REPRODUCTION
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Testosterone and tusks: maturation and seasonal reproductive patterns of live, free-ranging male dugongs (*Dugong dugon*) in a subtropical population

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Abstract

Knowledge of male reproductive status and activity in free-ranging animals is vital to understanding reproductive patterns and population dynamics. Until now, almost all information regarding reproductive behavior of the dugong, a cryptic marine mammal, has relied on post-mortem examination. We examined the relationships between body length, tusk eruption (secondary sexual characteristic), seasonality, and group association on fecal testosterone metabolite concentrations in 322 free-ranging dugongs (159 males, 163 females) in subtropical Moreton Bay, Australia. Fecal testosterone concentrations demonstrated biologically meaningful differences in testicular activity between sexes and across reproductive/age classes, and were correlated with circulating concentrations in serum. Male dugongs have a pre-reproductive period that persists until a body length of 240 cm is achieved. Puberty apparently occurs between 240 and 260 cm body length when fecal testosterone levels increase fourfold (>500 ng/g) over juvenile levels, and is associated with tusk eruption. However, social maturity may be delayed until male dugongs are larger than 260 cm with well-developed tusks. In mature males, the lowest (<500 ng/g) fecal testosterone concentrations occur in the austral autumn months with maximal concentrations in September–October, coincident with the onset of a spring mating season. During spring, solitary mature males had fecal testosterone concentrations double those of mature males sampled within groups, potentially suggesting a mating strategy involving roving of reproductively active males. This study demonstrates that single-point physiological data from individuals across a population have value as indicators of reproductive processes. Our approach provides an efficacious non-lethal method for the census of reproductive status and seasonality in live male dugongs.

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Introduction

Reproductive biology influences the viability of vulnerable populations through its effects on demography, movements, and population genetics, so that its study is crucial to conservation programs. Although the dynamics of mammalian populations are focused largely on female reproductive rates (Caswell 2001), studies have increasingly illustrated the importance of the male's reproductive output in population processes (e.g. Milner-Gulland *et al*. 2003, Rankin & Kokko 2007). Male mammals can have substantial effects on population dynamics when a reduction in female fecundity is attributed to declining fertilization rates associated with a low proportion of sexually mature males or restrictions on male breeding activity (e.g. seasonality). In a free-ranging wildlife population, gaining an understanding of the male reproductive state often relies on assessing outward signs, such as external morphological features or behavioral characteristics, and this can be particularly challenging when studying cryptic species such as fully aquatic marine mammals.

Dugongs (*Dugong dugon*) are a long-lived species with low reproductive rate, and their limited reproductive potential has been identified as a major contributing factor to the vulnerability of populations (Heinsohn *et al*. 2004). Furthering our knowledge on dugong reproductive biology throughout their tropical–subtropical range has been highlighted as a priority for effective management and conservation of dugong populations (Marsh *et al*. 2012). As with many species of marine mammals, our understanding of the basic reproductive biology of male dugongs has relied on post-mortem studies of retrieved carcasses (Marsh *et al*. 1984*a*, Kwan 2002). No quantitative information has been collected on reproductive status and/or activity of live, free-ranging males, but only anecdotal or glimpsed behavioral observations at the water surface (e.g. Anderson & Birtles 1978, Preen 1989). A further challenge to conducting reproductive studies on live males is that dugongs are testicond

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mammals, with their testes permanently concealed within the abdomen (Marsh *et al*. 1984*a*), and exhibit no obvious sexual dimorphism in body size or morphology. However, male dugongs do possess a pair of tusks (permanent incisors) that erupt after puberty (initiation of spermatogenesis; Marsh 1980, Marsh *et al*. 1984*a*), and also occasionally in females older than 40 years (Marsh *et al*. 1984*b*). This dimorphism in tusk eruption indicates a non-feeding function in dugongs, and eruption associated with maturity suggests a secondary sexual characteristic with a possible reproductive role.

Sexual maturity in male dugongs has been previously defined based on testis weight, seminiferous tubule diameter, and the relative proportion of tubules in various stages of spermatogenesis (Marsh *et al*. 1984*a*, Marsh 1995, Kwan 2002). However, information obtained from the histological analysis of dugong gonads (testes and/or epididymides) has been spatially biased toward the tropical regions of northern Queensland (Townsville and Mornington Island, Marsh *et al*. 1984*a*), Torres Strait in Australia (Mabuiag Island, Kwan 2002), and southern Papua New Guinea (Daru, Marsh 1995), where carcasses from drownings and/or indigenous harvest have been more readily available. Even at a relatively fine spatial scale among these studied tropical populations, life history parameters of dugongs are prone to spatial variability (Marsh & Kwan 2008). Moreover, post-mortem studies have been hampered by small sample size in the pubertal age/size range (Marsh 1995), making more accurate estimates of length or age at sexual maturity difficult. Male dugongs in tropical populations attain sexual maturity at a range of body lengths between 2.2 and 2.5 m (Marsh *et al*. 1984*a*). Within a free-ranging subtropical population, a substantial proportion of dugongs (∼30%; Lanyon JM (2012), unpublished observations) fall within this size range of uncertain reproductive status, leaving a deficiency in demographic data. Furthermore, precocious sexual maturation has been recorded in waters around Mabuiag Island in Torres Strait, with two males as small as 1.9 m having fully spermatogenic testes (Kwan 2002). This suggests that there is a need to obtain more precise reproductive data for male dugongs throughout their geographic range, particularly in non-tropical regions.

Moreover, gonadal activity of mature male dugongs also appears to be variable, so that not all males in a population produce spermatozoa continuously or synchronously (Marsh *et al*. 1984*a*, Marsh 1995, Kwan 2002). In the tropics, male dugongs with spermatogenic testes have been sampled predominantly in the latter half of the year (austral winter–spring), consistent with a diffuse breeding season (Marsh *et al*. 1984*a*, Marsh 1995, Kwan 2002). It has been suggested that mature male dugongs with regressed testes and aspermic seminiferous tubules may be symptomatic of prolonged periods of sexual inactivity (Marsh *et al*. 1984*a*). However, the timing of male reproductive cycles and the extent of inactivity have not been thoroughly investigated since carcass analysis provides data on the individual male at time of death only. Further, if food availability and/or quality plays a role in the reproductive cycles of dugongs (Marsh *et al*. 1984*b*, Lanyon *et al*. 1989, Marsh & Kwan 2008), variation in these parameters might be expected between geographic regions. For example, it is reasonable to expect that life histories of dugongs in non-tropical regions with a more pronounced seasonality in seagrass growth and nutrient availability (Preen 1995) may be different from those in the tropics. Yet until now, no life history investigations have been conducted for subtropical populations.

Since most reproductive processes are hormone-dependent, endocrine analysis is a useful means of non-lethally assessing reproductive status. Testosterone is a steroid hormone necessary for the development of secondary sexual characteristics, and influences male sexual behavior, increases aggressive behavior, and promotes seasonal changes in accessory sex glands when circulating concentrations peak prior to the mating season (Wingfield *et al*. 1990, Lincoln 1998, Buck & Barnes 2003, Pelletier *et al*. 2003). Moreover, the use of excreta to measure testosterone metabolites offers an approach to study logistically challenging, fully aquatic species like sirenians. For another sirenian species, the physiological relevance of using fecal samples to measure testosterone levels was demonstrated by showing that the injection of exogenous GnRH to adult male Amazonian manatees (*Trichechus inunguis*) led to significant increases in excreted androgen concentrations (Amaral *et al*. 2009). Fecal testosterone concentrations have also been used to gain information on reproductive patterns in male Florida manatees (*Trichechus manatus latirostris*) in captive facilities and in the wild, confirming seasonal and gender variation in androgen production (Larkin *et al*. 2005). In dugongs, Lanyon *et al*. (2005) conducted a preliminary study to examine testosterone concentrations in fecal samples from seven male and three female wild dugongs. Testosterone concentrations in males were generally higher in adults (>2.5 m body length) and the lowest in immature dugongs (<2.2 m). However, the small sample size provided limited insight into the dugong's reproductive endocrinology.

Developing a better understanding of reproductive physiology (including sexual maturity and seasonality) in male dugongs will allow for the determination of life history parameters for real live populations. This paper describes an application of fecal testosterone analysis, to provide a non-lethal means of assessing the testicular activity of free-ranging male dugongs. Specifically, our objectives were to 1) validate an enzyme-immunoassay (EIA) for measuring fecal testosterone metabolite concentrations in dugongs; 2) determine fecal testosterone metabolite concentrations associated with sex, body size,

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and secondary sex characteristics; 3) determine if a multifactorial model incorporating fecal testosterone metabolite concentration, body size, and the presence of erupted tusks could be used to accurately differentiate mature males in the population; 4) characterize temporal patterns of androgen expression, particularly with respect to seasonality; and 5) investigate the testicular activity of mature males in different social associations. This study presents the first investigation of male reproductive activity in a live, free-ranging dugong population, and the first reproductive study of dugongs in the subtropics. As such, it serves as an important foundation for future life history and ecological studies.

Results

Study animals

A total of 322 individual free-ranging dugongs were sampled in Moreton Bay (159 males, 163 females). Based on body length measurements and previously reported reproductive size classification methods by Marsh *et al.* (1984*b*), 28 male dugongs were classified as juvenile (<220 cm body length) and likely to be immature; 84 as adult (≥250 cm body length) and probably mature; and 47 males of uncertain reproductive status (220–249 cm body length). Feces were collected from all 322 individuals, of which 66 dugongs (29 males, 37 females) were sampled out-of-water and had both feces and blood collected. Of the 29 males, 5 were juveniles, 14 were adults, and 10 were of uncertain reproductive status.

Evaluation of sexual maturity

Testosterone was detected in the serum of 65 out of the 66 dugongs from which blood was sampled during health assessments; one serum sample from an adult female had no detectable testosterone using the described assay methodology. Serum and fecal concentrations of testosterone were significantly and positively correlated ($R^2=0.58$, $P<0.001$), with $\log_{10}$-transformed fecal concentrations (ng/g) predicted by the linear equation: $y=0.60 \log_{10}$ serum testosterone (ng/ml) $+2.18$ (Fig. 1). Serum testosterone concentrations were significantly higher in male dugongs (2.72±0.70 ng/ml) than in females (0.08±0.01 ng/ml; $t_{64}=10.11$, $P<0.001$). The highest serum testosterone concentration in males (0.09–13.31 ng/ml) was over 33 times greater than the highest concentrations recorded in a female (0.00–0.44 ng/ml) and over 11 times greater than in a juvenile male (0.26–1.19 ng/ml). Adult males had significantly higher serum testosterone concentrations (4.79±1.23 ng/ml) than juvenile male (0.64±0.17 ng/ml) and female dugongs ($F_{2,52}=71.47$, $P<0.001$), with no significant difference in levels between juvenile males and females ($P>0.05$). During out-of-water sampling in

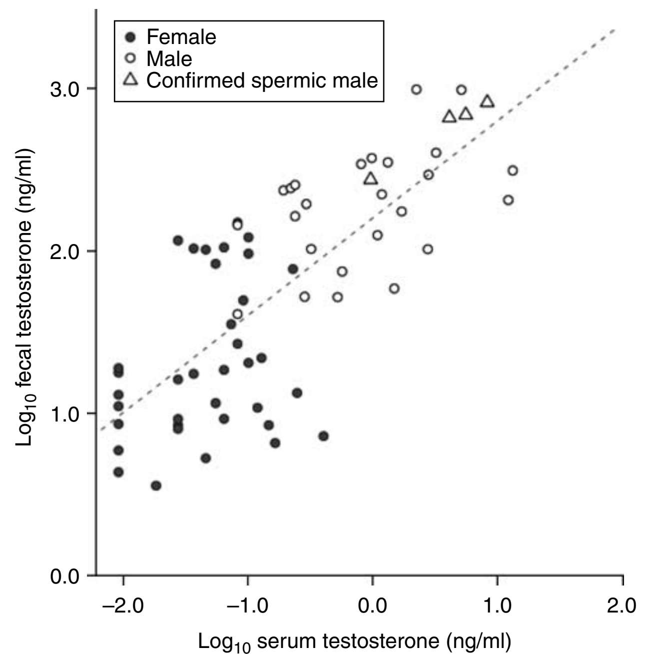

Figure 1 Comparison of serum ($\log_{10}$-transformed ng/ml) and fecal testosterone concentrations ($\log_{10}$-transformed ng/g) in individual live, free-ranging dugongs ($n=65$, excluding one female with no detectable levels of serum testosterone using the described EIA), for biological validation. Males ($n=29$) represented by open white circles, males that voluntarily ejaculated during health assessments ($n=4$) by open white triangles, and females ($n=32$) by closed gray circles. Dotted line represents the linear regression equation: $y=0.86x+2.21$.

late May–June, four adult males (Dugong MB08692 at 271 cm body length, MB08693 at 274 cm, MB10048 at 272 cm, and MB10055 at 276 cm) were confirmed as spermic due to passive discharge of semen or urine containing live spermatozoa. The volume of semen collected from these males ranged from <1 to 9 ml. Only two of these males produced samples considered as semen fluid with a high density of spermatozoa, and the other two males' samples were urine with spermatorrhea. Although these confirmed spermic males appeared to have serum testosterone concentrations (5.16± 1.66 ng/ml) greater than adult males that did not ejaculate and from which blood was also collected (3.28±1.23 ng/ml; $n=10$), the difference between the two groups was not significant ($t_{12}=-0.83$, $P=0.43$; Fig. 1). Fecal testosterone levels of these confirmed spermic males were twice the mean concentration (612±118 ng/g) of adult males that had sperm-free urine samples (309±84 ng/g; $t_{12}=-2.51$, $P=0.03$; Fig. 1), and were among the highest levels measured during May–June of any year. Two larger males sampled out-of-water, MB09881 at 290 cm and MB09896 at 280 cm, had lower fecal testosterone levels of 59 and 179 ng/g, respectively, and had sperm-free urine during sampling.

Testosterone metabolites (4–7100 ng/g) were measurable in all dugong fecal samples collected ($n=322$).

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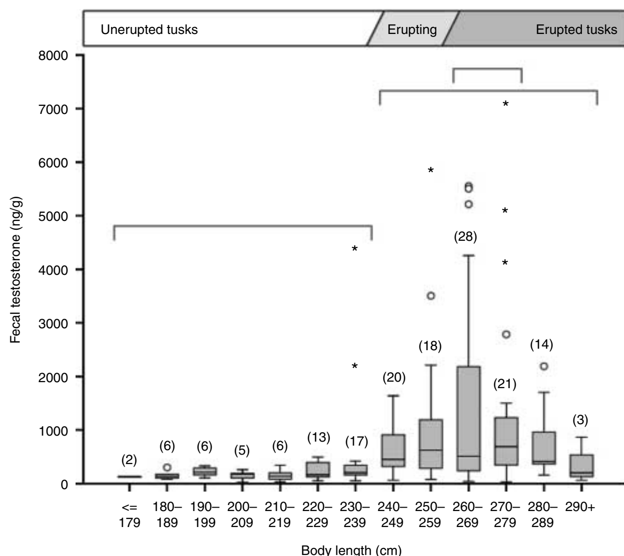

Figure 2 Fecal testosterone concentrations (ng/g) from all male dugongs (n=154) categorized in body length classes of 10 cm increments. For boxplots, the line inside the box indicates the median value, the height of the box encompasses the distance between the 25th and 75th quartiles, and the whiskers delineate extreme observations. Outliers are marked with an open circle (>1.5× interquartile range) and extreme outliers are marked with a star (>3× interquartile range). Size classes under different brackets are significantly different (P<0.05). Sample sizes are indicated in parentheses. Data on presence of erupted tusks in male dugongs by body length (see Fig. 4) are indicated above.

Males had significantly higher mean fecal testosterone concentrations (821±101 ng/g), by almost an order of magnitude than female dugongs (84±7 ng/g; t_{320}= 16.19, P<0.001). Adult males had significantly higher fecal testosterone concentrations than juvenile males and all females ($F_{3,304}$=103.18, P<0.001), with the highest adult male fecal testosterone concentration (7100 ng/g) measuring over 17 times the highest concentration recorded in a juvenile male (maximum 343 ng/g) and any female (maximum 415 ng/g). Mean fecal testosterone concentration in adult males (1200± 171 ng/g) was sevenfold greater than in juvenile males (163±16 ng/g). Juvenile male fecal testosterone concentrations were not significantly different compared to levels measured in females (P>0.05).

Males sampled in this study ranged in body length from 147 to 299 cm. In males, fecal testosterone concentration increased with body length (r=0.41, P<0.001), so that males >240 cm had significantly higher fecal testosterone concentrations than smaller males ($F_{12,146}$=3.84, P<0.001; Fig. 2). Males smaller than 240 cm body length had low (all <500 ng/g) concentrations of fecal testosterone (233±66 ng/g, n=55) with levels similar to females, and only ∼25% of the mean concentration recorded in larger (>240 cm) males (945±266 ng/g, n=104). In males >240 cm, the highest fecal testosterone concentrations (1425± 371 ng/g) were recorded in individuals with body lengths between 260 and 279 cm, with levels in these males significantly higher (at least 1.3-fold) than in all others (P<0.05; Fig. 2). Males >280 cm had lower fecal testosterone concentrations (639±140 ng/g, n=17) than males at body lengths between 260 and 279 cm that produced maximal concentrations. These largest (>280 cm) males had fecal testosterone concentrations statistically similar to males between 240 and 259 cm, but significantly higher than smaller males <240 cm (Fig. 2).

Fifty-eight percent of all male dugongs sampled had erupted tusks (n=53; body length range=228–290 cm), compared to only 9% of female dugongs (n=7; body length range=248–312 cm). All male dugongs >260 cm body length had erupted tusks and all males <238 cm body length had unerupted tusks (Fig. 3), excluding one small male at 228 cm in length with erupted tusks (Dugong MB09914). Male dugongs with body lengths between 238 and 260 cm had variable tusk eruption: 57% of dugongs (n=13) in this size group had erupted tusks, with a trend of a greater proportion of animals with erupted tusks with increasing body size (Fig. 3). Only three out of 159 males that were <240 cm body length had erupted tusks: Dugong MB09914 was 228 cm long, MB09814 was 238 cm, and MB08767 was 239 cm, and fecal testosterone levels for these dugongs were 393 ng/g (sampled August 2009), 282 ng/g (January 2009), and 127 ng/g (November 2008) respectively. One of these males, MB09814 (238 cm), had newly erupted tusks, i.e. with the tusk tips breaking through the gingiva.

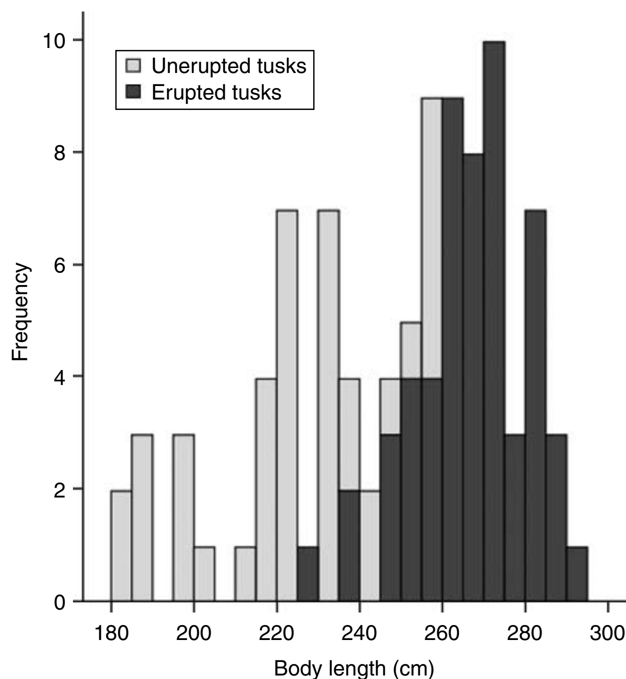

Figure 3 Frequency histogram of the body length (cm) of male dugongs, showing those with unerupted tusks (light bars; n=39) and erupted tusks (dark bars; n=55).

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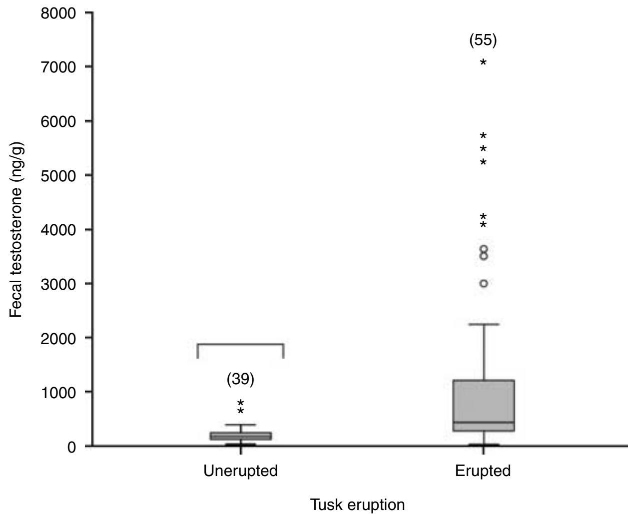

Figure 4 Fecal testosterone concentrations (ng/g) in male dugongs with unerupted and erupted tusks. For boxplots, the line inside the box indicates the median value, the height of the box encompasses the distance between the 25th and 75th quartiles, and the whiskers delineate extreme observations. Outliers are marked with an open circle (>1.5× interquartile range) and extreme outliers are marked with a star (>3× interquartile range). Groups under different brackets are significantly different ($P<0.05$). Sample sizes are indicated in parentheses.

Males with erupted tusks had significantly higher concentrations of fecal testosterone than males with unerupted tusks ($t_{90}=-5.44$, $P<0.001$; Fig. 4), but there was no significant relationship between testosterone level and tusk eruption in females ($t_{82}=-0.81$, $P=0.42$). Males with erupted tusks had an average of six times more fecal testosterone (1236±232 ng/g, $n=53$) than males with unerupted tusks (209±25 ng/g, $n=39$). Most males with unerupted tusks had fecal testosterone levels <500 ng/g ($n=37$); except for two males (identified as outliers, Fig. 4) with concentrations of 683 ng/g (Dugong MB09939 at 260 cm body length; sampled October 2009) and 822 ng/g (MB09961 at 252 cm body length; sampled December 2009). The latter male had swollen gingiva, suggesting that his tusks were close to eruption.

Principal components analysis (PCA) incorporating the variables of body morphometrics (body length and maximum girth), fecal testosterone concentration, and an index of tusk eruption (unerupted or erupted) revealed two components, PC1 and PC2, explaining 53 and 36% of the variance respectively. Both components showed a number of strong loadings, with all variables loading substantially on only one component. Body morphometrics loaded strongly (pattern coefficients >0.94; structure coefficients >0.96) on PC1. Fecal testosterone concentration and the presence of erupted tusks loaded strongly (pattern coefficients >0.83; structure coefficients >0.87) on PC2. Each male dugong was plotted as a function of its loadings for PC1 and PC2, and classified into one of two groups, using a *K*-means classification function (Fig. 5). This classification method assigned male dugongs (total $n=94$ males with all variables measured) into two groups: Group 1 contained 42.4% of males ($n=55$), and included 100% of males previously categorized as juveniles ($n=18$), 11.3% of adults ($n=6$) and 71.4% of males of uncertain reproductive status ($n=15$); Group 2 contained 57.6% of males ($n=39$), and included 88.7% of males previously categorized as adults ($n=49$) but excluded all juveniles ($n=0$), and contained 28.6% of males of uncertain reproductive status ($n=6$; Fig. 5). Group 1 was considered to be composed of immature males and Group 2 was considered to be of mature males.

Discriminant function analysis (DFA) with cross-validation procedures indicated that 91.5% of males were correctly classified (86 out of 94) into each reproductive group (immature and mature), with a classification error rate of 8.5% (eight out of 94 males). Individuals showing a discrepancy in the classification of their reproductive state included three males with erupted tusks at small (228–239 cm) body lengths (MB08767, MB09814, and MB09914, discussed above as outliers) with mean fecal testosterone concentration of 267±77 ng/g (range 126–393 ng/g), and conversely, five males with unerupted tusks at large (249–260 cm) body

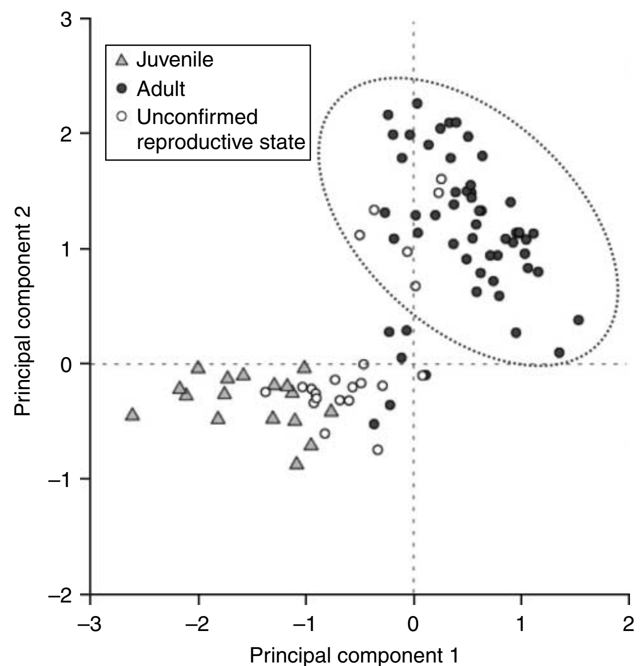

Figure 5 Component matrix of the principal component analysis, showing the factor scores of each male dugong by reproductive status. Juvenile males (≤220 cm body length) are represented by solid light gray triangles, adult males (>250 cm) by solid dark gray circles, and males of uncertain reproductive status (221–250 cm) by open white circles, according to reproductive size classification by Marsh *et al.* (1984*a*). Dotted line marks the zero coordinate for each principal component axis. Males encompassed by the circle were classified as reproductively mature by *K*-means classification function (i.e. Group 2).

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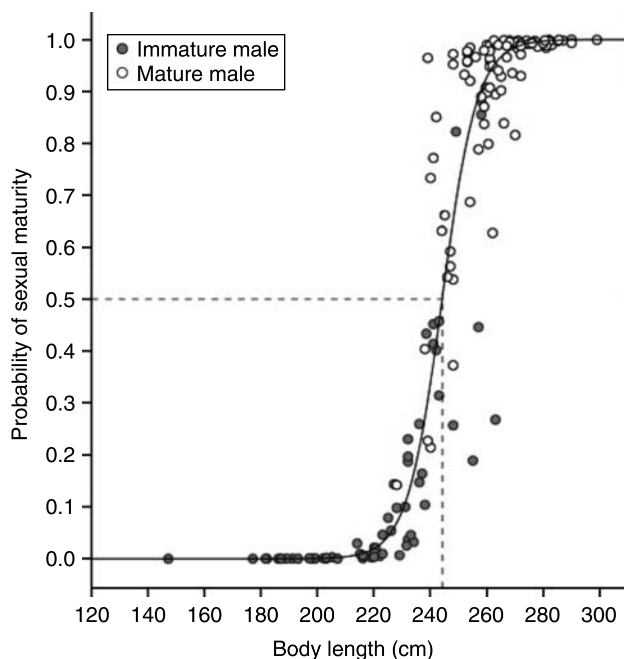

Figure 6 Probability of male dugong sexual maturity with increasing body length (cm), showing predicted immature (represented by closed gray circles; $n=64$) and mature males (represented by open white circles; $n=95$) based on the DFA classification. Body length corresponding to 50% probability of male sexual maturity is reached at 244 cm, as indicated by the dotted line.

lengths (MB08703, MB08754, and MB08758 as well as MB09939 and MB09961, discussed above as outliers) with mean fecal testosterone concentration of 498± 108 ng/g (range 284–822 ng/g). Standardized canonical Discriminant Function weightings identified body length (0.68) as the most important character defining these groups. Using DFA to classify all males sampled in this study (total $n=159$), 64 males were classified as immature (Group 1) with body lengths ranging between 147 and 257 cm and 95 males classified as mature (Group 2) were 239–299 cm long. Therefore, attainment of sexual maturity in male dugongs occurred over a range of sizes between 239 cm (smallest classified mature male) and 257 cm (largest classified immature male). Figure 6 shows the probability of group membership as a mature male (Group 2) with increasing body length, with a 50% probability of sexual maturity at body length of 244 cm. The range of fecal testosterone concentration was 23–498 ng/g for males classified as immature and 30–7100 ng/g for mature males. Over six years of sampling, we suggest that 40.3% of males were immature and 59.7% of males were mature from the sampled live population in Moreton Bay.

Temporal patterns

Male dugongs were sampled in all months of the year (except April) over six years (July 2005–June 2011). Fecal testosterone concentration in males did not vary across years ($F_{6,129}=0.46$, $P=0.84$), but showed highly significant temporal variation across months ($F_{10,129}=4.86$, $P<0.001$; Fig. 7). Mature males exhibited significant differences in fecal testosterone concentrations across months ($F_{10,82}=7.05$, $P<0.001$), but no differences were found in immature males ($F_{10,54}=1.72$, $P=0.10$). In mature males, elevated fecal testosterone concentrations (225–7100 ng/g; Fig. 7) were recorded from August to November of each year. The highest fecal testosterone concentrations were recorded in the months of September and October (2276±523 ng/g) and these were significantly higher (mean eightfold) than in the months from December to May in which the lowest concentrations occurred (277±89 ng/g; all $P<0.05$; Fig. 7). This temporal variation in fecal testosterone concentrations in mature males was significant across seasons ($F_{3,89}=14.51$, $P<0.001$). In the austral spring (September–November), mature males had significantly higher (1703±243 ng/g, mean threefold) fecal testosterone concentrations than in summer (December–February; 391±71 ng/g), autumn (March–May; 300± 84 ng/g), and winter (June–August; 856±244 ng/g) seasons (all $P<0.05$; Fig. 7). No seasonal differences in fecal testosterone concentrations were found in immature male dugongs (summer, 175±27 ng/g, $n=13$; autumn, 183±32 ng/g, $n=10$; winter, 187±34 ng/g, $n=12$; spring, 341±76 ng/g, $n=29$; $F_{3,60}=1.67$,

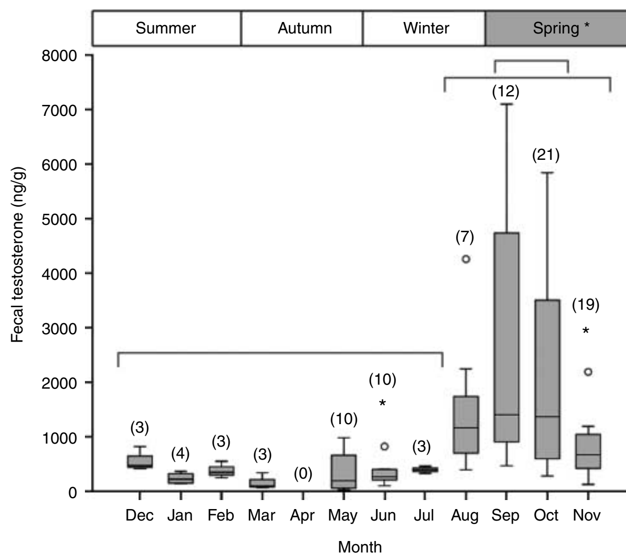

Figure 7 Temporal variation in fecal testosterone concentrations (ng/g) of mature male dugongs by month and austral seasons (showing both mature males by closed gray bars and immature males represented by open white bars). For boxplots, the line inside the box indicates the median value, the height of the box encompasses the distance between the 25th and 75th quartiles, and the whiskers delineate extreme observations. Outliers are marked with an open circle (>1.5×interquartile range) and extreme outliers are marked with a star (>3× interquartile range). Groups under different brackets and seasons marked with an asterisk are significantly different ($P<0.05$). Sample sizes are indicated in parentheses.

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$P=0.18$). In very large mature males (>280 cm body length) that showed a decline in testosterone production relative to other mature size classes (i.e. >240 cm, discussed above), seasonal differences in fecal testosterone concentrations were still significant, although concentrations for each season were lower (summer, 312 ± 77 ng/g, $n=3$; autumn, 146 ± 44 ng/g, $n=3$; winter, 404 ng/g, $n=1$; spring, 909 ± 197 ng/g, $n=10$; $F_{3,13}=5.60$, $P=0.01$) than the mean values for all mature males. Differences in fecal testosterone concentration between immature and mature males was the greatest during spring ($t_{79}=7.46$, $P<0.001$) when mature males had concentrations, on average, five times higher than immature males; this compared to differences between immature and mature males during winter (fourfold higher; $t_{30}=3.21$, $P=0.003$) and summer (twofold higher; $t_{21}=2.84$, $P=0.01$), but there was no significant difference between immature and mature males recorded during autumn ($t_{21}=0.39$, $P=0.70$).

Repeat fecal samples were collected from 19 individual male and 14 female dugongs over the period of this study and were assigned to two seasonal periods: summer–autumn (December–May) or winter–spring (June–November): there were insufficient samples to compare all four separate seasons. Each of these dugongs was sampled twice and one male (MB05410) was sampled four times. For individual males sampled between seasonal periods ($n=9$), fecal testosterone concentration was significantly higher in the winter–spring period compared to summer–autumn ($t_8=-3.01$, $P=0.02$; Fig. 8). The mean increase in fecal testosterone concentration was 538 ng/g with a 95% confidence interval ranging from 126 to 949 ng/g. The effect size was large, with 35% of the variability in fecal testosterone concentration explained by season. However, the differences in an individual's fecal testosterone concentrations between summer–autumn and winter–spring were significant only in mature ($t_8=-3.01$, $P=0.02$), and not in immature males ($t_{153}=-72.81$, $P<0.001$; Fig. 8). The smallest change in fecal testosterone concentration between seasonal periods was recorded in dugong MB06554 (Fig. 8) who was sampled as a large attendant calf with mother in both December 2006 (summer–autumn) and October 2007 (winter–spring), and showed little change in fecal testosterone between sampling periods (104 and 77 ng/g respectively). One mature male, MB05410, was sampled four times between 2005 and 2009 with the following temporal variation in fecal testosterone concentrations: 756 and 1190 ng/g in July 2005 (winter); 263 ng/g in December 2006 (summer); and 1831 ng/g in August 2009 (winter). For males repeatedly sampled within seasonal periods (i.e. sampled twice in summer–autumn or sampled twice in winter–spring; $n=10$), there was no significant difference in fecal testosterone concentration between sampling events ($t_{10}=0.80$, $P=0.45$). Fecal testosterone concentrations in female dugongs showed no significant trend across ($t_5=-1.23$, $P=0.27$) or within seasonal periods ($t_7=0.00$, $P=1.00$).

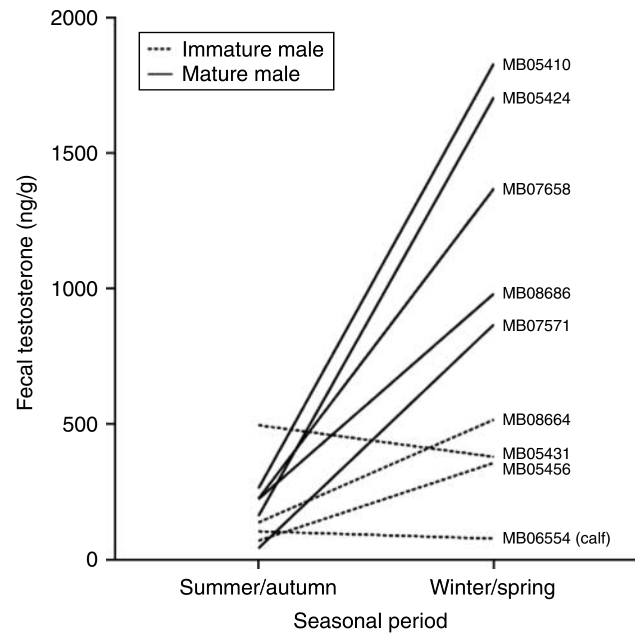

Figure 8 Seasonal changes in fecal testosterone concentrations of nine male dugongs sampled on two occasions over 2005–2011 during both summer–autumn (December–May) and winter–spring (June–November). Immature males are represented by a broken line ($n=4$); and mature males by a solid line ($n=5$).

Group association effects

In Moreton Bay, most of the sampled dugongs (68%, $n=186$) were found in association with other dugongs rather than as solitary individuals ($n=86$; $X^2=34.47$, $P<0.001$). Season significantly influenced group association in dugongs ($X^2=10.82$, $P=0.01$), with animals associating more frequently in groups over winter (72%, $n=38$) and spring (74%, $n=99$) than during autumn months (46%, $n=17$), with summer not significantly different from other seasons (67%, $n=32$). However, seasonal influences on dugong group dynamics were different between sexes. For females, group association was influenced by season ($X^2=8.01$, $P=0.04$), with females over spring associating in groups more frequently (80%, $n=48$) than occurring alone (20%, $n=12$). This was significantly different from the trend over autumn when females had similar frequencies of being found as solitary individuals (52%, $n=11$) or associated with other dugongs (48%, $n=10$). Summer and winter months showed similar likelihoods of females being associated in groups (68%, $n=19$ and 71%, $n=17$ respectively) or as individuals (32%, $n=9$; and 29%, $n=7$). However, for male dugongs, group association was not influenced by season ($X^2=4.18$, $P=0.24$), with males equally likely to associate (mean 62%, $n=92$) or occur as solitary animals (mean 38%, $n=47$) across all seasons.

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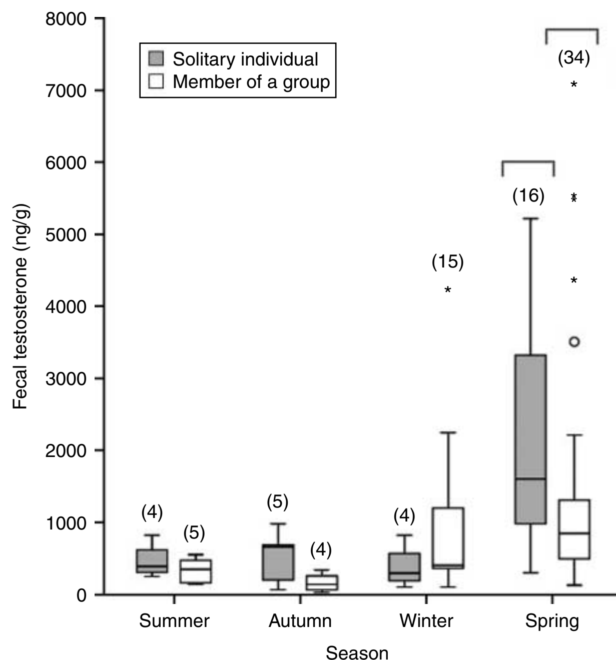

Figure 9 Seasonal differences in fecal testosterone concentrations of mature male dugongs according to social association, i.e. solitary individual or member of a group. For boxplots, the line inside the box indicates the median value, the height of the box encompasses the distance between the 25th and 75th quartiles, and the whiskers delineate extreme observations. Outliers are marked with an open circle (>1.5× interquartile range) and extreme outliers are marked with a star (>3× interquartile range). Groups under different brackets are significantly different ($P<0.05$). Sample sizes are indicated in parentheses.

Mature male dugongs had significantly different fecal testosterone concentrations across seasons and these were correlated with group association ($F_{3,84}=2.66$, $P=0.05$; Fig. 9). In spring, lone mature males had significantly higher fecal testosterone concentrations (2328±407 ng/g) than males encountered within a group (1429±286 ng/g; $F_{1,49}=7.01$, $P=0.01$); although five males sampled within herds (out of 34 males sampled in herds over spring) had high concentrations (>3000 ng/g) and were identified as outliers (Fig. 9). In all other seasons (summer–winter), there were no differences in fecal testosterone concentrations of mature males found within a group or as singletons (all $P>0.20$). For immature males, there were no differences in fecal testosterone concentrations between lone dugongs or members of a herd ($F_{1,52}=0.00$, $P=0.97$) and no seasonal interaction ($F_{3,52}=1.71$, $P=0.18$), suggesting that within all seasons, solitary immature males had fecal testosterone concentrations similar to those immature males within a group.

Discussion

Variation in fecal testosterone levels was strongly linked to life history in male dugongs. This study provides the first assessment of testicular activity across all ontogenetic stages of free-ranging dugongs, determined by changes in fecal testosterone metabolite concentration. Reproductively mature adult males had significantly higher fecal (up to 17-fold) and serum (up to 33-fold) testosterone concentrations than juvenile males or females, suggesting that the EIA applied here successfully detected biologically relevant differences in testicular activity similar to fecal androgen studies in other large free-ranging mammals (e.g. Dloniak *et al.* 2004, Ganswindt *et al.* 2005, Rolland *et al.* 2005). Furthermore, the close correlation between testosterone concentrations in serum and fecal samples demonstrated that hormones eliminated via the digestive tract of the dugong are representative of circulating concentrations, lending further validity to the use of fecal hormone techniques to examine reproductive endocrine activity in this wildlife species.

Attainment of sexual maturity

Fecal testosterone metabolite concentrations were positively correlated with body size in male dugongs, most likely indicating enhanced gonadal steroidogenesis that occurs in association with growth and testicular development. Male dugongs with a body length >240 cm had more than four times the concentration of fecal testosterone than smaller and probably younger males. Testosterone secretion may be synchronous with both testicular maturation and testicular activity in male dugongs, with further evidence from post-mortem studies that the relative volume of Leydig cells in dugong testes increases with progressing spermatogenesis of the seminiferous epithelium (Marsh *et al.* 1984*a*). Similarly, higher circulating testosterone concentrations have been shown to reflect sexual maturation in other marine mammals (reviewed by Atkinson (2008)), including cetaceans (Desportes *et al.* 1994, Kjeld *et al.* 2006, Atkinson & Yoshioka 2007) and pinnipeds (Atkinson 1997) for which maturity has been confirmed by histological examination of the gonads.

The present study has delineated statistically significant body size ranges over which reproductive maturity and sexual activity are achieved by male dugongs in Moreton Bay. The attainment of reproductive maturation was characterized by significant elevations in testosterone production along with eruption of tusks. All male dugongs smaller than 240 cm body length had relatively low fecal testosterone concentrations (<500 ng/g) and unerupted tusks, suggesting that in Moreton Bay, most males <240 cm are prepubescent and non-reproductive. Fecal testosterone concentrations higher than 500 ng/g were only seen in male dugongs longer than 240 cm, suggesting that this is the approximate body size at which functional spermatogenesis commences. Testosterone concentration and eruption of tusks were variable in the intermediate body size class of

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240–260 cm, indicating that the timing of puberty is somewhat variable in this location.

Patterns of testosterone production observed in this study confirm that testicular maturity precedes but is closely associated with the emergence of tusks in male dugongs, with tusked males having fecal testosterone concentrations an average of sixfold higher than males with unerupted tusks. Furthermore, only two males out of 159 examined had high fecal testosterone concentrations (>500 ng/g) but unerupted tusks (although one male had tusks close to eruption), so that individuals approaching 240 cm body length may be showing signs of early puberty. This is consistent with histological studies suggesting that tusks do not erupt until after mature sperms first appear in the testes and epididymides of male dugongs (Marsh 1980, Marsh *et al*. 1984*a*, Kwan 2002). However, the absence of erupted tusks in small males with functional testicles presumably diminishes their sexual prowess (discussed below).

In Moreton Bay, male dugongs predicted to be newly mature based on testosterone concentration and tusk eruption were at 81% of full-grown asymptotic length (i.e. reaching sexual maturity when ∼244 cm long with a growth potential of ∼300 cm during a male's lifespan), suggesting that the acquisition of reproductive maturity in male dugongs may occur relatively late in life (>10 years of age; Lanyon JM (2012), unpublished observations), similar to other long-lived mammal species (Desportes *et al*. 1994, Lincoln 1998). We found no physiological evidence in Moreton Bay of males having the degree of precocious maturation as has been reported elsewhere. In Moreton Bay, the smallest potentially mature male had a body length of 228 cm, erupted tusks but fecal testosterone level (393 ng/g) within the immature range; although this low testosterone may also be due to sampling during the non-breeding season rather than to sexual immaturity (see below). This small-tusked male may be precocious for the Moreton Bay population because all other males showed signs of maturity at body sizes >240 cm. At a large regional scale over northern Australia, dugongs have been considered to attain sexual maturity over a broad size range of 190 to 250 cm (Marsh *et al*. 1984*b*, Kwan 2002). It appears that male dugongs in subtropical Moreton Bay may need to reach a larger body size (>240 cm) before they begin to mature sexually, compared to those from northern tropical waters. Of all of the previously sampled populations, body size of males at maturity in Moreton Bay may be most similar to males in Townsville whose testes are also in active spermatogenesis from 240 cm body length (Marsh *et al*. 1984*a*). However, differences in maturation may be most protracted when comparing dugongs from Moreton Bay, at the southern-most limit of dugong distribution, with lower-latitude populations. In Torres Strait, male dugongs are considered to reach sexual maturity at smaller sizes (the smallest mature males recorded were two individuals, 191 and 193 cm long) and earlier ages (determined by counting the annual dentinal growth layers of the tusks: Marsh 1980) compared with other locations (Kwan 2002). Torres Strait has extensive and plentiful seagrass beds (Kwan 2002, Marsh & Kwan 2008), and seagrass nutrient availability may be more persistent in this tropical region compared to the subtropics (Lanyon *et al*. 1989), allowing puberty to occur earlier. Such spatial differences in the sexual maturation of dugongs are likely to be resource-dependent (Marsh & Kwan 2008), and warrant further investigation into growth rates and body condition differences between populations.

The ability to distinguish sexual maturity across a representative set of live male dugongs in a free-ranging population has significant application in population dynamics. Using a multifactorial approach, we have been able to determine the likelihood of sexual maturity for each individual male dugong in this study, providing new information for population structure. Defining sexual maturity in males is complex (Perrin & Reilly 1984); however, the criteria used in this study (i.e. fecal testosterone concentration, body length, and tusk eruption) have effectively and non-lethally determined this life stage in live dugongs. Given the importance of determining the reproductive structure for population management, we believe that our results using single fecal samples and/or body length measures will be useful for field researchers. However, it is important to note that for single sampling events, low testosterone concentrations do not necessarily always equate to sexual immaturity (i.e. when considering variation with breeding season, discussed below), whereas higher concentrations are always consistent with sexually mature or maturing dugongs.

Sexual activity of mature males

Large male dugongs ≥260 cm body length, all had erupted tusks, and had higher overall concentrations of testosterone compared to all other males. Erupted tusks and large body size may allow male dugongs to be reproductively competitive in a potentially promiscuous mating system (Preen 1989), with a significant increase in testosterone production assisting aggressive behavior as well as spermatogenesis in actively breeding males. From anecdotal behavioral observations and body scarring on dugongs, it is probable that male dugongs use their tusks during physically competitive and aggressive interactions with other males (Preen 1989) and/or during mating attempts to roll the females for ventrum-to-ventrum copulation (Anderson & Birtles 1978). In dugongs, there may be an important distinction between physiological maturity at body lengths >240 cm (testicular maturation and initiation of spermatogenesis) and social maturity at ≥260 cm (large body size, tusk eruption, development of accessory sex glands, and sexual behavior), which is necessary for reproductive success. It is likely that

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although sexually mature male dugongs are capable of producing sperm, they may not have the capacity to mate successfully until further growth and development, as has been found in other marine mammals (Anderson & Fedak 1985, Desportes *et al*. 1993) and ungulates (Lincoln 1989, 1998, Pelletier *et al*. 2003). Large mature male dugongs with well-developed tusks may presumably experience more reproductive challenges and successful copulations than their smaller younger counterparts. Accordingly, there may be an association between elevated testosterone levels and social dominance in individual male dugongs, similar to patterns of testosterone variation reported in their phylogenetic relatives, the Asian (*Elephas maximus*; Lincoln & Ratnasooriya 1996) and African elephant (*Loxodonta africana*; Rasmussen *et al*. 1996, Ganswindt *et al*. 2005, Hollister-Smith *et al*. 2007), and rock hyrax (*Procavia capensis*; Koren *et al*. 2006).

Interestingly, the very largest male dugongs (>280 cm body lengths, i.e. 11% of all males sampled) had significantly lower mean testosterone concentrations compared to other mature males, suggesting that older male dugongs may have reduced testicular steroidogenesis and a consequent decline in reproductive function. In some other male mammals (e.g. North Atlantic fin whales older than 40 years (Kjeld *et al*. 2006)), decreases in testosterone production may accompany aging (Blottner *et al*. 1996, Lincoln 1998, Zirkin & Chen 2000). Reduced testosterone may result through spermatogenic failure or senescence, or individual variation in the timing and duration of spermatogenesis, with numerous causal factors including nutritional state, exposure to receptive females, or social status. Reproductive quiescence in male dugongs has also been suggested from testicular histology, where some old males may enter sterile cycles on a long-term or permanent basis (Marsh *et al*. 1984*a*). The possibility of reproductive senescence could not be thoroughly assessed in this study, and is poorly understood in other male marine mammals (Atkinson & Yoshioka 2007). However, the methodology described here on live animals allows repeat sampling of individuals and has the potential for more complete characterization of reproductive activity with age.

Reproductive seasonality

Mature male dugongs in Moreton Bay showed spring elevations in fecal testosterone, indicating that male dugongs in this population are seasonal breeders. Longitudinal monitoring of the same dugong was not possible in this study; nonetheless, a number of recaptured mature and immature individuals of both sexes provided insight into seasonal and inter-annual fluctuations, which agreed with the seasonal reproductive patterns observed across the entire population. Our results are consistent with the patterns of testicular activity seen in other seasonally breeding male mammals, where the testes do not remain uniformly active throughout the year (reviewed by Lincoln (1989)). Seasonal increases in androgens can influence both sexual physiological changes including gonad size, total sperm count, semen volume, and sperm motility (Schroeder & Keller 1989, Blottner *et al*. 1996, Mogoe *et al*. 2000) and behavioral traits, such as reproductive motivation, aggression, social status, and dispersal (Creel *et al*. 1997, Cavigelli & Pereira 2000, Buck & Barnes 2003, Dloniak *et al*. 2004): all aspects of male reproduction that promote successful breeding. It is unclear which mechanisms correlate with seasonal androgen expression in dugongs, but there is evidence that both physiological and behavioral variations may be occurring in males.

Maximum testosterone production of mature dugongs occurred during September–October each year, when testosterone concentrations were approximately four times higher than in all other months. This suggests that androgenic and potential spermatogenic activity is highest during the spring mating season in Moreton Bay. If testosterone expression in dugongs is related to testicular physiology similar to that reported for other male marine mammals including pinnipeds and cetaceans, then seasonal increases in testosterone secretion may be followed 1–2 months later by physiological manifestations such as increases in testicular volume and/or seminal quality (Schroeder & Keller 1989, Noonan *et al*. 1991, Neimanis *et al*. 2000, Atkinson 2008). If this time lag is applied to male dugongs in Moreton Bay, maximal testosterone concentrations in September may be followed by increased testicular function and/or spermatozoa competence during October and November, potentially increasing the likelihood of impregnation during these months. Considering the predominance of neonatal calves observed in Moreton Bay in December and January (Lanyon 2003) and an estimated 14-month gestation period (Boyd *et al*. 1999, Kwan 2002), it is probable that most successful conceptions occur over spring (September–November), i.e. the period of highest testosterone, leading to the observed seasonal calving. Furthermore, histological studies of dugong gonads from the tropics show a similar temporal peak in male spermatogenic activity, with a greater number of post-mortem males sampled in October showing active testicular tissues with advanced stages of germ cell production and a high density of spermatozoa in the epididymal tissue (Marsh *et al*. 1984*b*, Kwan 2002). Declining testosterone production in mature males over autumn months in this study concurs with a preponderance of adult male dugongs with regressed, resting, or intermediate testes between February and May in northern Australia (Marsh *et al*. 1984*a*, Kwan 2002).

Active spermatogenesis in early winter (late May–June) was confirmed by the four mature male dugongs

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that produced sperm during sampling. This collection occurred prior to the main peak in testosterone production, which is associated with the commencement of the breeding season. However, seasonal reproduction requires that males have adequate numbers of viable sperm when females enter estrus, and hence, the spermatogenic cycle must be initiated months before breeding (Atkinson 1997). Germ cell proliferation in male dugong testes has been evident from May onwards in north Australian populations (Marsh 1995, Kwan 2002). Notably, testosterone concentrations of confirmed spermic males in this study were higher than other adult males sampled at the same time from which sperm was not obtained, though concentrations were low compared to the highest levels in mature males over spring months. Semen quality and sperm counts were not measured in each of these samples, and the volumes of seminal fluid (<1–9 ml) discharged by these males were considerably less than a semen sample passively collected from a captive male dugong (∼150 ml; S. Gilchrist (2011), personal communication) in September when testosterone concentrations are maximal. It is probable that ejaculate quality in male dugongs varies with testosterone concentration and season similar to other seasonal breeders (Schroeder & Keller 1989, Blottner *et al*. 1996) and spermatogenesis may in fact cease over autumn, when testosterone concentrations are similar to levels in immature males, the extent of which may vary between individuals.

Furthermore, elevation of male testosterone measured in spring months coincides with aggressive dugong interactions in Moreton Bay that have only been observed in late October and early November (Preen 1989). In these vigorous and injurious encounters, male dugongs engage in physically combative competitions for estrous females (Preen 1989), and wounding from tusk rakes is common on both males and females (Burgess EA & Lanyon JM (2012) unpublished observations). Injuries related to mating interactions in Hawaiian monk seals have been found to coincide with the period of highest testosterone in males (Atkinson & Gilmartin 1992), and a similar association may also be found in dugongs. In male mammals, increasing testosterone levels mediate changes in mating behavior and physical aggression (Wingfield *et al*. 1990, Creel *et al*. 1997, Lincoln 1998, Strier *et al*. 1999, Buck & Barnes 2003, Pelletier *et al*. 2003, Ganswindt *et al*. 2005), with males showing a peak in behavior coincident with maximal testicular activity or shortly afterwards (Lincoln 1989). The physiological mechanisms driving intraspecific aggression in dugongs are probably similar to those found in other species, such that male dugong testosterone levels may reach a physiological maximum to promote responses to agonistic challenges during breeding (Wingfield *et al*. 1990, Strier *et al*. 1999).

Male dugong mating strategy

Dugongs form large herds (>10 dugongs but up to 300) during the spring and summer in Moreton Bay (Lanyon 2003). Female dugongs in this study were more frequently associated with herds during spring months, whereas males were equally likely to be encountered in groups or as solitary individuals. Hence, the formation of dugong groups over spring in Moreton Bay appears to be largely influenced by the females. Little is known about dugong social structure and spatial organization, but anecdotal accounts suggest that mating may be polygynous and promiscuous (Preen 1989, Boyd *et al*. 1999) but vary regionally (Anderson 2002). When testosterone production increased in mature males during spring months (i.e. apparent breeding season), lone mature males found away from herds had fecal testosterone concentrations twice the level measured in males associated with groups. It is possible that the reproductive state of male dugongs may influence the social system through roving movements and dispersion of breeding males. In Moreton Bay, mating herds similar to those seen in Florida manatees (Hartman 1979) have been observed (Preen 1989). However, compared with Florida manatees, dugongs may exhibit more violent competition among males to gain access to estrous females within herds (Preen 1989). Male dugongs in breeding condition and with high testicular activity may leave herds due to intensity of male competition for females within the group, or alternatively these lone male dugongs may be purposefully roving between herds to gain access to unrelated females for mating opportunities. In some terrestrial mammals, including spotted hyenas (*Crocuta crocuta*) and chacma baboons (*Papio hamadryas*), fecal androgen concentrations in immigrating adult males were often higher than in natal adult males (Dloniak *et al*. 2004, Beehner *et al*. 2006), suggesting that increased testosterone production is associated with male roving status similar to what may be occurring here. Further, a few males (15%) within dugong herds had elevated testosterone concentrations above group mean values, suggesting a higher level of testicular activity in a minority of males associated with herds. These males may have experienced prolonged high testosterone production in response to more frequent interactions with other males and receptive females as proposed under the ‘challenge hypothesis’ for polygynous males (1990), and may potentially be associated with male dominance and rank acquisition (e.g. Creel *et al*. 1997, Beehner *et al*. 2006).

Understanding the connection between social dynamics and reproduction is vital when elucidating species’ life history, and often requires long-term behavioral data. The present results provide new insights into dugong group associations and the potential role of androgens in mediating roving and sociality in male dugongs, and suggest that androgen-group interactions

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in free-ranging populations can be a useful tool to further elucidate the mating strategies of cryptic species.

Materials and Methods

Data collection

Live, free-ranging dugongs were sampled in subtropical Moreton Bay (27° 19′ S, 153° 24′ E), an embayment at the southern limit of the dugong's range in eastern Australia, over six years from July 2005 to June 2011. Dugongs were sampled year-round across all austral seasons: summer (December–February), autumn (March–May), winter (June–August), and spring (September–November). Dugongs were sampled opportunistically during boat-based line transects across their principal feeding areas on the Eastern Banks of Moreton Bay, at high tide when they were foraging (Lanyon 2003). When a dugong was encountered, it was recorded as being either solitary or a member of a group. Dugongs of both sexes and all ages (except neonates) were captured and restrained at the water surface for a short period (5–6 min; Lanyon *et al*. 2006). Each dugong was tagged for identification (Lanyon *et al*. 2002) and sexed (Lanyon *et al*. 2006). Body size of each dugong was based on total body length measured in a straight line from snout to fluke notch, and maximum girth measured at the umbilicus position. Dugongs were checked for the presence of erupted tusks by lifting the oral disk and examining the front of the premaxilla. Tusks were scored as either erupted or unerupted for both male and female dugongs ($n=164$).

Reproductive classification of male dugongs

Male dugongs were grouped into reproductive size classes based on body length, following the size classification of Marsh *et al*. (1984*b*) that used histological examination of testis and/or epididymis to confirm sexual maturity: body lengths <220 cm were considered likely to be reproductively immature (juvenile), showing narrow, closed seminiferous tubules (i.e. no lumen) with an absence of spermatids or spermatozoa, and a single testis weight of <20 g; body lengths ≥ 250 cm were probably mature (adult), showing open seminiferous tubules with various phases of spermatocyte maturation and a single testis weight of more than 30 g; and body lengths between 220 and 249 cm body length were of variable reproductive status.

Fecal and serum sample collection

Fresh fecal samples (~ 4 g) were collected from each dugong by inserting a soft latex tube into feces held in the distal part of the rectum. Fecal samples (uncontaminated by seawater) were transferred into zip-lock plastic bags and kept on ice before being frozen, and stored at -20 °C until analyzed. A total of 357 fecal samples were collected representing 322 individuals, with 33 dugongs sampled on more than one occasion ($n=35$ fecal samples) within the study period.

To investigate circulating hormone concentrations, blood samples were collected from 66 dugongs (37 females, 29 males) as part of out-of-water health assessments. These annual health assessments were conducted in May–June over four consecutive years: 19–23 May 2008 ($n=13$), 11–17 May 2009 ($n=17$), 11–17 June 2010 ($n=20$), and 29 May–2 June 2011 ($n=16$). Dugongs were captured and sampled as described above, and then lifted clear of the water onto the rear deck of a research vessel for comprehensive medical examination (Lanyon *et al*. 2010). For each dugong, a blood sample was collected from the brachial arteriovenous plexus on the pectoral flipper, within 5–10 min of arrival on deck. A 21-gauge 3.8-cm /1.5-in needle (or 5-cm /2-in needle in the case of large adults >270 cm body length) fitted to a 20-cm (14-in) extension set and Luer fitted Vacutainer collar was used for blood collection. Blood was collected in 10-ml red-top clot-activator Vacutainer tubes and serum was separated by onsite centrifugation (1790 ***g*** for 15–20 min) within 30 min of collection. Serum samples were stored frozen in cryovials until analysis. When a dugong was first brought on deck, a clean plastic Frisbee was placed underneath the genital orifice. This device was intended to act as a urine receptacle, but also collected voluntary semen ejaculate from male dugongs. Before the dugong was released between 25 and 50 min later, the urine was decanted into a sterile container and examined for the presence of sperm under field microscopy (400× magnification).

Steroid extraction and analysis

Fecal samples were dried at 55 °C overnight prior to being pulverized and mixed. Powdered feces were weighed out to the nearest 0.20 ± 0.01 g in a 5-ml glass scintillation vial (Sigma–Aldrich) and then mixed with 4.0 ml of 80% methanol (Crown Scientific, Brisbane, QLD, Australia). This mixture was vortexed briefly, and placed on a rotating shaker overnight (minimum of 12 h). The following morning, samples were centrifuged for 15 min at 1500 ***g***. The supernatant fraction was decanted into vials and stored at -20 °C. Fecal extracts were diluted (1:30) in assay buffer (pH 7.0) prior to hormone analysis, and extreme low and high concentrated samples were re-diluted to 1:5 and 1:420 as needed.

For measurement of testosterone (and metabolites) from both serum and fecal extracts, we used a single-antibody EIA following Walker *et al*. (2002). Testosterone polyclonal antibody R156/7 and the corresponding HRP conjugate were obtained from C Munro (University of California, Davis, CA, USA). The testosterone antibody had the following cross-reactivities as tested by Gudermuth *et al*. (1998): testosterone (100%), 5α-dihydrotestosterone (29%), androstenedione (0.5%), and with six other steroids, including estradiol, progesterone, and cortisol (all ≤ 0.1%).

The EIA plate (96 wells) was coated with testosterone antibody solution (1:10 000) and incubated overnight at 4 °C. The following day, the plates were washed to remove unbound antibody. Immediately after this wash, 50 µl of standards (1.9–1000 pg/50 µl; Sigma–Aldrich), controls, and dugong samples (undiluted serum or diluted fecal extract) were loaded as duplicates on to the plate, and then 50 µl of HRP-testosterone conjugate (1:15 000) were added to each well. After incubating for 2 h at room temperature, plates were washed and 100 µl of color substrate solution were added to each well. Plates were read when the optical density of the maximum binding wells

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reached 1.0, using a microplate reader with Revelation Software (Dynex MRX II, Q-lab Pty Ltd, Brisbane, QLD, Australia).

The assay was validated biochemically for dugongs by demonstrating 1) parallelism between serially diluted extracts (1:1–1:2048) and the standard curve ($R^2=0.99$, $P<0.001$), and 2) significant recovery of exogenous testosterone (92.3%) added to a pool of dry feces prior to extraction. To monitor precision and reproducibility, low (65% binding) and high (30% binding) quality control samples were run on each plate. Inter-assay coefficient of variation (CV) was 13.3 and 10.8% for low and high controls respectively. Intra-assay CV was 3.5%; calculated from the variation of measurements between duplicates, except when there was a large discrepancy (CV >10%) in which case the sample would be assayed again.

Statistical analysis

For those dugongs repeatedly sampled during the project, we used the data from the first fecal sample collection to ensure that individual dugongs were represented only once in statistical analyses. However, data from duplicate fecal samples were used to investigate variation within individual dugongs between seasonal periods (36 fecal samples representing 33 dugongs). We used simple descriptive statistics (mean ± s.e.m., range) to summarize the data set. Testosterone concentrations were transformed using the common logarithm, $\log_{10}$, to adjust for a skewed, non-normal distribution. Levene tests were conducted for all variables to check for homogeneity of variance, using Welch's correction for unequal variances. A linear regression analysis was applied to the matched serum and fecal samples to determine the correlation between testosterone concentrations in these two media. Testosterone metabolite concentrations in the fecal and serum samples were compared between sexes, two reproductive size classes of male dugongs (juvenile and adult; after Marsh *et al*. 1984*b*), and against tusk state (unerupted and erupted) using an independent *t*-test or one-way ANOVA. If significant differences were found, *post-hoc* Tukey tests were conducted. We also tested for differences in serum and fecal testosterone concentration between confirmed spermic males and adult males that did not ejaculate during health assessments using an independent *t*-test. A linear correlation analysis was performed to examine the relationship between fecal testosterone metabolite concentration and body length in males.

Sexual maturity in male dugongs was characterized using multifactorial models incorporating body size, hormonal changes, and the presence of erupted tusks. PCA was conducted with two variables of body morphometrics (body length, maximum girth; both in cm), $\log_{10}$-transformed fecal testosterone concentration (ng/g), and an index of tusk eruption (1, unerupted tusks; 2, erupted tusks), using a correlation matrix ($n=92$ male dugongs with all variables measured). Prior to performing PCA, the suitability of data for factor analysis was assessed. Inspection of the correlation matrix revealed the presence of many coefficients of 0.3 and above. The Kaiser–Meyer–Olkin value was 0.6 and Bartlett's test of Sphericity reached statistical significance ($P<0.001$), supporting the factorability of the correlation matrix. PCA revealed the presence of two components with eigenvalues exceeding 1, and an inspection of the scree plot showed a clear break after the second component; it was decided to retain two components for further analysis. To aid in the interpretation of these two components, oblimin rotation was performed with the two factors showing low inter-correlation ($r=0.14$). We used graphical representation of the PCA in order to visualize discrete groups of males that might represent immature and mature dugongs. Male dugongs were assigned to each group using a non-hierarchical classification procedure (*K*-means cluster). This method was based on establishing a predetermined number of groups (in this case, two) and assigning dugongs to one of the groups according to their loads on the two axes of the PCA, by means of an iterative process that minimizes intra-group variance and maximizes between-group variance. Using the results of the classification, a DFA was conducted to classify dugongs as immature or mature on the basis of body length, maximum girth, presence of erupted tusks, and fecal testosterone concentration. A cross-validation technique was used in the analysis, which permitted an estimation of error (i.e. inconsistencies) in this classification methodology. For the estimation of mean size at onset of sexual maturity for male dugongs, a logistic function was fitted to the probability of group membership as a mature male by body length.

A two-way ANOVA was used to evaluate the effects of temporal variability (sampling years and months), and the interaction of these variables on fecal testosterone concentration in male dugongs. Seasonal effects on fecal testosterone concentration for each male reproductive state (immature or mature; as predicted by DFA) were compared using an independent *t*-test or one-way ANOVA. For each season, the difference in fecal testosterone concentration between immature and mature males was compared using *t*-test analysis. To specifically test for differences in fecal testosterone concentration within an individual between seasonal periods, we performed a paired-sample *t*-test for those dugongs sampled on more than one occasion (i.e. using duplicate fecal samples from 35 individuals). For this analysis, we compared fecal testosterone concentrations in June–November (winter–spring) to concentrations in December–May (summer–autumn); these periods were distinguished by *post-hoc* analysis.

Group associations of dugongs were investigated using χ^2 analysis to compare the frequencies of individuals found as solitary animals or associated with a herd between sexes and across seasons. We investigated the variation in fecal testosterone concentration with group association (solitary individual or member of a group) in both immature and mature males using a two-way ANOVA. All analyses were conducted using the statistical program SPSS (version 19.0 for Macintosh, SPSS, Inc., Chicago, IL, USA). A difference of $P<0.05$ was taken as significant for all statistical tests.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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