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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 current 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. Secondarily, 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