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Evaluation of respiratory vapour and blubber samples for use in endocrine assessments of bottlenose dolphins (*Tursiops* spp.)

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ABSTRACT

Blubber and respiratory vapour ('blow') are now commonly used for endocrine studies on cetaceans, primarily because they can be obtained using minimally invasive methods. For many species, these samples have yet to be validated for these purposes. The objective of this study was to examine the performance of blow and blubber hormone monitoring, relative to serum hormone monitoring, for evaluating the reproductive and adrenal condition of captive bottlenose dolphins (*Tursiops* spp.). Eighteen bottlenose dolphins were sampled five times for serum and blow and twice for blubber throughout a one-year period. Concentrations of progesterone, testosterone, oestradiol and cortisol were measured in each sample type. Hormone levels were examined in relation to dolphin age, sex, reproductive status, season, time of sample collection (morning/afternoon) and collection type (in- or out-of-water sampling). Patterns in hormone levels were similar for serum and blubber. For instance, in both sample types, progesterone levels were significantly higher in pregnant (serum: 34.10 ± 8.64 ng/mL; blubber: 13.01 ± 0.72 ng/g) than in non-pregnant females (serum: 0.32 ± 0.09 ng/mL; blubber: 1.17 ± 0.10 ng/g). This pattern was not detected in blow, primarily because seawater contamination, nylon sampling materials and variable sample volumes influenced measured concentrations. In addition, the respiratory water content of a blow sample is known to affect measured hormone levels. Two methods were trialled to control for variability in sample volumes and dilution: (1) normalising blow hormone concentrations relative to urea nitrogen levels (a potential endogenous standard), and (2) measuring the relative proportions (i.e. ratios) of blow hormones. These correction measures had little influence on blow hormone results. Further refinement of blow hormone monitoring methods is required before they can be used for reproductive or adrenal assessments of bottlenose dolphins. Blubber, on the other hand, should be a suitable proxy for serum when attempting to classify pregnancy status and male maturity in these species.

1. Introduction

Cetacean physiology has generally been studied post-mortem (Chittleborough, 1954; Kellar et al., 2013b) or by examining captive animals (Sawyer-Steffan et al., 1983; Yuen et al., 2009). Considerably less physiological research has been conducted on free-swimming cetaceans due to the difficulty of locating, capturing and collecting blood from most species (reviewed in: Amaral, 2010; Hunt et al., 2013). Several alternatives to the use of blood have been developed over the last thirty years. Among these, the most frequently employed methods are remote tissue biopsying, the collection of faeces and, more recently, the sampling of respiratory vapour ('blow'). A key benefit of these sampling techniques is that they pose minimal animal welfare concerns and cause minimal disruption to the behaviour of animals (Amaral, 2010; Hunt et al., 2013).

Remote tissue-biopsying is the most widely used technique for

Abbreviations: BSA, bovine serum albumin; CV, coefficient of variation; E2, oestradiol; EIA, enzyme-immunoassay; F, cortisol; *g*, gravity; GLMM, generalised linear mixed model; IgG, Immunoglobulin G; IW, in-water; MHz, megahertz; OOW, out-of-water; P4, progesterone; PBS, phosphate buffered saline; QLD, Queensland; SE, standard error of the mean; SMI, sperm motility index; T, testosterone; UN, urea nitrogen

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collecting biological samples from wild cetaceans but is typically limited to adults because of the wounds caused by darting. One of the advantages of this method is that both skin and blubber (adipose tissue) can be collected simultaneously. The collection of multiple tissues increases the potential number of analyses that can be performed, such as genetics on skin (Dalebout et al., 1998) and toxicology on blubber (Yordy et al., 2010), and the scope of information that can be obtained from an animal. Given that blubber accumulates and retains lipophilic molecules, such as steroids, it has also often been used for studying the endocrinology of wild cetaceans (Mansour et al., 2002; Trana et al., 2016; Vu et al., 2015). The major outcome of this research is that elevated blubber progesterone (P4) levels appears diagnostic of pregnancy in all species studied to date (Clark et al., 2016; Kellar et al., 2006; Kellar et al., 2013a,b; Kellar et al., 2014; Mansour et al., 2002; Trego et al., 2013), including common bottlenose dolphins (*Tursiops truncatus*; Kellar et al., 2017; Pérez et al., 2011). Fewer studies have examined links between blubber hormone levels and male reproductive parameters. However, blubber testosterone (T) appears to be a marker of male sexual maturation and reproductive seasonality in short-beaked common dolphins (*Delphinus delphis*; Kellar et al., 2009) and humpback whales (*Megaptera novaeangliae*; Vu et al., 2015). Adrenal stress has also been examined in a number of species, including short-beaked common dolphins (Kellar et al., 2015), belugas (*Delphinapterus leucas*; Trana et al., 2016) and bottlenose dolphins (Champagne et al., 2017, 2018), by measuring blubber cortisol (F) levels after a natural or pharmacologically induced stress event. It has not been determined whether subtler endocrine patterns, such as seasonal patterns of glucocorticoid secretion, can be detected when using blubber. Further, control sample experiments have not been performed to examine whether the blubber hormone extraction procedure itself could influence the detected signals.

In addition to blubber, blow samples have been used for reproductive and adrenal endocrine studies on several cetacean species (Burgess et al., 2018; Richard et al., 2017; Thompson et al., 2014; Tizzi et al., 2010). Obtaining repeatable blow hormone measurements has proved difficult, however, due to variability in respiratory water dilution. Human breath studies have suffered similar issues and attempts to compensate for this using endogenous markers for dilution have failed (reviewed in: Effros, 2010). Urea nitrogen (UN) has been the most frequently trialled endogenous standard, primarily because it diffuses into pulmonary tissues and fluids (Effros et al., 1992) and is maintained in a relatively narrow concentration range in healthy mammals (Haschek et al., 2009). Despite the failed attempts in human studies, there is gathering interest in using UN as a potential marker for dilution in cetacean blow (Burgess et al., 2018; Thompson et al., 2014). Cetaceans possess relatively high UN levels when compared to other mammals (Medway and Geraci, 1965; Myers, 1920), meaning that UN should be present in blow at detectable levels. Thus far, results have been successful for a study on north Atlantic right whales (*Eubalaena glacialis*; Burgess et al., 2018), but were only listed 'inconclusive' for a study on belugas (Thompson et al., 2014). Examining blow hormone ratios, rather than absolute levels, has been proposed as an alternative way to account for the variable levels of dilution (Hunt et al., 2014). Hormone ratios have proved effective for reproductive studies in humans (reviewed in: Sollberger and Ehlert, 2016) and some wild vertebrates (Hogan et al., 2013; Velloso et al., 1998), but have yet to be used for reproductive or adrenal endocrine studies of cetaceans.

Several previous blow hormone studies have also raised concerns that some common sampling materials, and potentially seawater contamination, can affect hormone measurements (Burgess et al., 2016; Thompson et al., 2014). When sampling free-swimming cetaceans, seawater contamination levels can vary depending on the individual's surfacing-diving behaviour, the sampling approach and the collection device used. Existing blow collection devices have varied slightly between studies, with most being composed of an empty dish covered with a nylon material (Hogg et al., 2009; Hunt et al., 2014). Nylon seems to be favoured due its widespread availability, low cost and capacity to adsorb liquid. The ability to 'capture' and retain large volumes of blow vapour is essential for studies on free-swimming cetaceans, which can rarely be repeatedly sampled. Yet, currently there is a limited understanding of how different levels of seawater contamination or sampling materials could affect hormone measurements.

Few attempts have been made to compare blow or blubber hormone levels to those in serum, or to information on the reproductive state or adrenal condition of live cetaceans. Further, existing blow hormone studies have focussed on collection techniques, and control measures, that are only practicable in a captive or wild-capture setting. The motivation for this study was to evaluate the efficacy of blow and blubber hormone monitoring under conditions that reflect the sampling of free-swimming cetaceans (e.g. single exhalation, no cleaning of a blowhole prior to collection). Here, we used captive bottlenose dolphins as a model species to (1) determine whether patterns in blow and blubber steroid hormone (P4, T, oestradiol [E2], F) measures are similar to those in serum, (2) evaluate if and how blow and blubber hormone levels vary in relation to biological, environmental and sampling-related factors, and (3) trial and evaluate the performance of blow hormone ratio values, and UN normalised blow hormone levels as alternative measures to absolute blow hormone levels.

2. Materials and methods

2.1. Study animals

Sampling occurred in eight sampling periods (lasting between 1 and 3 days) between June 2015 and August 2016 at SeaWorld Australia on the Gold Coast, Queensland (QLD). Samples were collected from eleven female and seven male bottlenose dolphins (*T. truncatus*, *T. aduncus* and hybrids), using voluntary, in-water procedures (n = 42) and out-of-water, capture-restraint procedures (n = 32; Table 1). There was minimal delay between the capture of an animal and the collection of the first sample (i.e. either blood or blow; range: 27 s to 2 min 53 s).

Both adult and juvenile dolphins were sampled in this study. The maturity status of a dolphin was classified using known ages of some individuals and their body lengths (Kemper et al., 2014; Wang and Yang, 2009; Wells and Scott, 2009). Information about a dolphin's reproductive history was also used to confirm sexual maturity for some individuals (e.g. the production of viable sperm, previous pregnancies).

All dolphins were housed in sand-bottom outdoor enclosures filled with natural seawater (18–26 °C), which ranged in size from 1500 to 8000 m^3. The activity demands for each animal varied daily and seasonally due to changes in performance and interaction schedules. Dolphins were maintained in small social groups consisting of either two males or one to four adult females. Group composition was occasionally altered (independent of this study) for social enrichment, training and breeding purposes, with only one adult male allowed access to females at any time. From the start of the study (June 2015) until mid-December 2015 hormonal contraceptives were orally administered (Altrenogest 0.044 mg/kg body mass; Table 1) to six adult females to suppress reproductive activity.

2.2. Blood collection

In-water (IW) blood samples (n = 45) were collected from dolphins that were positioned in dorsal recumbency with their flukes voluntarily resting on the pool edge or trainer's lap. Out-of-water (OOW) sampling (n = 29) occurred with the animal manually restrained on a floating platform—completely out of the water—for a maximum of 15 min (mean ± SE: 8 min 7 s ± 51 s). Blood was collected via caudal venipuncture using a 23-gauge butterfly needle and extension set. The venipuncture site was cleaned before inserting the needle by swabbing with a solution of chlorhexidine in methylated spirits (0.5% w/v in 70% w/v alcohol solution). Blood was drawn into a 10 mL syringe and

Table 1
Demographics, sex and species of study animals and their corresponding sampling schedule.

ID	Species	Month-year						
		Jun-15	Aug-15	Dec-15	Feb-16	Mar-16	May-16	Aug-16
Adult females								
TTF1	*Tursiops truncatus*	–	a, d^{+UN}	a, d^{+UN}	a, d $^{+UN}$	–	–	–
TTF2	*T. truncatus*	–	a, d*^	a^^{UN}	a, b, d *^{UN}	–	a^{UN}	a, b, d*
TTF3	*T. truncatus*	–	–	a, d^{+}	a, d $^{+UN}$	–	a, d^{+UN}	a, d^{+}
TTF4	*T. truncatus*	–	a, d*^^{UN}	a^^{UN}	a ^{UN}	a, b, d*	a^{UN}	a, b, d*
TTF5	*T. truncatus*	–	a, d*^^{UN}	a^	a, b, d*	–	a	a, b, d*
TTF7	*T. truncatus*	–	a, d*^^{UN}	a^	a, b, d*^{UN}	–	a^{UN}	a, b, d*$^{+}$
TXF1	*T. truncatus* × *aduncus*	–	a, d*^	a^^{UN}	a, d*^{UN}	–	a, d^{+UN}	a, b, d*$^{+}$
TXF2	*T. truncatus* × *aduncus*	–	a, d *^^{UN}	a^	a ^{UN}	a, b, d*	a^{UN}	a, b, d*
Juvenile females								
TAF1	*T. aduncus*	–	a*	–	–	–	–	–
TAF2	*T. aduncus*	–	a*	–	–	–	–	–
TTF6	*T. truncatus*	–	a, d*^{UN}	a ^{UN}	a, b, d*^{UN}	–	a	a, b*
Adult males								
TTM1	*T. truncatus*	a	a, c	a, c* ^{UN}	a, c ^{UN}		a, c ^{UN}	a, b*
TTM2	*T. truncatus*	–	a, c ^{UN}	a, c ^{UN}	a, c ^{UN}	–	a, c ^{UN}	a, b*
TTM3	*T. truncatus*	–	a, c	a, ^{UN}	a, c ^{UN}	–	a, c ^{UN}	a
TTM4	*T. truncatus*	–	a, c	a, c	a, b, c*^{UN}	–	a, c ^{UN}	a, b*
TTM5	*T. truncatus*	–	a, c	a, c ^{UN}	a, c^{UN}	–	–	–
Juvenile males								
TAM1	*T. aduncus*	–	–	–	a*	–	–	a, b*
TAM2	*T. aduncus*	–	–	–	a*	–	–	a, b*

a: serum and blow collected; b: blubber collected; c: semen collected; d: ovarian and/or uterine ultrasound performed.
$^{+}$ pregnant at time of sampling. *out-of-water sampling period. ^receiving Altrenogest at time of sampling. ^{UN}blow sample included in urea nitrogen trial.

transferred into 4 mL serum clot activator Vacuette® tubes (Greiner Bio-One). Samples were left to clot at room temperature for a minimum of 30 min and then centrifuged for 10 min at 3000 × *g*. The serum was then aspirated and transferred into 2 mL cryovials (Nunc™, Australia) and stored at −20 °C until analysis.

2.3. Blow sample collection and steroid hormone extraction

Blow samples were collected (n = 74), and steroid hormones extracted, according to previous protocols (Hogg et al., 2005; Hunt et al., 2014) with modifications to the collection device and the preservation method. A single exhalation was collected into an inverted 250 mL polystyrene sample container (LS10065-UL, ProSciTech, Australia) filled with a 30-cm^2 piece of nylon tulle (100% Nylon, Wholesale Wedding Supplies, Australia). The nylon tulle had been sanitised by two rounds of alternate rinsing and drying with distilled water and absolute ethanol (> 99%, Merck Millipore, Australia). A further 10-cm^2 piece of sanitised nylon tulle was secured, using parafilm®, as a barrier to the opening of the container.

Blow samples were collected within 3 min (mean ± SE: 2 min 6 s ± 11 s) of the initial needle stick during each sampling bout. During IW sampling, blow samples were collected via operant conditioning, with a two-finger tap around the blowhole used to cue an exhalation. OOW samples were captured opportunistically from the animal while restrained, usually before blood sampling commenced. Subjective notes were recorded for each blow sample, specifically the strength of the exhalation. Samples collected from a 'loud' audible exhalation, with large visible droplets in the specimen container, and brown or green particles, were classified as 'strong'. Other samples collected from a quiet exhalation, with no visible droplets, were classified as 'weak'. The mass or volume of a sample was not directly measured. After an exhalation, both pieces of nylon tulle were forced to the bottom of the container and submerged in 15 mL of absolute ethanol. The container cap was then replaced, sealed with parafilm® and shaken for 1 min. Samples were placed on ice in a cooler (1 – 2 h) and then transferred to −20 °C.

Following overnight storage, ethanol-preserved blow samples were decanted into 50 mL conical tubes (BD Falcon, Australia). The nylon tulle was then centrifuged at 3000 × *g* for 5 min and the extracted fluid added to the original sample. Samples were dried under compressed air at 37 °C (Ratek DBH10). The drying process left an organic residue at the bottom of the tube, which was reconstituted in 1.5 mL of assay buffer (NaH_2PO_4 [5.42 g], Na_2HPO_4 [8.66 g], NaCl [8.70 g], BSA [1.00 g], ProClin 150 [1 mL], H_2O Mili-Q [1000 mL], pH [7.0]). Each tube was then vortexed at high speed for 1 min to ensure even mixing and then centrifuged for 1 min at 3000 × *g*. Samples were then aliquoted into 1.5 mL centrifuge tubes (Thermo Scientific, Australia) and stored at −80 °C until analysis.

2.4. Blubber sample collection and steroid hormone extraction

Tissue biopsies (n = 19) were collected from twelve dolphins (female: n = 7; male: n = 5) during OOW examinations in February, March and August 2016. Biopsies were collected within 10 min of the initial needle stick (mean ± SE: 6 min ± 13 s). Seven of these animals were sampled in both summer and winter, with the remaining five dolphins sampled in only one of these seasons (Table 1). Tissue samples were collected using 8 mm hand driven biopsy punches (Paramount Surgimed Ltd) delivered postero-lateral of the dorsal fin to a maximum tissue depth of 20 mm. These samples were placed into sterile specimen containers (70 mL, Sarstedt) and frozen at −20 °C.

Steroid hormones were isolated from blubber using an organic solvent extraction method modified from previous studies (Kellar et al., 2006; Trego et al., 2013). A small amount of blubber (75 ± 5 mg), immediately adjacent to the skin, was dissected into small pieces (~2 mm^3) and placed into 2 mL homogenisation tubes (Cat. No. 522S, Daintree Scientific, Australia). These tubes were filled with 1 g of 1 mm silica carbide beads (Daintree Scientific, Australia) and 1 mL of phosphate buffered saline (PBS; D8662, Sigma-Aldrich Ltd, Australia). Tissue samples were then mechanically homogenised for three 5-min cycles (Mini-Beadbeater-16, BioSpec), cooling on ice for 5 min between each cycle. The homogenate and beads were then decanted into 5 mL polypropylene tubes (LBS504N, ThermoFisher, Australia). A 1 mL volume of 100% ethanol:acetone (4:1) was then added to both the polypropylene and homogenisation tubes. Homogenisation tubes were then vortexed for 1 min at high speed to remove any remaining blubber

residue. This mixture was aspirated and transferred into its corresponding polypropylene tube. All polypropylene tubes were then vortexed for 1 min at high speed and centrifuged for 1 min at 3000 × *g*. The liquid fraction (PBS:ethanol:acetone) was decanted into a new 5 mL tube and dried under compressed air at 37 °C (Ratek DBH10D). After drying, 2 mL of diethyl ether (> 99%, Sigma-Aldrich) was added to each tube and the vortexing, centrifuging and drying steps were repeated. The resulting residue was re-suspended in 1 mL of acetonitrile (> 99%, Sigma-Aldrich). Samples were then vortexed for 1 min and centrifuged for 1 min at 3000 × *g*. A 1 mL volume of hexane (> 99%, Sigma-Aldrich) was then added to each tube, and the vortexing step repeated. Each tube was then centrifuged for 5 min at 3000 × *g*, with the top layer (hexane) then removed and discarded. A further 1 mL of hexane was added to each tube, and the samples vortexed and centrifuged as in previous steps. The hexane layer was again discarded and the final acetonitrile-lipid mixture was dried under compressed air at 37 °C. The final steroid hormone residue was reconstituted in 1 mL of assay buffer and vortexed on high for 1 min to ensure even mixing. Blubber hormone extracts were stored at −80 °c until analysis.

Too few blubber samples were collected to conduct extraction efficiency tests. The extraction technique used here has, however, been examined for humpback whale blubber samples (Mingramm, 2018). In this case, extraction efficiency was found to vary for each of the different parent hormones (mean recovery: 95% [P4], 126% [T], 72% [E2], 104% [F]). The amount of parent hormone recovered was highly consistent between duplicate samples (CVs: 8.2% [P4], 3.1% [T], 4.2% [E2], 0.92% [F]).

2.5. Blow and blubber control experiments

To test for 'false' signals from the blubber extraction process, control samples were prepared using homogenisation tubes containing no tissue. These samples were then extracted and analysed as if 'real' blubber samples. For blow, there were several other control samples prepared. Firstly, to assess whether the collection dish or storage tubes influenced hormone measurements, empty specimen containers and conical tubes were filled with 15 mL of ethanol and then treated as if 'real' blow samples. Next, seawater contamination control samples were prepared by filling 50 mL conical tubes with 2 mL of seawater, eluting with 15 mL of ethanol and then analysing after the extraction/drying/reconstitution process. To identify whether the source of seawater had an influence on concentrations, duplicate samples were collected and analysed from two different sites: dolphin enclosures, and nearby coastal waters (Palm Beach, QLD, Australia). As part of this experiment it was also necessary to determine whether hormones could be detected in the raw seawater samples. Raw seawater samples (i.e. not extracted) were therefore included in assays for each hormone. Finally, blow sampling kit controls were prepared to assess the combined effects of sampling materials, seawater contamination and the extraction process. Blow sampling kit controls were made using three different masses of the nylon tulle sampling material (low: 0.6 ± 0.05 g, medium: 1.2 ± 0.05 g, high: 2 ± 0.05 g). Each of these sampling kits was then also spiked with different volumes of seawater (either no seawater, 0.02 mL, 0.2 mL, or 2 mL). The masses of nylon and volumes of seawater used were chosen to reflect both the sampling conditions/descriptions of previous studies (Hogg et al., 2009; Hunt et al., 2014) and the range of perceived contamination levels we observed in a pilot study on humpback whales. One to four replicate samples were produced for each possible nylon mass/seawater volume combination (n = 29). These control samples were extracted in ethanol, dried and reconstituted in assay buffer as per 'real' samples.

2.6. Assessment of male dolphin reproductive and maturity status

Semen was collected from adult males to investigate links between testicular activity and T levels. Five dolphins were sampled between August 2015 and May 2016, using established operant-conditioning techniques (Keller, 1986). First, animals were instructed to roll over onto their dorsal surface near to the pool edge. The animal was then cued, with tactile stimulation, to extrude its penis from the genital groove. Once erect, a gloved hand (Nitrisoft, Nitrile examination glove) was used to grasp the penis and direct the ejaculate into a dry, and clean, large plastic jug. The ejaculate was then decanted into a sterile specimen container (70 mL, Sarstedt).

Dolphin semen characteristics were examined using published methods for these species (Robeck and O'Brien, 2004; Yuen et al., 2009). To determine the initial quality of a sample there were a number of criteria that were subjectively assessed: volume (mL), colour, turbidity, viscosity, contamination, pH and percentage of live/dead sperm cells (using a eosin-nigrosin stain procedure). All criteria that required microscopic observations/counts were made using a light microscope (Olympus, Tokyo, Japan) with a pre-warmed stage. Only non-contaminated samples with a high proportion (i.e. > 50%) of live cells were included in this study. For these samples, sperm total motility (%), sperm progressive motility (%), sperm kinetic rating (0 – 5 scale; 0: no forward movement; 5: fast progressively forward movement) and sperm concentration (million sperm per mL of semen) were also assessed. Sperm counts were conducted with a Neabauer haemocytometer using raw semen that had been diluted at 1:100 with 5% saline. Sperm motility was examined at × 400 magnification using raw semen that was diluted 1:1 (vol/vol) with pre-warmed porcine semen extender (Androstar® Plus, Minitube). Motility was assessed for one hundred sperm in three fields of view. Sperm were classified as progressively motile, non-progressively motile or immotile. Sperm total motility was calculated as progressively motile + non-progressively motile. A sperm motility index (SMI) was calculated as SMI = progressively motile × kinetic rating (Robeck and O'Brien, 2004).

2.7. Assessment of female dolphin reproductive status

Females were classified as either juvenile, 'Altrenogest', anoestrus, 'cycling' or pregnant for each sampling round. Classifications were made using physiological, morphometric and behavioural measures. The animal husbandry staff recorded any observations of socio-sexual interactions (e.g. mating), abnormal behaviours (e.g. regurgitating food) or other displays that could indicate oestrous activity (e.g. mounting, beak to genital contact, ventral surface rubbing, poor responsiveness to instruction) as described elsewhere (Muraco and Kuczaj, 2015; Robeck et al., 2005). Females were classified as oestrous cycling in two scenarios:

(1) oestrous behaviours had been observed in the three weeks before sampling and the individual showed increased serum P4 levels relative to other times, or (2) oestrous behaviours were observed within the three weeks after sampling was conducted and the individual had shown increased serum E2 levels relative to other sampling periods. This resulted in a forty-two day window, which is slightly longer than the reported cycle lengths for these species (Brook, 1997; Robeck et al., 2005). A few opportunistic attempts were also made to visualize ovarian structures during OOW sampling (as per: Brook, 2001; Robeck et al., 2005). Ultrasounds were conducted using a real-time B-mode ultrasound (Logiq book XP, GE Healthcare) with a 2.0 MHz transducer (wide footprint convex linear probe). The ovaries were located by placing the transducer perpendicular to the flank, at the level of the genital slit, and moving dorsally or cranially. Uterine ultrasounds were also used to diagnose pregnancies, track foetal development and predict parturition

(as per: Lacave et al., 2004). All non-pregnant adult females that were not receiving Altrenogest and that did not meet the criteria for cycling were classified as anoestrus.

2.8. Analysis of biological samples

Blow and blubber hormone extracts and serum samples were analysed for steroid hormone levels using enzyme-immunoassays (EIAs) that have already been used, and validated for serum, for these species (O'Brien and Robeck, 2012; Steinman et al., 2016) and described elsewhere (Munro and Stabenfeldt, 1984; Munro and Lasley, 1988; Munro et al., 1991). The antibodies used in these EIAs (CL425 [P4], R156/7 [T], R9472 [E2], R4866 [F], Coralie Munro, UC Davis) are known to cross-react with several other hormone metabolites (see: Young et al., 2004; Thompson et al., 2012; Knott et al., 2013) that may be present in each sample. Therefore, unless otherwise specified, a reference to 'steroid' or 'hormone' levels relates to the concentration of a parent hormone and its metabolites.

The EIAs were performed as previously described with minor modifications; specifically, microtitre plates were pre-coated with goat anti-mouse IgG (A008-25MG, Arbor Assays, USA) for P4 assays and goat anti-rabbit IgG (A009-25MG, Arbor Assays, USA) for T, E2 and F assays. The only other modification was a change in antibody and horseradish peroxidase conjugate dilutions. Antibody dilutions were 1:80 000 (P4), 1:120 000 (T), 1:185 000 (E2) and 1:80 000 (F). The horseradish peroxidase conjugate was diluted at 1:400 000 (P4), 1:400 000 (T), 1:320 000 (E2) and 1:120 000 (F). EIAs were evaluated for colour development using a Biotek Reader ELx808 (Gen5™ software (Biotek, USA) with read and reference wavelengths of 405 nm and 540 nm [P4, T] or 630 nm [E2, F]). Inter-assay coefficients of variation (CVs) for high controls were 9.8% (P4), 7.8% (T), 7.5% (E2) and 6.4% (F). Inter-assay CVs for low controls were 14% (P4), 16% (T), 14% (E2) and 18% (F). Individual sample results were accepted if intra-assay (duplicate well) CVs were < 10%. Blow hormone levels were expressed as nanogram of hormone per millilitre of extract. Blubber concentrations were expressed as nanogram of hormone per gram of blubber (wet weight).

Parallel displacement of serial dilutions of pooled serum, and blow extracts, relative to the standard curves, was demonstrated (P4: R^2 = [0.95, 0.99]; T: R^2 = [0.99, 0.97]; E2: R^2 = [0.96, 0.99]; F: R^2 = [0.98, 0.99], respectively). Parallelism was also demonstrated for serial dilutions of blubber extracts relative to the P4 standard curve (R^2 = 0.98); there were insufficient volumes of blubber extracts to conduct parallelism tests for all other assays. For all samples, parallelism occurred over a limited range (from neat to 1:8), indicating that hormone concentrations were low but within the reliable range of all four EIAs. Consequently, serum, blow and blubber extracts were initially analysed 'neat'. High concentration samples were re-diluted and analysed again until their results fell into the middle of the standard curve.

The potential for substances within each sample type to affect hormone measurements was also examined. Firstly, pooled serum and pooled blow samples were made by combining and mixing 100 µl volumes from 13 different samples. In total, there were four blow and serum pooled samples made in this way (i.e. one blow and serum pooled sample for each assay). There was insufficient blubber to make these pooled samples and to conduct these hormone recovery/accuracy tests. 'Test samples' were then made for each assay by spiking 100 µl volumes of each EIA hormone standard with an equal volume of either serum or blow. Each assay was then run with a set of serum and blow test samples, and pooled serum and blow samples (to determine background concentration). 'Expected values' were calculated as half the concentration of the EIA hormone standard plus half the concentration of the pooled sample; 'observed values' were calculated as the measured concentration for test samples minus half of the pooled sample (background) concentration. Linear regressions of expected vs. observed results were then generated using values that fell within the linear range of the assay (~20%–80% binding). All relationships between observed and expected values were significant for blow (P4: $F_{(1,4)}$ = 268.66; T: $F_{(1,3)}$ = 535.00; E2: $F_{(1,3)}$ = 2204.32; F: $F_{(1,3)}$ = 864.44; all $p < 0.001$) and serum (P4: $F_{(1,3)}$ = 233.09; T: $F_{(1,3)}$ = 802.99; E2: $F_{(1,3)}$ = 662.36; F: $F_{(1,3)}$ = 310.99; all $p < 0.001$). The slope coefficients for blow T, E2 and F indicated good accuracy (95% CIs [0.97, 1.21], [0.99, 1.10], [1.01, 1.26], respectively). Conversely, slope coefficients for blow P4 (0.62, 0.88), and all serum hormones were significantly different from one (P4 [0.55, 0.65], T [0.49, 0.69], E2 [0.25, 0.30], F [0.40, 0.58]), indicating an under or over-estimation of the actual parent hormone concentration.

UN was trialled as an endogenous marker for blow dilution. A random, stratified (by sex) selection of dolphin blow samples (n = 38) and a random set of blow sampling kit controls (n = 6) were selected for the UN trial (Table 1). Concentrations of UN were measured using commercial colorimetric detection kits (K024-H1, Arbor Assays®, USA). Normalised blow hormone levels were expressed as nanogram of hormone per milligram of UN in the sample (i.e. ng/mg [UN]).

2.9. Statistical analyses

Statistical analyses were undertaken using *R* version 3.4.0 (R Development Core Team [2017]) with significance determined at *p*-values < 0.05. Mean ± standard error (SE) values of all hormone measures (absolute concentrations, blow hormone ratio values and UN normalised blow hormone levels), were summarised for groups of interest (e.g. reproductive states).

Given that blow samples were dried and reconstituted in a constant volume of assay buffer, the initial sample volume was expected to have an influence on measured hormone concentrations. 'Collection type' (i.e. IW or OOW sampling) and 'exhalation strength' (i.e. strong or weak) were identified as two possible factors that might influence sample volume. Permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) was used to determine whether these differences in blow hormones (model 1) and UN levels (model 2) were likely to be significant. The dissimilarity metric used for these analyses was the Euclidean distances between data points. Due to the limited sample sizes 'individual subject' was not included in the model, meaning that the data was not entirely independent. As both models had an unbalanced design, type II tests were applied using the 'RVAideMemoire' package (Hervé, 2017). An assumption of PERMANOVAs, which is particularly important for models with unbalanced designs, is that there is a similar degree of within-group multivariate dispersion. This was evaluated using the distance-based tests described by Anderson (2006) in the 'vegan' package (Oksanen et al., 2017).

Following these analyses, generalised linear mixed models (GLMMs) were used to determine if hormone concentrations were influenced by biological and environmental factors, and whether these effects were consistent for each sample type (Table 2). 'Individual subject' was included in all models, as a random effect, to account for repeated sampling of dolphins. Each model also contained several fixed effects that had been selected *a priori* (Table 2). These predictors included 'sample type' (serum, blow and blubber), 'collection type' (IW or OOW sampling), 'life history class' (juvenile male, juvenile female, adult male and adult female), 'season' (winter or summer), 'collection time' (8:00–12:00 [morning] or 12:00–16:00 [afternoon]), 'pregnancy status' (pregnant or non-pregnant adult female), 'ovarian condition' (oestrous cycling female or non-cycling adult female), 'male age class' (juvenile or adult) and 'sampling date' (month-year). All GLMMs included interactions between 'sample type' and the other fixed effects in the model (Table 2). These interaction terms were used to assess whether patterns in hormone levels were consistent for serum, blow and blubber. Each GLMM examined a separate set of data to test slightly different hypotheses; there was no 'universal null hypothesis' being tested across all models. For this reason, alpha values were not adjusted (see: Armstrong, 2014). All response variables were non-normally distributed and right-skewed; GLMMs were therefore fitted with a Gamma distribution and a log-link function, using the 'lme4' package (Bates et al., 2015). Evidence for multicollinearity between predictor variables

Table 2
Structure of statistical models for assessing relationships between hormones and biological, sampling and environmental variables.

Model #	*Response Variable*	*Fixed predictors*	*Random predictors*	*Samples used*	*Type of analysis*	*Notes*
1	P4, T, E2, F (multivariate)	Collection type + exhalation strength	–	Blow samples from all dolphins (n = 74)	PERMANOVA	• Exhalation strengths were "strong" or "weak" only.
2	UN	Collection type + exhalation strength	–	Random sub-set of blow samples (n = 38)	Permutational ANOVA	
3	P4	Sample type + pregnancy status + (sample type × pregnancy status)	Individual subject	Serum, blow and blubber from adult females (n = 96)	GLMM	
4	E2	Sample type + ovarian condition + (sample type × ovarian condition)	Individual subject	Blow and blubber samples from adult females (n = 50)	GLMM	• Non-oestrous cycling females included pregnant females, anoestrus females and those receiving Altrenogest. • Serum hormone levels were not considered as they were used to help classify ovarian condition.
5	T	Sample type + age class + (sample type × age class)	Individual subject	Serum, blow and blubber from all males (n = 60)	GLMM	
6	T	Sample type + sampling date + (sample type × sampling date)	Individual subject	Serum and blow samples from adult males (n = 48)	GLMM	• Sampling dates in 2015 were June, August and December, and in 2016 were February, May and August
7	F	Sample type + collection type + life history class + season + collection time + (sample type × collection type) + (sample type × life history class) + (sample type × season) + (sample type × collection time)	Individual subject	Serum, blow and blubber from all dolphins (n = 165)	GLMM	• Season refers to winter (June, August, May) or summer (December, February, March).

was examined via inspection of the generalised variance inflation factor, using the 'car' package (Fox and Weisberg, 2011). Residual plots were examined for heterogeneity, autocorrelation and over-dispersion. Coefficient estimates, standard errors, Wald z- or t-statistics and associated *p*-values were examined for model parameters. Likelihood-ratio tests were used to examine if a predictor had a significant impact on the fit of a model. Tukey's tests were then used, *post-hoc*, to conduct comparisons between each level of a predictor variable.

2.10. Ethics of experimentation

The procedures used in this study were approved by the University of Queensland's Animal Ethics Committee for Native/Exotic Wildlife and Marine Animals (approval number SVS/458/14), and were compliant with the requirements of the *Animal Care and Protection Act 2001* (QLD, Australia), and the current *Australian code for the care and use of animals for scientific purposes* and the *Australian code for the responsible conduct of research*. All OOW sampling was conducted under direct supervision of the SeaWorld veterinarian.

3. Results

3.1. Control sample measurements and sampling effects

A key objective of this study was to determine whether reproductive (P4, T and E2) and adrenal steroid hormones (F) could be accurately quantified in blow and blubber samples. To achieve this, it was necessary to identify other sampling-related variables that might influence hormone measurements in these samples. Each of the target hormones was quantifiable in extracted blow and blubber samples, and in serum. Signals were also detected in ethanol extracted seawater samples and nylon sampling kits but not in blubber extraction controls, tube/container controls or raw seawater samples. The hormone signals detected in extracted seawater samples were similar for samples collected from dolphin enclosures and coastal waters (mean ± SE ng/mL; F: [0.50 ± 0.21, 0.37 ± 0.20]; P4: [0.10 ± 0.00, 0.09 ± 0.00]; T: [0.36 ± 0.14, 0.27 ± 0.12]; E2: [0.19 ± 0.03, 0.18 ± 0.04], respectively). Detected signals, in all blow control samples, increased in value with the volume of seawater added (Fig. 1). A similar effect was also found for nylon tulle, with measurements increasing along with the mass of nylon material used (Fig. 1). Overall, F and T measurements were the most affected by seawater and nylon sampling materials (Fig. 1). Six blow sampling kit controls were also analysed for UN concentrations, with no detectable levels found in any of these samples.

Sample volume was also expected to have an influence on dolphin blow hormone levels. However, this was not directly tested due to all samples being eluted with ethanol immediately after collection (for hormone extraction). 'Exhalation strength'—a proxy for volume—was recorded instead and found to have a marked influence on the measured hormone content ($F_{(1,71)} = 3.27$, $p = 0.050$) and, to a lesser extent, UN content of a sample ($F_{(1,35)} = 2.59p = 0.10$). The hormone and UN content of a sample also appeared to be influenced by 'collection type' (F = [15.76, 3.44], p = [< 0.001, 0.050], respectively). Though, individual effects

(i.e. repeated measures) were not considered in these previous four models, which might have slightly biased these results. Overall, 'strong' exhalations (n = 41) were typically recorded during IW sampling and 'weak' exhalations (n = 33) observed during OOW sampling (Chi-square contingency test: $\chi 2 = 24.68$, $p < 0.001$). Together, this led to large differences in concentration between 'strong' IW samples (F: 0.37 ± 0.03 ng/mL; P4: 0.34 ± 0.06 ng/mL; T: 0.41 ± 0.05 ng/mL; E2: 0.77 ± 0.11 ng/mL; UN: 4.96 ± 1.14 mg/dL) and 'weak' OOW samples (F: 0.16 ± 0.02 ng/mL; P4: 0.11 ± 0.01 ng/mL; T: 0.13 ± 0.02 ng/mL; E2: 0.21 ± 0.03 ng/mL; UN: 0.65 ± 0.47 mg/dL).

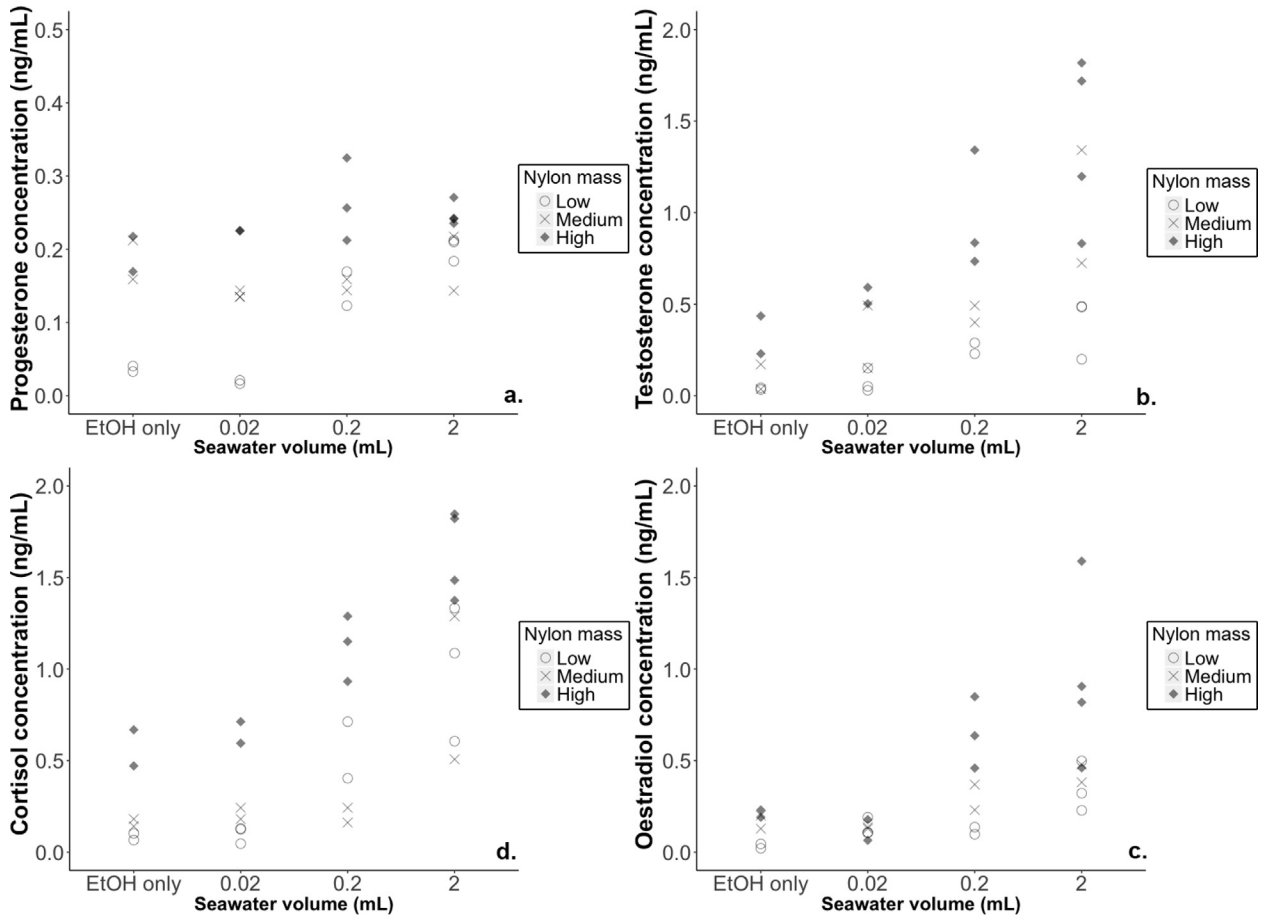

Fig. 1. Detected levels (ng/mL) of progesterone (a.), testosterone (b.), oestradiol (c.) and cortisol (d.) in blow sample collection and extraction controls. Hormone levels were measured in control sampling kits made of three different masses of nylon tulle ('low': 0.6 ± 0.05 g; 'medium': 1.2 ± 0.05 g; 'high': 2 ± 0.05 g). Sampling kits treated with ethanol for hormone extraction and either no seawater (two control samples for all three masses of nylon), 0.02 mL of seawater (n = 2 [low mass], 3 [medium mass], 2 [high mass]), 0.2 mL of seawater (n = 2 [low mass], 2 [low mass], 3 [high mass]) or 2 mL of seawater (n = 3 [low mass], 2 [medium mass], 4 [high mass]). Statistical comparisons between each group were not performed.

3.2. Patterns in reproductive hormones

There were several groups of dolphins that displayed relatively similar patterns in hormone levels for each sample type (Table 3). For instance, juveniles (n = 3), anoestrus females (n = 5) and those receiving Altrenogest (n = 6) all displayed low P4 levels in blow, blubber and serum (Table 3). In contrast, pregnant females possessed markedly elevated P4 levels in each of these sample types (Fig. 2; Table 3). Despite this, the interaction between 'pregnancy status' and 'sample type' did have a significant impact on the fit of the P4 model

Table 3
Mean ± SE of reproductive hormone levels in the serum, blow and blubber of female bottlenose dolphins in different reproductive states. The reproductive state of each female varied over the course of the study.

Sample	*Hormone*	*Female Reproductive State*				
		Juvenile(n = 3)	*Altrenogest(n = 6)*	*Anoestrus(n = 5)*	*Cycling(n = 4)*	*Pregnant(n = 4)*
Serum (n = 46)	*P4 (ng/mL)*	0.09 ± 0.02	0.15 ± 0.03	0.11 ± 0.02	0.87 ± 0.23	34.10 ± 8.64 [a]
	E2 (ng/mL)	0.16 ± 0.06	0.58 ± 0.21	0.94 ± 0.22	1.99 ± 0.58	0.23 ± 0.07
Blow(n = 46)	*P4 (ng/mL)*	0.16 ± 0.02	0.16 ± 0.01	0.14 ± 0.03	0.21 ± 0.03	0.60 ± 0.20
	Normalised P4 ((ng/mg[UN])	0.96 ± 0.87	0.63 ± 0.21	0.18 ± 0.04	0.42 ± 0.29	0.22 ± 0.06
	E2 (ng/mL)	0.47 ± 0.20	0.44 ± 0.15	0.55 ± 0.15	0.69 ± 0.15	0.67 ± 0.23
	Normalised E2 ((ng/mg[UN])	1.73 ± 1.60	1.12 ± 0.36	0.89 ± 0.48	1.35 ± 0.93	0.39 ± 0.20
	P4:T ratio value	0.77 ± 0.27	1.49 ± 0.26	0.55 ± 0.09	4.07 ± 3.53	2.31 ± 0.81
	P4:E2 ratio value	0.51 ± 0.15	0.84 ± 0.25	0.47 ± 0.10	0.37 ± 0.05	1.05 ± 0.30
	E2:T ratio value	1.76 ± 0.51	2.50 ± 0.40	1.42 ± 0.21	19.07 ± 17.62	2.16 ± 0.51
Blubber [b] (n = 13)	*P4 (ng/g)*	0.83 ± 0.11[c]	–	1.27 ± 0.17	1.20 ± 0.06	13.01 ± 0.72 [a]
	E2 (ng/g)	2.88 ± 1.36[c]	–	2.92 ± 0.46	5.04 ± 1.42	1.56 ± 0.78

[a] Pregnant females displayed significantly higher serum (Tukey's: z = 8.2, $p < 0.001$) and blubber P4 levels than other adult females (Tukey's: z = 3.05, $p = 0.022$).

[b] Blubber was collected from 1 juvenile female (twice), 4 anoestrus females, 3 cycling females and 2 pregnant females;3out 7 were sampled in different reproductive states over the study period.

[c] Results from 1 juvenile female only (sampled twice).

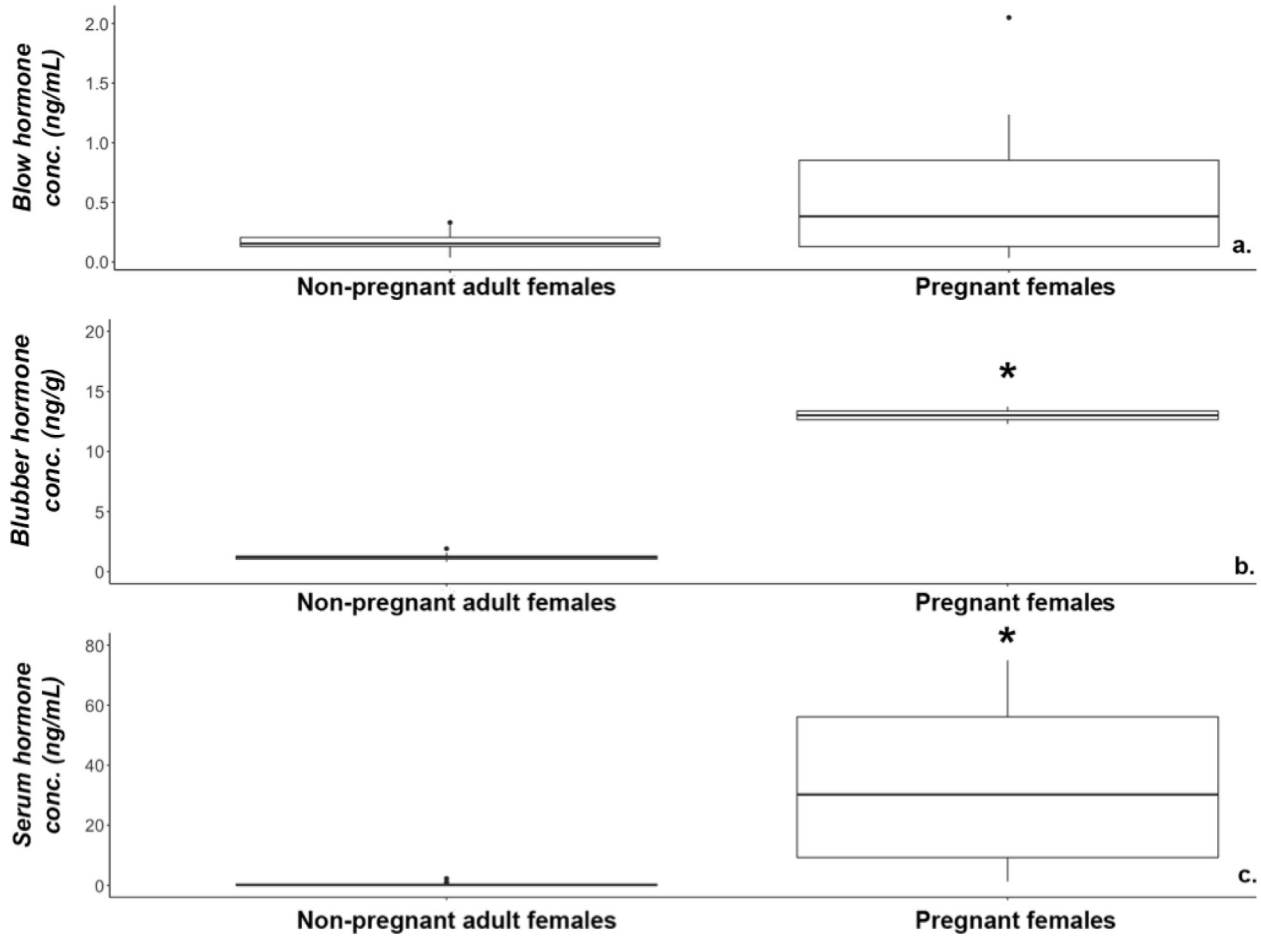

Fig. 2. Progesterone levels in the blow (a.), blubber (b.) and serum (c.) of pregnant (n = 10 [serum/blow], 2 [blubber]) and non-pregnant adult females (n = 29 [serum/blow], 9 [blubber]). Boxplot displays median value and interquartile range, with whiskers representing values within the 1.5 × interquartile range. Values beyond this range plotted as individual points. * Progesterone levels were significantly higher ($p < 0.05$) in pregnant than in non-pregnant adult females.

($\chi^2 = 67.47$, $p < 0.001$). Subsequent *post-hoc* comparisons (Tukey's tests) revealed that P4 levels differed significantly between pregnant and non-pregnant females but only for serum (34.10 ± 8.64, 0.32 ± 0.09 ng/mL, respectively; z = 8.2, $p < 0.001$) and blubber (13.01 ± 0.72, 1.17 ± 0.10 ng/g, respectively; z = 3.05, $p = 0.022$). Differences in blow P4 levels were not significant ($p = 0.61$). Overall, the lowest serum and blubber P4 measurements for pregnant females were greater than the highest measurements for non-pregnant females (Fig. 2).

There were eight adult female dolphins that were sampled during a confirmed or suspected oestrous cycle. For two females, follicles were visualised using ovarian ultrasounds, confirming that they were in the follicular phase at the time of sampling. These individuals had markedly elevated serum E2 levels

(3.09 ± 0.41 ng/mL) when compared to 'non-cycling' adult females (0.55 ± 0.13 ng/mL). The remaining six females were classified as 'cycling' using a combination of visual observations (i.e. for key behaviours such as repeated rubbing of their ventral surface on solid surfaces) and through monitoring of their serum E2 and P4 levels. Oestrous behaviours were observed 4 days after one of these females had displayed an elevated serum E2 level (i.e. suspected follicular phase: 2.77 ng/mL) and 5 – 12 days before the other females (n = 5) had displayed elevated serum P4 levels (i.e. suspected luteal phase: 1.12 ± 0.31 ng/mL). Given the subjective nature of these classifications, and the low sample sizes present, there was no attempt to statistically distinguish between the individual phases of an oestrous cycle.

The interaction between 'ovarian condition' and 'sample type' failed to have a significant impact on the fit of the E2 model ($p = 0.46$). After the interaction term was removed, only 'sample type' was found to have a significant impact on the model fit ($\chi2 = 73.78$, $p < 0.001$). This indicated that blow and blubber E2 levels did not differ significantly between oestrous cycling females and non-cycling adult females (Table 3). Blow also differed markedly from serum in that its E2 concentrations were highest in periods after oestrous behaviours had been observed (i.e. suspected luteal phase; 0.83 ± 0.41 ng/mL) as opposed to prior to oestrous displays

(i.e. suspected follicular phase; 0.54 ± 0.08 ng/mL). Patterns in blow P4 levels were less variable and related poorly to the timing of oestrous behaviours (before: 0.20 ± 0.03 ng/mL; after: 0.22 ± 0.06 ng/mL). For blubber, patterns in hormone levels throughout an oestrous cycle could not be determined, as all samples were collected in the two weeks following a suspected oestrus event (i.e. the luteal phase).

For males, adult dolphins were found to possess higher T levels than juveniles in all sample types (Table 4; Tukey's tests: z = -7.53, $p < 0.001$ [serum], z = -3.47, $p = 0.0059$ [blow], z = -2.93, $p = 0.035$ [blubber]). These results remain tenuous as only two juvenile male dolphins (both *T. aduncus*) were sampled throughout the study. The interaction between 'sample type' and 'age class' was also found to have a significant impact on the fit of the T model ($\chi2 = 10.07$, $p = 0.0065$), meaning that there were slightly different patterns detected for each sample type. For adult males, there was also a significant interaction between 'sample type' and 'sampling date' ($\chi2 = 31.62$, $p < 0.001$), which indicated that there was seasonal variability in concentrations. Both serum and blow T concentrations increased between August 2015 (winter) and February 2016 (summer).

Table 4
Mean ± SE of testosterone levels in the serum, blow and blubber of juvenile and adult male bottlenose dolphins.

Sample	*Hormone*	*Juvenile male (n = 2)*	*Adult male (n = 5)*
Serum	*T (ng/mL)*	0.65 ± 0.05 (n = 4)	10.71 ± 1.14 (n = 24)*
Blow	*T (ng/mL)*	0.11 ± 0.10 (n = 4)	0.33 ± 0.06 (n = 24)
	Normalised T (ng/mg[UN])	–	0.33 ± 0.07 (n = 14)
	P4:T ratio value	3.68 ± 2.36 (n = 4)	1.05 ± 0.17 (n = 24)
	E2:T ratio value	7.13 ± 5.83 (n = 4)	2.09 ± 0.36 (n = 24)
Blubber	*T (ng/g)*	1.15 ± 0.06 (n = 2)	6.59 ± 0.84 (n = 4)*

*Significantly higher ($p < 0.05$) concentration than found in juvenile males.

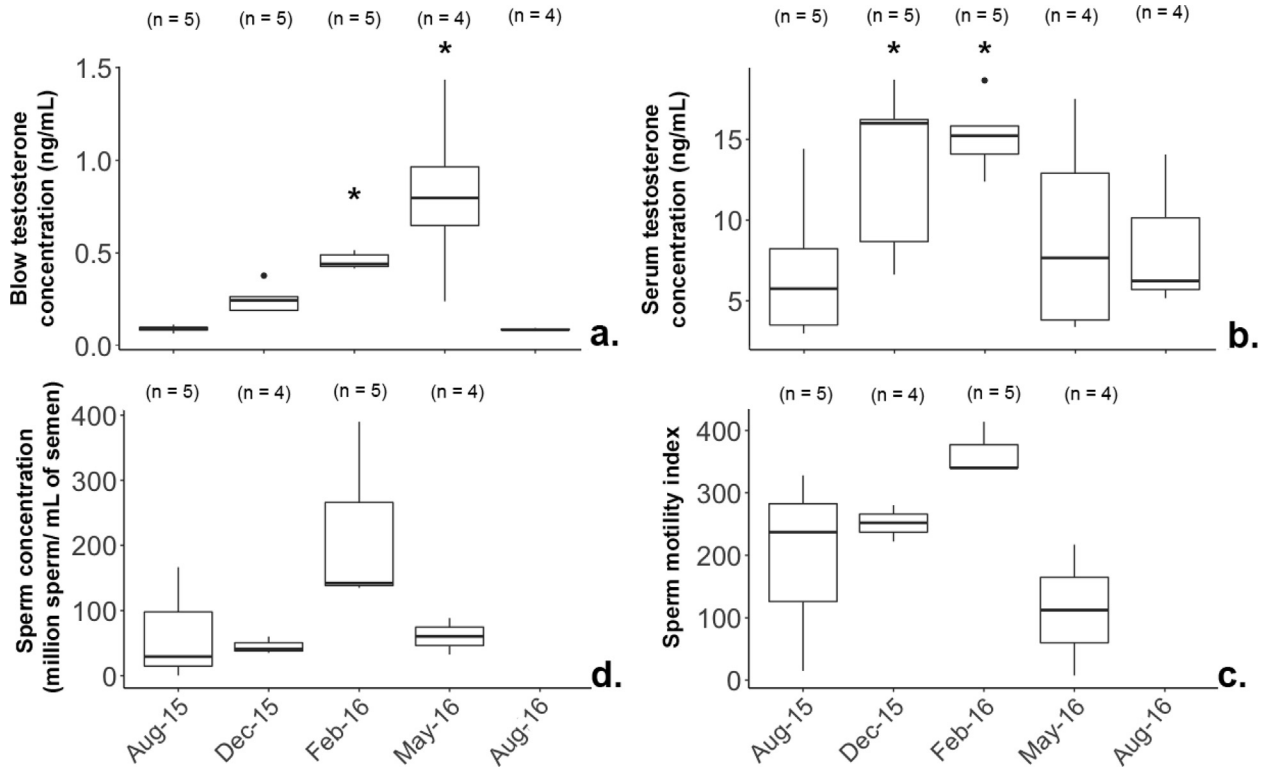

Fig. 3. Seasonal variation in testosterone levels (a: blow; b: serum), sperm motility (c.) and sperm concentration (d.) for adult male bottlenose dolphins. Semen was collected from four adult males between August 2015 and May 2016 only. Boxplot displays median value and interquartile range, with whiskers representing values within the 1.5 × interquartile range. Values beyond this range plotted as individual points. * Blow and serum testosterone levels were significantly higher (Tukey's *post-hoc* tests, $p < 0.05$) than in months not marked with an asterisk. All months marked with an asterisk had statistically similar levels of testosterone.

However, in contrast to serum, blow T levels were higher again in May of 2016 (Fig. 3). The seasonal changes in serum T levels and, to a lesser extent, blow T levels were consistent with those observed for semen characteristics (SMI and sperm concentration; Fig. 3). However, SMI and sperm concentration values were markedly more variable between sampling dates.

Blow hormone ratio values and UN normalised hormone levels were highly variable within each sex, age class and reproductive group (Tables 3 and 4). Both measures were unsuitable for discriminating between pregnant and non-pregnant females, and between adult and juvenile males—the two most significant reproductive endocrine patterns observed in the other sample types (Tables 3 and 4).

3.3. Patterns in cortisol

In this section, patterns in F levels were examined with respect to collection type (a potential stressor), the season of sampling, the time of day (i.e. morning or afternoon) and the animal's life history class. Interactions between these predictors and 'sample type' were also evaluated to determine whether patterns were consistent between serum, blow and blubber. Two of these interaction terms had a significant effect on the fit of the F model: (1) 'sample type' *x* 'sampling time' ($\chi 2 = 8.27$, $p = 0.016$), and (2) 'sample type' *x* 'collection type' ($\chi 2 = 91.66$, $p < 0.001$). Serum concentrations were significantly higher when samples were collected OOW (Tukey's: $z = 12.87$, $p < 0.001$) when compared to those collected IW; the opposite pattern was observed for blow (Table 5). A significant diurnal pattern was also observed for serum F levels (Tukey's: $z = 5.49$, $p < 0.001$), with concentrations being highest in the morning collection periods (Table 5). For dolphins that were sampled IW, F levels were around four times higher in the morning (7.12 ± 1.11 ng/mL) than in the afternoon (1.76 ± 0.23 ng/mL). In contrast, neither collection type nor the time of day had significant effects on F levels in blow or blubber. In the case of blubber, however, all samples had been collected OOW and only four individuals were sampled in both a morning and afternoon collection period.

The interaction between 'sample type' and 'life history class' approached statistical significance ($p = 0.089$), indicating that demographic trends in F levels might differ between serum, blow and blubber. However, this could not be fully evaluated given the low sample sizes and inherent biases the sampling design. In particular, all blubber and juvenile male samples were collected OOW. The only

Table 5
Mean ± SE of cortisol measurements in the serum, blow and blubber of female and male bottlenose dolphins sampled using voluntarily procedures and capture and restraint procedures.

Collection Type	Time	Sex (n)	Serum	Blubber	Blow				
			F (ng/mL)	*F (ng/g)*	*F (ng/mL)*	*Normalised F (ng/mg [UN])*	*F:P4 (ratio value)*	*F:T (ratio value)*	*F:E2(ratio value)*
In-water	*Morning*	*Female (n = 10 [serum/blow])*	4.33 ± 1.27[b]	–	0.44 ± 0.07	0.16 ± 0.08	1.69 ± 0.46	1.14 ± 0.25	0.62 ± 0.15
		Male (n = 19 [serum/blow])	8.58 ± 1.46[b]	–	0.36 ± 0.04	0.32 ± 0.11	1.70 ± 0.20	1.78 ± 0.52	0.82 ± 0.17
	Afternoon	*Female (n = 15 [serum/blow])*	1.73 ± 0.25	–	0.32 ± 0.05	0.84 ± 0.51	1.51 ± 0.18	1.29 ± 0.22	0.86 ± 0.12
		Male (n = 1 [serum/blow])	2.07	–	0.04	–	0.15	0.28	0.13
Out-of-water	*Morning*	*Female (n = 3 [serum/blow], 3 [blubber])*	39.53 ± 13.44[a,b]	3.07 ± 0.64	0.18 ± 0.11	–	1.37 ± 0.74	0.73 ± 0.35	0.52 ± 0.13
		Male (n = 8 [serum/blow],4 [blubber])	27.11 ± 6.50[a,b]	2.61 ± 0.48	0.19 ± 0.05	1.08[c]	1.67 ± 0.48	7.91 ± 6.36	1.50 ± 0.34
	Afternoon	*Female (n = 18 [serum/blow],10 [blubber])*	23.14 ± 4.02[a]	3.55 ± 0.39	0.19 ± 0.03	0.55 ± 0.13	2.21 ± 0.39	6.87 ± 5.54	1.19 ± 0.27
		(n = 2 [serum/blow], 2 [blubber])	23.32 ± 19.41[a]	2.39 ± 0.15	0.15 ± 0.00	–	4.00 ± 0.34	1.82 ± 0.01	2.14 ± 0.55

[a] Serum concentrations significantly higher in OOW samples (Tukey's: $z = 12.87$, $p < 0.001$).
[b] Serum concentrations significantly higher in morning samples (Tukey's: $z = 5.49$, $p < 0.001$).
[c] n = 1.

significant life history-related trend occurred during OOW sampling, with juvenile males displaying lower F levels (serum: 25.32 ± 8.77 ng/mL; blow: 0.13 ± 0.02 ng/mL; blubber: 2.12 ± 0.20 ng/g; Tukey's: $z = -3.10$, $p = 0.0098$) than adult males (serum: 27.02 ± 9.64 ng/mL; blow: 0.26 ± 0.09 ng/mL; blubber: 2.74 ± 0.44 ng/g).

In addition to these interaction effects, there was a small, yet significant, seasonal influence on F levels in all sample types (Wald: $t = -2.52$, $p = 0.012$). When sampling was conducted IW, mean F levels were relatively similar in winter (i.e. May, June, August; serum: 5.20 ± 1.99 ng/mL; blow: 0.36 ± 0.04 ng/mL) and summer (i.e. December, February, March; serum: 5.20 ± 1.99 ng/mL; blow: 0.36 ± 0.04 ng/mL). Conversely, when sampling was conducted OOW, F levels were slightly lower in winter (serum: 22.22 ± 3.67 ng/mL; blow: 0.15 ± 0.05 ng/mL; blubber: 2.61 ± 0.21 ng/g, respectively) than in summer (serum: 38.88 ± 4.62 ng/mL; blow: 0.29 ± 0.02 ng/mL; blubber: 4.08 ± 0.43 ng/g).

Blow F ratios values were typically greater than one in OOW samples and less than one for IW samples (Table 5). This indicated that F was the dominant hormone present in blow samples when sampling was conducted OOW—a pattern consistently seen for serum. In contrast, when F levels were normalised relative to UN levels, values were similar for IW and OOW samples. Other patterns that were observed for serum F levels, such as sex-related and diurnal trends, were not detected when blow hormones were normalised against UN levels or expressed relative to the concentration of other hormones (Table 5).

4. Discussion

Blubber and blow hormone monitoring have emerged as potentially viable non-lethal techniques for assessing the physiological condition of wild cetaceans, but for many species they have yet to be validated for these purposes. In this study we conducted some of the preliminary biochemical and physiological validations necessary to use these samples for reproductive and adrenal endocrine assessments of bottlenose dolphins. The analytical methods used here appeared suitable for endocrine monitoring, with several expected patterns detected in serum, including diurnal and stress-related changes to cortisol levels, pregnancy-related increases in progesterone and elevated testosterone levels in sexually mature males. However, for blow and blubber, there were several other issues or limitations found. Blow was unsuitable for monitoring the endocrine condition of dolphins as endogenous hormone levels were masked by other sampling-related factors. Blubber hormone monitoring, on the other hand, is limited by animal welfare concerns associated with repeated sampling and by the time it takes for this tissue to express circulating hormone signals. Several gross endocrine patterns could, however, be detected in the blubber of these species. In particular, age-class related differences in male testosterone levels, seasonal variation in cortisol levels and pregnancy-related differences in progesterone levels. Though, some of these blubber hormone results remain uncertain due to low sample sizes, particularly for juvenile male dolphins.

The progesterone levels measured in pregnant females and the cortisol levels measured in acutely 'stressed' animals (out-of-water sampled) were lower than previous reports for these species (Champagne et al., 2017, 2018; Kellar et al., 2017; Pérez et al., 2011). In the case of cortisol, this is most likely due to small discrepancies in capture procedures (the stressor), the duration between capture and sampling, and individual variation. In the case of progesterone, lower levels could be due to all pregnant females being sampled for blubber in the first trimester and our inclusion of two pregnant *T. truncatus* × *aduncus* hybrids. However, for both hormones, the use of different extraction and assay techniques might have also contributed to these results. In particular, the use of PBS rather than more polar solvents (e.g. ethanol) during the initial homogenisation of a sample would be expected to lead to poorer hormone extraction efficiency. However, this should have been offset by the subsequent ethanol:

acetone extraction step, which would have sequestered any lipid residue that remained in the tube. Further, the decision to use PBS was based primarily on our observations that other solvents (ethanol, diethyl ether) seemed to affect the texture of tissue samples, possibly through dehydration, resulting in incomplete homogenisation. Despite this different approach to hormone extraction, differences between pregnant and non-pregnant female progesterone levels were similar to previous studies. Thus, our blubber hormone extraction and analysis procedure appears suitable for detecting major endocrine patterns in bottlenose dolphins.

With regards to both cortisol and testosterone, there was slight seasonal variation in serum, and often blubber, concentrations. Cortisol levels in both of these sample types were slightly higher in summer than in winter. Though, accurately assessing these seasonal patterns would require further sampling of the same individuals across multiple months for several years. This type of seasonality is atypical for cetaceans, which usually show limited changes in adrenal activity (Kellar et al., 2015; St Aubin et al., 1996) or display the highest cortisol levels during periods with the lowest water temperatures (Funasaka et al., 2011; Orlov et al., 1988; Suzuki et al., 2003). For adult males, this summer period also coincided with peaks in serum testosterone levels, sperm motility and sperm concentration. Given that most cycling and mating occurred at this time it is possible that elevated testosterone and cortisol could be due to changes in behaviour and gonadal activity. Similarly, seasonal changes could partially be driven by the varying activity demands (e.g. performances, interactions), and nutritional status and health, of animals throughout the year. Cortisol and testosterone are also known to increase in concentration after an animal is captured (Champagne et al., 2018). However, variability in capture-restraint times is unlikely to have had a marked influence on our results as all samples were collected within a relatively short period (i.e. 8 min 7 s ± 51 s). Further, there was no evidence of variability in these times when compared across seasons. A final possibility is that elevated air temperatures in summer could enhance a dolphin's peripheral circulation (to lose heat) and lead to higher stress levels when captured and restrained out-of-water. Together, these factors might influence the secretion of certain hormones, including cortisol, or their rate of integration into blubber.

Delayed hormone expression in blubber is a possible reason why oestradiol levels were marginally higher in females sampled within 5 – 12 days after a suspected non-conceptive oestrus event. Given that these females were suspected to be in the luteal phase of an oestrous cycle, we expected progesterone to be the dominant reproductive hormone. Recent studies have identified that the integration of cortisol into bottlenose dolphin blubber may occur over several hours or possibly even days (Champagne et al., 2017, 2018). It is possible that this timeframe is even longer for oestradiol when considering the partition coefficients observed in other mammalian species (see: Pardridge and Mietus, 1979). A similar pattern in oestradiol levels was also found for blow. However, this particular result was likely due to sampling-related effects, particularly as many complex lipophilic molecules, including some steroid hormones, are known to rapidly integrate into respiratory fluids (i.e. within an hour; Pennington, 1981; Vichyanond et al., 1989). Nonetheless, for many cetacean species, a current research priority is to clarify hormone integration and clearance rates in different tissues and fluids. This could be achieved by repeatedly sampling dolphins following an exogenous hormone challenge or after a known oestrus event.

Other patterns that had been detected in blow, including the relatively high progesterone levels in pregnant females and the seasonal variability in male testosterone levels, were likely driven by sampling-related effects. Though, the lack of significant pregnancy-related differences in blow progesterone levels might also be an artefact of our small sample sizes. Blow hormone levels were strongly influenced by 'collection type' and 'exhalation strength', both of which were markers for sample volume. This effect was particularly apparent for adult males, which displayed distinctly lower testosterone levels when sampled out-of-water in August 2016. Similarly, juvenile males, which consistently displayed 'weak' exhalations and were always sampled out-of-water, displayed lower blow hormone levels than all other dolphins. In addition to variable sample volumes, blow hormone measurements were strongly influenced by seawater contamination levels. Seawater was expected to dilute samples at the time of collection but also produced 'false' endocrine signals when extracted with ethanol, dried and reconstituted in assay buffer. This blow hormone extraction procedure could amplify any hormones that were present in raw seawater. However, this would only occur if the volume of seawater contamination exceeded the volume of assay buffer used (i.e. 1.5 mL). Such levels of contamination are possible when sampling free-swimming animals but are unlikely to occur when sampling captive dolphins. Thus, it is more likely that seawater constituents either physically or chemically interfere with antigen-antibody binding, which could be directly tested by spiking serial dilutions of assay standards with extracted seawater samples. A further possibility is that seawater amplifies the signals produced by blow sampling materials, potentially by assisting with the extraction of chemical components from nylon fabric. This source of interference could be minimised or eliminated in future studies by using alternative materials, such as empty polystyrene dishes (Burgess et al., 2016), or by subtracting control sample results from blow hormone measurements (Burgess et al., 2018). The signal-to-noise ratio of blow samples could also be improved by collecting multiple exhalations from the animal (as per: Richard et al., 2017; Thompson et al., 2014). However, this particular modification is more difficult to implement in studies on free-swimming cetaceans due to permit/animal ethics restrictions and because many cetaceans will often display avoidance behaviour after a sampling attempt (Noren and Mocklin, 2012). Whether other blow hormone extraction and analytical techniques (e.g. chromatography-mass spectrometry) are less sensitive to these sampling-related effects should be investigated.

Overall, it is likely that the endogenous hormone signals in many blow samples were completely masked by sampling-related effects. These issues could not adequately resolved by expressing blow hormone concentrations relative to UN or as ratio values. UN could not be used as an endogenous standard because its concentration was influenced by sample volume (due to the extraction process) and seawater contamination (via sample dilution), as well as the expected variability in respiratory water content. Quantifying sample volume should therefore be considered an essential first step in any future blow hormone study. Hormone ratio values, which are not sensitive to sample volume, were also unsuitable for discriminating between different reproductive states, age classes and sexes of dolphins. The poor results for ratio values was primarily due to each hormone measurement being affected by seawater and nylon sampling materials to a different extent. The use blow hormone ratio values, and UN as a marker for dilution, should be revisited once issues of variable samples volumes, seawater contamination and nylon interference are resolved.

4.1. Conclusion

Here, we have demonstrated that reproductive and adrenal steroid hormone levels can be quantified in the blow and blubber of captive bottlenose dolphins. Patterns in blubber hormone levels were similar to those in serum. For blow, on the other hand, hormone measurements were strongly influenced by a range of sampling-related factors, such as seawater contamination and variable sample volumes. Until further control tests are performed, it is not possible to determine the extent to which our blow hormone measurements reflect a 'real' biological signal as opposed to these sampling-related effects. Given the inaccuracy of blow hormone measurements obtained here, in a captive setting, blow sampling is unlikely to be suitable for studying the endocrinology of wild bottlenose dolphins. In contrast, blubber should be suitable for detecting pregnancy, and possibly male sexual maturity, in these

species. Further sampling of adult male dolphins is warranted to confirm whether blubber testosterone levels vary seasonally with changes in testicular activity. Additional work is also required to determine whether blubber is suitable for detecting certain temporally restricted endocrine events (i.e. lasting minutes to hours), such as acute stress and oestrus. A critical step in these studies is producing more accurate estimates for hormone integration and clearance times, particularly for reproductive hormones. Overall, this work will help clarify the potential applications and limitations of using blubber hormone measurements as indicators of cetacean physiological condition.

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Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.12.015.

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Further reading

[dataset] Mingramm, Fletcher. 2018. Captive bottlenose dolphin serum, blubber and respiratory vapour endocrine database. Mendeley Data, v1. https://doi.org/10.17632/ndjf74krgc.1.