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Validation of Heterologous Radioimmunoassays (RIA) for Growth Hormone (GH) and Insulin-Like Growth Factor (IGF)-I in Phocid, Otariid, and Cetacean Species

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Abstract

Growth hormone (GH) is a homeorhetic hormone that coordinates diverse physiological processes to partition nutrients toward lean tissue accretion and milk production, and to inhibit utilization of nutrients by adipose tissue, especially during periods of energy deficiency. Due to age-related declines in GH, GH is negatively correlated with overall growth rate and lipid accretion but positively associated with protein accretion. Circulating GH influences peripheral tissues directly or indirectly and is primarily mediated via an insulin-like growth factor (IGF)-I. Similar to GH, age and nutrition influence IGF-I secretion. In general, IGF-I is positively associated with growth rate and accretion of protein. Since purified pinniped or cetacean GH and IGF-I are not available, heterologous assay techniques must be employed to quantify concentrations of these hormones. Critical to the reliability of heterologous assays are the proper execution of validations to demonstrate the accuracy and precision of the assay for each new species of interest. In this study, we developed and validated heterologous radioimmunoassays for seven marine mammal species using porcine and human antisera for GH and IGF-I quantification, respectively. Sensitivity, recovery of mass, assay precision, parallelism, and dilution linearity were determined for each assay and species. Both assays exhibited excellent parallelism and linearity. Appropriate concentrations of hormone were quantified throughout the range of the standard curve. Importantly, using these assays, we have demonstrated that serum concentrations of GH and IGF-I change with age and nutrient intake in these species. Validation of these assays allows evaluation of metabolic hormones that may provide unique perspectives on physiological regulation of differential growth and nutrient allocation for diverse marine mammal species.

Key Words: assay validation, growth hormone, insulin-like growth factor-I, phocid, otariid, cetacean, multi-species, radioimmunoassay

Introduction

Radioimmunoassays (RIA) are a tool commonly used by endocrinologists to quantify hormone concentrations. The RIA is a competitive binding assay that requires the hormone of interest to compete with a radio-labeled hormone for binding with a limited quantity of primary antisera (Ab) (Berson & Yalow, 1968). A key assumption with RIA analysis is that the primary Ab has equal affinity for the radio-labeled protein and the protein of interest being measured in the unknown sample (Berson & Yalow, 1968; Davies, 2005). In the case of heterologous assays, unless the protein of interest is 100% homologous among species, the primary Ab may preferentially bind the radioactively labeled protein at a rate greater than binding to the unknown. The degree of preferential binding will determine the accuracy, precision, and therefore the usefulness of a given RIA to quantify a protein of interest in a new species.

Improper or failure to perform validations of each new species of interest can result in erroneous results. Concentrations may appear inconsistent with the physiologic status of an animal or may appear unchanged after a dramatic change in physiologic status has occurred. These types of results could lead to flawed interpretations of experiments and illustrate the critical importance for rigorous and appropriate assay validations to be performed each time a new species assay is developed (Hammond et al., 2005).

The somatotropic axis is important in the regulation of growth, development, and regulation of energy and protein metabolism. The somatotropic axis is a multilevel hormonal system consisting of neuro-peptides from the hypothalamus, including growth hormone releasing factor (GRF) and somatostatin (SRIF), growth hormone (GH) produced from the anterior pituitary gland, and insulin-like growth factor (IGF)-I and IGF binding proteins (IGFBP) produced from the liver and peripheral tissues such as bone, skeletal muscle, and adipose tissue (Tuggle & Trenkle, 1996). Hypothalamic

neuro-peptides regulate GH secretion from somatotropes. In mammals, GRF stimulates release of GH, while SRIF inhibits GRF-induced GH secretion (Zinn et al., 1994; Tuggle & Trenkle, 1996; Rigamonti et al., 2001).

Growth hormone is a homeorhetic agent that coordinates diverse physiological processes, directly or indirectly, to partition nutrients toward lean tissue accretion and/or milk production and to inhibit utilization of nutrients by adipose tissue (Burton et al., 1994; Etherton & Bauman, 1998). In general, GH stimulates muscle protein accretion and inhibits the accretion of adipose (Burton et al., 1994). In mammals, GH reduces uptake of fatty acids into lipid and increases nitrogen retention in muscle, leading to an increase in growth rate and protein accretion while reducing fat accumulation (Bauman & Vernon, 1993; Mikel et al., 1993). Furthermore, GH facilitates utilization of energy stores from adipose to sustain lean body mass, especially in times of energy deficit (Eigenmann et al., 1985). In adipose, GH increases lipolysis and decreases lipogenesis by reducing insulin sensitivity, by down-regulating fatty acid synthase, or by phosphorylating transcription factors that mediate lipid storage (Kersten, 2001). Due to the overall age-related decline in GH, concentrations of GH are negatively correlated with overall growth rate and lipid accretion but positively associated with protein accretion (Corpas et al., 1993; Govoni et al., 2003). However, at a given age and nutritional status, animals with greater concentrations of GH have an overall faster growth rate characterized primarily by increased protein accretion with relatively little lipid deposition (Zinn et al., 1986; Mikel et al., 1993; Connor et al., 1999; Rausch et al., 2002; Govoni et al., 2003).

Many of the growth promoting effects of GH are mediated by IGF-I produced primarily in the liver (Etherton, 1991). Circulating GH interacts with membrane receptors in liver and other peripheral tissues to stimulate production of IGF-I (Carter-Su et al., 1996). In general, concentrations of IGF-I are positively associated with growth rate and accretion of protein (Umpleby et al., 1994; Davis & Simmen, 1997; Rausch et al., 2002). Administration of IGF-I in humans increases whole body protein metabolism by increasing protein synthesis and inhibiting proteolysis (Fryburg, 1994). In mammals, IGF-I can also increase muscle hypertrophy by stimulating satellite cell proliferation (Fiorotto et al., 2003), and greater IGF-I concentrations are associated with increased longissimus muscle area, indicating that IGF-I stimulates protein accretion and plays an important role in muscle development (Connor et al., 2000).

Both IGF-I and GH are responsive to nutrient intake and are key regulators in the allocation of energy for tissue growth and maintenance. For example, compared with animals fed *ad libitum*, restricted-fed animals have reduced growth rate, coupled with reduced IGF-I and increased GH (Rausch et al., 2002). The increased GH is a mechanism utilized to mobilize energy stores from adipose tissue via GH-induced stimulation of lipolysis to spare utilization of protein (Carrel & Allen, 2000). The decrease in IGF-I concentration reduces the anabolic influence of IGF-I and spares nutrients for maintenance (Bauman & Vernon, 1993). With the key role that both GH and IGF-I play in growth, development, and nutrient allocation, development of assays to study these hormones is needed to accurately assess how each hormone may be involved in marine mammal growth and development.

Both GH and IGF-I are key metabolic hormones that play a pivotal role in nutrient allocation for growth and development. Investigation of these hormones in species with differential growth rates and life history strategies may provide an interesting perspective on the physiological regulation of growth. However, before such investigations are undertaken, an assay technique must be developed and validated in multiple marine mammal species. Since purified pinniped or cetacean GH and IGF-I are not available, we developed and validated heterologous RIA for seven marine mammal species using porcine and human Ab to GH and IGF-I, respectively. The species used for assay validations included two families within the Order Carnivora (Family Phocidae and Otariidae) and one species from the Order Cetacea (Family Monodontidae). These species were chosen based on our interest in studying growth physiology of species with diverse life-history strategies and the availability of the appropriate quantities of serum necessary to perform validations. To validate these two assays for each species, sensitivity, recovery of mass, intra- and inter-assay precision, parallelism, and dilution linearity were determined (Davies, 2005).

Materials and Methods

GH Radioimmunoassay

Methods for a porcine RIA described by Barb et al. (1991) were followed for iodination and quantification of GH with minor modifications. Since purified marine mammal GH was not available, a rabbit-anti-porcine GH Ab was used (AFP422801Rb, A. F. Parlow, National Hormone & Peptide Program, Torrance, CA, USA) as the primary Ab.

GH Assay Buffers—Multiple buffers were used for the iodination of porcine GH and the GH RIA. The GH dilution buffer (0.1 M sodium bicarbonate at pH 8.0) was used to reconstitute purified

lyophilized porcine GH (AFP10864B, National Hormone & Peptide Program) for the iodination and standard curve preparation. A 0.5 M sodium phosphate buffer and 0.05 M phosphate buffer at pH 7.5 were used for the iodination. Phosphate buffered saline (PBS) (0.01 M, pH 7.5) was the GH assay buffer used to make all other buffers. Bovine serum albumin (BSA) was diluted with GH assay buffer to create 1% BSA buffer solution. Lyophylized rabbit-anti-porcine antisera, used for primary Ab (AFP422801, National Hormone & Peptide Program), was reconstituted with double-distilled water (1:50) and then diluted with GH assay buffer supplemented with 2% normal rabbit serum (NRS) to a stock primary Ab dilution of 1:1600. Primary Ab buffer, used to further dilute the primary Ab stock, contained GH assay buffer with 0.5% NRS. A secondary Ab buffer (GH assay buffer with 0.04 M EDTA) was used to dilute the secondary antisera to rabbit γ-globulin produced in goats (Calbiochem EMB Biosciences, San Diego, CA, USA).

GH Iodination—Purified lyophilized porcine GH was reconstituted to 1 μg μl^{-1} with GH dilution buffer. Free ^{125}I (PerkinElmer, Waltham, MA, USA; specific activity 17.4 Ci/mg, 643.8 GBq/mg as reported by PerkinElmer) was used for iodination of GH within 1 wk of release date. Included in the iodination reaction were 5 μg of porcine GH and 1 mCi of ^{125}I diluted with 0.05 M sodium phosphate buffer for a final reaction volume of 200 μl. The reaction was catalyzed in an Iodo-Gen reaction tube (Pierce Biotechnology, Rockford, IL, USA) and allowed to proceed for 9 min with regular, gentle agitation. The reaction mixture was then added to a size exclusion polyacrylamide gel column to separate ^{125}I labeled porcine GH from unlabeled protein and unbound ^{125}I.

The column was prepared with P-60 polyacrylamide gel (Bio-Rad, Hercules, CA, USA) hydrated with 0.05 M sodium phosphate buffer. The column was rinsed sequentially with 2 ml 1% BSA buffer, 2 ml 0.5 M phosphate buffer, and 6 ml 0.05 M phosphate buffer before the reaction mixture was applied to the column. The P-60 medium gel excludes particle size 90 to 180 μm with a fractionation range of 3,000 to 60,000 Daltons. Size exclusion gel chromatography impedes the flow of small particles, which are trapped in the gel beads, and allows larger molecules to flow through the column without hindrance.

Once reaction vial contents were absorbed into the column, a 0.05 M phosphate buffer was continually added to maintain column hydration. Fractions were collected in 0.5 ml increments into vials containing 0.5 ml 1% BSA buffer. A Cobra II Series Auto Gamma Counter (Packard Instruments Company, Meriden, CT, USA; efficiency 80%) was used to determine peak radioactivity in the collection tubes. Each tube was counted for 1 min to determine counts per minute (cpm). Total binding analysis was then performed on the fractions with the greatest radioactivity to identify optimal fractions for use in the RIA. Fractions with greater than 30% initial total binding were considered appropriate for use in the assay. ^{125}I-GH was stored at 4° C and produced reliable results for up to 6 wks, provided that the total binding was greater than 15%.

Radioimmunoassay for GH—Purified lyophilized porcine GH was reconstituted as described above and diluted to 250 ng/ml stock standard solution with 1% BSA buffer. Further dilution of 250 ng/ml stock to a 50 ng/ml working standard solution was performed with GH assay buffer. A standard curve was prepared through serial dilution of porcine GH with GH assay buffer for a concentration range from 25 ng/ml to 0.2 ng/ml. Total count (TC), nonspecific binding (NSB), total bound (TB), standards, high and low internal controls, and unknown samples were analyzed in quadruplicate. Standard porcine serum with high and low GH concentrations were used for internal control tubes. Primary Ab was added to unknown, standard, and TB tubes in a 1:2 ratio with a final dilution of 1:80,000 with primary Ab buffer. The NSB and TB tubes received 200 μl of GH assay buffer instead of sample, and NSB tubes received primary Ab buffer without Ab. The volume of sera added (200 μl) to tubes was adjusted with the GH assay buffer to ensure that the hormone concentration was within the linear portion of the standard curve. Glass test tubes containing primary Ab, assay buffer, and standard or unknown sample were vortexed and incubated for 24 h at room temperature (approximately 25° C).

After incubation, 100 μl of ^{125}I-GH was added to all tubes with approximately 10,000 cpm per tube (diluted with 1% BSA buffer). After addition of labeled ^{125}I-GH, tubes were vortexed and incubated at room temperature for 24 h. Secondary Ab was diluted with secondary Ab buffer, and 100 μl was added to all tubes except TC at a final dilution of 1:125. Tubes were again vortexed and incubated for 24 h at room temperature. Finally, 3 ml of 1% BSA buffer was added to all tubes, except TC. Tubes were vortexed and centrifuged 2,100 × g at 4° C for 30 min. Supernatant was poured off, and tubes were allowed to dry inverted over night. The radioactivity of the remaining pellet was quantified for 1 min in a gamma counter.

The percentage of ^{125}I-GH bound (B/Bo) to the primary Ab was calculated for a concentration range from 0.2 ng/ml to 25 ng/ml. The NSB cpm was subtracted from both the cpm of the standard or unknown sample (B) and the cpm of TB tubes

(Bo) to calculate percentage bound (B/Bo). The value was plotted vs the log of the concentration of purified GH in each standard point to establish a standard curve. The linear regression equation was then used to predict the GH concentration in unknown samples based on the percentage of bound ^{125}I-GH in each tube. Values are reported as ng of hormone per ml of serum after adjustment for volume of serum assayed. Replicates of the same sample were averaged. If the percent error between replicates was greater than 10%, or if concentration was outside the standard curve, the sample was reanalyzed.

IGF-I Radioimmunoassay

Assay protocols outlined by Johnson et al. (1996) for bovine RIA were followed for IGF-I quantification with minor modifications. Since the structure of IGF-I is highly conserved among species and is virtually identical among mammalian species (Foyt et al., 1991), we used rabbit-anti-human IGF-I antisera as primary Ab for quantification of IGF-I (AFP4892898, National Hormone & Peptide Program).

IGF-I Assay Buffers—The IGF-I assay buffer was prepared with 0.05 M Tris, 0.01 M EDTA, and 0.1% Tween 20 at a pH of 7.5, filtered through a 0.22 μm cellulose acetate filter (before the addition of Tween) and then degassed. The extraction buffer contained BSA diluted to 0.05% with IGF-I assay buffer. The 0.25% BSA buffer was prepared with BSA diluted to 0.25% with IGF-I assay buffer. Secondary Ab buffer (IGF-I assay buffer and 4% polyethylene glycol [PEG 8000]) was used to dilute the secondary antisera to rabbit γ-globulin produced in goats (Calbiochem EMB Biosciences, San Diego, CA, USA). Carrier solution was prepared with 1% NRS diluted with IGF-I assay buffer.

Acid Extraction—Quantification of IGF-I in serum may be performed on total or free (unbound) portions of circulating IGF-I. The majority of IGF-I circulates bound to IGFBP which modulate the interactions of IGF-I with target tissue receptors and regulates the clearance of IGF-I, therefore controlling the biological activity of IGF-I (Jones & Clemmons, 1995; Firth & Baxter, 2002). Quantifying only the free fraction may significantly underestimate biologically active IGF-I (Zapf et al., 1986). We used a glycylglycine hydrochloric acid extraction technique to separate IGF-I from its constituent binding proteins and to quantify total serum IGF-I concentrations (Breier et al., 1991; Johnson et al., 1996). Johnson et al. (1996) reported a linear relationship between the gold standard, acid gel filtration (Sephadex G-50 chromatography in 1 M acetic acid) extraction method and the glycylglycine hydrochloric acid extraction method. Further, the glycylglycine extraction method had an extraction efficiency > 85%, which is sufficient to remove IGFBP interference from IGF-I quantification (Breier et al., 1991).

To initiate acid extraction, 0.2 M glycylglycine (pH 2.1) was added to serum in a 1:1 ratio in disposable polystyrene test tubes, vortexed, and then incubated at room temperature for 36 h. After incubation, an extraction buffer was added to each serum extraction tube in 1 ml increments for a total of 3 ml and vortexed vigorously between additions. Extracted serum (25 μl) was added to polypropylene test tubes as described below.

Radioimmunoassay for IGF-I—A standard curve was prepared through serial dilution of human IGF-I (Lot #01, National Hormone & Peptide Program) with 0.25% BSA buffer for a concentration range from 1,280 ng/ml to 20 ng/ml. Total count, NSB, TB, standards, high and low internal controls, and unknown samples were analyzed in quadruplicate. Standard bovine sera with high and low IGF-I concentrations were used for internal control tubes. Extracted serum (25 μl) was added to disposable polypropylene test tubes containing 175 μl of 0.25% BSA buffer for a total volume of 200 μl. Purified lyophilized primary Ab (AFP4892898, National Hormone & Peptide Program) was reconstituted with double-distilled water (1:5), and then further diluted to a 1:50 primary Ab stock with IGF-I assay buffer. Primary Ab was then diluted to 1:125,000 with IGF-I assay buffer and added to unknown sample, standard, and TB tubes in a 1:2 ratio. Volume of sera added to tubes was adjusted with 0.25% BSA buffer to ensure that hormone concentration was within the linear portion of the standard curve. The TB tubes received 200 μl of 0.25% BSA buffer instead of the sample, and NSB tubes received 300 μl of 0.25% BSA buffer in place of Ab and the sample. Tubes were vortexed and incubated for 24 h at room temperature.

Human IGF-I labeled with ^{125}I (PerkinElmer, Waltham, MA, USA; specific activity 215 uCi/ug, 7.9 MBq/ug as reported by PerkinElmer) was reconstituted with double-distilled water. When stored at 4° C ^{125}I-IGF-I produced reliable results for up to 6 wks, provided that the total binding was greater than 15%. Reconstituted ^{125}I-IGF-I was diluted with 0.25% BSA buffer and added to all tubes (100 μl) with approximately 10,000 cpm/tube. Tubes were vortexed and then incubated for 48 h at 4º C.

Secondary Ab (400 μl) was added to all tubes, except TC; vortexed; and incubated for 1 h at 4º C followed by the addition of 100 μl of the carrier solution to all tubes, except TC. Tubes were then vortexed and incubated for 1 h at 4º C.

After incubation, tubes were then centrifuged at 1,834 × g and 4º C for 30 min. After centrifugation, the supernate was gently aspirated from each tube without disrupting the pellet. The remaining radioactivity in the pellet was quantified with a gamma counter for 1 min.

The percentage of ^{125}I-IGF-I bound (B/Bo) was calculated for a concentration range from 20 ng/ml to 1,280 ng/ml. The value was plotted vs the log of the concentration of purified IGF-I in each standard point to establish a standard curve. The linear regression equation was then used to predict the IGF-I concentration in unknown samples based on the percentage of bound ^{125}I-IGF-I in each tube. Values are reported as ng of hormone per ml of serum after adjustment for volume of serum assayed. Replicates of the same sample were averaged. If the percent error between replicates was greater than 10%, or if concentration was outside the standard curve, the sample was re-analyzed.

Animals and Samples

Species used for assay validations included two families within the Order Carnivora—the Family Phocidae: grey seal (*Halichoerus grypus*), harbor seal (*Phoca vitulina*), harp seal (*Pagophilus groenlandicus*), and hooded seal (*Cystophora cristata*), and the Family Otariidae: Northern fur seal (*Callorhinus ursinus*) and Steller sea lion (*Eumetopias jubatus*)—and one species from the Order Cetacea—Family Monodontidae: beluga or white whale (*Delphinapterus leucas*). These species were chosen based on availability of appropriate quantities of serum necessary to perform validations, and due to our interest in studying the developmental, nutritional, gender, species, and growth effects of components of the somatotropic axis in these diverse marine species. Validations of heterologous RIA are critical for each new species studied to determine if analysis provides accurate and precise measures of hormone concentration. Sera samples from adult animals were collected as a part of routine veterinary care of collection animals housed at Mystic Aquarium & Institute for Exploration (MAIFE) in Mystic, Connecticut, unless otherwise noted. After collection, sera were frozen and stored at -80° C until analysis. Components of the somatotropic axis have been shown to be stable for up to 9 y in frozen sera (Yoshinori et al., 2005).

Cetacean—Blood was collected from one male and two female adult beluga or white whales (n = 3) permanently housed at MAIFE, and serum was pooled for validations. To test effect of time of day on hormone concentrations, whales were sampled at 0930 and 1330 h. Paired Student's t-test was used to evaluate time of day effects on hormone concentrations. Due to the small sample size, values were considered significant at $p \leq 0.10$.

Phocid—Four species of "true seals" commonly rehabilitated at MAIFE—the grey seal, the harbor seal, the harp seal, and the hooded seal were sampled to validate GH and IGF-I assays in phocids.

One serum pool was made for each of the four phocid species, except the harbor seal for which four separate serum pools were prepared to determine if substances that may interfere with the hormone quantification vary due to age or gender. An adult male harbor seal (n = 7) serum pool was created from serum collected from three individuals at MAIFE and four free-ranging harbor seals from Alaska. The Alaskan harbor seal samples were collected in collaboration with the National Marine Mammal Laboratory (NMML) in Seattle, Washington. The adult female harbor seal (n = 7) serum pool consisted of three animals from MAIFE and four free-ranging animals from Alaska. The male pup (n = 9) serum pool contained serum from six stranded pups rehabilitated at MAIFE and three harbor seal pups rehabilitated at The Marine Mammal Center (TMMC) in Sausalito, California. The female pup (n = 8) serum pool consisted of serum from rehabilitated harbor seal pups (n = 4, MAIFE; n = 3, TMMC) and one free-ranging Alaskan pup. All pups were less than 4 mo of age.

The other phocid serum pools were created from serum collected from stranded pups rehabilitated at MAIFE. The grey seal (n = 5) serum pool consisted of serum collected from three pups (< 2 mo of age) and two juveniles (< 24 mo of age). Sera from juvenile harp seals (n = 7; < 24 mo of age) were combined for use in the validation. The hooded seal (n = 4) serum pool was made from serum combined from juveniles (< 24 mo of age).

To explore the changes in hormone concentration associated with nutritional status, samples were collected from one individual from each of the four species of "true seals" rehabilitated at MAIFE. Serum and mass of individuals were collected weekly throughout the rehabilitation process (approximately 8 wks). Animal care and feeding were based on protocols outlined by Gage (2002) and Richmond et al. (2008). Caloric intake was recorded daily and reported as average daily intake for a given week. Briefly, animals were tube fed formula on a mass specific basis for approximately 4 wks, then weaned to a whole fish diet also fed on a mass specific basis.

Otariid—Northern fur seals and Steller sea lions were used to validate GH and IGF-I assays. Northern fur seal (n = 4) serum pools were made from serum collected at MAIFE (two males and two females). Four separate serum pools were created for Steller sea lions to evaluate whether substances that may interfere with hormone quantification vary due to age or gender. Adult

male and adult female serum pools were created from archived serum from MAIFE (four males and five females). Pup serum samples were collected from 1-mo-old free-ranging pups (four males and four females) in Alaska in cooperation with the Alaska Department of Fish and Game (ADFG).

Validation Parameters

Sensitivity—The sensitivity was determined by quantifying the lowest concentration of hormone that can be measured accurately (Davies, 2005). This value corresponds to the lowest detectable concentration within the linear portion of the standard curve at approximately 90% B/Bo.

Recovery of Added Mass—Additional hormone mass was added to marine mammal serum with the addition of known quantities of a distinct serum pool or porcine or human standard for GH and IGF-I assays, respectively (Davies, 2005). Recovery of added mass was calculated by dividing the measured concentration of hormone by the quantity of hormone added. Three different quantities of added mass, throughout the range of the standard curve, were determined for each serum pool. Values are expressed as the average percentage recovery ± SD.

Precision—Inter- and intra-assay coefficient of variation (CV) were used to evaluate assay precision (Davies, 2005). The CV was calculated by dividing the SD of reference sample replicates (four to six replicates) by the average of those replicates and multiplied by 100. Inter-assay CV was determined in all species and the assay internal controls with the exception of the Northern fur seal GH for which serum was limited. Inter-assay CV was evaluated in two to four replicate assays.

Parallelism and Dilution Linearity—Pooled serum was serially diluted with assay buffer to determine if detection of hormone for each species was similar throughout the concentration range of the assay or standard curve (Davies, 2005). If the serum pool did not contain enough native hormone to be diluted twice and still remain within the detection limits of the assay, standard hormone was added to the serum pool. This was only necessary for the harp seal in the IGF-I assay. Linearity was determined by linear regression analysis of observed hormone concentrations vs expected hormone concentration. Expected hormone concentration was calculated based on the original concentration measured in undiluted serum. Serially diluted pools with a correlation coefficient (R^2) and slope of approximately one were considered linear.

Results

Importantly, cross reactivity of GH antiserum with other prolactin family proteins was low (A. F. Parlow, pers. comm.). In addition, human-IGF-I antiserum has been used in rat, bovine, and porcine species, and the cross reaction to human-IGF-II was less than 1% (A. F. Parlow, pers. comm.).

GH RIA Validations

The sensitivity of the assay was 0.2 ng/ml. Average recovery of mass added was between 90 and 102% in all species evaluated (Table 1). Average concentrations and SD of serum pools are presented in Table 2. Assay precision measured by intra-assay CV was less than 10% except when hormone concentrations were at the lower end of detection for the assay < 1 ng/ml (Table 2). Intra-assay CV were less than 10% in all species except beluga whales with 15.0 and 12.0% CV for low and high pools, respectively. Inter-assay CV ranged from 3.6% in Steller sea lions to 17.7% in beluga whales (Table 2). All inter-assay CV were less than 15% except in beluga whales (17.7%) and grey seals (16.9%). Serially diluted pooled sera from all species were parallel to the standard curve (Figure 1). Linearity was excellent for all species (> 99%) except beluga whales and harp seals (96.0 and 92.0%, respectively) (Figure 2). In both harbor seals and Steller sea lions, validation parameters were similar among serum pools from different sexes and age categories. This confirms that comparisons among different physiological states may be performed because these assays quantify hormone concentrations similarly regardless of gender or age.

IGF-I RIA Validations

The sensitivity of the assay was 20 ng/ml. Average recovery of mass added was between 93 and 103% in all species evaluated (Table 1). Average concentrations and SD of serum pools are presented

Table 1. Recovery of added mass for growth hormone (GH) and insulin-like growth factor (IGF)-I assays in seven marine mammal species

Species	GH	IGF-I
Otariid		
Steller sea lion	102 ± 7.4	99 ± 3.6
Northern fur seal	97 ± 14.9	95 ± 9.7
Phocid		
Grey seal	93 ± 3.3	103 ± 12.6
Harbor seal	95 ± 4.2	93 ± 3.1
Harp seal	96 ± 0.9	93 ± 8.0
Hooded seal	90 ± 0.7	102 ± 12.5
Cetacean		
Beluga whale	97 ± 7.4	97 ± 7.8

Note: Values presented are expressed as a percentage of mass recovered by mass added and shown as mean ± SD.

Table 2. Precision of growth hormone (GH) and insulin-like growth factor (IGF)-I assays estimated by percentage of coefficient of variation (CV)†

	GH				IGF-I			
	Mean (ng/ml)	SD	Intra-assay CV	Inter-assay CV	Mean (ng/ml)	SD	Intra-assay CV	Inter-assay CV
Porcine or Bovine‡								
High	5.0	0.42	3.9	8.5	149.3	16.1	2.0	10.8
Mid	2.1	0.18	2.8	8.7	--	--	--	--
Low	0.6	0.07	4.8	11.4	60.8	9.5	6.4	15.6
Cetacean								
Beluga whale high	1.7	0.33	11.2	17.7	264.5	33.8	9.0	12.8
Beluga whale low	0.6	0.07	12.2	--	176.6	16.2	11.1	9.2
Otariid								
Northern fur seal high	3.7	0.24	6.4	--	386.3	33.0	7.3	8.5
Northern fur seal low	1.0	0.03	2.6	--	73.2	8.1	9.9	11.1
Steller sea lion high	10.7	0.39	4.0	3.6	238.4	15.4	5.1	6.4
Steller sea lion low	5.9	0.45	2.0	7.6	173.0	13.4	5.7	7.8
Phocid								
Grey seal high	10.4	1.76	4.9	16.9	157.2	7.2	4.9	--
Grey seal low	0.7	0.07	9.4	--	45.9	1.6	7.9	3.5
Harbor seal high	7.9	0.62	4.7	7.8	309.6	24.6	10.9	--
Harbor seal low	0.2	0.02	7.5	--	39.7	5.1	7.4	12.9
Harp seal high	21.6	2.52	4.0	11.7	139.6	10.8	7.7	--
Harp seal low	3.5	0.23	6.5	--	12.5	1.7	5.1	13.3
Hooded seal high	21.6	3.17	6.4	14.7	--	--	--	--
Hooded seal low	1.5	0.16	10.7	--	54.9	4.1	7.3	7.7

†The CV was calculated by dividing the SD of reference sample replicates by the average of those replicates and multiplied by 100.

‡ Pooled porcine serum was used for the GH assay, while pooled bovine serum was used for the IGF-I assay for internal controls.

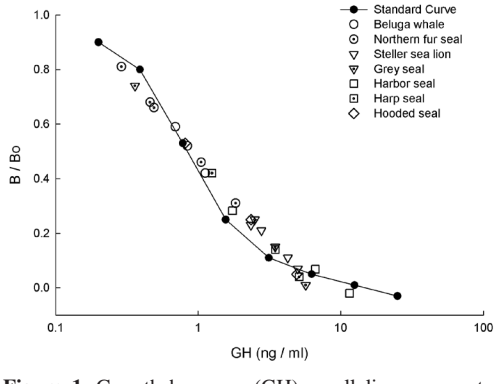

Figure 1. Growth hormone (GH) parallelism; concentrations of GH in marine mammal serum was parallel to the standard curve when serially diluted. Greater quantities of serum elicited a greater displacement of labeled porcine-GH compared with smaller quantities of serum in a manner parallel with the standard curve.

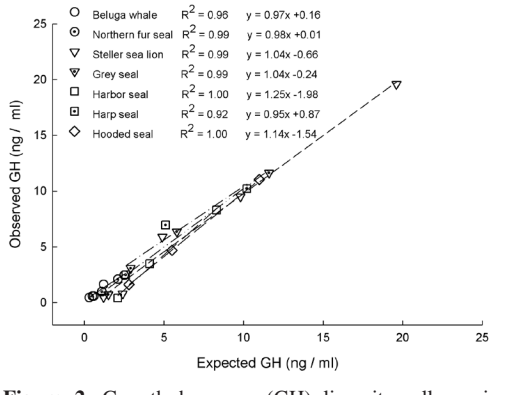

Figure 2. Growth hormone (GH) linearity; all species exhibited significant linear regressions with correlation coefficients and slopes close to 1.0. Linear regression equations and correlation coefficients (R^2) for each species pool are displayed on the graph.

in Table 2. Assay precision measured by intra-assay CV was less than 10% except when concentrations were at the lower end of detection of the assay: > 80% B/Bo (Table 2). Intra-assay CV were less than 10% in all species except beluga whales, which had a CV of 11.0%. Inter-assay CV ranged from 3.5% in grey seals to 15.6% in the bovine low pool. All marine mammal inter-assay CV were less than 15%. Serially diluted pooled sera from all species were parallel to the standard curve (Figure 3). Linearity was excellent (≥ 99%) for all species except hooded seals (97.0%) (Figure 4). In hooded seals, the slope of observed IGF-I concentration vs expected concentration was < 1 (slope = 0.68); however, the correlation coefficient was good (R^2 = 0.97). Concentrations of IGF-I in hooded seal serum were low (55 ng/ml), which may have contributed to the reduced slope. In both harbor seals and Steller sea lions, validation results were similar among serum pools regardless of age or gender of the serum pool tested. This confirms that comparisons among different physiological states may be performed because these assays quantify hormone concentrations similarly regardless of gender or age.

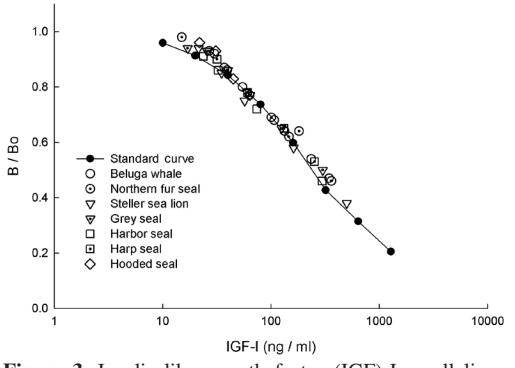

Figure 3. Insulin-like growth factor (IGF)-I parallelism; concentrations of IGF-I in marine mammal serum was parallel to the standard curve when serially diluted. Greater quantities of serum elicited a greater displacement of labeled human-IGF-I compared with smaller quantities of serum in a manner parallel with the standard curve.

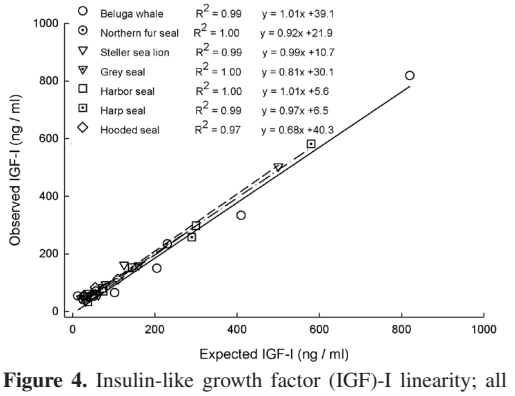

Figure 4. Insulin-like growth factor (IGF)-I linearity; all species, except hooded seals, exhibited significant linear regressions with slopes and correlation coefficients close to one. Linear regression equations and correlation coefficients (R^2) for each species pool are displayed on the graph.

Animal Experiments

In beluga whales, there was little variation in IGF-I concentration during the day (p = 0.61), while GH concentration declined in the afternoon (p = 0.10) (Figure 5).

Rehabilitated phocid species arrived at MAIFE thin and in a low nutritive state (Richmond et al., 2008). Intake increased and animals gained body mass throughout rehabilitation (Figures 6A & B). Concentrations of GH were greatest at nutritional nadir upon arrival and declined gradually with re-alimentation (Figure 6C). In contrast, IGF-I

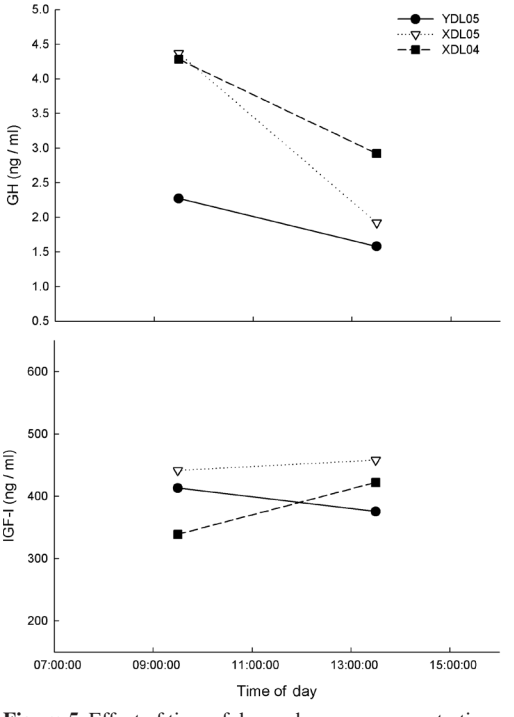

Figure 5. Effect of time of day on hormone concentrations; beluga whales were sampled at two time points during the same day to evaluate the effect of time of day on hormone concentrations. Each animal is represented by a unique symbol and a line connects their morning hormone concentration with the afternoon concentration. Concentrations of growth hormone (GH) declined in the afternoon (p = 0.10), while insulin-like growth factor (IGF)-I concentrations remained constant (p = 0.61).

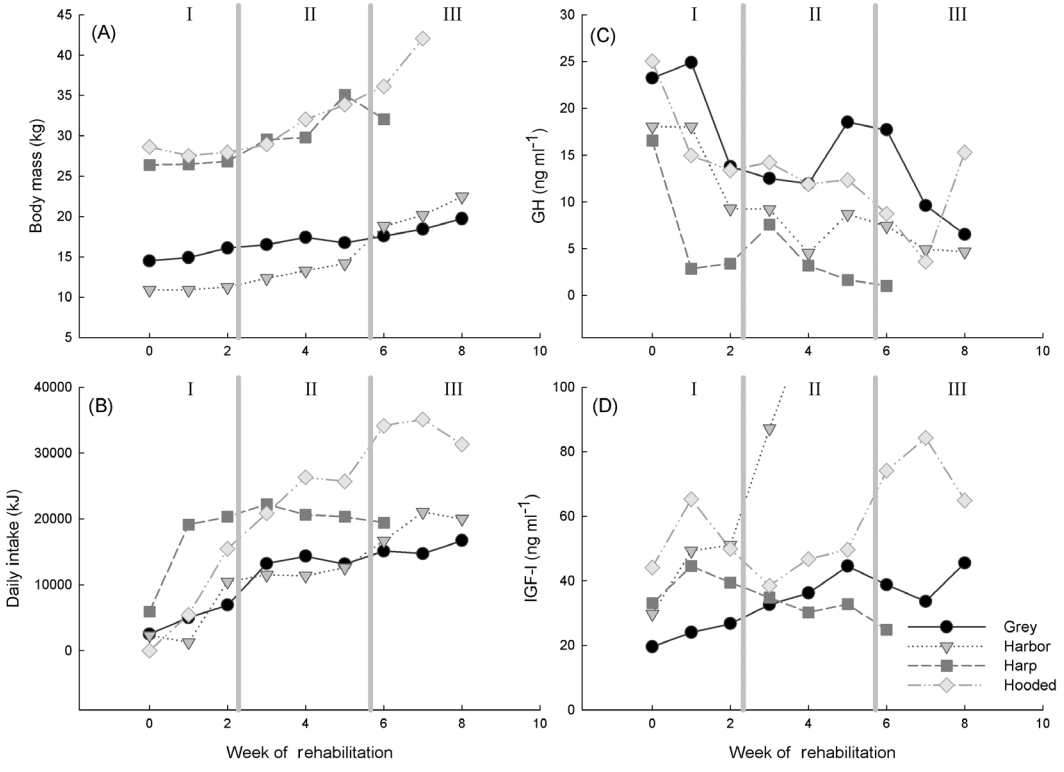

Figure 6. Changes in (A) body mass, (B) daily intake, (C) growth hormone (GH), and (D) insulin-like growth factor (IGF)-I concentrations in four species of stranded seals throughout the rehabilitation process; daily intake was reported as average values for a given week. The three phases of rehabilitation are delineated by vertical grey bars and identified with Roman numerals. Individuals were initially fed formula (Phase I), weaned (Phase II), and then changed to a solid fish diet (Phase III). Lines connect data points from individual animals.

concentrations were initially low and increased with re-alimentation (Figure 6D).

Hormone concentrations in harbor seal and Steller sea lion adult, pup, male and female serum pools were measurably different. Generally, GH was greater in pup serum pools (harbor seal: male 5.8 and female 6.9 ng/ml; Steller sea lion: male 9.7 and female 6.2 ng/ml) compared with adult serum pools (harbor seal: male 0.2 and female 4.7 ng/ml; Steller sea lion: male 0.8 and female 3.8 ng/ml), and IGF-I was greater in adult serum pools (harbor seal: male 297.1 and female 240.7 ng/ml; Steller sea lion: male 502.6 and 220.1 ng/ml) compared with pup serum pools (harbor seal: male 44.1 and female 31.2 ng/ml; Steller sea lion: male 161.6 and female 169.7 ng/ml) in both species.

Discussion

Radioimmunoassays are a common tool used by endocrinologists to quantify hormone concentrations. The RIA is a competitive binding assay that requires the hormone of interest to compete with a radio-labeled hormone for binding with a limited quantity of primary Ab. To develop a homologous RIA, large quantities of highly purified protein hormone are required to create a standard curve and a competitive radio-labeled ligand. Once purified protein is obtained, a primary Ab specific to the hormone of interest must be developed. This is a labor- and time-intensive process, especially when considering development in multiple species.

Heterologous assays are often chosen over homologous assays due to the difficulty in developing primary Ab for multiple species and the complexity of obtaining large quantities of purified hormone. Heterologous RIA use a primary Ab, protein standard, and radioactive labeled protein from a species different than the species of interest. Since the Ab is raised against an antigen from a different species, usually human, mouse, or rat, concentrations are often expressed as immunoreactive equivalents (i.e., human equivalents). This is an important distinction since actual bioactive quantities of a given protein may be different

from the quantities measured using a heterologous assay. These types of assays provide a relative measure that is often comparable with the actual quantity, but this limitation is important to note. Essential to using a heterologous assay is validation of the assay in each species of interest to ensure accuracy and precision of measurements. For RIA, sensitivity, parallelism, and percent recovery are the minimum validation parameters that must be evaluated (Davies, 2005).

While the GH protein is highly variable among species (Scanes & Campbell, 1995), previously published research was successful in measuring GH concentration in northern elephant seals (Ortiz et al., 2003) with a commercially available kit containing a similar porcine GH Ab to the one used in this study. Since the structure of IGF-I is highly conserved among species and is virtually identical among mammalian species (Foyt et al., 1991), anti-human IGF-I antisera is commonly used as primary Ab for quantification of IGF-I in many different mammalian species. Commercially available kits provide reliable results as long as appropriate validations are performed; however, costs associated with commercial kits are often prohibitive to large endocrine studies (>100 samples). The methods outlined in this study are less expensive than commercial kits if large numbers of samples are analyzed. The two assays that were chosen to validate in seven marine mammal species have been used for many years as homologous assays for quantifying porcine and bovine GH and IGF-I concentrations, respectively (Barb et al., 1991; Johnson et al., 1996). Mean percent recovery was high, close to 100%, for both assays in all species measured, indicating that these assays consistently recover a high proportion of hormone in serum. The intra-assay CV was low, indicating that precision and repeatability within an assay is high. Inter-assay CV values were also consistent, indicating a high degree of precision across repeated assays. Even though inter-assay CV was not determined for Northern fur seals, there is no reason to suspect the variation would be greater than what was observed in other species, especially given the low intra-assay CV.

An important consideration when evaluating hormone concentrations is that the standard curve is only accurate in predicting concentration in the linear portion of the curve. Binding plateaus at the low and high end of the curve and, therefore, concentrations cannot be accurately measured because very small changes in the percentage of bound ligand will result in large changes in predicted concentration. Serum volumes should be adjusted to keep concentrations within the linear portion of the curve to ensure accurate assessment of hormone concentrations.

Beluga whales exhibited a slightly greater intra-assay CV for GH, which may suggest that this Ab may not bind as well to beluga GH compared with pinniped GH. However, this CV was less than 15% in very low concentrations of GH, and within range of homologous assays using this and similar Ab (Barb et al., 1991; Ortiz et al., 2003). Samples in this study were from captive adults. Concentrations of GH in free-ranging adults and especially in young, growing animals will likely be much greater. In adult whales, increased GH secretion may be associated with seasonal changes in prey type and availability if those changes resulted in a negative energy balance (Seaman et al., 1986; Breier, 1999). Young, growing animals typically have greater GH concentrations compared with other age classes and are associated with an elevated growth rate (Harrell et al., 1999; Govoni et al., 2003).

Both assays exhibited excellent parallelism and linearity. Appropriate concentrations of hormone were quantified throughout the range of the standard curve. There was relatively less IGF-I recovered than expected in the serum pool from hooded seals. This may have been due to the low concentration of IGF-I in that serum pool and/or the increased variation at the low end of the curve.

Importantly, using these assays, we have demonstrated that serum concentrations of GH and IGF-I change with age, gender, and nutrient intake, indicating that we can quantify differences in these hormones with changes in the physiological state of marine mammals (Richmond, 2008). In beluga whales, the difference in GH concentrations when serum was collected in the morning vs the afternoon may suggest a responsiveness of GH to nutrient intake. Concentrations of GH were greater in the morning after an overnight fast, and then declined in the afternoon after the morning meal. Further analyses should be conducted to elucidate changes in the diurnal pattern of GH secretion and the response of GH to nutrient intake. Rehabilitated seals provide a unique model to study the effects of nutrient intake on the somatotropic axis because they arrive at facilities in a naturally fasted state (Richmond et al., 2008). For example, upon arrival at MAIFE, rehabilitated phocid species were thin and in a low nutritive state. Seals likely had been fasting for several days. The initial hormone concentrations, therefore, represent a nutritional nadir (see Figure 6). Similar to domestic species, GH declined and IGF-I increased with increased nutrient intake (Breier, 1999; Harrell et al., 1999; Rausch et al., 2002).

Differences in serum pools of harbor seals and Steller sea lions illustrate the ability of these assays to quantify age-related differences in hormone concentrations. Young sea lions and harbor seals

had GH concentrations that were greater than conspecific adults. Increased GH concentration is usually associated with an elevated growth rate that is characteristic of a young growing animal (Etherton & Bauman, 1998).

In domestic species, the somatotropic axis is a good indicator of nutritional status and a predictor of future growth rate (Breier, 1999; Harrell et al., 1999; Govoni et al., 2003). In addition, these metabolic hormones are key to many physiological processes, including lean tissue and adipose accretion, lactation, and energy partitioning (Etherton & Bauman, 1998). These types of data may be useful in studying the role of the somatotropic axis in energy partitioning and energy conservation, and the physiological regulation of growth and development in species with unique and varied life history traits. Validation of these assays in multiple species of marine mammals opens significant and varied opportunities for intra- and inter-species comparisons.

From an applied perspective, once baseline values of these hormones are quantified and once we understand the normal developmental pattern, we will be able to develop a model using the somatotropic axis to assess nutritional status of individuals and populations of marine mammals. This tool will be critical in the coming years as the effects of environmental change on these species and their habitats become even more evident. Changes in prey availability will continue to influence young, growing animals most severely and may influence long-term survival and fecundity of older animals (Horning & Trillmich, 1999; Muelbert et al., 2003). A model to assess nutritional status and the effects of nutritional deficit on wildlife will be an important tool for managers to assess and maintain healthy sustainable populations of marine mammals.

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