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Cortisol changes in bottlenose dolphins in the dolphin interactive program

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Abstract

Several kinds of animals are utilized in animal-assisted interventions (AAIs) for humans, which release stress and/or provide motivation for muscle movement. It is important to monitor AAI animals' stress levels to ensure their welfare. In the present study, we analyzed blood cortisol levels to evaluate the stress levels of Common bottlenose dolphin (*Tursiops truncatus*) in a dolphin interactive program (DIP). We sampled nine female dolphins that were used in the DIP in Motobu Genki village, Okinawa, Japan. Plasma cortisol levels were measured by ELISA, and the data were analyzed by comparing differences between seasons and before and after the DIPs on a given day. The average plasma cortisol level was 8.7±5.0 ng/ml and ranged from 1.6–29.3 ng/ml. Comparing before and after the DIP sessions on a given day, the average cortisol level of five dolphins decreased significantly. In comparison among seasons, average cortisol levels were significantly higher in the high visitor season than in the rising transition season (between the low and high visitor seasons) in two out of three animals. Regarding the falling transition season (between the high and low visitor seasons), two out of three animals tended to remain high in cortisol levels. From these results, we conclude that DIP induced little acute stress on dolphins. On the other hand, we showed the possibility of chronic stress in DIP-participating dolphins in the high visitor season. Further studies are needed to elucidate whether dolphins experience chronic stress by combining cortisol sampling with stress behavior monitoring.

Key Words: bottlenose dolphin, cortisol, dolphin interactive program

Introduction

Human and animal interactions often occur with domestic animals. It has been suggested that interactions with domestic animals are beneficial to human health. Animal-assisted interventions (AAIs) are popular in hospitals and nursing homes[1,18,23)]. The animals used in AAIs must not harm people, have no infectious diseases, be trainable, and so on. Most AAI animals are small animals, such as dogs[7,18,22,25)]; however, large horses are also utilized, as horse riding activates motor muscles and promotes mental stimulation in humans[17)]. Dolphins are also utilized for the same purpose: to promote muscle movement under imbalanced conditions and provide mental

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stimulation by physically interacting with dolphins[13].

Selye (1956)[35] defined stress as a physiological response to environmental stress. There are two physiological responses to stress. The first is controlled by the sympathetic-adrenal-medullary (SAM) axis, and the second is controlled by the hypothalamus-pituitary-adrenal (HPA) axis. Both axes respond to stress, but the SAM-related physiological reaction occurs before the HPA-related reaction. HPA axis activation releases steroid hormones from the adrenal cortex to induce a systemic response to stressors. Glucocorticoids, such as cortisol and corticosterone, are well-known stress markers activated by the HPA axis, which is produced in the adrenal cortex and circulates systemically via the blood. Glucocorticoids are also deposited in hair, nails, feces, urine, and saliva, and these levels increase when animals experience psychological and/or physical stress. It has been reported that glucocorticoids are present in a variety of animals not only on land but also in the sea[2,37]. Some studies have reported that circulating glucocorticoid levels follow a circadian or diurnal rhythm not only in domestic animals but also in captive wild animals (dogs[31], cows[12], horses[6], pigs[33], elephants[20], harbor seals[28], bottlenose dolphins[40]). Glucocorticoid levels increase in response to stress; then, negative feedback regulates glucocorticoid levels under acute stress conditions[35]. Under chronic stress conditions, HPA in dogs becomes hypoactive or dysregulated, and dog salivary cortisol levels change significantly less than they do under nonstress conditions[9].

The cortisol level in blood is a good biomarker of acute stress and is sometimes used as an indicator of poor animal welfare. Monitoring animals who participate in AAI programs is important not only for animal welfare but also for preventing harm to humans. In cetacean species, cortisol has been utilized as a stress marker. Blood cortisol levels are increased by restraint stress[19,21,30]. Noda et al. (2007)[27] noted that the blood cortisol level increased fivefold compared to the normal level when bottlenose dolphins experienced transportation stress. Ugaz et al. (2013)[43] reported that bottlenose dolphins reared in limited spaces showed much higher salivary cortisol levels than those reared in open spaces. Therefore, monitoring blood cortisol levels is a good method to evaluate the stress level of dolphins participating in the dolphin interactive program (DIP).

In this study, we evaluated the plasma cortisol level to understand the reactions of dolphins to DIP.

Materials and Methods

Bottlenose dolphin and Dolphin interactive facility

We surveyed the blood cortisol level of nine nonpregnant female Common bottlenose dolphin (*Tursiops truncatus*) reared in Motobu Genki village, Okinawa, Japan (https://owf.jp/english/)[23]. The facility is located on the coast, and the dolphin pools were partitioned along the coast. The water temperatures were 22–23 ℃ from December to April, 25 ℃ in May and November, 27 ℃ in June and October, and 28–29 ℃ in July, August, and September (https://ja.climate-data.org/)[8]. All of the rearing dolphins were nonpregnant female. Dolphins participated in the DIP up to three times a day. Dolphins were fed several times at morning and evening health checks, at their training, at DIP sessions, and at changing pools.

There are three types of DIPs: dolphin school plus (DS+), which involves touching a dolphin from the pier; swim with a dolphin (SWD), which involves touching a dolphin in the water; and royal swim (RS), which involves swimming with a dolphin (Table 1).

Blood sampling and cortisol measurement by ELISA

Dolphin blood samples were collected for

Table 1. Dolphin interactive program*

Program	Subtitle and Contents†	Remarks
DS+ (Dolphin School plus)	a) Guests interact with dolphins using toys and touch a dolphin on the DOC b) Guests touch a dolphin on the PLAT when the dolphin is close enough	2 sessions, No swimming, 30 minutes
SWD (Swim with a dolphin)	a) DS+ b) DORSAL (Dolphins assist a guest with floating at the PLAT and the other place in the pool for one session) c) Guests swim holding the dolphin's dorsal fin for approximately 15 m during two sessions at any place in the pool	3 sessions, 30 minutes
RS (Royal swim)‡	a) DS+ b) SWD c) HUMAN (Dolphin jumps over the guest) d) BELLY (Guests swim with the dolphin while laying on dolphin's belly for 10 m) e) Double DORSAL (Guest swim holding two dolphins' dorsal fins for approximately 10 m)	3 sessions, 30 minutes

† DOC: A stage on the surface; PLAT: A stage under water at human knee depth
‡ Each session is performed by different dolphins
* Between each session, dolphins are released into their enclosures for a couple of minutes

blood work during a morning/evening regular health check, and the remaining samples were provided for our study. The sampling dates and the season or months varied: samples were collected in July/August/September/October 2015 and June/December 2016. The number of samples was also different, but most were collected in summer. Most of the blood samples (n = 69) were taken in the regular morning health check at approximately 8:00 am before the program started. In addition, 23 samples were taken in the evening health check at approximately 4:00 pm 20 min after the program ended. Prior to blood sampling, one or two fish were fed to the dolphins as husbandry training. Samples were taken from the fluke by venipuncture under husbandry training. Each dolphin's reaction or attitude before and during sampling was also recorded. Blood samples were mixed with EDTA-2Na to prevent coagulation, and plasma was separated by centrifugation at 3,000 rpm for 15 min. Separated samples were stored at -20 ºC until cortisol measurement. Dolphin plasma samples were diluted 32 times, and the cortisol level was quantified using a Cortisol ELISA kit (Arbor Assays, Ann Arbor, MI, USA). Standards and plasma samples were loaded in duplicate and read with an iMarkTM Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm wavelength. The cortisol concentration was calculated in MyAssays HP (myassays.com)[24]. The intra- and interassay precision (CV%) for duplicate samples was 8.2% and 13.3%, respectively.

Analysis and statistics

Information on the subject animals that participated in DIP and the number of blood samples for each month is shown in Table 2. Same-day paired samples from five dolphins (CAY, MA, SP, PA and CH) were compared to analyze plasma cortisol differences before (morning) and after (evening) DIP on a given day. A paired t-test was utilized for this analysis, and $P < 0.05$ was considered to indicate a significant difference. We also analyzed the relationships between cortisol level and the presence of dolphin aversive reactions to each blood sampling. The dolphin sample information was listed together with cortisol change results (Table 4), i.e., collection dates and the presence or absence of aversive reactions to each blood collection.

July and August were the most active (high) seasons with the highest number of visitors (Table 3). Despite the limited sample size, we compared seasonal cortisol level changes between the

Table 2. Bottlenose dolphin information and blood sample profiles

ID	Age (2015)	Total sample number	Sampled month and sample number					
			June	July	August	September	October	December
CAY	15	17	6	1	2	4	3	1
MA	14	17	6	2	2	4	1	2
SP	12	16	5	2	3	5	0	1
PA	11	12	4	1	1	3	1	2
CH	9	9	4	0	3	0	0	2
LE	20	6	0	2	2	2	0	0
SU	15	6	0	2	2	0	0	2
AN	9	5	0	1	1	3	0	0
CR	9	4	0	1	2	1	0	0

Table 3. Monthly participants in the dolphin interactive program

	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR
Season*	Mod		Trans	High		Trans		Low				Trans
Mean	1,147	1,137	1,509	3,109	3,351	1,390	1,001	386	393	305	225	900
SD	330	120	145	159	27	319	86	48	66	89	27	132
n/day	38	37	50	100	108	46	32	13	13	10	8	29

* Mod: moderate season; Trans: transitional season; High: high season; Low: low season
Mean: mean number of people who participated in the DIP, SD: standard deviation, n/day: average number of turnouts per day.

transitional season and high season. Regarding transitional seasons, June is the month in which the number of human participants increases, and September is the month in which participation decreases. Therefore, we compared the cortisol changes to analyze the stress of dolphins in the high season (July/August) and in two transitional seasons (June and September). We did not include the low visitor season and moderate visitor season in the comparison due to the small sample size. To analyze cortisol changes in all three seasons, we chose CAY, MA, and SP because only those three dolphin samples covered the target seasons. The samples from July and August were combined to increase the sample size. Only plasma samples taken in the morning were used for this comparison to compare the results under the same conditions. The mean cortisol differences in those seasons were compared individually. For statistical analysis, ANOVA was used to perform comparisons of DIP participants in the high season and transition season. The α-values for multiple comparisons were adjusted to α=0.017 using sequential Holm's procedure to control for the type one familywise error rate. The statistical analyses were performed with R software (The R Foundation for Statistical Computing, Vienna, Austria).

Results

1) Cortisol levels in all dolphins

Plasma cortisol levels were different in each dolphin; the mean level was 8.7±5.0 ng/ml, and the range was between 1.6 and 29.3 ng/ml (Fig. 1).

2) Plasma cortisol changes before and after all sessions on a given day

The changes in plasma cortisol levels before and after the DIP session are shown in Table 4. Almost all dolphins tended to show lower cortisol levels after the DIP session than before the session, except for one same-day paired sample in CAY (Jun/20/2016). The mean (±SD) plasma cortisol levels of the five dolphins were 7.6±4.8

Table 4. Cortisol level (ng/ml) before (Pre) and after (Post) the DIP session

	Sampling date	Cortisol level (ng/ml)		Contents and number of DIP		Unwilling†
		Pre (8 am)	Post (4 pm)	AM	PM	
CAY	Jun/13/2016	2.8	2.7	DS+/DS+ (2)	– (0)	non
	Jun/20/2016	4.6	9.4*	DS+/RS (2)	DS+ (1)	non
MA	Jun/13/2016	4.4	2.3	SWD/RS (2)	DS+ (1)	non
	Jun/20/2016	5.2	1.8	SWD/RS (2)	SWD (1)	both
SP	Jun/13/2016	4.8	4.3	SWD/RS (2)	DS+ (1)	pre
	Jun/20/2016	4.2	2.9	SWD/RS (2)	DS+ (1)	both
PA	Jun/20/2016	9.2	2.2	SWD/RS (2)	– (0)	both
	Dec/05/2016	15.1	8.1	DS+/RS (2)	DS+ (1)	both
CH	Jun/20/2016	16.3	8.7	SWD/RS (2)	– (0)	non
	Dec/05/2016	8.3	4.2	DS+ (1)	– (0)	non

* The postsession plasma cortisol level increased compared to the presession plasma cortisol level.
† Dolphin was unwilling to undergo blood sampling; non: not observed; pre: before the daily session; both: both before and after the daily session.

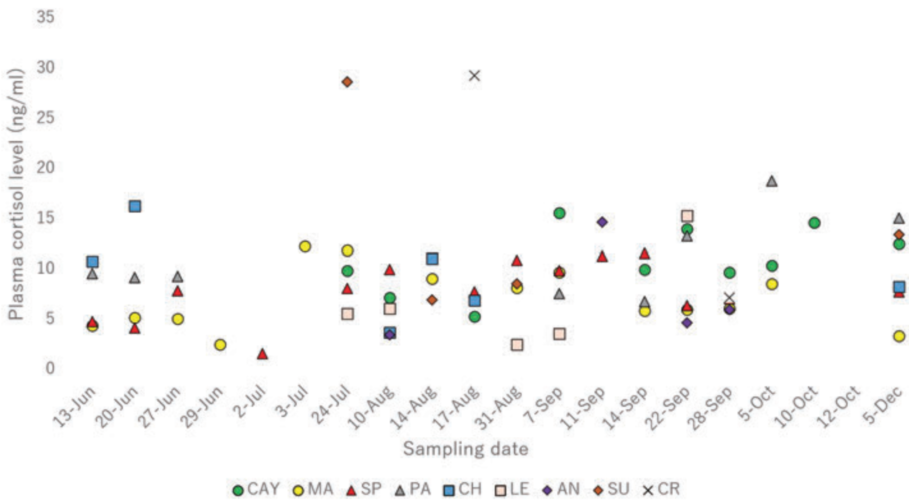

Fig. 1. Plasma cortisol levels (ng/ml) in all obtained dolphin samples.
Letters indicate months; Jun: June, Jul: July, Aug: August, Sep: September, Oct: October, Dec: December. Symbols indicate each dolphin ID.

ng/ml before sessions and 4.7±2.8 ng/ml after sessions. There were significant differences before and after sessions ($P = 0.018$).

During blood sampling, dolphins sometimes show aversive behavior in response to blood collection (Table 4). MA, SP and PA were sometimes unwilling to undergo a blood draw before and/or after the session on a day; however, their cortisol levels did not increase after the session. Especially in one same-day paired sample in SP (Jun/13/2016), she was unwilling to undergo blood sampling only before the session; however, her cortisol levels were almost the same before and after the session on that day (Table 4).

3) The cortisol changes in three dolphins compared among the visitor number status seasons

The mean cortisol level in CAY was 6.1±6.2 ng/ml in June, 7.5±2.3 ng/ml in July/August, and 12.3±2.9 ng/ml in September (Fig. 2). In CAY, the mean cortisol level in September tended to be higher than that in July/August ($P = 0.068$), although there were no significant differences among seasons. The mean cortisol level in MA

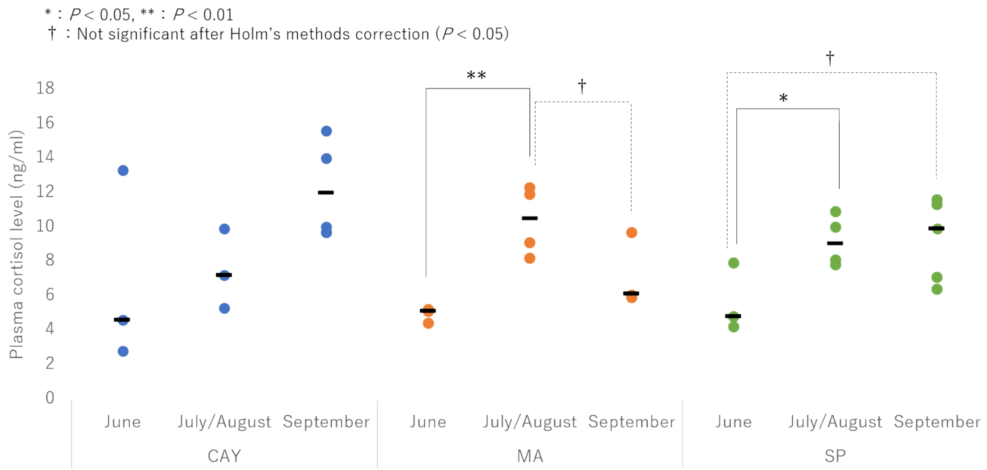

Fig. 2. Cortisol levels in June, July/August, and September in CAY, MA and SP. Black bars indicate the median values of each group.

was 4.2±1.6 ng/ml in June, 10.4±2.0 ng/ml in July/August, and 6.9±1.8 ng/ml in September (Fig. 2). There were significant differences between June and July/August ($P = 0.008$) and nearly significant differences between July/August and September ($P = 0.044$, not significant after Holm's methods correction) in MA. The mean cortisol level in SP was 5.9±1.7 ng/ml in June, 9.2±1.5 ng/ml in July/August, and 9.3±2.4 ng/ml in September (Fig. 2). There were significant differences between June and July/August ($P = 0.012$) and nearly significant differences between June and September ($P = 0.048$, not significant after Holm's method correction) in SP.

Discussion

We evaluated the stress levels of captive bottlenose dolphins in a DIP by surveying plasma cortisol level changes and factors that affect dolphin stress.

Suzuki (2001)[39] reported that the blood cortisol level of bottlenose dolphins (230 samples) was 11.0±9.2 ng/ml according to radioimmunoassay (RIA). Suzuki et al. (2003)[40] also reported that the blood cortisol level was 3.8±1.8 ng/ml in two female bottlenose dolphins in the morning according to the same RIA method. Thomson and Geraci (1986)[41] reported a level of 10.9 ng/ml in a relaxed situation. However, the serum cortisol level was high, at 17–30 ng/ml[19] and 28±10 ng/ml[30], when bottlenose dolphins were captured and blood was sampled. The results of the present study showed a wide range of cortisol levels (range: 1.6–29.3 ng/ml, mean: 8.7±5.0 ng/ml) that overlapped with those in previous studies, even if there were differences in the measurement methods between previous studies (RIA) and the present study (ELISA).

From the results of the comparisons before and after sessions, four out of five dolphins showed a cortisol decrease. When summing the measurements of the five dolphins, cortisol levels were also significantly lower in the evening (after all sessions ended) than in the morning (before session start). Additionally, the DIP in Motobu Genki village comprises three thirty-min sessions per individual per day to minimize the burden on the dolphins. Considering these facts, we suggested that there was little stress on dolphins induced by DIP.

We further discuss several factors that could affect the cortisol level before and after DIP. It is known that cortisol levels increase immediately after the Trier Social Stress Test (approximately 3 minutes) in humans. The cortisol levels peak 10 minutes after stress release and then gradually decrease and return to baseline in approximately 70 minutes[16]. In the present study, the effects of the sessions are considered to remain even in cortisol levels of blood samples taken after the session because blood was collected 20 minutes after the DIP sessions.

Glucocorticoids are affected by food intake, and it has been reported that there is an increase in the fasted state[4,34]. In the present study, dolphins were frequently fed throughout the day. In addition, dolphins were fed immediately before blood collection, but it was only one or two fish. Therefore, it is considered that the effect of food intake on the cortisol level is small.

In our study, dolphins sometimes showed an aversive reaction to blood sampling; however, cortisol levels did not seem to increase when dolphins showed aversive reactions. It was difficult to determine why dolphins were unwilling to undergo blood sampling on that day. The sampling difficulty experienced by keepers makes dolphins reluctant to draw their blood. Struggling with blood sampling may cause an increase in cortisol. In silver fox, it has been reported that cortisol levels increase due to stress caused by physical restraint[21]. In Motobu Genki Village, blood sampling is conducted under husbandry training. Furthermore, body temperature was measured every day in the same posture as when blood was collected, and the trainers did not restrain dolphins excessively. Therefore, it is considered that there was no excessive stress due to blood sampling.

The plasma cortisol level in the daytime is also affected by the circadian rhythm, according to Judd and Ridgeway (1977)[15]. Suzuki et al. (2003)[40] reported diurnal changes in serum cortisol levels in bottlenose dolphins that were higher in the morning (9 am) than in the evening (6 pm). It is considered that the cortisol levels of the dolphins in the present study followed the circadian rhythm.

Summer is the peak season for tourists in Okinawa and DIP participants in Motobu Genki village. The number of visitors starts increasing in June and decreases to baseline by September (Table 3). Therefore, the high season is considered July and August, and June and September are considered transition seasons. The cortisol levels in the three dolphins (CAY, MA, and SP) were compared between the transition seasons and high season. In MA and SP, the stress level might increase, reflecting the number of DIP participants because the mean cortisol level in July/August was significantly higher than that in June. For CAY, the mean cortisol level was not significantly changed between July/August and June. According to the mean cortisol level in September, the stress levels in MA tended to decrease. On the other hand, the mean cortisol levels in CAY and SP showed an increasing tendency in September, although there were no significant differences. For CAY and SP, the effects of the high season may have remained as chronic stress even in September. Regarding MA, the levels may have tended to decrease in September due to internal factors such as stress tolerance that are different from other individuals. However, the cause could not be identified only from the individual information obtained from the keeper.

Orlov et al. (1988)[29] reported that the blood cortisol level was 32.7±2.5 ng/ml in spring and 23.5±3.9 ng/ml in autumn. Funasaka et al. (2011)[11] reported that daily mean concentrations of serum cortisol during the spring equinox (March, 6.1±0.5 ng/ml) were higher than those in the summer solstices (June, 3.7±0.3 ng/ml) and winter (December, 3.1±0.4 ng/ml) in four adult male bottlenose dolphins. There might be some seasonal or periodic effects on blood cortisol levels, but the exact mechanism is unknown. One-year monitoring is needed to enhance our study.

In humans, the dynamics of salivary cortisol levels in chronic psychological stress conditions have been reported. The cortisol awakening response (CAR) is increased in occupational stress and general life stress and dull in those showing fatigue, burnout, and posttraumatic stress disorder[26]. If an increased cortisol level is associated with a large number of visitors, it can be interpreted as chronic stress rather than acute stress. Long-term monitoring is required to assess chronic stress in dolphins.

The effects of long-term continuous blood sampling on dolphins are unknown. Therefore, sampling methods for assessing chronic stress need to be established. The candidates for noninvasive stress markers are skin[3], feces[5], and saliva[32,43]. A stress behavior assessment would also be helpful to demonstrate psychological reactions. Not only terrestrial animals but also marine mammals express their feelings or emotions with behaviors or vocalizations. Trone et al. (2005)[42] noted that play behavior changed significantly in dolphins in a DIP, while the other behaviors did not. Esch et al. (2009)[10] studied whistle sounds as another stress indicator. Stress assessments should be performed using these multiple markers.

In the present study, the mean cortisol levels of five dolphins were significantly lower after the session than before the session. We conclude that there was little effect of acute stress on dolphins induced by DIP. On the other hand, we showed the possibility of chronic stress in DIP-participating dolphins in the high visitor season; however, additional stress evaluations are needed to detect chronic stress in dolphins. Continuous stress monitoring with adequate biomarkers will reveal dolphin stress levels in detail, which will contribute to developing a suitable format for DIPs in the future.

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