Testosterone Trends Within and Across Seasons in Male Humpback Whales (*Megaptera novaeangliae*) from Hawaii and Alaska

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Abstract

Understanding reproductive profiles and timing of reproductive events is essential in the management and conservation of humpback whales (*Megaptera novaeangliae*).
Yet compared to other parameters and life history traits, such as abundance, migratory trends, reproductive rates, behavior and communication, relatively little is known about variations in reproductive physiology, especially in males. Here, an enzyme immunoassay (EIA) for testosterone was validated for use in biopsy samples from male humpback whales. Analyses were conducted on 277 North Pacific male humpback whale blubber samples, including 268 non-calves and 9 calves that were collected in the Hawaiian breeding grounds and the Southeast Alaskan feeding grounds from 2004-2006. Testosterone concentrations (ng/g) were significantly different between non-calves sampled in Hawaii (n=182) and Alaska (n=86, $p<0.05$) with peak testosterone concentrations occurring in the winter (January-March) and the lowest concentrations occurring in the summer (June-September). Fall and spring showed increasing and decreasing trends in testosterone concentrations, respectively. Blubber testosterone concentrations in non-calves and calves sampled in Alaska were not significantly different. Blubber and skin from the same individual biopsies (n=37) were also compared, with blubber having significantly higher testosterone concentrations ($p<0.05$) than skin samples. We found variability in testosterone concentration with age, suggesting that male humpbacks reach peak lifetime testosterone concentrations in the breeding grounds around age 8-25 years. The testosterone profile of male humpback whales follows a predictable pattern for capital breeders, where testosterone begins to increase prior to the breeding season, stimulating the onset of spermatogenesis. Incorporation of reproductive hormonal profiles into our overall understanding of humpback whale physiology will shed additional light on the timing of reproduction and overall health of the recently delisted Hawaii distinct population segment (DPS).

Keywords: testosterone, reproduction, health, *Megaptera novaeangliae,* humpback whale

1. Introduction

Understanding reproductive trends is an essential component in long-term monitoring of any species. Knowledge of the temporal and spatial nuances surrounding reproductive events is critical for assessing population growth rates and allows managers to create effective strategies for mitigation of anthropogenic disturbances during these reproductively sensitive times. In addition, significant deviations from the reproductive timeline of a healthy, growing population could be indicative of wider marine ecosystem changes. Of the mysticete species, the humpback whale (*Megaptera novaeangliae*) is arguably the most extensively studied (Clapham, 1996; Gabriele et al., 2017; Pack et al., 2017). Yet, compared to other parameters and life history traits, such as abundance, migratory trends, reproductive rates, behavior and communication (Baker et al., 1985; Barlow et al., 2011; Chittleborough, 1965; Clapham et al., 1992; Clapham and Mayo, 1990; Craig et al., 2003, 2002; Gabriele et al., 2007; Helweg and Herman, 1994; Tyack and Whitehead, 1983), relatively little is known about variations in reproductive physiology, especially in males (Chittleborough, 1955; Vu et al., 2015).

Age at sexual maturity for humpback whales is known to vary by population. On the US east coast humpback whale males and females attain sexual maturity at approximately 5 years of age, with the age at first calving occurring between 5-7 years (Chittleborough, 1965; Clapham et al., 1992). In the North Pacific, female sexual maturity is thought to be attained later, where the mean age of first calving is ~11.8 years (Gabriele et al., 2010, 2007). It is unknown whether males in the North Pacific age at sexual maturity is the same as is reported in whaling literature, yet one known aged male (8 years) has been observed singing in Glacier Bay National Park (Gabriele personal communication, 2018).

Reproduction in all but the Arabian Sea population of humpback whales (Mikhalev, 1997) is based around an annual migration from high latitude nutritionally productive feeding grounds to low latitude warm breeding grounds on which all but calves-of-the-year fast (Baker et al., 1985; Chittleborough, 1965; Katona and Beard, 1990), although occasional feeding on some breeding grounds has been observed (Gendron, 1993). Humpback whales of both sexes and all age classes migrate between feeding and breeding grounds with migratory timing a function of sex, age class, and reproductive and nutritional condition (Chittleborough, 1965; Craig et al., 2003; Straley et al., 1994). The exact triggers for the initiation of migration from the feeding grounds to the breeding grounds are still debated and may involve several interacting factors such as photoperiod, hormonal state, body condition and food availability (Baker et al., 1985; Craig et al., 2003). While still on the feeding grounds, humpback whale males begin to exhibit aggressive behavior toward conspecifics and have been heard singing in late fall to early winter

(Gabriele and Frankel, 2002; Straley et al., 1994). On the breeding grounds, male humpback whales, presumably prospecting for mating opportunities, often singly escort lone females, as well as those with a calf (Craig et al., 2002; Mobley and Herman, 1985). When two or more escorts are present, they typically compete with each other through physical displays and aggression for spatial proximity, and presumably mating access to the female, (Clapham et al., 1992; Herman et al., 2007; Tyack and Whitehead, 1983) with larger males tending to attain the role of principal escort (i.e. the male defending the position closest in proximity to the female) (Pack et al., 2012; Spitz et al., 2002). Also on the breeding grounds, lone male humpbacks and occasionally those accompanying mother-calf pairs produce a complex, ordered and hierarchically organized series of vocalizations termed “song” (Payne and McVay, 1971), that may be repeated for hours (Helweg and Herman, 1994). Individual males within a breeding area sing asynchronously (Au et al., 2006). Although portions of a song may change within and between a breeding season, all males on the same breeding area tend to converge on the same rendition of song (Garland et al., 2011; Payne and Payne, 1985). Cultural transmission of song may also occur across breeding areas (Noad et al., 2000).

While the absolute functions of song are still debated (Herman, 2017), it has been proposed that singing may be stimulated by male hormonal changes (Clark and Clapham, 2004; Herman, 2017; Straley et al., 1994), as occurs in birds singing seasonally (Marler et al., 1988; Nottebohm et al., 1987). Likewise, even though the act of successful male-female copulation has yet to be witnessed (Herman et al., 2007; Pack et al., 2002), the types of associations involving male humpbacks and their behavior in the breeding grounds (Clapham, 1996; Clapham and Mayo, 1990; Craig et al., 2003, 2002, Pack et al., 2012, 2009; Spitz et al., 2002) are likely to be associated with hormonal changes. Morphological studies of male gonads and examination of sperm count and fertility in male humpbacks reported that male humpback whales taken by whalers on breeding grounds had higher sperm counts than males on the feeding grounds (Chittleborough, 1955). However, a complete understanding on how reproductive hormone levels vary within and between breeding and feeding grounds is lacking.

Testosterone is one of the main androgens in mammals. Released by the Leydig cells in the testes and to a lesser extent from the adrenal glands, testosterone triggers spermatogenesis, can alter behavior, affects both primary and secondary sexual development such as muscle mass and sex drive, and indicates the onset of sexual maturity (Atkinson and Yoshioka, 2007; Sharpe et al., 1992). As such, testosterone levels have a direct effect on reproductive success in males (Kita et al., 1999). Higher testosterone levels have been linked to increased aggression in male mammals (Bouissou, 1983), the ability for males to move upward in social hierarchies (Beehner et al., 2006) and altered behavior in the breeding season, such as roving (Burgess et al., 2012). Conventional thinking holds that in seasonal breeders, serum testosterone concentrations exhibit a cyclical trend, reaching a peak before mating begins, and then falling post-mating season (Schroeder and Keller, 1989). This seasonal trend holds true for three previously studied cetacean species. In the Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) testicular endocrine function increases in the spring (*i.e.,* the onset of breeding season), before testosterone concentrations reach a maximum in the summer (Funasaka et al., 2011). Similarly, in the North Atlantic fin whale (*Balaenoptera physalus*) and North Atlantic minke whale (*Balaenoptera acutorostrata*), increasing testosterone concentrations were observed prior to the breeding season (Kjeld et al., 2006, 2004). Thus far, only one published paper has examined seasonal trends of testosterone in male humpback whales. Focused on the Mexico distinct population segment (DPS; a DPS is a vertebrate population or group of populations that is discrete and significant in relation to the entire species), which exhibits feeding fidelity in California and Washington, a recent study found that testosterone exhibits a yearly parabolic trend with the highest concentrations occurring in the breeding season (Vu et al., 2015). To date, no study has examined testosterone concentrations or trends for the Hawaii DPS of male humpback whales, despite this being the primary breeding grounds of North Pacific humpback whales (Barlow et al., 2011).

The purpose of the present study was to compare concentrations of testosterone in male humpback whales in both the breeding grounds of Hawaii and the feeding grounds of Southeast Alaska (which contain large numbers of whales migrating to and from Hawaii) (Barlow et al., 2011; Calambokidis et al., 2008), in order to test the assumption that testosterone concentrations are higher during the breeding season than the feeding season. Blubber is the current gold standard for understanding hormonal trends in free-ranging large, cetaceans and is thought to be a good approximation of current circulating hormones in blood serum (Champagne et al., 2017). Specific objectives of this project were to determine from blubber 1) if testosterone concentrations are spatially and temporally dependent, 2) if age class correlates with testosterone concentration, and 3) if testosterone concentrations vary between blubber and skin samples.

2. Materials and Methods

2.1. Study areas

Humpback whale males of the Hawaii DPS that exhibit feeding fidelity to Southeast Alaska (SEAK) were examined in this study (Figure 1). Blubber and skin biopsy samples were collected from two locales: 1) Southeast Alaska, including Sitka Sound (57.0˚N 135.5W˚), Chatham Strait (56.95˚N 134.62˚W), Frederick Sound (57.13˚N 134.10˚W), Lynn Canal (58.4˚N 134.8˚W) and waters west of Prince of Wales (55.95˚N 132.48˚W), and 2) the Hawaiian islands, specifically the Au'au, Kalohi and Pailolo channels between Maui, Moloka'i, Lana'i and Kaho'olawe (20.89˚N 156.68˚W) and off the North Kohala Coast of Hawai'i Island (19.98˚N 155.87˚W).

2.2. Sample collection

2.2.1. Biopsy sampling

Samples were collected during Structure of Populations, Levels of Abundance, and Status of Humpbacks (SPLASH) project (Calambokidis et al., 2008). SPLASH was an international collaborative study of humpback whales across different North Pacific feeding and breeding grounds including Hawaii and Southeast Alaska from 2004-2006. Following SPLASH protocols, tissue samples of humpback whales were obtained using a hollow stainless-steel-tipped retrievable floating dart fired from either a crossbow or modified pneumatic rifle while paralleling the whale from a small vessel usually at a distance of 10-20m. Tissue samples were retrieved and removed from the dart tip with sterile tweezers and placed in 1.5ml cryovials or the whole tip was placed in a sterile container for later processing. The sample was kept cool while in the field and, once extracted from the biopsy tip, the samples were frozen at -20˚ or -80˚C in each researcher's respective lab and eventually archived at the National Marine Fisheries Service (NMFS) Southwest Fisheries Science Center (SWFSC) Marine Mammal and Turtle Division.

2.2.2. *Sample selection*

Samples used in this study (n=277) were randomly selected from the pool of samples collected during SPLASH in Hawaii and Alaska when whales were present in these waters to capture the cyclical variation in physiological parameters of humpback whales throughout their migration. Samples were classified according to the sample type (skin or blubber), location where the biopsy was obtained (Alaska or Hawaii), date of collection (day, season). Seasons were defined as follows: fall (September 16-January 15), winter (January 16-March 15), spring (March 16-June 15), and summer (June 16-September 15).

2.3. *Data collected about each whale*

2.3.1. *Photographic identification using natural markings*

Identification photographs (photo-id) of the tail flukes of tissue-sampled humpback whales were collected either prior to or after the biopsy was obtained. Humpback whales can be identified by the unique black and white pigmentation patterns on the ventral surface of their flukes along with the distinctive trailing edge (Katona et al., 1979). To verify and link the biopsy to a specific whale, dorsal fin photos were also collected during the fluke id and biopsy processes. Whales with a photograph were matched to regional catalogs and to the SPLASH catalog. Consequently, an individual whale may have multiple identifying numbers but the unifying number across both areas is the SPLASH ID.

2.3.2. *Determining age-class and reproductive status for an individual whale*

Age-class of whales was determined from field notes that accompanied the samples. Calves were designated based on their small size (ca. < 5 m) (Pack et al., 2017, 2009) and close spatial association with an adult-sized whale (i.e. its mother) that displayed nurturant behavior (e.g. shielding the small-sized whale with its pectoral fin) (Gabriele et al., 2017; Glockner-Ferrari and Ferrari, 1985). All other whales were considered non-calves. Sighting histories from regional databases of individual humpback whales with a regional identification number that was matched to an individual SPLASH ID were used to determine whales of known age or a minimum age for whales whose exact age was unknown. Whales of known age were first sighted as calves. The minimum age of a whale who was photographed prior to the SPLASH project as an adult was calculated as the number of years from the earliest sighting to the most recent sighting plus two years (to account for the individual's year as a calf and year as a yearling). For example, the known age of a whale photographed during the study in 2006 who was originally photographed in 1994 as a calf would be 12 years, whereas the minimum age of a whale photographed in 2006 who was originally photographed as an non-calf in 1994 would calculate as 14 years. Minimum age thus represents a conservative estimate of age.

2.4. *Sex and genetic identification*

Oregon State University Cetacean Conservation and Genomics Laboratory conducted genetic analyses and sex determination on the samples as part of the post-collection aims of the SPLASH effort (Baker et al., 2013). Each whale was given a unique genetic ID which was used to match whales under one SPLASH ID when photographs were of too poor quality to do so.

2.5. Hormone extraction

Hormone extraction methods were modified from those described in Mansour et al. (2002) and Kellar et al. (2006). Sub-samples contained only one type of pure tissue (i.e. either blubber or skin) from a single biopsy. Blubber and skin samples were weighed and recorded weights were between 0.12 g and 0.20 g. Samples were homogenized using a Teflon hand tool in 500 µl of 100% ethanol. They were then processed at 3,000 rcf in a refrigerated centrifuge for 15 minutes and 500 ul of supernatant was poured into sterile 12 x 75 mm borosilicate disposable glass culture tubes. This step was repeated to obtain 1,000 µl of collected supernatant. Supernatants were evaporated under compressed air. Two ml of ethanol:acetone (4:1) were added to the residue, vortexed, and centrifuged (15 min). The supernatant was transferred to a new glass culture tube and evaporated. To this new residue, 1 ml diethyl ether was added and the samples were again vortexed, centrifuged, transferred to clean glass tubes, and evaporated. Acetonitrile (1 ml) was added and samples were vortexed before 1 ml of hexane was added and vortexed. Samples were centrifuged (15 min) and the solvents formed two immiscible layers with hexane on top. The acetonitrile layer was collected and re-extracted with 1 ml hexane, centrifuged (15 min), and the final acetonitrile layer was aspirated and evaporated.

2.6. Enzyme immunoassay (EIA)

Testosterone concentrations were measured using Enzo Life Science kit (ADI-900-065) and procedures were performed according to the manufacturer's protocol. Assay plates were read by a plate reader (Chromate, Awareness Technologies) at 405 nm. Manufacturer cross-reactivity with other steroids was as follows: 19-hydroxytestosterone (14.64%), androstenedione (7.20%), dehydroepiandrosterone (0.72%), estradiol (0.40%) and less than 0.001% for all other steroids analyzed. Assay parallelism and accuracy tests were performed in order to validate use of humpback whale blubber for measuring testosterone in EIA. A pooled blubber sample for male humpback whales was created to validate the testosterone assay. Serial dilutions (neat to 1:16) of the pool exhibited displacement parallel to that of the standard curve and proved accurate ($y=3.40 + 0.90x$, $r^2=0.99$) in the amount of testosterone measured. Inter-assay coefficient of variation for three assay controls were 16%, 8%, and 9%, respectively and intra-assay coefficient of variation fell below 10%. The lower limit of detection (LD) was 3.9 pg/ml with 62 out of 277 samples (22%) falling below this threshold. Substitution in the form of $LD/\sqrt{2}$ was performed for these 62 samples, a process that is accepted if less than 25% of samples are substituted and there is only one LD (Croghan and Egeghy, 2003; LaFleur et al., 2011; US EPA, 2000).

2.7. Statistical Analyses

Temporal and spatial differences in blubber testosterone concentrations were analyzed using a Welch's t-test or a one-way ANOVA in the programming language Python (Python Software Foundation. Python Language Reference, version 3.6.6. Available at http://www.python.org). If a significant result ($p<0.05$) was found in the ANOVA test, a Tukey's Honestly Significant Difference (HSD) test was performed to determine which groups differed significantly from each other. The spatial and temporal range of variation in testosterone concentration was depicted by boxplots which show the mean and nominal range of the data inferred from the upper and lower quartiles, as well as outliers in the data. T-tests (Welch's t-test and paired t-test), ANOVA, Tukey's HSD test and boxplot analyses were also performed to examine any difference between calves and non-calves and between blubber and skin sample types. Additionally, a Pearson Correlation Test was conducted to determine any potential relationships between blubber and skin testosterone concentrations.

3. Results

A total of 277 tissue samples (268 male non-calves, 9 male calves) were analyzed for testosterone. Ten individually identified whales were sampled in consecutive years in both Alaska and Hawaii.

3.1. Testosterone concentration by location and season

Testosterone concentration in blubber samples from non-calf humpback whales was significantly different from whales sampled in Hawaii (n=182, 0.96 ± 0.70 ng/g (mean ± standard deviation)) than those sampled in Alaska (n=86, 0.15 ± 0.40 ng/g) (Welch's t-test, $p<0.05$, Figure 2). When binned by season, the concentrations of testosterone from highest to lowest were winter (n=128, 1.10 ± 0.74 ng/g), spring (n=53, 0.65 ± 0.52 ng/g), fall (n=31, 0.44 ± 0.64 ng/g), and summer (n=57, 0.07 ± 0.08 ng/g) (Figure 3). Testosterone concentrations were not significantly different between fall and spring, whereas all other pairings of seasons were significantly different (n=268, $p<0.05$, ANOVA and Tukey's HSD test).

Spring was the only season during which biopsies were collected from whales in both Alaska and Hawaii. The median date of collection for whales biopsied in the spring in Alaska was June 2nd, whereas the median date of collection in Hawaii was March 31st. Whales in spring (n=52) located in Alaska (n=4, 0.06 ± 0.02 ng/g) had significantly different testosterone concentrations than whales that were located in Hawaii (n=48, 0.70 ± 0.51 ng/g, $p<0.05$, Welch's t-test, Figure 4).

When examined on a monthly time scale (combining data from Hawaii and Alaska), testosterone concentrations showed a parabolic relationship, peaking in January and February, declining to the lowest levels in June and July, and increasing as fall progressed (n=268, Figure 5). When only Hawaii samples were considered, a peak testosterone concentration occurred in January followed by a decrease in testosterone concentration over the course of the breeding season (Figure 6). Furthermore, the testosterone concentrations of four whales who were biopsied twice during the same breeding season in Hawaii all decreased from the earlier to the later sample (i.e. as the breeding season progressed) (Figure 7).

3.2. Testosterone concentration from individual whales biopsied in both Hawaii and Alaska

Tissue samples were obtained for 10 individually identified whales in both Hawaii and Alaska in consecutive years with three individuals (470736, 474074, 474110) having replicate samples in one or more sampling locations for a total of 24 blubber samples (Table 1). For all but one individual, testosterone was higher in Hawaii (12 biopsies, 0.73 ± 0.43 ng/g) than Alaska (12 biopsies, 0.09 ± 0.09 ng/g). The exception was whale 470452 who showed higher testosterone when located in Alaska, rather than Hawaii. Examination of the accompanying field notes did not provide any indication as to why this might be, other than this sample was the latest collected (on Oct 25th) for the 10 whales biopsied in Alaska.

3.3. Testosterone concentration in blubber and skin

Blubber and skin samples from the same whales (n=37) were compared, with blubber samples having significantly different testosterone concentrations than skin samples (paired t-test, $p<0.05$). When blubber and skin samples were also binned by geographic location, testosterone concentration was significantly different only for Hawaii blubber samples (n=20, 0.88 ± 0.48 ng/g) versus Hawaii skin (n=20, 0.35 ± 0.38 ng/g), with no significant difference detected between Alaska blubber (n=17, 0.14 ± 0.26 ng/g), Alaska skin (n=17, 0.07 ± 0.02 ng/g), and Hawaii skin ($p<0.05$, Figure 8).

3.4. Testosterone by age

While there were not enough data to conduct a robust statistical analysis of difference in calves from Alaska (n=7) and Hawaii (n=2), it appears from plotting the data that testosterone concentrations were similar in each location. There was enough data to determine that testosterone concentrations in non-calves and calves from Alaska were not significantly different (p=0.14, Figure 9).

The exact age was available from long term sighting data for 17 of the sampled whales (i.e. because they were first sighted as calves) and minimum age was calculated for 56 sampled whales. Whales who were first sighted as adults during the SPLASH effort were not included in analyses as there were no data from which to calculate a minimum age. Figure 10a depicts whales whose exact age was known, and Figure 10b depicts whales whose minimum age was determined from multiple sightings. For each graph, a 2nd order parabolic curve best fit the data (Minimum Age $R^2 = 0.29$ and 0.03 for Hawaii and Alaska, respectively; Exact Age $R^2 = 0.09$ and 0.20 for Hawaii and Alaska, respectively) and indicates that male humpbacks retain a relatively low testosterone concentration throughout their lives during the feeding season, but reach the highest levels of testosterone concentrations from ages 8-25, peaking around age 15. This preliminary finding was reached without controlling for sighting date within each season due to small samples sizes.

4. Discussion

Male humpback whales exhibited higher testosterone concentrations in the Hawaiian breeding grounds than in the Alaskan feeding grounds; a trend that was observed at both the group level and within individuals sampled in both locations (Table 1, Figure 2). This finding supports previous morphological studies of humpback whale testes in the Southern hemisphere, which found increased sperm counts in male whales killed in commercial whaling on the breeding grounds when compared with those killed on feeding grounds (Chittleborough, 1955). It is also consistent with and expands upon an earlier study of testosterone concentration based on 35 blubber samples of the Mexican DPS of humpback whales which found that testosterone levels were at their lowest June-September and were the highest October-April, with peak testosterone occurring January-February (Vu et al., 2015).

For male humpback whales in the present study, testosterone concentrations were at their lowest during the feeding season and began to increase toward the end of the feeding season in Alaska prior to beginning their migration to Hawaii (Figures 3 & 5). Chittleborough (1955) found that fewer sperm were present earlier in the breeding season (season = June-October, Southern hemisphere) and that sperm presence began to increase toward the end of the season (July and August). Our results complement Chittleborough’s findings and suggest that male humpback whales begin spermatogenesis prior to leaving the feeding grounds (Figure 5). This makes reproductive sense, so that humpback whale males are equipped with the gametes needed for a successful breeding season when they reach the breeding grounds or locations where they may breed enroute to these grounds (Craig and Herman, 1997). Increasing testosterone concentrations before the onset of the breeding season has been observed across other mammalian species (Blottner et al., 1996; Funasaka et al., 2011; Kjeld et al., 2006, 2004; Tsubota et al., 1997). Testosterone is required for spermatogenesis (Weinbauer and Nieschlag, 1990), which is known to take 61 days in bulls (Amann, 1970) and 74 days in humans (Amann, 2008).

The increase in testosterone towards the end of the feeding season (Figure 5) may stimulate or cue the start of male singing, which in the breeding grounds is clearly an important component of the humpback whale mating system (Herman, 2017) and has also been recorded toward the end of the feeding season in Alaskan waters and on feeding grounds or during migration in other populations (Chariff et al. 2001; Clark and Clapham, 2004; Gabriele and Frankel, 2002a; Straley et al., 1994). Clark and Clapham (2004) go so far as to suggest the “breeding area” encompasses the feeding area, migratory route and breeding grounds as based on the prevalence of song. In addition, Tyack (1981) using Nishiwaki (1962) whaling data, compared singing bout lengths in males and ovulation of female humpbacks and concluded that singing is likely related to reproductive behavior as singing bouts were at their lowest when ovulation was at its highest (IE, males spent less time singing/searching for mates). However, direct studies on the relationship between hormone levels, in either male or female humpback whales, have not been examined. Given the variation in testosterone concentrations of males shown in the present study as well as variability in song production on the feeding grounds and breeding grounds (Au et al. 2000), future studies should examine how hormones vary with the timing of singing in male singers.

No significant difference was found in testosterone concentrations between non-calves and calves in Alaska (Figure 9). Calves in both locations had relatively low testosterone concentrations, with the exception of one of the two calves in Hawaii who had a testosterone concentration of 1.05 ng/g (the other calf had a concentration of 0.03 ng/g). This outlier, however, is not surprising as most mammalian young exhibit high levels of reproductive hormones at birth, which immediately begin to taper off and remain low until sexual maturity is reached (Challis et al., 2001; Dhakal et al., 2011).

Our findings on the variability of testosterone concentrations and age (Figure 10a, 10b) suggest that male humpbacks reach peak lifetime testosterone concentrations in the breeding grounds between the ages of 8 and 25 years. However, this does not imply that males are not fertile beyond this age. For example, while other mammals may undergo senescence (Beehner et al., 2009; Nussey et al., 2013), male humpbacks have been observed in reproductive roles (singing and escorting) over periods of 20 years (Herman et al., 2013). It is unclear whether reproductive senescence occurs. However, Chittleborough (1955) found no evidence of any decline in testis weight or spermatogenetic activity in physically mature males, suggesting that the oldest/biggest whales still had the gametes necessary for breeding. In the present study, age data on 73 individual whales was obtained. From males of known and minimum estimated age, it appears that testosterone concentration during the breeding season reaches a maximum around 8-25 years of age and then begins to decline, reaching levels similar to those found on the feeding grounds when the whales are >30 years of age (Figure 10a,10b). This suggests that humpback whale males reach peak reproductive capacity around 10 to 20 years of age and that fertility may decline as whale's age. It is important to note that the estimated whale ages are based on a minimum age, and that the actual ages of individuals may be far older. In order to more fully understand how hormone concentrations vary between age classes, additional samples of known aged calves, juveniles (age 2-5 years) and male humpbacks older than 30 years of age are needed.

We found that testosterone concentrations were significantly higher in blubber than in skin ($p<0.05$), with only a weak positive correlation detected ($r=0.64$, Pearson Correlation Test, Figure 8). This indicates that testosterone concentrations were not consistent between types of tissue thus, testosterone concentrations in skin tissue should not be compared to testosterone concentrations in blubber. It should also be noted that from examination of captive bottlenose dolphins (*Tursiops truncatus*), hormones in blubber can be used as a proxy for circulating hormones in the blood serum (Champagne et al., 2017). As such we recommend that future studies continue to use blubber in hormonal analysis of free ranging cetaceans.

Male mammals often exhibit aggression toward competitors in order to access mates (Campagna et al., 1988; Herman et al., 2007; Tyack and Whitehead, 1983), yet aggressive behavior and its relationship to testosterone has not been examined in humpback whales. Increased male aggression in mammalian species is often accompanied by an increase in testosterone (Bouissou, 1983), with the most successful animals often having the highest testosterone (Beehner et al., 2006). During the humpback whale breeding season, individual fecund females are often the focus of competing males within so called "competitive groups" (Clapham et al., 1992; Tyack and Whitehead, 1983). Mature male humpbacks have relatively long residency periods on the breeding grounds (Craig et al., 2001) allowing them to compete over extended periods of time.

In the current study peak testosterone concentrations occurred between January and February (Figure 5) which would suggest that peak reproductive potential (i.e. greatest concentration of gametes) in males occurs during March and April based on the timeline of spermatogenesis in other species (Amann, 2008, 1970). Males who undergo spermatogenesis earlier in the season, perhaps while still on the feeding grounds are at a mating advantage as they are able to breed with early arriving females on the breeding grounds. Our data alone cannot resolve the exact timing of peak breeding, but it suggests a trade-off between physical fitness and reproductive fitness, as males who leave the feeding grounds earlier may have better mating success, but may also be in poorer nutritional condition. In order to properly understand the role that testosterone plays in group dynamics on the breeding grounds, additional blubber samples are needed from individual males of varying ages who participate in specific behavioral groups (e.g. competitive versus non-competitive) and different behavioral roles (e.g. principal vs secondary escorts).

Trends in migratory timing have been well documented (Baker et al., 1985; Craig et al., 2003; Gabriele et al., 1996; Mann et al., 2000), but the impetus to leave the feeding grounds remains unclear. Some researchers have proposed that nutritional state, body condition and food availability (Brodie, 1975), photoperiod (Baker, 1978), or hormonal levels are responsible for timing of migration, whereas others postulate that it is likely a combination of all of these factors (Craig et al., 2003). The present study indicates that testosterone may play a role in the motivation to commence migration, as found in other mammalian species (Stern, 2009), or is a correlate of one or more of the factors noted above. In order to definitively answer these questions, increased sampling effort in Alaska in the late fall and spring is needed. This would include sampling in Alaska during the winter to measure hormones in whales who fail to migrate, in both spring and fall to understand if the migration timing of humpbacks is shifting, and in years of anomalous environmental occurrences, such as the Northeast Pacific marine heatwave of 2013-2015 (Peterson et al., 2015).

Capturing natural variation within a species is important in its own right, but access to long-term datasets is essential in management decisions. For example, an established long-term monitoring program for North Atlantic right whales (*Eubalaena glacialis*) documented a decline (and subsequent increase) in stress-related fecal hormone metabolites (Rolland et al., 2012) in the aftermath of the September 9th, 2001 terrorist attack due to a mandatory reduction in shipping traffic. As a result, slower shipping speeds and alternative shipping routes have since gone into effect to better protect these whales (Laist et al., 2014).These datasets allow managers to see if changes in a certain metrics are anomalous or are part of natural variation.

While several DPS's of humpback whales in the North Pacific, including the Hawaii DPS were recently delisted from an endangered status (under the Endangered Species Act, NMFS 2016) events over the last few years have some researchers questioning the health of the population. Glacier Bay National Park biologists have consistently monitored humpbacks whales in Glacier Bay and Icy Strait since 1985 and have documented a decline in the local abundance of humpbacks beginning in 2014 to present day, as well as a decrease in the overall crude birthing rate (CBR), with the lowest CBR ever recorded over the 33-year monitoring program occurring in 2016 (Neilson et al., 2017). In addition, over the last few years, an increasing number of humpback whales have been present on the feeding grounds of Sitka, AK in winter and spring, perhaps suggesting a delayed or absent southern migration (Straley et al., 2018). There are also fewer whales present off west Maui and Hawaii Island (HMMC 2018; Kugler et al. 2017) and an increasing number of 'skinny' whales returning to the feeding grounds (Neilson et al., 2017; Straley et al., 2018). Reported strandings of humpbacks in Alaska for 2016 were higher than the previous 16-year average and unusual mortality events (UME) were declared for Alaska and British Columbia large whales in 2015 and Atlantic humpback whales in 2017 (NMFS, 2017), suggesting that environmental conditions may be changing or global humpback whale populations may be reaching carrying capacity.

The present study represents a key step in creating additional tools for monitoring physiological changes in humpback whales across time. The results of this study collectively suggest that males i) begin to undergo spermatogenesis before they reach the Hawaiian breeding grounds, ii) experience peak testosterone concentrations during January and February on the breeding season, iii) show decreased testosterone concentrations coinciding with the end of the breeding season and migration to feeding grounds, and iv) are at their peak fertility at 8 to 25 years of age.

This study is another demonstration of how non-lethal techniques in combination with long-term life history data can aid in our better understanding of the physiology and behavior of humpback whales. With their high site fidelity, abundant numbers, coastal presence and role as a top predator, humpback whales can serve as important marine sentinels, providing a lens into ecosystem conditions and processes as they are unequivocally linked to the marine resources they depend on. With their high lipid content and preference for lower trophic species, such as forage fish and euphausiids, any fluctuations shown at the humpback whale population level could be cause for concern in both important commercial fish stocks and humans (Bossart, 2011). A baseline dataset of hormonal biomarkers creates the opportunity for long term monitoring of humpback whale physiology. Shifts in the physiology of humpbacks could be indicative of any number of factors including: climate change, density dependent influences, shifts in prey abundance, quality and availability, or anthropogenic disturbances (Burek et al., 2008; Learmonth et al., 2006; Rolland et al., 2012; Straley et al., 2018). Regardless of cause, behavioral and longitudinal data of individually identified humpbacks combined with endocrine markers, provide a powerful tool in the assessment of physiology and life history states for responsible management and conservation of humpback whales.

Acknowledgements

The authors thank the many researchers who participated in the 2004-2006 SPLASH effort and allowed the use of their samples from which a rich sample set of blubber was available for this project; Kelly Robertson and Gabriela Serra-Valente of the National Marine Fisheries Service SWFSC for housing the archived samples and for their assistance in sample location and retrieval. This project would not have been possible without genetic sex identification conducted in the Oregon State University Cetacean Conservation and Genomics Laboratory. The authors also sincerely wish to thank Jennifer Cedarleaf for help in organizing data sheets and rummaging through databases to match samples across ID's; Janet Neilson for painstaking maintenance of accurate whale sighting history data in Southeast Alaska, and; Kendall Mashburn for guidance in laboratory techniques and assistance with processing of samples. Additional thanks are given to Wendell Raymond for his help in creating the sample location map. Funding for this project was provided by the Office of Naval Research (Research grant #N0014-14-1-0425), the Northern Gulf Applied Research Grant, the University of Alaska-Fairbanks Resilience and Adaptation Program, the National Marine Fisheries Service, and the University of Alaska-Fairbanks Cooperative Institute for Alaska Research.

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Table 1. Seasonal differences in testosterone concentrations of individuals (n=10) who were biopsied in both Hawaii and Alaska. Testosterone was higher when whales were in Hawaii (0.73 ± 0.43 ng/g) than in Alaska (0.09 ± 0.09 ng/g), with the exception of whale 470452. Field notes could not identify why whale 470452 had higher testosterone other than this sample was the latest collected (Oct 25th) for the 10 whales biopsied in Alaska.

Splash ID	Date	Hawaii	Date	Alaska
430109	4/10/05	0.10	7/24/04	0.01
430148	4/21/04	0.34	8/9/05	0.02
430228	1/22/05	1.24	7/7/04	0.14
430349	2/25/05	0.56	7/8/04	0.02
470452	1/7/05	0.13	10/25/04	0.25
470736	2/7/06	0.63	10/20/04	0.16
			7/7/04	0.01
474070	2/3/05	0.90	8/10/04	0.04
	2/23/06	1.54		
474074	2/7/05	0.89	7/23/04	0.04
474110	1/24/05	1.24	6/30/04	0.06
	2/9/05	0.42	10/15/05	0.09
430404	2/12/04	0.73	10/23/04	0.25

Figure Captions.

Figure 1. Blubber and skin biopsy samples were collected from two locales; A) throughout Southeast Alaska (n=86), including Sitka Sound (57.0˚N 135.5W˚), Chatham Strait (56.95˚N 134.62˚W), Frederick Sound (57.13˚N 134.10˚W), Lynn Canal (58.4˚N 134.8˚W) and waters west of Prince of Wales (55.95˚N 132.48˚W), and B) in the Hawaiian islands (n=182), specifically the Au'au, Kalohi and Pailolo channels between Maui, Moloka'i, Lana'i and Kaho'olawe and off the North Kohala Coast of Hawai'i Island (20.89˚N 156.68˚W and 19.98˚N 155.87˚W, respectively.

Figure 2. Testosterone concentrations (ng/g) by geographic location. Testosterone concentrations were significantly ($p<0.05$) higher when male humpbacks were in Hawaii (HI) than in Alaska (AK).

Figure 3. Testosterone concentrations binned by season, regardless of location. All seasons, except fall were significantly different from each other ($p<0.05$). Fall was not significantly different than spring but was significantly different than summer and winter ($p<0.05$).

Figure 4. Spring testosterone concentrations binned by location. Spring was the only season in which biopsy collection efforts obtained samples from both locations. Whales sampled in Hawaii (HI) during the spring had significantly higher testosterone than whales sampled in Alaska (AK) in the spring ($p<0.05$).

Figure 5. Mean monthly testosterone concentrations (ng/g). No samples were available for May, all other months have the sample size provided. Peak testosterone concentrations occurred on the breeding grounds between Jan-Mar, whereas the lowest concentrations were observed on the feeding grounds from Jun-Sep.

Figure 6. Testosterone concentrations of all non-calf biopsies (n=182) collected in Hawaii by month during the breeding season. Concentrations of testosterone decreased as the season progressed.

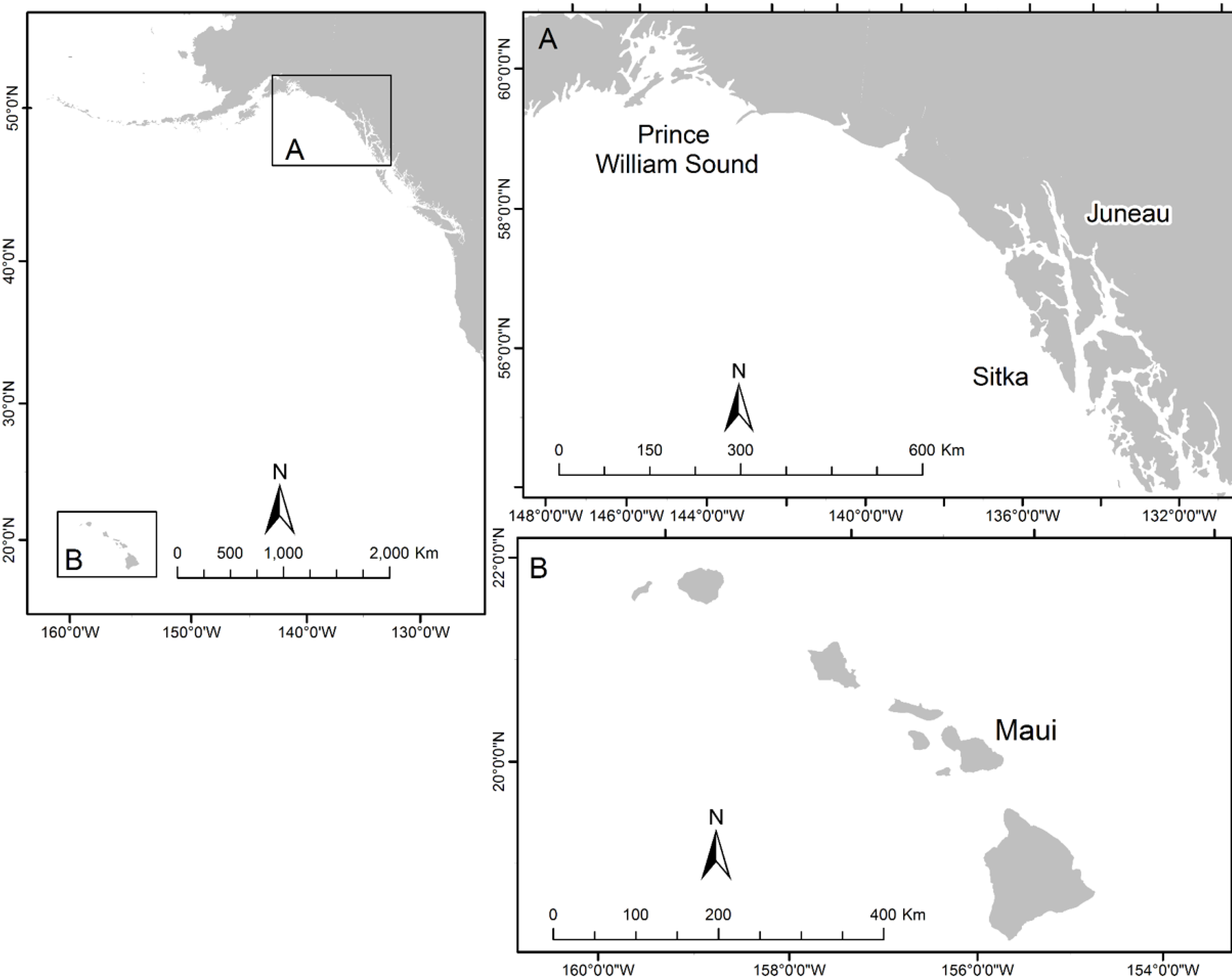
Figure 7. Blubber testosterone concentrations of individual whales that were biopsied twice during the same Hawaiian breeding season. Blubber testosterone concentrations decreased as

the season progressed in all individual whales. Black bars represent the first biopsy collected, grey bars represent the biopsy collected later in the same breeding season.

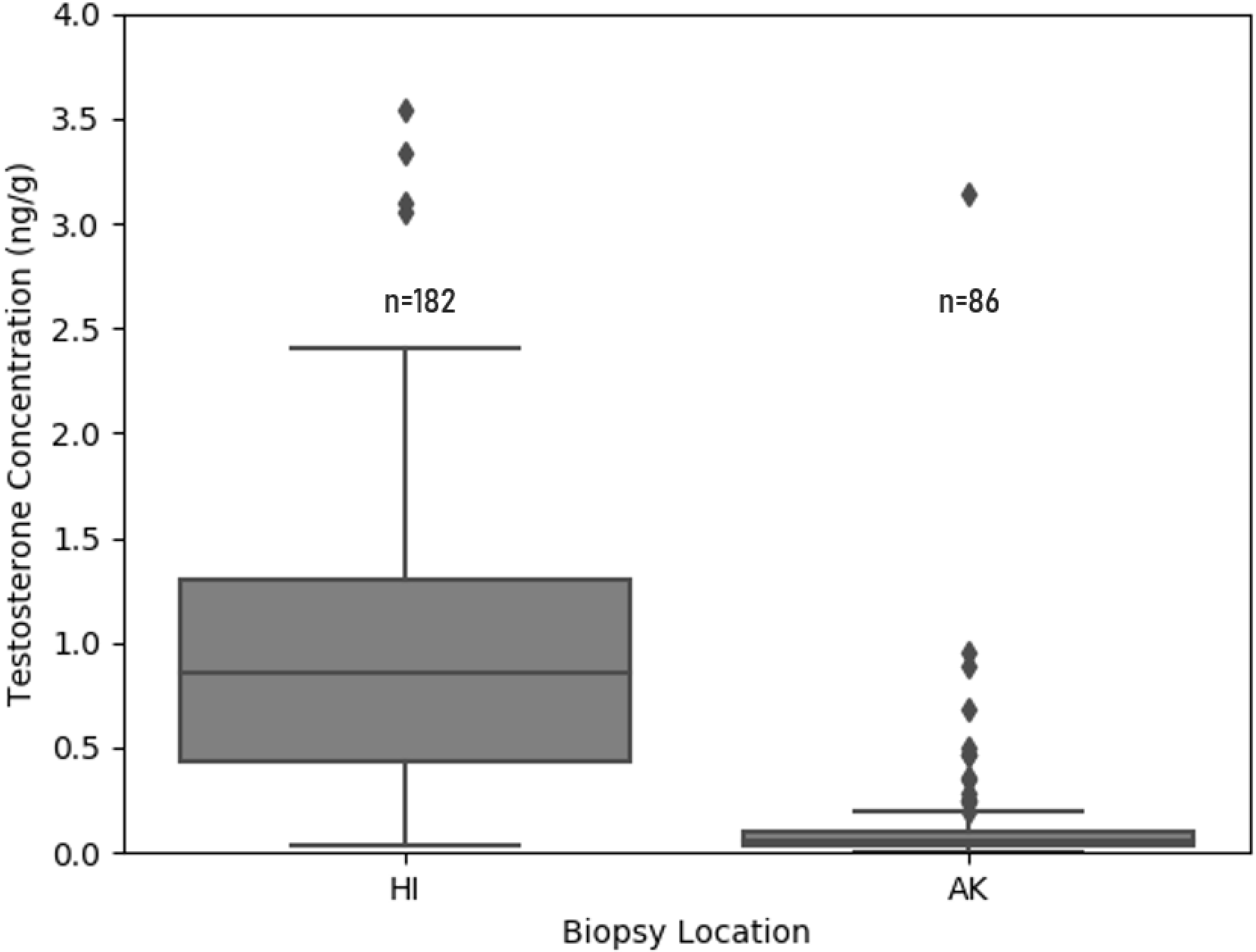
Figure 8. Testosterone concentrations in blubber (B) and skin (S). Mean testosterone concentrations were significantly higher in blubber than in skin for animals located in Hawaii ($p<0.05$). Animals in Alaska had very low testosterone concentrations and no difference between blubber and skin was detected (Tukey's HSD test). Labels are as follows: HI-B = Hawaii blubber, HI-S = Hawaii skin, AK-B = Alaska blubber, AK-S = Alaska skin.

Figure 9. Testosterone concentrations of calves and non-calves in Alaska. No significant difference was found between the two age classes ($p=0.14$) Labels are as follows: AK-C = Alaska calf and AK-A = Alaska non-calf.

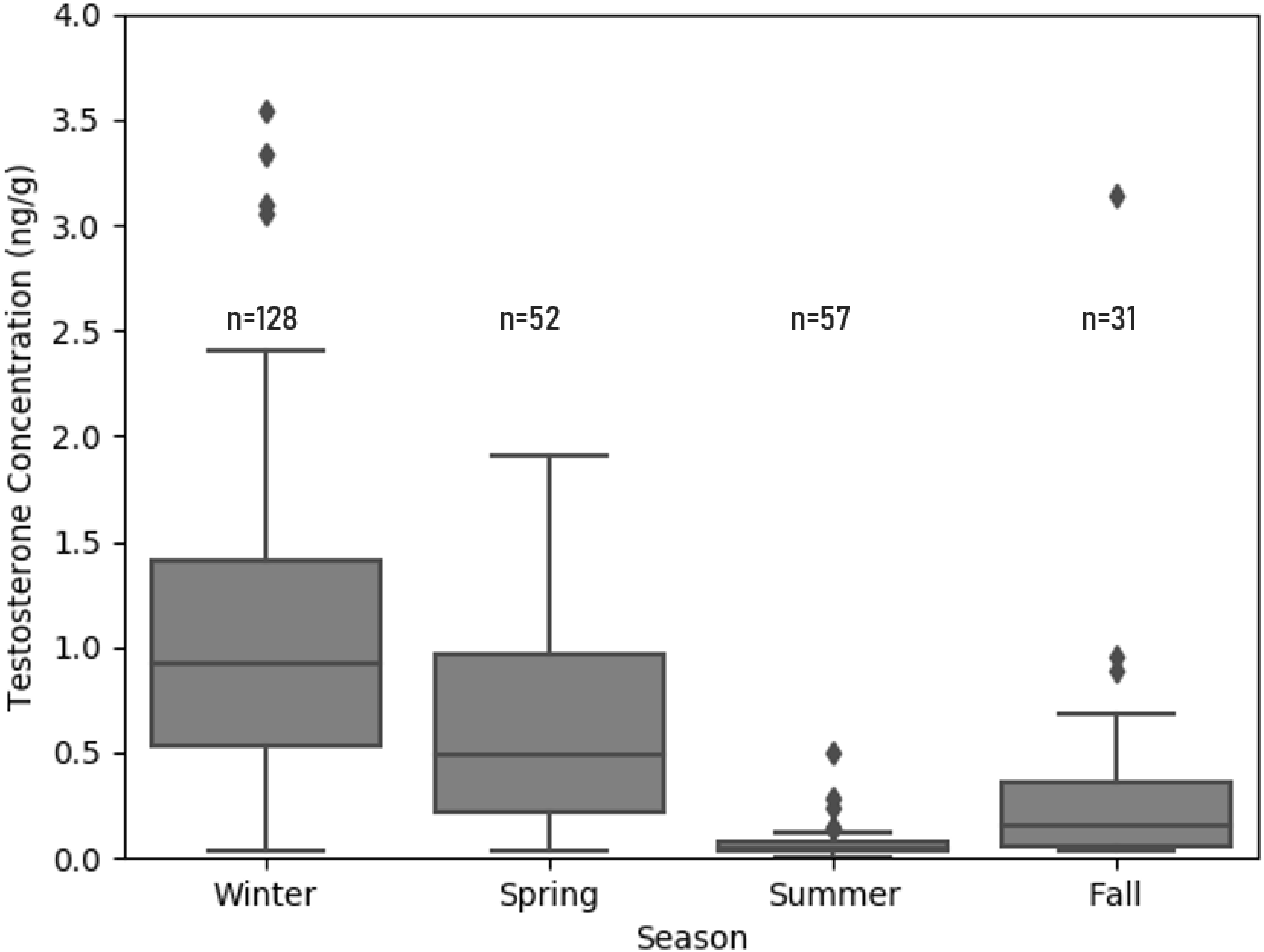
Figure 10 a & b. Testosterone concentration plotted against exact or estimated age of 74 whales. Paired age and testosterone concentration data suggest that testosterone levels remain consistently low on the feeding grounds and that humpback whale males may experience peak testosterone concentrations from 8-25 years of age on the breeding grounds. Whales were split by location into Hawaii (black circles) or Alaska (open circles) grounds based on where each biopsy was collected. A) Exact age of 17 whales (Hawaii=5, Alaska=12) who were first seen as calves, were compared to testosterone concentrations. A 2nd order parabolic curve best fit the data of each group (R^2= 0.09, 0.20 for HI and AK, respectively); B) Minimum age of 56 whales (Hawaii = 19, Alaska=37 individuals) were compared to testosterone concentrations. As these whales were first seen as full adults, two years were added to the year they were first seen to account for a year as a calf and a year as a yearling. A 2nd order parabolic curve best fit the data of each group (R^2 = 0.29, 0.03 for HI and AK, respectively).



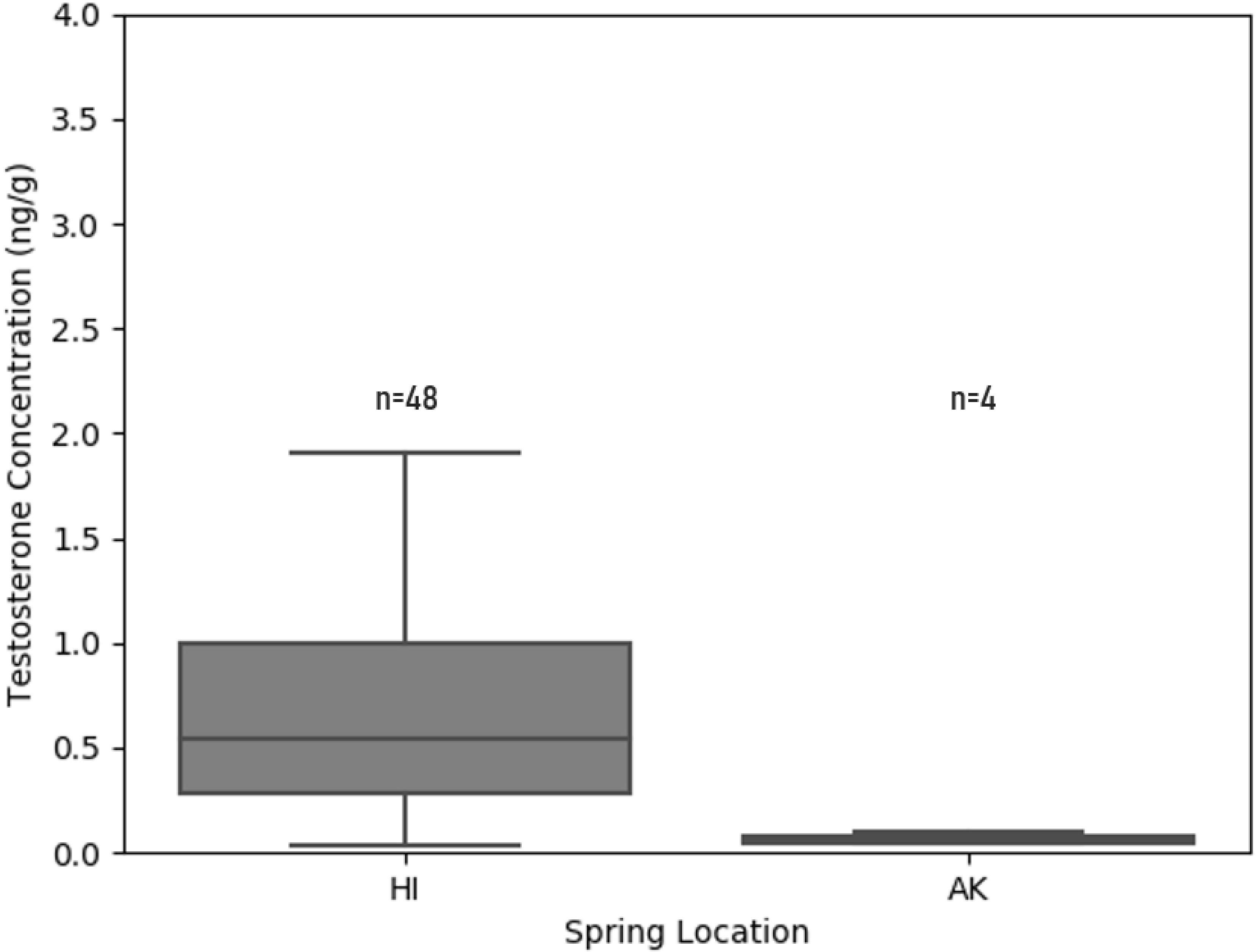
A
B
N
0
500
1,000
2,000 Km
50°0'N
40°0'N
30°0'N
20°0'N
160°0'W
150°0'W
140°0'W
130°0'W
Prince
William Sound
Juneau
Sitka
150
300
600 Km
60°0'0"N
58°0'0"N
56°0'0"N
148°0'0"W
146°0'0"W
144°0'0"W
140°0'0"W
136°0'0"W
132°0'0"W
Maui
100
200
400 Km
22°0'0"N
20°0'0"N
160°0'0"W
158°0'0"W
156°0'0"W
154°0'0"W



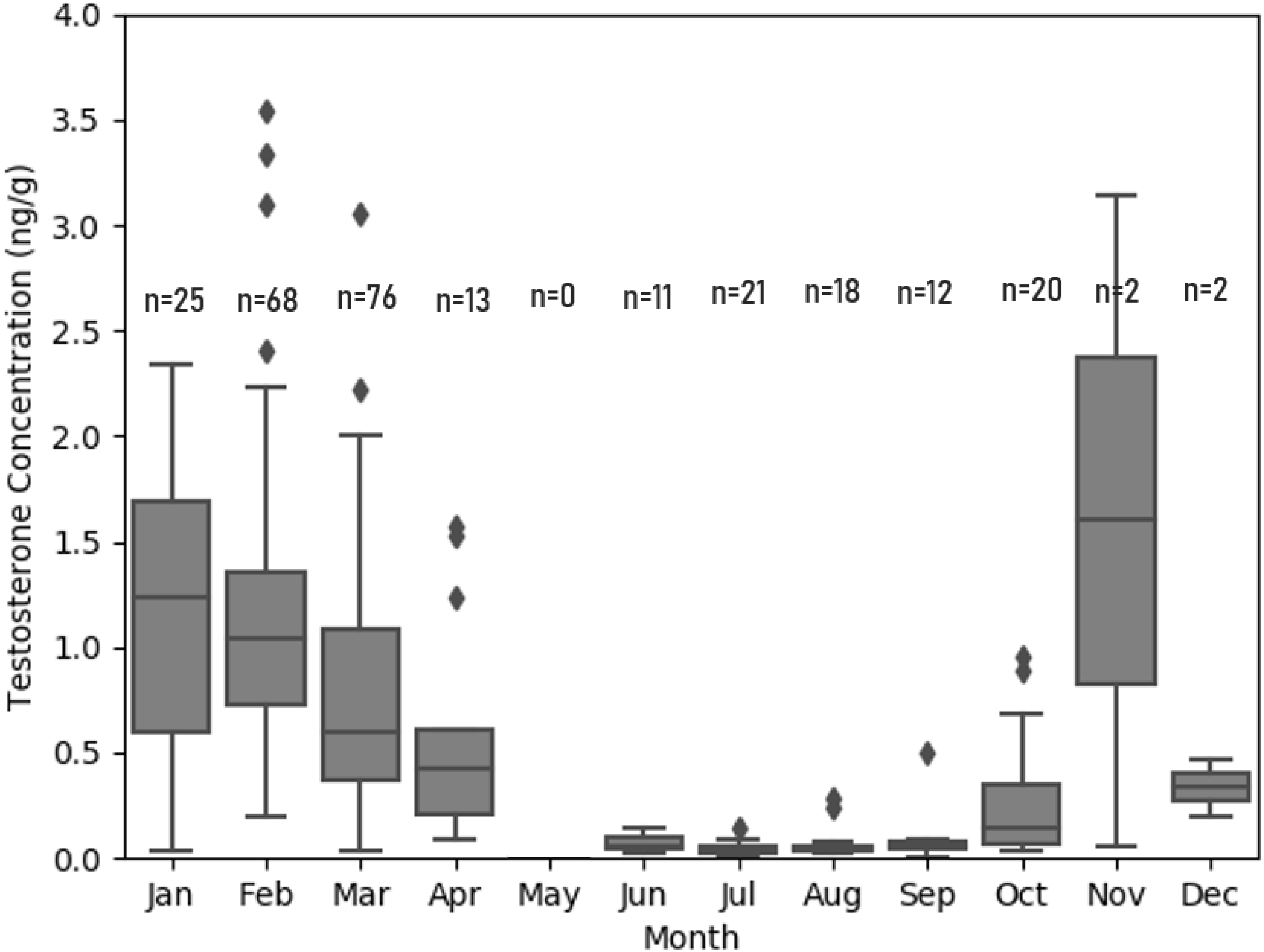
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Testosterone Concentration (ng/g)
n=182
n=86
HI
AK
Biopsy Location



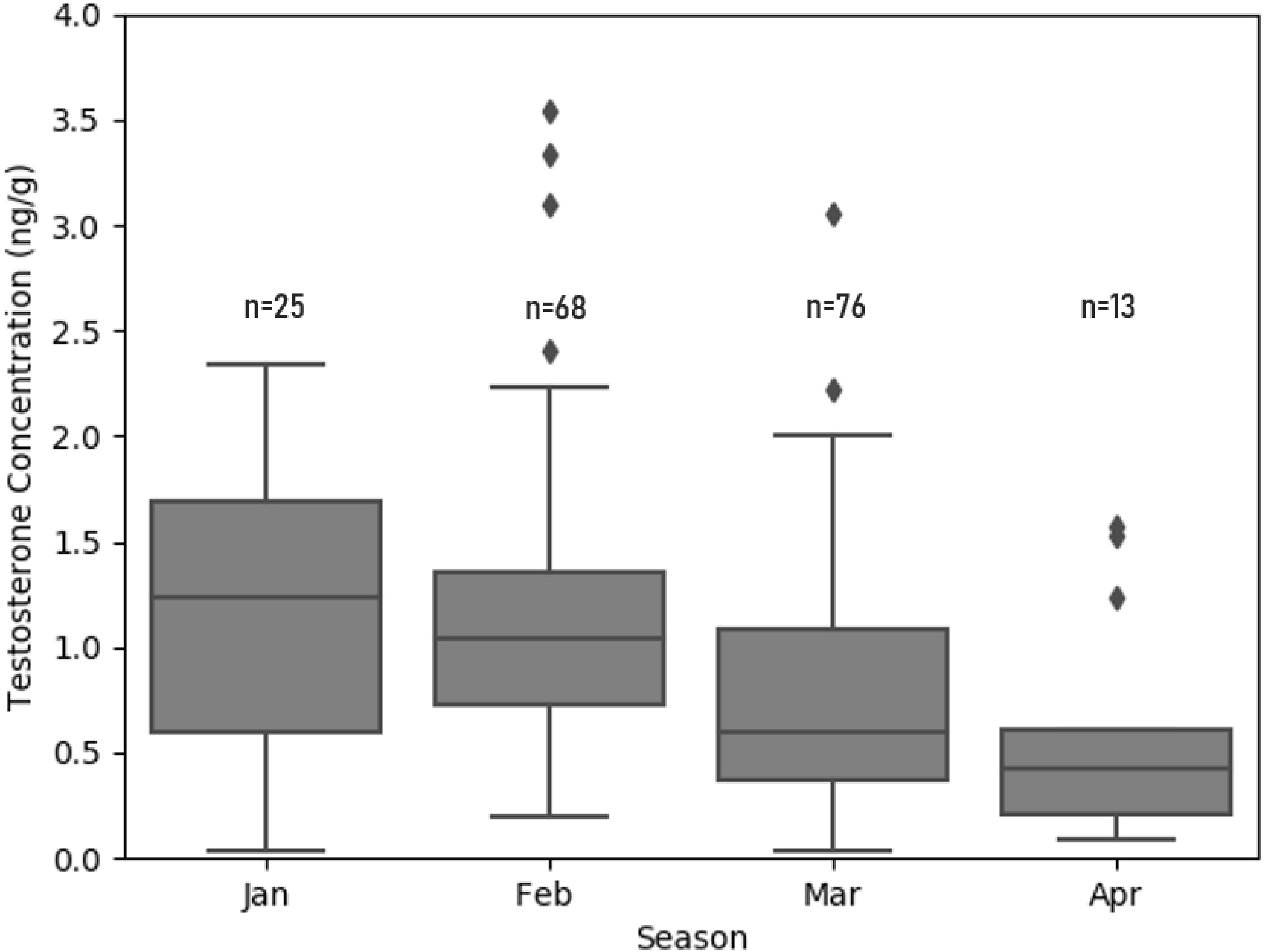
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Testosterone Concentration (ng/g)
n=128
n=52
n=57
n=31
Winter
Spring
Summer
Fall
Season

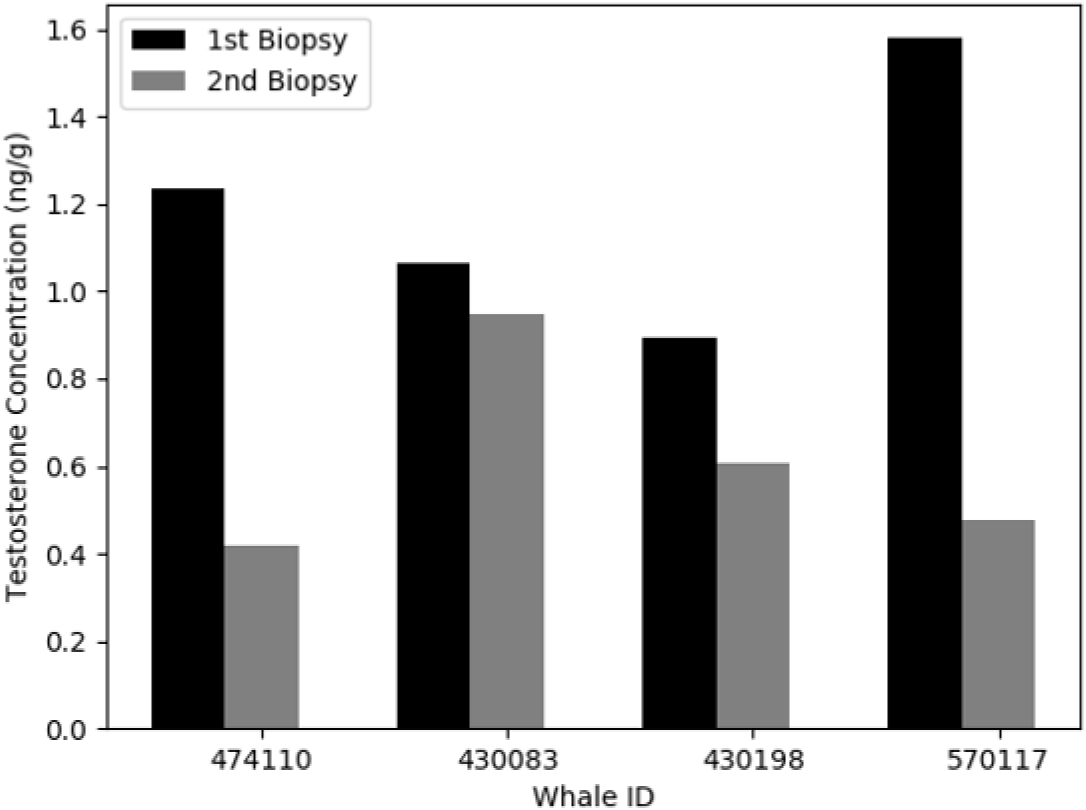


4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Testosterone Concentration (ng/g)
n=48
n=4
HI
AK
Spring Location

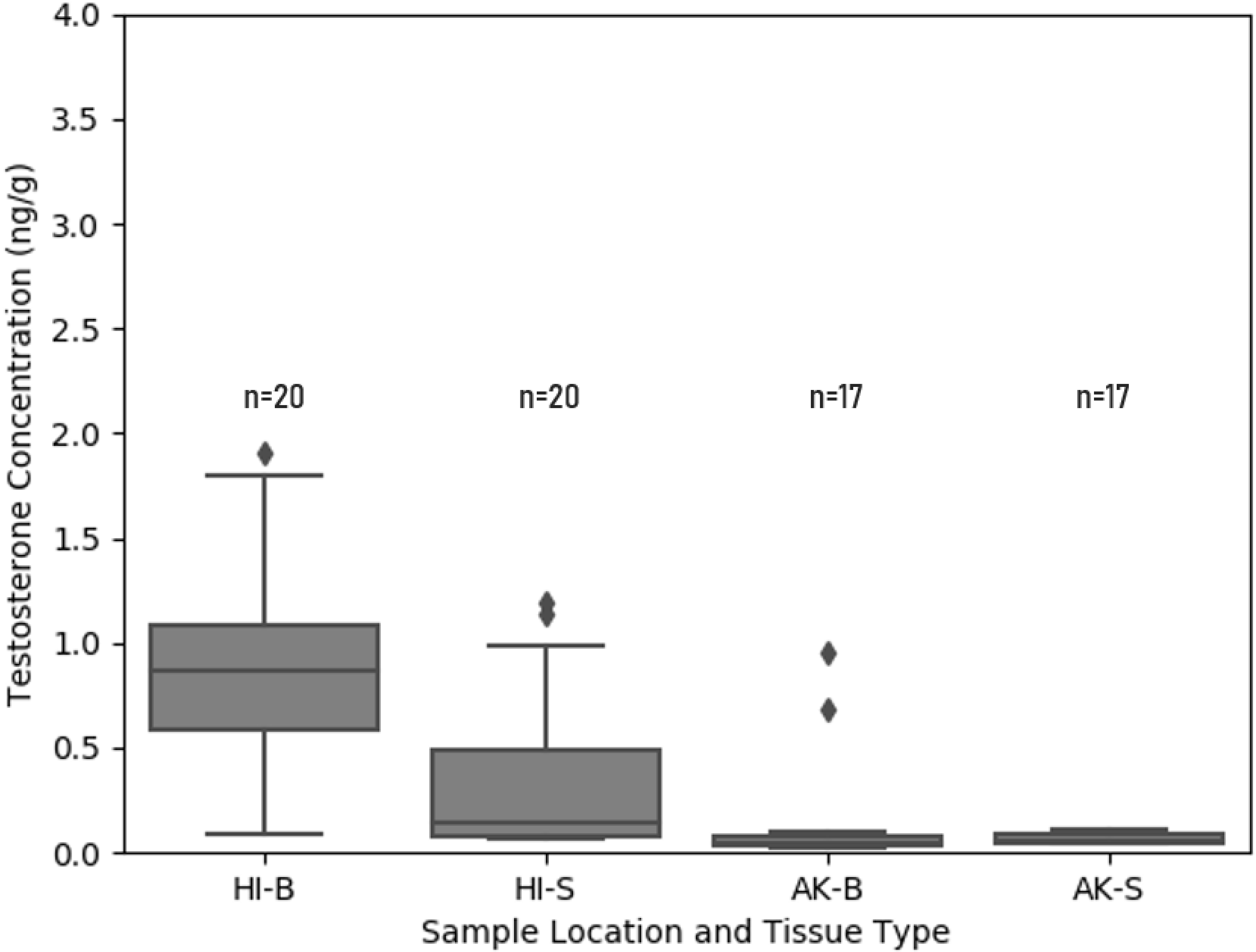


Testosterone Concentration (ng/g)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
n=25
n=68
n=76
n=13
n=0
n=11
n=21
n=18
n=12
n=20
n=2
n=2
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month

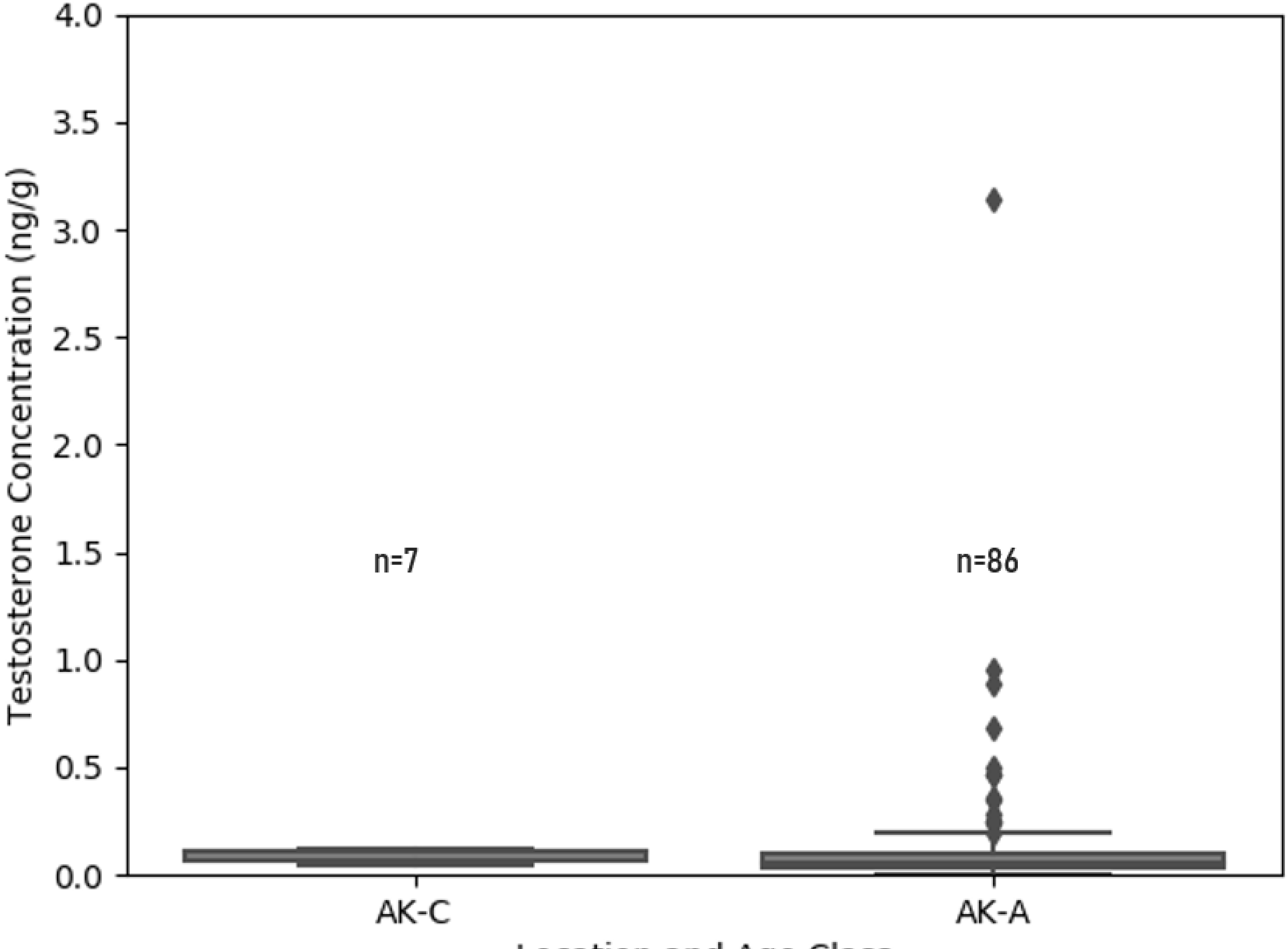

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Testosterone Concentration (ng/g)
n=25
n=68
n=76
n=13
Jan
Feb
Mar
Apr
Season

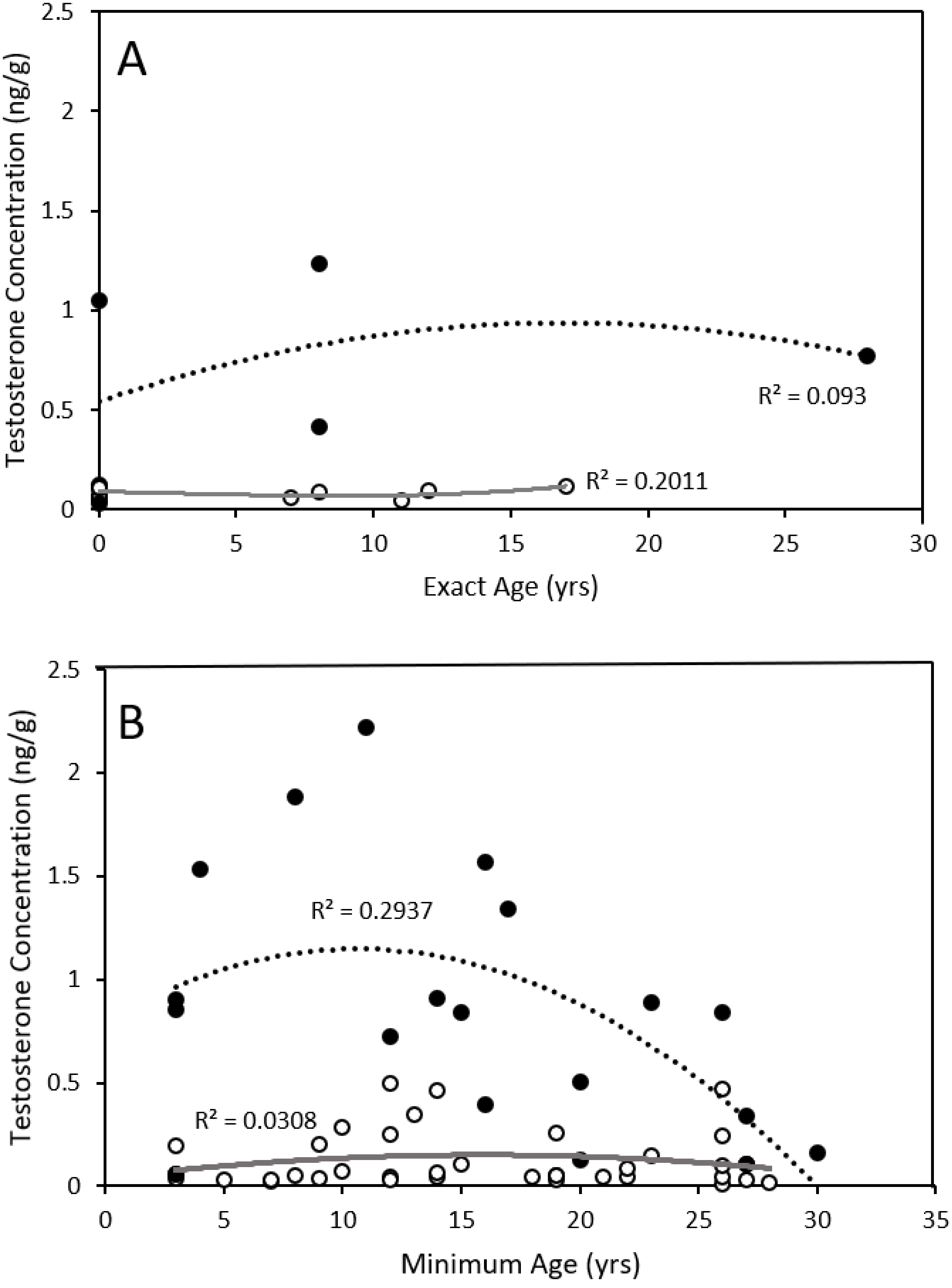


1st Biopsy
2nd Biopsy
Testosterone Concentration (ng/g)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
474110
430083
430198
570117
Whale ID



4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Testosterone Concentration (ng/g)
n=20
n=20
n=17
n=17
HI-B
HI-S
AK-B
AK-S
Sample Location and Tissue Type


4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Testosterone Concentration (ng/g)
n=7
n=86
AK-C
AK-A
Location and Age Class



A
Testosterone Concentration (ng/g)
Exact Age (yrs)
R² = 0.093
R² = 0.2011
B
Testosterone Concentration (ng/g)
Minimum Age (yrs)
R² = 0.2937
R² = 0.0308