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An immune response-based approach to evaluate physiological stress in rehabilitating loggerhead sea turtle

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ABSTRACT

The sea turtles maintaining in rescue centres can cause physiological stress to the animals with subsequent effects, such as the imbalance of immune system components. It is therefore crucial to investigate how innate immune responses are influenced by stress within recovery centers with the aim to use them as primary tools for the evaluation of the rescued specimens' health status and for developing more effective conservative strategies. In this study we monitored for the first time different immune responses in hospitalized Mediterranean loggerhead sea turtles (*Caretta caretta*) (n = 88), comparing them with free-ranging animals (n = 11). The final scope was to identify sensitive tools based on immune responses parameters that rescue centers operators could use to verify the stress condition of hospitalized specimens. Blood samples were obtained from loggerhead sea turtles hospidalized for different periods (≤2 months; > 2 months and ≤1 year; > 1 year) in various Italian rescue centers. Free-ranging turtles were captured in the South of Spain during a shipboard survey with a minimal invasive technique. Samples were analyzed for different stress-associated parameters (white cells count, heterophils:lymphocytes (H:L) ratio, respiratory burst, total antioxidant status (TAS), lysozyme). Free-ranging specimens showed lower values for most of the measured parameters. The highest values of TAS, lysozyme activity, respiratory burst and leukocytes profile were detected during the first 2 months of hospitalization, that resulted as the most critical period for the rehabilitation of turtles. After more than 1 year, immune values were similar to the values of free-ranging, indicating an acclimatization to captivity and health conditions amelioration. Moreover, monocytes low levels and eosinophils significant decrease in hospitalized animals indicated the absence of chronic inflammations and reduction of parasitic load during rehabilitation. Statistical analysis pointed out that lysozyme activity and eosinophyls count represent valid methods to diagnose physiological stress and inflammation in hospitalized loggerhead sea turtles.

1. Introduction

Loggerhead sea turtle (*Caretta caretta*) is a long-living omnivorous animal and the most abundant and widespread species of the Mediterranean Sea (Margaritoulis et al., 2003). Since 2015 the Mediterranean subpopulation is listed by the International Union for Conservation of Nature (IUCN) as "least concern". The current population is the result of decades of intense conservation programs (establishment of sea turtle facilities, European projects, nesting monitoring, national and international guidelines and laws) and the cessation of these programs would result in population decrease.

Among several measures of conservation, particular attention should be given to the maintaining of sea turtles in the rescue centres. The first Mediterranean rescue centre was opened in 1994, and after more than twenty years nearly fifty rescue facilities are distributed along the Mediterranean Sea coasts (Ullmann and Stachowitsch, 2015). Handling and transportation procedures of stranded animals and their maintaining in the rescue centers, combined with environmental threats (fisheries bycatch, boat strikes, intentional killing and entanglement in marine debris including ghost gears, degradation and reduction of nesting habitats) cause a physiological stress that must be considered when assessing the health status of captive and rehabilitating animals (Perrault et al., 2016). To the best of our knowledge, no data are available on the evaluation of stress levels in sea turtles hospitalized in Mediterranean rescue centres.

Physiological stress can cause the imbalance of some components of the immune system in rescued animals, for example adrenal hormones and infectious diseases increase, chronic inflammation (Flower et al.,

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2015; Hunt et al., 2016). Reptiles, and in general all vertebrates, possess both innate and adaptive immune system. The adaptive component involves cell-mediated and humoral mechanisms that generate an immunological memory; it responds slowly and specifically, while the innate branch represents the first line of non-specific and rapid defence and responds without previous exposure of the organism to invaders (Zimmerman et al., 2010). In reptiles, the innate system is more efficient then the humoral system and includes lymphocytes, heterophils, eosinophils, basophils, monocytes, macrophages, respiratory burst, natural antibodies, cytokines, complement system, antimicrobial peptides, etc. Up to now, studies on reptiles' immune system, and particularly of loggerhead sea turtles, are scarce and conducted on isolated leukocyte cultures (Rousselet et al., 2013a, 2017).

Measurement of hematological and biochemical analyte ranges are commonly used as a diagnostic technique to assess the health status of reptiles and many of these parameters can be used to evaluate stress levels. The most popular method to assess the physiological stress, as reported by some papers (Flower et al., 2015; Romero, 2004; Wikelski and Cooke, 2006), is the measurement of glucocorticoid hormones levels in plasma, such as corticosterone (Rousselet et al., 2013b). Although it has been proven to be a useful tool, the rapid change in hormone levels immediately after the capture makes it difficult to obtain baseline values. The use of haematological parameters such as relative white blood cells count (WBC) on blood smears is particularly useful in the conservation field because it represents an alternative method for measuring stress, due to rapid sampling and relative inexpensive analysis. Reptiles, such as the majority of vertebrates, have five types of WBCs: lymphocytes, heterophils (replaced with neutrophils in mammals), eosinophils, basophils and monocytes. Some authors report that reptiles have a sixth cell type, called azurophils (Hawkey and Dennett, 1989; LeBlanc et al., 2000). All these cells play a major role in protecting the body against both infectious disease and foreign invaders (Stacy et al., 2011). Although Casal and Oròs (2007) characterized the different types of leukocytes in *Caretta caretta*, only few papers investigated leukocytes profile variations in hospitalized turtles (Camacho et al., 2014; Flower et al., 2015; Hunt et al., 2016).

In presence of environmental stressors, the glucocorticoid hormones increase, causing changes in the leukocyte components: circulating lymphocytes adhere to the walls of blood vessels, and subsequently undergo transmigration from circulation into other tissues (lymphonodes, spleen, bone marrow and skin). This exodus causes a significant reduction in their circulating numbers. In contrast, glucocorticoids also stimulate an influx of neutrophils into the blood from bone marrow and attenuate the egress of neutrophils from the blood to other compartments (Davis et al., 2008). The proportion between heterophils and lymphocytes (called H:L ratio) seems to be conserved across all vertebrates (Cabagna et al., 2005; Chen et al., 2007; Davis and Maerz, 2008; Moreno et al., 2002; Pfaff et al., 2007; Witeska, 2005) and researchers consider it an alternative and fast approach to evaluating the stress responses (Davis et al., 2008).

Of all the circulating leucocytes, monocytes and heterophils are considered the cells with the major phagocytosis activity, the principal mechanism responsible for the elimination of invaders from the organism (Zimmerman et al., 2010). Following phagocytosis the expression of NADPH oxidase, a membrane-bound enzyme that generates intracellular reactive oxygen species (ROS), is the initial step of a process called respiratory burst, which kills ingested microorganisms through toxic molecules (Kuby et al., 2007; Levin et al., 2007). Leukocytic production of ROS is an ancient innate immune response observed in birds (Rodriguez et al., 2001; Terrón et al., 2004), reptiles (Mccoll and Daniels, 1988), and teleost fish (Panigrahi et al., 2004; Selvaraj et al., 2006), as well as in invertebrates such as insects (Bergin et al., 2005; Koike et al., 1991). This process seems to be exploited mainly by lymphocytes in *Caretta caretta* (Rousselet et al., 2013a) and by heterophils and monocytes in crocodilians (Merchant et al., 2009).

The respiratory burst permits to eliminate microorganisms from the organism, but it also increases the oxidants produced endogenously that cause physiological stress to the organism. Total antioxidants (enzymatic and non-enzymatic molecules) represent the first line of defence against the excessive production of ROS (Kefer et al., 2009). Among several methods used in ecotoxicology to investigate the oxidative stress, measurement of total antioxidant status (TAS) has been developed to determine the collective effect of antioxidant defence (enzymatic and non-enzymatic). This tool has been developed in humans (Miller et al., 1993) and over the years, applied in different phyla because of its fast, easy and versatile applicability on different tissues (Amado et al., 2009; Arukwe et al., 2015; Bashir et al., 2015; Bilham et al., 2013; Cohen et al., 2007; Zahran and Risha, 2014), but it has not been reported previously for any sea turtle species.

Phagocytes (i.e. macrophages, monocytes and heterophils), in addition to respiratory burst, release lysozyme into the mucus, saliva and plasma, an enzyme able to digest the surface of Gram-positive bacteria (Balfry and Iwama, 2004). It acts as a marker of pro-inflammatory responses and its activity is a measure of innate immunity with a key role in oxidative stress (Perrault et al., 2016). Alterations in lysozyme plasma levels of loggerhead sea turtle seem to be related also with some environmental contaminants (Day et al., 2007; Keller et al., 2006), but no literature exists in relation to physiological stress in sea turtles.

The goal of our study was to evaluate the physiological stress of rescued animals by comparison with free-ranging animals, and to evaluate the integrated immune responses in relation to different rehabilitation timeframes of Mediterranean loggerhead sea turtles. Our final aim was to identify sensitive tools based on immune responses that rescue centres operators could use to verify the stress condition of the hospitalized specimens.

2. Material and methods

2.1. Sampling

Between 2009 and 2017, about 98 blood samples were collected from loggerhead sea turtles rehabilitating in several Italian rescue centres (n = 88) and from free-ranging specimens (n = 11).

Free-ranging turtles were captured in the South of Spain during a shipboard survey with a very low invasive technique. When the divers saw a basking turtle, the boat stopped several hundreds of meters away, the divers approached the animal and carried it on the research vessel. After the annotation of morphological parameters, a blood sample was collected. At the end of the sampling, the animal was released into the sea.

Blood samples were also collected from animals recovered in different Italian rescue centres due to different circumstances such as entanglement lines, traumatic carapace injuries, malnutrition, picked floating at the surface and unidentified causes. Sick and injured loggerhead sea turtles were stabilized before the sampling performed during routine physical examinations, causing them minimal distress. The loggerhead sea turtles were manually restrained out of the water for the 30 min sampling period. Every rescue centre operates with authorization in derogation from DPR 357/2003 and following the ISPRA (Higher Institute for Environmental Protection and Research) decision. The cares and procedures carried out on the Italian rescued turtles for all the rehabilitation period are performed in accordance with routine veterinary practice and guidelines for conservation and rehabilitation of marine turtles (Mo et al., 2013).

The average curved carapace length (CCL) was 52.5 cm ± 14.0 (range 20.0–78.0 cm) and the average weight 22.2 kg ± 16.3 (1.3–65.5 kg). The duration of the hospitalization period (≤2 months; > 2 months and ≤1 year; > 1 year) was recorded for each turtle. Sex of the animals was not determined in most cases since they were too young, then this variable was not included in the statistical analysis. Blood samples (2–6 mL) were obtained from the dorsal cervical sinus using a disposable syringe and transferred to solvent-rinsed

glass vials with Teflon caps containing heparinized saline (heparin sodium). An aliquot of whole blood was used for the preparation of two blood smears for white cell count and a second aliquot was stored at −80 °C until use. The remaining whole blood was transferred in plastic tubes and centrifuged for 5 min at 5000 *g* to obtain plasma, stored at −80 °C until analysis.

2.2. Total and differential white blood cells (WBC) count

Air-dried blood smears were stained with Diff-Quick stain and two hundred leukocytes were counted and classified as lymphocytes, monocytes, eosinophils, heterophils or basophils according to the cellular morphology for loggerhead sea turtle described by Casal and Orós (2007). Thrombocytes, as suggested in Davis et al. (2008), were considered only in their activated form.

2.3. H:L ratio

The ratio between the number of heterophils and lymphocytes was measured from each differential WBC, in order to obtain H:L ratio, a numerical stress index.

2.4. Respiratory burst

The respiratory burst activity was evaluated as the presence of intracellular oxyradical produced by NADPH oxidase, and it was measured with the NBT assay, following the method of Secombes (1990), modified as follows. For each sample, 100 µL of whole blood were added in triplicate to a 96-well plate and incubated at 30 °C for 2 h to allow cell adhesion. Unattached cells were then washed off 3 times with L-15 medium. 100 µL L-15 medium supplemented with NBT (1 mg/mL) was then added to each well and the plate was incubated at room temperature for 1 h. After incubation, the plate was discarded and fixed with 100% methanol for 10 min. The plate was washed several times with 70% methanol and air-dried. 120 µL of KOH and then 140 µL of DMSO were added to each well in order to destroy cell wall and dissolve the crystals of formazan blue deriving from the reduction of NBT by the oxyradicals. Measurements were performed at 630 nm using an ELISA microplate reader (Microplate Reader Model 680XR, Bio-Rad) using KOH/DMSO as white. The respiratory burst activity was expressed as a reduction of NBT.

2.5. TAS

A commercial kit (Antioxidant Assay Kit, Sigma, St. Louis, MO) based on the method of Miller et al. (1993), modified as follows, was used to evaluate the TAS. A stock solution of 1,5 mM of Trolox, a water-soluble analogue of vitamin E, was used for the standard curve. Trolox was diluted in assay buffer for the preparation of the different standard curve points (0, 0.015, 0.045, 0.105, 0.21, 0.42 mM). Aliquots of each concentration (10 µL/well) were added to a 96-well plate in duplicate. For each sample 30 µL of plasma diluted 1:100 in assay buffer were added in duplicate to the plate. 20 µL of myoglobin were added to each well followed by 150 µL of chromogen [ABTS (2,2-Azino-di [3-ethylbenzthiazoline])] and the plate was incubated at room temperature for 4.5 min. Absorbance at 405 nm was measured using a microplate ELISA reader (Microplate Reader Model 680XR, Bio-Rad). The TAS was expressed as mM of Trolox by linear regression of the standard curve.

2.6. Lysozyme activity

The lysozyme activity was measured in plasma samples with a standard turbidity test described by Keller et al. (2006), modified as follows. Briefly, 1 mg/mL stock solution of hen egg white lysozyme (HEL, Sigma, St. Louis, MO) was prepared in 0.1 M phosphate buffer (pH 5.9) and serially diluted in phosphate buffer to produce the standard curve of 0, 0.3, 0.6, 1.25, 2.5, 5, 10, 20, 25 µg/mL. Daily, 50 mg of the lyophilized cells were solved in 0.1 M phosphate buffer to obtain a fresh solution of *Micrococcus lysodeikticus* (Sigma, St. Louis, MO). Each concentration of the standard curve (25 µL/well) was added to a 96-well plate in triplicate and 25 µL of each sample was added in quadruplicate to the same plate. The *M. lysodeikticus* solution (175 µL/well) was quickly added to three sample wells and to each of the standard wells. The blank was the fourth well-containing plasma with 175 µL of phosphate buffer, without *M. lysodeikticus*. Absorbance at 450 nm was measured using a microplate ELISA reader (Microplate Reader Model 550, Bio-Rad). The optical density (O.D.) was measured immediately (T_0) and after 5 min (T_5) and the activity expressed as HEL concentration (µg/mL) by linear regression of the standard curve.

2.7. Statistical analysis

Different statistical analyses were performed using STATA 14 software (StataCorp, 2015). In order to determinate the immune responses in free-ranging animals and rehabilitated sea turtles and for measuring several stress responses in relation to the different time of rehabilitation, non-parametric tests were performed (Gibbons, 1997) that being distribution-free procedures, no assumptions about the sampled population are called for. First, the Mann-Whitney two-sample statistic (Mann and Whitney, 1947; Wilcoxon, 1945) was used to test the hypothesis that free-ranging and hospitalized animals come from populations with the same distribution in order to test differences in shape and spread as well as just differences in medians. Second, a Kruskal-Wallis equality of populations rank test (Friedman, 1937) was used to evaluate statistically significant differences between groups defined on the basis of three different times of rehabilitation plus the free-ranging group. Specifically, we tested the null hypothesis that the population centers are all equal against the alternative hypothesis that at least one of the populations tends to exhibit larger values than at least one of the other populations. Then, a Dunn's pairwise comparison test was indeed used for multiple comparisons accounting for the fact that we would have to protect the family error rate against a smaller number of comparisons (Dunn, 1964). Nevertheless, various robustness checks of findings have been carried out. Results are reported and discussed in the Supplementary material. Indeed, while a non-parametric test being based on ranks is more robust to outliers, it is also true that it generally has less power than the corresponding parametric test. Moreover, if distributions to be compared have a different shape, the Kruskal-Wallis test can be used only to compare mean ranks. It means that if different groups have different shapes (one is skewed to the right and another is skewed to the left, for instance, or they have different variances), the Kruskal-Wallis test may give inaccurate results (Fagerland and Sandvik, 2009). Accordingly, we made a preliminary sensitivity analysis. First, we tested the normality of data, we detected if extreme values significantly affect the process of estimating statistics (e.g. the average and standard deviation of a sample). Then, we performed parametric tests by using a bootstrapping technique (Efron and Tibshirani, 1993) for computing standard errors so that bypass the no normality of data in order to compare the parametric results with the no parametric findings. Furthermore, we employed Spearman rank correlation test to evaluate the degree of correlation between variables. After examining preliminary results from the Spearman test, in our final results, we included only significantly different from zero correlations (statistical significance was set at $\alpha = 0.05$).

3. Results

Table 1 reports the $\chi^2_{(1)}$ statistics and the associated p-value for all the animals with respect to the 7 variables under evaluation in the current study[1]. Four out of 7 indicators (p-value < 0.05) differed from free-ranging to hospitalized animals. Based on the high rank sums for each of these variables, the stress level is clearly different between the

Table 1
Two-sample Wilcoxon rank-sum (Mann-Whitney) test (free-ranging vs hospitalized animals).

Variable	$\chi^2_{(1)}$ statistic*	p-value
HETEROPHILS	1.749	0.190
LYMPHOCYTES	4.299	0.038
H:L RATIO	4.220	0.040
EOSINOPHILS	9.142	0.002
MONOCYTES	0.012	0.912
THROMBOCYTES	0.022	0.882
LYSOZYME	18.685	< 0.001

* Mann- Whitney test.

free-ranging and the hospitalized group. The latter showed the highest values for all the investigated biomarkers, except for eosinophils and lymphocytes. Looking at the p-values we can also assert that lysozyme and eosinophils are the most important indicators to discriminate the stress level.

In Table 2 we report the results of the Kruskall-Wallis test and the Dunn's Pairwise tests for the different stress responses measurements in relation to the different rehabilitation periods[2]. We show only differences across pairs of groups that show a p-value lower than 0.10.

There was evidence (at different significant levels) of a difference between 2 or 3 pairs of groups in heterophils, eosinophils, lymphocytes and lysozyme, of a difference in only one pair of groups in thrombocytes, H:L ratio and respiratory burst. Basophils, monocytes and TAS only did not show any differences across groups. Box plots of all analyzed data are reported in Supplementary material (Figures S3-S6).

Fig. 1 reports the scatter plots of indicators pairs that showed significant Spearman correlation. Heterophils and thrombocytes, two classes of phagocytes, presented a negative correlation ($\rho = -0.81$ and $p < 0.01$ respectively), while a positive correlation was found between heterophils and respiratory burst activity ($\rho = 0.64$; $p = 0.01$). A negative correlation was registered between thrombocytes and respiratory burst ($\rho = -0.63$; $p = 0.01$).

4. Discussion

To our knowledge, this is the first study that has investigated the well-being of loggerhead sea turtles rehabilitating in different Italian rescue centres compared to free-ranging animals, by means of an integrated innate immune responses evaluation. The typical mammalian memory response is absent in reptiles and humoral immune system is slower and less robust; for these reasons they have potentiated the innate immune responses (Zimmerman et al., 2013). It is therefore crucial to better elucidate how innate immune responses are influenced by physiological stress within recovery centers for a proper evaluation of the health status of hospitalized species and for the development of more effective conservative strategies.

Heterophils and lymphocytes are the major effectors of the innate response in reptiles, i.e. nearly 80% combined and the remaining 20% is represented by a combination of eosinophils, monocytes and basophils (Raphael and Melkonian, 2004; Werner, 2007). The mean values of heterophils and lymphocytes observed in our study (70.55% together) are in line with those reported elsewhere in turtles species (Casal and Orós, 2007; Flower et al., 2015; Hunt et al., 2016; Zimmerman et al., 2013). The higher percentage of heterophils in hospitalized specimens than in free-ranging confirms the physiological stress condition of rehabilitating animals. Moreover, heterophils detected in animals hospitalized in the timeframe ≤2 months are statistically higher then heterophils detected in animals hospitalized for other periods, confirming their involvement in infections and inflammation protection (Stacy et al., 2011).

H:L ratio values of free ranging specimens are lower compare to the hospitalized ones. Moreover, H:L ratio and heterophils values present the same trend in animals along all the rehabilitation period, showing similar values to other reptiles species (Lance and Elsey, 1999).

To date, researchers have reported < 10% of eosinophils in healthy sea turtles (Camacho et al., 2014; Casal and Orós, 2007; Flower et al., 2015; Hunt et al., 2016; Keller et al., 2004). Although their function has not been well studied, the statistically significant increase observed in free-ranging eosinophils in comparison with all hospitalized specimens suggests their involvement in parasitic infections and other types of antigenic stimulation (Stacy et al., 2011), common of the natural environment. Moreover, the absence of statistical differences among the rescued animals highlight that investigated rescue facilities were free from pathogen agents and that a controlled environment helps turtles to reduce their parasitic load.

Monocytes usually compose 0%–5% of total leukocytes in sea turtles and their percentage increases during chronic antigenic stimulation, chronic inflammation, and bacterial or parasitic diseases (Davis et al., 2004; Flower et al., 2015). The results of this study highlight the absence of monocytes in all investigated groups (free-ranging and hospitalized), suggesting that the basal level of these cells in loggerhead sea turtles is close to 0%. The lack of increase in the monocytes values in hospitalized specimens indicates that the maintaining in a rescue center do not cause inflammation; these findings are in contrast with Flower et al. (2015) who reported that turtles in a long-term rehabilitation showed elevation in monocytes over time when compared with turtles in short-term rehabilitation.

To the best of our knowledge, the most abundant leukocytes in juvenile and adult rehabilitating loggerhead sea turtles were thrombocytes (Camacho et al., 2014; Casal et al., 2009), cells functionally comparable to platelets and primarily involved in hemostasis and wound healing. Contrarily, our results show that the most abundant component of WBC is represented by heterophils and that the percentage of thrombocytes increases during the rehabilitation. Recently, it has been shown that these cells are involved in inflammation, antimicrobial host defense and overall immune response (Ferdous et al., 2016), and this could explain our results. The positive Spearman correlation between the number of heterophils and the respiratory burst activity confirms that the major effectors of respiratory burst are heterophils, as suggested by Merchant et al. (2009), while the negative correlation between thrombocytes and respiratory burst suggests that thrombocytes are not involved in the production of superoxides. The function of basophils in reptiles is not completely understood although few studies (Campbell, 1995; Rupley, 1997) reported that their percentage increases with certain hemoparasitic and viral infections. Basophils percentage varies widely among reptile species and some authors reported that they are under-represented in marine turtles (Casal and Orós, 2007; Work et al., 1998), as recorded in our study.

The production of superoxides is an ancient innate defence mechanism widely diffused in all phyla (Merchant et al., 2009). To our knowledge, only Rousselet et al. (2013a, 2013b) evaluated the respiratory burst on sea turtle isolated leukocytes. However, due to the difficulty in establishing healthy leukocyte cultures and to the large blood volumes needed (Merchant et al., 2008), we investigated for the first time the respiratory burst in whole blood of loggerhead sea turtles. The statistical difference between the two periods of hospitalization (≤2 months; > 2 months and ≤1 year) suggests that the first period spent into the centres is particularly stressful for the animals, probably because of both health and captivity conditions. Further studies are needed to investigate the basal levels of the respiratory burst.

To our knowledge, this is the first study that reports TAS analysis on

[1] The test was not performed on basophils because their value was zero in free-ranging specimens (see figure S3 in Supplementary material).

[2] No statistical differences were detected among the different rescue centers, so we decided to not consider this variable. Results follow the trend regardless of the site of recovery (data not showed).

Table 2
Kruskal-Wallis and Dunn's Pairwise Comparison. A = free-ranging animals; B = duration of hospitalization ≤2 months; C = duration of hospitalization > 2 months and ≤1 year; D = duration of hospitalization > 1 year (p.values in brackets).

Variable	Kruskal-Wallis statistic	Dunn's Pairwise Comparison statistic				
	χ^2_3	A vs B	B vs C	B vs D	A vs C	A vs D
HETEROPHILS	17.330 (< 0.001)	−2.32 (0.0604)	3.20 (0.0041)	2.70 (0.0202)		
EOSINOPHILS	19.004 (< 0.001)	3.88 (< 0.001)		−2.43 (0.0452)		
THROMBOCYTES	13.141 (0.004)		−3.219 (0.0039)			
H:L RATIO	10.014 (0.018)	−2.669 (0.0228)				
BASOPHILS	6.269 (0.010)	n.a.				
LYMPHOCYTES	6.580 (0.087)	2.274 (0.0689)			2.318 (0.0613)	
MONOCYTES	0.586 (0.900)					
RESPIRATORY BURST	4.688 (0.096)	n.a.	2.149 (0.0474)		n.a.	n.a.
TAS	2.39 (0.496)	n.a.			n.a.	n.a.
LYSOZYME	23.210 (< 0.001)	−4.765 (< 0.001)			−2.887 (0.0117)	−2.658 (0.0236)

Note: n.a. = not attinent.

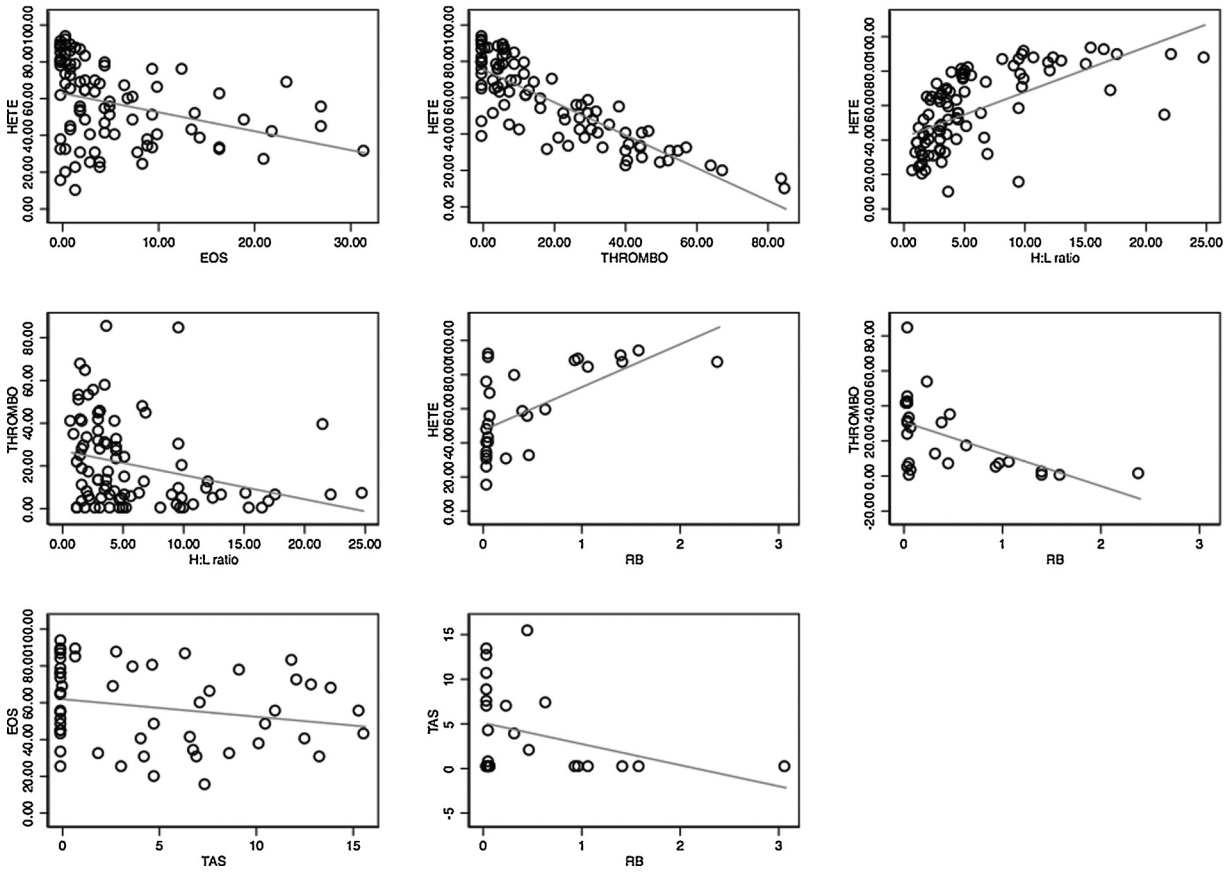

Fig. 1. Scatter plots of indicators pairs with significant Spearman correlation ($p < 0.05$).

Caretta caretta and no comparison can be made with studies on other reptile species (Arukwe et al., 2015; Baker et al., 2007; Treidel et al., 2016), due to the different methods used. Although no statistically significant differences among groups were found, the specimens hospitalized ≤2 months showed the highest mean values, similarly to lysozyme activity, respiratory burst and leukocytes profile, confirming the hypothesis that the first two months in rescue centres represent a critical period for the rehabilitation mainly for the immune status of the animals.

Plasma lysozyme is a pro-inflammatory response biomarker and a measure of innate immunity (Burton et al., 2002). It is secreted by granulocytes in response to foreign microorganisms and lyses gram-

positive bacteria (Balfry and Iwama, 2004). Few papers evaluated lysozyme activity in loggerhead sea turtles showing similar values to our data (Day et al., 2007; Keller et al., 2006). Two other authors (Rodgers et al., 2018; Walsh et al., 2010) investigated plasma and serum lysozyme activity in loggerhead sea turtles, but the reported values are not in line with our data. Among our results, free-ranging mean values are statistically lower (1.99 μg/mL, Table 1) with respect to values found in recovered specimens (10.74 μg/mL) and then they can be considered as reference group. Moreover, these data suggest that a temporary captivity could play a role for the enhancement of *C. caretta* general inflammation, in agreement with the findings of Flower et al. (2015). Since lysozyme basal values may differ because of genetic components, environmental causes or recent infections (Kiron et al., 1995), our free-ranging animals were sampled immediately after their capture, with fast manipulations, and not hospitalized. This allows us to consider the free-ranging values as the loggerhead sea turtles plasmatic lysozyme baseline values. The low invasive technique used to capture turtles could explain why our values are lower than other studies, in which turtles have been more stressed out probably because of capture with pound or trawl nets (Day et al., 2007; Keller et al., 2006). This is the first study that evaluates lysozyme activity in sea turtles or other reptiles subjected to rehabilitation. The highest mean levels of lysozyme activity were detected during the first two months of rehabilitation, presumably due to unstable health status and to rehabilitation-caused stress. This hypothesis is in agreement with the work of Demers and Bayne (1997) on rainbow trouts which upon manipulation (acute stress) showed an increase in plasma lysozyme levels compared to controls. The slight activity decrease after two months and after 1 year compared with the activity of shorter rehabilitation time is probably due both to the improvement of health conditions and to the acclimatization to captivity, as suggested by other studies performed on stress effect on hormonal and immune responses (Martin, 2009; Romero, 2004).

Statistical elaboration of our results suggests that lysozyme and eosinophils evaluation may represent the most valid tools to detect inflammation and physiological stress, permitting to consider them good health indicators, able to discriminate the different stress levels in rescued specimens. In the rescue facilities the health status of specimens could be affected by infectious disease, chronic inflammation and parasitic infections. The lysozyme activity, the first line of defense against bacteria and microorganisms, could represent the best tool to assess general immune health conditions because of its versatility and ease of use. Moreover, the absence of eosinophils in the WCB count could confirm that a controlled environment is free from parasites, as the national guidelines for rehabilitation sea turtle recommend.

5. Conclusions

The efforts done for sea turtles conservation, the improvements of their medical management, and the studies on their physiological parameters have greatly increased in recent years, especially thanks to the increasing involvement of veterinary surgeons and scientific researchers in sea turtle rehabilitation centres around the world. To assess the well-being of hospitalized animals in rescue centres during different time periods we have investigated the possibility to use integrated immune responses analysis, that require few milliliters of blood to obtain information concerning their physiological stress. Our results indicate that the first 2 months in the rescue center represent the critical period during the rehabilitation of the animals, while after more than 1 year the immune values are similar to those of free-ranging that resulted to be the lowest for all the measured parameters. Chronic inflammation was not enhanced and turtles presented a reduction of their parasitic load during rehabilitation. The evaluation of lysozyme activity and eosinophyls represent the most valid methods to diagnose physiological stress and inflammation in hospitalized loggerhead sea turtles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetimm.2018.11.013.

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