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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 tridentatus*) 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. In vivo production was proven by demonstrating that HPLC-fractionated plasma had peaks of immunoreactive 15α-T and 15α-P that co-eluted with standards through using previously developed radioimmunoassays for 15α-T and 15α-P. The possible functionality of 15α-T and 15α-P was 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 appeared 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.
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1. 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* (Bryan et al., 2003, 2004; Kime and Callard, 1982; 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; Bryan et al., 2004; Young et al., 2004a,b), and therefore may be functional hormones. In additional, 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 Callard, 1982; Kime and Rafter, 1981).

To properly interpret the significance of 15α-hydroxylated steroids, it must first be established in which species the unusual steroids are present. If 15α-hydroxylated steroids are only found in *Petromyzon* and *Lampetra* species,

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these steroids may represent a derived trait that evolved one or more times within the northern lamprey lineage. However, if 15α-hydroxylated steroids are common to all northern lamprey species, it suggests that perhaps 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 (Hardistry and Potter, 1971).

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, 2004). The emphasis on sea lamprey 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 Northern 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 (Almeida et al., 2000; Maitland, 1980), 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., Baker, 2004; Thornton, 2001), it is important to determine whether the physiological traits being investigated are common to all lamprey species, or are specific to sea lampreys.

Our hypothesis is that 15α-hydroxylated steroids are produced in the testes and circulated in the plasma of all holarctic 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 plasma 15α-P and 15α-T levels change in response to injection of lamprey GnRH I or lamprey GnRH III in lamprey species other than *P. marinus*.

2. Methods

All chemicals were obtained from Sigma unless otherwise noted. Taxonomic names are as designated by Gill et al. (2003). Reference 15α-hydroxylated steroids were provided by Dr. Ivan Černý (Bryan et al., 2003, 2004).

2.1. Animals

For in vitro experiments and blood drawing, silver lampreys (*Ichthyomyzon unicuspis*), chestnut lampreys (*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 experiment using silver lampreys, the experiment was carried out in May 2003 at the Hammond Bay Biological Station. The silver lampreys had an average weight ± standard error of 274.7 ± 5.30 g and were held at 12 ± 1 °C in flow-through tanks. For Pacific lamprey (*Entosphenus tridentatus*), the experiment was carried out in February 2003 at the USGS—Columbia River Research Laboratory in Cook, WA. Pacific lampreys 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 at 12 ± 1 °C in flow-through tanks, 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).

2.2. 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 (1,2,6,7-^{3}H[N])-progesterone or (1,2,6,7-^{3}H[N])-testosterone 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 and the media were 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 known elution points of 15α-hydroxylated and precursor standards, using 10 μg of standard in adjacent HPLC runs. To prevent contamination between HPLC runs, the column was flushed with solvent B (70% acetonitrile, 0.01% TFA; Bryan et al., 2003). Percent conversion to a given product was calculated by dividing the dpm found to co-elute 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 co-elution with 15α-P and 15α-T on HPLC, fractions were tentatively identified. From these fractions, an amount equivalent to 5000 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.

2.3. 15α-Hydroxylated steroid immunoreactivity in plasma

This experiment used spermiating male silver, chestnut, American brook, and Pacific lampreys (*E. 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 23 gauge heparinized syringes. The blood was held on ice 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).

2.4. GnRH experiments

There were three treatment groups with 16 lampreys per group. An initial blood sample was taken as described above 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 hormones synthesized by Bachem Peptide, King of Prussia, PA) in a dose of 100 μg/kg, or 0.9% saline for control animals. Injections were given using 25 gauge needles and total injection volume was 0.1 ml/50 g body weight. 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, paired *t* tests were used to compare plasma levels before and after injection for each treatment group.

3. Results

3.1. In vitro incubations

See Table 1 for conversion rates. All species synthesized 15α-P and 15α-T from [^{3}H]-P and [^{3}H]-T, as determined by co-elution on both HPLC (Figs. 1 and 2) and TLC. However, all species examined also converted [^{3}H]-P into an unknown product that eluted at 32 min 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 (46.1% conversion), 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 (58.9% conversion).

Male chestnut lampreys converted P into 15α-P (47.6% conversion), 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 (69.3% conversion).

Male American brook lampreys converted P to 15α-P (30.4% conversion), but there was more conversion (34.1%) to a product that eluted at 32–33 min on HPLC. T was converted to 15α-T (34.9% conversion), and was also converted (17.4%) to a product that eluted at 34 min on HPLC.

3.2. 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. 3).

For silver lampreys, immunoreactivity in the elution position of 15α-P was 31.6% of the total immunoreactivity. Immunoreactivity in the elution position of 15α-T was 66.9% of the total immunoreactivity.

For male chestnut lamprey plasma, the largest amount of immunoreactivity to 15α-P co-eluted with 15α-P. Immunoreactivity in the elution position of 15α-P was 34.1% of the total ir-15α-P. Immunoreactivity in the elution position of 15α-T was 96.3% of the total ir-15α-T, with the only peak of immunoreactivity corresponding to the elution time of 15α-T.

For male American brook lampreys, immunoreactivity in the elution position of 15α-P was 36.9% of the total

Table 1
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 co-elutes with 15α-P or 15α-T on high performance liquid chromatography (HPLC).

[b] Whether the in vitro product that co-elutes with standard on HPLC also co-elutes with standard on thin layer chromatography (TLC).

[c] Whether there is a peak of immunoreactivity in plasma extract that co-elutes 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.

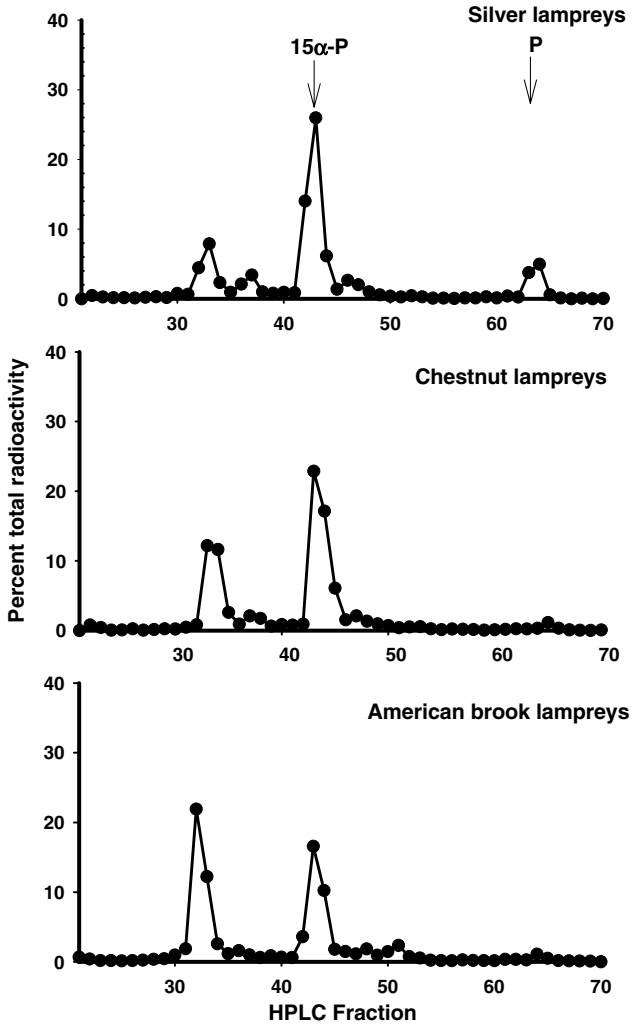

Fig. 1. In vitro products of metabolism of tritiated progesterone (P) by testicular tissue of silver lampreys, chestnut lampreys, and American brook lampreys. The *x*-axis is HPLC fraction and the *y*-axis is percent of total radioactivity. Arrows indicate the elution points of 15α-hydroxyprogesterone (15α-P) and P.

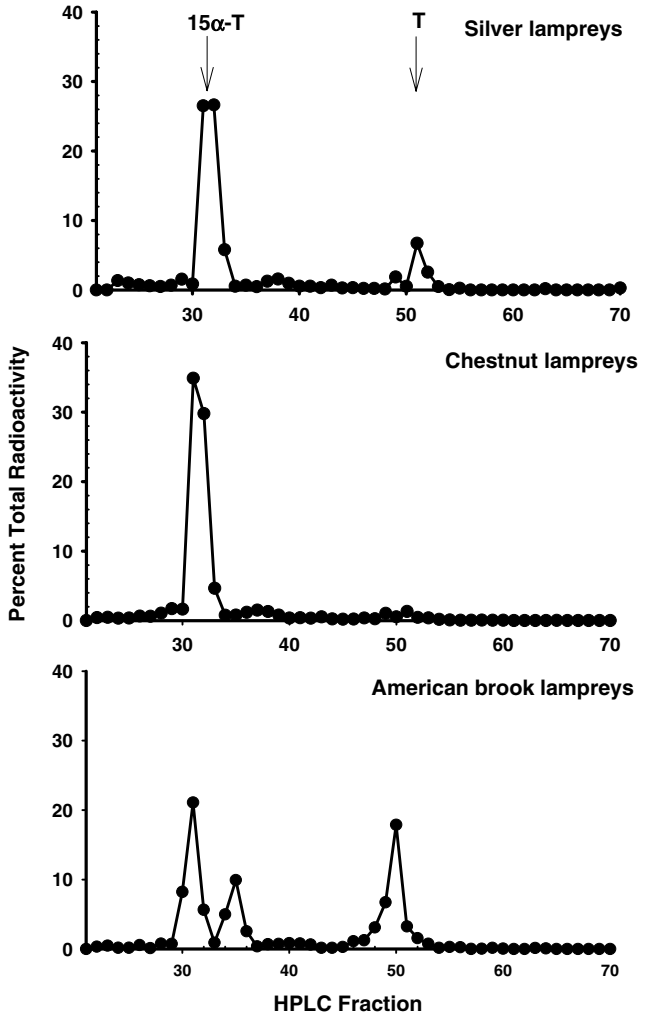

Fig. 2. In vitro products of metabolism of tritiated testosterone (T) by testicular tissue of silver lampreys, chestnut lampreys, and American brook lampreys. The *x*-axis is HPLC fraction and the *y*-axis is percent of total radioactivity. Arrows indicate the elution points of 15α-hydroxytestosterone (15α-T) and T.

immunoreactivity. A second large peak of immunoreactivity co-eluted with progesterone (25.2%). Immunoreactivity in the elution position of 15α-T was 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 21.7% of the total immunoreactivity. Immunoreactivity in the elution position of 15α-T was 53% of the total ir-15α-T. Levels of 15α-T in the fractionated plasma were very low, and other peaks were likely artifacts of the RIA.

3.3. GnRH experiments

For male silver lampreys, the ANOVA did not detect any significant differences among groups ($P > 0.05$). 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. 4).

4. 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 holarctic lampreys, *Ichthyomyzon* (Potter, 1980), it is likely that the common ancestor of the species examined in this study also produced 15α-hydroxylated steroids. However, from this research, it appears likely that there are differences in production and circulation of 15α-hydroxylated steroids among species, which may be indicative of functional endocrine differences among lamprey species.

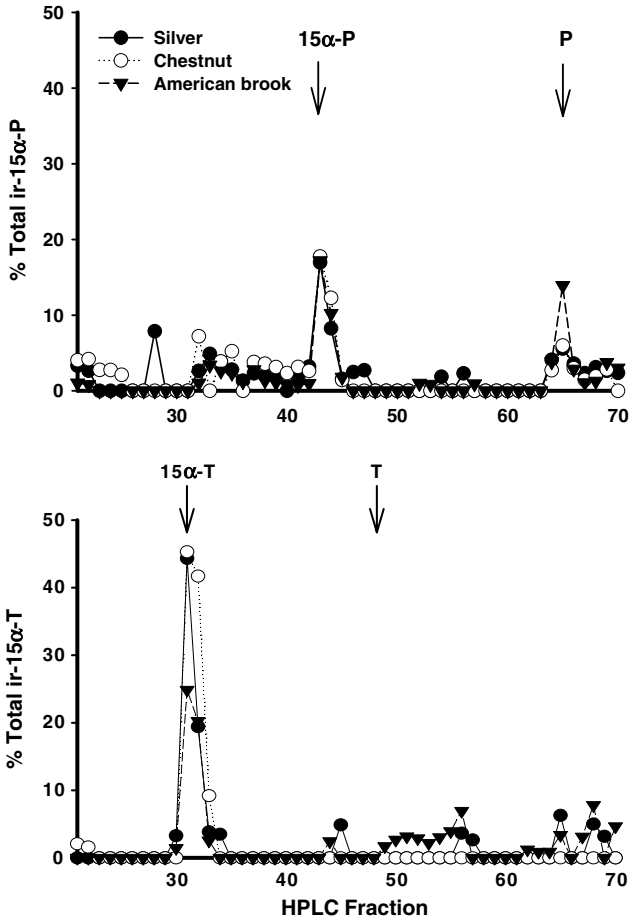

Fig. 3. Immunoreactivity (ir) detected in HPLC fractions of extracted pooled plasma. The *x*-axis is HPLC fraction and the *y*-axis is percent of total immunoreactivity. The upper graph shows ir-15α-hydroxyprogesterone (15α-P) in pooled plasma obtained from male silver, chestnut, American brook, and 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).

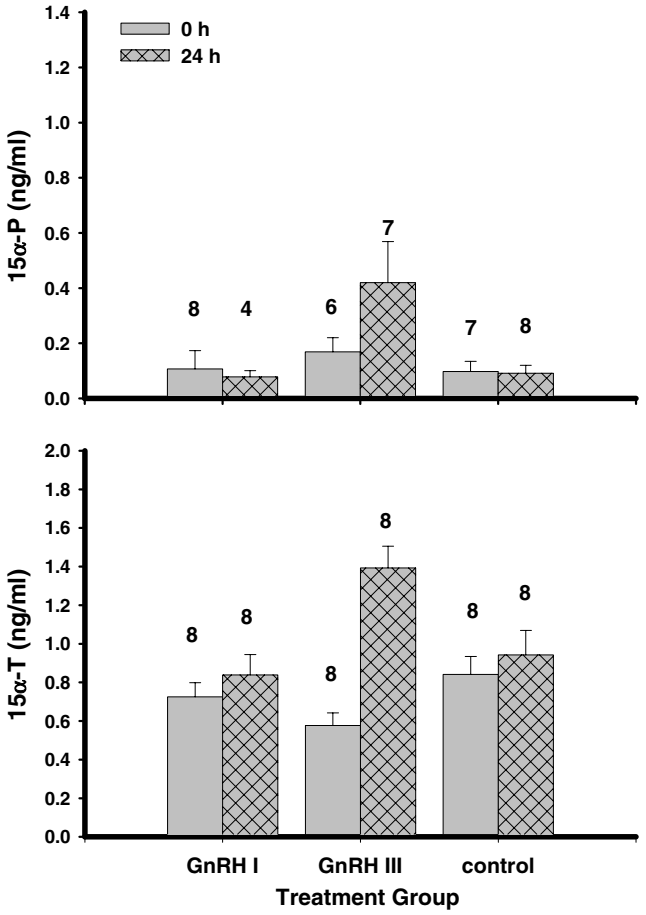

Fig. 4. Results of GnRH-injection experiment using male Pacific lampreys. Pacific lampreys were injected with GnRH I or GnRH III (100 μg/kg) or saline. Blood was sampled prior to and 24 h after injection, and was assayed for 15α-P (upper graph) and 15α-T (lower graph), with the number of animals (*n*) indicated above each bar. Injection with GnRH III resulted in significantly higher 15α-T plasma levels ($P < 0.05$) and a trend toward higher plasma levels of 15α-P ($P = 0.07$). Injection with GnRH I did not cause a significant change in plasma levels of either steroid.

All species examined formed 15α-P and 15α-T in vitro, and therefore it is suggested that the gonads of the species examined express 15α-hydroxylase enzyme(s) 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 in the species examined 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 (Bryan et al., 2004), 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. Metabolism of progesterone by lampreys has been shown to yield unidentified (unusual) products in several other studies (Kime and Rafter, 1981; Weisbart and Youson, 1975, 1977).

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, *L. fluviatilis* (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.

It is possible that the temperature at which the incubations were conducted had an effect on steroid conversion by affecting the concentrations and efficiencies of the steroidogenic enzymes. The optimum spawning temperature has not been determined for each of these species, but is thought to be an average of 18 °C (Manion and Hanson, 1980), although spawning for some species has been observed over a wide range (Pacific lamprey, 10–15 °C, Close et al., 2002; sea lamprey, 10–23 °C, Hanson and Manion, 1978). Only a few "point" observations are available for chestnut (16.5 °C, Case, 1970), silver (18.2 °C, Cochran and Lyons, 2004), and American brook lampreys (12.5 °C, Seagle and Nagel, 1982). Since all of the lampreys were acclimated to 12 °C and this temperature is near or within the range of spawning temperatures for all species involved, it most likely did not affect the results of the in vitro experiments.

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 co-elute with 15α-T, which elutes at 30 min, and that after incubating tissues with [^{3}H]-P for 4 h, we did not find evidence of T or 15α-T production using progesterone as a precursor.

Plasma concentrations of 15α-hydroxylated steroids were measured in response to injection with GnRH as a way to gauge possible functionality within the hypothalamal–pituitary–gonadal axis. Increased levels of endogenous GnRH have been correlated with spermiation and ovulation and increased levels of sex steroids (Sower, 2003). However, the direct relationship between 15α-hydroxylated sex steroids, or any sex steroids, and gonadal maturation has not yet been studied in lampreys.

The plasma concentrations of 15α-hydroxylated steroids responded differently to injections of GnRH in different species. There are several possible reasons for these differences. First, the injection and sampling scheme used for silver and Pacific lamprey was different than that used on sea lamprey (Bryan et al., 2004; Young et al., 2004a). Sea lampreys were given two serial injections, 24 h apart, and blood was sampled 8 and 24 h after the second injection. While 15α-T circulatory levels were elevated at both the 8 and 24 h sampling times (Young et al., 2004a), 15α-P circulatory levels were elevated at 8 h and declined significantly at 24 h, but were still above baseline levels (Bryan et al., 2004). In sea lampreys, it has been demonstrated that a single injection of either type of GnRH is sufficient to elevate plasma levels of P and E_2, although whether P plasma concentrations remained elevated at 24 h post-injection differed by study (Deragon and Sower, 1994; Gazourian et al., 1997, 2000; Sower, 1989; Sower et al., 1993). 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 was 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 spermiating. The Pacific lamprey experiment used very immature adult males. It is not known how GnRH affects steroidogenesis in gonads at different maturation stages, but it is likely that differing amounts or types of steroids would be produced, and this would be dependent on the amounts and types of steroidogenic enzymes present. In addition, the results observed in Pacific lampreys may be indicative of functional differences between the two types of GnRH (Sower, 2003).

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 6 months and a year prior to spawning, and overwinter in a fasting state in streams (Beamish, 1980; Close et al., 2002). This change in life cycle might affect the timing of physiological events related to reproduction, and may therefore be indicative of 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 (Silver et al., 2004), 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 levels of GnRH III increase in the brain during the reproductive season, while levels of GnRH I remain the same (Sower, 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).

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 phylogenetics tree (Gill et al., 2003). Most studies agree that *Ichthyomyzon* is the ancestral genus of all holarctic lampreys, and *I. unicuspis* (silver lamprey) is the extant ancestral species. The genus *Petromyzon*, which contains only one species, *P. marinus*, is derived from *Ichthyomyzon*, and the two genera are monophyletic in origin (Gill et al., 2003). Phylogenies based on dentition (Hubbs and Potter, 1971; Potter, 1980) divided genus *Lampetra* 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 and *Lampetra* is the most derived. *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 holarctic lampreys, since this enzyme is found in representatives of the most ancestral, most derived, and an intermediate genera within the Petromyzontidae family.

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 C7 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 determine the functions of hydroxylated steroids in Agnathans, and to discern the reasons for the existence of hydroxylated steroids in early vertebrates.

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