Optimal blood handling for measures of ecological immunology and diet choice

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Summary

In wildlife blood is a much used diagnostic tool to investigate health status and lately also to investigate foraging habitat and trophic position. Under field circumstances however, direct centrifugation of blood isn't always an option.
The aim of this study was to investigate whether or not delayed centrifugation influences the outcome of a range of common ecological assays.
We took single blood samples from 9 mallards (*Anas platyrhynchos*) and 9 Bewick's swans *(Cygnus bewickii)*, which we centrifuged after 0, 2 and 4 hours, to perform 4 commonly used ecological assays; antibodies to avian influenza, haptoglobin, hemolysis and hemagglutination and stable isotopes of Carbon and Nitrogen.
We also aimed to investigate whether the type of fluid component used (sera or plasma) influences the outcome of these assays.
Results of the assays showed no significant differences between different time points.
Type of fluid component did influence some outcomes. Significant differences were found for hemagglutination and stable isotopes assays.
Results of this study suggest that when performing a hemagglutination or stable isotopes assay only one type of fluid component should be used in order to get comparable results.

Contents

Introduction

Blood is commonly used for ecological research in wildlife For example, blood is often used during investigation of the health status of animals, including many immune and general health assays. Examples of these are: hemolysis and hemagglutination test, which is used to measure innate immunity [1], avian influenza antibody test, used to measure seroprevalence of antibodies to avian influenza [6], and the haptoglobin test, which is used to measure recent inflammatory processes [4]. And recently, blood samples from wildlife are being used to infer the habitat in which an individual has been foraging in the previous days and weeks through assessment of the concentration of various stable isotopes [14, 16]. Because the environmental variation in heavy isotope concentration is reflected in tissues of animals, this facilitates the investigation of trophic position and migratory patterns.
So by a single blood sample, it's possible to answer a wide range of questions.

However, since both health and diet investigations require the analyses of sera or plasma, this has to be achieved by centrifugation of the blood. While most researchers aim to minimize the time between collecting blood samples and separating the cells from the fluid fraction, under certain field conditions (such as handling a large volume of animals or working in remote locations) this may not be possible for a number of hours. Delayed separation of blood components has been seen to alter concentrations of plasma and sera glucose, phosphorus and potassium in human clinical studies [17,18], however, little is known of the temporal changes occurring in wildlife blood, particularly in relation to common health and diet analyses.

Given the widespread use of health and diet analyses in ecophysiology, and the potential for significant inter-species and inter-study differences in the time between taking and separating blood samples, we aimed to investigate whether delayed centrifugation may influence the outcome of a range of common ecological assays.
The ecological assays we plan to use include measures of:

- Disease exposure:
  Antibodies to endemic wildlife disease [Avian Influenza Viruses]
  Recent inflammatory response [Haptoglobin concentration]
- Innate Immune function:
  Natural and compliment antibodies
- Foraging habitat:
  Carbon and Nitrogen stable isotope concentration

In addition we aim to investigate whether the type of fluid component used (sera or plasma) influences the outcome of these assays. Plasma is the supernatant obtained following

centrifugation of unclotted whole blood, where as sera is the supernatant of blood that has been allowed to clot prior to centrifugation. Both are widely used in field studies; however, until today no direct comparison of the functional difference between the two fluids has been made.

Avian Influenza

In waterfowl infections with avian influenza are frequently present and waterfowl are considered a very important natural reservoir of avian influenza(reference). These infections mostly do not cause clinical signs in their hosts, but can be highly pathogenic to other birds, like chickens and turkeys [3].
Therefore understanding the natural reservoir of AI is very important in predicting potential outbreaks and the main reason why a lot of research is being done on avian influenza.
When the influenza virus becomes present in the body antibodies are produced in order to fight infection. Several tests, including an ELISA, can be used to measure the presence of antibodies and therefore these can be used to determine whether or not a bird has been exposed to the virus, and whether or not that specific variant is a potential risk.

Hemolysis and Hemagglutination (HLHA)

The HLHA test is a relatively easy way to measure two important features of the innate immune system, the complement system and natural antibodies.
Complement helps to fight infection in the body. Through a number of pathways, complement is activated to perform its basic functions [2].
Natural antibodies (NAbs) usually are mainly produced by white blood cells in the body [8]. Natural Antibodies differ from induced specific antibodies because they seem to occur in sera without appearance of any antigenic stimulation, by which they link innate and adaptive immunity [9]. Natural antibodies are able to bind to a large variety of exogenous antigens, including those on bacteria and viruses, thus neutralizing them [8].

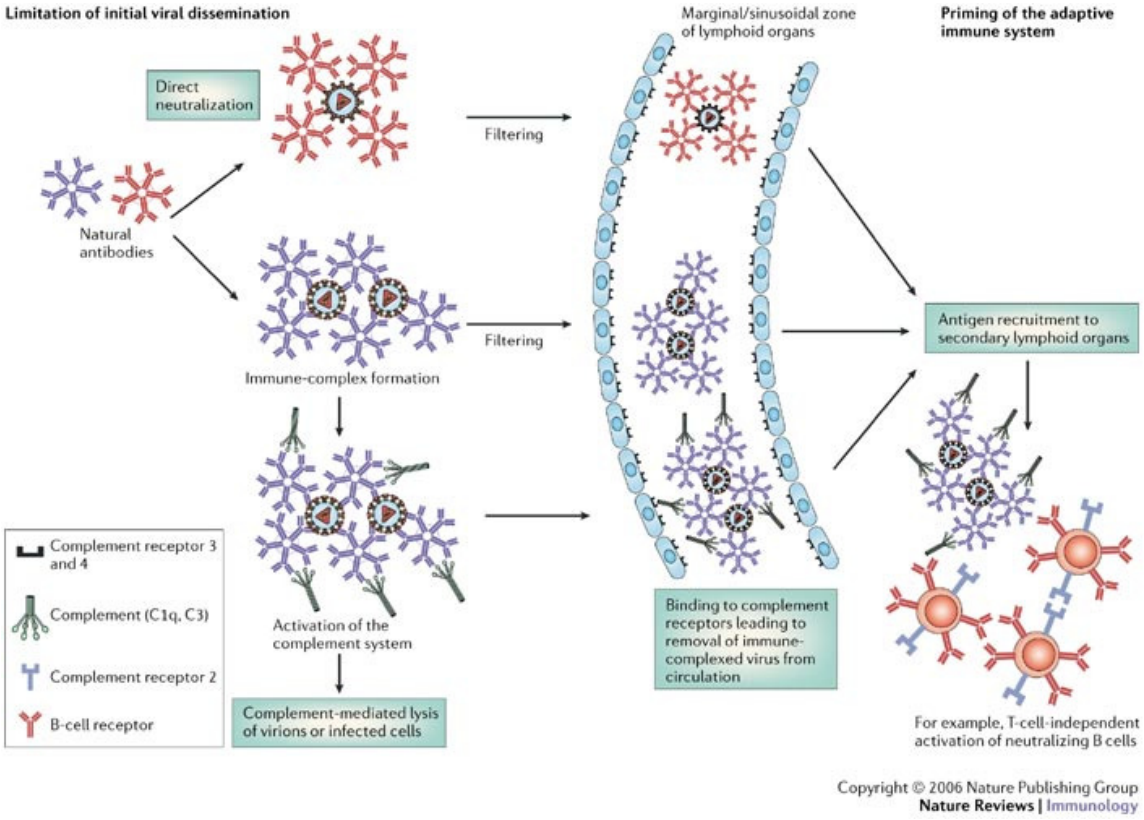


Fig. 1: Natural antibodies and complement From the following article: *Antiviral antibody responses: the two extremes of a wide spectrum* Lars Hangartner, Rolf M. Zinkernagel and Hans Hengartner **Nature Reviews Immunology 6, 231-243 (March 2006)**

In wildlife studies, determining immune function has become an important tool for evolutionary and ecological investigation, but the types of assays that can be performed on wild animals are limited. The stress of handling, the difficulty and unreliability of recapturing animals, the lack of specialized reagents and the small size of study groups all are constraining factors [1]. Therefore, the HLHA assay provides an easy way of assessing innate immune function in wildlife, with only a single and small blood sample [1] where lysis reflects binding of the blood to complement and natural antibodies, whereas agglutination reflects only the interaction with natural antibodies [1].
For instance, research has been done to evaluate immune strategies of red knots during an annual cycle using the HLHA assay [7] and in 2008 Buehler et.al. used the HLHA assay among other assays to identify different immune strategies in different environments [13]

Haptoglobin

Haptoglobin (Hp) is a protein which has various functions in the body and is found in both humans and animals.
Haptoglobin is synthesized by the liver and under normal conditions is found in only small amounts in the blood. The main function of Hp is to trap haemoglobin, which is freed during hemolysis. In order to do this, Hp binds to the haem portion and forms a haptoglobin-hemoglobincomplex, which is degraded in the liver by the RES and eliminated through the bile. This way Hp inhibits the oxidative effect of haemoglobin and prevents it from serving as a nutrient for invading pathogens.
In case of hemolysis the Hp concentration in the blood will drop quickly [4].

In case of an inflammatory response though, the Hp concentration in the blood will be high. During inflammation haptoglobin acts as acute phase protein, where it inhibits microbe iron uptake. In wildlife studies Hp is therefore used as a way of measuring recent inflammatory response.

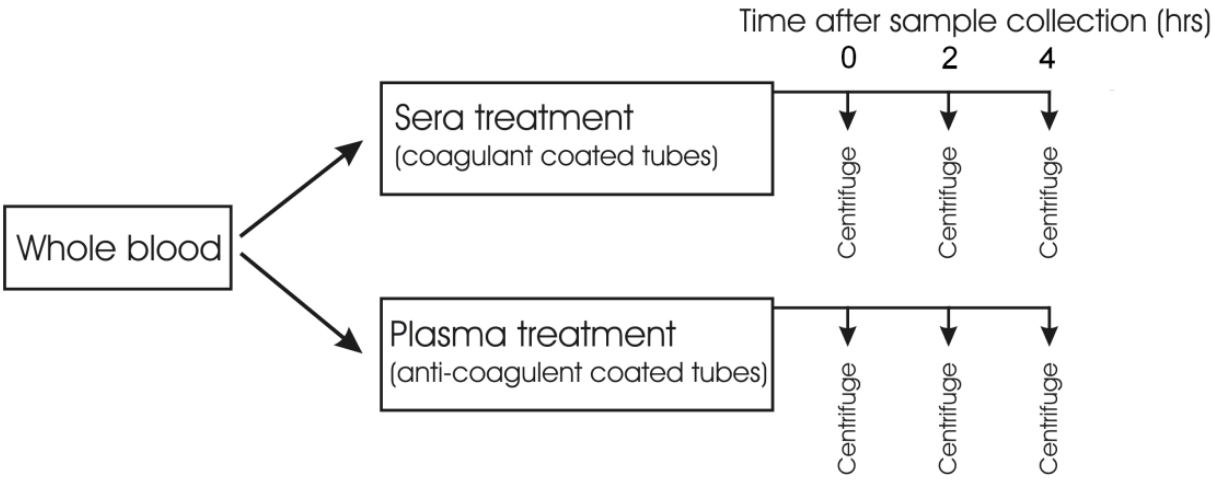
Stable Isotopes

Isotopes of elements differ in mass and are often fractionated from one another by biological, physical, or chemical processes. These fractionations result in natural variations in the isotopic compositions of compounds throughout the environment. When foraging, animals take up these compounds and isotopes are then incorporated in their tissues in a way that resembles the isotopic composition of their diet, hence stable isotopes provide a way of determining foraging habitats of animals.
The stable isotopes of C and N are relevant to ecological applications since they are used to track the input of these elements within food-webs. Because isotopic ratios change slightly in a predictable way when being incorporated in tissue, in the order of 2‰ [16], they are also used to determine the trophic positions of species.
By analyzing stable isotopes in tissue of animals, it becomes possible to determine what, when and where an animal ate and by this migratory patterns [14], trophic positions [16] and foraging areas [10] can be determined.

Materials and Methods

Experimental design

The experiment was conducted in two different waterfowl species, mallards (*Anas platyrhynchos*) and Bewick's swans *(Cygnus bewickii).* Approximately 3ml of whole blood (up to 1% of the circulating blood volume of a 5kg swan; approximately 5% of the circulating blood volume of a mallard) was collected from 9 individuals of each species from the vena saphena. Each sample was directly split into 6 separate experimental vials, 3 vials containing a commercially prepared anticoagulant for the plasma treatment (Greiner Bioone #450477) and 3 containing a commercially prepared clotting agent for the sera treatment (Greiner Bioone #450470).
The tubes were then stored at room temperature for 30 minutes, after which the first two blood samples, sera and plasma t = 0, were centrifuged. The remaining vials were then kept in the refrigerator at 4°C until centrifugation.
After centrifuging, the plasma and the sera were pipetted into labelled vials and frozen at -20°C. This procedure was repeated after 2 and 4 hours, respectively, each repeat using one plasma and one serum vial, according to the timetable below.



Animals

All 18 birds live in captivity at the NIOO Heteren and are used to being handled. The mallards live together in a group, whilst the swans live in pairs. For this experiment male and female animals were chosen at random.
After bleeding, the animals were returned to their regular housing in the waterfowl facility.

Blood analyses

Antibodies to avian influenza

Whether or not a sample contained antibodies to the nucleoprotein of avian influenza was tested using a comercially available ELISA kit (IDEXX FlockChek AI MultiS-Screen). We analysed the samples following instructions which were included. The test required 1/10 dilution of serum and plasma samples and 100 µL of each diluted sample. All samples were run in duplicate with supplied positive and negative controls.
Presence or absence of AI antibodies was then determined by the ratio of sample to negative (S/N) absorbance, recorded at 650 nm using a microplate reader. Samples with an S/N ratio ≥ 0.50 were considered negative, samples with S/N ratio < 0.50 were considered positive.

Hemolyse/Hemagglutination

We performed this assay as described by Matson et.al. 2005 [1].
We used U-bottom 96-well plates. First 25 µL of each sample was pipetted in columns 1 and 2 of the plate and 25 µL of PBS (Phosphate Bufferd Saline) are added to columns 2-12. Then a serial dilution (1:2) of the contents of column 2 through column 11 was made using a multichannel pipette. After that, 25 µL of a pre-prepared 1% rabbit blood cell suspension, was added to all wells. This suspension was made by diluting whole-rabbit blood stored in 50% alsevers solution in PBS. Then, we sealed each plate with ParafilmM en covered them with a polystyrene plate lid.
The plates were incubated in a 37 °C water bath for 90 minutes and upon completion of the incubation, the long axis of each plate is tilted to a 45° angle for 20 min at room temperature.
The first scans were made then, using a flatbed positive transparency scanner (Hp Scanjet G4050), at 600 dpi.
We left the plates for 70 minutes at room temperature and made the second scan. [1]
After that, all plates were scored for lysis and agglutination.

Haptoglobin

To determine haptoglobin concentration in the samples, we used the Phase Range Haptoglobin Assay kit (Tri delta diagnostics) following the 'manual' method.
The assay was performed at room temperature. Before starting, two reagents were made, following instructions, by using the substances in the kit and the calibrator was

serially diluted with PBS to generate a calibration standard curve. For the assay itself, 7,5 µL of the samples was used.
Absorbance was recorded at 630 nm, using a microplate reader (Synergy HT, BioTek).

Stable Isotope ratios of Nitrogen and carbon

Four microlitres of each sample were freeze-dried overnight at a temperature of -80 °C in tin cups (Tin Capsules D1002, Elemental Microanalysis Limited).
Samples were analyzed using an isotope ratio mass spectrometer.

Statistics

For the first aim we are specifically interested in whether the time between sampling and centrifuging alters the outcome of our ecophysiological tests. As such, these values, were compared in a repeated-measures ANOVA (separately for plasma and sera). Comparison between results of the ELISA antibody to avian influenza test were made using a Cochrane Q-test.
To assess any potential difference between plasma and sera we will compare the result of each treatment within each time point using a paired t-test. Results between plasma and sera in the avian influenza assay were compared using a McNemar's test.

Results

Delayed centrifugation

We obtained consistent results regardless of the time delay between obtaining the sample and centrifuging it. No significant differences in results for centrifugation at, 0, 2 and 4 hours, respectively, were found: p= 0.346 (HL, figures 3 and 4), p=0.769 (HA, figures 5 and 6), p= 0.597 (Hp, figures 7 and 8), p=0.507 (SI N figures 9 and 10), p=0.621 (SI C figures 11 and 12), using a repeated measures ANOVA No effect was found for the antibodies to AI test. All of the values were non-significant (p = 0.368, figures 1 and 2) when compared using a Cochrane Q-test

Fluid component

Significant differences (P<0.05) between treatment groups were found in swans for hemagglutination and stable isotope carbon. The haptoglobin assay also showed significance, but only at time 0 (table 1 and figures 3, 5, 7, 9, 11).

Assay	Time	N	P-value
Hemolysis	0	9	0.500
	2	9	1.000
	4	8	0.567
Hemagglutination	0	9	<0.001
	2	9	0.008
	4	8	0.011
Haptoglobin	0	9	0.004
	2	8	0.506
	4	8	0.060
Stable isotope Nitrogen	0	9	0.667
	2	8	0.142
	4	9	0.529
Stable isotope Carbon	0	9	<0.001
	2	8	0.001
	4	9	0.001

Table 1: Conducted assays and their p-values for Bewick's swans; red values represent significant results

In mallards, significant differences between serum and plasma were found for hemagglutination and stable isotopes carbon and nitrogen (table 2 and figures 4, 6, 8, 10, 12).

Assay	Time	N	P-value
Hemolysis	0	9	1.000
	2	7	0.689
	4	8	0.685
Hemagglutination	0	9	<0.001
	2	7	<0.001
	4	8	0.026
Haptoglobin	0	9	0.155
	2	8	0.185
	4	8	0.605
Stable isotope Nitrogen	0	9	0.014
	2	8	<0.001
	4	8	0.016
Stable isotope Carbon	0	9	<0.001
	2	8	<0.001
	4	8	0.008

Table 2: Conducted assays and their p-values for mallards; red values represent significant results

No significant differences were seen in the detectability of antibodies to the nucleoprotein of avian influenza between serum and plasma ($p>0.05$; McNemar's test; Figure 1 and 2).

The following figures show mean values of the plasma and serum results of the assays conducted with the swans and mallards.

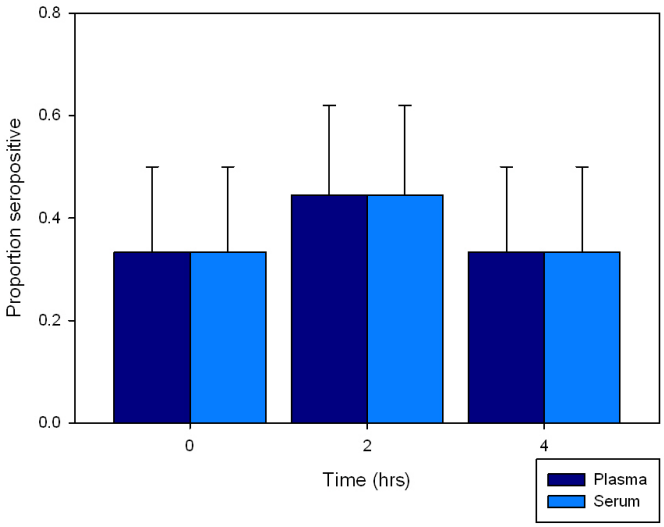


Fig. 1 Mean antibodies to avian influenza in Bewick's swans. Single blood samples, stored to be either serum or plasma and centrifuged at different times after sampling. Error bars represent standard error.

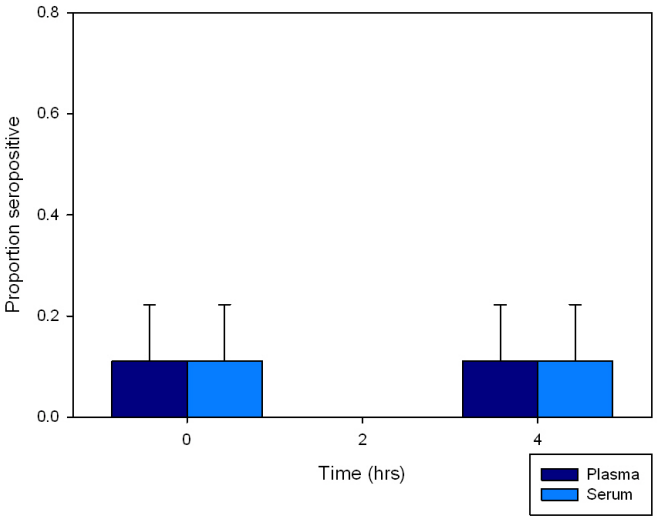


Fig. 2 Mean antibodies to avian influenza in mallards. Single blood samples, stored to be either serum or plasma and centrifuged at different times after sampling. Error bars represent standard error.

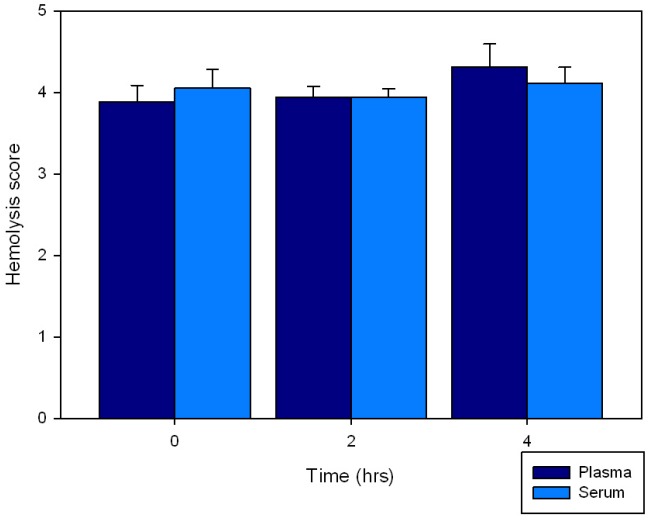


Fig. 3 Mean hemolysis scores in Bewick's swans. Single blood samples, stored to be either serum or plasma and centrifuged at different times after sampling. Error bars represent standard error.

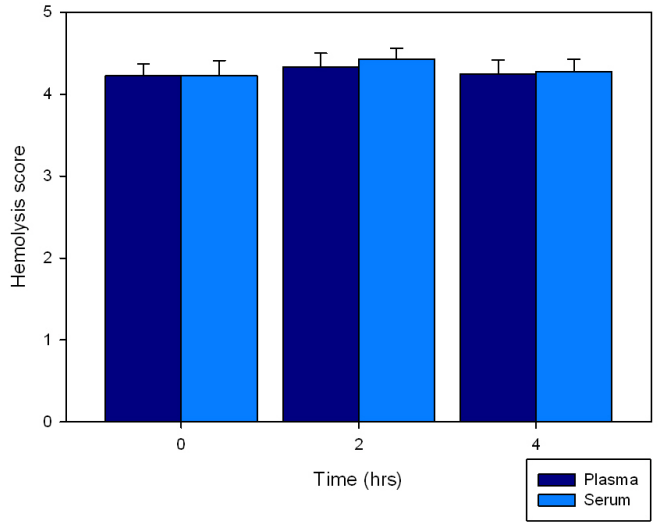


Fig. 4 Mean hemolysis scores in mallards. Single blood samples, stored to be either serum or plasma and centrifuged at different times after sampling. Error bars represent standard error.

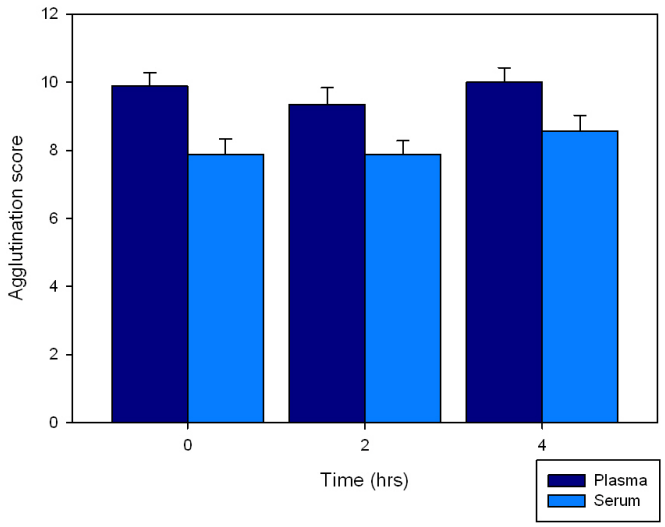


Fig. 5 Mean hemagglutination scores in Bewick's swans. Single blood samples, stored to be either serum or plasma and centrifuged at different times after sampling. Error bars represent standard error.

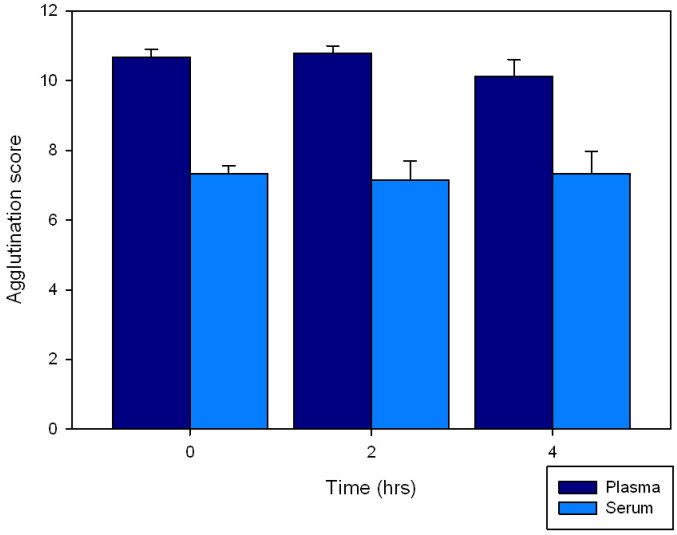


Fig. 6 Mean hemagglutination scores in mallards. Single blood samples, stored to be either serum or plasma and centrifuged at different times after sampling. Error bars represent standard error.

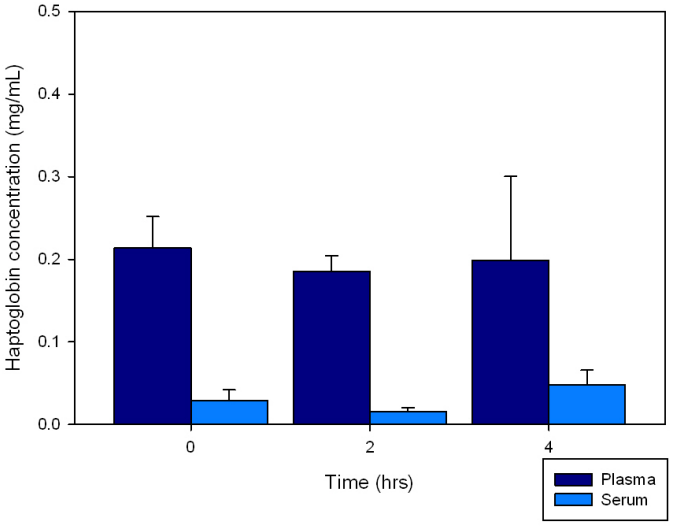


Fig. 7 Mean haptoglobin concentration in Bewick's swans. Single blood samples, stored to be either serum or plasma and centrifuged at different times after sampling. Error bars represent standard error.

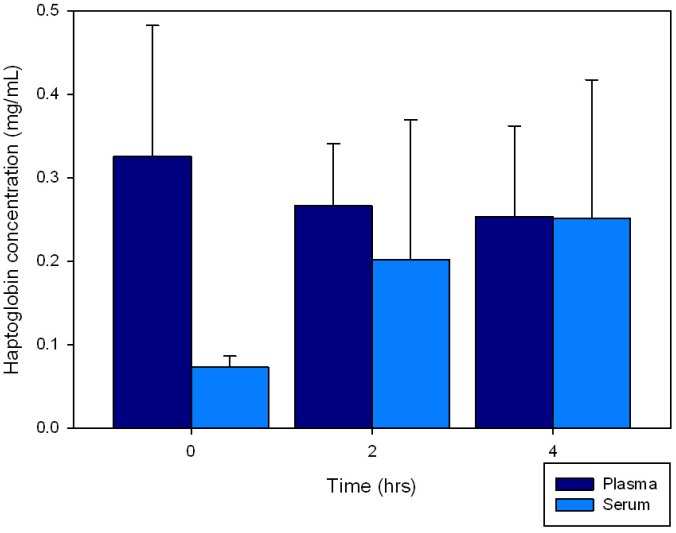


Fig. 8 Mean haptoglobin concentration in mallards. Single blood samples, stored to be either serum or plasma and centrifuged at different times after sampling. Error bars represent standard error.

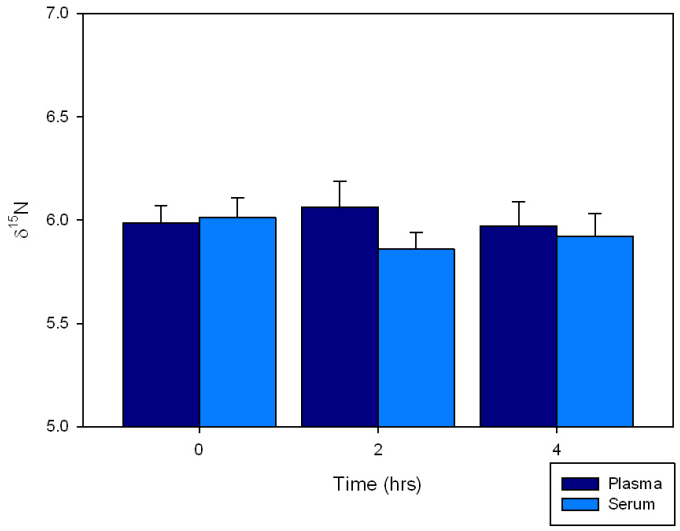


Fig. 9 Mean stable isotope values of Nitrogen in Bewick's swans. Single blood samples, stored to be either serum or plasma and centrifuged at different times after sampling. Error bars represent standard error.

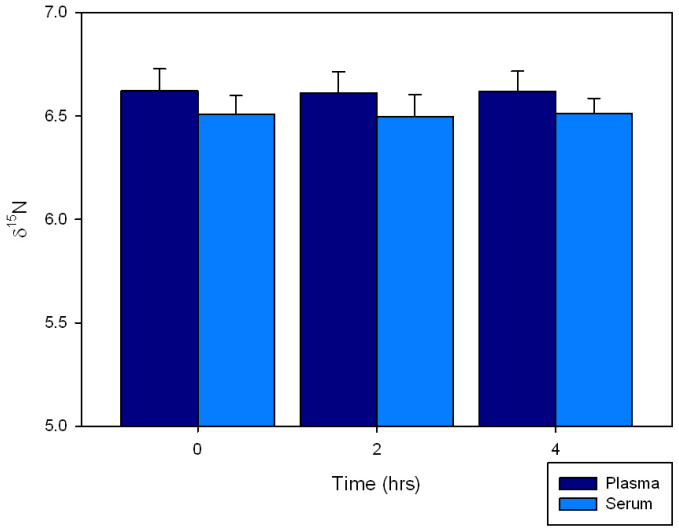


Fig. 10 Mean stable isotope values of Nitrogen in mallards. Single blood samples, stored to be either serum or plasma and centrifuged at different times after sampling. Error bars represent standard error.

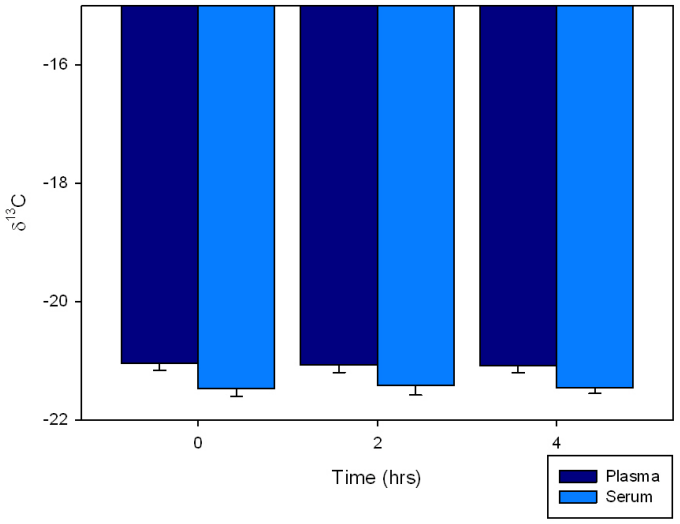


Fig. 11 Mean stable isotope values of Carbon in Bewick's swans. Single blood samples, stored to be either serum or plasma and centrifuged at different times after sampling. Error bars represent standard error.

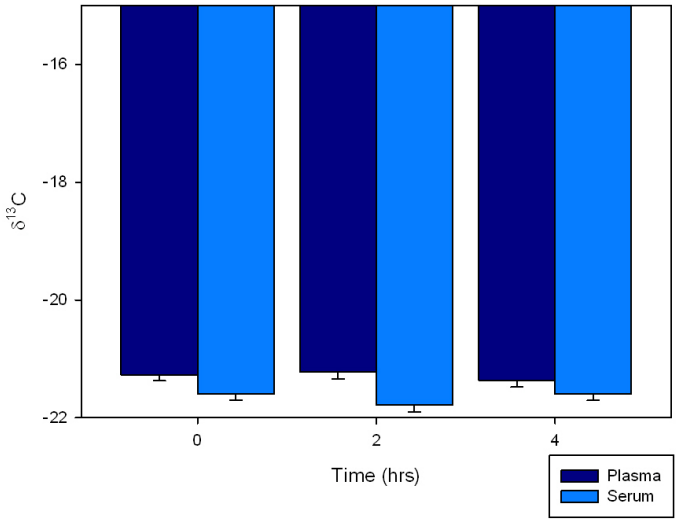


Fig. 12 Mean stable isotope values of Carbon in mallards. Single blood samples, stored to be either serum or plasma and centrifuged at different times after sampling. Error bars represent standard error.

Discussion

The results from this study show that when conducting the ecological assays described above, the results of these assays are consistent with the time between sampling and centrifugation for at least 4 hours when samples are refrigerated (4°C).

However, use of different fluid compartments does influence results.

Antibodies to avian influenza

This assay showed no differences in results between serum and plasma at all (figs. 1 and 2), no significant differences between treatment groups were found. Considering this, both serum and plasma can be used in order to perform this test.

Hemolysis / Hemagglutination

Results of the hemolysis assay showed no significant differences between fluid components. As mentioned above, this indicates that both serum and plasma can be used to perform this test. The hemagglutination assay did show significant differences between the two fluid components (table 1 and 2), both in mallards and swans, although in swans differences appear larger than in mallards.
Plasma hemagglutination was notably higher than serum hemagglutination both in Bewick's swans and mallards. Whiteman et.al. [12] showed the same differences in hemagglutination scores between birds from some islands as we saw between plasma (10.5) and serum (7). In a study of Buelher et.al. [13] using free-living birds, differences between two following years were found which are four times greater than differences between plasma and serum in mallards and ten times greater than in swans. This suggest that although inconsistent use of serum and plasma wouldn't fully account for the differences, it would certainly contribute to it. If similar studies were performed using plasma and serum inconsistently, this could influence interpretation of results. Our results therefore show the importance of using either serum or plasma.

Haptoglobin

The haptoglobin assay only shows a significant difference in swans and only at time point 0 (fig.1). However, looking at the results of the assays, this difference appears to result from only one high value in one swan, and may not represent a general trend, in which case the one significant difference found would be a measurement error.
In mallards there was no significance found at all, so in this species it shouldn't matter which fluid component is used when performing this assay.

Stable Isotopes

The results of the stable isotope assays clearly show significant differences between plasma and serum. Both in swans as in mallards stable carbon isotopes are significantly different, serum is more depleted in carbon than plasma. For stable nitrogen isotopes on the other hand significant differences between serum and plasma only applies to mallards (table 2 and figs. 9 and 10), so for this assay there is a difference between species.
Although not many studies have yet been performed using plasma or serum to determine stable isotopes, this may well happen in the future. Results in this study show the importance of consistent use of fluid components in order to gain comparable and therefore reliable results.

Conclusion

This study aimed to address the following questions: does delayed centrifugation of blood influence the outcome of a range of common ecological assays; and does the type of fluid component used (serum or plasma) influence the outcome of these assays?

We found that delayed centrifugation does not influence the consistency of a range of immune assays or stable isotope concentrations in the blood. Therefore, researchers in the field, who want to perform these specific tests, have at least 4 hours to centrifuge their samples.

We also found that measures of hemagglutination and the carbon stable isotope ratio were not consistent between fluid types, indicating that consistent use of fluid type would be necessary to gain reliable results from these assays.

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