Title:

Bottlenose dolphin calves have multi-year elevations of plasma oxytocin compared to all other age classes

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Abstract

Providing for infants nutritionally via lactation is one of the hallmarks of mammalian reproduction, and infants without motivated mothers providing for them are unlikely to survive. Mothers must maintain regular contact with infants both spatially and temporally while utilising their environment to forage, avoid threats and find shelter. However, mothers can only do this and maximise their reproductive success with some degree of co-operation from infants, despite their developing physical and cognitive capabilities. The neuropeptide hormone oxytocin (OT) triggers proximity-seeking behaviour and acts in a positive feedback loop across mother-infant bonds, stimulating appropriate pro-social behaviour across the pair. However, data on infant OT levels is lacking, and it is unclear how important infants are in maintaining mother-infant associations. The bottlenose dolphin (*Tursiops truncatus*) is a mammalian species that is fully physically mobile at birth and has multi-year, but individually variable, lactation periods. We investigated OT concentrations in mother-infant pairs of wild individuals compared to other age and reproductive classes. An ELISA to detect OT in dolphin plasma was successfully validated with extracted plasma. We highlight a statistical method for testing for parallelism that could be applied to other ELISA validation studies. OT concentrations were consistently elevated in calves up to at least 4 years of age with lactating mothers (12.1 ±0.9 pg/ml), while all mothers (4.5 ±0.4 pg/ml) had OT concentrations comparable to non-lactating individuals (5.9 ±0.5 pg/ml). Concentrations within infants were individually variable, and may reflect the strength of the bond with their mother. The OT system likely provides a physiological mechanism for motivating infants to perform behaviours that prevent long-term separation from their mothers during this crucial time in their life history. Elevated infant OT has also been linked to energetic and developmental advantages which may lead to greater survival rates.

Environmental or anthropogenic disturbances to OT release can occur during bond formation or can disrupt the communication methods used to reinforce these bonds via OT elevation. Variation in OT expression in infants, and its behavioural and physiological consequences, may explain differences in reproductive success despite appropriate maternal behaviour expression.

Keywords

Infant behaviour; maternal behaviour; mother-infant bonds; oxytocin; proximity seeking; separation

1. Introduction

Reproductive success in animal species that display parental behaviour is reliant on an individual's ability to maintain care-giving activities towards dependant infants while continuing to utilise habitat effectively for essential resources (Dietz et al. 1994, Hill et al. 2000, Tremblay and Cherel 2003, Macri and Wübel 2007, Robb et al. 2008). Mammalian infant survival is particularly dependant on repeated, consistent interactions with mothers during early life, as they are the primary individuals able to provide infants with milk via lactation. Mothers are stimulated to associate with their offspring and engage in care-giving behaviours towards them via both physiological systems and cognitive processes. After birth, the quality of care a mammalian mother gives to her infant(s) is the biggest factor determining their survival (Nowak et al. 2000). However, even the most motivated mothers cannot rear their young successfully if there is no co-ordination or co-operation from dependant infants (Fleming et al. 1999). Cognitive abilities in offspring undergo substantial development during the time they are reliant on their parents (Rice and Barone 2000, Branchi 2009). Therefore for infants, physiological systems promoting parental associations and appropriate behaviour towards care-givers may be crucial for averting separations or preventing behaviour that increases the likelihood of separation.

Oxytocin (OT) is a neuropeptide hormone that is vital for bond formation and the initiation and expression of maternal care in mammals (Gimpl and Farenholz 2001, Ross and Young 2009, Rilling and Young 2014, Jurek and Neumann 2018). In wild mammalian species it causes proximity seeking behaviours between mothers or adult care-givers and infants (meerkats (*Suricata suricatta*); Madden and Clutton-Brock 2010, grey seals (*Halichoerus grypus*); Robinson et al. 2015a, 2017). Oxytocin is released via a positive feedback mechanism, and interactions between individuals sharing a functional bond generate elevated peripheral OT levels in bond mates (Rilling and Young 2014, Nagasawa et al. 2015, Robinson et al. 2019). However, there is evidence that a mother's peripheral OT concentration becomes a less effective predictor of her proximity to dependant infants as they become more physically developed and mobile (Robinson et al. 2015a). Drivers of offspring behaviour within a mother-infant dyad may therefore play an important role in determining the success or failure of a reproductive event as infants develop across the dependant period. Despite this, thus far there are few studies on infant OT concentrations and their potential impacts on behaviour and development. Data on endogenous infant plasma OT levels prior to weaning have only been published for three species, and all show elevated plasma concentrations of the hormone compared to adults (human (*Homo sapiens*) babies; Leake et al. 1981, laboratory mice (*Mus musculus*); Higashida et al. 2010, grey seals; Robinson et al. 2019). Manipulation experiments on newly weaned grey seals under a month old have also demonstrated that elevated OT triggers proximity-seeking behaviour in young individuals (Robinson et al. 2017). However, of these three species, two have very short dependant periods (grey seals; approximately 18 days Fedak and Anderson 1982, mice; approximately 22 days Higashida et al. 2010) and human babies have only been studied during the first four days of life (Leake et al. 1981). For all of these species, infants additionally have limited physical ability to separate from mothers during the immediate post partum period. Therefore, to explore links between offspring OT concentrations and maintaining mother-infant associations, it would be ideal to obtain measurements from a species that is highly mobile from birth and that relies on maternal care for longer than a few weeks.

Species of the orders Artiodactyla and Perissodactyla typically produce highly developed, precocial young which are capable of independent locomotive movement soon after birth (Lent 1974). Infants are able to move independently to either stay alongside their mothers or to seek out a hiding spot to conceal themselves while mothers continue to feed (Fisher et al. 2002). Of the animals included in these two orders, the production of infants with highly developed locomotive skills is especially vital to cetaceans, which reproduce in aquatic environments and must swim to the surface to breathe as soon as they are born (Dearolf et al. 2000). Infants from Delphinid species face additional challenges driving their advanced mobility skills at early ages. Dolphin calves must constantly swim from birth, and during the first month of postpartum life mother-calf pairs show little typical sleep behaviour (Lyamin et al. 2005). Calves can ride their mother’s pressure wave to reduce the amount of time spent actively swimming, however they are regularly separated from each other, as mothers within dolphin species do not halt feeding behaviour during the early life stages of their calves as many migratory whale species do (Pomeroy et al. 2017). All dolphin species are carnivorous and mothers have to suddenly accelerate to facilitate prey capture throughout the dependant period. Such foraging behaviour increases opportunities for mother-calf separation and there is evidence that dolphin calves that are unable to successfully cope with frequent maternal separations have lower survival rates (Mann and Watson-Capps 2005). In addition to these spatial challenges, many species of dolphins typically do not wean their calves nutritionally until at least one year of age (Perrin and Reilly 1984), with some species taking much longer and with substantial individual variation (Mann et al. 2000). Dolphin species therefore present a unique model species to test whether elevated OT concentrations are present in mother-infant pairs. These pairs consist of two individuals that are physically able to rapidly separate from each other, but must reunite as calves are nutritionally dependent on mothers for a long, but variable, period of time.

The common bottlenose dolphin (*Tursiops truncatus*) is one of the best studied of all cetacean species as wild individuals are frequently individually identifiable, which has permitted many long-term studies on behaviour, life history and population dynamics across the globe (Würsig and Jefferson 1990). The Sarasota Dolphin Research Program (SDRP) based in the USA has studied the long-term, year-round resident community of common bottlenose dolphins (*T. t. truncatus*) that lives in the coastal waters off the west coast of Florida near Sarasota since 1970 (Wells 1991, 2014). Nutritional weaning ages for wild bottlenose dolphin calves typically range between approximately 2.7 - 5 years of age, with some individuals nursing for double this range (Mann et al. 2000). In the Sarasota community, calf independence occurs typically at 3 – 6 years of age, but substantial variation across different mother-calf pairs exists with some lactating mothers associating with their calves for 9 years (Wells et al. 1987, Connor et al. 2000).

The behavioural and population data collected by the SDRP is uniquely complemented by occasional health assessments of a subset of the resident community, with the shallow waters of Sarasota Bay facilitating safe capture, blood sampling and release of free-ranging wild dolphins (Wells et al. 2004). To complement the data collected from free-ranging individuals, it is possible to study dolphins under human care at zoological facilities. This enables researchers to obtain samples and measurements in minimally stressful conditions via training individual dolphins to co-operate with research protocols (Ramirez 2012). Many analytical protocols identifying biomarkers for studying the physiology of free-ranging cetacean populations rely on studying cetaceans under human care to develop and verify novel methodologies (Würsig et al. 2018). Zoo Duisburg (ZD) has several decades of experience maintaining small cetaceans under human care, and the dolphins at ZD have enabled research studying detailed aspects of individual physiology (Kastelein et al. 1993) and behaviour (Janik and Slater 1998). By utilising samples from both the SDRP and ZD, we were able to test if elevated OT is present in mother-calf pairs within a species that is highly mobile from birth and determine whether any elevation occurs throughout the variable lactation period of several years. If OT dynamics are important for driving associations between mothers and infants, concentrations within these individuals should be elevated for at least the duration of lactation, however long that may be within a specific dyad.

2. Methods

2.1. Ethics and permits

All animal procedures were performed under applicable national, and institutional guidelines. Capture and sampling work with wild bottlenose dolphins was conducted by the SDRP under National Marine Fisheries Service Scientific Research Permit Number 15543 issued to RSW, and under Mote Marine Laboratory Institutional Animal Care and Use Committee approvals renewed annually. All research received prior ethical approval from the University of St Andrews Animal Welfare and Ethics Committee. Plasma samples from the USA were transported to the UK for analysis under CITES export permits 14US39971B/9 and 16US98573B/9 and CITES import permits 528413/01 and 549036/01.

2.2. Study site and animals

Individual dolphins sampled for this study came from two sources, ZD where dolphins are held under human care, and wild individuals who were briefly captured, sampled and released by the SDRP as part of a long-term study that includes health assessments. Blood draws from six dolphins at ZD took place as part of routine veterinary assessments in 2014 and aliquots of plasma collected during this sampling opportunity were set aside for use in this study.

Fieldwork with wild dolphins was conducted on the resident bottlenose dolphin community near Sarasota Bay, Florida, USA in May 2014 – 2016 with the SDRP. Capture-release studies of this population have occurred periodically since 1970 and are accomplished by rapid encircling of study individuals with a net deployed from the rear of a nine-metre vessel, followed by immediate physical restraint by trained handlers and veterinarians (Wells and Scott 1990, Wells et al. 2004). Study animals were identified via individual markings on the dorsal fin prior to capture. Plasma samples and mass data were collected from all individuals except two in the 2014 cohort, which were not weighed. The numbers of individuals sampled are as follows; 19 in 2014, 14 in 2015 and 9 in 2016. This gave a total of 42 samples from wild dolphins, with two individuals sampled in both 2014 and 2015.

2.3. Plasma sampling

All plasma samples were collected from peripheral veins in the ventral surface of the tail fluke. Dolphins under human care were trained to present their flukes for venipuncture (Ramirez 2012) and wild dolphins were manually restrained for the procedure immediately after capture. The time intervals between 1. capture net deployment to restraining an individual, 2. restraining an individual to sample collection and 3. sample collection to sample freezing at -80ºC were recorded for all wild individuals to control for potential variable capture stress and sample collection or processing times in the statistical analysis. Prolonged restraint stress has been shown to impact on OT concentrations in some mammal species (e.g. prairie voles (*Microtus ochrogaster*), Grippo et al. 2009). However, validation studies on plasma collected from other wild marine mammal species (grey and harbour (*Phoca vitulina*) seals, Robinson et al. 2014) show that this variation only exists if unextracted plasma is used for OT detection, which was not the case in our study.

Plasma samples were drawn into lithium heparin or ethylenediaminetetraacetic acid (EDTA) vacutainers with no addition of aprotinin. Mean volume of plasma per sample was 2.7ml (range: 0.5-3ml). Samples were stored on ice immediately after collection until they could be spun, aliquoted and frozen at -20ºC (ZD) or snap frozen and stored at -80ºC (SDRP). Prior validation work has demonstrated no difference in OT concentrations across vacutainer type as long as extracted plasma is used for OT detection and no changes in OT levels have been detected in samples stored at -20ºC for at least two years (Robinson et al. 2014).

2.4. OT detection

All plasma samples were transported on dry ice to the University of St Andrews for OT analysis. Samples were analysed in duplicate for OT using an enzyme-linked immunosorbent assay (ELISA) (Enzo Life Sciences) with each sample under-going solid-phase extraction using Sep-Pak C18 columns (Szeto et al. 2011) prior to analysis. The protocol for extraction followed the manufacturer's instructions but included the modifications detailed in Robinson et al. (2014) to adapt the standard protocol for use with marine mammal plasma, which can block the Sep-Pak C18 columns if not sufficiently centrifuged after acidification. All OT detection in this study was performed using extracted samples. To determine recovery rates, a set of plasma samples (n=10) were first spiked with known quantities of oxytocin, then these samples were extracted and analysed using the same protocol as all other samples.

All ELISA plates were read using a BioTek ELx800 reader and the standard curve and assay results for all plates were then fitted using the calibFit package (Haaland et al., 2011) in R version 3.4.1. (R Development Core Team, 2012). As one of the aims of the current study was to successfully validate this ELISA plate for use in bottlenose dolphin plasma, all quality control information, including coefficients of variance, recovery rates and sample parallelism with the standard curve is given in the results section.

2.5. Statistical analysis

All analyses were performed using the statistical package R 3.4.1 (R Development Core Team, 2012).

As part of the validation of the OT ELISA kit, parallelism was tested for statistically by generating linear models for the serial dilutions (optical density plotted against natural log of percentage sample dilution) of the ELISA kit standards and extracted plasma samples and then testing for significant interactions between the regression lines using an ANOVA (Kershaw et al. 2017). Significant interactions between linear regression lines indicate that they cross, demonstrating that the lines are not parallel. The standard curve for the OT ELISA kit is a logistic curve rather than a straight line; therefore, to allow linear regression modelling, the natural log of the percentage sample dilution was used for parallelism analysis to give linear dilution lines rather than curved ones.

To investigate whether there was a context difference between capture methodology used to obtain blood samples, plasma OT concentrations from adult (>10 years) and juvenile (<10 years and independent of mother) individuals from the wild and zoological park locations were compared. No calves under human care were sampled; therefore, no calves were included in this stage of analysis. A two-way ANOVA compared individuals of the four age/sex classes (adult male, adult female, juvenile male and juvenile female) and OT levels from samples collected from wild individuals using established physical restraint methods and from individuals under human care trained to present tail flukes for blood sampling. Data were normally distributed with equal variance across groups; therefore, no transformation was required prior to the two-way ANOVA.

A generalised additive mixed model (GAMM) (Wood 2006a) was used to investigate whether social dyad type affected plasma OT concentrations. Explanatory variables explored in these models included the year of sampling (2014, 2015, 2016), the individual's sex, the social dyad the sampled individual was part of when sampled (calf with mother, mother with calf, male within a male alliance or lone individuals), whether other dyads were present when individuals were sampled (a mother-calf pair or a male alliance), the time from capture net deployment to restraining an individual (in seconds), the time from restraining an individual to sample collection (in seconds) and the time from sample collection until freezing at -80ºC (in seconds). The model was fitted using the multiple generalized cross validation library *mgcv* (Wood 2012). The identities of individuals were fitted as random effect smooths (Wood 2006b) as the same individuals were sampled in multiple years. The smoothing parameters were set by maximum likelihood to reduce the risk of overfitting associated with other methods (Wood 2011). The model was fitted with a Gaussian error distribution. Model selection was done by backwards stepwise elimination through examination of R^2 values, Akaike's information criterion (AIC) values, QQ and residual plots to identify the best model given the data assessing goodness of fit and parsimony.

It was not possible to include age of individuals in the main GAMM model as this was unknown for several adult individuals. Age was known for all calves sampled however, and the calves sampled included some that were old enough to be able to separate from mothers (at approximately 3-6 years of age (Wells et al. 1987)). Weaning events that result in infants becoming independent of their mothers have been previously shown to impact on plasma OT levels in grey seals (Robinson et al. 2019) and mice (Higashida et al 2010), therefore a Pearson correlation coefficient was calculated for calf age and plasma OT concentrations.

To investigate any relationship between body size and plasma oxytocin concentrations, Pearson correlation coefficients were calculated between weight and plasma oxytocin levels in calves (68 – 117kg, n = 13) and non-calf individuals (juveniles and adults, 124 – 291kg, n = 27). Calves and non-calves were investigated separately as age class and weight are strongly correlated, and therefore cannot be distinguished analytically within one analysis.

3. Results

3.1. Validation of an ELISA for OT detection in bottlenose dolphin plasma

The commercial ELISA kit was successfully validated for detecting OT in bottlenose dolphins using extracted plasma samples (ANOVA: $F_{1, 4} = 0.12$, $p = 0.75$) (Fig. 1). When using extracted plasma, recovery rates for the extraction and ELISA procedure were 112% (n = 10), intra-assay coefficient of variance (calculated across duplicates) for this assay was 2.9% and inter-assay coefficient of variance over the three plates used in this study was 7.3%.

3.2. Basal concentrations of OT in wild bottlenose dolphins and dolphins under human care

Mean basal plasma OT concentrations and the ranges detected for wild bottlenose adults, juveniles and calves and dolphins under human care are given in table 1.

Table 1. Mean and ranges of detected basal plasma OT concentrations in bottlenose dolphins by age class, sex and capture location.

Source	Age class	Sex	n	Mean plasma OT (pg/ml)	Range of plasma OT (pg/ml)
Wild	Adult	Male	10	5.62	2.6 – 7.5
		Female	15	4.58	2.3 – 8.3
	Juvenile	Male	2	7.8	5.1 – 10.5
		Female	1	4.4	na
	Calf	Male	4	10.2	9.2 – 11
		Female	10	12.9	4.2 – 18.4
Under human care	Adult	Male	1	2.5	na
		Female	3	5.4	3.7 – 7.1
	Juvenile	Male	0	na	na
		Female	2	3.9	3.4 – 4.5

There were no significant differences in plasma OT concentrations between individuals sampled in wild or non-wild contexts (ANOVA: $F_{1, 29} = 1.08$, $p = 0.31$) or between adults or juveniles of either sex (ANOVA: $F_{3, 29} = 2.415$, $p = 0.09$).

3.3. OT concentrations across social dyad types

Social dyad type, whether other dyads were present during capture and the individual identity smooth were retained in the final model investigating variation in plasma OT concentrations. The year of sampling, individual sex and three measures of different time intervals during the capture and sampling procedures had no significant impact on plasma OT concentrations and were removed from the final model. The social dyad type was the only variable that significantly affected plasma OT concentrations (GAMM: $R^2 = 0.62$, SI Table 1), with 'calves with mothers' having higher OT concentrations than all other individuals in the various dyad types (Fig. 2) ($p < 0.001$ for all comparisons, Table 2).

Table 2. Mean and ranges of detected basal plasma OT concentrations in bottlenose dolphins by social dyad type.

Social dyad type	n	Mean plasma OT (pg/ml)	Range of plasma OT (pg/ml)
Lone individuals	4	6.4	4.4 - 10.5
Males in alliances	10	5.62	2.6 – 7.5
Mothers with calves	14	4.5	2.3 – 8.3
Calves with mothers	14	12.1	4.2 – 18.4

3.4. Age driven variation in calf plasma OT

While there was a negative correlation between calf age and plasma OT concentrations, it was non-significant (Pearson correlation coefficient, $r = -0.39$, $p = 0.17$, Fig. 3).

3.5 Weight driven variation in plasma OT

There was no relationship between weight and plasma OT levels in calves (Pearson correlation coefficient, $r = -0.23$, $p = 0.4$, Fig. 4a) or juveniles/adults (Pearson correlation coefficient, $r = 0.17$, $p = 0.4$). Visual inspection of the data plots (Fig. 4) and investigation of behavioural observations and capture records indicated the presence of an outlier in the juvenile/adult cohort (see discussion section), and when this individual was removed there was a significant positive relationship between weight and plasma OT levels in these age ranges (Pearson correlation coefficient, $r = 0.5$, $p = 0.01$, Fig. 4b).

4. Discussion

4.1. Successful ELISA validation with extracted plasma

No endocrine study can proceed without confidence in the methods used to detect the hormone of interest. Analytical methods for hormone studies are usually developed with laboratory model animals, and any study looking to investigate wildlife species with the same methods must first verify that the methodology is still accurate in this new context. Here we demonstrate that OT in plasma from bottlenose dolphins can be accurately detected using a commercial ELISA. With the successful validation of plasma OT detection in this species, there is potential to further validate these analysis methods in other cetacean species or using other obtainable substrates (see De Mello and De Oliveira 2016) such as urine (Muraco et al. 2009, Steinman et al. 2016), saliva (Monreal-Pawlowsky et al. 2016) or exhaled respiratory vapour (Burgess et al. 2018), as both urine and saliva samples have previously been successfully used for OT detection in other mammalian wildlife species (e.g. Crockford et al. 2013, Leeds et al. 2018, Schaebs et al. 2019).

When collecting any kind of biological sample from study subjects there is the potential for sampling procedures to cause significant changes to the hormone of interest (Beerda et al. 1996). Both central and peripheral oxytocin release can occur in response to a variety of physical and psychological stressors under laboratory conditions (Neumann 2002, Landgraf and Neumann 2004), therefore it was necessary to investigate the potential for the capture and sampling methods used in this study to affect plasma oxytocin levels. Our data show that there was no relationship between any of the restraint durations experienced by free-ranging individuals and their plasma oxytocin concentrations, and additionally found no difference in peripheral oxytocin levels across free-ranging individuals and those under human care, who had been trained and habituated to blood draw procedures. These two findings demonstrate that the protocols used to sample wild dolphins were either not long enough in duration or were not acute enough physical or psychological stressors to cause peripheral oxytocin release in this species. This finding agrees with results from other wild marine mammal species that showed no impact of restraint time or the use of physical or chemical restraint on peripheral oxytocin levels in grey seals (Robinson et al. 2014). The types of stressors that have been documented to cause peripheral oxytocin changes thus far in the literature are far more extreme than the methods used in this study, and include long-term restraint (Grippo et al. 2009, CS Carter, personal communication), restraint in a supine position (Hashimoto et al. 1989), forced swimming tests (Wotjak et al. 1998) and noxious or conditioned fear stimuli (Onaka 2004). Peripheral oxytocin dynamics in relation to stress are additionally modulated or ‘buffered’ by the social context in which the stressor is experienced (Smith and Wang 2014) and as free-ranging individuals travelling in small groups were captured together, and were not separated outside of acoustic or visual range of each other during sampling, it is possible that social buffering of stress responses took place and prevented peripheral oxytocin changes during sampling.

When reporting validations, determining whether a dilution series of the sample substrate is parallel with the standard curve from the assay is one of several basic requirements to demonstrate the assay is reacting correctly and predictably to the sample (Plikaytis et al. 1994). Non-linearity in response to parallelism testing is an indication that problems are occurring when attempting to use a particular sample type with an assay. A common cause of these difficulties are matrix effects, where non-target substances present in the sample substrate bind either specifically or non-specifically to the reagents used as part of the ELISA protocol (Tu and Bennett 2017). Interference can also occur due to degradation of the target peptide by proteinases or alterations to protein conformation via exposure to chemicals during sample collection, such as chelation of plasma collected with ethylenediamine tetraacetic acid (EDTA) vacutainer tubes (Schwickart et al. 2014).

While conducting parallelism testing is vital, even in studies using such tests as part of validations there is substantial variation in what is reported. Published ways of confirming parallelism include plots with only visual confirmation (e.g., Sarkar and Prakash 2006), detecting linearity and inferring parallelism from this (e.g., Bienboire-Frosini et al. 2017) and statistical analysis proving parallelism (e.g., Burgess et al. 2018), even if only with a portion of the standard curve (e.g., MacLean et al. 2017a).

Statistically proving parallelism is the most reliable way to confirm its presence, and the method used in this paper from Kershaw et al. (2017) provides an irrefutable method for testing for parallelism across the entire curve. Currently, there is increasing interest in using OT ELISAs to investigate the social behavioural endocrinology of domestic (e.g., Bienboire-Frosini et al. 2017, Schaebs et al. 2019) and wild animal species (e.g., Leeds et al. 2018, Schaebs et al. 2019). Reporting methods for hormone detection that have both succeeded and failed validation checks will enable future studies to utilise the best methods when developing their own protocols with wildlife or domestic species.

4.2. OT dynamics in adult bottlenose dolphins

OT concentrations in adult dolphins did not vary across the sexes, and lactating mothers had comparable levels to all other adult classes. There was also no difference in the OT levels detected in wild individuals or those under human care. Adult dolphin OT levels in plasma (mean: 4.9pg/ml, range: 2.3-8.3pg/ml) were individually variable, but were comparable to levels reported in non-breeding grey seals (4.3pg/ml, Robinson et al. 2015a), rats (6.8pg/ml, Landgraf 1981), domestic dogs (9 -13 pg/ml, MacLean et al. 2017b), six other domestic animal species (ranging from 2.9 – 10.9 pg/ml, Bienboire-Frosini et al. 2017) and adult humans (0.1–23 pg/ml, reviewed in Szeto et al. 2011). Therefore, bottlenose dolphins have comparable basal OT levels to more traditional model animal species and humans.

Elevated peripheral OT levels are indicative of functional bonds between individuals (Strathearn et al. 2009, Crockford 2013 & Crockford 2014) and the neuropeptide acts via positive feedback loops within bonded pairs, linking proximity seeking behaviour, social interactions and elevated peripheral OT levels (Nagasawa et al. 2015, Robinson et al. 2019). Co-ordinated central and peripheral release of OT occurs in certain contexts (Neumann and Landgraf 2012), including during mother-infant interactions (Strathearn et al 2009). Additionally, both manipulation studies (Madden and Clutton-Brock 2010, Robinson et al. 2017) and correlations of endogenous peripheral OT and proximity seeking behaviours (Robinson et al. 2015a) demonstrate these aspects of mother-infant behaviour and physiology are linked. Therefore, elevated peripheral OT levels could act as a biomarker for an individual's motivation to remain in close proximity to bond-mates during critical periods.

Maternal dolphin OT levels at least 2 years postpartum were comparable to those reported in lactating human mothers within 5 days of birth (10.8 pg/ml, Dawood et al. 1981) and 1-4 months after birth (5.4 pg/ml, Drewett et al. 1982). Maternal dolphin values were only comparable to the lower range of OT values detected in lactating seals in the first 18 days postpartum (mean 8.2pg/ml, ranging between 3.5 – 25.5 pg/ml, Robinson et al. 2019). Unlike seals, there was no difference in OT levels between lactating and non-breeding individuals (Robinson et al. 2015a). This indicates that, unlike in seal mothers, there is no constant significant elevation of basal OT levels throughout lactation in dolphin mothers. The difference in maternal OT values may be due to the large difference in the time frame of dependency in these two species. Grey seal mothers only nurse their pups for 18 days before returning to sea, with nutritional weaning and independence occurring simultaneously (Pomeroy et al. 1999). High motivation to remain close to pups, and the consistently elevated OT levels this generates via positive feedback loops in mothers, may be required to ensure they stay together for this brief, important time. In dolphins however, constant motivation to stay with calves in mothers over several years could be maladaptive. Wild mothers must separate from calves frequently over the dependant period, especially during foraging bouts where rapid acceleration must happen to facilitate prey capture (Mann and Smuts 1998, Gibson and Mann 2008). In dolphin populations outside of Sarasota, mothers must also dive to depths calves cannot reach while feeding. OT release in dolphin mothers may instead be associated with specific infant stimuli such as the sight and sound of the calf, as seen in humans which also have long dependant periods with their infants (Strathearn et al. 2009, Seltzer et al. 2010). It is also possible that mothers have sustained elevations of OT during the immediate post-natal period, when 'imprinting' between mother and calf is thought to take place (Mann and Smuts 1998) and maternal signature whistling rates are high for the first few weeks after birth (Fripp and Tyack 2008). OT release in dolphin mothers, and the motivation to associate with bonded individuals that comes with it (Robinson et al. 2017), may therefore be tied to specific time frames, behaviours or social cues that individuals encounter when approaching and interacting with other conspecifics.

All dolphin species live in social groups, with different sexes and species showing a variety of social bond duration and function. Delphinids are social to enable them to exploit their environment most effectively (Tyack 1986) while avoiding predators or reducing predation risk (Heithaus et al. 2002). Types of social bonds within delphinids range from fission-fusion social systems seen in mother-calf groups of bottlenose dolphins (Gibson and Mann 2008), stable multi-year associations between male bottlenose dolphins within breeding alliances (Connor et al. 1992, Connor and Krützen 2015) to the life-long associations present in orca pods (Bigg et al. 1990). Bottlenose dolphins are able to recognise one another acoustically across several decades (Bruck 2013) and it is likely that individually distinct stimuli, such as signature whistles (Janik and Sayigh et al. 2013), can cause OT release in social contexts as seen in primates (Crockford et al. 2013, Wittig et al. 2014). OT release may then stimulate pro-social behaviours essential for staying together in vast marine environments, such as triggering reunions (Smolker et al. 1993) and could also stimulate behaviours that strengthen bonds such as group synchrony (Connor et al. 2006a), contact swimming (Connor et al. 2006b) or petting (Connor et al. 2006a). Social interactions and bonding represent a crucial aspect of the lives of all delphinid species, and OT release could enable individuals to link individual specific stimuli to a physiological reward via dopamine release in the brain (Strathearn et al. 2009), reinforcing and and maintaining the social bonds that are vital for these species to survive and thrive in the marine environment.

4.3. Elevated OT concentrations in infant bottlenose dolphins

Dolphin calves had approximately double the basal plasma OT levels compared to all other age classes, and this elevation was present in individuals 2 – 4 years old. In the one calf that was sampled in two consecutive years, basal OT levels were elevated at both measurement points. There are few studies to compare these values to, the one study reporting concentrations in mice pups are from unextracted plasma (Higashida et al. 2010) while the one study of human babies measured levels from only the first few days of life (Leake et al. 1981). Concentrations measured in dependant grey seal pups (8 – 52.2 pg.ml, Robinson et al. 2019) are comparable but range into much higher values than those found in dolphin calves, although individual variability is present in both data sets. An alternative measurement of peripheral oxytocin using saliva samples from human children 1-7 years of age also showed that the youngest infants (between approximately 1-2 years old) had much higher levels than older children (up to approximately 7 years old) (Nishizato et al. 2017). The elevation of calf OT levels could indicate the presence of functional mother-calf bonds and the individual variation in OT may indicate the 'strength' of that bond (Crockford et al. 2013).

Elevation of basal OT is not present in any other age class in this species and likely is associated with motivation for calves to interact with mothers or other bonded individuals. It has previously been demonstrated in both natural and experimental settings that elevated peripheral OT levels are associated with increases in proximity seeking behaviour, even at a young age (Madden and Clutton-Brock 2010, Robinson et al. 2015a, 2017). Calf survival is reliant on staying with mothers, or successfully reuniting with them when separations do occur, and functional mother-calf bonds that are regulated by elevated OT during the dependant period would provide physiological motivation for this to take place. The foraging ecology of bottlenose dolphins means that separations of calves from their mothers happens frequently throughout the prolonged dependant period seen in this species (Mann and Smuts 1998, Gibson and Mann 2008). It is important for these highly mobile infants to behave in an adaptive manner to assist reunions (Mann and Watson-Capps 2005). Mothers do play an important role in reunions (Kuczaj et al. 2015), but there is also evidence that calves can actively promote reunions via increased rates of whistling compared to mothers (Smolker et al. 1993). After the first two months of life, calves additionally become the primary instigators of reunions with their mothers and are responsible for modulating the distance separating the two (Owen 2001). Elevation of OT acting as part of a positive OT feedback loop may therefore enable successful co-ordination of calf behaviour with mothers while occupying environments that carry the risk of rapid separation from care-givers. It has been hypothesised that adults in cognitively complex, social species rely less on hormonal cues to perpetuate parental behaviour, as other neurological processes can instigate and perpetuate care giving behaviour (Broad et al 2006). However, infants of any species are still developing cognitively, so they may have to rely on hormonal cues, such as OT release, to stimulate appropriate behaviour towards caregivers.

There are potential alternative explanations for the high plasma oxytocin levels found in dolphin calves, however the supporting data and analysis presented here make them unlikely to be significant drivers of peripheral oxytocin dynamics. It is hypothetically possible that high peripheral oxytocin levels in young calves may be present simply because of allometry when compared to larger adults, however our data on oxytocin - weight relationships demonstrate this is not the case. There was no relationship between weight and oxytocin levels in calves or juveniles/adults. Additionally, when one juvenile outlier data point (see below) was removed from the juvenile/adult cohort, there was actually a significant positive relationship between weight and plasma oxytocin levels, indicating that larger adults have higher concentrations than smaller adults, the opposite of the expected relationship if allometry was driving peripheral oxytocin concentrations.

As previously discussed, restraint stress has been shown to cause changes to peripheral oxytocin levels in laboratory settings (Grippo et al. 2009) and, as the calves in the study had minimal prior experience of the capture and sampling process, they may have been stressed enough to cause the elevated plasma oxytocin levels observed in this age class. This is unlikely to be the case as there was substantial range in individual calf oxytocin levels which was not associated with any variation in capture or handling times, with the lowest (4.2pg/ml) being comparable to levels in other age classes despite this individual's inexperience with capture and sampling events. Five other individuals (three juvenile and two adults) also had no prior experience of the capture and sampling process, and all except one showed low peripheral oxytocin levels comparable to concentrations found in more experienced individuals. The one juvenile that did show elevated plasma oxytocin levels is an interesting outlier in the study (Fig. 4b, denoted by a triangle). This four-year-old male had only separated from his mother 2-3 months prior to capture and his high plasma oxytocin levels may have occurred as he was still transitioning physiologically to a solitary juvenile. It is likely that this individual had not been nutritionally dependent on his mother for years prior to the actual separation of the pair, however it is unknown whether changes in peripheral oxytocin are associated with nutritional weaning or with termination of mother-infant contact. While plasma oxytocin levels do fall immediately after infants separate from mothers (Robinson et al. 2017) they remain elevated above juvenile and adult levels for weeks after the separation occurs (Robinson et al. 2015b), and it is unknown how long it takes for infant levels to fall to juvenile or adult baseline concentrations. In addition to his newly independent status, this juvenile was also the only individual in the study that had a prior injury (stingray barbs embedded within the head) which were treated by veterinarians before release, and this could have been causing chronic pain and stress which may have impacted his health and physiology in comparison to the other individuals in the study.

OT, or OT-like peptides, are present in all vertebrate animals, and have been shown to influence parental behaviours in mammals (e.g., Robinson et al. 2015a), birds (e.g., Chokchaloemwong et al. 2013) and fish (e.g., O'Connell et al. 2012). Stimulation of appropriate infant behaviour to facilitate rearing success may be as important as parental behaviours, and all of the few studies that have documented peripheral infant OT thus far have found elevations of this hormone in dependant offspring. OT has also been linked to infant mass gain prior to weaning (Robinson et al. 2019), and may cause higher rates of infant survival via physical, developmental advantages. OT release in infants is likely to be dependent on the quality of bond with the care-givers, which in turn is dependant on the interactions after offspring are born, often during a finite period of time during which the bond between mother and infant(s) is made (Kendrick 2000). However, this period is vulnerable to disruption, and if physical contact or communication is prevented or interrupted during this time, then mother-infant bonding and associated OT release cannot take place. Many wildlife species, including bottlenose dolphins, live in regions with increasing anthropogenic disruption. Acoustic pollution and increasing human presence in marine environments are threats to cetacean populations (Würsig and Evans 2002) and the disruption they cause could potentially lead to failure of mother-infant bonds, resulting in low OT levels, less motivation for calves to associate with mothers and higher infant mortality in disturbed populations. Understanding the sensory modalities that mother-calf pairs rely on and how these interact with OT release would enable effective protective measures to be put in place during this vulnerable time in their life history.

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Author Contributions

KJR conceived and designed the study, RSW and VMJ organised and supervised the fieldwork and sample collection from individuals, RSW provided background life history and social data for the wild individuals, KT provided samples from individuals under human care, NH and VMJ provided essential laboratory equipment and funds, KJR performed all sample and data analysis, VMJ and KJR decided on the direction of the manuscript, KJR wrote the manuscript, all authors critically revised the manuscript and gave final approval of the version to be published.

Competing Interests

The authors declare they have no conflicts of interest.

Data Availability

Data access is available upon reasonable request.

Figure Captions

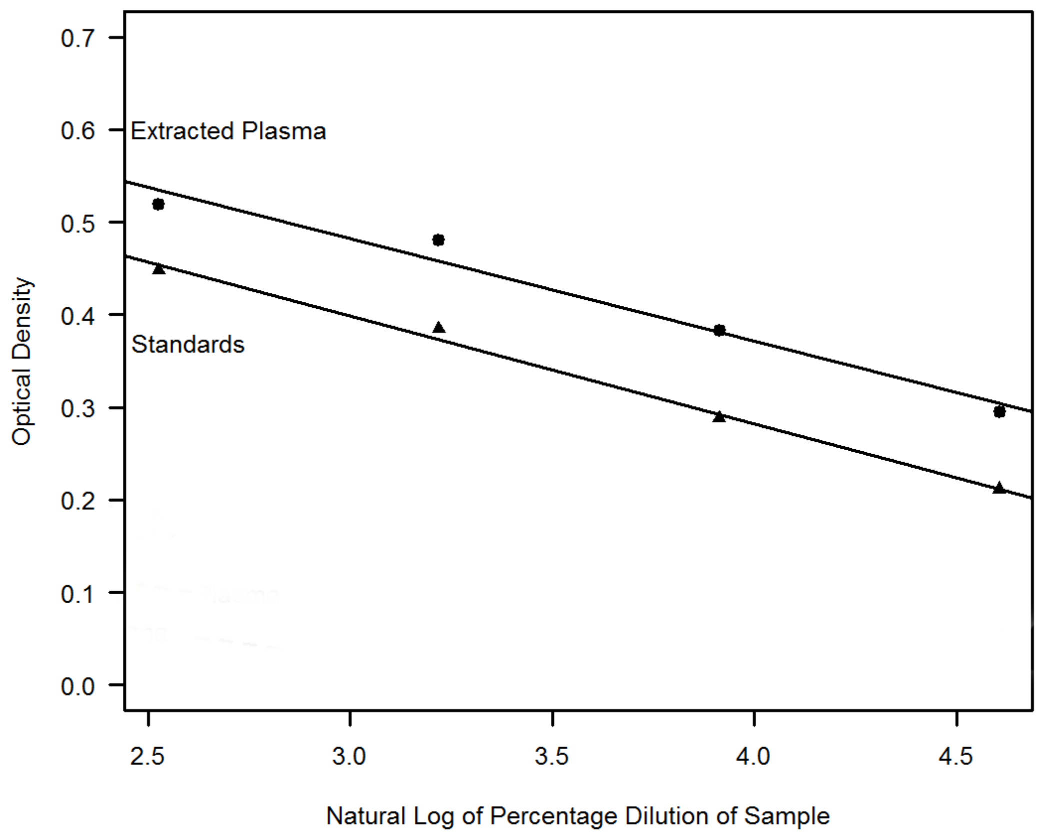

Fig. 1. Dilution lines testing for parallelism between the ELISA kit standards (solid triangles) and extracted bottlenose dolphin plasma (solid circles). Parallelism between the standards and extracted samples was confirmed by statistical comparison (see results, $p = 0.75$).

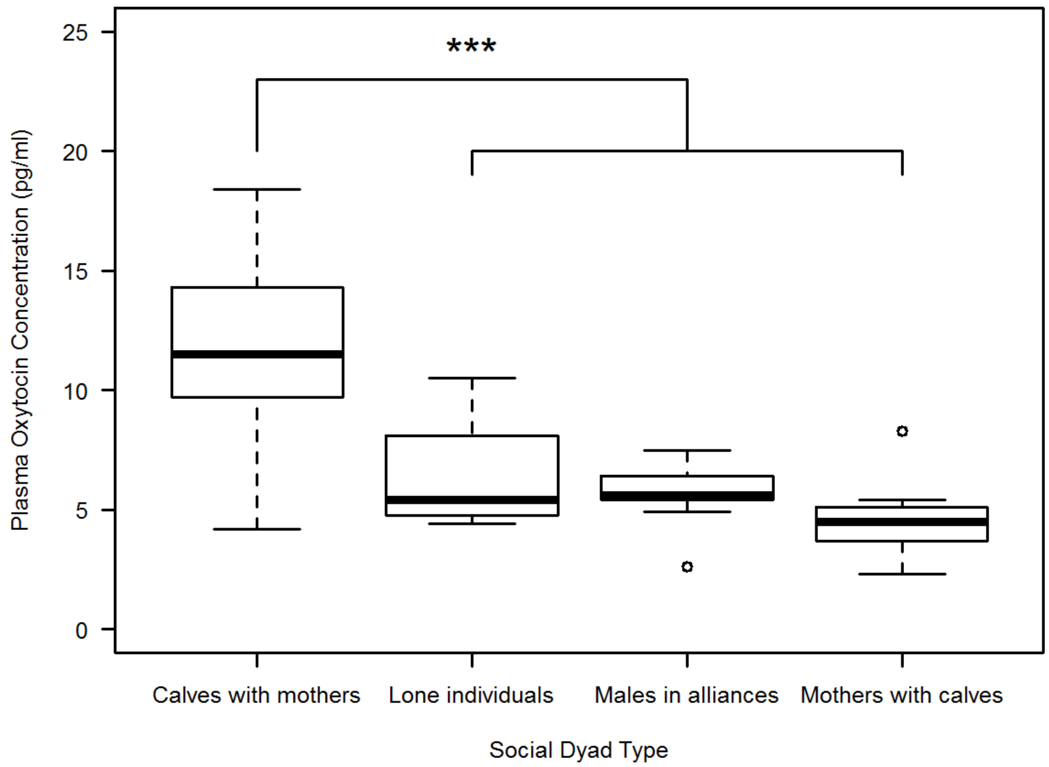

Fig. 2. OT concentrations in individuals in different types of social dyad (n = 14 calves with mothers, 4 lone individuals, 10 males in alliances and 14 mothers with calves) with median, upper and lower quartiles, 1.5x interquartile range and outliers shown. Significant differences between groups are denoted by asterisks, *** for $p<0.001$.

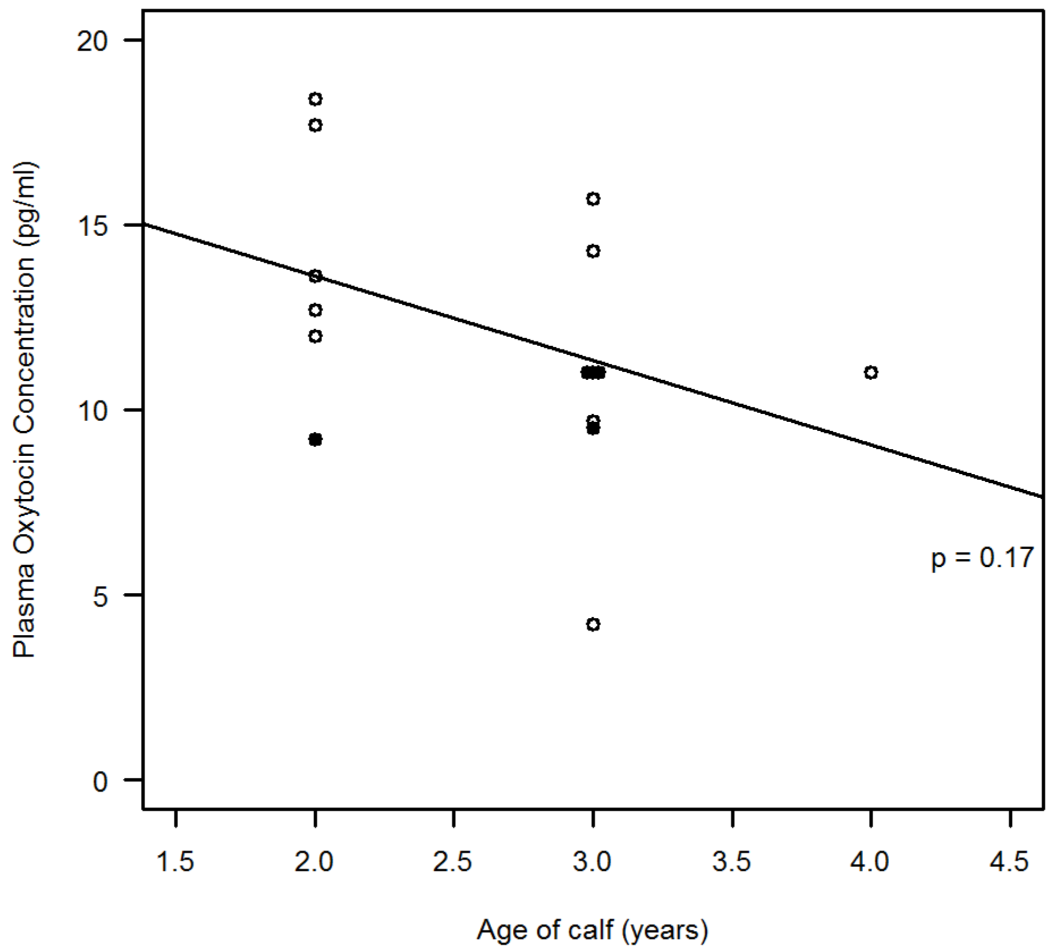

Fig. 3. The non-significant negative relationship between calf plasma OT concentrations (pg/ml) and calf age (years) with the Pearson’s correlation significance value. Males (n = 4) are shown with filled points, females (n = 10) are shown with open points.

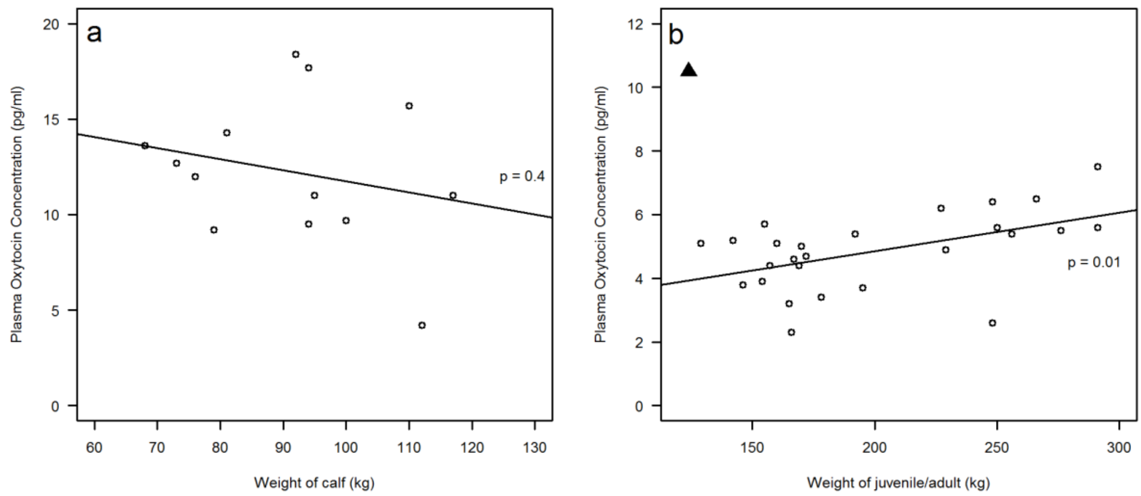

Fig. 4a. The non-significant negative relationship between calf plasma OT concentrations (pg/ml) and calf weight (kg) with the Pearson's correlation significance value. 4b. The significant positive relationship between juvenile/adult plasma OT concentrations (pg/ml) and juvenile/adult weight (kg) with the Pearson's correlation significance value. An outlier that was removed from the analysis is plotted with a triangle symbol. Including this outlier leads to a non-significant relationship.

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