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The effects of seasonal variation and physical activity on constitutive humoral immunity in barnacle geese (*Branta leucopsis*)

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Abstract

Background: Wild migratory birds are important vectors in the transmission of various zoonotic diseases, like West Nile disease and avian influenza. Transmission of these diseases is not only a risk to public health, but also results in economic damages. Development, maintenance, and use of the immune system is costly, and animals are limited in the number of resources they can invest. This results in a trade-off between the immune system and other physiological processes. Seasonal change and migration can both affect immune function.

Aim: Investigate the effects of seasonal change and physical activity on the constitutive humoral immune system.

Organisms: barnacle geese (*Branta leucopsis*)

Place of research: Wageningen University

Methodology: Wild geese of two populations (non-migratory Dutch and migratory Russian) were captured and kept in a common garden environment. Constitutive humoral immunity was measured across one year and before and after exercise trials using a hemagglutination-hemolysis assay.

Principal findings: It was found that hemagglutination decreased during autumn in the running Russian population. Hemolysis was found to be decreased during late winter in the running Dutch population. Within the running Russian population, hemolysis was found to be decreased in late winter and spring. When comparing immune function pre- and post-activity it was found that in the running Russian population hemagglutination was significantly lower pre-activity compared to post-activity during one trial. Hemolysis was found to be significantly lower pre-activity compared to post-activity in one exercise trial.

Conclusion: Constitutive humoral immune parameters differ across seasons and pre- and post-activity. Predicting how these parameters change based on previous research on different species is difficult. Genetic differences between populations likely play an important role in humoral immune defence.

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Contents

Introduction

Migratory birds play an important role in the transmission of a variety of diseases such as avian influenza and West Nile disease (Jourdain et al., 2007). As avian influenza causes not only the death of wild birds but also causes economic damages due to mortality in domesticated birds and the culling of farms, gaining new insights into how wild birds spread infectious diseases, including zoonotic ones, will aid in preventing the transmission of these (zoonotic) diseases (Burns et al., 2006). In order to defend against these diseases, birds need to develop and maintain their immune system.

Barnacle geese

Barnacle geese (*Branta leucopsis*) are medium sized black, white and grey geese found across northern Europe and Greenland (Norsk Polarinstitutt, 2018; Vogelbescherming Nederland, 2021). The barnacle geese residing In the Netherlands can be divided into two populations. One population uses the Netherlands as overwintering grounds and returns north in spring to breed. The second population remains in the Netherlands year-round (Jonker et al., 2013).

Immune defence and seasonal change

Immune defence requires many resources to develop, maintain and use, but animals are limited in the number of resources they can invest at any given time. Therefore, trade-offs between immune defence and other costly processes such as growth, migration and reproduction are created (Bichet et al., 2022; Lochmiller & Deerenberg, 2000). Ecological processes like (a)biotic factors as well as intra-specific constraints, interactions with pathogens and population genetics influence the complexity of the immune system (Schulenburg et al., 2008). The number of resources available to individuals varies throughout the year. During winter food availability is decreased and the lower temperature results in increased thermoregulatory demands. Other seasonal activities, such as breeding and raising offspring, and migration are all energetically expensive as well. Therefore, timing these activities to periods with high energy availability is crucial for survival (Nelson, 2004; Nelson & Demas, 1996). There is likely a genetic component to seasonal variation in constitutive immunity. When seasonal variations in environmental factors are somewhat predictable adjustments to physiological systems, such as immune defence, can be based on a genetic component. It is currently unknown which immune measures vary across seasons and which remain stable (Versteegh et al., 2014).

Constitutive humoral immunity in birds

The avian immune system can be divided into two categories: humoral immunity and cellular immunity. Humoral immunity consists of antibodies secreted by B lymphocytes and is controlled by the bursa of Fabricius, a lymphoid organ unique to birds (Sharma, 1991). The cellular immune response of birds includes T lymphocytes, macrophages, dendritic cells, and natural killer cells (Tieleman, 2018). The first line of defence of the immune system against pathogens is constitutive innate immunity. Constitutive innate immunity consists of both humoral (natural antibodies, complement and acute phase proteins) and cellular (macrophages, heterophils and thrombocytes) components (Tieleman et al., 2005). Natural antibodies are encoded in the germline genome and are thus passed on from one generation to the next. They are also present in naive (non-immunized) animals. The genotype-dependent expression of natural antibodies makes them very suitable to compare constitutive innate humoral immunity between species and within populations (Matson et al., 2005).

Effect of physical activity on immune defence

The migratory flights of birds are the most extraordinary feats of physical performance by animals and have a significant impact on the immune system (Bichet et al., 2022). During flight the energy expenditure can reach about 20 times the basal metabolic rate (van Dijk & Matson, 2016). During flight, foraging for food is not possible, and thus birds need to rely on previously acquired energy stores. This results in a decrease in body size and breast muscle during migration (Jenni & Jenni-Eiermann, 1998). In order to prepare for migration, several resource intensive processes, such as hyperactivity, moulting and increasing fat stores need to be completed. During this time the same birds will likely also need to build up their immune system to ward off disease (O'Neil & Ketterson, 2012; van Dijk & Matson, 2016). Because migratory preparation and immunoenhancement are both high in resource consumption and occur at the same time of year one would expect trade-offs to occur in favour of one of the two processes (O'Neil & Ketterson, 2012). Investing resources in migration could leave individuals vulnerable to disease at stopover sites and the destination. Conversely investing too heavily in the immune system could leave the individual unable to complete migration (O'Neil & Ketterson, 2012). Changes in the immune system can also occur after completing strenuous exercise, including flight. It was found that in European Starlings both the agglutination and lysis decreased after an endurance flight in a wind tunnel (Nebel et al., 2012). In pigeons, significant reductions in eosinophils and monocytes were found in pigeons after flight, but no significant change in hemagglutination was found (Matson et al., 2012). The effects of exercise, as well as the agglutination and lysis titers, differ between species (Matson et al., 2005; Nebel et al., 2012). Because of this the effect of seasonal change and physical activity on the constitutive innate humoral immune defence of barnacle geese is currently poorly understood.

Research questions

My research will address the following question: What is the effect of exercise on the constitutive innate humoral immune defence of barnacle geese from migratory and non-migratory populations? In order to answer this, two sub-questions need to be answered first:

1. To what extent do constitutive innate humoral immune defences differ between migratory and non-migratory populations barnacle geese across seasons?

2. To what extent do constitutive innate humoral immune defences differ before and after physical activity in barnacle geese across seasons?

Hypothesis

I expect that both the hemagglutination and the hemolysis of the migratory birds will be increased compared to the non-migratory birds during migratory preparation (spring and autumn). I suspect that this is due to increased investing in their immune system as a part of the migratory preparation that would occur in the wild. Immunoenhancement before migration would give greater protection to diseases at stop-over sites and the destination (O'Neil & Ketterson, 2012; Versteegh et al., 2014). I do not expect significant differences during the rest of the year.

I also expect that the hemagglutination and the hemolysis of birds just after physical activity will be decreased compared to before the exercise, due to the trade-off between immune response and strenuous exercise. Because constitutive immunity is maintained even in the absence of infection, it is likely to be important in the trade-off between constitutive humoral immunity and intense physical activity, such as migratory flight (Matson et al., 2012; Nebel et al., 2012).

Materials & Methods

Sample Collection

The serum samples used in this study have been collected from 16 barnacle geese captured from two different wild populations: non-migratory 'Dutch' geese and migratory 'Russian' geese. The geese have been kept together in a common-garden environment at the Netherlands Institute of Ecology (NIOO-KNAW). The non-migratory geese have been further divided into two groups: 'running' (exercised four times on a treadmill) and 'non-running' (not exercised). All the Russian geese were exercised and thus are designated as 'running'. In total there are three sample groups: 'running Dutch', 'non-running Dutch' and 'running Russian' (see Table 1). In total 189 serum samples were collected.

Table 1 Distribution of sampled individuals

Group	**Quantity of individuals**
Running Dutch	7
Non-running Dutch	4
Running Russian	5

The serum samples to be used for the hemolysis–hemagglutination assay were collected approximately once a month in: October (2019), January (2020), February (2020), March (2020), May (2020), June (2020), a "moult" period containing July and August, October (2020) and January (2021). Four exercise trials, each of which lasted for 2.5 hours, were scheduled several days after the blood collection on February, March, moult, and October. Each running goose experienced 2 hours of food fasting before the trial and the post-activity bloods were collected from 3 hours after the trial started, regardless of whether a bird managed to complete the planned 2.5 hours' trial or not. The 4 non-running Dutch geese were brought indoor for 5 hours of food fasting until the post-fasting blood samples were collected for each trial. Each goose has been assigned an ID code. The samples were labelled with the ID of the bird and the date the sample was taken. The samples were then stored in the freezer in the Lumen building at the Wageningen University & Research campus.

The hemolysis-hemagglutination assay

The hemolysis-hemagglutination assay used in this thesis is a slightly modified version of the one described by Matson et al. (2005). The main change to the protocol is the use of two 96-wells (each 8 rows by 12 columns) instead of one. First the plates were prepared by milk-blocking the wells, in order to reduce non-specific bonding. 25µL of 6 plasma samples was added to the 1st two columns of rows 2-7 and 25µL of chicken plasma to the 1st two columns of rows 1 and 8. Next the samples were serially diluted from columns 2-11 and columns 12-22 (second plate). After this a 1% RBC suspension of rabbit blood (Xebios) was added to all the wells. Finally, the plates were incubated in a water bath at 38 degrees for 90 minutes, cooled at room temperature for 20 minutes at a 45-degree angle, and scanned for scoring of the agglutination. After this the plates were left flat at room temperature for another 70 min and scanned again for scoring of the lysis. A final scan to score for lysis will be performed 24 hours (+/- 2 hours) post water bath removal.

Data analysis

The data analysis of this study was performed using the software R (version 4.2.2) and RStudio (version 2022.12.0).

To analyse the relationship between the humoral immune defence and seasonal change three linear mixed models (hemagglutination, hemolysis after 90 minutes, hemolysis after 24 hours) were created. The data set includes all three populations and seven sampling periods (stages).

$$Humoral\ immune\ defense \sim stage\ *\ pop\_running\ +\ (1|bird\_ID)$$

The explanatory variable "stage" representing the month the sample was collected with the exception of the "moult stage" samples which were collected when 9th primary feather of the bird regrew over 200 mm. The explanatory variable "pop_running" contains the population and treatment (Russian vs Dutch and running vs non-running) of the specific bird. Finally, the variable "bird_ID" represents each individual bird.

A second set of three linear mixed models were created to test the correlation between the humoral immune defence and physical activity. The second set of models includes the two "running" populations and the four stages in which exercise trials occurred.

$$Humoral\ immune\ defense \sim stage\ *\ pop\_running\ *\ type\ +\ (1|bird\_ID)$$

The explanatory variable "type" represents the moment of sampling (before or after exercise).

Post-hoc tests to check for interactions were done using pairwise comparison using the Tukey method for p-value adjustment.

Results

The hemagglutination-hemolysis assay

For the hemagglutination-hemolysis assay 176 samples were tested and scored. The hemagglutination scores of all samples ranged from 6 to 21. Hemolysis scores at 90 minutes ranged from 0 to 5 and the hemolysis scores after 24 hours ranged from 0 to 6.25.

Constitutive humoral immunity across seasons

Hemagglutination

The ANOVA showed that there is a significant difference in the hemagglutination scores across the seasons ($F = 3.4176$; $p < 0.01$) (**Figure 1**). A significant interaction between the sampling period and the populations was found ($F = 1.9652$; $p < 0.05$).

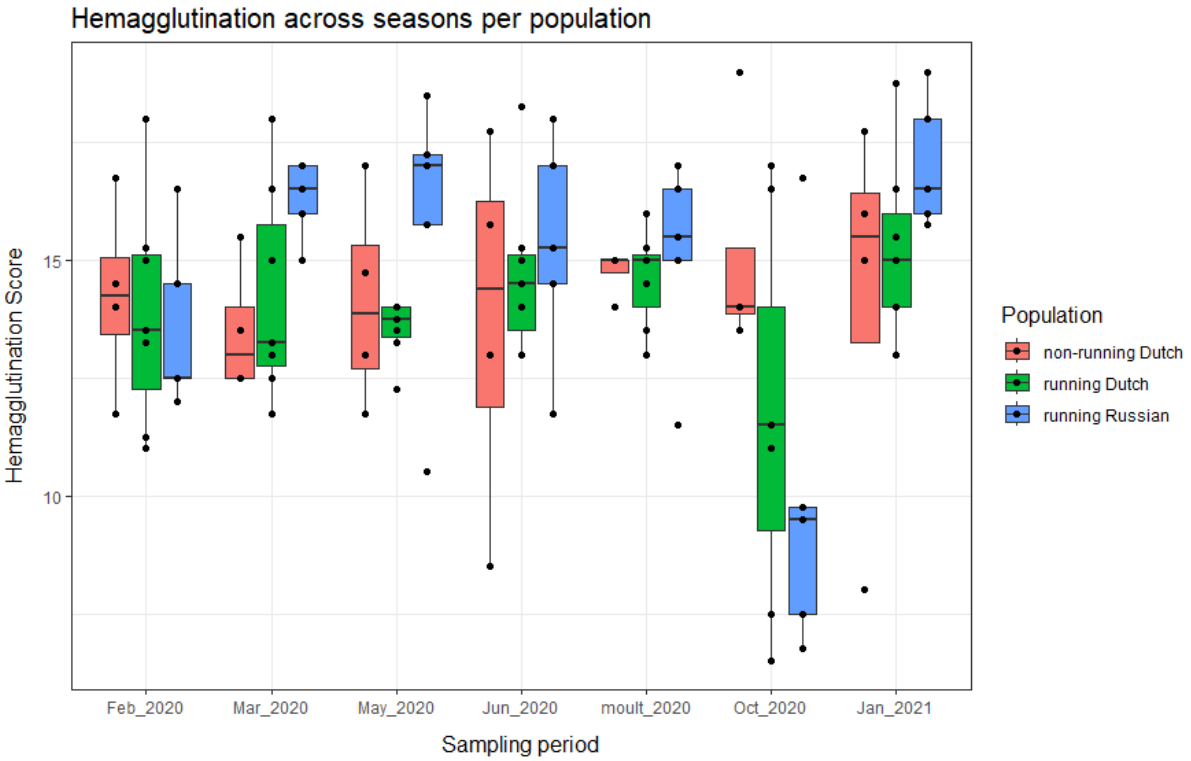

Figure 1 Hemagglutination scores of the three populations across seasons
Boxplot of hemagglutination scores of the three populations in each sampling period. Colours denote population. Horizontal black lines denote the median value. The boxes extend from the 25th to the 75th percentile. Dots denote observations.

Hemagglutination was stable across seasons for both the running and non-running Dutch populations. There is a decrease in hemagglutination in October in the running Dutch population, but this not significant. The decrease in October was significant for the running Russian population (**Table 1**)

Table 1 Significant results of the pairwise comparison between stages within each population

Population	**Compared sampling periods**		**p - value**
Running Russian	October	March	0.0012
	October	May	0.0040
	October	June	0.0120
	October	Moult	0.0181
	October	January 2021	0.0002

Hemolysis at 90 minutes

The ANOVA found significant differences in the hemolysis scores at 90 minutes between sampling periods (F = 5.7531; p = 3.792e-05) (Figure 2). A significant interaction between the sampling period and the populations (F = 2.9109; p < 0.01) was also found.

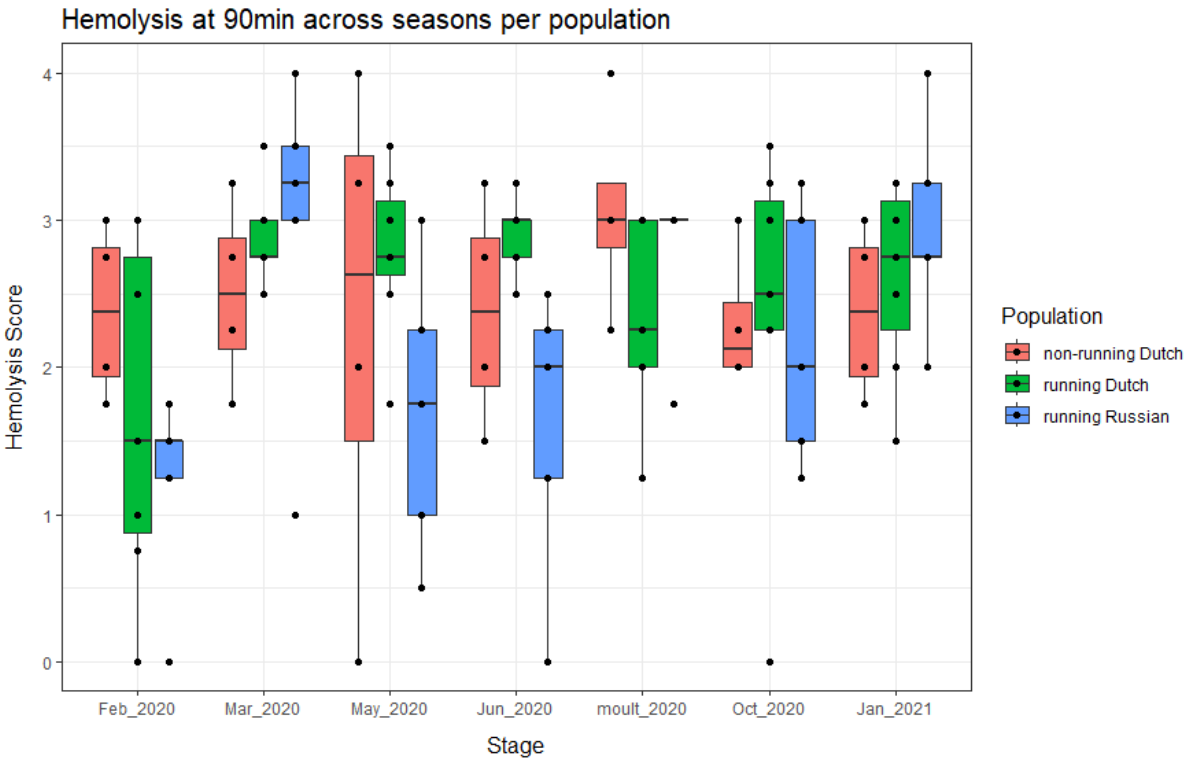

Figure 2 ***Hemolysis scores of the three populations across seasons***
Boxplot of hemolysis scores after 90 minutes of the three populations in each sampling period. Colours denote population. Horizontal black lines denote the median value. The boxes extend from the 25th to the 75th percentile. Dots denote observations.

For the non-running Dutch hemolysis was stable across seasons. For the running Dutch population hemolysis was low in February, then increased in March and remained at this level in May and June. It then dropped to levels similar to February for the rest of the measurements. The hemolysis of the running Russian was low in February, then increased in March. Hemolysis was lower during May and June. The scores increased during Moult and remained at similar levels during October and January 2021 (**Table 2**).

Table 2 Significant results of the pairwise comparison between stages within each population

Population	**Compared sampling periods**		**p - value**
Running Dutch	February	March	0.0107
	February	May	0.0277
	February	June	0.0107
Running Russian	February	March	0.0007
	February	Moult	0.0043
	February	January 2021	0.0007
	January 2021	May	0.0421
	January 2021	June	0.0207
	June	March	0.0207
	March	May	0.0421

Hemolysis at 24 hours

The ANOVA found significant differences in the hemolysis scores after 24 hours of the different sampling periods ($F = 4.5717$; $p < 0.001$) (Figure 3). No significant interactions were found.

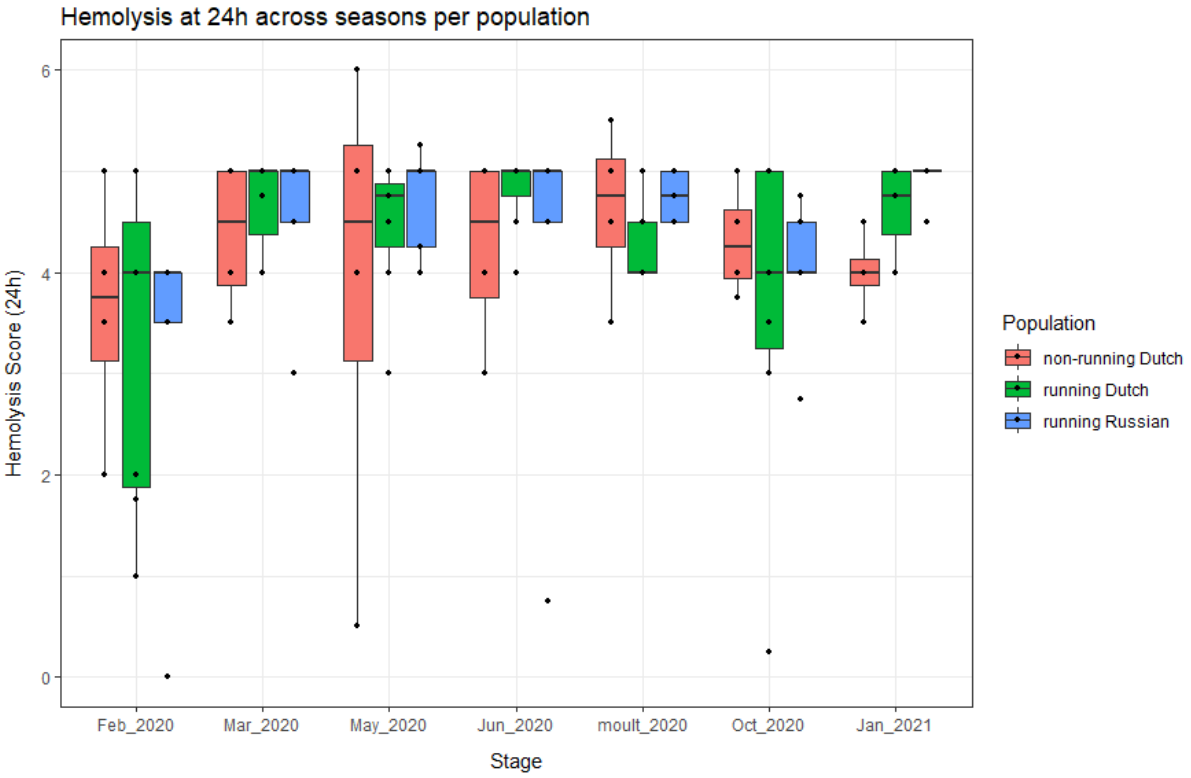

Figure 3 ***Hemolysis scores of the three populations across seasons***
Boxplot of hemolysis scores after 24 hours of the three populations in each sampling period. Colours denote population. Horizontal black lines denote the median value. The boxes extend from the 25th to the 75th percentile. Dots denote observations.

Hemolysis after 24 hours was low in February, then increased in March. Hemolysis remained at this level for the rest of the sampling periods (**Table 3**).

Table 3 Results of the pairwise comparison between stages.

Compared sampling periods		p - value
February	March	0.0029
February	May	0.0171
February	June	0.0108
February	Moult	0.0041
February	January 2021	0.0025

Constitutive humoral immunity before and after physical activity

Hemagglutination

The ANOVA found significant differences in hemagglutination scores between the sampling periods ($F = 4.6454$; $p < 0.01$) (**Figure 5** and **Figure 4**). Significant interactions were found between sampling period and type ($F = 5.2053$; $p < 0.01$), and between the populations and type ($F = 4.5691$; $p < 0.05$) (**Figure 6**).

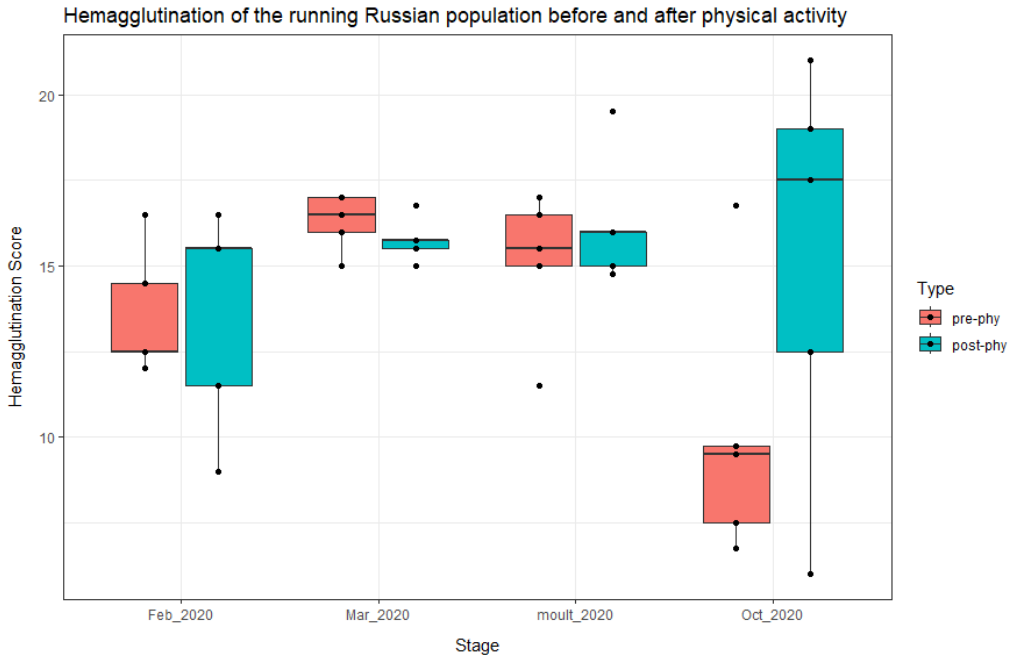

Figure 5 Hemagglutination scores of the running Russian population pre- and post-activity.
Boxplot of hemagglutination scores of the running Russian population for four exercise trials. Colours denote pre- and post-activity. Horizontal black lines denote the median value. The boxes extend from the 25th to the 75th percentile. Dots denote observations.

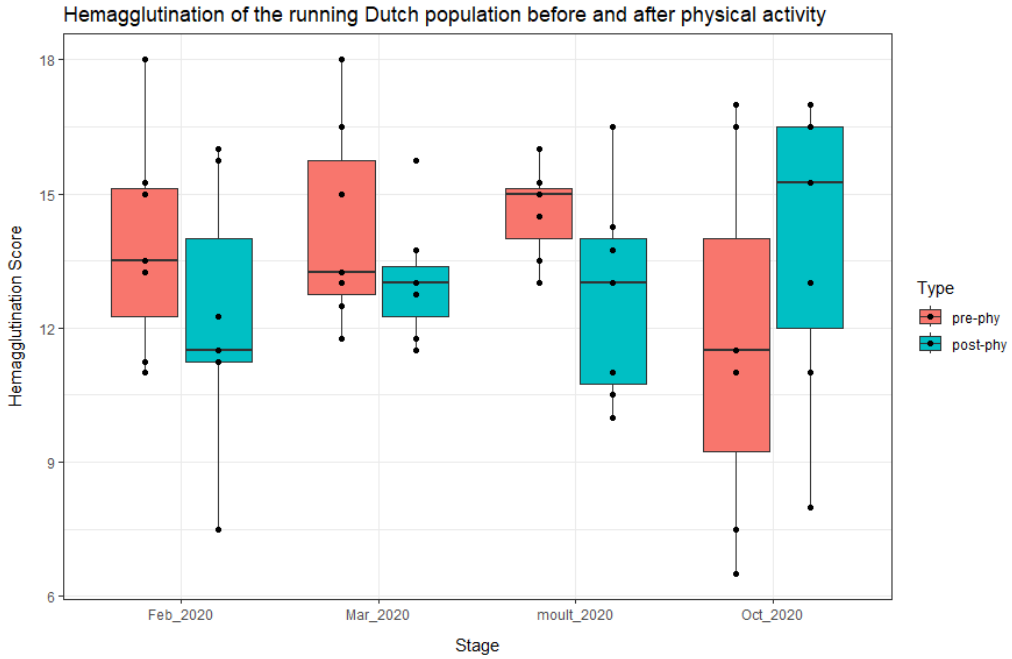

Figure 4 Hemagglutination scores of the running Dutch population pre- and post-activity.
Boxplot of hemagglutination scores of the running Dutch population for four exercise trials. Colours denote pre- and post-activity. Horizontal black lines denote the median value. The boxes extend from the 25th to the 75th percentile. Dots denote observations.

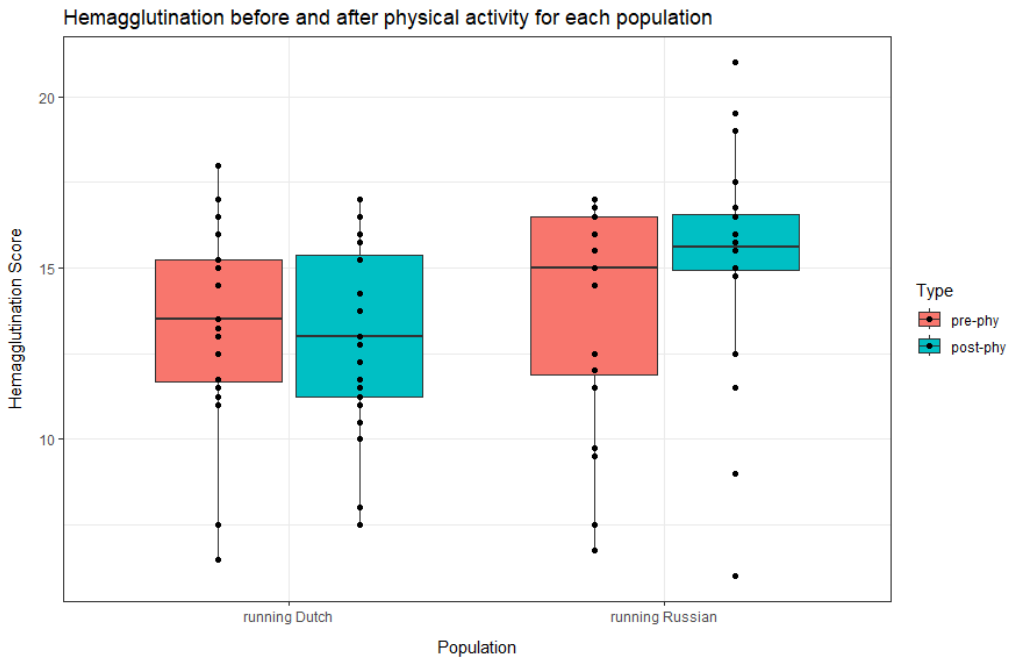

Figure 6 Hemagglutination scores of both populations before and after physical activity.
Boxplot of hemagglutination scores of both populations pre- and post-activity. Colours denote pre- and post-activity. Horizontal black lines denote the median value. The boxes extend from the 25th to the 75th percentile. Dots denote observations.

It was found that hemagglutination was at similar levels across three of the four trials for the running Russian population. In the fourth trial the pre-activity scores were significantly lower than the post-activity scores ($p < 0.01$). In the running Dutch population, the pre- and post-activity scores were similar for all four trials.

Hemolysis after 90 minutes

The ANOVA found significant differences in the hemolysis scores between the sampling periods (F = 5.6326; $p < 0.01$), as well as between pre- and post-activity (F = 7.3695; $p < 0.01$) (**Figure** and **Figure**). A significant interaction between the sampling period and type (F = 3.5113; $p < 0.05$) was also found (Figure 9).

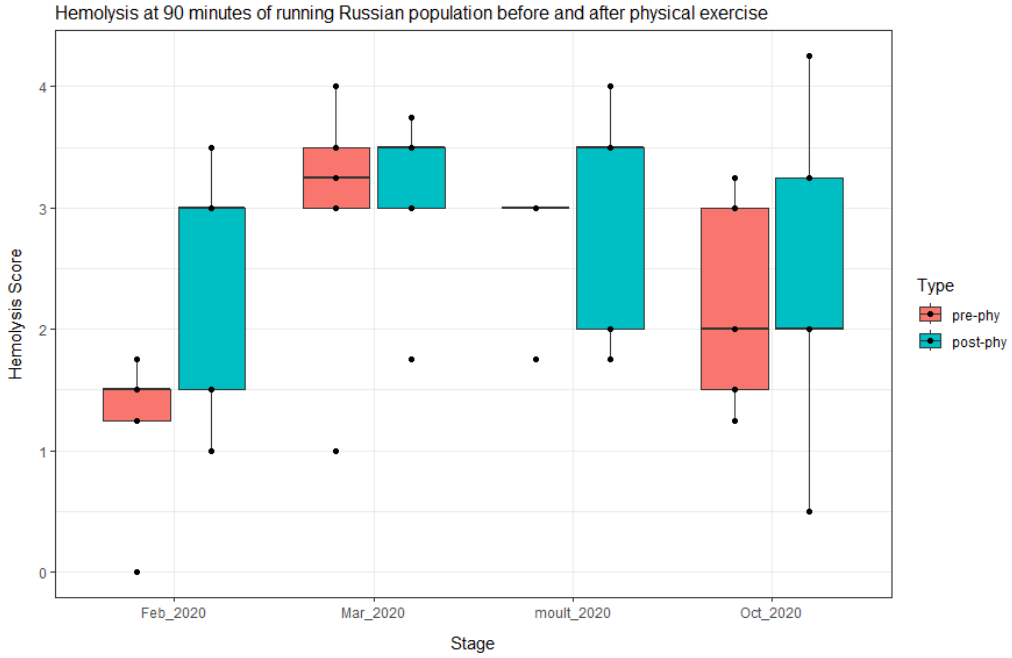

Figure 7 Hemolysis scores of the running Russian population pre- and post-activity.
Boxplot of hemolysis scores after 90 minutes of the running Russian population for four exercise trials. Colours denote pre- and post-activity. Horizontal black lines denote the median value. The boxes extend from the 25th to the 75th percentile. Dots denote observations.

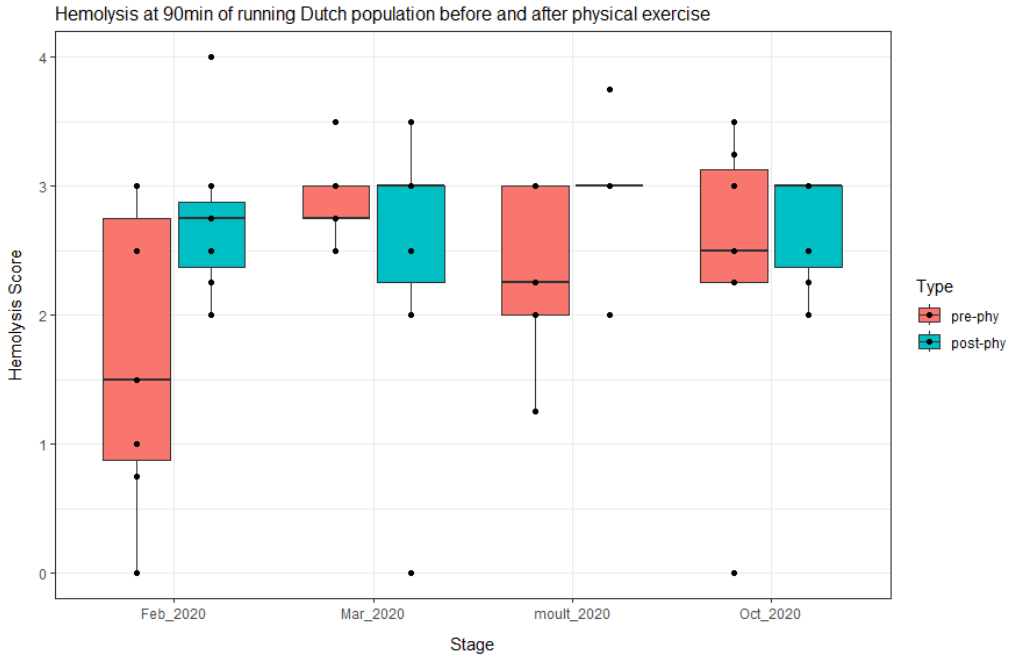

Figure 8 Hemolysis scores of the running Dutch population pre- and post-activity.
Boxplot of hemolysis scores after 90 minutes of the running Dutch population for four exercise trials. Colours denote pre- and post-activity. Horizontal black lines denote the median value. The boxes extend from the 25th to the 75th percentile. Dots denote observations.

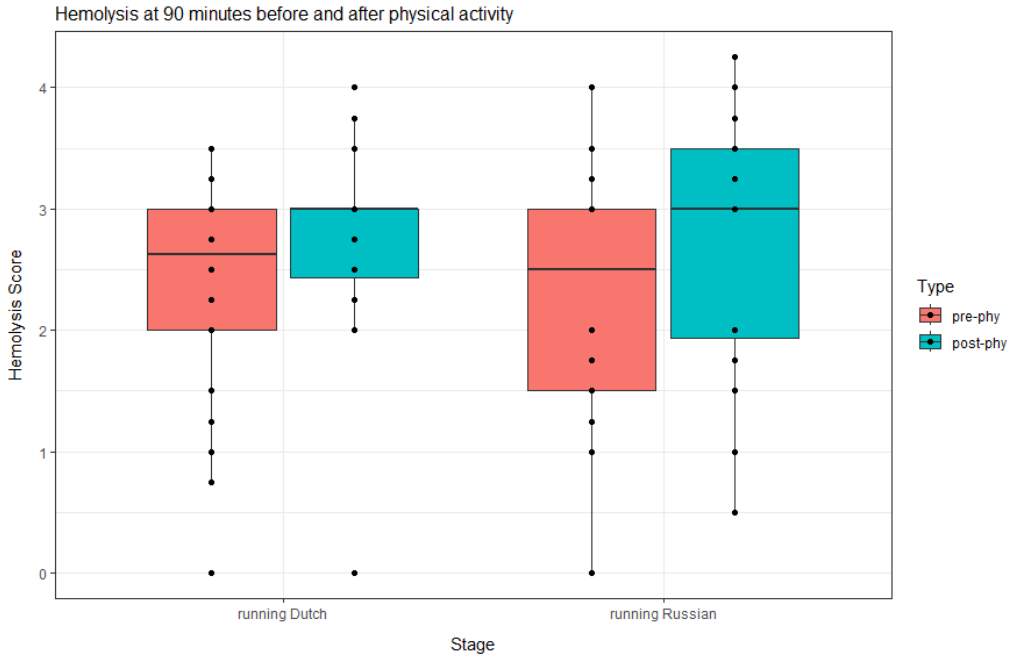

Figure 9 Hemolysis scores pre- and post-activity.
Boxplot of hemolysis scores after 90 minutes pre- and post-activity. Colours denote pre- and post-activity. Horizontal black lines denote the median value. The boxes extend from the 25th to the 75th percentile. Dots denote observations.

Hemolysis in both populations was significantly lower pre-activity in the first trial ($p < 0.001$), the scores increased post-activity. The scores remained at similar levels for the other three trials.

Hemolysis after 24 hours

The ANOVA found significant differences in the hemolysis scores between the sampling periods (F = 3.6635; $p < 0.05$) (Figure 10 and Figure 11). No significant interactions were found.

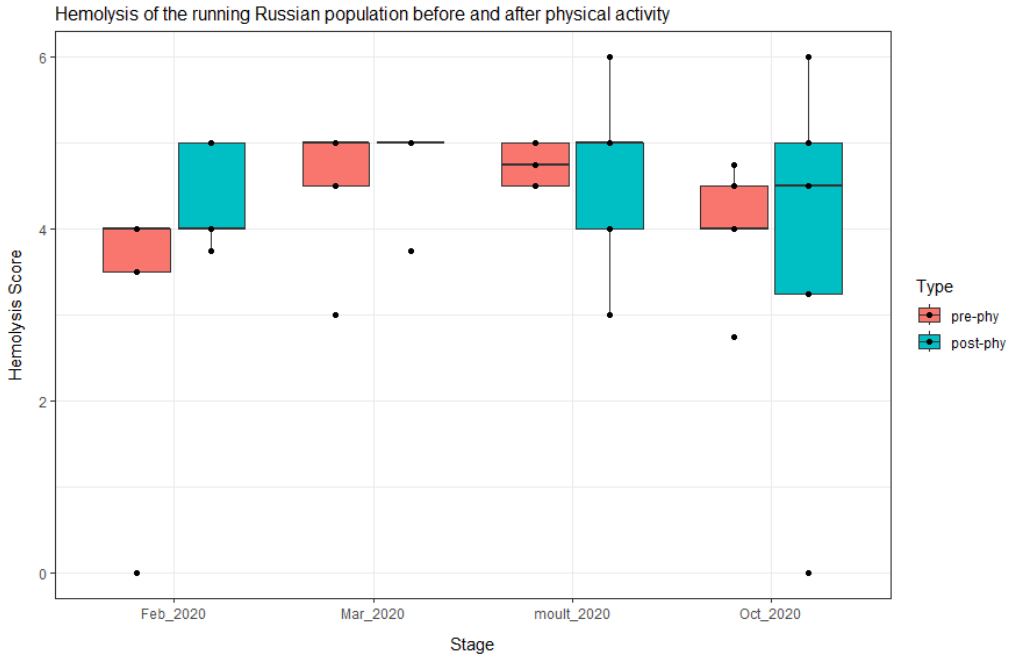

Figure 10 Hemolysis scores of the running Russian population pre- and post-activity.
Boxplot of hemolysis scores after 24 hours of the running Russian population for four exercise trials. Colours denote pre- and post-activity. Horizontal black lines denote the median value. The boxes extend from the 25th to the 75th percentile. Dots denote observations.

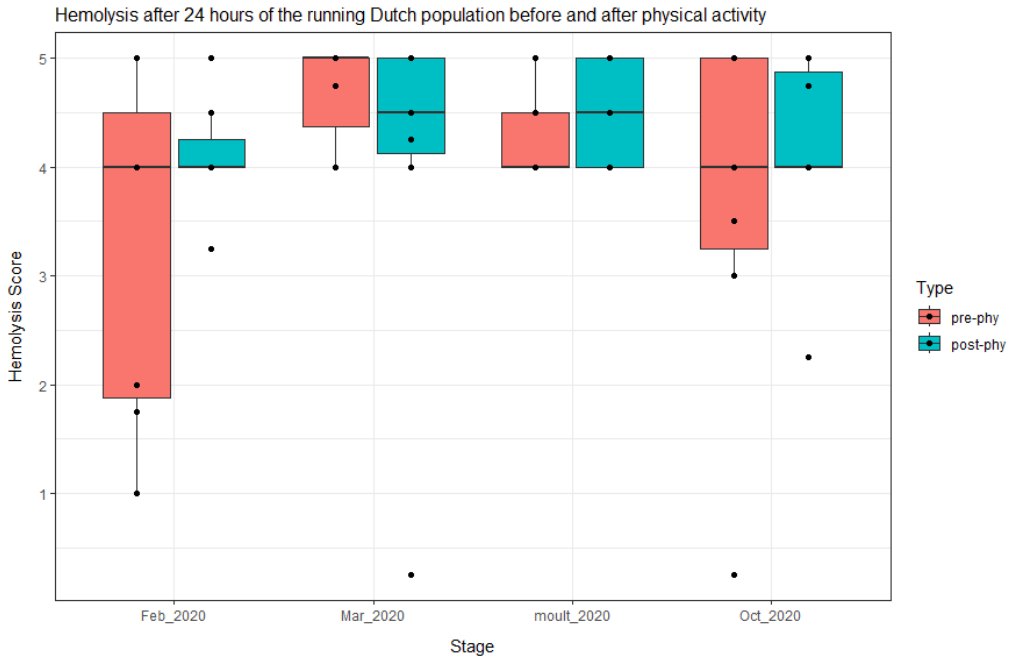

Figure 11 Hemolysis scores of the running Dutch population pre- and post-activity.
Boxplot of hemolysis scores after 24 hours of the running Dutch population for four exercise trials. Colours denote pre- and post-activity. Horizontal black lines denote the median value. The boxes extend from the 25th to the 75th percentile. Dots denote observations.

The hemolysis scores after 24 hours were significantly lower pre-activity compared to the post-activity scores ($p < 0.001$). In the next trial scores increased and remained at similar levels for the other trials.

Discussion

Immune function across seasons

The results show that hemagglutination significantly decreased during autumn in the running Russian population. A significant interaction between sampling period and population was also observed, showing a strong decrease in the running Russian population. It was also found that hemolysis after 90 minutes significantly differed between seasons. Here a significant interaction between sampling period and population, with a strong increase during early spring, was found. Hemolysis after 24 hours was found to be significantly decreased during February. It was hypothesized that the hemagglutination scores of the migratory (Russian) population would increase during spring and autumn. As our results contradict this hypothesis it cannot be accepted.

A study investigating differences in constitutive immune function in long- and short distance migrants found no difference in hemagglutination or hemolysis between early and late short distance migrants (Hegemann et al., 2022). Hegemann et al., (2022) suggest that investment into immune function is dependent on migration strategy (long– vs short-distance migration). This would explain the differences found between the migratory and non-migratory populations found in this thesis. A possible explanation for the contradictory results of this thesis could be that different parameters of immune function show different patterns in different species.

A study on the effect of environment and age on constitutive immune function in several subspecies Red Knots found no significant differences in immune function between the subspecies. This suggests that environmental factors play a more important part in determining constitutive immune function than genetics. It was concluded that both environmental factors, such as resource availability, and individual level factors influence immune function (Buehler et al., 2009). Similar results were found by Hegemann et al., (2012). Their study on immune function in free-living skylarks found that hemolysis and agglutination varied throughout the year, with lysis peaking during breeding and agglutination decreasing after breeding. Both lysis and agglutination also varied significantly between years. It was theorized that this is the result of variations in resource availability and parasite pressure among years. These environmental variations then affect trade-offs between immune function and other physiological systems. This would then result in immunological flexibility across seasons and among years. As the geese sampled for this study were kept in a common-garden environment the differences between the three populations cannot be explained by differences in environmental conditions.

In this thesis a significant difference in hemagglutination between the migratory (Russian) and non-migratory (Dutch) populations was found. This suggests that genetic differences between the two populations play an important role in regulating hemagglutination across seasons. Hemolysis after 90 minutes differed between the migratory and non-migratory populations, as well as between the two non-migratory populations (running and non-running). This indicates that in addition to genetic differences, physical activity likely affects hemolysis. Because the measurements used span one year it is not known if the result found remain consistent across multiple years.

Immune function before and after physical activity

The results found that hemagglutination before physical activity was decreased compared to after in one exercise trial for the migratory population, as well as a significant interaction between type and population. No significant difference in hemolysis between the migratory and non-migratory populations was found.

In their study Owen and Moore (2006) found that thrushes captured during migration had lowered immune function compared to non-migratory individuals of the same species. They found that the migratory thrushes had lower hematocrit, leukocyte and lymphocyte counts, and higher heterophil counts and H:L ratios. They suspect that if migration compromises the immune system this could result in increased susceptibility to disease and longer stays at stopover sites. While this study did not measure natural antibodies their results seem to contradict the results of this thesis. A possible reason for this discrepancy could be that the samples from Owen and Moore were taken from thrushes during their migration and the samples used in this thesis were collected just after physical activity. A study on the constitutive immune function of northern wheatears at stop-over sites found that both agglutination and lysis increased significantly within two days (Eikenaar et al., 2020). This suggests that stop-over sites are not only used to refuel, but also to recover during migrations.

A study on the effects of repeated wind-tunnel flights on humoral acquired induced immunity in red knots found no difference in antibody production when comparing flown individuals to non-flown controls. They theorized that flight is not as demanding as previously thought or that poor conditions in the wild, such as food constraints or adverse weather may induce stress and immunosuppression (Hasselquist et al., 2007). In their study Nebel et al. (2012) did find that both hemagglutination and hemolysis in European starlings were decreased after an endurance flight in a wind tunnel. They theorize that these decreases in immune function could be due to being in a migratory state or as a response to the physical activity due to adaptive trade-offs between immune function and migration.

Hemagglutination of the running Russian population was decreased pre-activity in the fourth trial, while hemolysis was decreased pre-activity in the first trial. In both cases the post-activity levels were similar to the levels of the other trials. The first trial occurred in late winter (February) and the fourth trial occurred in autumn. It could be that immune defence is downregulated during this time of year but increased post-activity possibly due to the exercise on the treadmill. The interaction found between both the sampling period and type as well as between population and type for hemagglutination suggests that both the time of year and genetics play a role in regulating hemagglutination. For hemolysis the interaction between sampling period and type suggest that the time of year plays a major part in the regulation of this part of the humoral immune system

Conclusion & Recommendations

In conclusion, that genetics play a role in how seasonal change and physical activity affect constitutive humoral immunity. While the agglutination and lysis were found to differ significantly in certain months it is difficult to draw general conclusions based on the results of one year of sampling. Additionally, I found that both genetics and season play important roles in the regulation of constitutive humoral immunity pre- and post-activity. It is similarly difficult to draw conclusions on the effects of physical exercise on constitutive humoral immunity based on previous research.

I recommend that future research increases the frequency of the sampling to every month to get a complete picture of the changes in the humoral immune defence across seasons. Additionally, I recommend increasing the sampling to multiple years, to determine if changes in constitutive humoral immunity are stable across years. Finally, I recommend for future studies to increase the sample size and sample an equal number of individuals per population, as well as create four groups in order to have control groups for both seasonal change and physical activity.

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