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Validation of a homologous canine relaxin radioimmunoassay and application with pregnant and non-pregnant Northern fur seals (*Callorhinus ursinus*)[☆]

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ABSTRACT

The primary objectives of this study were to validate a canine relaxin RIA for use in otariids and phocids and consider practical applications. For 6 captive Northern fur seal females, serum samples were grouped and examined according to pregnancy ($n = 13$), post-partum ($n = 8$) and non-pregnancy ($n = 6$), and, for 2 captive Northern fur seal males, serum samples were grouped and examined together regardless of age (2 mo–15 yrs, $n = 6$). Placental tissue was available for examination from one Northern fur seal, Steller sea lion and harbor seal. The validation process involved several steps using an acid-acetone extraction process to isolate a relaxin-containing fraction in pools of serum from each group of fur seals and placental tissue from each seal species. A relaxin-like substance was detected in extracts of pregnant, non-pregnant and male serum and placental tissue in a dose-responsive manner as increasing volumes of respective extracts or amounts of canine relaxin were introduced into the assay. In raw serum samples, mean immuno-reactive relaxin concentrations were higher ($P < 0.05$) during pregnancy than post-partum and non-pregnancy, and lower ($P < 0.05$) in male than female fur seals. During pregnancy, mean serum concentrations of relaxin progressively increased ($P < 0.05$) over Months 4–10 and, in serial samples collected from the same fur seals before and after parturition, mean concentrations were higher ($P < 0.06$) pre-partum than post-partum. In conclusion, validation of a homologous canine relaxin RIA for use in otariids and phocids resulted in the discovery of a relaxin-like substance in extracted and raw serum and placental tissue from Northern fur seals, a Steller sea lion and harbor seal. Distinctly higher immuno-reactive concentrations during pregnancy indicated the potential for relaxin to serve as a hormonal marker to differentiate between pregnant and non-pregnant or pseudopregnant pinnipeds.

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1. Introduction

In 1988 the US National Marine Fisheries Service (NMFS) declared the stock of Eastern Pacific Northern fur seals (*Callorhinus ursinus*) of the Pribilof Islands (St Paul and St George), Alaska as depleted under the Marine Mammal Protection Act (NMFS, 2007). The basis for the population decline is not fully known but likely involves a combination of natural (e.g., disease, predator-prey) and human-related (e.g., sea debris, endocrine disruptors) factors that have had a direct and indirect effect on reproduction. Our cur-

rent understanding of reproductive physiology in fur seals is rudimentary since most of what is known comes from a few early studies where seals were captured at sea and on land at different times during their reproductive cycles, euthanized and necropsied (reviews, Daniel, 1981; Boyd, 1991; Atkinson, 1997; Boyd et al., 1999). Apart from terminal studies, there are only a few later studies that have collected reproductive data in a longitudinal manner in other otariids (Gales et al., 1997; Greig et al., 2007; Villegas-Amtmann et al., 2009). To clarify the nature of the population decline and for planning mitigation strategies in Northern fur seals and other threatened otariid and phocid populations, there is a need to establish a contemporary and comprehensive data base of pinniped reproductive physiology using nonlethal techniques.

Implantation and development of the placenta is a pivotal event in the survival of the early embryo, yet it is one of the least understood aspects of reproduction in mammals (review, Cross et al., 1994).

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Implantation failure accounts for almost 80% of early embryonic loss that occurs in domestic animals (Roberts et al., 1990, 1992; King, 1991). Although the pregnancy loss rate in Northern fur seals pre- and post-embryonic diapause is not known, approximately 88% of 603 multiparous females collected at sea and examined post-mortem had evidence of implantation of which approximately 1.6% of the pregnant females apparently aborted a fetus (York and Scheffer, 1997). For the remaining fur seals, implantation either failed (i.e., no evidence of implantation) or the early embryo was overlooked.

Relaxin is a 6-kDa polypeptide related to the insulin family of hormones and growth factors (review, Park et al., 2005). In most species studied, the embryo/feto-placental unit is the greatest source of relaxin production. In some species (reviews, Hayes, 2004; Sherwood, 2004; Park et al., 2005), relaxin is also produced to a lesser extent by other reproductive organs in females (e.g., ovaries, uterus) and males (e.g., testes, prostate) as well as in non-reproductive organs (e.g., heart, kidneys). Relaxin is well known to have an effect in humans and laboratory and domestic animals during late pregnancy as a hormone responsible for relaxation of the cervix and pelvic ligaments in association with parturition (review, Dschietzig and Stangl, 2003). Less well known is the role relaxin plays during early pregnancy when non-placental sources of relaxin signal remodeling of the uterine stroma and vasculature and the production of factors that promote trophoblast adherence to and invasion of the endometrium (Dschietzig and Stangl, 2003; Hayes, 2004; Sherwood, 2004; Park et al., 2005). In this regard, disruption of relaxin production during early pregnancy has been associated with embryo/fetal loss in humans and primates (Stewart et al., 1993; Einspanier et al., 1999).

In veterinary medicine, immunoassay and detection of elevated systemic relaxin concentrations is a diagnostic indicator of pregnancy and differential measure to distinguish between pregnant and pseudopregnant dogs (Steinetz et al., 1989) since progesterone concentrations are not distinctly different between pregnant and non-pregnant bitches (review, Johnston et al., 2001). Reportedly (Sherwood, 2004), there has been limited (<76%) evolutionary conservation of the amino acid sequence of relaxin among the more than 25 species in which the sequence of relaxin is known. Nevertheless, canine as well as porcine and equine radioimmunoassays (RIA) have been used to detect immuno-reactive relaxin in association with pregnancy in the serum of spotted hyenas (Steinetz et al., 1997), llamas (Bravo et al., 1996), rhinoceroses and elephants (Steinetz et al., 2005), in the plasma of coyotes (Carlson and Gese, 2007), and in the serum and urine of domestic and non-domestic cats (Stewart and Stabenfeldt, 1985; de Haas van Dorsser et al., 2006). Immuno-reactive relaxin has also been detected in placental tissue of the spotted hyena (Steinetz et al., 1997). Considering these results, relaxin has been proposed as a potential hormonal marker of pregnancy in wild animal species (reviews, Steinetz et al., 2005, 2009).

Anatomically, the chorio-allantoic placenta of pinnipeds is comparable to many other carnivores (Harrison et al., 1952). For example, canids and pinnipeds have zonary, endothelio-chorial placentation (Craig, 1964; Miglino et al., 2006). Considering the evolutionary proximity of these species, a previously validated canine relaxin RIA (Steinetz et al., 1996) was chosen to attempt validation for use with pinnipeds and investigate the potential of relaxin to serve as a hormonal marker of pregnancy in otariids and phocids. It is expected that a relaxin RIA for use in pinnipeds will provide a nonlethal and repeatable approach to study the basic physiology of uterine–conceptus–ovarian interactions during the peri-implantation period at the end of embryonic diapause and serve as a diagnostic tool for detecting pregnancy status in captive and free-ranging otariids and phocids for conservation and reproductive management purposes.

The primary objectives of this study were to validate a canine relaxin RIA for use with serum from Northern fur seals, test the

hypothesis that immuno-reactive relaxin concentrations are higher in pregnant than in non-pregnant fur seals and consider practical applications. Secondly, placental tissue samples from a Northern fur seal, Steller sea lion (*Eumetopias jubatus*) and harbor seal (*Phoca vitulina*) were included in the study, in part, to supplement the assay validation process and provide preliminary information on the source of relaxin in otariids and phocids.

2. Materials and methods

2.1. Animals and serum samples

Northern fur seals from which the serum samples were collected and used in the present study were in residence at Mystic Aquarium (Mystic, CT, USA). All 6 females were captured and moved from St Paul Island, Alaska as young adults in the summer of 1984, whereas the 2 males were born in captivity in 1988 and 2003. Seals were housed and fed in compliance with the US Animal Welfare Act and standard aquarium management practices for pinnipeds, which included a US Department of Agriculture-approved Program of Veterinary Care for the regular collection of blood to monitor health status and to assess apparent health issues. Blood samples were collected periodically from 1989–2007 from males throughout growth and development (e.g., pre- and post-puberty) and females during various reproductive states (e.g., estrous cycle and pregnancy). Blood samples were taken under manual restraint from an interdigital vein in the webbing of the hind flipper using a 20-gauge, 2.54 cm needle on a 12 cc eccentric-tip syringe. Blood from each seal was placed in a 10-ml serum separator tube and processed for clinical chemistry. Remaining serum (1–2 ml) from each seal was transferred to a separate cryo-vial, labeled with seal identification and date, and archived at –60 °C. Archived serum samples were selected and shipped frozen to a laboratory at New York University School of Medicine (Tuxedo, NY, USA) and stored at –20 °C until assay.

Serum samples from adult female fur seals were grouped according to reproductive status: (1) pregnancy (blood collected from 4–12 mo of gestation, $n = 13$ samples), (2) post-partum (blood collected from 4 d–5 mo after parturition, $n = 8$ samples) and (3) non-pregnancy (blood collected over 12-mo reproductive cycles, $n = 6$ samples). Samples from male fur seals were grouped together regardless of age (blood collected from 2 mo–15 yrs, $n = 6$ samples). In addition, samples from the pregnancy group were subgrouped according to months of pregnancy: (1) Months 4–5 ($n = 2$ samples), (2) Months 7–8 ($n = 4$ samples) and (3) Months 9–10 ($n = 6$ samples). Month of pregnancy was determined retrospectively from day of parturition. The non-pregnancy group included samples from non-bred and bred seals and was defined according to the absence of a pup after a 12-mo reproductive cycle. Samples that were associated with a known pregnancy that was subsequently lost were not included in the non-pregnancy group. Since there was a limited amount of serum in individual samples, portions of serum were collected from multiple samples within respective groups and combined into separate pools for acid-acetone extraction to isolate a relaxin-containing fraction and test for immuno-reactivity in the validation process.

2.2. Placental tissue samples

Northern fur seal, Steller sea lion and harbor seal placentae were collected and made available according to respective permits issued under the US Marine Mammal Protection Act. From the California Marine Mammal Center (Sausalito, CA, USA), a placenta was collected after stillbirth from a stranded Northern fur seal in March 2007 from which a tissue sample was cut and frozen (–80 °C). From the Alaska SeaLife Center (Seward, AK, USA), placentae were

collected and frozen (-20°C) after necropsy of a recently deceased free-ranging Steller sea lion and after birth from a captive harbor seal in May 2008. The fur seal placental tissue sample and sea lion and harbor seal placentae were shipped frozen to a laboratory at New York University School of Medicine (Tuxedo, NY, USA) and stored at -20°C until further processing and assay.

Tissue samples from each seal placenta were processed for assay of relaxin in a comparable manner to that described for the spotted hyena placenta (Steinetz et al., 1997). Briefly, a 15 g sample previously cut from the fur seal placenta, and 250 g samples cut from a site most representative (darkest red) of the zonary or vascular portion of the sea lion and harbor seal placentae were homogenized in cold phosphate buffered saline (PBS), centrifuged and decanted to remove tissue fragments and fat. Supernatants of the placental tissue samples were set aside for acid-acetone extraction to isolate a relaxin-containing fraction and test for immuno-reactivity in the validation process.

2.3. Acid-acetone extraction of serum pools and placental tissue supernatants

Many polypeptides are precipitated by acidified acetone, but relaxin is a relatively small and highly stable protein that is soluble in acid-acetone (Steinetz et al., 1962). Extraction in acidified acetone separates small polypeptides from larger, acetone-insoluble proteins that are predominant in blood and other tissues, which could potentially interfere with immuno-reactivity in the relaxin RIA. The acid-acetone extraction process used herein has been described (Doczi, 1963) and previously used to isolate the relaxin-containing fraction from placental tissue of the spotted hyena to verify immuno-reactivity in the canine relaxin assay (Steinetz et al., 1997). Briefly, serum pooled from pregnant (2.0 ml), non-pregnant (8.5 ml) and male (7.0 ml) fur seals and supernatants of homogenized placental tissue from each seal species were mixed with water, concentrated HCl and 70% (v/v) acetone at -4°C for approximately 24 h. Afterwards, respective mixtures were filtered and precipitated with additional cold acetone (90%, v/v). Approximately 24 h later, the acetone supernatants were removed and respective relaxin-containing fractions were dried and reconstituted with assay buffer and set aside until assay.

2.4. Relaxin assay

Details of a previously validated canine relaxin RIA have been described (Steinetz et al., 1996). In the present study, 3 antibodies developed against relaxin were initially tested for immuno-reactivity to a relaxin-like substance in raw serum from a pregnant Northern fur seal: (1) rabbit polyclonal porcine relaxin antiserum R6 which reacts specifically with the receptor binding domain of all relaxins thus far sequenced (O'Byrne and Steinetz, 1976), (2) rabbit polyclonal canine relaxin antiserum No. 79888 which is highly reactive with relaxin in canids and felids (Steinetz et al., 1996; de Haas van Dorsser et al., 2006), and (3) rabbit polyclonal canine relaxin antiserum No. 80037 (Steinetz et al., 1996). Specific binding was approximately 12% with antiserum No. 80037 in pregnant fur seal serum compared to little or no detectable immuno-reactivity with the other antisera in the same pregnant fur seal sample. Hence, a homologous canine relaxin RIA was used in the present study which included canine antiserum No. 80037 as the primary antibody (working dilution, 1:5000) and synthetic canine relaxin as reference standard (0.1–12.5 ng/tube) and ^{125}I -labeled ligand (approximately 30,000 cpm/tube).

Verification of the canine relaxin RIA for use with serum and placental tissue from Northern fur seals and a Steller sea lion and harbor seal included several validation steps following acid-acetone extraction: (1) isolation and immuno-reactivity of a relaxin-

like substance in extracts from pools of pregnant, non-pregnant and male serum and placental tissue from each seal species, (2) specific displacement of radiolabeled canine relaxin from the canine relaxin antibody by increasing volumes (100 and 200 μl) of extract from pools of pregnant, non-pregnant and male serum and placental tissue from each seal species, and (3) additivity by adding increasing amounts of canine relaxin (0, 0.8 and 1.6 ng) to an extract of pooled male fur seal serum or assay buffer.

Extracts of pooled serum and placental tissue samples from Northern fur seals and a Steller sea lion and harbor seal and raw serum samples from individual fur seals were assayed for immuno-reactive relaxin using 100 or 200 μl of extracts in assay buffer or 200 μl of raw serum in duplicate. The results were expressed as the average specific binding or concentration of duplicates relative to the canine relaxin reference standard. Assay sensitivity was set at 90% of specific binding (approximately 0.6 ng/ml) and intra- and inter-assay coefficients of variation were 5% and 15%, respectively.

2.5. Statistical analyses

Prior to statistical analyses, the data were checked for extreme values using Dixon's outlier test (Kanji, 1993), tested for homogeneity and transformed if necessary. Analysis of variance was used to determine differences in serum concentrations of immuno-reactive relaxin among groups (pregnant, post-partum and non-pregnant and male fur seals) and months of pregnancy (4–5, 7–8, and 9–10). If there was a significant effect, independent *t*-tests were used to identify mean differences among groups or months. In serial samples collected repeatedly from the same fur seals before and after parturition, a paired *t*-test was used to compare mean differences in serum relaxin concentrations pre- and post-partum. A probability of $P \leq 0.05$ indicated that a difference was significant and probabilities between $P > 0.05$ and 0.1 indicated that a difference approached significance. Values are reported as mean $\pm$ SEM unless otherwise indicated.

3. Results

Validation results following acid-acetone extraction of pools of serum and placental tissue from Northern fur seals and a Steller sea lion and harbor seal are shown for each step in Table 1. The acid-acetone extraction process with the fur seal placental tissue sample yielded a relaxin-containing fraction that was sufficient to examine duplicate volumes of 100 μl but not 200 μl of extract (Table 1, Step 2). Compared with 250 g samples of placental tissue from the sea lion and harbor seal, a 15 g sample of placental tissue from the fur seal was inadequate to complete the dose-responsive displacement of radiolabeled canine relaxin from the canine antibody.

Mean immuno-reactive relaxin concentrations in raw serum samples from groups of pregnant, post-partum, non-pregnant and male fur seals are shown with statistical results in Table 2. For serial samples collected repeatedly from the same fur seals pre- and post-partum, individual and mean results are shown with statistical results in Table 3. Inspection of the individual results indicated relaxin concentrations declined after parturition in 3 of the 4 seals. In Seal A, the relaxin value during pregnancy in 1989 was uncharacteristically low compared to the concentration at approximately the same stage of gestation in the subsequent year, and in comparison to values in other seals in the pregnant group (Table 3). Consequently, the aberrant value reduced the statistical significance between mean concentrations of relaxin pre- and post-partum.

Mean immuno-reactive relaxin concentrations in fur seals sub-grouped according to months of pregnancy are shown with

Table 1

Validation steps for verifying a homologous canine relaxin RIA for use in otariids and phocids.

Step 1. Immuno-reactivity in relaxin-containing fractions following acid-acetone extraction of pooled serum and placental tissue from each seal species.				
		Relaxin		
Acid-acetone extraction		Pregnant pool	Non-pregnant pool	Male pool
Fur seal serum (ng/ml)		42.7	17.2	5.0
		Northern fur seal	Steller sea lion	Harbor seal
Placental tissue (ng/kg)		200	1600	292
Step 2. Specific displacement of radiolabeled relaxin binding in response to increasing volumes of acid-acetone extracts of pooled serum or placental tissue from each seal species.				
		Specific binding (%)		
Northern fur seal serum		Pregnant pool	Non-pregnant pool	Male pool
Extract volume (μl)				
100		59	75	83
200		44	61	76
Placental tissue		Northern fur seal	Steller sea lion	Harbor seal
Extract volume (μl)				
100		76	73	87
200		*	61	74
Step 3. Additivity and specific displacement of radiolabeled relaxin binding by adding increasing amounts of canine relaxin standard to an acid-acetone extract of pooled male Northern fur seal serum or assay buffer.				
		Specific binding (%)		
Canine relaxin (ng/ml)		Seal serum	Assay buffer	
0.0		66	100	
0.8		53	66	
1.6		46	52	

* Placental tissue sample (15 g) was insufficient to prepare an adequate amount of extract for assay at greater volume.

Table 2

Mean (±SEM) immuno-reactive relaxin concentrations in raw serum samples grouped according to pregnant, post-partum and non-pregnant females (6) and male (2) Northern fur seals.

Groups	No. samples	Relaxin (ng/ml) ¹
Pregnant	13	8.2 ± 1.5 ^a
Post-partum	8	3.6 ± 1.0 ^b
Non-pregnant	6	2.5 ± 0.4 ^b
Male	6	1.1 ± 0.2 ^c

¹Group effect, $P < 0.002$.^{a,b,c} Values differ, $P < 0.05$.**Table 3**

Individual and mean concentrations of immuno-reactive relaxin in raw serum from Northern fur seals pre- and post-partum for two consecutive reproductive cycles for Seal A and one reproductive cycle each for Seals B and C.

Seal	Yr	Pre-partum		Post-partum	
		Time	Relaxin (ng/ml)	Time	Relaxin (ng/ml)
A	1989	4 mo	3.7	1 mo	5.1
A	1990	5 mo	12.1	1 mo	4.2
B	1989	4 mo	7.4	4 d	2.3
C	1990	3 mo	10.1	5 mo	0.8
Mean ± SEM			8.4 ± 1.8 ^a		3.1 ± 1.0 ^b

^{a,b} Values differ, $P < 0.06$.

statistical results in Table 4. Although there was a significant effect of month as a result of a progressive increase in mean concentrations of relaxin during gestation, the data should be interpreted cautiously because of the small number of samples and concentrations that were near the sensitivity of the assay, especially during Months 4–5.

4. Discussion

The availability of archived serum samples collected from captive Northern fur seals and placental tissue from a free-ranging

Table 4

Mean (±SEM) immuno-reactive relaxin concentrations in raw serum samples from pregnant Northern fur seals (6) sub-grouped according to month of pregnancy. Month of pregnancy was determined retrospectively from day of parturition.

Serum sample	Months of pregnancy		
	4–5	7–8	9–10
Number	2	4	6
Relaxin (ng/ml) ¹	0.8 ± 0.0 ^a	7.1 ± 1.8 ^{b,x}	11.1 ± 2.1 ^{b,y}

¹Month effect, $P < 0.05$.^{a,b} Values differ, $P < 0.04$.^{x,y} Values differ, $P < 0.1$.

Northern fur seal, Steller sea lion, and captive harbor seal provided the opportunity to adapt and validate a homologous canine relaxin RIA for the discovery of a relaxin-like substance in pinnipeds. The pelagic nature of fur seals (Atkinson, 1997; Boyd et al., 1999) and regulatory criteria under the Marine Mammal Protection Act (NMFS, 2007) preclude systematic collection of blood samples from free-ranging seal pups, juveniles and adults during growth and reproduction. Aquariums have played a historic role in the conservation of fur seals, and periodic collection of blood samples is a routine part of management practices. Even though the number of captive fur seals that have been conditioned for the collection of blood is limited to a few seals and aquariums (e.g., Mystic, CT and Seattle, WA, USA), availability of numerous serum samples archived over multiple years can provide a reasonable distribution of samples for examination during pre- and post-puberty and various stages of the estrous cycle, pregnancy and post-partum. Although the effect of long-term cryopreservation on serum concentrations of relaxin is unknown, it is known that relaxin is a stable and soluble protein hormone in acid-acetone that can withstand exposure to extreme cold, heat and long periods at ambient temperature (Steinetz et al., 1962; Doczi, 1963). In the present study, freezer storage times and conditions were similar among archived pregnant, non-pregnant, and male fur seal

samples; therefore, any alteration (i.e., decline) in serum relaxin concentrations due to prolonged storage would presumably be proportional among samples. Thus, this study represents an unprecedented collaboration among a diverse group of scientists from government, academia and aquatic institutes to develop a comprehensive data base for reproductive hormones in Northern fur seals that may serve as a future source of reference for samples taken from captive as well as free-ranging fur seals and other otariids and phocids.

The canine relaxin RIA has been fully validated for use in dogs (Steinetz et al., 1996) and successfully validated and adapted for use in a variety of wildlife species primarily as a noninvasive approach to distinguish pregnant from non-pregnant or pseudopregnant animals (Steinetz et al., 2005, 2009). In the present study, acid-acetone extraction (Doczi, 1963; Steinetz et al., 1997) was used to isolate a relaxin-like substance in pools of serum from female (pregnant and non-pregnant) and male Northern fur seals and placental tissue from a Northern fur seal, Steller sea lion and harbor seal. Assay of the relaxin-containing fractions indicated relatively high, intermediate and low serum concentrations of immuno-reactive relaxin in pregnant, non-pregnant and male fur seals, respectively. Immuno-reactive relaxin was also detected in the placenta of each seal species. There was a dose-responsive displacement of radiolabeled canine relaxin from the canine antibody as increasing volumes of extracts from respective serum pools or placental tissue were introduced into the assay. A dose-responsive displacement of radiolabeled canine relaxin was also observed as increasing amounts of the canine relaxin reference standard were added to the relaxin-containing fraction of male fur seal serum. When compared with the same amount of canine relaxin added to assay buffer, specific binding was lower (12–34%) for the extract than buffer at each level, which was attributed to the additive effect of a relaxin-like substance in male seal serum.

Although the acid-acetone extraction process was inefficient requiring a relatively large volume (2–8 ml) of pooled serum from individual samples from pregnant, non-pregnant and male Northern fur seals, the process was effective and fundamental for validation of the canine relaxin assay for use with fur seal serum. It was not thoroughly known beforehand if components of raw serum would interfere with immuno-reactivity in the relaxin assay. Nevertheless, the extraction process was not practical for the relatively small volume of serum remaining in individual fur seal samples.

In raw serum samples, mean concentrations of immuno-reactive relaxin were significantly higher in pregnant than in non-pregnant or post-partum fur seals, and significantly lower in males than females. Temporally, there was a progressive increase in mean concentrations of relaxin from relatively low values during Months 4–5 to significantly higher values during Months 9–10. In serial samples collected from the same fur seals before and after parturition, mean relaxin concentrations were higher pre-partum than post-partum. The directional changes in serum relaxin from relatively high concentrations during pregnancy to low concentrations after parturition and during non-pregnancy in fur seals is similar to that reported in dogs (Johnston et al., 2001), horses (Ginther, 1992), pigs (Dlamini et al., 1995), laboratory rodents (Sherwood, 1994), camelids (Bravo et al., 1996) and wild animal species (Steinetz et al., 2005, 2009). Taken together, raw serum from pregnant, non-pregnant (post-partum) and male fur seals did not appear to interfere with detection of relaxin based on comparable relative changes among extracts of pooled serum from corresponding groups in the validation process. Moreover, the combined results supported the hypothesis that immuno-reactive relaxin concentrations are higher in pregnant than in non-pregnant Northern fur seals.

In the present study, the relatively low serum concentrations of immuno-reactive relaxin during Months 4–5 of pregnancy corresponds to the end of embryonic diapause, a period of delayed

implantation that reportedly ranges from 3–5 months in Northern fur seals (Daniel, 1981; Boyd, 1991; Atkinson, 1997; Boyd et al., 1999) following ovulation primarily in July (Craig, 1964). However, the lack of samples during diapause and small number of samples thereafter preclude a detailed understanding of any temporal relationship between relaxin and the peri-implantation period in fur seals at this time. Later, during pregnancy, serum concentrations of relaxin were relatively higher and apparently of placental origin. Although low, the source of relaxin in non-pregnant and male fur seals is not known. In species other than pinnipeds, reproductive (e.g., ovaries, uterus, mammary glands, testes, prostate) and non-reproductive organs (e.g., brain, heart, kidneys) have been identified as non-placental sources of relaxin (Hayes, 2004; Sherwood, 2004; Park et al., 2005). Future studies are needed to critically examine the changes in circulating concentrations of relaxin encompassing the end of embryonic diapause and identify and confirm placental and non-placental sources of relaxin in female and male Northern fur seals and other otariids and phocids of both genders.

Knowledge of relative changes in circulating concentrations of relaxin has both basic and applied implications. From a basic perspective, results of immunoassay of relaxin in combination with results from ultrasonic imaging of the uterus, conceptus and ovaries in pinnipeds (Adams et al., 2007) can further our understanding of functional and structural relationships at the end of embryonic diapause and survival of the early embryo during implantation in a non-lethal manner. From an applied perspective, assay of relaxin may be a more effective hormonal indicator than progesterone to distinguish between pregnant and non-pregnant or pseudopregnant otariids and phocids. In general, it is thought that the corpus luteum in pregnant seals is maintained throughout gestation, but may regress before parturition in some species of pinnipeds (Daniel, 1981; Boyd, 1991; Atkinson, 1997; Boyd et al., 1999). Comparatively, in non-pregnant seals, it is thought that functional and structural development of the corpus luteum is similar to that during the period of delayed implantation but, thereafter, it gradually regresses in the absence of placental luteotrophic support (Boyd et al., 1999). The condition of elevated concentrations of progesterone in non-pregnant seals that appears to mimic the condition in pregnant seals has been referred to as pseudopregnancy (Atkinson, 1997; Boyd et al., 1999), which seems comparable to the condition reported in dogs (Johnston et al., 2001). Until functional and structural development of the corpus luteum has been thoroughly characterized in pregnant and non-pregnant pinnipeds, the results of immunoassay of circulating concentrations of progesterone as a hormonal indicator of pregnancy will be equivocal. Alternatively, results of the present study indicate that elevated concentrations of immuno-reactive relaxin are pregnancy-specific; hence, relaxin has the potential to serve as an effective hormonal marker for diagnosing pregnancy in otariids and phocids as it is in canids and other species (Steinetz et al., 2005, 2009).

In conclusion, validation of a homologous canine relaxin RIA for use in otariids and phocids resulted in the discovery of a relaxin-like substance in extracted and raw serum and placental tissue from Northern fur seals, a Steller sea lion and harbor seal. Distinctly higher immuno-reactive concentrations during pregnancy indicated the potential for relaxin to serve as a hormonal marker to differentiate between pregnant and non-pregnant or pseudopregnant pinnipeds.

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