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Baseline Urinalysis of the Fully Marine, Herbivorous Dugong (*Dugong dugon*)

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Abstract

The dugong (*Dugong dugon*) is a fully marine mammal that grazes in nearshore seagrass meadows and whose health is vulnerable to human coastal activities. This study establishes urine baseline ranges for apparently healthy dugongs that can be used as health biomarkers. Voluntary urine samples (uncontaminated by seawater) were collected from 71 wild-caught dugongs in Moreton Bay, Australia, that were held out of water during an annual health assessment from 2008 to 2022. Urine was analysed for qualitative characteristics (colour, turbidity, odour), biochemistry through reactive urinalysis test strips, urine specific gravity (USG) by refractometer, electrolytes by flame photometry, sediment by microscopy, and bacterial culture. Urine of dugongs was typically pale yellow, clear to slightly cloudy, and mildly odorous. Urine was usually slightly alkaline (mean pH 8), and USG was low (mean 1.018). Urinalysis from dipstick indicated consistently negative readings for the presence of glucose, urobilinogen, bilirubin, ketones, and nitrites. Urinary protein was detected in 85% of sampled dugongs. Haemolysed red blood cells were recorded in > 85% of urine samples; microscopy indicated light haematuria (intact red blood cells) in 16% of samples. Seven percent of dugongs had detectable levels of leukocytes suggesting the possibility of mild urinary tract infection. Urinary sediment containing epithelial cells, keratinaceous debris, calcium carbonate crystalluria, and rare struvite crystals were typical. Light to moderate levels of bacteria were present in urine samples, with variable mixed growths, including *Halomonas aquamarina*, *Pseudomonas stutzeri*, *Photobacterium damselae*, *Psychrobacter* spp., and *Staphylococcus aureus*. Spermatozoa were present in the urine of 32% of the sexually mature males. Physical characteristics and chemistry of dugong urine showed some similarities to those of manatees and other herbivores. These baseline urinalysis data for healthy wild dugongs in a single population are valuable benchmarks against which dugongs of variable health status (including compromised dugongs) and from other localities may be compared.

Key Words: bacteria, biochemistry, cytology, dugong, *Dugong dugon*, health, sirenian, urinalysis, urine

Introduction

The dugong (*Dugong dugon*) is a long-lived, slow breeding, fully marine mammal that inhabits the warm coastal waters of the Indo-Pacific. The dugong is a threatened species, listed as "Vulnerable to Extinction" globally by the International Union for Conservation of Nature (Marsh & Sobtzick, 2019) but is likely "Endangered" in many localities. Dugongs feed on marine seagrasses in nearshore and sheltered offshore waters, and unlike their sirenian relatives (manatees), can live independently of fresh water (Marsh et al., 2011; Smoll et al., 2020). There is no evidence that dugongs practise mariposia (drinking of seawater), so it is likely they obtain their water requirements from consumption of seagrass and from metabolic oxidation of their diet (Smoll et al., 2020).

The seagrass habitats on which dugongs depend are threatened globally by anthropogenic impacts, including removal through coastal development and degradation through pollution (Tang & Hadibarata, 2022). Seagrass is susceptible to seasonal and year-to-year variation in productivity and abundance and also to extreme weather events such as storms, cyclones, and coastal flooding that may cause degradation or loss (Correia & Smee, 2022). In turn, these events may place nutritional stress on dugongs (Burgess et al., 2013; Lanyon, 2019). Extreme weather events that have degraded critical dugong habitat have led to apparent declines in local dugong populations (Marsh et al., 2011; Lanyon, 2019), either through emigration (Preen & Marsh, 1995),

mortality from starvation (Meager & Limpus, 2014), or reduced reproductive rate (Marsh & Sobtzick, 2019). There are likely other health effects on dugongs that are related to their coastal herbivorous habits (e.g., Haynes et al., 2005; Owen et al., 2012; Wong et al., 2019).

Health assessment of marine wildlife is an approach that is increasingly used to monitor individuals and populations (Walsh et al., 2018). During a health assessment, clinically informative biological tissues (e.g., blood, faeces, urine) are sampled and analysed against benchmark reference intervals for apparently healthy individuals. These samples can be effective physiological indicators that provide information about the health of an animal (Shultz et al., 2021). Urine is such a biomarker, whose properties can relay information regarding urinary tract or other infections; kidney dysfunction; bladder pathologies, including neoplasia or cystitis; diseases of the liver or gall bladder; dehydration; and nutritional state of wildlife when combined with physical examination and other diagnostic health indicators (Parrah et al., 2013; Callens & Bartges, 2015; Tighe & Brown, 2015). Urinalysis of marine mammals, including sirenians, has also been informative of physiological state and osmoregulatory homeostasis (Ortiz et al., 1998; Ortiz, 2001; Smoll et al., 2020). Some baseline urine parameters have been published for manatee species held in captive facilities, including West Indian manatees (*Trichechus manatus*; i.e., Antillean manatees [*Trichechus manatus manatus*]: Cabrias-Contreras et al., 2021; and Florida manatees [*Trichechus manatus latirostris*]: Manire et al., 2003) and Amazonian manatees (*Trichechus inunguis*; Pantoja et al., 2010, 2012). This study is the first to report on the biochemical, macroscopic, and microscopic characteristics of urine collected from free-ranging sirenians, and specifically from the fully marine dugong.

Methods

Urine Sampling

Urine samples were collected from wild, apparently healthy dugongs during an annual health assessment program conducted in Moreton Bay, southeast Queensland (27° 20.090 to 27° 24.870 S; 153° 21.260 to 153° 23.840 E) between 2008 and 2018, and in 2022 (sampling was not conducted in 2019 through 2021 due to COVID). The program was conducted annually in either late autumn through winter (May to June) or in austral spring (September to November). Each year, up to 20 dugongs were captured opportunistically from the Eastern Banks region of the bay (Lanyon, 2003), and health assessments were conducted on the deck of a research vessel (Lanyon et al., 2010). Dugongs were held out of water, unrestrained, and in the recumbent position for 30 to 60 min while vital signs were monitored and clinical samples (including blood, saliva, tears, faeces, urine) were collected (Lanyon et al., 2010). Sex, body morphometrics (body length, girth, weight), and body condition (on a 5-point scale from poor to excellent) were recorded (Lanyon et al., 2002). Sexual maturity (subadult or adult) and reproductive status (including pregnancy) were confirmed by faecal endocrinology (after Burgess et al., 2012a, 2012b). Each dugong was tagged for identification with a numbered titanium turtle tag applied to the trailing edge of the tail fluke and with a permanent genetag based on a dugong-specific microsatellite panel (Lanyon et al., 2002). A short-term paintstick (waterproof crayon) mark was applied to the dorsum upon release to avoid recaptures of individuals during the sampling week. Reference intervals for serum biochemistry and haematology of dugongs have been reported elsewhere (Lanyon et al., 2015, and Woolford et al., 2015, respectively).

To collect urine, each dugong was rolled slightly from the recumbent position to expose the ventral urogenital and anal regions so that these could be rinsed with fresh water and patted dry. A free-catch urine sample was collected into a plastic Frisbee® placed under the urogenital opening for the duration of time that the dugong was on deck, and another Frisbee® was placed under the anal opening to collect faeces and limit contamination of urine by faecal material (Figure 1). A dry towel was placed over the dugong's posterior dorsum to indicate the body region to remain dry during dousing of the animal for thermoregulation; this prevented contamination of urine by seawater. Immediately prior to release of the dugong, collected urine was transferred into a sterile specimen jar and its volume estimated.

Urinalysis

All urine samples collected between 2010 and 2022 were analysed immediately on site for physical characteristics, refractometry, and test strip biochemistry (see below). At least 1.5 ml of urine was needed to complete full urinalysis; any extra urine was stored frozen at -80ºC. In contrast, the smaller urine samples (< 1.5 ml) collected early in the program (2008 and 2009) were not analysed immediately but were archived frozen at -20ºC, then thawed briefly and analysed in April 2022. Consistent discrepancies in some biochemical urinalysis parameters between archived and recent samples suggested that > 10 y of freezing had introduced artefacts (see Daniels et al., 2013); all samples from 2008 and 2009 were consequently excluded from the final

Figure 1. Placement of plastic Frisbees® under urinogenital orifice (left) for collection of free urine and under anus (right) for collection of faeces in an adult male dugong (*Dugong dugon*). The dugong has been rolled slightly away to facilitate placement. The paintstick (crayon) marks on the flank correspond to midpoints of orifices to assist in locating Frisbees® for sample retrieval.

biochemical profile. Samples that were obviously contaminated by traces of seawater or faeces, or that included large quantities of semen, were also excluded from analysis.

Gross examination included assessing and categorising the physical characteristics of urine, including colour (colourless, pale yellow, straw yellow, dark yellow, other), turbidity (clear, slightly cloudy, cloudy, flocculent), and odour (none, slight, mild, strong). These urinalysis categories were broadly similar to those applied to other sirenians (Manire et al., 2003; Pantoja et al., 2012; Cabrias-Contreras et al., 2021), allowing for cross-species comparisons. Since these physical categories are somewhat subjective, all urine evaluators underwent training, and there was continuity of staff across years. Any other notable qualitative observations, such as presence of semen, faeces, or other potential contaminants in urine, were recorded.

Urine specific gravity (USG) was measured by refractometer after calibration of the device with distilled water. Although USG was also measured by test strip, we chose to report USG as measured by refractometer due to its superior accuracy (see Jiménez-Zucchet et al., 2019).

Biochemical urinalysis was conducted using Multistix Reagent Strips® (Siemens, Munich, Germany) by soaking the strip fully in urine, removing the strip, and blotting off excess urine through touching the strip edge to a paper towel. Urine parameter values were assessed against the manufacturer's recommended colour development times; qualitative results were recorded as positive or negative where appropriate; and if analytes were present, concentrations were measured according to the degree of colour change. Urine parameters measured by test strip included pH, leukocytes, blood, nitrites, ketones, bilirubin, urobilinogen, protein, and glucose.

Urine electrolyte content was analysed for a random subset of 20 samples, comprising five adult females, five adult males, and 10 subadult males. Concentrations of sodium (Na^+) and potassium (K^+) were measured using a BWB-XP flame photometer (BWB Technologies, Newbury, UK). Urea nitrogen and creatinine concentrations and electrolyte content (i.e., chloride, sodium, and potassium) of dugong urine were analysed in a random subset of 20 dugongs, and these results have been published separately in Smoll et al. (2020).

Analysis of urinary sediment, including culture of bacterial inclusions, was conducted for a randomly selected subsample of six dugongs sampled in 2022; other urinalysis data for 2022 samples are not reported here. Urine was pipetted into a 1 ml tube and spun in a microcentrifuge until sediment and supernatant separated. The sediment pellet was resuspended in a drop of supernatant and then spread onto a microscope slide and allowed to air dry; the remaining supernatant was discarded. One air-dried slide from cytospun urine and an entire urine sample from each of these six specimens were

sent to an external veterinary pathology laboratory for cytopathology and bacterial culture. The slides were examined under light microscopy at magnifications of 10x and 40x across at least five high power fields (hpf) of view. For each urine sample, the mean numbers of microscopic elements/hpf were quantitatively categorised as nil (0 elements/hpf), rare (> 0 to ≤ 2.5 elements/hpf), occasional (> 2.5 to < 5.0 elements/hpf), few (≥ 5.0 to < 7.5 elements/hpf), or many (≥ 7.5 elements/hpf).

Data Analysis

Urine data collected across annual health assessments were collated, nonsensical values were removed, and units were standardised. Qualitative results were reported as presence/absence or as counts and percentages where appropriate. Descriptive statistics for quantitative results included sample size, mean ± standard error, median, mode, range, and interquartile range (used as the baseline reference range), and these were calculated for the entire sample set of dugongs (pooled) and for separate sex, maturity, and season cohorts when sample size permitted; all basic analyses were conducted in Microsoft *Excel*. Blood and protein chemical urinalysis data recorded initially as a range were assigned the mid value of the range, trace blood was assigned a negative value (after Kelly et al., 2009), and negative results were treated as zero. Differences in urinalysis parameters between demographic cohorts of dugongs were identified using Exact Wilcoxon Rank Sum Tests and completed in *R Studio Desktop*, Version 2022.02.3+492.

Results

Urine Samples

Of the 202 wild dugongs sampled for health assessment in Moreton Bay over the decade 2008 to 2018, 106 (52%) individuals urinated voluntarily while on board the vessel. Of these 106 individual urine samples, 65 samples of sufficient volume that were uncontaminated (by seawater or faeces) were included in this analysis. Of these 65 dugongs, 48 were male and 17 were female. Urine samples separated according to maturity cohorts (confirmed by endocrine analysis) consisted of two subadult females, 17 subadult males (including one estranged calf), 15 adult females (including five pregnant females), and 31 adult males. The fewer number of usable female urine samples compared to males may be explained by the external morphology of female dugongs relative to the collection technique. The urinogenital and anal orifices of females are almost contiguous so that contamination of urine by faecal material was more likely to occur if the females and/or their Frisbees® moved while on deck. All care was taken to avoid this issue, including repositioning Frisbees® if necessary. This was even more problematic for subadult females with their relatively smaller perineum.

All 65 dugongs from which urine was analysed were assessed as "apparently healthy." On a 5-point ranked scale from excellent (5) to poor (1) body condition based on fatness, 62 dugongs were in good to excellent condition (ranking 3 to 5). Of the other three dugongs, one (MB14568; sampled in winter 2014) had fair body condition (rank 2), while the remaining two (MB11114 and MB11122; both sampled in winter 2011) had poor body condition (rank 1). Apart from their smaller girths, these three dugongs appeared to be healthy. Skin condition of all of the dugongs ranged from good to excellent (on a similar 5-point scale), and vital signs and behaviour both during the capture procedure and restraint period fell within normal ranges (Lanyon et al., 2010).

Urine was collected and analysed from 41 dugongs in May through July (i.e., the austral autumn–winter season), and from 24 dugongs between September and November (i.e., the austral spring–summer season). Sperm was present in the urine of 32% (10 of 31) of adult males: seven samples contained motile sperm while three sperm samples were non-motile. Motile, viable sperm were found in urine sampled in May, June, and September.

Table 1 outlines the key descriptive statistics for each of the measured urine parameters and includes sample size (n), mean ± standard error ($\bar{x}$ ± SE), median, mode, range, and interquartile range (IQR), as appropriate, for physical characteristics, urine test strip biochemistry, refractometry, and electrolytes. IQR was used as the baseline descriptive reference range for these urine parameters. An additional six urine samples (making 71 total urine samples) collected in 2022 were analysed for cytopathology and microbiology (see below). The sample set of dugongs in this study did not include recaptures of individuals.

Gross Examination: Physical Characteristics

The colour of dugong urine ranged from colourless (rank 1) to straw yellow (rank 3) for 60 of 61 dugongs: IQR = 1 to 3 (Table 1). Colourless urine was found in 30% of dugongs, pale yellow in 33%, and straw yellow for 36%. A single dugong (MB14555) had dark yellow (rank 4) urine. Mean urine colour for dugongs was 2.1 ± 0.11 (n = 61), equivalent to pale yellow (Table 1).

Urine turbidity ranged from clear (rank 1) to cloudy (rank 3) for 56 of 59 dugongs: IQR = 1 to 2 (Table 1). Most dugongs had clear urine (42%),

Table 1. Descriptive statistics (sample size [*n*], mean ± standard error [SE], median, mode, range, and interquartile range [IQR]/baseline range values) for qualitative urine parameters, urine test strip chemistry, specific gravity by refractometer, and electrolytes from dugong (*Dugong dugon*) urine ($n = 65$). All urobilinogen results were recorded as 0.1 to 1 mg/dL. Qualitative characteristics including colour (1 = colourless, 2 = pale yellow, 3 = straw yellow, 4 = dark yellow, 5 = other), turbidity (1 = clear, 2 = slightly cloudy, 3 = cloudy, 4 = flocculent), and odour (1 = none, 2 = slight, 3 = mild, 4 = strong). All blood measurements in urine refer to haemolysed blood only.

Physical characteristics	*n*	$\bar{x} \pm$ SE	Median	Mode	Range	IQR/Baseline range
Colour rating	61	2.10 ± 0.11	2	3	1-4	1-3
Turbidity rating	59	1.83 ± 0.11	2	1	1-4	1-3
Odour rating	59	2.66 ± 0.13	2	2	1-4	2-3
Urine specific gravity	51	1.018 ± 0.001	1.018	1.016	1.002-1.032	1.014-1.022
Biochemistry by dip strip						
pH	63	8 ± 0.12	8	8	5-9	7.5-9
Leukocytes (wbc/μL)	57	13.33 ± 9.06	0	0	0-500	0-0
Blood (rbc/μL)	61	76.23 ± 12.09	50	50	0-250	7.5-50
Nitrite (-/+)	56	--	0	0	0-1	0-0
Ketone (mmol/L)	62	0.04 ± 0.02	0	0	0-0.5	0-0
Bilirubin (-/+/++)	61	--	0	0	0-2	0-0
Urobilinogen (mg/dL)	61	0	0	0	0	0-0
Protein (g/L)	62	0.17 ± 0.04	0.15	0.15	0-2	0-0.15
Glucose (mg/dL)	62		0	0	0-100	0-0
Other analyses						
Urea nitrogen (mmol/L)	18	4.97 ± 1.24	3.85	--	0.8-19.6	1.63-5.4
Creatinine (mmol/L)	19	0.50 ± 0.13	0.3	0	0-1.97	0.04-0.81
Na^+ (mmol/L)	19	449.3 ± 36.7	479	--	60-665	382.5-547
K^+ (mmol/L)	19	46.16 ± 8.65	39	--	3-139	14.5-77.75
Cl^- (mmol/L)	19	474.95 ± 40.22	540	--	70-677	398.5-608.75

while 37% had slightly cloudy, 16% had cloudy, and 5% had flocculant ($n = 3$) urine. Apart from the adult males with a high percentage of spermaturia (which were removed from the final analysis), the three dugongs with the cloudiest urine comprised a pregnant female, a non-pregnant adult female, and an adult male. Mean urine turbidity for dugongs was 1.83 ± 0.11 ($n = 59$), equivalent to tending towards slightly cloudy (Table 1).

Dugong urine was mostly odorous, ranging from no odour (rank 1) to a strong pungent smell (rank 4): IQR = 2 to 3 (Table 1). Twelve percent of dugongs had urine with no noticeable odour, 34% had a slight odour, 30% had a mild odour, and 24% were strongly odiferous. All but one of the most odorous urine samples were from males; the female with malodorous urine was one of the three dugongs in poorest body condition (MB11114). Dugong urine had a mean odour of 2.7 ± 0.13 ($n = 59$), tending towards a mild odour (Table 1).

USG measured by refractometer ranged from 1.002 to 1.032, mean 1.018 ± 0.001 ($n = 51$): IQR = 1.014 to 1.022 (Table 1).

Biochemical Examination

Urinary pH ranged from 5 to 9, with a mean of 8 ± 0.12 ($n = 63$): IQR = 7.5 to 9 (Table 1). Slightly acidic urine occurred in four adult dugongs only (i.e., two dugongs with pH 5; two with pH 6.5). Protein levels in urine were low, ranging from 0 to 2 g/L, mean 0.17 ± 0.04 g/L ($n = 62$): IQR = 0 to 0.15 g/L (Table 1); 39% of dugongs had zero detectable protein. Ketone levels were zero in 92% of dugongs (57 of 62) and were detected at levels of 0.5 mmol/L in the remaining five dugongs, which were all sampled in winter months. Statistically, mean ketone level was 0.04 ± 0.02 mmol/L, and IQR = 0 to 0, suggesting a normal value of zero (Table 1). For bilirubin, 87% of dugongs had negative (zero) levels—that is, all below the detectable range of 0.5 mg/dL; 11% (7 of 62) had a value of 1 (+ present), and a single young adult male (MB18053) had a count of 2 (++): IQR = 0 to 0 mg/dL (Table 1). Urobilinogen was not detected in the urine of dugongs: all samples were below the detectable level of 0.1 mg/dL. Urinary nitrites ranged from 0 to 1 (i.e., absent to present). Of 56 dugongs, 52 (93%) had zero nitrites so that normal levels were zero. Four dugongs had detectable nitrite levels: a pregnant female (MB15711),

one that also had detectable ketones (MB13468), and two adult males in excellent condition. All dugongs, except one, had zero urinary glucose (i.e., 61 of 62 dugongs [98%]; Table 1). A single subadult male (MB12371) had a reading of 100 mg/dL glucose (i.e., the minimum detectable level by test strip).

Haemolysed blood was found in 85% of urine samples (63% had < 150 red blood cells [rbc]/μL and 21% had ~250 rbc/μL), while 15% of dugongs had zero detectable blood. Blood concentration ranged from 0 to 250 rbc/μL urine, and mode was 50 rbc/μL (n = 61): IQR = 7.5 to 50 rbc/μL (Table 1). Non-haemolysed blood was not detected in urine samples. Ninety-three percent (51 of 55 dugongs) had zero leukocytes (white blood cells [wbc]) in their urine; the four dugongs with detectable levels had up to 75 wbc/μL: IQR = 0 to 0 wbc/μL (Table 1).

Cohort Variation in Urine Biochemical Composition

Table 2 reports those urinary parameters that showed significant differences between demographic cohorts when cohort size was sufficient for statistical comparison. Mean urinary (haemolysed) blood content was significantly higher in adults (89.19 ± 0.05 rbc/μL) compared to subadults (55 ± 24.27), and also for females (138.24 ± 24.08) compared to males (52.27 ± 12.31). Mean USG was higher in males (1.02 ± 0.002) compared to females (1.012 ± 0.002), and in dugongs sampled in winter (1.021 ± 0.002) compared to spring (1.016 ± 0.001). Mean creatinine was significantly higher in males (0.66 ± 0.15 mmol/L) compared to females (0.04 ± 0.02). All three urinary electrolytes (Na^+, K^+, and Cl^-) were at significantly higher mean urinary levels in males compared to females (Table 2).

Table 2. Statistically significant results of an Exact Wilcoxon Rank Sum Test for differences in dugong urinalysis parameters between demographic cohorts by sex and maturity status: females denoted by [1] vs males denoted by [2]; subadult[1] vs adult[2]; spring season[1] vs winter season[2]; male subadult[1] vs male adult[2]. All results are expressed at significance level $p < 0.05$.

	n_1	n_2	$(\bar{x} \pm SE)_1$	$(\bar{x} \pm SE)_2$	W statistic	p
Female[1] vs male[2]						
Blood (rbc/μL)	17	44	138.24 ± 24.08	52.27 ± 12.31	617.5	< 0.001
Urine specific gravity	15	36	1.012 ± 0.002	1.020 ± 0.002	129.5	0.003
Creatinine (mmol/L)	5	14	0.04 ± 0.02	0.66 ± 0.15	9	0.014
Na+ (mmol/L)	5	14	300.4 ± 89.53	502.5 ± 28.78	13	0.044
K+ (mmol/L)	5	14	17.4 ± 9.46	56.43 ± 10	10	0.02
Cl- (mmol/L)	5	14	338.4 ± 96.09	523.71 ± 36.46	12	0.034
Subadult[1] vs adult[2]						
Blood (rbc/μL)	16	42	55 ± 24.27	89.19 ± 0.05	209	0.023
Winter[1] vs spring[2]						
Urine specific gravity	19	32	1.021 ± 0.002	1.016 ± 0.001	408	0.042
Male subadult[1] vs male adult[2]						
Blood (rbc/μL)	14	28	27.14 ± 17.3	65 ± 17.02	123.5	0.049

Table 3. Results of the urine cytopathology by microscopy and bacterial content by culture for a subset of six randomly selected dugong urine samples. Bacterial identification legend: HAA = *Halomonas aquamarina*, HAL = *Halomonas* sp., PHO = *Photobacterium damselae*, PSE = *Pseudomonas stutzeri*, PSY = *Psychrobacter* spp., STA = *Staphylococcus aureus*, VIA = *Vibrio alginolyticus*, VIB = *Vibrio* sp., and VIP = *Vibrio parahaemolyticus*. M = male; F = female. rbc = red blood cells. Note that there was insufficient urine for full cytopathology of dugong MB22256.

Specimen ID	Sex	rbc	$CaCO_3$ crystals	Struvite crystals	Epithelial cells	Fat	Casts	Sperm	Debris	Debris type	Bacteria cultured
MB22253	M	Nil	Few	Rare	Nil	Nil	Nil	Nil	Occasional		HAL, PHO
MB22254	F	Nil	Occasional	Rare	Few	Nil	Nil	Nil	Few	Keratinaceous, anucleate squames	HAL, VIA, PSY
MB22255	M	Nil	Occasional	Rare	Nil	Nil	Nil	Nil	Few	Keratinaceous, anucleate squames	HAA, VIB
MB22256	F	Nil	Nil	Rare	Nil	Nil	Nil	Nil	Few		HAL, VIA, VIP
MB22257	F	Few	Occasional	Nil	Nil	Nil	Nil	Nil	Many	Keratinaceous, lysed cells, amorphous debris	HAL, STA
MB22260	M	Nil	Occasional	Rare	Nil	Nil	Nil	Nil	Many	Keratinaceous, lysed cells	VIA, PSE

Urine Cytopathology

A range of sedimentary inclusions were recovered from the subsample of six dugong urine samples collected in 2022 (Table 3; Figure 2). These included occasional calcium carbonate crystals in five of the six samples; rare struvite crystals in five of the six samples (Figure 2a); and other crystals that may have included calcium oxalate (monohydrate and/or dihydrate), indinavir, and cystine (Figure 2b). Epithelial cells were sometimes present (Figure 2c), and cellular debris (Figure 2d) was present in all six samples, suggesting that these inclusions may be normal. Identifiable debris included keratinaceous anucleate squames, lysed cells, and/or amorphous debris (Table 3). One sample only showed microscopic evidence of red blood cells or haemoglobin (Figure 2d).

Urinary Microbiology

Each urine sample cultured for microbiology showed a moderate growth of mixed organisms that included the following bacteria: *Halomonas* unidentified species, *Halomonas aquamarina*,

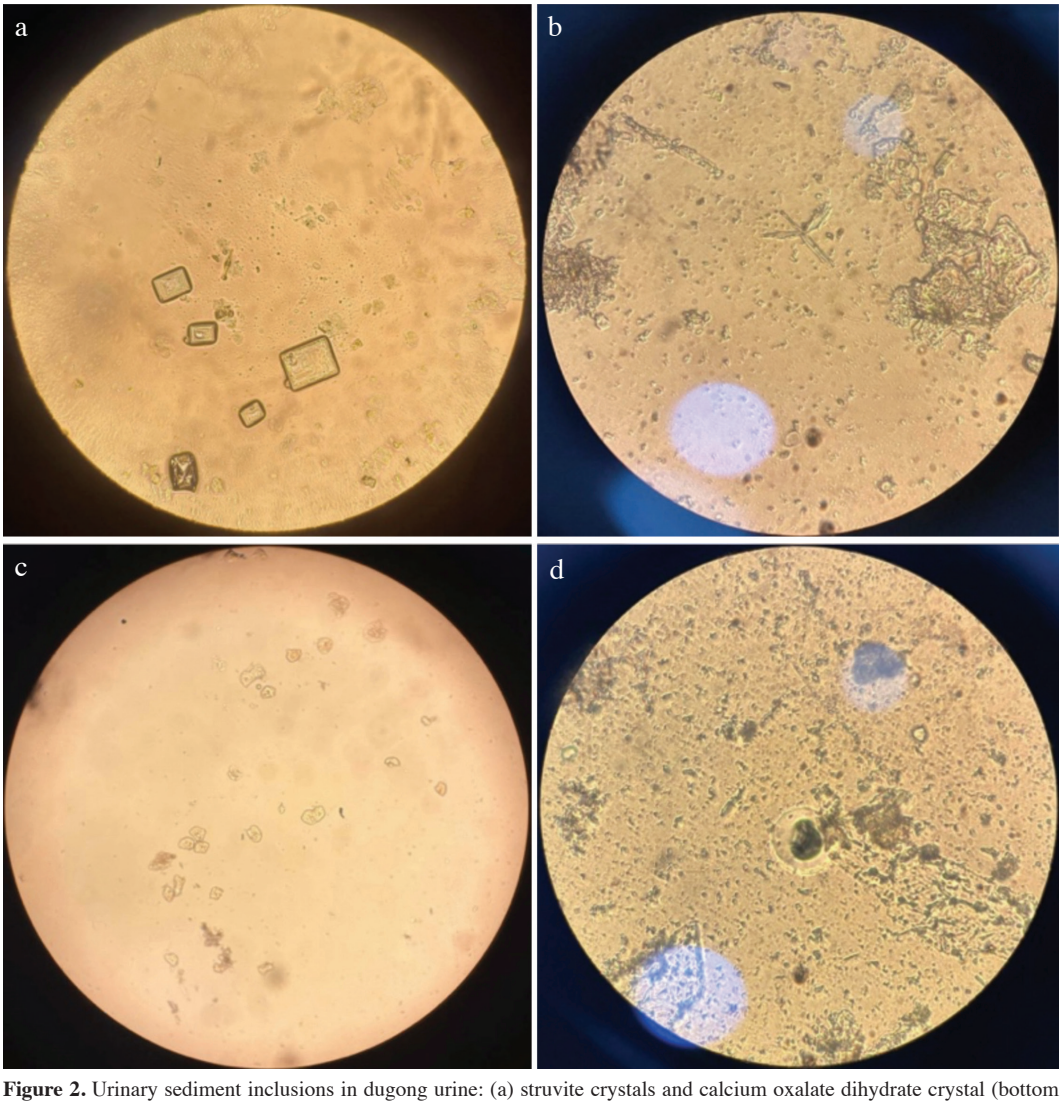

Figure 2. Urinary sediment inclusions in dugong urine: (a) struvite crystals and calcium oxalate dihydrate crystal (bottom left) (40x magnification); (b) urinary crystals, including possible indinavir stone in centre of field (100x magnification); (c) epithelial cells (40x magnification); and (d) red blood cell in centre of field and cellular debris (100x magnification).

Photobacterium damselae, *Pseudomonas stutzeri*, *Psychrobacter* unidentified species, *Staphylococcus aureus*, *Vibrio* unidentified species, *Vibrio alginolyticus,* and *Vibrio parahaemolyticus* (Table 3).

Discussion

Baseline urine parameters are reported herein for 65 apparently healthy, free-ranging, wild-caught dugongs of both sexes, and for subadult and adult dugongs. The urine of dugongs was typically pale yellow in colour, clear to slightly cloudy, and mildly odiferous. These characteristics are thus considered clinically normal for this species. The physical characteristics of dugong urine generally fall within the ranges described for manatees (Pantoja et al., 2012; Cabrias-Contreras et al., 2021) and for healthy herbivorous mammals (Parrah et al., 2013). However, the generally paler colour and less pungent urine of dugongs compared to manatees might suggest production of more dilute urine. There were no obvious trends in urine colour, turbidity, nor odour that could be related to age or sex of these apparently healthy dugongs. Interestingly, the one female dugong with strongly malodorous urine was in the poorest body condition (thinnest) of all sampled dugongs, suggesting dehydration associated with nutrient deficiency or some other health issue. It is possible that urine of dugongs that are health-compromised or in a low nutritional plane may vary in physical characteristics to dugongs in excellent condition.

Dugong urine was mildly alkaline with a mean pH of 8 and range of 7.5 to 9. The pH of dugong urine falls within the range reported for manatees (West Indian manatees: Manire et al., 2003; Amazonian manatees: Pantoja et al., 2012), including Antillean manatees in estuarine and marine habitats whose urinary pH tended higher (pH 8 to 8.3) than those in freshwater systems (pH 7.6) (Cabrias-Contreros et al., 2021). The range of pH measured in dugong urine is also consistent with pH measured in mammalian herbivores other than manatees (e.g., horses, sheep, cows). There are relationships between urine pH and dietary strategy, with carnivores' high protein diet producing more acidic urine (Parrah et al., 2013). Prevalence of acidic urine was low: four of the 63 dugongs had mildly acidic urine (pH 5 to 6.5), and the reasons for this are unknown. Causes of urinary acidosis may include deficiency or excess of dietary protein. Alternatively, respiratory or metabolic acidosis, perhaps even caused by pursuit and capture (Lanyon et al., 2012), may presumably lead to mild urinary acidosis.

Urinary specific gravity (USG) was low in dugongs, ranging from 1.002 to 1.032 with a mean of 1.018. This was slightly higher than both the mean and upper end of the range measured in Antillean (mean 1.01; Cabrias-Contreros et al., 2021) and Amazonian (mean 1.005; Pantoja et al., 2012) manatees. USG is directly proportional to urine and plasma osmolality and measures solute concentration and urine density, or the ability of the kidney to concentrate or dilute the urine over that of the plasma. The osmolality of dugong plasma and electrolyte content of dugong urine have been measured previously and are significantly higher than in West Indian manatees. This hypertonic urine presumably reflects the fully marine habitat of the dugong (Smoll et al., 2020). Furthermore, USG was higher in male dugongs than in females, particularly in males during the mating season. This result is consistent with the idea that foraging becomes a lesser priority for males than mating at this time of year (see Burgess et al., 2013).

Dugong urine was consistently negative for the presence of glucose, bilirubin, urobilinogen, ketones, and nitrites according to test strip analysis. These results are similar to those recorded for urine of both Antillean and Amazonian manatees (Cabrias-Contreros et al., 2021, and Pantoja et al., 2012, respectively); however, there were the odd outlier animals. Healthy mammals generally excrete little to no urinary glucose (Parrah et al., 2013); and in the present study, only one dugong had minimally detectable levels of urinary glucose. This subadult male (MB12371) was a recaptured docile animal in good body condition. If emotional glucosuria occurred under restraint conditions (Garcia-Navarro, 1996), this was not obvious behaviourally.

Urobilinogen was not detected in the urine of dugongs. This is the main urinary pigment that gives urine its characteristic yellow colour. The infrequency of darkly pigmented urine in dugongs suggested that urobilinogenuria (increased urine urobilinogen) was not an issue. Low detectable levels of bilirubin (i.e., +) were recorded in seven dugongs, and a single small adult male (MB18053) in excellent body condition had a count of 2 (++). There were no obvious indicators of liver dysfunction in this animal with respect to the liver enzymes AST, ALP, nor blood bilirubin.

Dugongs, similar to manatees, normally had zero levels of ketones; however, ketone levels were at detectable levels of 0.5 mmol/L in five of the dugongs. Interestingly, all dugongs with ketonuria were sampled in winter, a time when water temperature is low, seagrass nutrient and energy levels are low, and foraging involves increased movements/exercise, and, thus, when the risk of ketosis is highest. Other studies have recorded higher levels of urinary ketones in animals under nutritional stress—for example, around lactation or end of pregnancy when they mobilise large amounts of energy stores

following a negative energy balance (e.g., Parrah et al., 2013). This may be the case here.

Of 56 dugongs, four dugongs had detectable levels of urinary nitrites; the basis for these readings was not apparent. One of these dugongs was a pregnant female (MB15711), one was a dugong with detectable ketones (MB13468), and the other two were adult males in excellent condition. Since nitrites can be produced by bacteria present in urine, the presence of nitrite in urine may indicate urinary tract infection (e.g., Garcia-Navarro, 1996, as cited in Pantoja et al., 2012), and nitrites should be considered in relation to bacterial load.

Sixty-one percent of the dugongs had detectable but low levels of protein (0 to 2 g/L) in their urine samples. Similarly, low levels of protein (0 to 0.15 g/L) have been recorded in the urine of Amazonian manatees (Pantoja et al., 2012), suggesting that mild proteinuria may be non-pathological or normal in sirenians. Alternatively, proteinuria may be caused by renal disease, infection of the urinary tract, kidney malfunction, or even the presence of blood in the urine (see below); however, haemorrhage may need to be considerable and macroscopic before it manifests in this way (see Parrah et al., 2013).

The majority of dugongs (85%) had detectable levels of red blood cells in their urine, and all samples were haemolysed. Of the six dugongs whose urine sediment was analysed microscopically, only one had visible intact red blood cells. It is therefore possible that the urine test strips were picking up haemoglobin rather than intact red blood cells, similar to findings by Pantoja et al. (2012). Microhaematuria, as found in these dugongs, may be indicative of intravascular haemolysis or urinary tract infections, kidney stones, or even vigorous exercise. The prevalence and amounts of urinary blood were greater in females than males, and in adults compared to subadults; the underlying reasons are unknown, but the sexual and maturity biases may suggest factors related to reproduction such as oestrous and/or recent pregnancy. A broader analysis of population cohort differences as more urine samples become available may assist with interpretation of these patterns. Four dugongs (i.e., 7%) had detectable levels of white blood cells (leukocytes) in the urine, suggesting possible urinary tract infection. However, low levels of leukocytes may be normal in urine (Bossart et al., 2001). Interestingly, two dugongs with the highest urinary leukocyte readings also had high detectable levels of sperm; these dugongs were removed from the final analysis due to possible confounding influence.

All dugongs for which urinary sediment was analysed for cytopathology routinely showed crystalluria, with formation of calcium carbonate and calcium oxalate crystals (most commonly), and struvite crystals, but also the rare presence of a range of other crystals. Such crystals may form with super saturation of urine and/or ingestion of specific foods; and in some species of herbivores (e.g., horses; Matos & Matos, 1995), this can be a normal finding. Urinary crystals also occur commonly in Amazonian manatees (Pantoja et al., 2012). Seagrasses, including those frequently consumed by dugongs in the Moreton Bay study area (e.g., *Halophila ovalis*), have a relatively high calcium content (Wan Hazma et al., 2015), and this may presumably result in calcium-saturated urine. An alternative explanation is that the formation of urinary crystals/kidney stones may be indicative of pathology.

The cellular content of urinary sediment always included moderate amounts of anucleate squames, keratinaceous debris, and lysed cellular debris. Interestingly, the most abundant epithelial squamous cells were present in a single large mature female sampled in the mating season, and these may have originated from the urinogenital tract. Squamous cells are the type that have been most frequently observed in urine samples of marine mammals, presumably from the urinogenital or vascular system (Bossart et al., 2001), and heavy loads of such cells have also been found in the urine of adult female Amazonian manatees during the mating season, suggesting increased shedding of the vaginal epithelium during oestrous (Pantoja et al., 2012). Such inclusions appear to be normal in dugong urine.

All dugongs for which urinary sediment was analysed for microbiology had a mixed growth of bacteria present, usually in moderate amounts. Small amounts of bacteria in urine may be normal, may be the result of environmental contamination, or may be indicative of a urinary tract infection. All attempts were made to avoid cross-contamination of urine with faeces in this study. Some of the marine bacteria cultured from dugong urine samples are known to cause infection or disease in humans and in various marine animals. These include the low virulence *Pseudomonas stutzeri* that has been isolated from marine sediments (Bennasar et al., 1998); *Photobacterium damselae* (formerly *Vibrio damselae*) that causes infection in a variety of marine animals (Rivas et al., 2013); and *Psychrobacter* spp., a bacterial genus that has been found in a wide range of marine animals, including in the blowhole and skin of the Yangtze finless porpoise (*Neophocaena asiaeorientalis asiaeorientalis*; Zhang et al., 2022). *Vibrio* species present in dugong urine included *V. alginolyticus* and *V. parahaemolyticus*, both of which are major pathogens in a number of aquatic animals, including fish and invertebrates, causing vibriosis

(Droubogiannis et al., 2022), which may cause seafood poisoning in humans. *Vibrio* spp. have been documented previously during handling of marine mammals, including belugas (*Delphinapterus leucas*), sea otters (*Enhydra lutris*), and harbor porpoises (*Phocoena phocoena*), and have not been associated with apparent disease in these cases (Goertz et al., 2013; Gulland et al., 2022). Since *V. parahaemolyticus* has been identified as a marine mammal faecal pathogen, this may indicate some faecal contamination of urine in the single adult female or the presence of this bacterium elsewhere in the body. *V. vulnificus* has been previously detected in an aspirated tumour from a dugong in Moreton Bay (JML, unpub. data, 2006). *Halomonas* spp., including *H. aquamarina*, are marine pathogens that have been isolated from marine surface waters (ZoBell & Upham, 1944), and their pathogenicities appear to be unknown. *Staphylococcus aureus* was detected in the urine of one dugong. *S. aureus* is known to cause disease in small cetaceans and pinnipeds (Faires et al., 2009; van Elk et al., 2012), and there is some evidence of host species-specific strains (van Elk et al., 2012). The *S. aureus* strain present in the single dugong was not identified. A concurrent study by JML is characterising the pathogenic microbes of diverse dugong tissues, including the skin, gut, and urogenital and respiratory tracts, to build a more integrated microbiome profile for the species.

In summary, with respect to biochemical characteristics, dugong urine showed some similarities to the distantly related Amazonian and Antillean manatees, despite their different phylogenies, habitats, diets, and general lifestyles. Dugongs are obligate seagrass grazers, fully marine, and independent of fresh water, while manatees have differing degrees of dependence on freshwater sources and a broader diet. These nutritional and physiological differences are reflected in their variable metabolisms and life histories. The macroscopic and microscopic characteristics and biochemical reference ranges for urine from wild dugongs presented herein provide a physiological baseline against which to assess the health of wild and captive dugongs.

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