Bachem Peptide Company (King of Prussia, PA, USA) according to Sherwood et al. (1986) for GnRH I and Sower et al. (1993) for GnRH III.

In vitro experiments. The *in vitro* experiments to examine the capacity of mature river lamprey gonads to 15-hydroxylate steroids were carried out in April 2004. Incubations were carried out each using pooled gonadal tissue from two lampreys, and were carried out separately for lampreys held in captivity over the winter ("tank-held" lampreys) and for lampreys captured on the spawning ground ("fresh-caught" lampreys). Testis incubations were conducted as previously described (Bryan et al. 2003, 2004) using 0.5 g testis in 10 ml L-15 media for 4 h, with 25 μCi of tritiated testosterone (T) or tritiated progesterone (P), although 75 μCi was used for the incubation of testis from tank-held lampreys. The incubation was stopped by freezing, and the media was thawed and extracted as by Bryan (2003, 2004) using an activated Sep-pak (Waters Corporation, Milford, MA, USA). The identity of the 15α-hydroxylated steroid products was confirmed through coelution with standards on high performance liquid chromatography (HPLC) and thin layer chromatography (TLC), coelution of acetylated products and standard on TLC, and binding to specific antibodies raised against standards (Bryan et al. 2003, 2004). Although 15α-T and 15β-T elute less than one minute apart on HPLC, they

do not co-migrate on TLC, and therefore the two very similar molecules can be distinguished from one another.

Natural changes in plasma steroid concentrations. Blood was sampled using heparinized syringes from 20 tank-held lampreys each in December, February, and March, and from 32 lampreys in April. Blood was also sampled from 26 fresh-caught lampreys in April. The lampreys were killed so that their sex could be determined surgically, and gonadal-somatic index (GSI) could be recorded. The blood was centrifuged at 1000 *x* g for 20 min, the plasma pipetted off, and stored at -20° C. The plasma was assayed using radioimmunoassays (RIAs) for 15α-T (Bryan et al. 2003) and 15α-P (Bryan et al. 2004). Differences in steroid levels and GSI among months for tank-held lampreys of each sex were analyzed using an ANOVA, followed by multiple comparison tests using a Bonferroni adjustment if significant results were obtained. Correlations between GSI and steroid levels were also investigated.

Effect of GnRH on plasma steroid concentrations. The GnRH-injection experiment was performed using tank-held lampreys only. There were three treatment groups, each consisting of eight males, and each treatment was placed in a separate tank. Lampreys were given two serial injections, 24 h apart, of either GnRH I (100 μg/kg, Sherwood et al. 1986), GnRH III (100 μg/kg; Sower et al. 1993), or saline as a control. Blood was sampled 6 h after the second injection, processed to obtain plasma, and assayed as described above. The assay was further validated by pooling plasma from lampreys treated with GnRH I and GnRH III. The plasma was extracted using a Sep-pak and

fractionated using HPLC as described above. The HPLC-fractionated plasma was then assayed for both 15α-T and 15α-P, and immunoreactivity was compared to the known elution times of the steroids. Differences in steroid levels among treatment groups were investigated using ANOVA, and Bonferroni adjustments for multiple comparison tests were used to determine differences between specific groups if significant results were obtained.

Results

In vitro experiments. Males produced both 15α-T and 15α-P from ^{3}H-T and ^{3}H-P *in vitro*, but ^{3}H-P was converted into additional products as well (Fig. 21). The identity of the 15α-hydroxylated steroids was confirmed through coelution with 15α-T or 15α-P on HPLC and TLC, coelution of acetylated *in vitro* products and acetylated standard on TLC, and binding to specific antibodies raised against 15α-T.

Natural changes in plasma steroid concentrations. Tank-held males experienced changes in plasma steroid levels from the time of their migration to the time that spawning takes place (Fig. 22). 15α-T levels in males did not change significantly in the tank-held lampreys (ANOVA, d.f. = 3, 47, $P > 0.0500$) and the fresh-caught males had significantly less 15α-T than tank-held lampreys in April (t-test, d.f. = 25, $P = 0.0144$). 15α-P levels in tank-held males changed significantly (ANOVA, d.f. = 3, 47, $P < 0.0001$), and plasma levels of 15α-P were lower in December than in February ($P = 0.0050$), March ($P = 0.0030$), and April ($P < 0.0001$). Fresh-caught males had less 15α-P than tank-held males in April (t-test, d.f. = 25, $P = 0.0157$). Average tank-held male GSI

changed significantly among months (Fig. 23; ANOVA, d.f. = 3,47, $P < 0.0001$). Average male GSI was higher in March than in December ($P < 0.0001$) and in February ($P = 0.0020$), and was lower in April than in December ($P = 0.0440$), February ($P = 0.0060$) or March ($P < 0.0001$). GSI did not have significant correlations with either 15α-T or 15α-P.

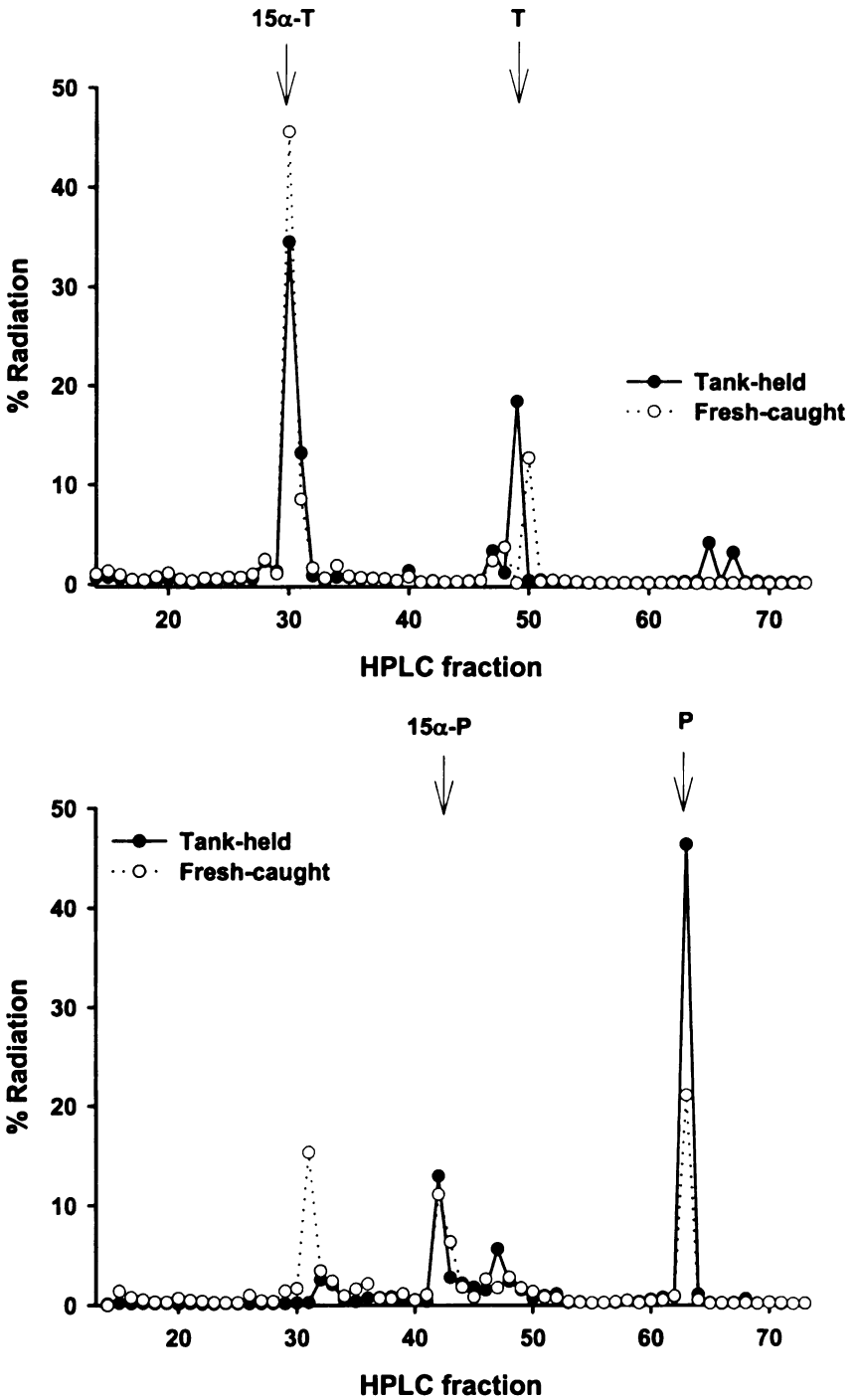

Figure 21: Results investigating *in vitro* and *in vivo* production of 15α-hydroxylated steroids in male river lampreys. The x-axis is HPLC fraction and the y-axis is percent of total radioactivity. Arrows indicate the known elution points of 15α-P, progesterone (P), 15α-T, and testosterone (T).

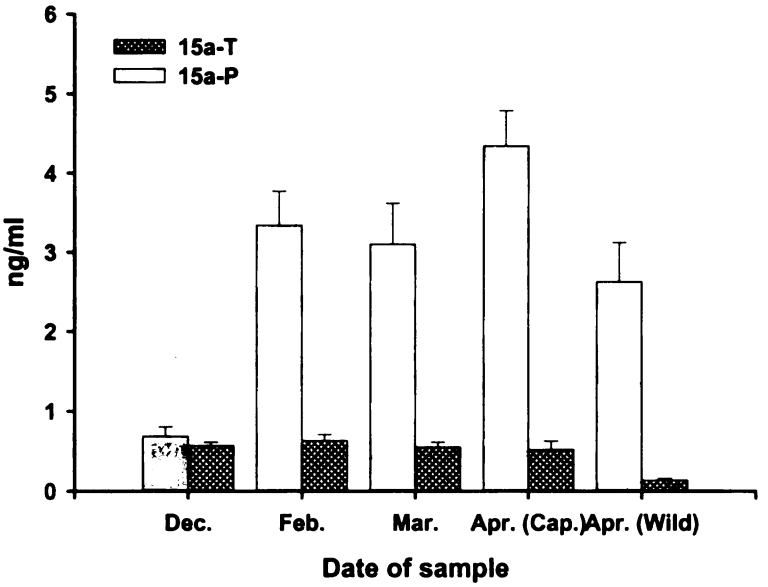

Figure 22: Changes in plasma steroid levels between the time of upstream migration and spawning in river lampreys. Plasma was obtained and assayed for 15α-hydroxytestosterone (15α-T) and 15α-hydroxyprogesterone (15α-P). The sex of the animals was confirmed surgically. The first four bars represent lampreys captured during their upstream migration and held at ambient light and temperature conditions, and the last bar represents lampreys that over-wintered in streams, and were captured while nesting or spawning. The error bars represent one standard error of the mean.

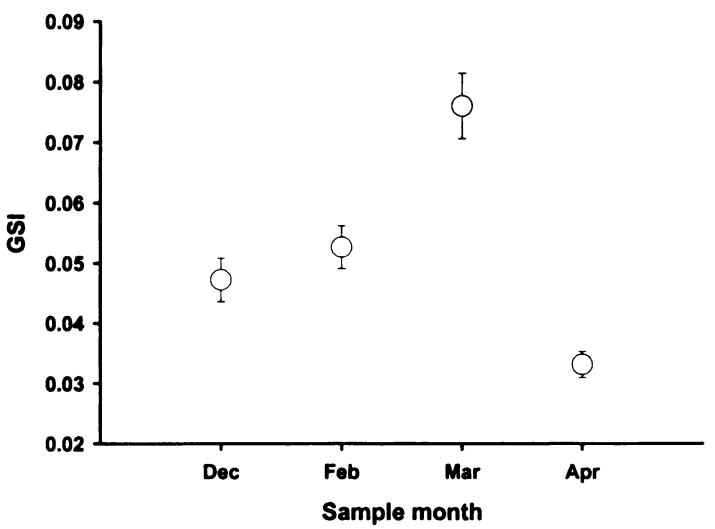

Figure 23: Mean GSI of tank-held male lampreys in different months. The error bars represent the standard error of the mean.

Effect of GnRH on plasma steroid concentrations. Neither type of GnRH had a significant effect on circulatory 15α-T levels. Control 15α-T levels were (mean ± standard error) 0.13 ± 0.03 ng/ml. However, both types of GnRH had significant effects on circulatory 15α-P levels in males (ANOVA, d.f. = 2,20, $P = 0.0010$), but there was no difference between the effects of the two different types of GnRH. Control levels of 15α-P in males were 1.86 ± 0.17 ng/ml, levels in lamprey treated with GnRH I were 3.72 ± 0.44 ng/ml, and levels in lampreys treated with GnRH III were 4.48 ± 0.61 ng/ml (Fig. 24).

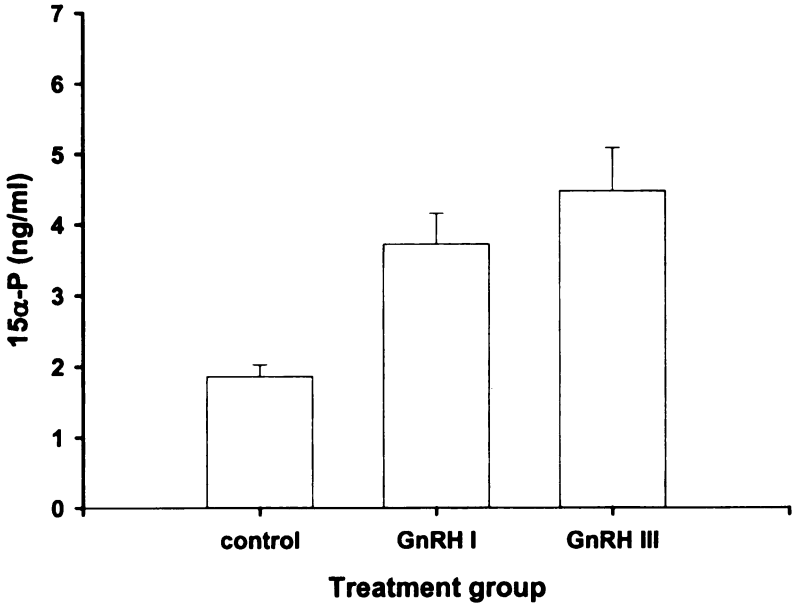

Figure 24: 15α-Hydroxyprogesterone (15α-P) plasma concentrations of male lampreys given two serial injections, 24 h apart of GnRH (100 μg/kg). Blood was sampled 6 h after the second injection. GnRH I and GnRH III both elicited significantly higher plasma levels of 15α-P than saline injections in male lampreys. Neither type of GnRH significantly increased plasma levels of 15α-hydroxytestosterone (15α-T) in male lampreys.

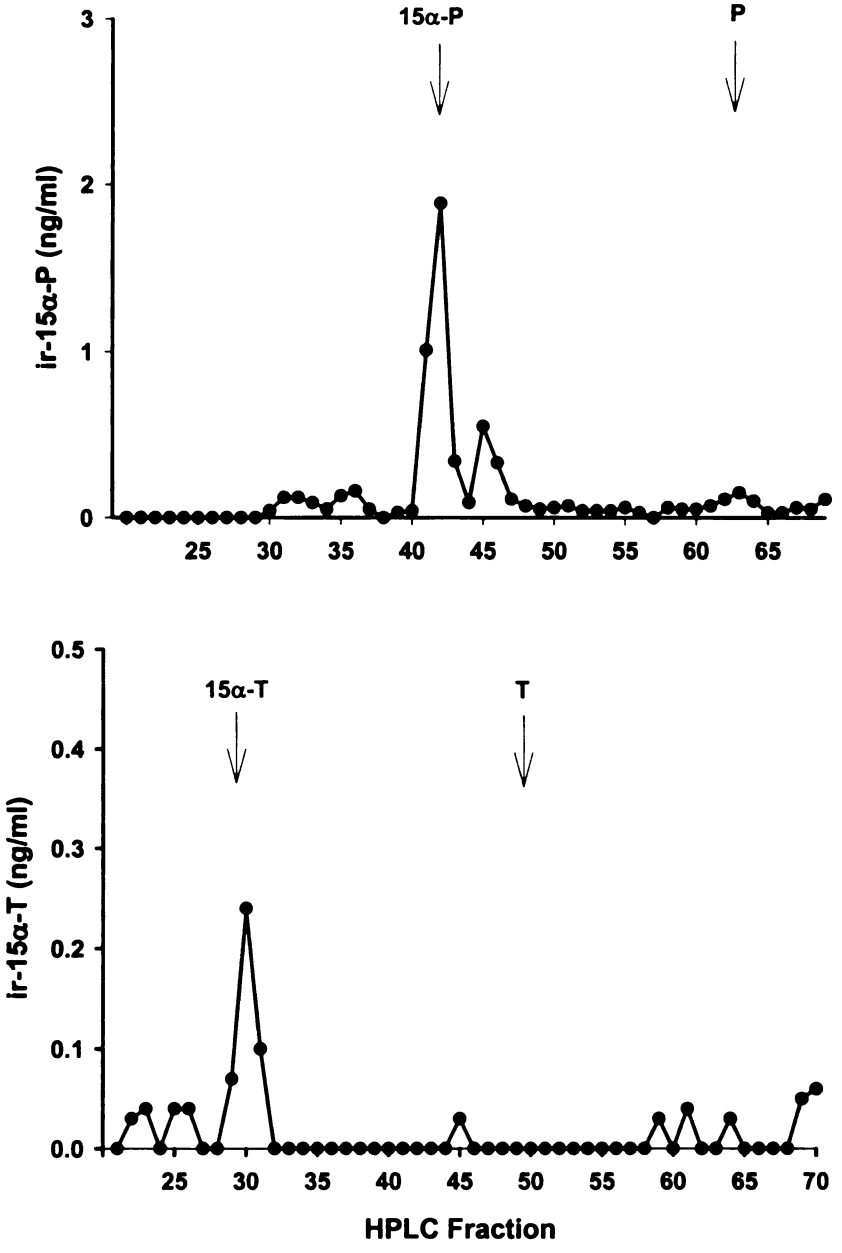

Figure 25: Immunoreactivity (ir) to antibodies raised against 15α-hydroxyprogesterone (15α-P) and 15α-hydroxytestosterone (15α-T) in HPLC-fractionated river lamprey plasma. Plasmas from lampreys injected with GnRH I or GnRH III were pooled, extracted, fractionated using HPLC, and the fractions assayed using RIA. Arrows indicate the known elution points of 15α-P, progesterone (P), 15α-T, and testosterone (T).

In HPLC-fractionated male river lamprey plasma, there were five peaks of ir-15α-P (Fig. 25). The largest of these peaks coeluted with 15α-P, and was 49.8% of the total immunoreactivy. Of the remaining peaks, one coeluted with P (64 min), and the others did not correspond with available standards, and were at 32, 37, and 46-47 min. There was one major peak of ir-15α-T, which coeluted with 15α-T and was 50.7% of the total immunoreactivity.

Discussion

Male European river lampreys appear to produce 15α-hydroxylated steroids in the gonads and circulate them in the plasma. Males produced both 15α-T and 15α-P *in vitro*, although other steroids were produced, and relatively large quantities of P were left unconverted. Additionally, although the putative 15α-T produced *in vitro* in males coeluted with standard on TLC in both its unaltered and acetylated forms, and bound strongly to antibodies raised again 15α-T, it also exhibited binding to antibodies raised against 15β-T. The binding to antisera raised against 15β-T may be due to contamination of the standard 15β-T which was used in raising antibodies with 15α-T (Bryan et al. 2003). In sea lampreys, it was found that the conversion of P to three other steroids, one of which was identified as 15α-P, changed as the reproductive season progressed, with the synthesis of 15α-P being greatest earlier in the reproductive season (Bryan et al. 2004). River lampreys may exhibit a similar phenomenon, and this may also explain the discrepancies in the results between this study and a previous one (Kime and Rafter 1981) which used river lampreys immediately after being captured during their upstream migration.

River lampreys have a semelparous, anadromous life cycle in which larvae are filter feeders which live in stream sediment, followed by a parasitic phase which lives in the ocean, and adults migrate to streams to spawn in gravel (Maitland 1980). The upstream spawning migration takes place in the fall, and lampreys overwinter in a fasting state in streams before spawning in spring (Maitland 1980). Levels of steroids changed in males during the time between their upstream migration in December and the time at which spawning occurs naturally in April. Plasma concentrations of 15α-P increased as the season progressed, but the significant increase occurred between December and February. Again, more research is necessary to determine the function of 15α-P, and further research may determine if it regulates final maturation in lampreys , as progestagens are known to do for many teleost fish species (review: Miura and Miura 2001). Levels of 15α-T did not change throughout this period.

The lower steroid levels found in fresh-caught lampreys in April are likely due to the stress of capture and transport, as plasma was sampled the day after they were removed from the river. Laboratory conditions for tank-held lampreys mimicked the natural photoperiod and temperatures that the fresh-caught lampreys would encounter. However, lampreys held in captivity since December were acclimated to being held in a tank.

Despite statistically significant changes in plasma steroid levels at different times of sampling, it is still unknown how, and if, these steroids function within the HPG axis. 15α-T levels did not change in response to either type of GnRH in males or females. If

15α-T does not have a physiological function near the time at which sexual maturity occurs, levels of this steroid may not be affected by GnRH. As maturation progresses, many teleost fish exhibit a shift in steroidogenesis from androgens (C19 steroids) to progestagens (C21 steroids) (Scott et al. 1983; Barry et al. 1990). However, at this time, it has not yet been determined whether 15α-T is a functional hormone, and its lack of response to GnRH may also suggest a lack of function. Additional experiments using lampreys at earlier life stages would be useful in determining whether the lack of response to GnRH seen in this study is stage-specific in river lampreys.

In contrast to its effect on 15α-T, both types of GnRH increase circulatory levels of 15α-P. The data obtained from this experiment are particularly puzzling, considering the data obtained from non-injected fish used to examine changes in steroid levels during the overwintering period. Tank-held males used to examine seasonal differences (no injections) had higher plasma concentrations of 15α-P than control males, and similar levels to GnRH-injected fish.

The results from the experiments using GnRH are different than those obtained using other species of lampreys. Using the same injection protocol as in this study, at 8 h after the second injection, male sea lampreys (*Petromyzon marinus*) exhibit a 2-5 fold mean increase in 15α-T levels (Young et al. 2004), and a 36 fold mean increase in 15α-P levels (Bryan et al. 2004). The disparity may be due to life history differences and associated physiological differences, as sea lampreys migrate upstream in the spring directly before spawning (Manion and Hanson 1980). Additionally, using a different injection protocol

in which lampreys were given one injection of GnRH and plasma sampled 24 h later, neither type of GnRH caused increased levels of 15α-T or 15α-P in male silver lampreys (*Ichthyomyzon unicuspis*) near spawning and only GnRH III caused increased levels of 15α-T and 15α-P in male Pacific lampreys (*Entosphenus tridentatus*) several months prior to spawning (Bryan et al. submitted).

Of the lampreys species thus far investigated, the testes of all of them have the capacity to produce 15α-hydroxylated steroids *in vitro*, and immunoreactive 15α-hydroxylated steroids have been detected in the plasma. Although the synthetic pathway to produce 15α-hydroxylated steroids seems functional in all male lampreys studied, the actual patterns and regulation of these synthetic pathways appears to differ among species. In particular, there appears to be vast differences among species in how GnRH treatment affects circulating levels of 15α-hydroxylated steroids. The differences in responses cannot be explained by phylogenetics, as sea lampreys and silver lampreys belong to a monophyletic group, and Pacific lampreys and river lampreys evolved later from a common ancestor (Gill et al. 2003). There are differences in life history in these species, as river and Pacific lampreys cease feeding and migrate upstream in the fall and overwinter in streams before spawning (Maitland 1980; Beamish 1980), while silver and sea lampreys migrate upstream in the spring and have a much short fasting period (Manion and Hanson 1980). Additionally, the feeding ecology of each of these species of lampreys differs as well, and based on dentition, silver and sea lampreys ingest host blood, while Pacific and river lampreys ingest host body tissues (Potter and Hillard 1987) although it appears that the pacific lamprey attacks ventrally and prefers to ingest body

fluids (Beamish 1980). The differences in results may also be, explained by differences in experimental protocol, or by the maturation stage at which the experiments took place.

Further research is needed to elucidate the function of the 15α-hydroxylated steroids in all lamprey species, and to better understand how life history differences among lamprey species affect reproductive endocrinology.

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APPENDIX

Young B.A., Bryan M.B., Sower S.A., Scott A.P., Li W. 2004. 15α-Hydroxytestosterone induction by GnRH-I and GnRH-III in Atlantic and Great Lakes sea lamprey (*Petromyzon marinus* L.). General and Comparative Endocrinology 136, 276-281.

15α-Hydroxytestosterone induction by GnRH I and GnRH III in Atlantic and Great Lakes sea lamprey (*Petromyzon marinus* L.)

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Abstract
The sea lamprey (*Petromyzon marinus* L.) represents one of the two most ancient classes of vertebrates and possesses a functional hypothalamus-pituitary-gonadal axis. However, the presence and functionality of androgens in the sea lamprey remain elusive. Recently, 15α-hydroxytestosterone (15α-T) has been found in sea lamprey gonads and blood plasma. In this study we examined changes of circulatory concentrations of 15α-T in response to gonadotropin releasing hormone (GnRH) treatments. Plasma concentrations of 15α-T in sea lamprey increased 2 to 5 times for all GnRH-injected sea lamprey compared to controls ($P < 0.0001$). However, there were no differences among responses 1) to the two forms of GnRH (lamprey GnRH I or lamprey GnRH III), 2) to the doses delivered (50, 100, or 200 μg/kg), or 3) between post-injection sample intervals (8 or 24 h). Between lampreys from the Atlantic Ocean and Great Lakes sites, two of seven GnRH form and dosage comparisons showed between-site differences, but were not believed to represent an overall between-site difference. These are the first data to show a response of a C19 steroid to GnRH stimulation in sea lamprey.

Introduction

The sea lamprey (*Petromyzon marinus* L.) is among the most primitive of extant vertebrates and has a complex life cycle (Hardisty and Potter 1971). Sea lampreys begin their lives in freshwater as blind, filter-feeding ammocoetes (larvae). After three to seven years, metamorphosis occurs and the ammocoetes become sexually immature, free-swimming juveniles that migrate to the sea (Atlantic Ocean) or lakes (Great Lakes). During an approximate 15-month parasitic phase, gametogenesis progresses and spermatogonia proliferate and develop into primary and secondary spermatocytes in males. After migration to freshwater streams, both sexes undergo final reproductive maturation. The semelparous life cycle of the sea lamprey ends soon after spawning in streams. The sea lamprey is the only jawless fish in which the hypothalamus-pituitary-gonadal (HPG) axis has been established (Sower 1998; Sower and Kawauchi 2001). Therefore, further knowledge regarding the reproductive physiology of sea lampreys can aid in understanding the evolution of endocrine controls for reproduction seen in more modern vertebrates.

The neuroendocrine components of the reproductive endocrine system are highly conserved among vertebrates, including lampreys (Thornton and DeSalle 2000; Sower 1998; Sower and Kawauchi 2001; Thornton 2001). However, the roles of gonadal steroids appear to differ in lampreys. Several groups have detected very low concentrations of immunoreactive testosterone in plasma of male and female sea lampreys, but have been unable to demonstrate that it increases in response to injections of lamprey gonadotropin releasing hormone (GnRH) and heterologous gonadotropin(s),

or that its circulatory concentrations and profiles are sexually dimorphic (Katz et al. 1982; Sower et al. 1985a, 1985b; Linville et al. 1987). In addition, Ho et al. (1987) demonstrated estrogen binding, but not androgen binding to receptors in lamprey testis. These studies were later supported by a study that identified the cDNA that appeared to encode a putative estrogen receptor, but did not identify any cDNA sequences that are homologous to typical vertebrate androgen receptors in sea lampreys (Thornton 2001). these findings support the hypothesis (Sower 1990, 1998) that cyclostomes may not use androgens as male reproductive hormones.

One other possible explanation for the absence of experimental evidence for typical androgens and their corresponding receptors is that lamprey androgens are structurally different from those identified in other vertebrates. Kime and Rafter (1981) and Kime and Callard (1982) showed that the testis of *Lampetra fluviatilis* and *Petromyzon marinus* have the capacity to hydroxylate testosterone at the C15 position, forming 15α-hydroxytestosterone (15α-T). This has recently been confirmed by two studies. Lowartz et al. (2003) found steroids produced *in vivo* and *in vitro* by sea lampreys that have elution times corresponding to 15α-hydroxylated steroids when analyzed with High Performance Liquid Chromatography (HPLC). Bryan et al. (2003) used HPLC, Thin Layer Chromatography (TLC), and microchemical analysis to further show that sea lampreys produce 15α-T *in vitro*. Bryan et al. (2003) also developed a radioimmunoassay (RIA) for 15α-T and showed this steroid to be present in the blood plasma of male sea lampreys (and to a lesser extent in females). Despite the identification of 15α-T in sea lampreys, the function of this steroid has yet to be

characterized.

This study was designed to determine a potential endocrine role of 15α-T in the sea lamprey through induction by GnRH, thus showing this sex steroid to be a possible component of the well-established and functional hypothalamus-pituitary-gonadal (HPG) axis of the sea lamprey (Sower and Kawauchi 2001). In particular, the effects of lamprey GnRH I (Sherwood et al. 1986) and GnRH III (Sower et al. 1993) on 15α-T plasma concentrations were measured. Using adult male sea lampreys, an experiment was designed to test: 1) the effects of the administration at different doses and at different times post-treatment of exogenous lamprey GnRH I and GnRH III on 15α-T plasma concentrations; and 2) to determine if there was a difference in response between adult prespermiating male sea lampreys from the Great Lakes or Atlantic Ocean.

Methods

Pre-spermiating, adult male sea lampreys were collected during late-spring (May) spawning migrations. Anadromous lampreys were trapped at the Cocheco River fish ladder in Dover, New Hampshire, USA. Great Lakes lampreys were trapped at the mouths of two Lake Huron tributaries, the AuGres and AuSable rivers in Michigan, USA. Atlantic Ocean lampreys were held at the University of New Hampshire (Durham, NH) and Great Lakes lampreys were held at the Hammond Bay Biological Station (USGS-BRD, Millersville, MI) in tanks containing approximately 160 L of continuous-flow water for at least two days prior to treatment applications. Water at the New Hampshire site came from the Oyster River while the Michigan site used Lake Huron water.

Temperature during acclimation and experimental periods was maintained at 16 °C (±1 °C) for both sites. Lamprey were weighed and measured for use in subsequent dosage calculations.

Lamprey GnRH I and lamprey GnRH III were synthesized by American Peptide Company (Sunnyvale, CA) and dissolved in 0.6% saline less than 30 minutes prior to administration. Each site used seven treatments, twelve lampreys per treatment, with two timed injections, and two timed bleedings as done previously (Sower 1989; Deragon and Sower 1994; Gazourian et al. 2000). Each group of 12 lamprey were injected intraperitoneally with either 0.6% saline (control), lamprey GnRH I (50, 100, or 200 μg/kg body weight), or lamprey GnRH III (50, 100, or 200 μg/kg body weight). The two injections, 48 h apart, were administered beginning at 8:00 am. Blood samples (0.5-1.0 ml) were collected 8 h and 24 h after the second set of injections by cardiac puncture using heparinized syringes. After centrifugation of blood samples, plasma was collected and stored at -80 °C until analyzed for 15α-T by RIA according to Bryan et al. (2003). The RIA for 15α-T was conducted using raw plasma. The antibody raised against 15α-T was used at a dilution of 1:100,000 and radiolabel was dispensed so that there were 7,000 dpm per tube. In the absence of standard, the antibody bound approximately 50% of the available radiolabel. The sensitivity of the RIA was 39 pg/ml plasma and had a detectable range from 2 - 500 pg/tube.

The reliability of the 15α-T RIA, when applied to plasma from GnRH-injected lampreys was validated in three ways. First, 5 ml of pooled plasma from GnRH-treated lampreys

were extracted using a solid phase extraction cartridge (Sep-Pak C18; Waters, Milford, MA), fractionated using HPLC (Bryan et al. 2003), and 20 µl of each fraction then assayed for 15α-T using RIA. This was done in order to confirm that immunoreactivity was restricted to the expected elution position of 15α-T and that production of no new immunoreactive compounds had resulted from GnRH induction. Second, dilutions of pooled plasma from GnRH-treated lampreys were combined with labeled 15α-T in the absence of antibody. This was done to confirm the absence of plasma binding proteins (which if they were present, might interfere with the RIA). Third, standard 15α-T at a range of dilutions was added to plasma from GnRH-injected lamprey and assayed at volumes of 25 µl, 50 µl, and 100 µl (total volume brought to 100 µl with assay buffer). This was done to establish parallelism within the assay.

Results were analyzed at multiple treatment levels. Violations of normality and variance equality precluded the use of a factorial analysis of variance (ANOVA). For comparisons among several treatment groups, Kruskal-Wallis (K-W) tests were used. T-tests or Wilcoxon signed-ranked tests (W) were used for simple group comparisons. There were 14 interval comparisons (7 treatments x 2 sites) between the 8 h and 24 h samples. If those comparisons proved not significant, then the two intervals were pooled. Second, comparisons of the three dosage levels for GnRH I and GnRH III were made. Third, differences between the two forms of lamprey GnRH were evaluated by dose and site. Finally, between-site differences were assessed by GnRH treatment and dose.

Results

During the course of the experiment, 24 of the 168 lampreys died. Mortalities among treatments appeared random with the highest number (50%) seen in a control treatment. Lamprey size differed between the sites where Great Lakes sea lampreys had a mean length of 509 mm and mean weight of 243 g while Atlantic sea lampreys had an approximate mean length of 700 mm and an approximate mean weight of 800 g.

The RIA of HPLC fractions from GnRH-injected male plasma revealed a single peak of immunoreactivity corresponding to the known elution position of 15α-T (Figure 26). The test for binding proteins in the plasma was negative. The test of parallelism returned a line with a slope equal to the known values and an $r^2 = 0.86$.

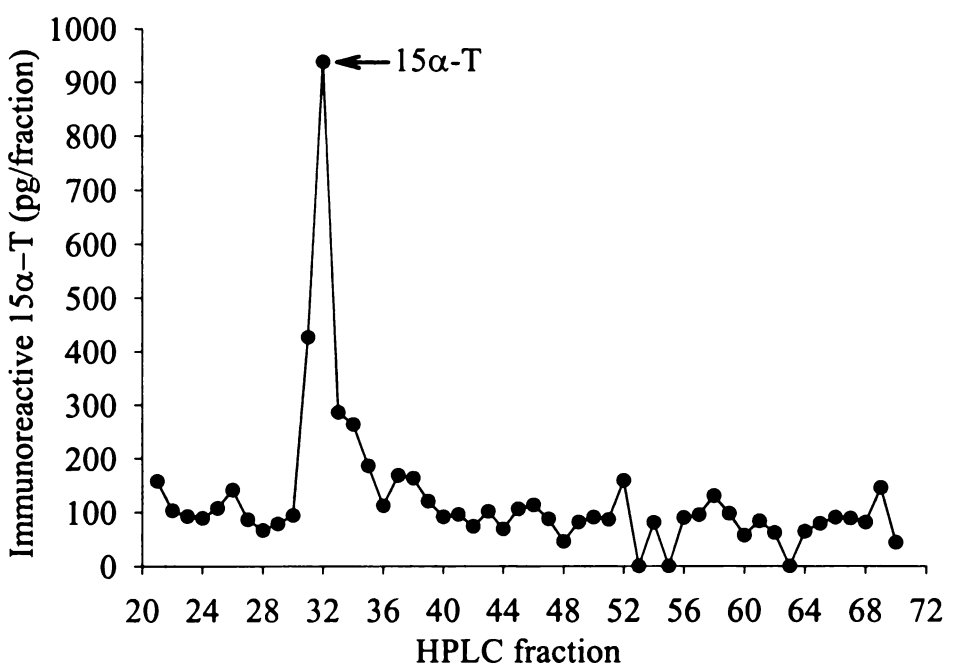

Figure 26: Radioimmunoassay of HPLC-fractionated plasma from GnRH-injected male sea lampreys. The elution point of 15α-hydoxytestosterone (15α-T) standard is indicated with an arrow.

Plasma analyzed using RIA yielded 15α-T concentrations ranging from 0.07 to 4.52 ng/ml and one extreme value of 7.97 ng/ml that was excluded from analyses. Average standard reference curve error was 13% at the midpoint and 29% at the low end. The data were not normally distributed (Shapiro-Wilk, $P < 0.0001$) at both sites and variation among the Atlantic lamprey data ($\sigma^2 = 1.20$) was more than double that of the Great Lakes site ($\sigma^2 = 0.55$). Treatment subset groupings of data did favor some parametric comparison tests, but non-parametric tests were necessarily used in most cases.

The 14 interval comparison tests showed three significant differences between the two sample times (8 and 24 h). This proportionally small number of differences was believed to not represent a true interval difference and led to the pooling of these two time intervals for further analyses. There was also no evidence of dose response differences within Great Lakes-GnRH I ($P = 0.82$), Great Lakes-GnRH III ($P = 0.58$), Atlantic-GnRH I ($P = 0.11$), and Atlantic-GnRH III ($P = 0.19$) groups. Additionally, no significant difference in 15α-T concentrations was found between lampreys injected with GnRH I or GnRH III at the Great Lakes site ($P = 0.93$), nor at the Atlantic Site ($P = 0.16$).

Only two of seven GnRH form and dosage specific treatment comparisons differed between the two sites. The GnRH III, 50 μg/kg treatment differed between sites (W, $P = 0.018$) where median 15α-T plasma concentrations were 2.04 ng/ml at the Atlantic site and 1.26 ng/ml at the Great Lakes site. The control groups (saline-injected) also differed (W, $P = 0.006$) where median 15α-T plasma concentrations for Atlantic sea lampreys

were 0.44 ng/ml and 0.29 ng/ml for Great Lakes sea lampreys. The ratios of 15α-T concentrations in Great Lakes versus Atlantic sea lampreys were similar for both GnRH-injected lampreys (1:1.31) and control group lampreys (1:1.52).

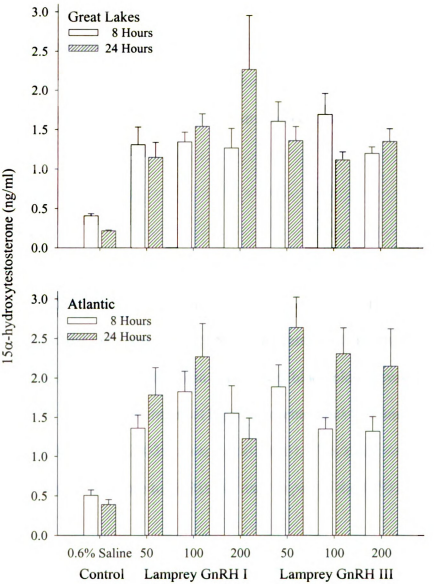

Figure 27: Differences in 15α-hydroxytestosterone blood plasma concentrations for male sea lampreys per site, GnRH form, dosage, and time interval. The X-axis labels indicate the GnRH form and dosage in μg/kg of lamprey body weight. Bars represent mean treatment values and error bars show mean standard error.

The effect of GnRH injections (regardless of dose or form) was evaluated by comparing 15α-T concentrations between all GnRH-injected lampreys of each site to their corresponding saline-injected control group. Because the Great Lakes control group showed some differences between time intervals, the comparisons were made per interval and with combined interval data. Large differences in sample size from pooling data led to GnRH-specific and dosage-specific comparisons. At both sites and for every level of classification (interval, dose, and GnRH form), GnRH-injected lampreys always had significantly higher (K-W, $P < 0.0001$) plasma concentrations of 15α-T than control groups (Figure 27).

Discussion

The results showed that both GnRH I and GnRH III elicited increases in 15α-T production and release in the blood plasma of adult male sea lampreys. GnRH-injected sea lampreys had 2 to 5 times greater 15α-T concentrations than their respective controls. The identity and presence of 15α-T in sea lamprey blood plasma was only recently confirmed and shown to be present at higher concentrations than immunoreactive testosterone (Bryan et al. 2003; Lowartz et al. 2003). This study now shows that 15α-T has a physiological response to GnRH and may be part of the HPG axis as induced by GTH.

The response of 15α-T further adds to the growing understanding of the unique endocrine system of the sea lamprey. To date, research has demonstrated the effects of hypothalamus and pituitary hormones on 17β-estradiol and progesterone production in

sea lampreys (Sower and Kawauchi 2001). GnRH I and GnRH III directly stimulate the pituitary and increase steroidogenesis (Gazourian et al. 2000). *In vitro* receptor localization studies have identified areas of the pituitary with an affinity for GnRH I and GnRH III (Knox et al. 1994) and estrogen receptor sites in the testis (Ho et al. 1987). In male sea lampreys, immunoreactive 17β-estradiol (E_2) has been found in blood plasma (Sower et al. 1983; Sower 1989; Katz et al. 1982), whose concentrations increase in response to injections of lamprey GnRH I, GnRH III, and GnRH analogs (Sower et al. 1983, 1985b, 1993; Sower 1989; Deragon and Sower 1994; Gazourian et al. 1997). Additionally, receptors for E_2 have been found in the testis (Ho et al. 1987). Concentrations of 15α-T showed the same proportional (injected versus control) increased responses to GnRH I and GnRH III as E_2 responses have shown (Young, unpubl. data). Expected receptor gene sequences for androgens were not found in the testis of lamprey (Thornton 2001), but the author indicated that does not exclude the possibility that very different receptor sequences exist for androgens, including 15α-T, in sea lampreys. Finding this response of 15α-T now requires further research to reveal whether it, like E_2, has receptor binding sites in the testis, thus supporting the hypothesis that C19 steroids may have an androgenic role in the sexual development and maturation processes of sea lampreys.

A possible explanation for the absence of change in 15α-T concentrations between the 8 h and 24 h sampling times may be that the duration selected was too short to observe the progression of increase and was not long enough to observe the pending decrease. Previous studies have shown that final maturation was accelerated by one or more

injections of GnRH (Sower 1989). The first injection is believed to activate the system, making it more sensitive to further injections. Sower (1989) shows how a single GnRH injection maintains the concentration of E_2 and progesterone (P) after 24 h, whereas after two successive GnRH injections, concentrations began to decrease after 24 h. The results from this study appear to indicate that for 15α-T responses, the two successive GnRH injections did not push the lampreys past the initial activation stage, thus the samples at the 8 h and 24 h sample times showed no differences in concentration.

The absence of dose-dependent responses indicates that the minimum GnRH doses used in this study were sufficient to induce the maximum 15α-T response in sea lamprey. Deragon and Sower (1994), using four successive injections, found E_2 concentrations to increase within 4 h, yet found no difference in responses between 100 and 200 μg/kg doses. Gazourian et al. (1997), using four successive injections, found that concentrations of both E_2 and P rose after 4 h and began to descend after 24 h. Gazourian et al. (2000), using a single injection and doses of 50 and 100 μg/kg, found similar elevated E_2 and P responses after 4 h, but a less pronounced decline after 24 h and that the 50 μg/kg dosage elicited lower responses in some instances. All studies found that higher temperatures generally produced higher concentrations of steroids. Based on the experimental design of these previous studies, the injection regime, sampling intervals, and temperature used in this study were expected to elicit differential 15α-T concentration responses between intervals and among doses. The fact that differences were not seen in these treatments as were seen for E_2 and P in other studies, suggests that the production of 15α-T may be regulated differently than the production of estrogens

and progestogens.

Within the range of doses used in this study, lamprey GnRH I and III were equipotent in increasing concentrations of 15α-T. Sower et al. (1993) first identified GnRH III and found that it elicited E_2 responses similar to those of GnRH I. Other studies have also shown that GnRH I and GnRH III are equally effective in elevating E_2 and P concentrations in sea lamprey (Sower 1998). Gazourian et al. (1997) showed that GnRH I and GnRH III were equipotent in stimulating steroidogenesis and inducing ovulation in females. Only Deragon and Sower (1994) found different responses to the two GnRH forms where GnRH III in males was more potent than GnRH I in its ability to induce spermiation and increase the concentrations of E_2 and P. The elevated concentrations of 15α-T seen in response to both GnRH I and GnRH III indicate that, at the doses examined, the different GnRH forms appear to have equal effects on this steroid and are similar to the responses of estrogens and progestogens.

The two GnRH form and dose-specific comparisons that differed between sites are not believed to represent an overall between-site difference. There was less than 39% difference in median plasma concentrations between sites for both of these two comparisons. This is low when compared to the over 72% difference in median plasma concentration between GnRH-treated lampreys and their respective controls. Although lamprey size and environmental conditions do differ between sites, the majority of the data support the conclusion that there is minimal if any difference in 15α-T plasma concentrations between the two sites.

The 15α-hydroxylated form of testosterone found in sea lampreys is unconventional and may be indicative of more specialized functions necessary in this ancient vertebrate. There have been 15α-hydroxylated steroids found in other organisms, but not as sex hormones (Levy et al. 1965; Giannopoulos et al. 1970; Brown et al. 1979). A possible explanation for the use of 15α-T by lampreys is the assumption that a hematophagous parasite needs different forms of hormones than its hosts to avoid interaction with exogenous hormones. However, because sea lamprey only have a short parasitic phase while the majority of their life cycle is spent as larvae with immature gonads, the reason for needing hydroxylated testosterone and its specific functions are not yet known. Though the origins and function of 15α-T may be in question, its placement in the HPG axis and response to lamprey GnRH add significant information toward the further understanding of the reproductive endocrinology of this ancient fish.

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