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Monitoring platelet function in marine mammals: Intracellular Ca^{2+} mobilization as a biomarker of platelet activation

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ABSTRACT

Platelet functionality plays a crucial role in marine mammals. Alterations in platelet function can result from stress, pathologies, or exposure to xenobiotics, among others. The early detection of platelet function abnormalities is essential in these species to prevent advanced pathology and mitigate potential risks. Our main objective was to establish a range of physiological values of platelet function in bottlenose dolphins (*Tursiops truncatus*), beluga whales (*Delphinapterus leucas*), sea lions (*Otaria flavescens*) and walruses (*Odobenus rosmarus*). Intraplatelet Ca^{2+} mobilization using adenosine diphosphate (ADP) as a platelet agonist was used as a platelet function biomarker, adapting the methodology previously described by us in dolphins (Felipo-Benavent et al., 2022) to the rest of the species. The assay was also adapted to a seal (*Phoca vitulina*). Numerical indicators of intraplatelet Ca^{2+} mobilization kinetics were established, and statistical analyses were performed to compare the effects of species, sex, age, aquarium and species. Significant differences were observed between species, being the platelets of the sea lions the more reactive to the agonist. This work demonstrates the usefulness of this assay in the diagnosis or monitoring of animals with hemostatic diseases, showing two clinical cases in which intraplatelet calcium mobilization values were altered in marine mammals suffering haemorrhages. This assay may also serve as a means to monitor environmental changes and their potential impact on the health of marine mammal populations.

1. Introduction

Platelets of marine mammals can be affected by different physiological and pathological conditions, stress and diving disturbances (Venn-Watson et al., 2008; Yu and Xia, 2013; Skinner et al., 2015; De Mello and Da Silva, 2019; Barratclough et al., 2020). In addition, marine mammals are exposed to environmental contaminants found to interfere with platelet activation in humans and mice, including heavy metals (Goodwin et al., 1995; Kostka et al., 1997; Watson et al., 2016; Nontarach et al., 2016; van Rensburg et al., 2019) or polychlorinated biphenyls (Raulf and Konig, 1991). However, no studies on the effects of environmental contaminants on platelet activation responses in marine mammals are available to our knowledge.

Abnormalities of platelet activation may lead to thromboembolic or hemorrhagic disorders, even at normal platelet counts (Bourguignon et al., 2022). Detecting early alterations of platelet function in marine mammals is relevant, as they do not show signs of weakness until pathology is advanced, to avoid attracting their natural predators. Therefore, the implementation of laboratory assays of platelet activation would be a significant advance for early diagnosis and monitorization of

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hemostatic disorders in mammal species in aquariums. Furthermore, such routine- and preventive clinical assays could also be applied to environmental veterinary studies on wild animals.

Early markers of platelet activation are a good starting point to study platelet function. Specifically, mobilization of intraplatelet Ca^{2+} is relevant because it precedes shape change, aggregation, granule secretion and expression of procoagulant activity of the platelets (Assinger et al., 2015; Bourguignon et al., 2022). Stimulation with platelet agonists, like adenosine diphosphate (ADP), results in a fast and reversible rise in cytosolic Ca^{2+}, due to Ca^{2+} uptake from the extracellular space and/or Ca^{2+} release to the cytosol from internal stores, such as the dense tubular system, granules, mitochondria or the internal face of cytoplasmic membrane (Monteiro et al., 1999; Bourguignon et al., 2022).

Flow cytometric analysis of Ca^{2+} mobilization has been widely used in human medicine to diagnose and monitor a number of hemostasia disorders related to abnormal platelet activation, including hypertension (Labiós et al., 2006; Alexandru et al., 2008; El Haouari and Rosado, 2009), diabetes mellitus (Ishii et al., 1991; Watala et al., 1998; Alexandru et al., 2008) leukaemia (Okuma et al., 1982), psychological disorders (Kusumi et al., 1994; Das et al., 1995), neurodegenerative diseases (Rao et al., 1996) or congenital conditions like Gray platelet syndrome (Mori et al., 1989). On the other hand, catecholamines like epinephrine and norepinephrine can exert an activating effect on platelets under acute stress conditions (Lages et al., 1981; Salamah et al., 2019; Alarayyed et al., 1997; Zoccarato et al., 1991; Hjemdahl and Wallén, 1997; Butt et al., 2005; Cimminiello et al., 1989; Feoktistov et al., 1992)

We have developed and applied for the first time to bottlenose dolphins (*Tursiops truncatus*) a flow cytometry assay of intraplatelet Ca^{2+} mobilization (Felipo-Benavent et al., 2022), as a suitable biomarker of platelet activation in whole blood samples of marine mammals. This test is an adaptation from a previous kinetic flow cytometry (FCM) method developed by our group and repeatedly used for assessing human platelet activation (Monteiro et al., 1999, 2003, 2022; García-Martínez et al., 2004; Melo-Ferraz et al., 2021). Briefly, the assay involves using the platelet-specific antibody CD41 that cross-recognizes human and dolphin platelets, and the Ca^{2+-}sensitive dye Fluo-4 (added to samples in its cell-permeable form Fluo-4 AM) to follow in real time the kinetics of intraplatelet Ca^{2+} mobilization upon activation of platelets with ADP (Felipo-Benavent et al., 2022). In the present work, we have extended this method to study intraplatelet Ca^{2+} mobilization in several marine mammal species. We have specifically modified the assay for its application to beluga whales (*Delphinapterus leucas*), sea lions (*Otaria flavescens*), seals (*Phoca vitulina*) and walruses (*Odobenus rosmarus*).

To our knowledge, this is the first time that intraplatelet Ca^{2+} mobilization has been studied as a platelet function biomarker in individuals of the aforementioned species, which represents a significant advance for the management and monitoring of their health status, as well as for the research of the hemostatic process in marine mammals.

2. Materials and methods

2.1. Animals

All the animals were from the Oceanogràfic Aquarium of the City of Arts and Sciences (Valencia, Spain) and from the Mundomar Aquarium (Benidorm, Spain). For this work, we have carried out for 3 years a continuous study of healthy animals belonging to the different species. Whenever possible, animals from two different aquariums have been used. Supplementary Table 1 summarizes the species, age, sex, weight and home aquarium for each individual included in this study.

Animal management and experimental procedures were approved by the Animal Care Committees of the Oceanogràfic and Mundomar Aquariums (Committee reference OCE-6-17).

2.1.1. Bottlenose dolphins (Tursiops truncatus)

Fifteen bottlenose dolphins from the Oceanogràfic Aquarium (Valencia, Spain) and five dolphins from the Mundomar Aquarium (Benidorm, Spain) were included in the study. Sixty-six blood samples were collected over 3 years from the whole dolphin population.

2.1.2. Beluga whales (Delphinapterus leucas)

Three beluga whales from the Oceanogràfic Aquarium were included in the study: two adults and one calf. To carry out this pilot study, 20 total samples were analyzed, proportionally distributed among the three animals.

2.1.3. Walruses (Odobenus rosmarus)

Three adult female walruses housed in the Oceanogràfic Aquarium were included in the study. The three animals were siblings of the same age. For the pilot study, five samples of each animal were studied, rendering 15 samples of the species.

2.1.4. Sea lions (Otaria flavescens)

Two sea lions from Mundomar Aquarium and six from the Oceanogràfic Aquarium were included in the study. Nine samples from sea lions were analyzed.

2.1.5. Seals (Phoca vitulina)

One seal from the Oceanogràfic Aquarium was sampled three times to adapt the methodology to this species.

2.2. Blood sampling

For the platelet assay, we obtained citrated whole blood from healthy animals. Animals were previously trained to collaborate voluntarily with trainers and veterinarians for blood collection. In cetaceans, blood samples were collected from a vein at the ventral surface of the caudal fin, while in pinnipeds blood was collected from interdigital veins of caudal flippers. The blood sampling procedure for pinnipeds (walruses, sea lions and the seal) and cetaceans (bottlenose dolphins and beluga whales) involved the use of specific equipment. Specifically, a Butterfly needle with a gauge size of 21G, known as Venofix® and manufactured by Fa.Braun, was utilized. This type of needle is commonly used for blood collection as it provides a suitable balance between patient comfort and effective sample extraction.

In addition to the Butterfly needle, single-use syringes with a capacity of 10 ml, supplied by Covetrus, were employed for blood collection in both pinnipeds and cetaceans. These syringes are designed for a one-time use, ensuring sterility and minimizing the risk of contamination during the blood sampling process.

By employing these specific tools, the researchers aimed to maintain consistency and standardization in the blood sampling procedure across all marine mammal species studied. This allowed for accurate and comparable measurements of intraplatelet Ca^{2+} mobilization, which was a key parameter of interest in the study.

Samples were analyzed in the Cytomics Laboratory at the Príncipe Felipe Research Center (CIPF, Valencia, Spain) within 2 h after being obtained.

2.3. Reagents and solutions

Antibody CD41-PE, clone P2 was from Beckman Coulter (Cat. No: A07781). Fluo-4 AM (Life Technologies, Cat #F14201), the cell-permeable form of Fluo-4, was prepared as 1 mM stock solution in DMSO, aliquoted and kept at −20 °C. Adenosine 5′-diphosphate (ADP) was from Sigma-Aldrich (Cat # A5285). The stock solution was 10 mM in PBS, aliquoted and kept at −20 °C. Modified Tyrode's Buffer (137 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4 and 10 mM HEPES, pH 7.4) was prepared weekly and stored at 4 °C. Before starting each experiment, 0.35% BSA and 5.5 mM glucose were added,

and the buffer kept at room temperature until use.

2.4. Intraplatelet Ca^{2+} mobilization study in marine mammals

The methodology for the analysis of intraplatelet Ca^{2+} mobilization in marine mammals is detailed in our previous work (Felipo-Benavent et al., 2022). Briefly, citrated whole blood was diluted 1:10 in modified Tyrode's Buffer containing BSA and glucose and then incubated with 5 μM Fluo-4 AM at 37 °C and 5% CO_2 for 15 min in the dark. To identify platelets, 25 μl of blood preloaded with Fluo-4 AM were incubated with 5 μl of CD41-PE for 15 min in the dark at room temperature. Finally, samples were diluted with 500 μl of modified Tyrode's Buffer containing BSA and glucose and transferred to an Eppendorf microtube.

2.5. Flow cytometry setup

All the experiments were performed on a CytoFLEX S Flow Cytometer (Beckman Coulter, California, United States) using the cytometer-interfaced CytExpert software (Beckman Coulter, California, United States). The flow cytometer was set up to measure forward scatter (FSC) and side scatter (SSC) signals, Fluo-4 fluorescence (FL1, exc 488 nm/em 525 nm), CD41-PE fluorescence (FL10, exc 561 nm/em 585 nm). All the signals were set to logarithmic amplification. Time was included as a cytometric parameter with stop condition at 10 min. Single free platelets were identified using the CD41-PE and FSC signals (Fig. 1(1A-E)). Then, intraplatelet Ca^{2+} mobilization kinetics was assessed by activating platelets with 24 μM ADP (Fig. 1(3A-E)). The changes in Fluo-4 fluorescence intensity (expressed in Fluorescence Arbitrary Units, or FAU) refflect the changing levels of intraplatelet cytosolic Ca^{2+}. For extracting numerical parameters from this type of plot, we define analytical regions covering the whole length of the fluorescence axis along the Time axis, i.e. time-windows. In the real-time experiment, the cytometer software automatically displays the selected statistical data for each time window as it is filled with events. The original time-windows can be modified after the run, if necessary, for more accurate numerical analysis. For each region, we registered the mean time (in seconds) and the mean fluorescence intensity (MFI) of Fluo-4, and these numerical values were exported for creating diverse kinetic plots using standard graph software.

Off-line data analysis and display were performed using CytExpert software (Beckman Coulter, California, United States) or FlowJo™ software (Becton Dickinson, New Jersey, USA).

2.6. Obtaining physiological intraplatelet Ca^{2+} mobilization reference values

In order to detect eventual alterations in platelet activation, a previous descriptive study of normal response for each species first was necessary. For this purpose, we calculated the following quantitative parameters from the platelet-related data generated by our flow cytometry assay.

2.6.1. Platelet count

This parameter is directly derived from the volumetric absolute counting system of the CytoFLEX cytometer and is expressed as the number of single $CD41^+$ events per μL of whole blood.

2.6.2. Parameters related to the intraplatelet Ca^{2+} rise induced by 24 μM ADP

a) Ratio between peak and baseline Fluo-4 fluorescence intensity (RPB): The fold that platelet Ca^{2+} increases after ADP addition.
b) Differential between peak and baseline Fluo-4 fluorescence intensity (DPB): The absolute difference in platelet Ca^{2+} concentration between the activated and resting platelets.

2.6.3. Parameters related to the recovery of resting intraplatelet Ca^{2+}

a) Slope of the Fluo-4 fluorescence curve from the peak to the end-point (SPE): The rate of recovery of cytosolic Ca^{2+} concentration after platelet activation.
b) Ratio between end-point and baseline Fluo-4 fluorescence intensity (REB): The fold that platelet Ca^{2+} remains elevated above the Ca^{2+} concentration in resting platelets at 10 min post-activation.
c) Differential between end-point and baseline Fluo-4 fluorescence intensity (DEB): The absolute difference between cytosolic Ca^{2+} concentration remaining at 10-min post-activation and in resting platelets.

2.7. Statistical analysis

The mean, median and standard error of the median (SEM) were calculated for each parameter in all the species. The results were considered statistically significant when p-value <0.05. To evaluate differences in these parameters between animals per aquarium, sex and age we used a *t*-test in Graphpad Prism 5. To evaluate global differences between species, the distribution of the data for each parameter studied (platelet count, RPB, DPB, REB, DEB, and SPE) was tested using Shapiro-Wilk test. In cases where the data distribution was normal the One-Way ANOVA test was utilized. When the comparison resulted statistically significant, the Tukey's post-hoc test was required for the multiple comparison analysis. In cases where the data distribution was not normal, the Kruskal Wallis test was used for the multiple comparision analysis.

3. Results

3.1. Flow cytometric detection of intraplatelet Ca^{2+} mobilization in platelets activated with ADP

In line with our previous findings with whole blood platelets in humans (Monteiro et al., 1999, 2003, 2022; García-Martínez et al., 2004; Melo-Ferraz et al., 2021) and bottlenose dolphins (Griffeth et al., 2014; Felipo-Benavent et al., 2022), the human-reacting monoclonal antibody CD41 (Clone P2) was found to identify platelets also in other marine mammals, both Cetacean and Pinnipeds. CD41 molecule is the integrin alpha IIb chain (GPIIb) which is non-covalently associated with the integrin beta 3 chain (GpIIIa) in the GpIIb/IIIa complex, the fibrinogen receptor involved in platelet aggregation (Jackson, 2007). This result supports the interest of systematic searching for suitable immunophenotypic FCM markers for marine mammal blood cells among commercially available anti-human antibodies, as shown for leukocyte immunophenotyping by Nouri-Shirazi et al. (2017).

As shown in Fig. 1 (1A-E) single platelets of all the species could be clearly identified and distinguished from other blood cells by their specific expression of CD41 and morphology (Forward Scatter signal). Platelets bound-to or coincident with erythrocytes and leukocytes were detected as events positive for CD41-PE with highest FSC signals, while platelet aggregates were excluded by means of FSC-height vs. FSC-area signals (Fig. 1 (2A-E)).

For performing the FCM assay (Felipo-Benavent et al., 2022), the baseline of Fluo-4 fluorescence is registered for 50–60 s. At this time, 24 μM ADP (Fig. 1(3A-E)) is added to the sample vial while the cytometer runs the sample up to 600 s. As exemplified in Fig. 1 (3A-E), in all the species tested an almost immediate rise in Ca^{2+} induced by ADP was observed, reflecting Ca^{2+} mobilization from internal stores. The subsequent decrease in Fluo-4 fluorescence indicates re-uptake of cytosolic Ca^{2+} into intraplatelet stores or extrusion across plasma membrane (Monteiro et al., 1999, 2003, 2022; García-Martínez et al., 2004; Melo-Ferraz et al., 2021). For a better comparison of the individual Ca^{2+} kinetics in each species, we have generated simplified plots of the changes of Fluo-4 fluorescence (FAU) in linear scale versus time (Fig. 2).

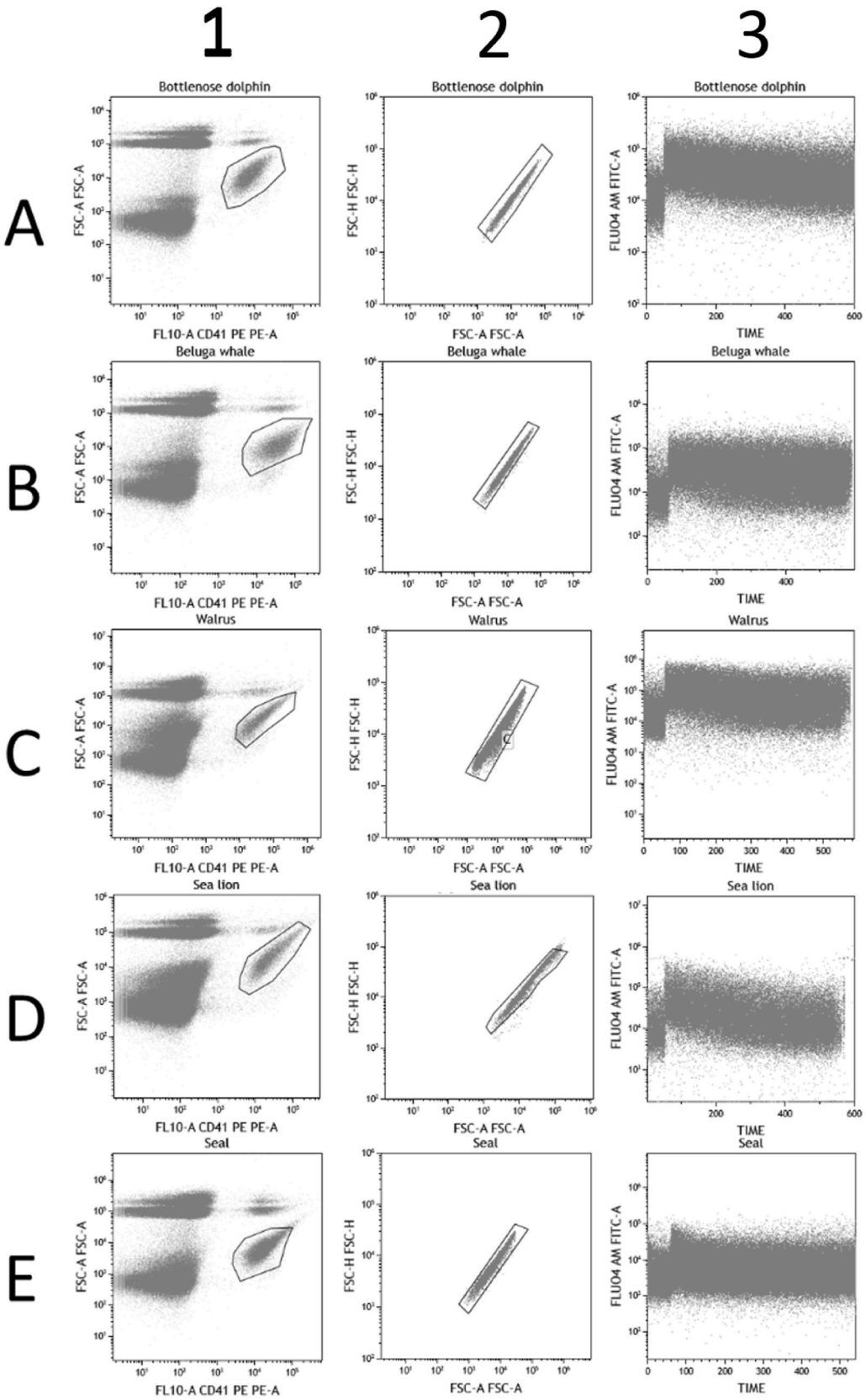

Fig. 1. Analysis by flow cytometry of intracellular Ca^{2+} mobilization in whole blood platelets activated by 24 μM ADP. The graphs show one representative example of the responses observed in (A) Bottlenose Dolphins; (B) Beluga Whales; (C) Walruses; (D) Sea lions; (E) Seal. The graphs are similar for individuals of the same species. (1) Identification and gating of platelets as $CD41^{+}/FSC^{low}$ events. (2) Aggregate exclusion by analysis of FSC-Height vs. FSC-Area of $CD41^{+}/FSC^{low}$ events. The gate region includes only free platelets. (3) Kinetic plot of Ca^{2+} mobilization generated by representing Fluo-4 mean fluorescence intensity per single platelet (Y-axis) versus Time (X-axis). Fluo-4 baseline fluorescence is recorded for 50 s and then 24 μM ADP is added to the sample vial. Changes of Fluo-4 fluorescence are recorded up to 600 s, and reflect the changes of intraplatelet Ca^{2+} concentration induced by ADP.

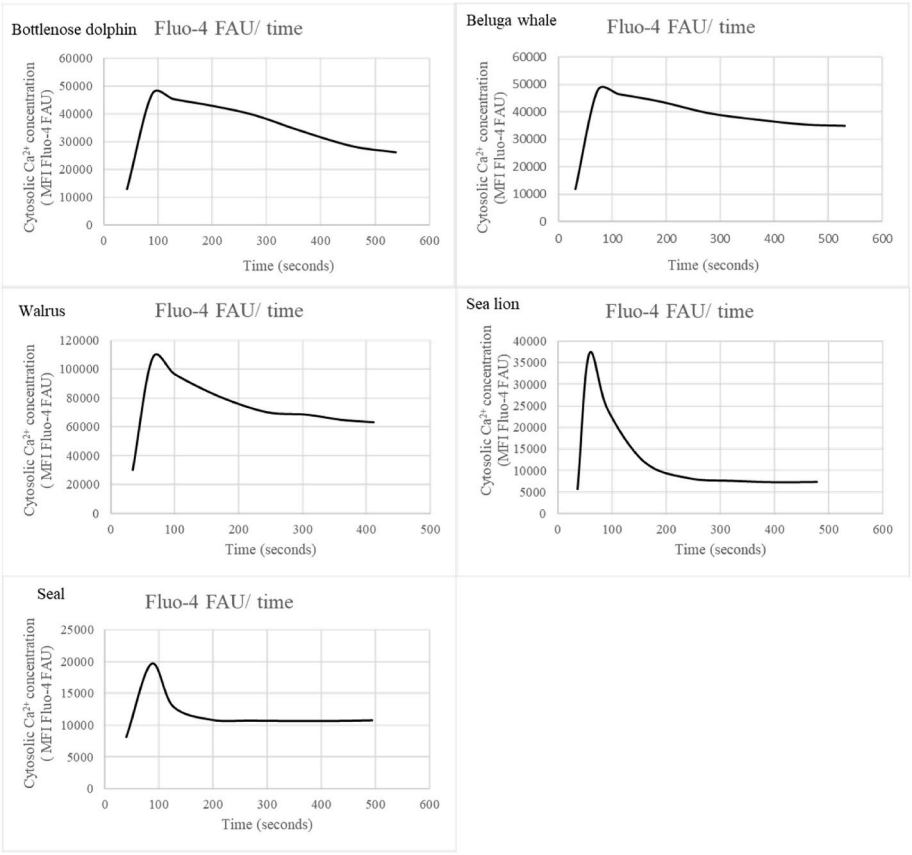

Fig. 2. Morphological rendering of the plots of intracellular Ca^{2+} mobilization in whole blood platelets activated by 24 μM ADP. The graphs show one representative example of the responses shown in Fig. 1 for single platelets of (1) Bottlenose Dolphins; (2) Beluga Whales; (3) Walruses; (4) Sea lions; (5) Seal. The graphs are similar for individuals of the same species. Graphs were generated by exporting the raw cytometric data into FlowJo software.

We have found differences in the general kinetics of platelet Ca^{2+} mobilization among species. Thus, intraplatelet Ca^{2+} released by ADP signaling remained elevated for longer time in cetaceans (bottlenose dolphins and beluga whales) than in pinnipeds (sea lions, walruses and seal), which showed faster recovery of Ca^{2+} to basal levels. Within families, cetaceans exhibited quite a similar Ca^{2+} mobilization kinetics, but among pinnipeds, the highest rise and fastest recovery was clearly observed in sea lions. As indicated before, we had access only to one seal, so the result obtained in this species should be considered as inconclusive.

3.2. Defining numerical indicators of intraplatelet Ca^{2+} mobilization in healthy marine mammals

To asess the reproducibility and sensitivity of our method for comparative and diagnostic studies of platelet function in marine mamals, it was required to define numerical indicators of normal activation of platelets in healthy individuals of each species. To that end, clinically healthy animals were systematically sampled along 3 years, and intraplatelet Ca^{2+} response to ADP addition was assessed by applying the descriptors of platelet activation previously validated in our work on dolphins (Felipo-Benavent et al., 2022).

In Fig. 3 we present an overlay plot of the average results of Ca^{2+} kinetics obtained for all the species investigated, which allow to easily visualize the general differences among species. This graph shows the time points and the Fluo-4 fluorescence data that are necessary for calculating the numerical indicators we apply for objective comparison of Ca^{2+} mobilization kinetics, as described in Material and Methods. Table 1 shows the median ± SEM of the Fluo-4 fluorescence intensity values in single platelets for each species at the baseline, at the peak of

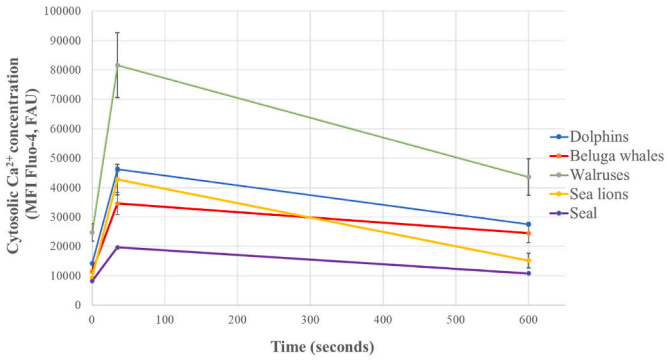

Fig. 3. Graphical description of the changes in the intraplatelet Ca^{2+} concentration induced by 24 μm ADP in marine mammal species. The graph shows the time points (basal, peak, end point) and the Fluo-4 fluorescence data used for calculating the numerical indicators for objective comparison of Ca^{2+} mobilization kinetics. The mean fluorescence intensity of Fluo-4 (MIF) in fluorescence arbitrary units (FAU) for each species is represented at time points: (1) baseline (i.e., resting platelets); (2) maximal Fluo-4 MIF (i.e., the Ca^{2+} peak after stimulation with ADP); (3) 600 s after stimulation with ADP (i.e., the end-point of analysis, including Ca^{2+} recovery to intraplatelet reservoirs). For each species, the values at each point are the mean of those obtained in all sampled animals (66 samples from 20 bottlenose dolphins, 20 samples from 3 beluga whales, 15 samples from 3 walruses, 9 samples from 8 sea lions, 1 sample from 1 seal).

maximum Fluo-4 fluorescence after ADP addition and at the end-point of the curve (600 s). As it can be seen, walruses had higher cytosolic Ca^{2+} concentrations than the other species at all points of the curve, while in

Table 1
Numerical description of the changes in the intraplatelet Ca^{2+} concentration induced by 24 μm ADP in marine mammal species.

SPECIES	FLUO-4 MEAN FLUORESCENCE INTENSITY (FAU)		
	Baseline	Peak	End-point
Bottlenose Dolphins (*Tursiops truncatus*) n = 20; 66 samples	14213 ± 241	46179 ± 836	27553 ± 610
Beluga Whales (*Delphinapterus leucas*) n = 3; 20 samples	11353 ± 1909	34593 ± 3695	24470 ± 3200
Walruses (*Odobenus rosmarus*) n = 3; 15 samples	24726 ± 2936	81613 ± 10996	43591 ± 6241
Sea lions (*Otaria flavescens*) n = 8; 9 samples	9415 ± 1290	42754 ± 5168	15150 ± 2428
Seals *(Phoca vitulina)*: n = 1; 3 samples	8156 ± 3155	19669 ± 3623	10796 ± 3519

Kinetic analysis of Ca^{2+} mobilization was performed as described in Material and Methods. The time points are shown in Fig. 3. Data are the Median ± SEM of the Fluo-4 mean fluorescence intensity in fluorescence arbitrary units (FAU) for all the identical experiments performed in each species.

all cases the end-point Fluo-4 remained higher than at baseline, indicating that after proximatly 600 s of platelet stimulation with ADP cytosolic Ca^{2+} had not returned to the resting levels.

The ranges of the physiological values for the six calculated indicators of intraplatelet Ca^{2+} mobilization in each species, and the effects of location, sex and age are detailed and discussed below (Tables 2–6).

3.2.1. Cetaceans

The indicators related to platelet concentration and Ca^{2+} mobilization in healthy bottlenose dolphins (*Tursiops truncatus*) are presented in Table 2. No significant differences in any indicator were found among the dolphins from both aquariums, per total group, age or sex. For this reason, all the adult animals could be grouped as a single population. However, calves presented a significantly higher free platelet count than adults and a lower DPB value, which was not accompanied by significant changes in the rest of indicators related Ca^{2+} mobilization kinetics.

The physiological intraplatelet Ca^{2+} mobilization values in beluga whales (*Delphinapterus leucas*) are detailed in Table 3. As in dolphins, no significant differences in any indicator were found related to age or sex. The calf examined had a higher free platelet count than the adults (p = 0,014).

3.2.2. Pinnipeds

The physiological intraplatelet Ca^{2+} mobilization values in walruses (*Odobenus rosmarus*) are detailed in Table 4. As all the walruses were females and of the same age, no sex or age differences could be investigated in this species.

No statistically significant differences were observed in intraplatelet Ca^{2+} mobilization between sea lions (*Otaria flavescens*) from both aquariums, which means that they could be considered a single population. No significant differences were observed between males and females (Table 5). As no calves were sampled for this pilot study, no age differences were assessed.

The intraplatelet Ca^{2+} mobilization results obtained from a healthy seal (*Phoca vitulina*) are shown in Table 6. These results cannot be considered representative of the population but allowed us to estimate how this process works in small pinnipeds. The objective of using samples from this single individual was to set up the methodology in seals.

3.3. Differences in intraplatelet Ca^{2+} mobilization among marine mammal species

Shapiro Wilk test determined that the data for the parameters Platelet count, RPB, DPB and REB, presented normal distribution, so One Way ANOVA test was used to evaluate global differences between species and Tukey's post-hoc was used for multiple comparison analysis. On the other hand, Shapiro Wilk test determined that the data of the DEB and SPE parameters did not present a normal distribution, so the Kruskal Wallis test was used to evaluate global differences between species.

Table 2
Physiological intraplatelet Ca^{2+} mobilization values after activating platelets with ADP in healthy Bottlenose dolphins (*Tursiops truncatus*) under human care.

Group of Animals	Free platelets/ μL	RPB	DPB	REB	DEB	SPE
Dolphins Oceanogràfic n = 15; 61 samples	54000 ± 4800	3.45 ± 0.25	31243 ± 3889	1,97 ± 0,12	11321 ± 1272	−38,4 ± 6
Dolphins Mundomar n = 5; 5 samples	38000 ± 5600	3,4 ± 0,7	42430 ± 8621	1,6 ± 0,1	17881 ± 7616	−66,5 ± 20
Total Group of Dolphins n = 20; 66 samples	50800 ± 4200	3,4 ± 0,2	34040 ± 3675	1,9 ± 0,1	12954 ± 2091	−50 ± 7
Females Oceanogràfic n = 9; 39 samples	50000 ± 5600	3,5 ± 0,3	36071 ± 5247	1,8 ± 0,2	12054 ± 1651	−47 ± 11
Females Mundomar n = 4; 4 samples	38000 ± 5600	3,4 ± 0,7	39187 ± 10313	1,7 ± 0,4	10387 ± 1747	−66,5 ± 20
Total Group of Females n = 13; 43 samples	46400 ± 4400	3,5 ± 0,3	37029 ± 4589	1,7 ± 0,1	11541 ± 1242	−60 ± 9
Total Group of Males n = 7; 23 samples	60400 ± 8600	3,3 ± 0,2	28488 ± 6003	1,57 ± 0,2	15579 ± 5663	−31 ± 9
Adult dolphins Oceanogràfic n = 11; 46 samples	49000 ± 5200	3,50 ± 0,03	35485 ± 4376	1,9 ± 0,1	13024 ± 1317	−54 ± 9,5
Adult dolphins Mundomar n = 5; 5 samples	38000 ± 5600	3,4 ± 0,7	39187 ± 10313	1,6 ± 0,1	10387 ± 1747	−66,5 ± 20
Total Adult Dolphins n = 16; 51 samples	46200 ± 4200	3,5 ± 0,3	37655 ± 3957	1,9 ± 0,1	14542 ± 2443	−49 ± 7
Calves n = 4; 15 samples	68400 ± 8600 p = 0,028*	3,3 ± 0,3	19578 ± 5219 p = 0,046*	1,9 ± 0,3	6606 ± 1529	−24,5 ± 17

Kinetic analysis of Ca^{2+} mobilization was performed as described in Material and Methods. The time points are shown in Fig. 3. Data are the Median ± SEM for all the identical experiments performed. DPB and DPB values are expressed as Fluo-4 mean fluorescence intensity in fluorescence arbitrary units (FAU). The values per parameter are compared for the whole population, origin, age and sex using *t*-test. p-value was only shown when differences were statistically significant (*) Statistically significant difference between calves/adults. There were not representative samples from calves and males from Mundomar.

Fig. 4 presents the statistical significances of each numerical indicator of intraplatelet Ca^{2+} mobilization among all the species studied. This figure allows a better comparison of the activation response than that provided by the simple visual inspection of the Ca^{2+} mobilization kinetics depicted in Figs. 1–3. The break down of the comparisons between species in the studied parameters is detailed below:

3.3.1. Platelet count

One Way ANOVA shows significant differences in the platelet count of the different species (p = 0,0018). Using the Tukey post-hoc analysis

Table 3
Physiological intraplatelet Ca^{2+} mobilization values after activating platelets with ADP in healthy Beluga whales (*Delphinapterus leukas*) under human care.

Group of Animals	Free platelets/μL	RPB	DPB	REB	DEB	SPE
Beluga whales n = 3; 20 samples	**77320 ± 5452**	**3.6 ± 0.3**	**27136 ± 3068**	**2.5 ± 0.2**	**14738.3 ± 2816**	**−34.7 ± 4.8**
Males n = 2; 11 samples	79040 ± 8400	3.8 ± 0.4	27740 ± 4803	2.68 ± 0.4	4182 ± 3912	−36.2 ± 8
Female n = 1; 9 samples	73480 ± 6560	3.5 ± 0.5	26397 ± 3789	2.4 ± 0.3	15419 ± 4289	−32.8 ± 5
Adults n = 2; 15 samples	69600 ± 4120	3.6 ± 0.4	28971 ± 3651	2.58 ± 0.3	16322 ± 3559	−36.2 ± 5.8
Calf n = 1; 5 samples	98000 ± 12000 p = 0.0145*	3.7 ± 0.5	21630 ± 5359	2.3 ± 0.4	9987 ± 3181	−30.2 ± 9.4

Kinetic analysis of Ca^{2+} mobilization was performed as described in Material and Methods. The time points are shown in Fig. 3. Data are the Median ± SEM for all the identical experiments performed. DPB and DPB values are expressed as Fluo-4 mean fluorescence intensity in fluorescence arbitrary units (FAU). The values per parameter are compared for the whole population, age and sex using *t*-test. p-value was only shown when differences were statistically significant (*) Statistically significant difference between calves/adults.

Table 4
Physiological intraplatelet Ca^{2+} mobilization values after activating platelets with ADP in healthy walruses (*Odobenus rosmarus*) under human care.

Group of Animals	Free platelets/μL	RPB	DPB	REB	EB	SPE
Walruses n = 3; 15 samples	70460 ± 4622	3.5 ± 0.3	70062.5 ± 6994	1.8 ± 0.1	23554.2 ± 3460	−111 ± 14.6

Kinetic analysis of Ca^{2+} mobilization was performed as described in Material and Methods. The time points are shown in Fig. 3. Data are the Median ± SEM for all the identical experiments performed. DPB and DPB values are expressed as Fluo-4 mean fluorescence intensity in fluorescence arbitrary units (FAU).

we obtained three different categories (A, B and AB). Bottlenose dolphins correspond to category A, presenting the lowest platelet count, and seals, category B, presenting the highest count. Category AB includes the species of beluga whales, walruses and sea lions, which present similar values between them but different from the other categories (bottlenose dolphins and seals), being the intermediate platelet counts (Figss. 4–1).

3.3.2. Parameters related to the intraplatelet Ca^{2+} rise induced by 24 μM ADP

a) Ratio between peak and baseline Fluo-4 fluorescence intensity (RPB):

One Way ANOVA shows significant differences in the RPB of the different species (p = 0,025). Using the Tukey post-hoc analysis we obtained three different categories (A, B and AB). Category A corresponds to the seal, which presents the lowest RPB value. Category B corresponds to sea lions, with the highest RPB value. Bottlenose dolphins, beluga whales and walruses are grouped in category AB with similar intermediate RPB values between them but different from the other categories (seals and sea lions) (Figss. 4–2). This means that after the stimulation with ADP, the platelets of the sea lions experiment a greater initial Ca^{2+} mobilization from the reservoirs to the cytosol than the rest of the species. The seal presents the lowest cytosolic Ca^{2+} increase, although these data must be taken with caution sampling only one individual. Cetaceans and walruses present a lower initial Ca^{2+} mobilization than sea lions and similar between them.

Table 5
Physiological intraplatelet Ca^{2+} mobilization values after activating platelets with ADP in healthy sea lions (*Otaria flavescens*) under human care.

Group of Animals	Free platelets/μL	RPB	DPB	REB	DEB	SPE
Sea lions Oceanogràfic n = 6; 6 samples	77200 ± 8860	4.9 ± 0.4	36875 ± 4590	1.6 ± 0.2	5800 ± 2379	−164 ± 16
Sea lions Mundomar n = 2; 3 samples	84400 ± 200	4 ± 0.2	22729 ± 727	1.7 ± 0.15	5537 ± 1409	−114.6 ± 18.-7
Total Group Sea lions n = 8; 9 samples	71120 ± 9440	4.6 ± 0.35	31362 ± 3693	1.6 ± 0.1	5413 ± 1414	−148 ± 13-.26
Females n = 5; 5 samples	82600 ± 7400	4.7 ± 0.5	32537 ± 4901	1.7 ± 0.2	7156 ± 2701	−150.2 ± 23
Males n = 3; 4 samples	62800 ± 100	4.5 ± 0.4	34674 ± 8606	1.4 ± 0.1	3366 ± 427	−154 ± 15

Kinetic analysis of Ca^{2+} mobilization was performed as described in Material and Methods. The time points are shown in Fig. 3. Data are the Median ± SEM for all the identical experiments performed. DPB and DPB values are expressed as Fluo-4 mean fluorescence intensity in fluorescence arbitrary units (FAU). The values per parameter are compared for the whole population, origin, and sex using *t*-test. No statistical differences were found for Ca^{2+} mobilization parameters between sea lions from different aquariums or sex.

Table 6
Physiological intraplatelet Ca^{2+} mobilization values after activating platelets with ADP in one healthy seal (*Phoca vitulina*) under human care.

Animal	Free platelets/μL	RPB	DPB	REB	DEB	SPE
Seal n = 1; 3 samples	79700 ± 0,6	2.41 ± 0,05	11513 ± 0,6	1.32 ± 0,06	2640 ± 0,6	−73.24 ± 0,6

Kinetic analysis of Ca^{2+} mobilization was performed as described in Material and Methods. The time points are shown in Fig. 3. DPB and DPB values are expressed as Fluo-4 mean fluorescence intensity in fluorescence arbitrary units (FAU).

b) Differential between peak and baseline Fluo-4 fluorescence intensity (DPB):

Taking the absolute values of cytosolic Ca^{2+} concentration before stimulation and in the peak, walruses mobilize more quantity of Ca^{2+} than the rest of the species, although the difference between cytosolic Ca^{2+} in the resting platelets and in the peak is less than in sea lions (RPB). This is because, as previously mentioned, walruses physiologically present more cytosolic Ca^{2+} in platelets than the rest of the species at all times (baseline, peak and end-point) (Table 1). One Way ANOVA shows significant differences in the DPB of the different species ($p < 0,0001$). Using the Tukey post-hoc analysis we obtained two categories (A and B). Category B corresponds to walruses, with the highest DPB value. Bottlenose dolphins, beluga whales, sea lions and the seal are grouped in category A, presenting similar values of DPB between them but different from the walruses, being the lower values of DPB (Figs. 4–).

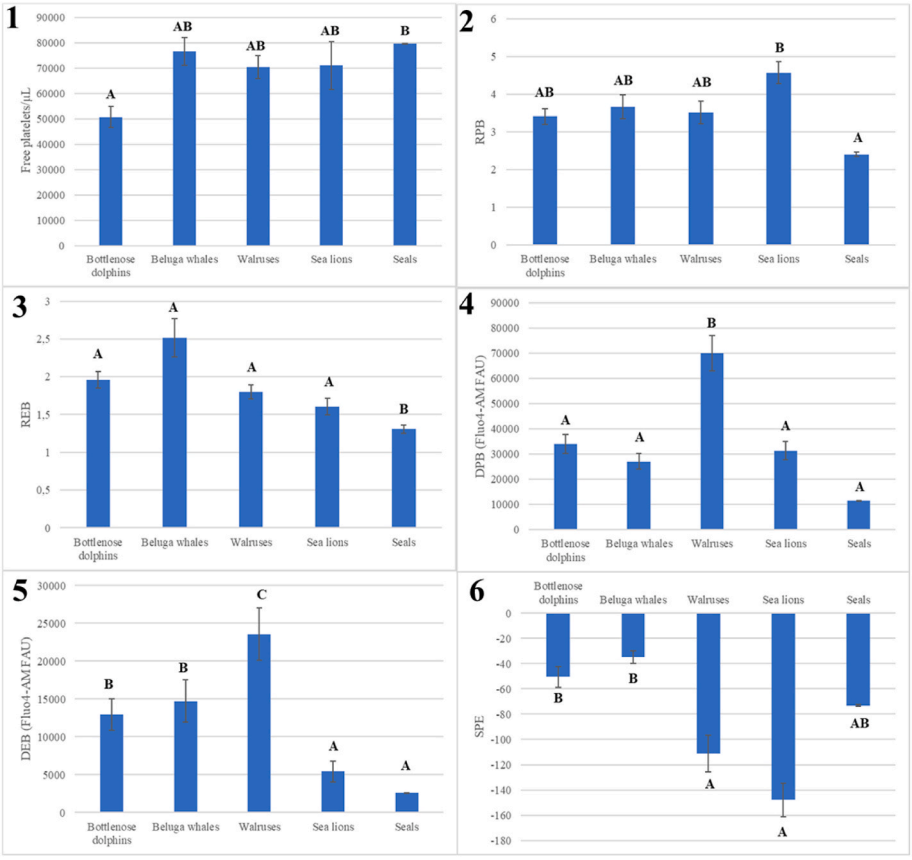

Fig. 4. Statistical comparison of the different indicators of intraplatelet Ca^{2+} mobilization between the different species using One Way ANOVA with post-hoc Tukey for platelet count, RPB, DPB and REB and Kruskal Wallis for DEB and SPE. Significant differences were found for all the studied indicators: (1) platelet count ($p = 0{,}0018$), with three categories (A, B and AB); (2) RPB ($p = 0{,}025$) with three categories (A, B and AB); (3) REB ($p < 0{,}0001$) with two categories (A and B); (4) DPB ($p < 0{,}0001$) with two categories (A and B); (5) DEB ($p = 0{,}0001$) with three categories (A, B and C); (6) SPE ($p < 0{,}0001$) with three different categories (A, AB and B).

3.3.3. *Parameters related to the recovery of resting intraplatelet Ca^{2+}*

a) Ratio between end-point and baseline Fluo-4 fluorescence intensity (REB):

One Way ANOVA shows significant differences in the REB of the different species ($p < 0{,}0001$). Using the Tukey post-hoc analysis we obtained two different categories (A and B). Category B corresponds to the seal, which presents the lowest REB value. Category A includes bottlenose dolphins, beluga whales, walruses and sea lions with similar REB values between them but different from the seal (Figss. 4–3). This means that in seals, cytosolic Ca^{2+} is recovered to its reservoirs more effectively than in other species, presenting similar cytosolic Ca^{2+} concentrations at baseline and end-point (Table 1 and Fig. 3). The rest of the species present a lower Ca^{2+} reuptake rate, presenting a final cytosolic Ca^{2+} concentrations much higher than those of the resting platelets (Figss. 4–3).

b) Differential between end-point and baseline Fluo-4 fluorescence intensity (DEB):

Evaluating the reuptake of cytosolic Ca^{2+} towards the reservoirs using absolute values, we observe that walruses mobilize the most quantity of Ca^{2+}. Again, this is due to the physiological higher cytosolic Ca^{2+} concentration of walruses' platelets than the other species at all points. Kruskal Wallis test indicates differences in the DEB of the different species ($p < 0{,}0001$)and categorizes the species into three groups (A, B and C). Category A corresponds to sea lions and the seal, which present similar values between them but different from the other species, being the lowest DEB values. Bottlenose dolphins and beluga whales are grouped in B category, with similar and intermediate DEB values and different to categories A and C (Figss. 4–5). Category C corresponds to walruses, with the highest DEB value. This means that cetaceans move more quantity of Ca^{2+} during platelet activation than small pinnipeds in absolute values. Walruses' platelets, for their part, are the ones that handle the greatest amount of Ca^{2+}in absolute values, but this does not mean that the changes in cytosolic Ca^{2+} concentration between baseline, peak and end-points are greater in these species, as has been observed evaluating the ratios RPB and REB.

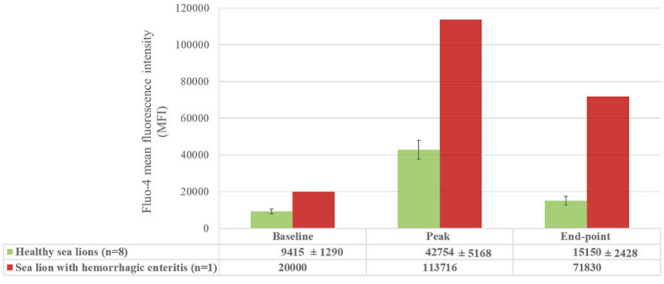

Fig. 5. Cytosolic Ca^{2+} concentration at the baseline, peak and end-points after platelet activation in a sea lion with hemorrhagic enteritis and healthy sea lions population, given by the Fluo-4 mean intensity fluorescence (MFI). Fluo-4 MFI is higher in the sick animal at all points, especially at the peak after platelet stimulation with ADP. The absolute values of MFI are detailed in the chart table.

c) Slope of the Fluo-4 fluorescence curve from the peak to the end-point (SPE):

Kruskal Wallis indicates differences in the SPE of the different species ($p < 0,0001$) and categorizes them into three different groups (A, AB and C). Category A corresponds to sea lions and walruses, which present similar values between them, being the lowest SPE value. It means a steeper rate curve of Ca^{2+} reuptake into the reservoirs. Category C involves bottlenose dolphins and beluga whales, with the highest SPE values, meaning a slower rate of Ca^{2+} reuptake. Category AB correspond to the seal, with intermediate rates of Ca^{2+} reaptake (Figss. 4–6). These results can be easily verified visually observing Fig. 2.

In short, the seal is the one that most effectively recovers Ca^{2+} towards the reservoirs (REB) at 600 s post-stimulation, but moves less quantity of Ca^{2+} than the rest of the species. Sea lions present the fastest reuptake of Ca^{2+} to the reservoirs, although the final concentration is not as close to baseline concentration as in the seal. For their part, cetaceans' platelets carry out the Ca^{2+} reuptake slowly and walruses present intermediate values between cetaceans and small pinnipeds.

All these results suggest that cetaceans are more homogeneous than pinnipeds regarding the kinetics of intraplatelet Ca^{2+} mobilization induced by ADP. Pinnipeds show significant and complex interspecies differences in several indicators. Thus, after platelet activation, RPB was higher in sea lions than in the rest of species, although DPB was greater in walruses. This indicates that sea lions have more intense response of Ca^{2+} mobilization than other pinnipeds and than cetaceans. Also, Ca^{2+} recovery rate to internal reservoirs was more efficient in seals but faster in sea lions, as indicate the value of SPE.

3.4. Application of intraplatelet Ca^{2+} mobilization measurement to veterinary control in real clinical cases

After establishing the physiological intraplatelet Ca^{2+} mobilization values per species, it was possible to detect alterations in several clinical cases of animals with hemostatic disorders.

3.4.1. Clinical case 1: sea lion with hemorrhagic enteritis

The first case we present is a sea lion presenting hemorrhagic enteritis as a consequence of an accidental ingestion of a foreign body. During the process, our group measured the intraplatelet Ca^{2+} mobilization as a platelet activation biomarker. The animal presented some different values from those of healthy sea lions, established in point 3.2 (Table 5).

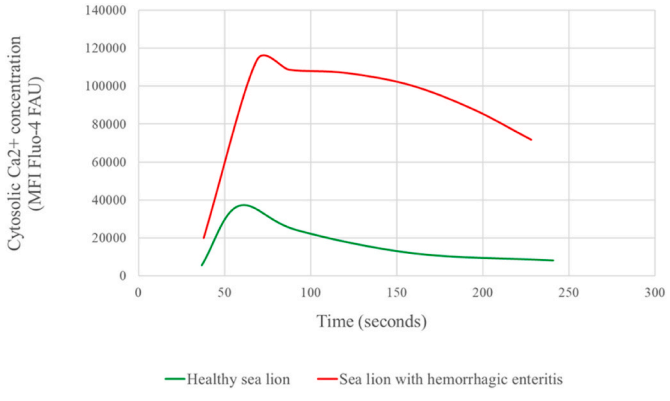

Fig. 6. Cytosolic Ca^{2+} concentration changes over time (MFI of Fluo-4 FAU) after activating platelets with ADP in samples from a healthy sea lion (green) and a sea lion with hemorrhagic enteritis (red). The animal with a hemorrhagic disorder presented a higher platelet cytosolic Ca^{2+} concentration in the resting state, a greater platelets response to ADP and a lower final Ca^{2+} recovery to intraplatelet reservoirs at the end-point than healthy animals. The healthy animal values is representative of the mean values of the total population of healthy sea lions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

On the one hand, in the sick animal the resting platelets presented a higher basal cytosolic Ca^{2+} concentration comparing to that observed in the healthy individuals (Figs. 5 and 6). It could indicate greater basal platelet activity in response to the hemorrhage. The possibility that the animal also suffered some stress as a result of the discomfort of the pathology must also be taken into account. As previously mentioned, acute stress has been related to platelet activation in other mammals (Zoccarato et al., 1991; Alarayyed et al., 1997; Butt et al., 2005).

When platelets were stimulated with ADP, the release of Ca^{2+} from reservoirs to the platelet cytosol was greater in the sick animal than in the healthy population (RPB and DPB) (Table 7), showing a more reactivity in platelets during the bleeding. This result can be easily observed in Figs. 5 and 6.

Regarding the Ca^{2+} reuptake into the reservoirs, the sick animal presented a less effective reorganization of the Ca^{2+} to the internal organelles, showing a greater difference between baseline Ca^{2+} in resting platelets and final levels. Despite this fact, the speed of Ca^{2+} reuptake is greater in the sick animal (SPE) (Table 7). Since the cytosolic Ca^{2+} concentration after platelet stimulation is much higher in the sick animal, despite the reuptake rate is higher than in healthy animals (SPE), it does not get as close to basal cytosolic levels as platelets from healthy animals at the end-point.

In conclusion, the platelets of the sick animal presented a different pattern of intraplatelet Ca^{2+} mobilization than those of healthy sea lions. This demonstrates the efficacy of this assay to detect alterations in platelet function in animals suffering diseases related to hemostasis, in this case, an hemorrhage.

3.4.2. Clinical case 2: Bottlenose dolphin with acute hemorrhagic gastritis

The second case we present is a dolphin with acute hemorrhagic gastritis. During the process, our group measured the intraplatelet Ca^{2+} mobilization as a platelet activation biomarker. The animal presented some different values from those of healthy dolphins, established in point 3.2 (Table 2).

The sick dolphin platelets showed a greater reactivity than those of the healthy dolphins when activated, presenting a 55.8% higher RPB than healthy population (Table 8).

Despite the fact that more Ca^{2+} is initially mobilized (RPB), it is then redistributed into the reservoirs just as efficiently (done by a similar REB) or even more (done by a lower DEB) in the sick animal than in the healthy ones, because the reuptake rate is higher in it than in the healthy dolphins (SPE) (Table 8).

In conclusion, applying the assay to measure intraplatelet Ca^{2+} mobilization, alterations in platelet function can be detected in the different species. We have demonstrated that animals with diseases related to hemostasis could present different values of Ca^{2+} mobilization from those established as physiological for its species in this same work.

It suggests that the adaptation of this assay to marine mammals is useful for diagnosing and monitoring platelet functionality disorders,

Table 7
Intraplatelet Ca^{2+} mobilization values after activating platelets with ADP in a sea lion with hemorrhagic enteritis and in healthy sea lions.

Animals	RPB	DPB	REB	DEB	SPE
Healthy sea lions n = 8; 9 samples	4.6 ± 0.35	31362 ± 3693	1.6 ± 0.1	5413 ± 1414	−148 ± 13.26
Sea lion with hemorrhagic enteritis n = 1; 1 sample	**5.6**	**93713**	**3.6**	**51827**	**−243**

Kinetic analysis of Ca^{2+} mobilization was performed as described in Material and Methods.The animal suffering a hemorrhage presented higher values of all the parameters of Ca^{2+} mobilization, indicating a more reactive platelets during the process.

Table 8
Intraplatelet Ca^{2+} mobilization values after activating platelets with ADP in a dolphin with acute hemorrhagic gastritis and in healthy dolphins.

Group of Animals	Free platelets/ µL	RPB	DPB	REB	DEB	SPE
Dolphins under human care n = 20; 66 samples	50,800 ± 4200	3,4 ± 0,2	34,040 ± 3675	1,9 ± 0,1	12,954 ± 2091	−50 ± 7
Dolphin with acute haemorrhagic gastritis n = 1; 1 sample	54,200	**5,3**	**42981**	1,8	**7654**	**−94,2**

Kinetic analysis of Ca^{2+} mobilization was performed as described in Material and Methods. The animal suffering a hemorrhage presented higher values of RPB, DPB and SPE, with a lower DEB value than healthy animals, indicating a more reactive platelets and a more efficient Ca^{2+} reuptake.

which may help to apply solutions early improving preventive medicine.

4. Discussion

Agonist-induced Ca^{2+} mobilization is a good indicator of platelet functionality in humans (Monteiro et al., 1999; Assinger et al., 2015; Bourguignon et al., 2022) but to our knowledge no application in veterinary clinics has been published. So much so that it has been used as a biomarker of platelet function in various studies with mice, humans, or rabbits (Ngo et al., 2018; Watala et al., 1998; Shiraishi et al., 2010). The data presented here have been obtained as a result of our adaptation to marine mammals of an existing FCM method to measure intraplatelet Ca^{2+} mobilization, repeatedly used in humans and mice (Monteiro et al., 1999, 2003, 2022; García-Martínez et al., 2004; Melo-Ferraz et al., 2021). Such protocol has been recently applied to dolphins by our group (Felipo-Benavent et al., 2022), and extended here to other cetacean and pinniped species. This accomplishment represents a pioneer methodological advance to study hemostasis in marine mammals, and an important contribution for health management in aquariums and in the wild.

The utility of a cross-reacting anti-human monoclonal antibody (CD41, Clone P2) to identify platelets in bottlenose dolphins (Griffeth et al., 2014; Felipo-Benavent et al., 2022) and other cetaceans and pinnipeds supports the interest of assessing commercially available anti-human antibodies for FCM analysis of marine mammal blood cells, as shown for leukocyte immunophenotyping in bottlenose dolphins (Nouri-Shirazi et al., 2017). CD41 protein is the integrin complex alpha-IIb/beta-3 (GPIIβ/IIIα), the receptor for fibronectin, fibrinogen, plasminogen, prothrombin, thrombospondin and vitronectin. CD41 recognizes the sequence R-G-D in a wide array of ligands and following activation, CD41 allows platelet/platelet interaction through binding of soluble fibrinogen (Hagemeyer and Peter, 2010). The P2 antibody used by us reacts with GPIIβ in intact complex with GPIIIα, but not with GPIIβ or GPIIIα alone (Phillips et al., 1988).

Human GPIIβ is encoded by the ITGA2B gene (www.genecards.org, 2023). This gene was present in the common ancestor of animals and currently 191 orthologs have been described, of which 87 in placental mammals, including some Cetacean species (www.ensembl.org, 2023). However, no information on ITGA2B gene orthologs in Pinnipedes is available. On the basis of protein sequence alignment, Table 9 shows that CD41 in six Cetacean species has a high degree of similarity (81.07–86.46%) with Human CD41. Two of those species are included in our platelet study, namely Bottlenose dolphin (85.49% similarity) and Beluga whale (81.07% similarity). The similarity with Human CD41 of Cetacean CD41 is within the range of the similarity with Human CD41 of Human and Non-Human Primate CD41 (81.65–99.52), and higher than the CD41 similarity of laboratory species frequently used as

Table 9
Similarity of CD41 protein sequence from selected species with the human CD41 sequence.

Species	Gene Identity	Similarity of protein sequence alignment (%)
Bottlenose Dolphin (Tursiops truncatus)	ENSTTRG00000010524	***85.49***
Beluga whale (Delphinapterus leucas)	ENSDLEP00000010874	***81.07***
Blue whale (*Balaenoptera musculus*)	ENSBMSG00010016509	86.46
Sperm whale (*Physeter catodon*)	ENSPCTG00005018981	85.59
Narwhal (*Monodon monoceros*)	ENSMMNG00015016602	84.91
Vaquita (*Phocoena sinus*)	ENSPSNG00000018166	85.11
Human- and Other Primates (23 species)	–	81.65–99.52
Mouse (*Mus musculus*)	ENSMUSG00000034664	80.45
Pig (*Sus scrofa*)	(ENSSSCG00000063008)	78.86

Gene identity indicates the Ensembl code for the human ITG2BA gene and its orthologues in each species. Sequence alignment and similarity data have been calculated by the BLAST tools embedded in the Ensembl portal (https://www.ensembl.org/Homo_sapiens/Gene/Compara_Ortholog?db=core;g=ENSG00000005961;r=17:44372180-44389649).

cardiovascular and immunological models, namely the mouse (80.45% similarity) and the pig (78,86% similarity).

By using CD41 as a suitable platelet marker, we have found interesting differences in the resting concentration of circulating platelet among the five marine mammals studied. On the one hand, dolphins presented the lower platelet count of all species tested (Fig. 4), while beluga whales, walruses and sea lions had platelet counts in a similar range, which is in accordance with earlier reports (Levin, 2019; Lauderdale et al., 2021). On the other hand, in both cetacean species investigated, blood platelet concentration was significantly higher in calves than in adults. Several previous works have described a higher platelet concentration, or wider ranges with a higher upper limit, in young cetacean individuals (De Mello and Da Silva, 2019; Hall et al., 2007) in accordance with the inverse association between age and platelet count in humans (Le Blanc and Lordkipanidzé, 2019), cattle (Gonzalez-Garduño et al., 2023), chicken (Hong et al., 2021) but not in Guinea pigs (Spittler et al., 2021) or Breton horses raised in Brasil (Da Conceição et al., 2022). In all cases, this parameter remained within the established reference range for these species (De Mello and Da Silva, 2019; Hall et al., 2007).

Marine mammal platelets have been previously studied by different groups, mainly from a hematological or morphological point of view (Patterson et al., 1993; Schaefer et al., 2011; Field and Tablin and 2012; Ponomareva et al., 2017). Platelet functional studies, however, are infrequent in these species. In previous works conducted with samples from killer whales or elephant seals, platelet activation has been studied by aggregometry, membrane lipid composition evaluation or thromboxane production. All these assays require sample washing, fixation or centrifugation (Patterson et al., 1993; Field and Tablin and 2012). In our functional FCM assay, citrated whole blood is diluted in specific buffer and labeled with fluorescent markers, reducing sample manipulation and ensuring near-physiological conditions. This methodology demonstrates consideration for platelet function and is applicable for veterinary clinical purposes as well as research studies focused on the physiology, physiopathology, or in vitro toxicology of marine mammals (Felipo-Benavent et al., 2022).

Our FCM assay of intraplatelet Ca^{2+} mobilization is performed on a quantitative basis and provides numerical parameters that contribute a rational description of the rise in cytosolic Ca^{2+} (namely, RPB and DPB) and the subsequent return of intraplatelet Ca^{2+} to resting values (namely, REB, DEB and SPE). It should be remarked that most of these

numerical descriptors are normalized indicators that allow to compare objectively the kinetics of Ca^{2+} response to ADP stimulation in different specimens or under different conditions. Moreover, our previous work has shown that these indicators reduce interindividual variability, and that the FCM assay has a good reproducibility, when carefully performed (Felipo-Benavent et al., 2022).

Whenever possible, our study has included animals from two different aquariums, and sex- or age differences have been also investigated (Supplementary Table 1). The results are more robust in dolphins because more animals, from two aquariums, and more samples are included. The data obtained with a single specimen of seal are anecdotical and must be considered only as a preliminar study mainly used for set up the assay in this species.

Within each species, we did not find any significant differences in Ca^{2+} response to ADP related to the age, sex and aquarium. Extending the study to animals from other centers would be highly relevant for adding these values to existing repertories of biochemical and cellular ranges in marine mammals (Wells et al., 2004; Nouri-Shirazi et al., 2017; Lauderdale et al., 2021).

The discussion of our results regarding the kinetics of platelet Ca^{2+} response to ADP is complex since, to our knowledge, this type of study is new in marine mammals. Our statistically validated observations reveal both intraorder and interorder differences in marine mammals. Marine mammals, which encompass various taxonomical orders, are a diverse group of organisms. This group consists of fully aquatic species (cetaceans) as well as semi-aquatic species (pinnipeds), each of which has adapted its biochemistry and physiology to suit their unique aquatic environment. These adaptations differ not only among marine mammal species but also from their terrestrial ancestors and relatives (Atkinson et al., 2015). Nonetheless, cetaceans and pinipeds present a similar pattern of intraplatelet Ca^{2+} mobilization and similar to that observed in humans too (Monteiro et al., 2003). Specifically, cetaceans presented more homogeneous pattern of Ca^{2+} mobilization induced by ADP than pinnipeds, which showed more interspecies differences. Sea lions showed a more reactive response to ADP than the rest of species, mobilizing more Ca^{2+} into the cytosol after stimulation (RPB). The subsequent Ca^{2+} reuptake to the reservoirs was more efficient in seals but faster in sea lions. Cetaceans presented the slowest recovery of intraplatelet Ca^{2+}, specially in dolphins. To establish more robust reference values for intraplatelet Ca^{2+} mobilization parameters in comparative physiological studies, further studies in animals from other aquariums and from the wild would be required.

The physiological values of the indicators of Ca^{2+} mobilization provide references to detect early alterations of platelet function caused by pathologies or by stress-associated conditions, including anthropogenic- and natural stressors (Thompson and Romano, 2019). This has been demonstrated in this work, where two different clinical cases of animals with haemorrhages were presented. In both cases the bioindicator of intraplatelet Ca^{2+} mobilization was altered, presenting several parameters out of the physiological range established for each specie. Testing routinely this platelet function biomarker can be a very useful tool in the early diagnosis and monitoring of hemostasis related diseases.

The discussion on stressors affecting marine mammals encompasses both common stressors applicable to captive and wild individuals, as well as distinctions between these settings. Understanding these stressors is essential for effective management and welfare considerations, taking into account the constant human interaction or lack of environmental complexity in the aquariums or the predation risk, environmental fluctuations, exposure to natural pathogens and diseases, and disturbances caused by human activities in the wild (Agusti et al., 2022). This would be of special relevance for the growing interest in the consequences of stress in marine mammals and the adaptative mechanisms that different species may invoke (Atkinson et al., 2015), most specially in relationship with diving physiology and the interferences of stressors there on, which are associated to disturbances of immunological and hormonal parameters (Thompson and Romano, 2015), including catecholamine overproduction (Thompson and Romano, 2019) which is known to induce activation of human platelets under acute stress conditions (Lages et al., 1981; Salamah et al., 2019; Alarayyed et al., 1997; Zoccarato et al., 1991; Hjemdahl and Wallén, 1997; Butt et al., 2005; Cimminiello et al., 1989; Feoktistov et al., 1992). Interestingly, platelets are affected by hyperbaric pressures (Olszanski, 1999; Brett et al., 2019; Scheiber et al., 2022) and platelet activation has been associated to the onset and the consequences of decompressive sickness in humans (Pontier et al., 2012; Barak et al., 2020; Cialoni et al., 2022) and rabbits (Cao et al., 2020), which are linked to decreased platelet count and generation of circulating platelet-derived microparticles. In contrast to terrestrial mammals, the platelets of marine mammals, including Killer whale (Patterson et al., 1993) and seal (Field et al., 2001) are not activated by simulated- or real diving to great depths and excessive platelet reactions during or after diving are not observed. While this has been suggested to depend intrinsecally on the elevated cholesterol content of these platelets, as compared to human platelets, other platelet-related mechanisms may be involved. Interestingly, both bottlenose dolphins and killer whales (*Orcinus orca*) lack coagulation factor XII (Hageman factor), resulting in longer clotting duration (Robinson et al., 1969). It has been proposed also that other processes related to the hydrodynamics and physiology of air-bubble management might explain totally or in part the lower impact of gas emboli pathology in marine mammals (Fahlman et al., 2021). It is expectable that our Ca^{2+} mobilization assay may provide a new and convenient experimental tool for studying platelet activation either in physiological settings or in experimental models of hyperbaric pressure and decompression.

In a quite different context, the intraplatelet Ca^{2+} mobilization assay presented here could be easily applied to assess the quality and efficacy of platelet-rich plasma (PRP) preparations used for regenerative medicine in marine mammals. PRP is an autogenous source for growth factors based on platelet concentration, which can be obtained by centrifuging whole blood collected in sodium citrate anticoagulant. As shown for dolphins (Griffeth et al., 2014) and sea lions' infusion of PRP is a biological therapy enhancing wound healing through the secretion of growth factors stored in platelet granules (Griffeth et al., 2014). Currently, there are well-defined PRP concentration protocols for humans and most domestic companion animal species but there is no clear centrifugation protocol for obtaining PRP in most marine mammal species (Morón-Elorza et al., 2021). Our assay would be useful for monitoring the suitability of the delicate platelet-separation procedures required and the ability of PRP to undergo appropriate activation, as a necessary step to release the intraplatelet growth factors.

In conclusion, the intraplatelet Ca^{2+} mobilization assay in marine mammals has the potential to serve as a valuable tool for monitoring environmental changes. By assessing platelet functionality, this assay can provide insights into the health and well-being of marine mammals in response to environmental stressors. Deviations from baseline values can indicate the impacts of contaminants, toxins, and other environmental stressors on platelet function. Additionally, shifts in intraplatelet Ca^{2+} mobilization patterns can reflect changes in prey availability, habitat degradation, and other environmental factors. Regular monitoring of intraplatelet Ca^{2+} mobilization in wild populations can help track changes over time and establish correlations between environmental changes and platelet functionality alterations. This information can inform conservation strategies and aid in the protection of marine mammal populations in the face of evolving environmental conditions.

Therefore, incorporating routine measurements of platelet function in marine mammals would improve comparative studies and preventive veterinary medicine. Our new intraplatelet Ca^{2+} mobilization assay could, therefore, be a useful diagnostic tool in aquariums and in free-range animals for the early detection of hemostatic pathologies and to improve health monitoring. In this regard, the data provided in this study could be useful as a quantitative reference for detecting alterations of platelet function in marine mammals, which could be related to

different pathologies, exposure to xenobiotics and other stress conditions.

Author contributions

All authors contributed to the writing, review and editing of the manuscript. T.A.A, M.V., and C.R. were responsible of the blood sampling and sanitary control of the animals. M.F.B, performed the flow cytometry measurements; M.F.B, A.M.R., C.R.G and J.E.O performed the interpretation of the results. M.F.B., D.G.P and C.R.G contributed to the first draft of the article and performing tables and figures. J.E.O and A. M.R. contributed to the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2023.105080.

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