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Contribution of Chicken GnRH-II and Lamprey GnRH-III on Gonadotropin Secretion

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1. Introduction

Proper gonadal function in mammals depends on gonadotropins secreted from the pituitary gland in a pulsatile manner. The hypothalamus, in turn, controls the secretion of gonadotropins by the pulsatile secretion of gonadotropin releasing hormone (GnRH) into the portal circulation of the pituitary. The idea of brain control over the hypophysis was first postulated by Geoffrey Harris during the late 1940s and early 1950s (Harris, 1948). The evidence available at that time indicated that stimulation of the central nervous system, but not direct stimulation of the pars distalis or neural lobe, caused the release of adenohypophysial hormones. During the 1950s and 1960s, trophic substances were extracted from the brain of different species (Guillemin, 2005). These releasing factors obtained from the median eminence affected secretions of the pars distalis. The search for these releasing factors was lead by McCann, Schally and Guillemin (Guillemin and Rosenberg, 1955; McCann and Fruit, 1957; Rumsfeld and Porter, 1962; Saffran et al., 1955).

Andrew Schally and his team isolated and synthesized for the first time the decapeptide GnRH, after the extraction of more than 250,000 pig hypothalami (Matsuo et al., 1971; Wade, 1978). Initially Schally and coworkers postulated that one hypothalamic hormone LH-RH/FSH-RH or simply GnRH controls the secretion of both LH and FSH from the pituitary gland (Schally et al., 1971). The terms luteinizing hormone-releasing hormone, and gonadotropin-releasing hormone have been widely adopted, and most journals allow the use of both names and abbreviations (Schally, 2000). In addition to GnRH, the occurrence of other two GnRH isoforms was first reported in chickens more than 10 years after of Dr. Schally's initial report (King and Millar, 1982; Miyamoto et al., 1982; Miyamoto et al., 1984). To date, several structural variants of GnRH have been identified in diverse vertebrates (Barran et al., 2005; Millar et al., 2004). These isoforms have various functions, including paracrine, autocrine, neuroendocrine, and neurotransmitter/neuromodulatory roles in the

central and peripheral nervous systems (King and Millar, 1995; Millar and King, 1987; Sealfon et al., 1997; Skinner et al., 2009).

	1	2	3	4	5	6	7	8	9	10
Mammal (mGnRH)	p	Glu	-His	-Trp	-Ser	Tyr	Gly	-Leu	-Arg	-Pro-Gly
Chicken-I (cGnRH-I)	p	Glu	-His	-Trp	-Ser	Tyr	Gly	-Leu	-Gln	-Pro-Gly
Chicken-II (cGnRH-II)	p	Glu	-His	-Trp	-Ser	-His	Gly	-Trp	-Tyr	-Pro-Gly
Lamprey-III (IGnRH-III)	p	Glu	-His	-Trp	-Ser	-His	-Asp	-Trp	-Lys	-Pro-Gly

Table 1. Amino acid (AA) sequence of GnRH isoforms. The bolded regions represent the conserved NH₃- and COOH- terminal residues. The numbers represent the relative position of each AA in the GnRH peptide (1 represents the N-terminal AA)

The nomenclature used to distinguish different GnRH isoforms between mammalian and non-mammalian species have been described using a variety of phylogenic and genomic synteny analyses (Kim et al., 2011; Millar et al., 2004; Roch et al., 2011; Tostivint, 2011). For the purpose of this book chapter, we adopted the nomenclature based on the species in which they were first discovered, depicted in Table 1, and described elsewhere (Millar et al., 2004).

In addition to the classically described mammalian form of GnRH (for extensive review see (Barb et al., 2001; Clarke, 2002; Esbenshade et al., 1990; Kaiser et al., 1997; McCann et al., 2002; Millar, 2005; Millar et al., 2008)), chicken GnRH-II (cGnRH-II) and lamprey GnRH-III (IGnRH-III) are of particular significance because they may coordinate the control of LH and FSH secretion in some vertebrate species.

2. GnRH receptors

The coordination of gonadotropin secretion is also modulated by the interaction of the GnRH peptide with its receptors. The GnRH receptor (GnRHR) has the characteristic feature of a classical seven-transmembrane G-protein-coupled receptor (Millar, 2003, 2005; Neill, 2002). Four vertebrate GnRHR lineages have been proposed using genome synteny and phylogenic analyses; nonmammalian type I, nonmammalian type II, nonmammalian type III/mammalian type II, and mammalian type I (Kim et al., 2011). For the purpose of this book chapter, we refer to the type I and type II GnRHRs as the mammalian type I and mammalian type II GnRH receptor, respectively.

There is evidence that in the rhesus monkey (*Macaca mulatta*) and the marmoset (*Callithrix jacchus*), the type I GnRHR has high affinity for mGnRH and lower affinity for cGnRH-II, and the type II GnRHR has high affinity for cGnRH-II and lower affinity for mGnRH (Millar et al., 2001; Neill, 2002). In fact, when COS-1 or COS-7 cells were transfected with the type II GnRHR, the potency was high for cGnRH-II and low for mGnRH. Nonetheless, in vivo and in vitro work in rhesus monkey (Densmore and Urbanski, 2003; Okada et al., 2003) and pigs

(Neill et al., 2002), using specific type I GnRHR antagonists (Antide and Cetrorelix) suggest that cGnRH-II can also stimulate gonadotropin secretion via the type I GnRHR (Neill et al., 2004).

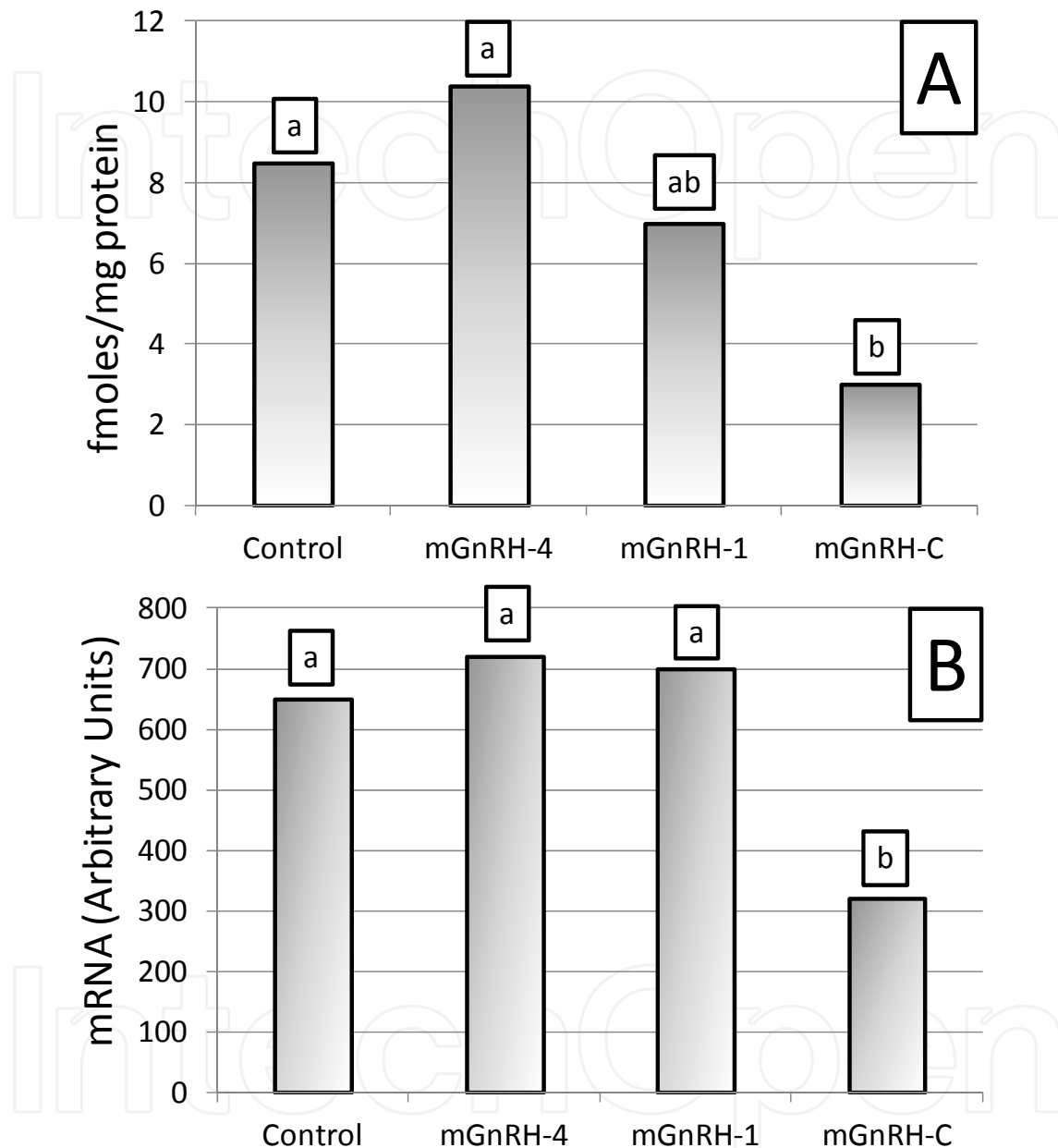


Figure 1. Concentrations of the type I GnRHR (A) and expression of the mRNA for the type I GnRHR (B) in the pituitary gland of anestrous cows that were treated for 13 days with 2 μ g of mGnRH infused (i.v.) continuously during 1 h (mGnRH-C), during 5 min once every hour (mGnRH-1), or during 5 min once every fourth hour (mGnRH-4) or with saline (control). Different letters indicate significant differences ($P < 0.1$ for A and $P < 0.05$ for B). Adapted from (Vizcarra et al., 1997).

In the pig, as well as other mammals, type I GnRHRs are characterized by the absence of a carboxyl-terminal tail (Kakar et al., 1992; Millar et al., 2004; Neill et al., 2004; Tsutsumi et al., 1992; Weesner and Matteri, 1994). The tail-less type I GnRHR is associated with a resistance

to rapid desensitization and ligand-induced internalization (Blomenrohr et al., 1999). When COS-1 cells expressing the type I receptor were incubated with a maximal dose of a mGnRH agonist, [³H]-inositol phosphate (IP) accumulated for 90 min indicating the failure of mGnRH desensitization during the experimental period (Neill, 2002). From a practical standpoint, we have demonstrated that administration of mGnRH at different frequencies differentially regulates the concentrations and the expression of the type I GnRHR (Vizcarra et al., 1997). Concentrations and expression of the type I GnRHR were reduced when mGnRH was infused continuously compared with those in control cows (Figure 1). However, when mGnRH was given as a pulse every hour or every fourth hour, concentration and expression of the type I GnRHR were not different from those in control cows. Our data indicates that pulsatile mGnRH does not influence concentrations of the type I GnRHR or type I GnRHR mRNA, but continuous infusion of mGnRH dramatically reduces the concentrations and expression of the type I GnRHR in the pituitary gland (Vizcarra et al., 1997).

The type II GnRHR has only 41% identity with the type I receptor and in contrast to the type I GnRHR, the type II receptor has a C-terminal cytoplasmic tail that is important for cell surface expression and agonist binding. The type II GnRHR has been cloned in several vertebrate species; whereas the type I GnRHR has been identified only in mammals (Kim et al., 2011). The tail of the type II GnRHR is phosphorylated upon agonist-binding followed by internalization and desensitization of the receptor (Blomenrohr et al., 1999). As with the type I receptor, the type II couples to the Gq α protein and, consequently, mediates the intracellular production of inositol trisphosphate (Cabrera-Vera et al., 2003; Millar and Newton, 2010). In contrast to the type I GnRHR, desensitization of the type II GnRHR in rhesus monkeys takes about 60 minutes, reflecting the properties of the cytoplasmic tail (Neill, 2002).

3. Chicken GnRH-II

Phylogenetic evidence indicates that cGnRH-II (initially isolated from the chicken brain) is an ancient form of GnRH that has been structurally conserved for over 100 million years of evolution, suggesting that its neural functions may have an important significance (Powell et al., 1994; Rastogi et al., 1998).

In birds, two forms of GnRH (cGnRH-I and cGnRH-II; Table 1) have been reported (King and Millar, 1982; Miyamoto et al., 1982; Miyamoto et al., 1984) and only indirect measurements of the GnRH pulse generator is available by measuring plasma LH concentrations in frequent samples or in pituitary extracts (Chou and Johnson, 1987; Sharp and Gow, 1983; Wilson and Sharp, 1975). In addition, we have reported the episodic nature of gonadotropin secretion in the mature fowl (Vizcarra et al., 2004). Gonadotropin secretion in chickens is characterized by a pulsatile pattern with LH pulses being more frequent and having greater amplitude than FSH pulses (Figure 2). Furthermore, we observed that there was a lack of synchrony between the episodic release of LH and FSH. Only 23% of the LH pulses were associated with FSH episodes, suggesting that in the adult male fowl LH and FSH secretion are regulated independently (Vizcarra et al., 2004).

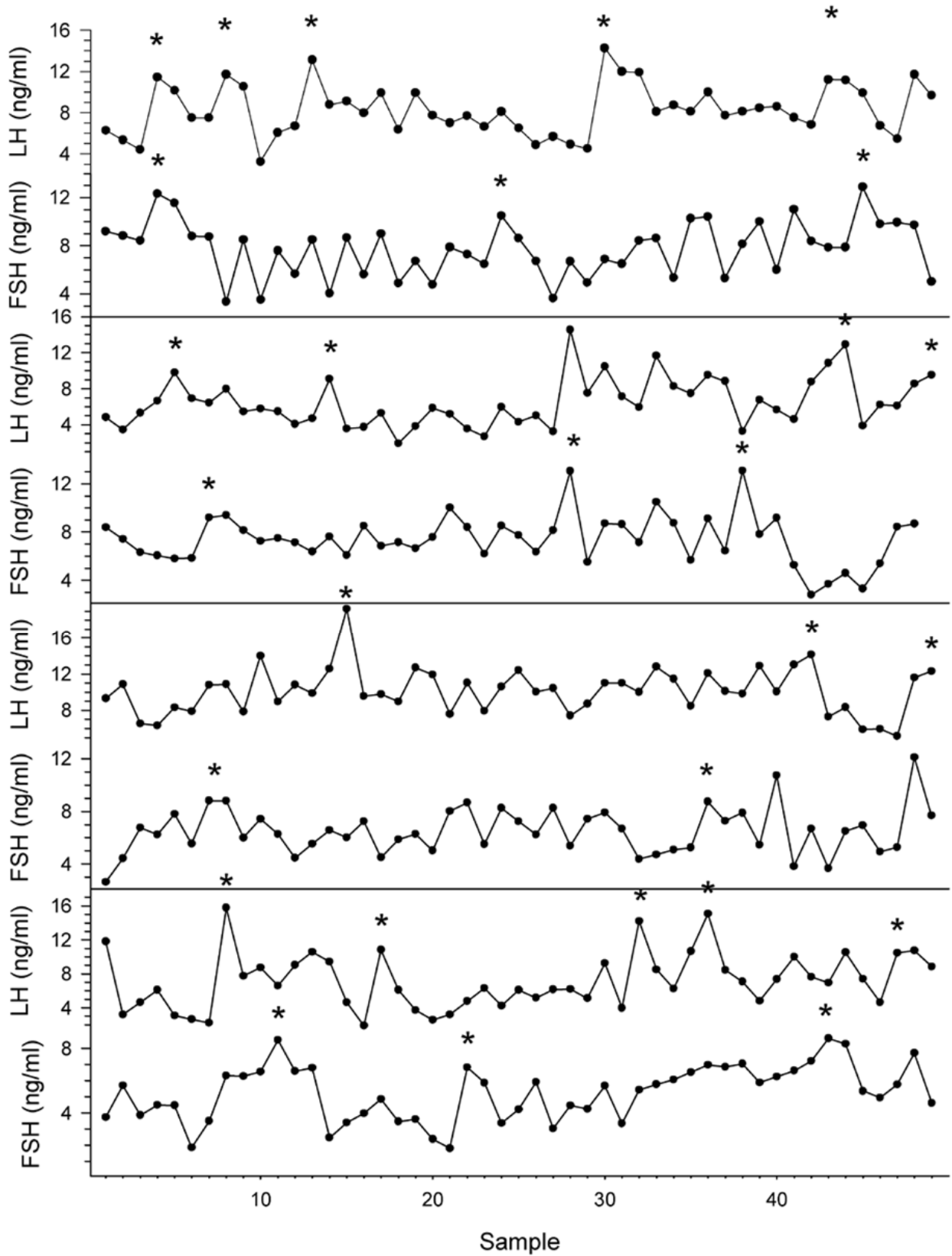


Figure 2. Pulsatile secretion of LH and FSH in plasma of four birds. Blood samples were obtained every 10-min for 8 h. Asterisks indicate the presence of a pulse of LH or FSH, as determined by Pulsar. Adapted from (Vizcarra et al., 2004)

Both cGnRH-I and -II stimulates gonadotropin release *in vivo* and *in vitro* in the chicken (Hattori et al., 1986). However, cGnRH-II was not found in the median eminence of the white-crowned sparrow (*Zonotrichia leucophrys gambelii*), suggesting that in these species cGnRH-II does not regulate pituitary gonadotropin secretion (Meddle et al., 2006). Although concentrations of FSH in small cockerels were not affected by cGnRH-I challenge (Krishnan et al., 1993), most of the evidence indicates that cGnRH-I is the prime regulator of gonadotropin release in chickens (Katz et al., 1990; Sharp et al., 1990). Active immunization against cGnRH-I but not against cGnRH-II was associated with decreased concentration of LH in laying hens (Sharp et al., 1990). We also evaluated the effect of active immunization against cGnRH-I and cGnRH-II in adult broiler breeder males (Vizcarra et al., 2000). At 10 weeks of age, males (10 per treatment), received a primary immunization against cGnRH-I, cGnRH-II, BSA, or were not immunized. Peptides were conjugated to BSA and emulsified in Freund's incomplete adjuvant and diethylaminoethyl-dextran. Booster immunizations were given at 3, 6 and 14 weeks after the primary immunization. Titers were increased in cGnRH-I but not in cGnRH-II treated birds compared with BSA immunized males (Figure 3). Concentrations of LH and FSH in frequent samples were not affected by treatment; however, testis weight was significantly decreased in cGnRH-I birds compared to the other treatments (Figure 4).

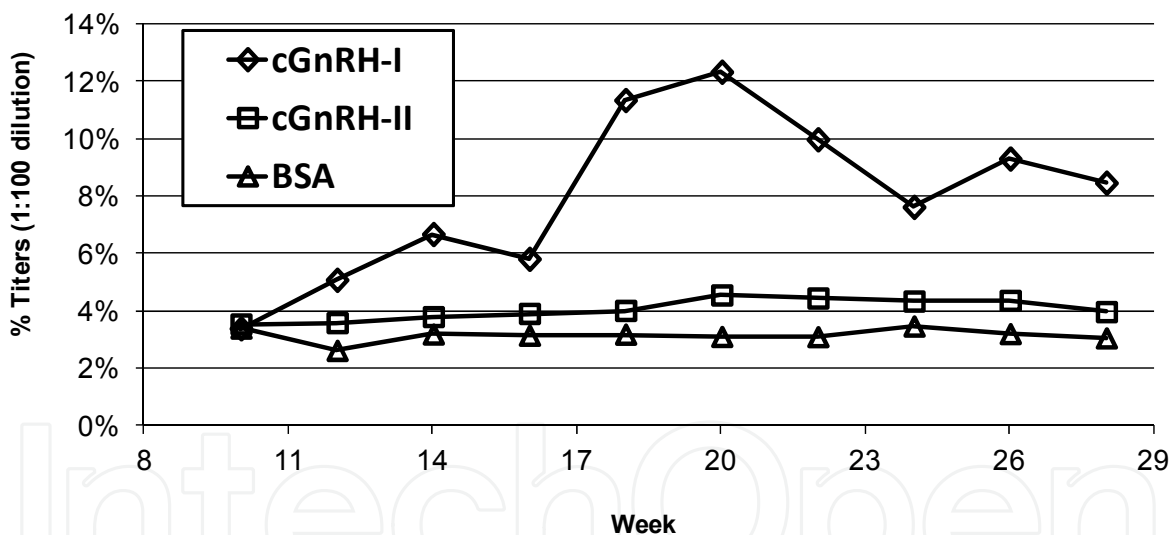


Figure 3. Antibody titers of male broiler breeders immunized against cGnRH-I, cGnRH-II, and BSA. Titers were increased ($P < 0.05$) in cGnRH-I but not in cGnRH-II treated birds compared with BSA immunized males.

There is evidence of a behavioral role attributed to cGnRH-II in birds that may be independent from cGnRH-I. Intracerebroventricular (ICV) infusion of cGnRH-II induced copulation solicitation in the female white-crowned sparrow, and social interactions in the house sparrow (*Passer domesticus*) may be regulated by cGnRH-II (Maney et al., 1997; Stevenson et al., 2008). In the mature male Zebra finch (*Taeniopygia guttata*) the number of cGnRH-II neurons is significantly reduced during the non-breeding season as compared with the breeding season (Perfito et al., 2011). A similar behavioral role of cGnRH-II has

been reported in mice (Kauffman and Rissman, 2004a). However, this information is questionable due to the lack of a functional cGnRH-II peptide and the lack of a functional type II GnRHR in mice (Stewart et al., 2009).

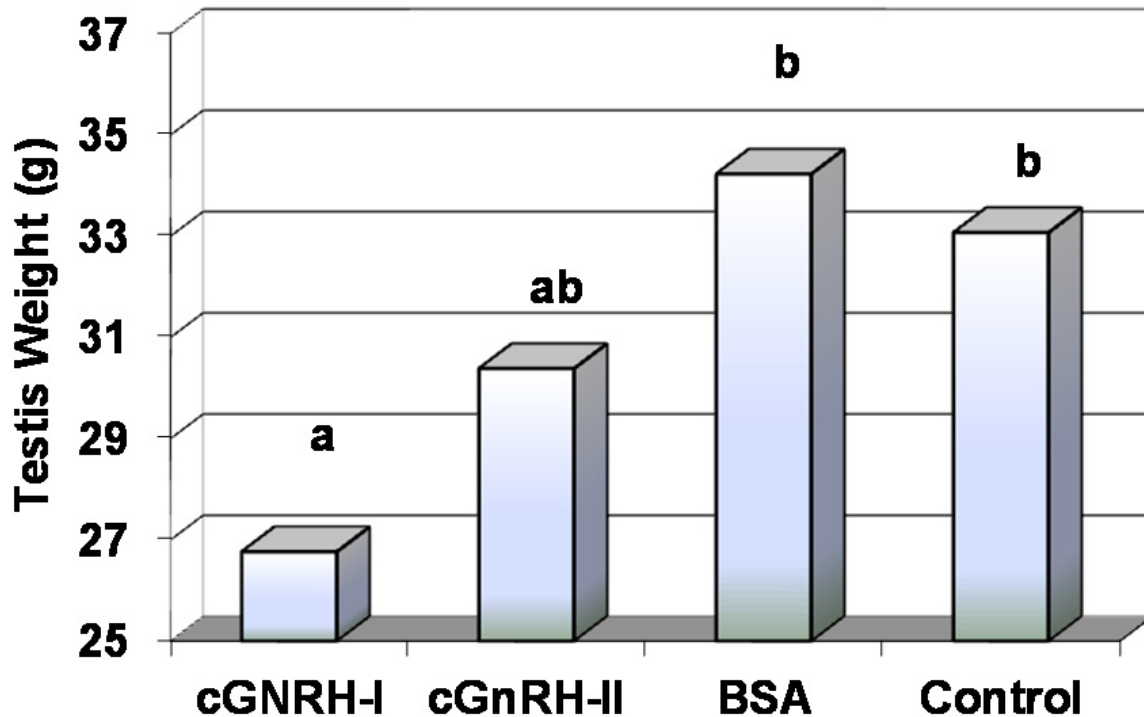


Figure 4. Testis weight of male broiler breeders immunized against cGnRH-I, cGnRH-II, BSA, and not immunized (Control) birds. Different letters indicate significant differences ($P < 0.05$)

Among primates, the rhesus monkey (*Macaca mulatta*), is one of the few species studied to date that possess a functional cGnRH-II peptide and type II GnRHR (Stewart et al., 2009). In these species, cGnRH-II is expressed in the hypothalamic median eminence (Urbanski et al., 1999), and has the ability to stimulate gonadotropin secretion (Lescheid et al., 1997). The rhesus hypothalamic cells that express mGnRH and cGnRH-II have a differential distribution pattern. In contrast to mGnRH, the axonal projections of cGnRH-II have a direct input in the neural lobe of the pituitary gland, raising the possibility that both forms of GnRH may play different physiological roles in the regulation of gonadotropin secretion (Urbanski et al., 1999). When cultured pituitary cells from male rhesus monkeys were incubated with cGnRH-II, LH and FSH were significantly increased. However, the *in vitro* effect of cGnRH-II on gonadotropin secretion was less potent than that of mGnRH (Okada et al., 2003). In contrast, *in vivo* exogenous doses of mGnRH and cGnRH-II in female rhesus monkeys were equally potent at stimulating LH release with little effect on FSH secretion (Densmore and Urbanski, 2003). In males and females rhesus monkeys, cGnRH-II mRNA expression in the mediobasal hypothalamus (MBH) significantly increased in adult animals compared with prepubertal macaques (Latimer et al., 2001). Since the MBH is associated with the pre-ovulatory LH surge and overall reproductive development (Spies et al., 1977), it is possible that cGnRH-II may play a role in the onset of puberty and sexual behavior.

Estrogen significantly increases cGnRH-II expression in the MBA (Densmore and Urbanski, 2004), while the same steroid significantly decreases mGnRH expression (Densmore and Urbanski, 2004; El Majdoubi et al., 1998). The positive and negative feedback mechanism of estrogen on the reproductive axis may be explained by the presence of the two GnRH isoforms present in the brain of the rhesus monkey. As noted above, few primate species are known to possess a functional cGnRH-II peptide and associated type II GnRH receptor. For instance, in the Chimpanzee (*Pan troglodytes*) the genes encoding the cGnRH-II peptide and the type II GnRH receptor contains a premature stop codon (Ikemoto and Park, 2006; Stewart et al., 2009); therefore there is a disruption of the ligand and the receptor. Information on regard to the GnRH-II system obtained in the rhesus monkey should not be generalized to other primate species.

Although human possesses a functional cGnRH-II peptide, the type II GnRH receptor is disrupted by a frame shift and premature stop codon (Pawson et al., 2005). The type II GnRHR gene remains active and an alternative splicing (GnRHR-II-reliquum) is expressed in gonadotropes that contains the type I GnRHR (Millar et al., 1999; Pawson et al., 2005). Simultaneous transfection of the type I GnRHR and GnRHR-II-reliquum into COS-7 cells resulted in reduced expression of the type I GnRHR, suggesting a modulator role of the GnRHR-II reliquum on the type I GnRH receptor (Pawson et al., 2005).

In the Musk shrew (*Suncus murinus*), cGnRH-II was identified by HPLC and radioimmunoassay (RIA), and the presence of a functional peptide subsequently reported (Dellovade et al., 1993; Rissman and Li, 1998; Stewart et al., 2009). Although there is evidence that the type II GnRHR may mediate behavioral effects of cGnRH-II in the Musk shrew (Kauffman et al., 2005), a functional type II GnRHR has not been reported (Stewart et al., 2009). Nevertheless, ICV infusion of cGnRH-II but not mGnRH stimulated sexual behavior in nutritionally challenged female musk shrews (Temple et al., 2003). When musk shrews were exposed to different levels of caloric intake, cGnRH-II mRNA expression was modulated by feed intake (Kauffman et al., 2006; Kauffman and Rissman, 2004b). These data suggest a role of GnRH-II in both feeding and sexual behavior.

Among domestic animals, the pig is the only relevant livestock species that expresses both a functional mGnRH and cGnRH-II peptide and the associated cognate functional type I and type II GnRHR (Stewart et al., 2009). In bovine and ovine species the cGnRH-II peptide and the type II GnRHR receptor are functionally inactivated (Morgan et al., 2006) and in equine species, the type II GnRHR is functionally inactivated (Stewart et al., 2009).

Very little information on the effect of cGnRH-II on gonadotropin secretion is available in pigs. Treatment of pig pituitary cells with nanomolar concentrations of cGnRH-II consistently stimulated a 15-20 fold increase in LH secretion, while FSH secretion was more variable, ranging from none to a 4-fold stimulation (Neill et al., 2002).

We conducted studies to evaluate the effect of active immunization against cGnRH-II on gonadotropin secretion and testicular function in boars (Bowen et al., 2006). A synthetic cGnRH-II peptide, where the common pGlu-His-Trp-Ser sequence at the N-terminal was

suppressed (see table 1) and a Cys residue was incorporated, was used in the conjugation process. Antibody titers were detectable in GnRH-II immunized animals four weeks after primary immunization (bottom panel Figure 5). Titers continued to increase as booster immunizations were given with limited to no cross-reactivity between mGnRH and cGnRH-II. No mGnRH specific antibodies were detected in control animals. Antibody titers against mGnRH were measured to determine if cGnRH-II antibodies recognized mGnRH. None of the animals produced antibodies that recognized mGnRH. However, when a plasma sample from a cow previously immunized against mGnRH was used, antibody titers were significantly higher (Figure 5; inset upper panel). None of the animals produced antibodies that recognized mGnRH, indicating that animals immunized against cGnRH-II produced antibodies that recognized only their own specific amino acid sequence. Active immunization against cGnRH-II significantly decreased gonadotropin secretion when compared with control barrows (Figure 6). These data suggest that the two GnRHs and GnRHRs systems, along with differences in signaling pathways, provide the potential for differential gonadotropin secretion in pigs.

GnRH antagonists, of which thousands have been formulated, cause an immediate and rapid reversible suppression of gonadotropin secretion. The principal mechanism of action of GnRH antagonists is competitive receptor occupancy of GnRHRs (Herbst, 2003; Huirne and Lambalk, 2001). The first generation of mGnRH antagonists contained replacements for His at position 2 and for Trp at position 3 (Huirne and Lambalk, 2001). The inhibitory activity increased after incorporation of a D-amino acid at position 6, but increased histamine-releasing activity resulted in anaphylactic reactions. The third generation antagonists have low histamine-releasing potency by replacing the D-amino acid at position 6 by neutral D-ureidoalkyl amino acids (Huirne and Lambalk, 2001). In pigs a third-generation antagonist (Cetrorelix) has been used *in vivo* and *in vitro* (Neill, 2002; Zanella et al., 2000). The ability of cetrorelix to inhibit [³H]-IP accumulation in response to cGnRH-II was evaluated in COS-1 cells that were transfected with the type II GnRHR. Increased concentrations of cGnRH-II in the media resulted in no inhibition of IP, and when cetrorelix was tested for agonist activity with the type II GnRHR, no activity was observed even at large doses (Neill, 2002). These data suggest that cetrorelix is a potent and specific antagonist to the type I GnRH receptor.

Daily intramuscular (*i.m.*) doses of cetrorelix decreased gonadotropin secretion in intact and castrated boars and gilts (Wise et al., 2000; Zanella et al., 2000; Ziecik et al., 1989). Administration of low doses (5 µg/kg of body weight; BW) of cetrorelix resulted in a decline of LH but had no effect on FSH concentrations, while doses of 10 µg/kg BW of cetrorelix were sufficient to inhibit FSH secretion (Wise et al., 2000; Zanella et al., 2000). Larger doses (20 µg, 50 µg, and 1 mg/kg BW) of cetrorelix also resulted in a significant decrease in LH concentrations with varied responses in FSH secretion (Moran et al., 2002; Wise et al., 2000; Ziecik et al., 1989). The lack of a consistent reduction of FSH secretion in pigs treated with the type I GnRHR antagonist may be associated with the presence of two GnRHs and two GnRH receptors, together with the differences in their signaling in swine species.

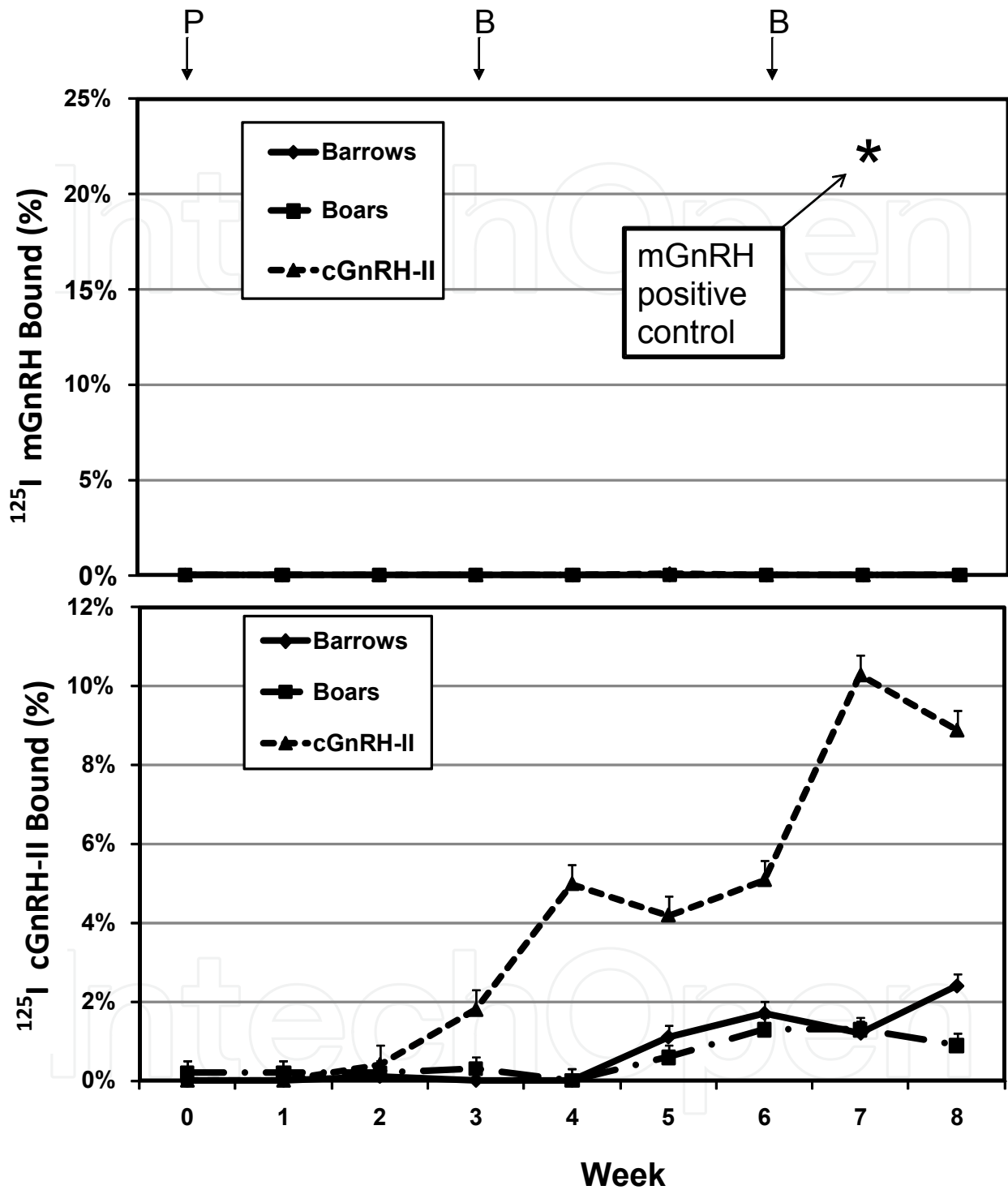


Figure 5. Effect of immunization on antibody titers against mGnRH and cGnRH-II in control barrows and boars immunized against BSA, and intact pigs immunized against cGnRH-II ($n = 12/\text{treatment}$). Antibody titers increased in animal immunized against cGnRH-II after the first booster immunization. Arrows indicate the times at which primary (P) and booster (B) immunizations were given. Plasma from a cow previously immunized against mGnRH (Vizcarra et al., 2011) was used as a positive control (inset). Adapted from (Bowen et al., 2006).

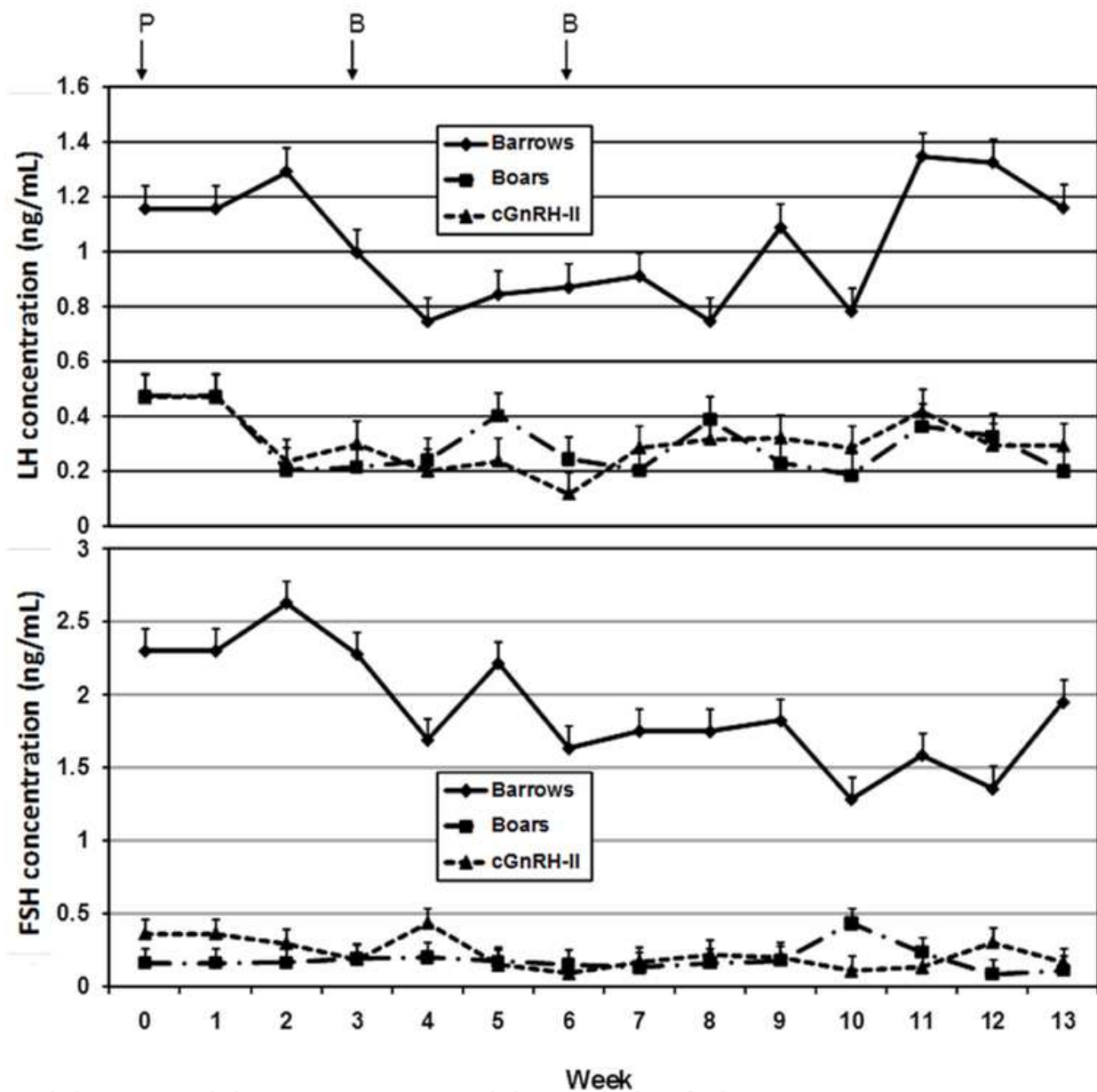


Figure 6. Concentrations of LH and FSH in weekly samples of control barrows and boars immunized against BSA, and intact pigs immunized against cGnRH-II ($n=12/\text{treatment}$). There was a treatment effect for LH ($P < 0.01$) and a treatment \times week interaction for FSH ($P < 0.03$), resulting in gonadotropin concentrations that were greater in control barrows compared with boars and cGnRH-II pigs. Arrows indicate the time at which primary (P) and booster (B) immunizations were given. Adapted from (Bowen et al., 2006).

Antagonist for the type II GnRHR have also been developed and tested in cells expressing rat GnRH (Maiti et al., 2003), human endometrial cells (Fister et al., 2007), and mice (Kim et al., 2009). However, all of these species have lost a functional type II GnRHR (Stewart et al., 2009) and data from these experiments are questionable. A type II GnRHR knockdown swine is being developed (Desaulniers et al., 2011). This animal model may provide new cues on the relative contribution of the type II GnRHR in pigs.

Taken together, GnRH-II is the most ancient and conserved member of the GnRH family, it is expressed in several vertebrates, and has the ability to control gonadotropin secretion in species that have a functional GnRH-II and type II GnRHR.

4. Lamprey GnRH-III

Sower and coworkers (Sower et al., 1993), reported the isolation of lGnRH-III from the sea lamprey (*Petromyzon marinus*). Although lGnRH-III is not a natural ligand of the type I or type II GnRHR, the lGnRH-III receptor shares different characteristics of both type I and type II GnRHRs (Silver et al., 2005; Silver and Sower, 2006). The presence of lGnRH-III (or a related analog) have been reported in brain extracts from humans, sheep, cows and rats (Dees et al., 1999; Hiney et al., 2002; Yahalom et al., 1999; Yu et al., 2000), suggesting a biological activity of this GnRH isoform in several species. However, to date, the gene expression of lGnRH-III has not been reported in mammalian species. There are indications that lGnRH-III might have antiproliferative effects on different types of cancer. Several GnRH analogs are used to treat various forms of cancer (Schally et al., 2001). Among these isoforms, lGnRH-III has a substantial antiproliferative effect on several cancer cell lines (Heredi-Szabo et al., 2006; Lovas et al., 1998; Mezo et al., 1997; Palyi et al., 1999),

The physiologic role played by lGnRH-III on gonadotropin secretion in mammalian species is controversial. Although lGnRH-III is a weak GnRH agonist, early research in mammalian species suggested that lGnRH-III can selectively stimulate the secretion of FSH without changing concentration of LH.

In rodents, lGnRH-III significantly increased FSH concentrations in a dose-dependent manner when using anterior pituitaries at 10^{-9} to 10^{-4} M concentrations. In contrast, LH concentrations were affected only when the highest doses of lGnRH-III (10^{-6} to 10^{-4} M) were used (Yu et al., 1997). Intravenous (i.v.) infusion of lGnRH-III also increased FSH without changes in LH concentrations (Yu et al., 1997). Subsequently, data from the same laboratory reported the isolation of a FSH-releasing factor (RF) obtained from the stalk-median eminence of rats. The FSHRF was associated with lGnRH-III, and had the ability to interact with a putative receptor to selectively release FSH (McCann et al., 2001; Yu et al., 2002; Yu et al., 2000). These data and that from other non-traditional sources (McCann and Yu, 2001) suggest that lGnRH-III is a potent and specific FSH-releasing peptide. However, other lines of research have raised questions about the ability of lGnRH-III to selectively secrete FSH in rodents (see below).

The presence of lGnRH-III in the brain of rats was identified by immunocytochemistry (Dees et al., 1999), and subsequently localized in the dorsomedial preoptic area (POA) of the brain and colocalized with mGnRH (Hiney et al., 2002). However, lGnRH-III was not detected in rats and other rodents by reverse-phase-HPLC followed by RIA, or by performing two successive HPLC steps to prevent the coelution of GnRH peptides (Gautron et al., 2005; Montaner et al., 1999; Montaner et al., 2001). When rats were infused (i.v.) with doses of lGnRH-III or mGnRH, gonadotropin secretion was increased in a dose-dependent manner with a greater increase in LH than FSH concentrations. The potency of lGnRH-III

was 180 to 650 fold weaker than that of mGnRH (Kovacs et al., 2002). Similarly, when rat pituitary cells were perfused with lGnRH-III or mGnRH (10^{-9} to 10^{-6} M), lGnRH-III was 1,000 fold less active in releasing LH than mGnRH (Lovas et al., 1998). Moreover, when rat pituitary cells were perfused with doses (10^{-7} to 10^{-5} M) of lGnRH-III, gonadotropin secretion was increased without any indication of a selective secretion of FSH (Kovacs et al., 2002). These data is in agreement with *in vitro* results obtained from rat hemipituitaries incubated with doses (10^{-9} to 10^{-7} M) of lGnRH-III (Montaner et al., 2001). The contradictory results obtained by different laboratories, may be explained by experimental condition, the influence of the presence or absence of steroid in the *in vivo* models, and data interpretation (Kovacs et al., 2002).

Undoubtedly, more research is needed to clarify the existence of lGnRH-III or a FSHRF that may be involved in the differential secretion of gonadotropins in mammals. In addition to the information provided above, other areas of investigation have stressed the need to reconsider the traditional conjecture that a single GnRH molecule controls reproduction (Igarashi and McCann, 1964; McCann et al., 1983; Padmanabhan and McNeilly, 2001). Briefly, lesions to the median eminence (ME) of castrated male rats suppressed LH but not FSH pulses, while animals with posterior to mid-ME lesions had no FSH pulses but maintained LH episodic releases (Marubayashi et al., 1999). Similarly, ablation of the dorsal anterior hypothalamus of ovariectomized rats suppressed FSH pulses but not LH (Lumpkin et al., 1989). These results raise the possibility that another form of GnRH may contribute nontraditionally to the control of reproductive function or may take part in an important neuroendocrine role. The nature of episodic FSH secretion in portal blood cannot be accounted completely by changes in GnRH secretion (Padmanabhan et al., 1997). When male rats were administered GnRH antiserum and/or GnRH antagonists, pulsatile FSH release was maintained while LH was abolished, giving further credence to the view that reproductive function may be regulated by more than one GnRH neuronal system (Culler and Negro-Vilar, 1987). We have observed that GnRH pulse frequency and amplitude differentially regulates LH and FSH gene transcription and serum concentrations of LH and FSH in cattle (Vizcarra et al., 1997). However, this mechanism of FSH secretion does not preclude the existence of other GnRH releasing factors. It is also possible that the concerted action of local pituitary factors and peripheral steroids could lead to a pulsatile FSH pattern. For instance activins, inhibins, and follistatins may provide an autocrine-paracrine regulation of FSH release at the pituitary level (Baird et al., 1991; DePaolo et al., 1991; Mather et al., 1992; Nett et al., 2002; Padmanabhan et al., 2002; Padmanabhan et al., 1997; Padmanabhan and McNeilly, 2001).

Data obtained in the late 1990's, using the rat model, inspired other laboratories to investigate the use of lGnRH-III in domestic species. Since this peptide was able to selectively stimulate FSH secretion in rats, several researches evaluated the potential use of lGnRH-III in different livestock species. Using similar techniques as those reported in rats, lGnRH-III (or a closely related peptide), was also extracted from sheep stalk-median eminence using a Sephadex G-25 column (Lumpkin et al., 1987; Yu et al., 2000), and from bovine brain samples using HPLC (Yahalom et al., 1999). However, as noted above, the gene

expression of lGnRH-III has not been reported in mammalian species. The ability of lGnRH-III (obtained from bovine midbrain tissue) to release LH was evaluated in cultured rat pituitary cells. The potency of lGnRH-III was only about 2% of that of mGnRH, suggesting that lGnRH-III is a weak agonist of mGnRH (Yahalom et al., 1999). When lGnRH-III (0.25 and 0.5 mg) was infused (i.v.) during the luteal phase of the estrous cycle of crossbred heifers, FSH concentrations were increased without changes in LH concentrations. At higher doses (2.0 and 8.0 mg) both FSH and LH were increased compared with basal concentrations. In contrast, a dose of 0.5 mg of lGnRH-III elicited a significant increase in LH with no changes in FSH secretion at day 20 of the estrous cycle. Authors suggested that the selectivity of lGnRH-III in cattle depends on the dosage and the stage of the estrous cycle (Dees et al., 2001).

In sharp contrast to the observations described above, no differential gonadotropin secretion was reported in ovariectomized cows (exposed to different steroid replacement therapy) when infused with doses (0.055, to 1.1 mg/kg BW) of lGnRH-III. Higher doses (4.4 mg/kg BW) released LH but not FSH. Similarly, *in vitro* doses (10^{-7} to 10^{-6} M) of lGnRH-III elicited a non-selective increase of LH and FSH, while lower doses (10^{-9} to 10^{-8} M) were not associated with gonadotropin secretion in bovine adenohypophyseal cells (Amstalden et al., 2004).

A clear and unbiased interpretation of the discordant results observed in cattle (Amstalden et al., 2004; Dees et al., 2001) is difficult. Reagents (RIAs) used in both laboratories to evaluate LH and FSH were provided by the National Hormone and Pituitary Program. Thus, it is unlikely that differences can be attributed to the ability of a particular RIA to detect FSH concentrations (Amstalden et al., 2004). The ovariectomized cow model, with estradiol and progesterone replacement therapy, used in one experiment (Amstalden et al., 2004) may provide a better animal model compared with intact cows. As noted above, it is well established that ovarian follicular peptides such as actvin, inhibin and follistatin regulate FSH secretion; therefore, intact animals could be influenced by ovarian secretions that may act as a confounding factor.

Along the same lines described for rats and cattle, there is contradictory evidence on the involvement of lGnRH-III on gonadotropin secretion in pigs. Infusion (i.m.) of lGnRH-III in barrows differentially stimulated FSH secretion within 1 h after treatment (Kauffold et al., 2005). On the other hand, when boars were actively immunized against lGnRH-III, concentrations of both LH and FSH were decreased without any evidence of a differential regulation of gonadotropin secretion (Bowen et al., 2006). We (Barretero-Hernandez et al., 2010) also evaluated the effect of infusion (i.v.) of different doses of lGnRH-III on the release of LH and FSH in pigs. Barrows were used to evaluate the effect of 0.1, 1.0 or 10.0 $\mu\text{g}/\text{kg}$ BW of exogenous lGnRH-III on LH and FSH secretion (Figure 7). Blood samples were taken at 10-min intervals for 6 h, starting 2 h before treatments were applied. Relative concentrations of FSH after lGnRH-III infusion did not influence mean concentration of FSH at any of the doses; however, 10.0 $\mu\text{g}/\text{kg}$ BW had a significant effect on LH secretion. We conclude that lGnRH-III is a weak GnRH agonist, and at high doses, lGnRH-III has the ability to release LH but not FSH in barrows. Similar findings were also obtained in gilts that were infused (i.m.) with a synthetic lGnRH-III product (Brussow et al., 2010).

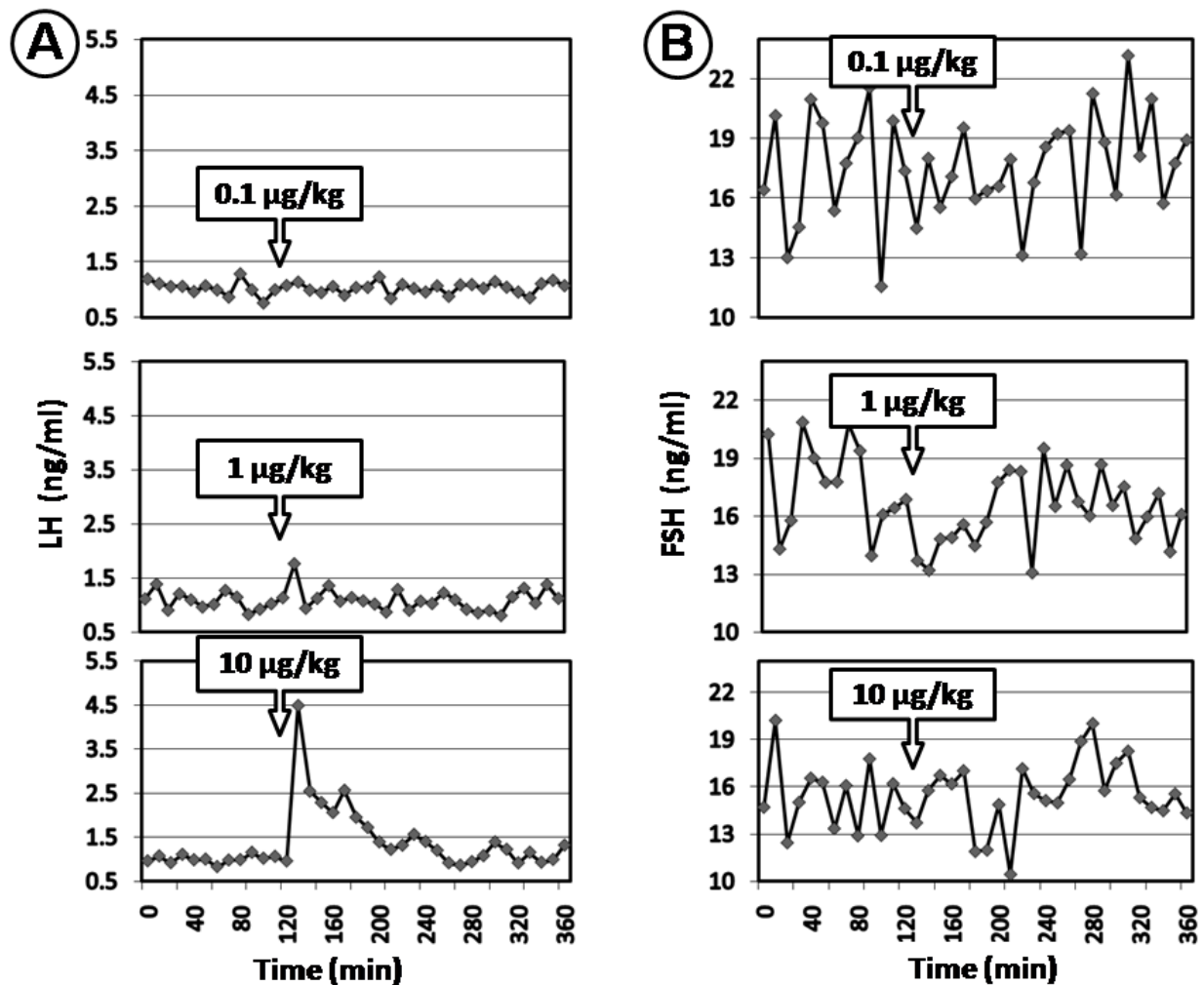


Figure 7. Mean concentrations of LH (A) and FSH (B) in serum at 10-min intervals before and after (arrows) 0.1, 1.0 or 10 µg of lGnRH-III were given intravenously. Only a dose of 10 µg/kg BW elicited a significant LH increase that was considered to be associated with exogenous lGnRH-III infusion (n = 6 animals per treatment). Adapted from (Barretero-Hernandez et al., 2010).

Taken together, the gene expression of lGnRH-III and its receptor has not been reported in mammalian species. Although early work in rats, cows and pigs suggested a selective release of FSH via lGnRH-III, the bulk of the evidence does not support a contribution of lGnRH-III on the selective release of FSH. It is possible that a different peptide (closely related to lGnRH-III) may be associated with FSH release.

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