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Research article

Circulating concentrations of thyroid hormones and cortisol in wild and semi-natural Yangtze finless porpoise (*Neophocaena asiaeorientalis*)

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Our understanding about how environmental and biological variables may influence circulating thyroid and adrenal hormones in free-ranging cetaceans is limited. As such, we used liquid chromatography–mass spectrometry to determine concentrations of circulating cortisol and thyroid hormones (THs; tT3, tT4) in 132 Yangtze finless porpoises (YFPs) located in Poyang Lake, (PL, $n = 92$) and Tian-E-Zhou Oxbow reserve (TZO, $n = 40$). For overall hormone comparisons, animals were partitioned by age [juvenile and adult (male and non-pregnant, non-lactating female)], sex, season (winter or spring) and geographical location. Geographically, during winter, circulating THs were significantly higher in the PL versus TZO population. Seasonally, within PL, THs were significantly higher in the winter versus spring season. Animals were further binned into groups as follows: juvenile male (JM) and juvenile female (JF), adult male (AM), non-pregnant adult female, pregnant female and non-pregnant lactating female. Intra-group comparisons between locations showed a significant increase in JM THs at PL. Significant increases in THs during winter compared to spring were detected between JM and JF groups. Mean comparisons of cortisol within and between locations for each group identified a significant increase for TZO AM versus TZO pregnant female and JM and JF. Seasonally, in PL, only JF has significantly higher cortisol in winter versus spring. Finally, we established reference values of THs and cortisol for YFPs in different geographical locations. These references are important baselines from which the effects of environmental and biological variables on THs and cortisol may be evaluated.

Key words: Cortisol, endangered species endocrinology, hormone reference values, thyroid hormone, Yangtze finless porpoise

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Introduction

Phylogenetically, the highly vascularized thyroid gland is a unique and highly conserved gland found throughout vertebrates (Norris, 2007). Thyroid hormones (THs), including 3, 5, 3′-tri-iodothyronine (T3) and thyroxine (T4) directly regulate metabolic activity in the liver, brain, skeletal muscle, pancreas and fat (Suzuki *et al.*, 2018). Furthermore, THs influence many aspects of cellular differentiation, growth rates, reproductive function, the cardiovascular, immune and nervous system and the ageing process (Eales, 1988; Choksi *et al.*, 2003; Ishikawa and Kitano, 2012). In cetaceans, the role of THs in thermoregulation is of particular importance due to the high thermal conductivity of their aquatic environment (Suzuki *et al.*, 2018). Studies have shown inconsistent results within and between cetacean species when investigating TH concentrations. These inconsistencies are possibly due to factors such as relatively small sample sizes, including females with unknown pregnancy status, differences in the environmental conditions, lack of longitudinal samples within the studies and variation between the clinical laboratories (St. Aubin and Geraci, 1989; Costa *et al.*, 1993; St. Aubin *et al.*, 1996; Rosa *et al.*, 2007; Fair *et al.*, 2011; West *et al.*, 2014).

Characterizing the normal fluctuations of circulating TH concentrations in unique and poorly studied species is an essential first step towards being able to detect abnormalities indicative of poor health (Fair *et al.*, 2011). Previous studies have been suggested deviations in the THs normal serum concentrations as a useful biomarker for endocrine disrupting chemical exposure (Tabuchi *et al.*, 2006), iodine deficiency (Delange, 1994), nutritional stress (Wasser *et al.*, 2017) and genetic disorders (De Felice and Di Lauro, 2004). In addition to environmental factors (season, temperature, pollutants, etc.), TH concentrations in other species (bottlenose dolphin, harbour seal, domestic animals, humans) are influenced by several biological variables including sex, age and reproductive status (Fisher, 1996; Feldman and Nelson, 2004; Tabuchi *et al.*, 2006; Kapelari *et al.*, 2008; Colodel *et al.*, 2010; Fair *et al.*, 2011; West *et al.*, 2014). However, few studies in cetaceans have investigated the influence of these biological variables and environmental factors on circulating THs concentration (St. Aubin *et al.*, 1996; Rosa *et al.*, 2007; Fair *et al.*, 2011; West *et al.*, 2014; Robeck *et al.*, 2019).

The Yangtze finless porpoise (YFP; *Neophocaena asiaeorientalis ssp. asiaeorientalis*) is a critically endangered freshwater cetacean that is native to the Yangtze River, Dongting and Poyang Lakes of China (Wang *et al.*, 2013; Mei *et al.*, 2014). Due to the disturbances of various anthropogenic activities, the wild population of the YFP has suffered significant population decline in the past few decades (Zhao *et al.*, 2008; Mei *et al.*, 2012; Huang *et al.*, 2020). According to the latest survey conducted in 2017, the population is currently estimated at approximately 1012 individuals, including 445 in the main stem of the Yangtze, 110 in the Dongting Lake and 457 in the Poyang lake (PL), (Huang *et al.*, 2020). PL is the most important refuge for protection of this endangered species due to its status as the largest freshwater lake in China, which holds almost half of the natural population of YFPs. However, YFPs in PL are exposed to several escalating anthropogenic stressors including dredging, heavy shipping, acoustic pollution, oil spills, chemical pollution, overfishing, illegal fishing and the use of harmful fishing tools can cause morbidity and mortality of YFP (Wang, 2009; Nabi *et al.*, 2017a; Mei *et al.*, 2021). In addition to the PL, some animals were moved from the main stem of Yangtze River and introduced to the Tian-E-Zhou Oxbow (TZO, a 21-km-long old course of the Yangtze River) in 1990, as the first large-scale *ex situ* conservation practice for this or any other cetacean species (Wang, 2015). Since the translocation, the population within TZO has increased steadily and, based on a survey conducted in 2015, is currently estimated at approximately 80 animals (unpublished data). However, while the population within the TZO had reached an apparent carrying capacity, it appears that all of the remaining natural populations are deteriorating due to continuous exposure to several escalating anthropogenic stressors in their habitats (Nabi *et al.*, 2020). Unlike PL, there is no dredging, shipping and fishing in the TZO over a certain period every year; however, water is apparently more polluted in TZO than in PL due to nearby farmland drainage (Nabi *et al.*, 2017a; Nabi *et al.*, 2020). Due to their critically endangered status, close monitoring of the individuals within the main populations via regular health assessments have been conducted (Nabi *et al.*, 2017a,b; Zeng *et al.*, 2017; Nabi *et al.*, 2018a,b; Zeng *et al.*, 2019) and reference ranges for several haematologic and biochemical parameters across age and reproductive states have been developed (Nabi *et al.*, 2019). However, our knowledge about TH endocrinology, specifically the influence of biological and environmental variables on the circulating THs concentration in the YFP, is limited due to their aquatic nature, logistic and ethical reasons and potential stress-induced physiological injuries (e.g. cardiac injury) that can occur during chasing and obtaining samples from this critically endangered species (Xiao *et al.*, 2018; Câmara *et al.*, 2020).

Like THs, glucocorticoids are essential mediators of metabolism and sustaining body homeostasis in response to several intrinsic and extrinsic stressors (St. Aubin *et al.*, 1996; Hart *et al.*, 2015). Escalating anthropogenic stressors (dredging, vessel traffic, acoustic pollution and chemical pollutants) are increasing challenges to cetacean populations including YFP, which may not be capable of adapting to such extreme pressures in a short time. A few studies in cetaceans have examined adrenal responses to captivity, cold temperature, capture and restraint (Thomson and Geraci, 1986; Ortiz and Worthy, 2000; Houser *et al.*, 2011), and variations due to age, sex and season (St. Aubin *et al.*, 1996; Suzuki et al., 2003). Cortisol has been considered an essential biomarker for measuring stress levels and overall population health (St. Aubin and Dierauf 2001). Abnormally high or low levels of cortisol are associated with compromised health (Kellar *et al.*, 2015). It is therefore essential to obtain reference levels of cortisol and understand the effects of biological and environmental effects on cortisol in endangered populations like YFPs for better conservation and management.

From our previous investigations, we detected physiological differences between the TZO and PL populations, which may indicate that the two populations have experienced different environmental conditions, anthropogenic pressures or both (Nabi *et al.*, 2017a; Nabi *et al.*, 2018b). However, the interplay between external stressors (anthropogenic and natural environmental) and their potential effect on metabolic processes as reflected by TH and glucocorticoid concentrations have never been investigated. Moreover, this work will help establish baseline concentrations of these hormones, which can then be used to help identify physiologic abnormalities that might manifest in the future. Therefore, the goal of this study was to describe THs and cortisol in the wild and semi-natural population of YFPs. Specific objectives were as follows: (i) to determine the influence of age, sex, reproductive status and season on the THs and cortisol concentration; (ii) to investigate the effect of escalating anthropogenic stressors on the circulating THs and cortisol in PL and TZO; and (iii) to develop initial reference concentrations of THs and cortisol for wild and semi-natural YFP populations relative to sex, age and reproductive states.

Materials and methods

Ethical approval

Ethical approval (NSFC-31430080) for the study was obtained from the Ministry of Agriculture of the People's Republic of China. The research ethics committee of the Institute of Hydrobiology, Chinese Academy of Science reviewed and approved the animal handling and blood sampling procedure. The whole study strictly adhered to Chinese law and ethical guidelines for biodiversity.

Animal chasing and blood sampling

Animals in the PL were sampled both in the spring season (2010 and 2015) and winter season (2011), whereas animals in the TZO were sampled only in the winter season (2015). Both in the TZO and PL, the 'sound chase and net capture' method was used to chase and capture the YFPs (Hua, 1987). Detailed information of animal chasing, catching, transportation, handling, blood sampling and release are explained by Hao *et al.* (2009) and Nabi *et al.* (2018a). In brief, the randomly selected animals for each capture ($n = 5$–10/day) were slowly chased (<10 km/h) by several parallel fishing boats using an engine noise (4.5 hp). The animals were confined to one section using soft nets to avoid injuries. Captured animals were then transported to the medical boat, kept on a sponge mattress and gently restrained for physical measurement and blood sampling. Within 10 min after the animal was restrained, ~10 ml of blood was collected aseptically from the dorsal major vein of the tail fluke using a disposable 10-ml syringe (Gemtier, G/Ø/L: 21/0.7/31 mm, 201 502, Shanghai, China). Blood was centrifuged (Eppendorf AG, 22332, Hamburg, Germany) for 15 min at 1500 g. Serum was transferred immediately into cryotubes (Fisher Scientific, Pittsburgh, PA, USA) and stored in a liquid nitrogen kettle for later analysis. During the capture events, the blood sampling procedure and the timing of blood collection were consistent for both populations. All the animals were immediately released gently into their environment after blood sampling.

Animal partitioning

Animals were divided into age groups based on total body length (Gao and Zhou, 1993; Hao *et al.*, 2007) and sex as follows: juvenile males (JMs, <138 cm), adult males (AMs, ≥138 cm), juvenile females (JFs, <130 cm) and adult females (AFs, ≥131 cm,). AFs were further classified based on physiologic status as non-pregnant non-lactating AF, pregnant females (PF) and lactating female (LF). Pregnant plus lactating females (P&L) as determined by ultrasonography (LOGIQ Book XP, NY, USA) of the reproductive tract and presence of milk in the mammary glands were combined with the pregnant group. This combination was made due to the small number of animals within this group and evidence with other cetaceans (Robeck *et al.*, 1993; West *et al.*, 2007) that pregnancy can only occur when the frequency of lactation drops below a threshold whereby it becomes physiologically inconsequential and estrus activity resumes. A total of 132 YFPs were sampled from the TZO ($n = 40$) and PL ($n = 92$) during dolphin capture–release health assessment surveys. They were further classified as follows: TZO (JM, $n = 9$; JF, $n = 4$; AM, $n = 14$; AF, $n = 4$; PF, $n = 4$; LF, $n = 3$; P&L, $n = 2$) and PL in the spring (JM, $n = 9$; JF, $n = 13$; AM, $n = 9$; AF, $n = 6$; PF, $n = 5$; LF, $n = 3$; P&L, $n = 2$) and winter season (JM, $n = 12$; JF, $n = 8$; AM, $n = 8$; AF, $n = 6$; PF, $n = 11$).

Hormone analysis by high-performance liquid chromatography–tandem mass spectrometry

Chemicals

The reference substances of total [t] tT3, tT4 and cortisol were purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). high performance liquid chromatography (HPLC)-graded methanol and acetonitrile were purchased from Merk KGaA, Germany, and the C18 and primary secondary amine (PSA) packings were purchased from ANPEL Laboratory Technologies Inc. (Shanghai, China).

Sample preparation

To a 200-μl serum sample, 1-ml HPLC-graded methanol was added and vortexed (Digital Vortex Mixer, Thermo Fisher Scientific, USA) for 30 s. Then, the supernatant was separated

Table 1: Results of three-way ANOVA for hormone concentrations comparing sex, age group (juvenile and adults) and season

Analysis	Hormone	Effect	DF	*F*-value	Pr > F
Location	Total T3	Main effect	**4**	**2.29**	**0.0702**
(Winter only)		Location (PL vs. TZO)	**1**	**5.35**	**0.0242**
		Sex	1	0.02	0.1997
		Age group	2	0.07	0.9305
	Total T4	Main effect	**5**	**4.31**	**0.0039**
		Location	**1**	**8.89**	**0.0041**
		Sex	**1**	**5.32**	**0.0245**
		Age group	2	0.50	0.6072
Season	Total T3	Main effect	**5**	**4.52**	**0.0028**
(PL only)		Age group	2	0.31	0.7350
		Sex	1	0.37	0.5425
		Season	**1**	**15.49**	**0.0002**
	Total T4	Main effect	**4**	**4.45**	**0.0031**
		Age group	2	0.08	0.9263
		Sex	1	0.96	0.3317
		Season	**1**	**16.83**	**0.0001**

Adult PFs or LFs were excluded from the analysis.

after centrifuging (TG-16G; Hunan Kaida Scientific Instrument Co., Ltd, China) at 12000 g for 5 min at 4°C and added 10 mg C18 packing and 10 mg PSA packing to the residue and vortexed for 30 s. The mixed solution was centrifuged again at 12000 g for 5 min at 4°C. The supernatant was evaporated to dryness under a gentle steam of nitrogen. The residue was re-dissolved in 200 μl methanol and was filtered by a 0.22-μm filter membrane. Finally, the sample (2 μl) was injected into the high-performance liquid chromatography–mass spectrometry system.

Instruments and conditions

The analysis was performed by a liquid chromatography-triple quadrupole mass spectrometer (Agilent, USA). Separation step was performed on an Agilent1260 HPLC system using an Agilent Poroshell 120 EC-C18 column (2.1 × 150 mm, 2.7 μm). The mobile phase consisted of methanol with 0.1% formic acid, at a volume ratio of 20:80, with isocratic elution at a flow rate of 0.3 ml/min. The temperature of the column was maintained at 35°C, and the injection volume was 2 μl. The mass spectrometer was operated in the positive ionization mode, and quantification was performed using the multiple-reaction-monitoring mode. The monitored ion transitions were 651.7 → 605.7, 777.6 → 731.6 and 363.1 → 327.1 (m/z); fragmentor voltages were130, 150 and 120 V; and the collision energies were 24, 22 and 14 eV, for tT3, tT4 and cortisol, respectively.

Data analysis

Data were analysed using Stata® version 14 (StataCorp LP, College Station, TX, USA). Hormone data were first evaluated for normality and homogeneity of variance and transformed (log or square root) if necessary. Initially, a separate three-way (factorial) analysis of variance (ANOVA) for each mean hormone concentration (tT3, tT4 and cortisol) was performed to determine if location (PL versus TZO during the winter), age group (only non-pregnant, non-lactating AFs were used to represent AF animals), sex or season (PL during the winter or spring) and their interactions effected the concentration variance. After the initial analysis, interactions that were considered non-significant ($P > 0.15$) were removed from the final model and the analysis was repeated. Additional analyses for each hormone concentration between each group (JM, JF, AM, AF, PF, LF) within each location during winter and then PL during the spring was performed using a one-way ANOVA with Šidák corrections. Significance was set at $P \leq 0.05$.

TH and cortisol mean concentration differences between season and location within each animal group were evaluated using a two-sided *t*-test using Welch's adjustments for unequal variances (Welch, 1947). Finally, Pearson pairwise correlation coefficients were used to determine if changes in THs and cortisol concentrations across all seasons and locations were correlated. Significant correlation coefficients ($P \leq 0.05$) were classified as follows: weak, $|r| \geq 0.1$ to <0.30; moderate,

|r| ≥ 0.3 to <0.50; strong, |r| ≥ 0.5 to <0.75; very strong, |r| ≥ 0.75 (Acock, 2016).

Once the need to partition the data by sex, age group, pregnancy or location was established through three-way ANOVA test as described above, reference intervals were calculated using a nonparametric bootstrap-based procedure (Linnet, 2000; International AIDS Vaccine Initiative, 2008). Using bootstrap resampling techniques (1000 reps), the mean 2.5, 50 and 97.5 percentiles from the 1000 replicates of each group were determined along with the 95% bootstrapped confidence intervals (CIs) for the mean of the 50th percentile, respectively.

Results

Thyroid hormones

Significant differences were detected in both tT3 and tT4 by location and season, while only tT4 during the winter was affected by sex of the animal. No differences were detected between different locations or seasons between juveniles and adults. While interactive terms were initially included, no interactions were significant, and they were removed from the final model (Table 1).

For location during the winter, overall mean and standard error of the mean (± sem) comparisons indicated a significant ($P < 0.03$) increase in concentrations in both tT3 and tT4 at PL (tT3, 0.25 ± 0.01 ng/ml; tT4, 18.5 ± 1.6 ng/ml) as compared to concentrations at TZO (tT3, 0.19 ± 0.02 ng/ml; tT4, 11.5 ± 1.6 ng/ml; Figure 1). For seasonal changes at PL, winter concentrations in both tT3 and tT4 were significantly ($P < 0.0001$) increased compared to the spring (tT3, 0.16 ± 0.02 ng/ml; tT4, 9.1 ± 1.6 ng/ml; Table 2; Figure 1).

Mean comparisons within each location and season for each group (JM, JF, AM, AF, PF, LF) did not find any significant differences (Table 2, Figure 1). However, intra-group comparisons between location found means differences in JM tT3 and tT4 between PL (tT3, 0.26 ± 0.03 ng/ml; tT4, 16.0 ± 2.8 ng/ml) and TZO (tT3, 0.16 ± 0.05 ng/ml; tT4, 7.2 ± 3.5 ng/ml; Table 2; Figure 1). Significant intra-group differences at PL between winter and spring were also detected within JM tT4 (winter, 16.0 ± 2.8 ng/ml; spring, 10.0 ± 4.0 ng/ml), JF tT3 (winter, 0.29 ± 0.02 ng/ml; spring, 0.14 ± 0.03 ng/ml), JF tT4 (winter, 21.6 ± 3.8 ng/ml; spring, 8.4 ± 2.8 ng/ml), PF tT3 (winter, 0.24 ± 0.02 ng/ml; spring, 0.13 ± 0.04 ng/ml) and, finally, PF tT4 (winter, 19.9 ± 3.2 ng/ml; spring, 8.8 ± 4.7 ng/ml; Table 2; Figure 1).

Cortisol

Significant differences in cortisol were detected in both location, season and between juveniles or adults (Table 2). For location samples (only in winter), a significant interaction was detected between location and sex, but sex alone was not significant. Sex was also not significant within PL animals and between seasons (Table 3).

Since samples were only collected during the winter at TZO, location comparison against PL were only done within this season. The analysis indicated that mean (± sem) concentration of cortisol were significantly increased ($P = 0.002$) at PL (16.5 ± 0.63 ng/ml) versus TZO (13.98 ± 0.76 ng/ml). Mean comparisons within and between locations for each group (JM, JF, AM, AF, PF, LF) indicated a significant increase for TZO AM (18.5 ± 0.09 ng/ml) compared to

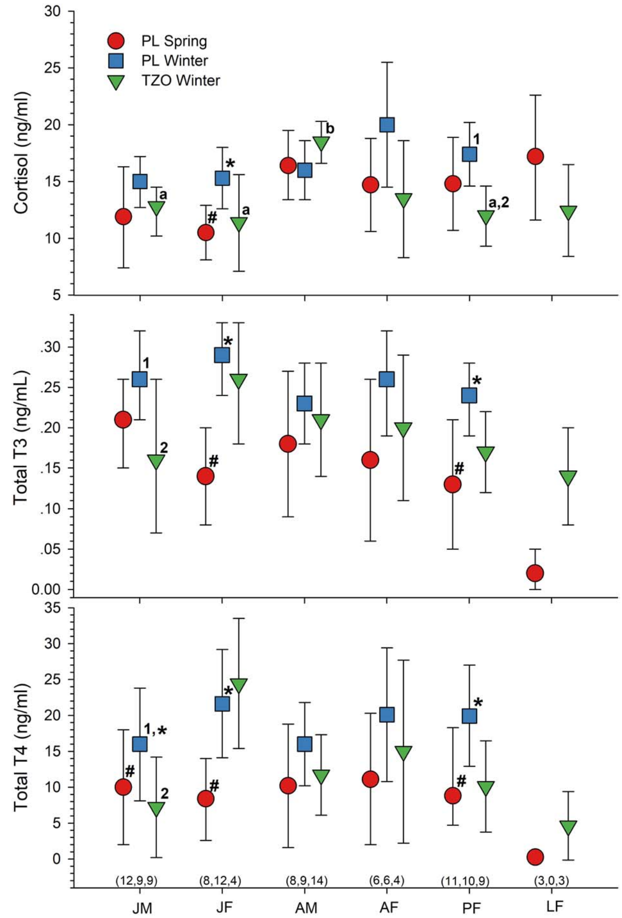

Figure 1: Serum cortisol and TH hormone concentrations (ng/ml) in male and female YFP located in PL of the Yangtze river system from 2010 to 2015 during winter and spring and the semi-reserve TZO in 2015 during winter. Mean comparisons (one-way ANOVA with Šidák correction) were made separately within each location during winter (PL and TZO) and spring (PL) for JMs and AMs, JFs and AFs (non-pregnant, non-lactating), PFs (lactating and non-lactating) and LFs (non-pregnant). Inter-location (winter: PL vs TZO) differences (1,2) and inter-season differences (PL only: *,#) within each group were determined using two-sided *t*-test with unequal variances using Welch's approximation. Numbers of samples for each group are on x-axis of the bottom graph and are same for each hormone/group combination.

Table 2: Serum TH concentrations (free T4 ng/ml; free T3 ng/ml) in male and female YFPs from animals located in PL of the Yangtze river system from 2010 to 2015 and located in the semi-reserve TZO during the winter of 2015

			Juvenile		Adult		Females	
Winter		Statistic	Male	Female	Males	Females	Pregnant	Lactating
		n	12	8	8	6	11	—
	Free T3	Mean	**0.26**[1]	**0.29**[*]	0.23	0.26	**0.24**[*]	
PL[1,*]		SEM	0.03	0.02	0.03	0.03	0.02	
		95% CI	0.21 to 0.32	0.24 to 0.33	0.18 to 0.28	0.19 to 0.32	0.19 to 0.28	
	Free T4	Mean	**16.0**[1,*]	**21.6**[*]	16.0	20.1	**19.9**[*]	—
		SEM	2.8	3.75	2.9	4.6	3.2	
		95% CI	8.8 to 23.8	14.1 to 29.2	10.2 to 21.8	10.8 to 29.4	13.5 to 26.3	
		n	9	4	14	4	6	3
	Free T3	Mean	**0.16**[2]	0.26	0.21	0.20	0.17	0.14
TZO[2]		SEM	0.05	0.04	0.03	0.05	0.024	0.29
		95% CI	0.07 to 0.26	0.18 to 0.33	0.14 to 0.28	0.11 to 0.29	0.12 to 0.22	0.08 to 0.20
	Free T4	Mean	**7.2**[2]	24.4	11.7	15.0	10.1	4.6
		SEM	3.5	4.5	2.8	6.3	2.5	2.4
		95% CI	0.2 to 14.2	15.4 to 33.5	6.1 to 17.3	2.2 to 27.7	5.1 to 15.1	−0.13 to 9.4
Spring								
		n	9	12	9	6	7	2
PL[#]	Free T3	Mean	0.21	**0.14**[#]	0.18	0.16	**0.13**[#]	0.03
		SEM	0.03	0.03	0.04	0.05	0.04	0.02
		95% CI	0.15 to 0.26	0.08 to 0.20	0.09 to 0.27	0.06 to 0.26	0.05 to 0.21	0.00 to 0.06
	Free T4	Mean	**10.0**[#]	**8.4**[#]	10.2	11.1	**8.8**[#]	0.3
		SEM	4.0	2.8	4.3	4.5	4.7	0.01
		95% CI	2.0 to 18.0	2.6 to 14.0	1.6 to 18.8	2.0 to 20.3	0.0 to 18.3	0.24 to 0.3

Mean comparisons (with Šidák corrections) within each location and season were between JMs and AMs, JFs, AFs (non-pregnant, non-lactating), PFs (lactating and non-lactating) and LFs (non-pregnant). Inter location (winter: PL vs. TZO) differences ([1,2]) and inter season differences ([*,#]) (PL only) within each group were determined using two-sided *t*-test with unequal variances using Welch's approximation.

Table 3: Results of three-way ANOVA for cortisol concentrations comparing animal groups [JMs and AMs, JFs and AFs (non-pregnant, non-lactating), PFs (lactating and non-lactating) and LFs (non-pregnant)], by location (PL and TZO) within winter and season within PL

Analysis	Effect	DF	*F*-value	Pr > F
Location samples	Main effect	**4**	**3.62**	**0.0104**
	age group	**2**	**6.43**	**0.0030**
	Location (PL vs TZO)	**1**	**9.80**	**0.0445**
	sex	1	0.75	0.3909
	Location by sex	**1**	**4.97**	**0.0296**
Season (PL only)	Main effect	**4**	**4.53**	**0.0027**
	age group	**2**	**5.26**	**0.0076**
	Season	**1**	**8.13**	**0.0058**
	Sex	4	0.00	0.9635

Table 4: Serum cortisol concentrations (ng/ml) in male and female YFPs located in PL of the Yangtze river system from 2010 to 2015 during winter and spring and the semi-reserve TZO in 2015 during winter

		Juvenile		Adult		Females	
	Statistic	Male	Female	Males	Females	Pregnant	Lactating
Winter	*n*	12	8	8	6	11	0
PL	Mean	15.0	15.3∗	16.0	20.0	17.4	—
	SEM	1.11	1.35	1.28	2.7	1.39	
	95% CI	12.7 to 17.2	12.6 to 18.0	13.4 to 18.6	14.5 to 25.5	14.6 to 20.2	
	n	9	4	14	4	6	3
TZO	Mean	12.8[a]	11.4[a]	18.5[b]	13.5[a,b]	12.0[a]	12.4[a,b]
	SEM	1.3	2.1	0.92	2.6	1.3	2.0
	95% CI	10.2 to 14.5	7.1 to 15.6	16.6 to 20.3	8.3 to 18.6	9.3 to 14.6	8.4 to 16.5
Spring	*n*	9	12	9	6	7	3
PL	Mean	11.9	10.5[#]	16.4	14.7	14.8	17.2
	SEM	2.20	1.19	1.53	2.04	2.04	2.7
	95% CI	7.4 to 16.3	8.1 to 12.9	13.4 to 19.5	10.6 to 18.8	10.7 to 18.9	11.6 to 22.6

Mean comparisons were made separately within each location during winter and spring for JMs and AMs, JFs and AFs (non-pregnant, non-lactating), PFs (lactating and non-lactating) and LFs (non-pregnant). For location (winter), overall cortisol for PL was significantly increased ($F = 3.13$, $P = 0.002$) compared to TZO, but no intra-group differences were detected. However, within TZO significant differences ($P \leq 0.05$) in mean concentrations between groups were detected and designated by differing superscripts (a,b,c). For PL, overall winter was significantly increased ($t_{df=89} = 2.76$, $P = 0.007$) compared to spring, with significant ($P \leq 0.05$) intra-group seasonal differences (within PL) indicated by differing superscripts (*,**#**). In addition, within each season no inter-group differences were detected.

TZO JM (12.8 ± 1.3 ng/ml), JF (11.4 ± 2.1 ng/ml) and PF (12.0 ± 1.3 ng/ml; Table 4; Figure 1). No inter-group differences were detected within the PL location during the winter. Differences within animal groups between locations were only significant for PF (PL, 17.4 ± 1.4; TZO, 12.0 ± 1.3). For the seasonal differences at PL, only JF was significantly different between winter (15.3 ± 1.4 ng/ml) and spring (10.5 ± 1.2 ng/ml; Table 4; Figure 1).

Hormone correlations and reference intervals

Across all locations and season, tT3 and tT4 had very strong significant correlation ($r = 0.78$, $P < 0.0001$). However, only a weak significant correlation was detected between cortisol and tT3 ($r = 0.20$, $P = 0.02$), but not between cortisol and tT4. When partitioned by location, no significant relationship between cortisol and either tT3 and tT4 was detected at TZO, but within PL and similar to the overall analysis, a positive weak correlation between cortisol and tT3 ($r = 0.26$, $P = 0.01$) was detected and an additional one between cortisol and tT4 ($r = 0.23$, $P = 0.03$). Further divisions within PL to look within winter and spring periods did not result in any significant correlations between cortisol and TH being detected. For reference intervals, mean concentration percentiles (2.5, 50 and 97.5 percentiles) within groups (between and within season and location) were partitioned based on significant mean differences. The values are then presented in Tables 5 and 6.

Discussion

Geographical location

In this study, we observed statistically significantly higher tT3 and tT4 in the PL versus TZO YFP population during the winter. These results were like our previous work, using samples collected from a small group of males only ($n = 42$) divided from both populations, whereby serum TH concentrations were significantly higher in PL YFPs males compared to TZO YFP males (Nabi *et al.*, 2018a). There could be several explanations for the significantly higher tT3 and tT4 in the PL YFP population. Firstly, unlike TZO, PL is a seasonal lake and during the winter there are significant decreases in size that result in a narrowed main channel that concentrates vessel traffic within a decreasing YFP habitat. This increased concentration of vessel traffic, an activity that has been demonstrated to alter normal YFP behaviour (Peng *et al.*, 2015; Nabi *et al.*, 2018c), could be expected to result in an increase in physiological stress within the animals. Activation of the stress response would then trigger an increase in, and, if chronic (>1 to 2 weeks in duration; Sapolsky *et al.*, 2000), an alteration of, metabolic requirements (Romano et al., 2004; Lyamin *et al.*, 2011; Mullur *et al.*, 2014). In support of this theory that habitat encroachment could be causing a stress response within the YFP PL population, we found a significant, positive correlation between cortisol and THs only in animals from PL. While only a weak correlation, this would be the first evidence demonstrating a direct link

Table 5: Serum TH (tT3 ng/ml and tT4 ng/ml) reference concentrations, bootstrap (1000 reps) mean 2.5th, 50th and 97.5th percentile for the YFPs (*Neophocaena asiaeorientalis ssp. asiaeorientalis*) located in PL of the Yangtze river system from 2010 to 2015 and the semi-reserve TZO in 2015 during the winter and spring

Group	TH hormone	Group	No. of YFP	Mean 2.5th	Mean 50th (95% CI)*	Mean 97.5th
PL (winter)	Free T_3	All	45	0.11	0.25 (0.22 to 0.28)	0.41
		JM	12	0.14	0.28 (0.18 to 0.37)	0.42
		JF, AM, AF	22	0.14	0.25 (0.20 to 0.29)	0.38
		PF	11	0.03	0.25 (0.22 to 0.28)	0.33
TZO (winter)	Free T_3	All#	37	0.02	0.19 (0.15 to 0.23)	0.44
		JM	9	0.03	0.14 (0.01 to 0.27)	0.44
		JF, AM, AF	22	0.02	0.20 (0.10 to 0.29)	0.38
		PF	9	0.63	0.19 (0.13 to 0.25)	0.21
		LF	3	0.09	0.15 (0.08 to 0.22)	0.19
PL (winter)	Free T_4	All	45	1.91	20.4 (15.6 to 25.2)	43.5
		JM & AM	20	3.1	14.1 (5.6 to 22.7)	44.8
		JF, AF	14	1.8	22.0 (16.1 to 28.0)	34.0
		PF	11	2.7	22.5 (15.0 to 30.1)	36.2
TZO (winter)	Free T_4	All#	37	0.03	11.5 (2.4 to 20.6)	31.9
		JM & AM	23	0.03	5.1 (−7.5 to 17.6)	25.6
		JF, AF	8	3.3	22.2 (10.5 to 34.0)	31.9
		PF	6	3.1	9.6 (3.0 to 16.1)	19.0
		LF	3	1.2	3.6 (−2.1 to 9.4)	9.2
PL (spring)	Free T_3	ALL#	43	0.02	0.14 (0.08 to 0.20)	0.40
		LF	3	0.01	0.03 (0.00 to 0.06)	0.05
PL (spring)	Free T_4	ALL#	43	0.03	3.1 (−1.4 to 7.6)	34.0
		LF	3	0.25	0.26 (0.25 to 0.26)	0.26

*The 95% bootstrap (1000 reps) CIs for 50th percentile.#Does not include LF

between glucocorticoids and TH in cetaceans. Chronic and acute increases of cortisol can have a direct effect on TH production primarily by affecting the release of TSH (thyroid-stimulating hormone; for review see Mullur *et al.*, 2014). In addition, chronic stimulation of the stress response can induce a hypermetabolic state characterized by increased lipolysis and an increasing resting energy expenditure (Sapolsky *et al.*, 2000; Champagne *et al.*, 2015). If continued over many years, this altered metabolic demand could result in a metabolic syndrome, a syndrome that has been hypothesized to have occurred with old age in one population of aquarium-housed dolphins (Venn-Watson *et al.*, 2013).

A second potential influence on TH concentration differences between PL and TZO YFP populations is that water within TZO has been determined to contain high concentrations of pesticides and other pollutants from the nearby agriculture land and poultry farms (Nabi *et al.*, 2017a; Nabi *et al.*, 2018b). Cetaceans are known to bioaccumulate and biomagnify pesticides found within prey they have ingested, and these toxins may interfere with the TH system (Fair *et al.*, 2010; Schwacke *et al.*, 2012). Pesticides exposure in cetaceans could suppress thyroid gland physiology by disrupting TH synthesis, clearance and transportation and cause glandular fibrosis that would adversely affect thyroid gland function (Das *et al.*, 2006; Schnitzler *et al.*, 2008; Schwacke *et al.*, 2012).

Finally, significantly higher TH concentrations found in the PL YFP population could represent an allostatic response (Romero, 2002) to the lower water temperature in the PL (9.7°C) versus TZO (14°C), a phenomenon that has been observed in the bottlenose dolphins (Fair *et al.*, 2011). Exposure to cold weather has been shown to increase circulating serum T_4 and T_3 concentrations, rates of deiodination and enhanced biliary excretion of T4 to T3 to produce endogenous heat for sustaining thermoregulation in some species (Wartofsky and Burman, 1982; Glinoer, 2005; Fair *et al.*, 2011). Conversely, lowered TH concentration during summer

Table 6: Serum cortisol (ng/ml) reference concentrations, bootstrap (1000 reps) mean 2.5th, 50th and 97.5th percentile for the YFPs (*Neophocaena asiaeorientalis ssp. asiaeorientalis*) located in PL of the Yangtze river system from 2010 to 2015 and the semi-reserve TZO in 2015 during the winter and spring

Location	Group	No. of YFP	Mean 2.5th	Mean 50th (95% CI)*	Mean 97.5th
PL Winter	All	45	9.0	15.6 (14.3 to 17.0)	28.4
	JM & JF	20	9.3	14.2 (12.3 to 16.0)	22.6
	AM & AF	14	10.3	16.0 (12.7 to 19.2)	28.9
	PF	11	9.0	17.8 (14.6 to 21.1)	25.5
TZO Winter	ALL	40	3.5	14.7 (12.9 to 16.5)	23.5
	JM & JF	13	3.4	13.8 (10.7 to 16.8)	17.5
	AM & AF	18	9.4	17.5 (15.4 to 19.5)	23.5
	PF & LF	9	7.3	10.9 (7.8 to 14.0)	16.4
PL Spring	ALL	46	0.79	14.7 (12.4 to 16.9)	22.4
	JM & JF	21	0.14	11.6 (8.1 to 15.1)	19.5
	AM & AF	15	5.1	16.2 (13.1 to 19.3)	20.9
	PF & LF	10	6.6	15.9 (12.2 to 19.6)	22.5

*The 95% bootstrap (1000 reps) CIs for 50th percentile.

reduces endogenous heat production to prevent overheating (Hudson, 1981).

Similar to THs, we observe a significant increase in the cortisol level of the PL YFPs population versus TZO population in the winter season. Nabi et al. (2018a), using samples collected during previous health examinations from a subset of males ($n = 43$) divided between both locations, found significantly higher serum cortisol levels in the PL YFP males versus TZO males. In both cases, the increase in cortisol secretion observed in PL YFP could be in response to increased dredging and commercial vessel traffic. For cetaceans regardless of age, and as discussed above, for TH differences, acoustic pollution is a potent stressor and higher cortisol level in response to noise has been reported in the North Atlantic right whales (*Eubalaena glacialis*) and bottlenose dolphin (Romano *et al.*, 2004; Wright *et al.*, 2007; Rolland *et al.*, 2012). However, it is hard to differentiate the glucocorticoids response of a wild animal to its environmental stressors versus the stress-induced response during handling and blood sampling (Fair *et al.*, 2014; Atkinson *et al.*, 2015; Champagne *et al.*, 2018; Steinman et al., 2020). Although we have used the same method for capturing and handling in both populations, we suggest considering the combined effects of environmental and handling stress in the interpretation of cortisol concentrations obtained herein (Fair *et al.*, 2017).

Seasonality

In PL YFP population, we found significantly higher serum tT3 and tT4 in the winter season compared to the spring season indicating the effect of temperature on THs. Generally, THs enhance cellular metabolism by inducing adenosine triphosphate (ATP) synthesis and utilization that ultimately leads to thermogenesis, while in some mammals including bottlenose dolphin, THs activity decline in winter, therefore, in cetaceans, the role of THs in response to cold adaptations is not well understood (Suzuki *et al.*, 2018). Not accounting for the effects of hormones assay methodological variation, it appears that seasonal secretory patterns of THs concentration are species dependent (St. Aubin and Geraci, 1989; Oki and Atkinson, 2004; Rosa *et al.*, 2007). For example, in harbour seals (*Phoca vitulina*), circulating tT4 and tT3 concentrations increases during winter (Oki and Atkinson, 2004), but in beluga whales (*Delphinapterus leucas*) and Steller sea lions (*Eumetopias jubatus*) both circulating levels of tT4 and tT3 increases in summer and decreases in winter (St. Aubin and Geraci, 1989; Myers *et al.*, 2006; Flower *et al.*, 2015), while no seasonality for circulating THs have been reported in bowhead whales (*Balena mysticetus*) and bottlenose dolphin (*Tursiops truncatus*) (St. Aubin *et al.*, 1996; Rosa *et al.*, 2007). For cortisol hormone, only JF showed a significantly higher level in winter versus spring season. Non-significantly increased cortisol concentrations have been reported in the winter in zoo-based killer whales (*Orcinus orca;* Suzuki *et al.*, 2002, 2003), but two larger studies with zoo-based killer whales within water temperature-controlled enclosures found no change (O'Brien *et al.*, 2017; Steinman and Robeck, 2020). This matches with other cetacean species including both wild and semi-domesticated bottlenose dolphin and a harbour seal that did not exhibit any seasonal change in cortisol secretion (Oki and Atkinson, 2004; St. Aubin *et al.*, 1996). The lack of seasonality in cortisol may either be the result of a species difference, a lack of extreme seasonal environmental changes or handling stress (Oki and Atkinson, 2004).

Age- and sex-dependent changes in serum THs and cortisol

In the TZO, only AM showed a significantly higher level of cortisol versus JM, JF and PF possibly suggesting the effect of age and sex. This is similar to a recent study that demonstrated that male killer whales show a significant increase with age in serum cortisol while non-pregnant nor pregnant females did not (Robeck *et al.*, 2017; Steinman and Robeck, 2020). However, other studies in bottlenose dolphins reported no significant influence of age and sex on the circulating cortisol level (St. Aubin *et al.*, 1996; Hart *et al.*, 2012; Fair *et al.*, 2014). Although caution should be used when comparing wild-captured health assessment studies versus conditioned sample collection typically found in zoo-based population studies.

We did not observe an effect of age associated with either circulating tT3 or tT4, while an effect of sex was reported in tT4 concentrations. In cetaceans, sex- and age-related differences in THs have been found in several species, though results vary. In most of cetacean species including bottlenose dolphins (St. Aubin *et al.*, 1996) and beluga whale (St. Aubin *et al.*, 2001), TH concentrations decreased with maturation and postnatal development and varied depending on the sex of an individual. Similarly, AM Amazon river dolphin (*Inia geoffrensis*) showed significantly lowered THs than immature males while no age-dependent changes were reported between JF and AF (Robeck *et al.*, 2019). With advancing age, serum T3 can decrease due to less responsiveness of TSH secretion to TRH levels (Fisher *et al.*, 1977). The reason that we did not see any age-dependent changes is unknown, and future analysis for free T3, T4, rT3 (reverse T3) and TSH may provide more information concerning TH physiology in YFP.

Furthermore, this study provides the first reference values of circulating cortisol, tT3 and tT4 for YFPs living in the wild and semi-natural environment (Tables 5 and 6). In this study, the reference value for cortisol concentrations in AM was significantly higher than the other groups possibly indicating that males have a different stress response to similar environmental conditions than females and this may indicate that other factors, possibly social, are influencing the increased glucocorticoid secretion in males (Trumble *et al.*, 2018). Unlike non-pregnant and pregnant females, cortisol hormone in YFP increases with age in male similar to male killer whales, humpback whales and blue whales (Robeck *et al.*, 2017, Trumble *et al.*, 2018; Steinman and Robeck, 2020). However, we did not observe significant variations in the tT3 and tT4 reference values across the age groups and reproductive states; therefore, further studies are required on a large sample size to understand the TH physiology across the ontogenetic development.

Species comparisons of THs and cortisol

In YFPs, circulating concentrations for tT3 levels were below the range values reported for bottlenose dolphin (St. Aubin et al., 1996; Fair *et al.*, 2011), beluga whale (St. Aubin and Geraci, 1988), manatee (Ortiz *et al.*, 2000), harbour seal (Renouf and Noseworthy, 1991; Oki and Atkinson, 2004) and Stellar sea lion (Myers *et al.*, 2006). However, tT4 levels were in the range of values reported for manatee (Ortiz *et al.*, 2000), harbour seal (Oki and Atkinson, 2004) and Stellar sea lion (Myers *et al.*, 2006) but lower than the bottlenose dolphin (St. Aubin *et al.*, 1996; Fair *et al.*, 2011) and beluga whale (St. Aubin and Geraci, 1988). Furthermore, tT3 concentrations were significantly lower than tT4, which is like the Amazon River dolphins (Robeck *et al.*, 2019), Stellar sea lions (Myers *et al.*, 2006) and bottlenose dolphins (Fair *et al.*, 2011) supporting the finding in other mammalian species that T4 act as a prohormone reserve for conversion to the biologically active form T3 (Brent, 2012).

Serum cortisol levels of YFPs was in the range of values reported for killer whale using enzyme immunoassay (O'Brien *et al.*, 2017, Steinman and Robeck, 2020; Steinman *et al.*, 2020) but slightly higher than the values reported for bottlenose dolphins using radioimmunoassay in resting condition (Champagne *et al.*, 2018). However, in stressed condition, cortisol level was higher in bottlenose dolphin compared to YFPs (Champagne *et al.*, 2018). When comparing hormones, the influence of several factors like collection technique (wild caught versus voluntary zoo based) captivity, age, environment and phylogenetic homogeneity and methodological differences must be considered (Fair *et al.*, 2011).

Conclusions

This study investigated the effects of geographical location, season and biological variables (age, sex, reproductive status) on circulating concentration of THs and cortisol in free-ranging and semi-natural population of YFPs. Significant differences in THs and cortisol concentration were associated with geographical location and season (THs). No age-related significant differences were observed in the THs concentration, while cortisol significantly increased with age in males. The elevated cortisol and TH concentrations found in PL versus TZO population could indicate enhanced metabolism and thermogenesis in response to a relatively colder environment and increased physical activities while avoiding seasonal concentrations of vessel traffic and dredging. Lowered THs in the TZO may also be a result of long-term exposure to endocrine-disrupting chemicals. This is the first large-scale report in YFPs about THs and cortisol concentrations, which will provide a critical base of information for population monitoring, health assessment, investigating the effects of environmental and biological variables and comparisons with other freshwater cetaceans that are currently threatened with extinction.

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