Evaluation of the Effects of Administering Ultradiluted *Avena sativa* and *Echinacea angustifolia* on the Hematological Parameters of Magellanic penguins (*Spheniscus magellanicus*) during the Reproductive Period

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Abstract

Background and Aims Penguins are seabirds that manifest physiological and immunological alterations during the reproductive season. The objectives of this study were to evaluate the laboratory parameters of male and female Magellanic penguins and to determine the penguins' hematological response to homeopathic medicines during that reproductive period.

Methods Penguins ($N = 18$), kept under human care in a zoological park setting, were evaluated during the reproductive period and were divided randomly into two groups: a group subjected to treatment with *Echinacea angustifolia* 6cH and *Avena sativa* 6cH ($N = 8$; four breeding couples), and a placebo control group ($N = 10$; five breeding couples). The investigators were blinded to treatment allocation. Two-way ANOVA was performed to determine whether the experimental group (control or verum) and the sex of the animal had any significant effect on the variation of each hematological parameter between the samples. One-way ANOVA was performed on hematological parameters for which the sex did not present a significant effect. The significance level was $p \leq 0.05$.

Results Significant effects were seen regarding the following: mean corpuscular volume (MCV), in which the verum group showed an increase (29.78 ± 52.95 fL) while the control group showed stability/reduction (-3.08 ± 46.36 fL) ($p = 0.049$); proportion of heterophils, in which the verum group showed a less marked increase ($8.38 \pm 12.53\%$) than that of the control group ($18.00 \pm 9.37\%$) ($p = 0.010$); lymphocyte concentration, in which the verum group showed less marked reduction ($-4.39 \pm 2.21 \times 109$ cells/L) than that of the control group ($-1.56 \pm 2.76 \times 109$ cells/L) ($p = 0.001$); and proportion of lymphocytes, in which the verum group showed a less marked reduction ($-6.75 \pm 10.35\%$) than that of the control group ($-17.3 \pm 8.73\%$) ($p = 0.002$).

Keywords
- homeopathy
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Conclusion Comparison of samples collected before and during the reproductive period showed that, regardless of group allocation, there were differences in the effects on MCV, heterophils and lymphocytes. Treatment with *Echinacea angustifolia* and *Avena sativa* resulted in maintenance of lymphocyte levels in Magellanic penguins during the breeding period, thus aiding these birds' immunity.

Introduction

Magellanic penguins (*Spheniscus magellanicus*) follow a well-established annual life cycle. In the natural environment, the stages of the cycle have the following sequence: settlement (September–October), incubation (October–November), rearing of the chicks (November–January), independence of the chicks (January–February), plumage change (February–April), and winter migration (April–September). Each phase of this cycle has distinct energy demands and involves physiological and immunological changes. The periods of settlement, incubation and rearing of the chicks involve greater physiological effort, and these are believed to be the most fragile moments of the penguins' life cycle.[1]

Physiological changes related to reproduction begin while penguins are still migrating. Gonadal reactivation is stimulated through the increase in daylight hours, which in turn induces penguins to return to their reproductive colonies and start nesting activities and then laying and incubating the eggs.[2]

Although male and female Magellanic penguins share the incubation and chick-rearing tasks, differences between males and females regarding the concentrations and proportions of circulating leukocytes can be seen over the course of the reproductive period. These differences suggest that there may be sexual differences in exposure or response to external factors such as stress or pathogens, or in relation to internal physiological factors (hormone concentrations and physiological resources mobilized for egg-laying).[3,4]

Homeopathic medical practice aims to balance the organism. Moreover, focusing on animal welfare can be considered as an alternative or complement to conventional medication. Homeopathy has other advantages, such as low cost, easy administration, and lack of side effects, adverse reactions or accumulation of residues from high doses and/or chronic administration of medications. Because homeopathic treatment is administered orally (food or water), it does not require physical containment of animals, thus minimizing the stress of the treatment.[5] Homeopathy applied in veterinary medicine can present excellent practical results, supporting certain concepts such as those of the minimum therapeutic dose, and it can present advantages over conventional medicine.[6–8]

The aims of the present small study were to evaluate the laboratory parameters of Magellanic penguins through blood collection, with interpretation of the blood counts before and during the reproduction period, and also between males and females, and to determine the action of the homeopathic medicines *Echinacea angustifolia* 6cH and *Avena sativa* 6cH on the hematological response of penguin couples during the breeding period.

Methods

Subjects

Nine breeding couples (nine females and nine males) of adult Magellanic penguins (*Spheniscus magellanicus*) that were being kept in the penguin enclosure at "Sabina Escola Parque do Conhecimento", located in the city of Santo André, SP, Brazil, were evaluated. These penguins were maintained as a single social group, in an enclosure consisting of 33 m^2 of dry area and 81 m^2 of pool containing 110,000 L of salt water. The limit on the number of penguins was 28 individuals, and they had free access to both the pool and the dry area. The dry area contained artificial burrows as nests, and sterilized hay was provided for the couples to make their nests by themselves. The penguin enclosure was photoperiod-controlled throughout the year, through an artificial lighting system with timers that simulated the photoperiod of the natural environment of this species (Patagonia, Argentina). The sex of these penguins had been previously determined through PCR, from blood sampling.

The penguins were analyzed during the reproductive period. We performed convenience sampling, the animals being randomized into pairs and then divided randomly into two groups of couples. Though the experimental design had intended the use of five couples in the verum group, one couple had to be excluded from the study due to the impossibility of sampling in the second stage of the experiment, resulting in a verum group comprising four couples and a control group comprising five couples.

This study was authorized by the Ethics Committee for Animal Use (CEUA) of Santo Amaro University, under the number 16.2/2020 (►**Supplementary Material 1**, available online only), and was also authorized through the Brazilian Ministry of the Environment's Authorization System for Activities with Scientific Purposes (SISBIO), under the number 75384–1 (►**Supplementary Material 2**, available online only).

Experimental Design

The verum group was treated with *Echinacea angustifolia* 6cH and *Avena sativa* 6cH in sucrose globules that were administered orally in the fish that were fed to each penguin. The control group received globules (placebos) consisting of the same inert vehicle but without the homeopathic preparation. These globules (verum or placebo) were administered

to the animals twice a day in the first 10 days of each month during the trial period (September and October).

The procedures were performed blindly: the investigators were blinded to treatment allocation. The globules destined for each experimental group were identified through the use of pots with different colors, a procedure that was organized by a volunteer who was not involved in the study. This volunteer recorded in writing which colored pot corresponded to the globules for the verum and control groups, and this information was only revealed to the researchers after the end of the experiment, when the hematological analyses had already been completed. The globules for the two groups did not have any perceptible characteristics (e.g., texture, color or odor) that would allow their differentiation.

Two blood collections were performed: the first collection was done before the beginning of the reproductive period and the administration of the treatments; and the second collection was done after the second egg of each couple had been laid.

Hematological Analyses

Blood collection (up to 2 mL) was performed by means of puncturing the metatarsal vein of the penguin while it was under physical containment, without sedation. Blood smears were performed using fresh blood, and samples were placed in lytic heparin microtubes and sent immediately to the laboratory.

The complete blood counts were evaluated following routine procedures, using the manual counting method, with dilution of 1:200 in Natt-Herrick solution.[9] The following hematological parameters were measured: total erythrocyte count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total plasma protein, and total and differential leukocyte counts (a leukogram comprising heterophils, eosinophils, basophils, lymphocytes, and monocytes).

Statistical Analysis

Boxplots were used to represent the distribution of the hematological results (minimum, 1st quartile, median, 3rd quartile, maximum, and extreme values). The change between collections (Δ variable) was calculated for each hematological variable by subtracting the value in the final collection from the value in the initial collection. Two-way ANOVA was used to determine whether the group (control vs. verum) and the sex of the penguin (male vs. female) had any significant effect on the change between collections regarding each hematological variable. In the case of hematological parameters in which sex did not present a significant effect on the difference between collections, the analysis of variance was re-done using the experimental group as the only independent variable (one-way ANOVA). The significance level adopted was $p \leq 0.05$ for all tests. Due to the predominance of null values, the proportion and concentration of basophils were not compared using statistical tests.

Results

The comparisons of hematological results between the control and verum groups are presented in ►**Table 1**.

Sex had a significant effect on the change between collections for two variables: hematocrit ($p < 0.001$) and hemoglobin concentration ($p = 0.005$). These two variables did not present any significant effect in the experimental group regarding the change between collections ($p = 0.089$). Females showed a tendency for hematocrit to be reduced during the experiment ($-6.18\% \pm 5.63\%$), while males showed a trend toward stability/increase ($0.56\% \pm 4.78\%$; ►**Fig. 1A**). Females also showed a more intense tendency toward reduced hemoglobin concentration during the experiment (-2.86 ± 1.91 g/dL) than did males (-1.14 ± 1.45 g/dL; ►**Fig. 1B**). The other hematological variables did not present any significant effect from sex on the change between collections.

The control and verum groups showed a significant difference in the change between collections for the following variables: MCV ($p = 0.049$), proportion of heterophils ($p = 0.010$), proportion of lymphocytes ($p = 0.002$) and lymphocyte concentration ($p = 0.001$). The verum group showed a trend toward increased MCV (29.78 ± 52.95 fL), while the control group showed a trend toward stability/reduction (-3.08 ± 46.36 fL; ►**Fig. 2A**). The verum group showed a trend toward increased proportion of heterophils ($8.38\% \pm 12.53\%$) that was less marked than in the control group ($18.00\% \pm 9.37\%$; ►**Fig. 2B**). The verum group showed a trend toward reduced proportion of lymphocytes ($-6.75\% \pm 10.35\%$) that was less marked than in the control group ($-17.30\% \pm 8.73\%$; ►**Fig. 2C**). The verum group also showed a trend toward reduced lymphocyte concentration ($-1.56 \pm 2.76 \times 10^9$ cells/L) that was less marked than in the control group (-4.39 ± 2.210^9 cells/L; ►**Fig. 2D**).

Discussion

Moreno et al showed that delayed reproduction occurred in a population of wild penguins that was related to hematological indications of health problems and reduced T-cell dependent immunocompetence. If penguins' reproductive success is affected by their health status, it can be expected that those that are unsuccessful with regard to reproduction would have poorer health than that of penguins with successful reproduction.[10]

An MCV of 172.0 ± 53.0 fL and an MCHC of $26.41 \pm 0.02\%$ were obtained in a study on 27 blood samples from Magellanic penguins undergoing rehabilitation in Espírito Santo,[11] while in the present study these values were an MCV of 230.16 ± 34.76 fL to 259.94 ± 28.34 fL and an MCH of $70.63 \pm 11.9\%$ to $78.01 \pm 10\%$. The verum group showed a difference in MCV at the beginning and end of treatment: 230.16 ± 34.76 fL and 259.94 ± 28.34 fL, respectively.

Before the reproductive period, the penguins of the present study had a mean hematocrit of $51.70\% \pm 3.71\%$, while the value was $49.80\% \pm 2.74\%$ during the reproductive period. Thus, there was no risk to animal health and no

Table 1 Summary of hematological results (mean ± standard deviation) obtained from the two samples collected (beginning and end of the experiment) for the two experimental groups (control and verum)

	Control group		Verum group		Change between collections (Δ)		Significance (*p*)	
	Beginning	End	Beginning	End	Control	Verum	Group	Sex
Erythrocytes (10^{12} cells/L)	2.19 ± 0.24	2.12 ± 0.24	2.29 ± 0.38	1.86 ± 0.35	−0.07 ± 0.33	−0.43 ± 0.61	0.130	–
Hematocrit (%)	51.70 ± 3.71	49.80 ± 2.74	51.63 ± 3.62	47.68 ± 6.87	-1.90 ± 4.56	−3.95 ± 8.13	0.089	<0001*
Hemoglobin (g/dL)	16.50 ± 1.55	14.75 ± 1.44	16.58 ± 1.40	14.26 ± 2.15	−1.75 ± 1.88	−2.31 ± 2.02	0.330	0.005*
MCV (fL)	241.3 ± 31.54	238.22 ± 31.56	230.16 ± 34.76	259.94 ± 28.34	−3.08 ± 46.36	29.78 ± 52.95	0.049*	–
MCH (pg)	75.98 ± 9.75	70.63 ± 11.9	73.94 ± 12.21	78.01 ± 10.88	−5.35 ± 14.83	4.08 ± 19.29	0.096	–
MCHC (g/dL)	31.68 ± 3.64	29.62 ± 2.52	32.20 ± 2.85	29.96 ± 1.77	−2.06 ± 3.33	−2.24 ± 3.58	0.875	–
TPP (g/L)	63.40 ± 6.67	61.20 ± 5.27	67.50 ± 10.89	65.50 ± 9.90	−2.20 ± 7.27	−2.00 ± 15.64	0.958	–
WBC (10^9 cells/L)	16.59 ± 3.83	11.19 ± 4.85	17.76 ± 4.68	14.74 ± 5.67	−5.40 ± 4.17	−3.03 ± 5.79	0.149	–
Heterophils (10^9 cells/L)	7.27 ± 1.19	6.93 ± 2.59	10.71 ± 3.98	9.60 ± 3.59	−0.34 ± 2.84	−1.11 ± 4.19	0.503	–
Lymphocytes (10^9 cells/L)	8.05 ± 3.55	3.66 ± 3.52	5.75 ± 1.22	4.19 ± 2.99	−4.39 ± 2.21	−1.56 ± 2.76	0.001*	–
Eosinophils (10^9 cells/L)	0.55 ± 0.46	0.41 ± 0.39	0.58 ± 0.88	0.33 ± 0.36	−0.14 ± 0.61	−0.25 ± 0.89	0.643	–
Monocytes (10^9 cells/L)	0.72 ± 0.82	0.19 ± 0.20	0.72 ± 0.43	0.59 ± 0.48	−0.53 ± 0.86	−0.13 ± 0.54	0.100	–
Basophils (10^9 cells/L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.06	0.00 ± 0.00	0.02 ± 0.06	–	–
Heterophils (%)	46.10 ± 11.71	64.1 ± 12.96	58.88 ± 8.46	67.25 ± 12.7	18.00 ± 9.37	8.38 ± 12.53	0.010*	–
Lymphocytes (%)	46.80 ± 10.21	29.5 ± 12.73	33.13 ± 4.88	26.38 ± 11.69	−17.3 ± 8.73	−6.75 ± 10.35	0.002*	–
Eosinophils (%)	3.30 ± 2.36	4.00 ± 3.89	3.63 ± 5.48	2.00 ± 1.93	0.70 ± 4.35	−1.63 ± 5.45	0.151	–
Monocytes (%)	3.80 ± 3.61	2.40 ± 3.44	4.38 ± 2.97	4.25 ± 2.66	−1.40 ± 5.52	−0.13 ± 3.36	0.410	–
Basophils (%)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.13 ± 0.35	0.00 ± 0.00	0.13 ± 0.35	–	–

Abbreviations: TPP, total plasma proteins; WBC, white blood cells.
*denotes significant *p*-value.

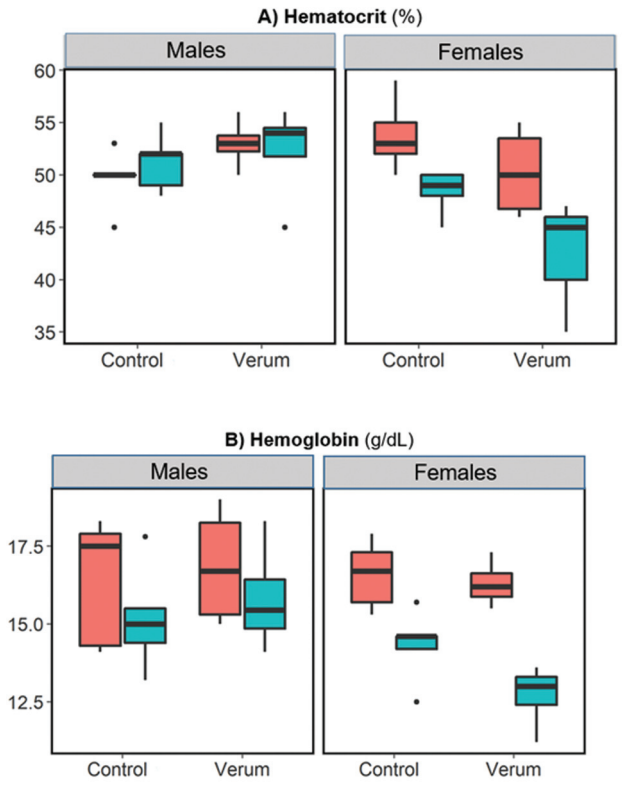

Fig. 1 Boxplots comparing hematological results of blood samples taken at the beginning (*red*) and then at the end (*blue*) of the experiment in male and female penguins. Figure A, hematocrit; Figure B, hemoglobin.

difference during the reproduction period, since 48.00% ± 4.15% is considered to be a healthy value.[4] In general, the results obtained in this study were within what would be expected for the species, based on the reference values of Gallo et al.[4]

Some aspects of reproduction were previously correlated with variation in female health and condition in a seabird population, and parental quality in this and other species may be linked to health.[3]

Free-living females may present alterations to cell parameters according to the reproductive phase, such that at the time of laying eggs the hematocrit level is reduced.[3] Moreover it was observed among free-living male penguins that the lymphocyte counts decreased during reproduction.[12] The findings from the present small study corroborate this, such that the hematocrit levels and red blood cell counts remained similar in males and showed a decrease in females during the reproduction period. A highly significant difference in the initial leukogram was also observed between reproductively experienced and inexperienced couples, such that higher white blood cell counts were observed in late couples without reproductive experience.[3]

In Galápagos penguins (*Spheniscus mendiculus*) there was no difference in white blood cell counts between the sexes. However, females presented higher eosinophil levels than males.[13] In African penguins (*Spheniscus demersus*), there was no difference in leukocyte count between the sexes, but there was a difference regionally.[14] The result obtained in the present study corroborates this, in that no difference in white blood cell count was observed but there was a difference in red blood cells between the sexes of the penguins.

In a study on African penguins, the hematocrit, hemoglobin, and red blood cell counts were significantly higher in males than in females.[14] The findings of the present study corroborate this, in that the hematocrit and red blood cell levels remained similar in males and showed a decrease in females during the reproductive period. This difference had already been seen in another study, in which hematocrit levels were also higher in male Magellanic penguins.[3]

Regarding the proportion of heterophils, the verum group showed a trend toward increased levels (8.38% ± 12.53%) that was less accentuated than that of the control group (18.00% ± 9.37%). This may have been related to immunomodulation. Regarding the proportion of lymphocytes, the verum group showed a decreasing trend (−6.75% ± 10.35%) that was less marked than that of the control group (−17.30% ± 8.73%). In general, the heterophil/lymphocyte (H/L) ratio is interpreted as an indication of stress: the higher the proportion (i.e., when heterophils are high and lymphocytes are low), the more "physiological stress" the bird is facing (which may be stress due to a psychological stimulus of fear/discomfort or exposure to pathogens or parasites). Thus, if the verum group is showing a less intense increase in heterophils and a less intense reduction in lymphocytes, this means that the H/L ratio of the verum group is more stable, whilst the control group is varying more vigorously in relation to an increase in the H/L ratio (thus suggestive of greater physiological stress).

Lymphopenia may be observed as a result of an excess of endogenous or exogenous corticosteroids.[15] In the present study, there was a significant difference in lymphocyte counts between the groups, such that in the control group there was a more marked reduction than in the verum group. This supports the hypothesis of a lower percentage of endogenous corticosteroids in this group. Homeopathic medicines are known to cause immunomodulation in the animal organism.[6] This was corroborated in previous studies through findings that chicks presented higher lymphocyte levels when treated with a homeopathic complex composed of *Echinacea angustifolia* 6cH, *Avena sativa* 6cH, *Calcarea carbonica* 30cH, *Pyrogenium* 30cH, *Arsenicum album* 30cH, *Colibacillinum* 30cH, *Lachesis muta* 30cH and *Nux vomica* 30cH.[16]

It is known that the medications chosen for the present study (*Echinacea angustifolia* and *Avena sativa*) have shown positive results in other studies. For example, in an experiment with newborn pigs, it was observed that use of *Echinacea angustifolia* had an immunostimulant effect that promoted growth and provided a non-specific degree of protection, thus increasing the average daily weight gain of newborns until weaning.[17]

Use of *Echinacea and Belladonna* in mice was found to result in modulation of peritoneal inflammatory reactions and in cytoprotective action on leukocytes. This modified cell migration to an inflammatory focus and hence made the

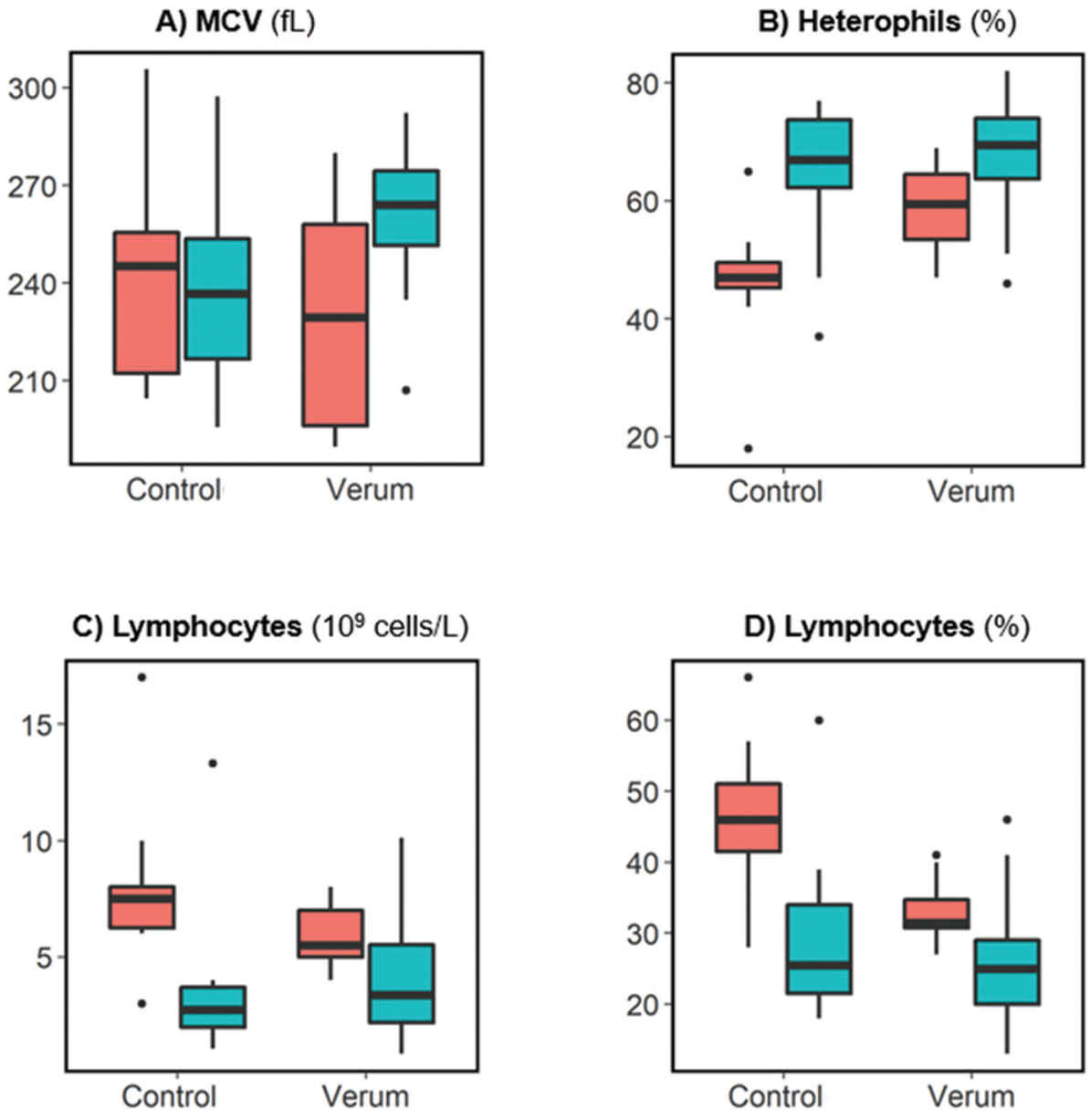

Fig. 2 Boxplots comparing (A) mean corpuscular volume (MCV), (B) relative proportion of heterophils, (C) total lymphocyte concentration, and (D) relative proportion of lymphocytes in blood samples taken at the beginning (*red*) and then at the end (*blue*) of the experiment.

process more acute, through increasing the migration of polymorphonuclear cells and reducing the migration of lymphocytes and macrophages.[18]

In a study on Magellanic penguins presenting pododermatitis of different degrees, homeopathy gave rise to improvement of the condition and provided rapid and effective therapy.[19]

In Magellanic penguins treated with *Carduus marianus*, there was an average decrease in ALT values from 141.2 ± 61.5 U/L to 65.3 ± 28.7 U/L, along with an average decrease in AST values from $394.7 \pm$ to 113.7 U/L to 223.2 ± 116.7 U/L. The response to homeopathic treatment was similar to that of conventional treatment at previous check-ups, but without side effects and with easy administration.[20]

Use of homeopathic remedies in veterinary medicine for wild animals has shown positive results, even after the animal's disease or behavioral alteration had been unsuccessfully treated with conventional medicinal products. Use of homeopathic medications has also been found to decrease the financial cost of treating an animal undergoing rehabilitation and to diminish the stress that the animal might experience before receiving the appropriate medication.[21,22]

Conclusion

Magellanic penguins may present significant blood count variation during the reproductive season, and homeopathic treatment with *Echinacea angustifolia* and *Avena sativa* was able to modulate these hematological changes. In particular,

it is noteworthy that the treatment attenuated the increase in the heterophil-lymphocyte ratio. This suggests that an immunomodulation effect occurred, which reduced the cellular response to the physiological stress associated with the reproductive period.

Supplementary Material

Supplementary file 1. Authorization by the Ethics Committee for Animal Use (CEUA) of Santo Amaro University.
Supplementary file 2. Authorization by the Brazilian Ministry of the Environment's Authorization System for Activities with Scientific Purposes (SISBIO).

Conflict of Interest
None declared.

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