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Evidence of melatonin secretion in cetaceans: Plasma concentration and extrapineal HIOMT-like presence in the bottlenose dolphin *Tursiops truncatus*

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ABSTRACT

The pineal gland is generally believed to be absent in cetaceans, although few and subsequently unconfirmed reports described the organ in some species. The recent description of a complete and photographed pineal body in a bottlenose dolphin (*Tursiops truncatus*) prompted us to examine a series of 29 brains of the same species, but no gland was found. We then decided to investigate if the main product of the gland, melatonin, was nevertheless produced and present in the plasma of this species. We collected plasma and serum samples from a series of captive bottlenose dolphins for a period of 7 months spanning from winter to summer and we determined the indoleamine concentration by radio-immunoassay (RIA). The results demonstrated for the first time a quantitative assessment of melatonin production in the blood of a cetacean. Melatonin levels were comparable to those of terrestrial mammals (5.15–27.74 pg/ml daylight concentration), with indications of both seasonal and daily variation although the presence of a circadian rhythm remains uncertain. Immunohistochemical analyses using as a marker hydroxyindole-*O*-methyltransferase (HIOMT, the key enzyme involved in the biosynthesis of the hormone), suggested extrapineal melatonin production by the retina, the Harderian gland and the gut. The enzyme was unequivocally localized in all the three tissues, and, specifically, ganglion cells in the retina showed a very strong HIOMT-immunoreactivity. Our results suggest that further research might reveal unexplored aspects of melatonin production in cetaceans and deserves special attention and further efforts.

1. Introduction

The presence of a distinct pineal gland (*epiphysis cerebri*) in cetaceans has been long discussed with no definite conclusions. No pineal gland was reported in the Amazon river dolphin (*Inia geoffrensis*) [17], in the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) and the spinner dolphin (*Stenella longirostris*) [3], in the short-beaked common dolphin (*Delphinus delphis*) [37], in the dwarf sperm whale (*Kogia sima*) [39], and in the fetal narwhal (*Monodon monoceros*) [20]. A "rudimentary" pineal gland was observed by Fuse [14] in the finless porpoise (*Neophocaena phocaenoides*), while the presence of a well-formed gland was described at least in some specimens of beluga (*Delphinapterus leucas*) and bottlenose whale (*Hyperoodon ampullatus*) [25], sperm whale (*Physeter macrocephalus*) [38], and harbor porpoise (*Phocoena phocoena*) [4]. However, the presence of a pineal gland is apparently not constant among all the individuals of a given species. A pineal gland was found in six humpback whales (*Megaptera novaeangliae*) [15], whereas both Breathnach [6] and Pilleri [44] did not find any in the same species. In the sei whale (*Balaenoptera borealis*) Pilleri [45] described a pineal gland "intimately bound with choroid plexus under the splenium of the corpus callosum", but Arvy [3] reported it to be absent. Duffield et al. [12] were able to find a pineal body only in one out of 11 specimens of bowhead whale (*Balaena mysticetus*) harvested by Alaskan Eskimos. In an adult blue whale (*Balaenoptera musculus*) no pineal gland was found in a study [43], but the presence of the organ was reported in a fetal specimen by others [21]. The common bottlenose dolphin, *Tursiops truncatus*, a widely studied cetacean, represents an emblematic case. Neither McFarland et al. [34] nor Ridgway [50] were able to detect a pineal body in several brains of this species. On the contrary, Morgane and Jacobs [36] observed a pineal gland "present up to the adult stage", and an unmistakable image of a large pineal gland in a pregnant female has been published recently [28]. Variations of the size in the gland during pregnancy were described in bats [18], squirrels [5] and humans [65], but the gland does not appear or disappear completely.

The unpredictability of the presence of a pineal organ in cetaceans has never been fully explained. From the evolutionary point of view, a specific organ should be present (or absent) in a given species even if rudimentary, but its appearance only in random individuals is enigmatic. Furthermore, the pineal gland is well

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developed in other non-cetacean marine mammals, specially *Phocidae* [9] and *Otariidae* [35], in which it reaches a remarkably large size with high melatonin plasma levels in newborns [54].

Prompted by the puzzling data present in the literature we examined a series of brains of *T. truncatus* by gross dissection to evaluate systematically the possible presence of a pineal gland. Given that no report is present in the literature about the secretion of melatonin in this species, we also studied for the first time its plasma concentration in captive individuals. Finally, we investigated extrapineal melatonin production in three tissues indicated in the literature as the most probable sites for other mammals: the retina [58], the Harderian gland [41], and the gastrointestinal tract [24]. To this effect we chose the enzyme hydroxyindole-*O*-methyltransferase (HIOMT) as a marker, due to its terminal position in the biosynthetic pathway of the hormone, and its high specificity for melatonin-producing tissues [2].

2. Materials and methods

2.1. Brain samples

The Mediterranean Marine Mammal Tissue Bank (MMMTB, http://www.mammiferimarini.sperivet.unipd.it/eng/index.php), established in 2002 and hosted by the Department of Experimental Veterinary Science of the University of Padova, contains over 2500 samples of cetacean organs, including a collection of more than forty formalin-fixed brains, 29 of which belong to bottlenose dolphins. Twenty-nine of these latter brains were cut into serial coronal sections 1 cm thick; one additional brain was cut into sagittal sections 1 cm thick. All sections were examined for the presence of a pineal gland in the epithalamus. Structures possibly identified as pineal glands were to be removed, washed in phosphate buffer saline (PBS) pH 7.4 overnight, immersed in a cryoprotective solution (30% sucrose in PBS), frozen and then cut into serial cryostat sections (8–10 μm thick).

A few bovine pineal glands were removed at the slaughterhouse, fixed in 10% buffered formalin overnight, processed for cryostat sectioning and used as a positive control for immunohistochemistry (see below).

2.2. Specimens and sampling design for melatonin quantification

A total of twelve captive bottlenose dolphins of known age, maintained into two distinct dolphinaria, A (latitude 41°37′N; $n = 4$) and B (latitude 37°07′N; $n = 8$) were involved in the present study (Table 1). Bottlenose dolphins held in captivity are constantly monitored under a strict ethical code enforced by European laws (see below). Therefore sampling was scheduled to overlap veterinary medical health controls and because of this the experimental design was slightly different in the two facilities, due to the different temporal availability of the veterinary staffs and the necessity to coordinate and integrate the sampling effort with routine activities.

The animals that we sampled from have been living in the same facility from birth or at least for several years. They could not be constrained into artificial light:dark cycles, due to the unfeasibility of such procedure in their quarters. Complete darkness in the pools after sunset is impossible to achieve, since there is always a minimal nocturnal artificial lighting. Although the dolphin pools are seldom directly illuminated, lights streams in from adjacent sectors of the parks in which the dolphinaria are located. Light intensity was not monitored, but we relied upon sunrise/sunset time gathered from the U.S. Navy *on-line* service (http://aa.usno.navy.mil/).

Water is generally heated in the dolphin pool during winter time. Temperature range therefore varies from approx. 18 °C (coldest winter peak) to 27 °C (warmest summer peak).

Dolphins are usually fed at fixed hours during the day, plus unscheduled meal sections in which feeding is a way to positively enforce required behaviors. Cooperation during the sampling sessions is routinely accompanied by feeding rewards. Furthermore, while blood was withdrawn from the vessels of the fluke of one animal, the others were kept at distance by rewarding them with fish. When blood is sampled, the animal stands still at the surface and floats only with the pectoral fins. Blood sampling from the flukes cannot be performed in the dark or with minimal red light. A permanent catheter cannot be inserted, because of the continuous high mobility of the flukes. Here we stress that these are the normal operational setting for routine medical samplings.

For the assessment of seasonal variations of melatonin concentration, one sample was scheduled to be drawn at approx 10:00 during the first week of each month (December 2008 to June 2009, group A; February to August 2009, group B, respectively, see Table 2). For the assessment of daily variations, sampling took place at approx 10:00 and 17:30, for three subsequent days during the second week of December and June in dolphinarium A, and for two subsequent days during the first week of February and August in dolphinarium B, respectively (see Table 3).

Each blood sample was drawn from the ventral surface of the tail flukes into vacuum EDTA tubes, while trained dolphins offered voluntarily their flukes. The plasma was then separated after centrifugation for 20 min at 2800*g* (3500 rpm) and stored at −20 °C.

Table 1
List of specimens with identification name, sex and age (in years).

	Sex	Age
A group		
RM1	M	17
RM2	M	11
RM3	M	10
RM4	F	7
B group		
PT1	F	27
PT2	M	49
PT3	M	14
PT4	F	31
PT5	F	41
PT6	F	13
PT7	F	13
PT8	F	10

F, female; M, male.

Table 2
Melatonin concentration (in pg/ml) in blood samples used for seasonal variation assessment.

	A group						
	DEC	JAN	FEB	MAR	APR	MAY	JUN
RM1	12,90	10,57	11,54	9,64	7,13	7,95	12,67
RM2	13,25	11,64	11,93	12,91	11,78	13,03	13,35
RM3	18,04	17,57	20,57	23,01	18,11	20,86	19,20
RM4	20,37	24,33	22,63	25,35	18,38	21,50	16,99
	B Group						
	FEB	MAR	APR	MAY	JUN	JUL	AUG
PT1	16,36	21,65	21,36	22,31	14,76	10,94	27,56
PT2	14,21	9,59	14,72	14,16	7,60	12,04	15,24
PT3	11,55	17,65	14,58	11,58	8,73	10,26	25,43
PT4	12,26	12,72	14,77	15,11	8,25	9,77	24,53
PT5	6,02	11,38	14,73	13,88	5,15	6,89	19,35
PT6	15,13	21,71	19,47	16,03	11,35	27,74	/
PT7	/	16,53	15,84	/	7,61	10,77	/
PT8	/	/	/	19,48	11,03	23,04	20,70

/, Not determined; DEC, December; JAN, January; FEB, February; MAR, March; APR, April; JUN, June; JUL, July; AUG, August.