Los Alamos NATIONAL LABORATORY EST.1943

LA-UR-15-24264 (Accepted Manuscript)

Bacteria-killing ability of fresh blood plasma compared to frozen blood plasma

Jacobs, Anne C
Fair, Jeanne Marie

Provided by the author(s) and the Los Alamos National Laboratory (2018-01-03).

To be published in: Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology

DOI to publisher's version: 10.1016/j.cbpa.2015.10.004

Permalink to record: http://permalink.lanl.gov/object/view?what=info:lanl-repo/lareport/LA-UR-15-24264

Disclaimer:
Approved for public release. Los Alamos National Laboratory, an affirmative action/equal opportunity employer, is operated by the Los Alamos National Security, LLC for the National Nuclear Security Administration of the U.S. Department of Energy under contract DE-AC52-06NA25396. Los Alamos National Laboratory strongly supports academic freedom and a researcher's right to publish; as an institution, however, the Laboratory does not endorse the viewpoint of a publication or guarantee its technical correctness.

RESEARCH LIBRARY

Bacteria-killing ability of fresh blood plasma compared to frozen blood plasma

Anne C. Jacobs[a,1] and Jeanne M. Fair[b]

a) Department of Biology, University of California, Riverside, CA 92521, USA

b) Los Alamos National Laboratory, Global Security – Emerging Threats, Mailstop K404, Los Alamos, NM 87545, USA

1) Present Address: Department of Biology, Allegheny College, Meadville, PA 16335, USA

Corresponding Author

Anne C. Jacobs

Email: ajacobs@allegheny.edu

Running title: Repeatability of Bactericidal Assay

M.S. has 18 pages, 1 figure

© 2015. This manuscript version is made available under the Elsevier user license
http://www.elsevier.com/open-access/userlicense/1.0/

Abstract

In recent years, the bacteria-killing assay (BKA) has become a popular technique among ecoimmunologists. New variations of that assay allow researchers to use smaller volumes of blood, an important consideration for those working on small-bodied animals. However, this version of the assay requires access to a lab with a Nanodrop spectrophotometer, something that may not be available in the field. One possible solution is to freeze plasma for transport; however, this assumes that frozen plasma samples will give comparable results to fresh ones. We tested this assumption using plasma samples from three species of birds: chickens (*Gallus gallus*), ash-throated flycatchers (*Myiarchus cinerascens*), and western bluebirds (*Sialia mexicana*). Chicken plasma samples lost most or all of their bacterial killing ability after freezing. This did not happen in flycatchers and bluebirds; however, frozen plasma did not produce results comparable to those obtained using fresh plasma. We caution researchers using the BKA to use fresh samples whenever possible, and to validate the use of frozen samples on a species-by-species basis.

Keywords: ecological immunology, microbicidal ability of blood, repeatability

Introduction

Eco-immunology, or the study of how immune function trades off with other aspects of life history, has become a popular field (Sheldon and Verhulst, 1996; French, 2009). To properly study these trade-offs, however, researchers must have reliable ways of measuring immune function in captive and wild animals. Several techniques are available, particularly for birds, each with benefits and drawbacks (Norris and Evans, 2000; Salvante, 2006). Recently, measuring the antimicrobial capacity of blood has become a popular field technique in eco-immunology (Matson et al., 2006; Millet et al., 2007; Forsman et al., 2008; Merrill et al., 2014). This assay, also known as the bacteria-killing assay (BKA), provides a measure of innate immune response by quantifying the ability of proteins in the plasma (such as complement, natural antibodies, and lysozyme) and/or phagocytic cells to kill bacteria (Matson et al., 2006; Millet et al., 2007). Some positive aspects of this assay are that it is relatively simple to carry out, requires only small volumes of blood, and is easy to interpret.

The BKA was adapted from an assay used in humans to assess the bactericidal ability of leukocytes (Keusch et al., 1975), and was modified to measure immune response in birds by Millet et al. (2007). These methods have been readily adapted for other vertebrate taxa as well, including amphibians (e.g. Venesky et al., 2012; Gervasi et al., 2014) and mammals (e.g. Schneeberger et al., 2013). The original form of the assay involves mixing blood or plasma with a common microbe that has the potential to cause disease (e.g. *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*) and plating out the resulting mixture on agar plates (Millet et al., 2007). Researchers then measure the number of microbes killed by comparing the results to a control plate in which microbes were not mixed with blood. However, Liebl and Martin (2009) devised an alternative version of the assay, which uses a Nanodrop spectrophotometer to

measure bacterial growth in lieu of counting bacterial colonies on plates. The advantage of this modified assay is that it requires smaller blood volumes; the disadvantage is that it requires access to laboratory equipment, such as the Nanodrop, and therefore cannot be carried out by field researchers working in remote areas with little or no access to electricity.

When describing the modified version of the antimicrobial assay, Liebl and Martin (2009) also conducted a test to determine if frozen samples could be used in the assay. They reported that, after approximately three weeks in the freezer, the antimicrobial capacity of the samples dropped to zero. However, other studies have used frozen plasma samples and reported viable results (e.g. Morrison et al., 2009; Merrill et al., 2014). For example, Morrison et al. (2009) used nestling tree swallows plasma that had been frozen at -80°C for 6 months. They stated in the methods that the results obtained using frozen plasma were similar to those obtained using fresh plasma. The Morrison et al. (2009) study used the plate method to measure bacterial growth rather than the Nanodrop method; however, the two assays should measure the same thing, albeit by different means. Thus the question of whether frozen plasma can produce usable results must be further addressed.

Here, we examine the repeatability of the bacteria-killing (BKA) assay described by Liebl and Martin (2009) using fresh and frozen samples to determine whether frozen samples can still yield useful results. We took plasma samples from three different species of birds, ran the BKA using the fresh samples, and then froze the remaining plasma for several weeks before running the assay again. We predicted that frozen plasma would produce results comparable to fresh plasma.

Materials and Methods

We collected fresh blood from domestic chickens (*Gallus gallus*; $n = 11$), ash-throated flycatchers (*Myiarchus cinerascens*; $n = 7$), and western bluebirds (*Sialia mexicana*; $n = 22$). Adult chicken samples came from a backyard poultry collection in northern New Mexico. Bluebird and flycatcher blood was obtained from wild birds that were monitored as part of the Los Alamos National Laboratory's Avian Nestbox Network. This network includes several hundred nestboxes placed on trees at various sites throughout Laboratory's property and the surrounding areas. Ash-throated flycatchers and western bluebirds are the two most common species inhabiting these boxes (Fair et al., 2003). All work was approved by the Los Alamos National Laboratory Animal Care and Use Committee and conducted in accordance with the Guidelines to the Use of Wild Birds in Research (Fair et al., 2010). For further details on the system, see Fair et al. (2003) and Jacobs et al. (2013).

All chicken and flycatcher blood samples were collected during the summer of 2012; bluebird blood samples were collected in the summers of 2011 ($n = 6$ samples) and 2012 ($n = 16$ samples). During the breeding season, adult bluebirds ($n = 11$) were captured using mist nest placed in front of active nestboxes. We also obtained samples from nestling bluebirds between the ages of 9-21 days while they were still in the nest. For further details about bluebird sampling, see Jacobs et al. (2015). All flycatcher samples came from nestling flycatchers between 9-14 days old, and all the chicken samples came from adult females. To collect blood from the bluebirds and flycatchers, we first swabbed the collection site with 70% ethanol, then used a sterile needle to puncture the wing vein and collected 20 – 50 µL in a heparinized microhematocrit tube. On chickens, we used a sterile syringe to draw blood from the wing vein. We then transferred the blood into a heparinized tube until processing.

Samples were briefly stored on ice before processing. All blood samples were taken back to the laboratory for processing the same day as collection. We spun blood down in a centrifuge for five minutes and collected the plasma for use in the assay. We used enough fresh plasma to run the initial bactericidal assay (see below), then immediately placed the remaining plasma in individually-marked tubes in the freezer at -80°C. After samples had been in the freezer for a minimum of 19 days (average time frozen = 30 days, range 19-53 days), we removed and allowed them to thaw in a bucket of ice and reran the assay. We chose this length of time based on data from Liebl and Martin (2009) which suggested that plasma loses its ability to kill *E. coli* after roughly 20 days in the freezer. Assay protocols were identical for both fresh and frozen samples.

The Bacteria Killing Assay

To measure the ability of plasma to kill bacteria, we used the methods described in Liebl and Martin (2009). We used *Escherichia coli* (ATCC #8739) supplied in pellets containing $1x10^6$ to $1x10^7$ microorganisms per pellet (Epower Microorganisms, MicroBioLogics, St. Cloud, MN). Each pellet was reconstituted in 40 mL of sterile 1X phosphate buffered saline (PBS) at 37°C. This stock solution was then diluted down to make a working solution of $2x10^5$ microorganisms per mL. We made a fresh stock solution every week and kept all bacterial solutions at 4ºC at all times.

1.5 µL of plasma was mixed with 34.5 µL PBS and 12.5 µL of the bacterial working solution. This mixture was then incubated for 30 minutes at 37°C. After the initial incubation, we added 250 µL of sterile tryptic soy broth. We also prepared a sterile blank, which contained 48.5 µL of PBS with 250 µL of tryptic soy broth, and a positive control, which contained 36 µL PBS,

12.5 μL of the bacterial working solution, and 250 μL of tryptic soy broth. All samples were incubated for 12 hours at 37°C after the addition of the tryptic soy broth. We used a Tecan Infinite 200 (Tecan Group Ltd., Männedorf, Switzerland) to measure the absorbance of the samples at 300 nm. We calculated the antimicrobial activity of the plasma as 1 – (absorbance of sample/absorbance of positive control). We ran all samples in triplicate when we had sufficient plasma volumes and in duplicate when we did not and we averaged the results from each replicate to get a single value for each individual.

Statistical Analysis

We tested all data for normality using a Shapiro-Wilks test. Frozen BKA values were non-normally distributed and could not be transformed to meet assumptions of normality due to a large number of zero values. We had bluebird samples from two years (2011 and 2012); however, bacteria killing ability of the fresh plasma did not differ between years ($F_{1,21} = 1.24$, $P = 0.28$). Likewise, we had bluebird samples from both adult birds and nestlings; however, BKA values did not differ significantly with age ($F_{1,21} = 0.19$, $P = 0.66$). We therefore pooled samples for all analyses.

To calculate repeatability for the individual replicates for both fresh samples and frozen samples, we used the methods described in Lessells and Boag (1987). We then tested whether results obtained from frozen samples correlated with results obtained from fresh samples using a Spearman rank correlation. We also compared the average BKA values of fresh and frozen plasma using a Wilcoxen signed rank test.

Different species showed different patterns in terms of the ability of the frozen plasma to kill bacteria (see below). We therefore reran the analysis on each species separately. To

149 determine whether storage time affected our results, we used a linear regression to test for a
150 relationship between how many hours we had stored the fresh blood before processing it and the
151 BKA of fresh plasma. We were missing data on collection time for three individuals; thus our
152 sample size was reduced to $n = 37$ for this test. We also ran this analysis on bluebirds only; we
153 did not test for a relationship between assay timing and BKA in chickens because all samples
154 were collected and processed at roughly the same time, and we did not run this test for
155 flycatchers because missing data reduced our sample size to $n = 4$. Alpha levels are set at $\alpha =$
156 0.05, and all values are reported as means ± standard deviation.
157
158 **Results**
159 When examining the repeatability of the replicates used, both fresh and frozen replicates
160 showed significant variation among rather than within individuals and moderate to high
161 repeatability (fresh plasma: $P < 0.001$, $R = 0.40$; frozen plasma: $P < 0.001$, $R = 0.70$). However,
162 we found no correlation between the BKA of fresh plasma and the BKA of frozen plasma when
163 pooling all species ($r_s = 0.06$, $n = 40$, $P = 0.70$). The number of days a sample had been frozen
164 did not affect the magnitude of the difference between fresh and frozen samples ($r_s = -0.18$, $n =$
165 40, $P = 0.25$). BKA values for frozen samples tended to be lower than those of fresh samples
166 (fresh BKA mean = 0.29 ± 0.21; frozen BKA mean = 0.13 ± 0.16; $z = 3.58$, $n = 40$, $P = 0.0002$).
167 However, this was due to a large number of frozen samples in which the BKA value dropped to
168 zero (33%), particularly among chickens (see below).
169
170 *Within-species comparison of fresh and frozen plasma*

In chickens, the BKA of frozen plasma dropped close to zero in most samples (Fig 1A), and there was no correlation between the killing ability of fresh plasma and frozen plasma (r_S = 0.50, n = 11, P = 0.12). In bluebirds and flycatchers, plasma samples retained some ability to kill bacteria; however, the BKA values of frozen plasma were not similar to those of fresh plasma in either species (bluebirds: r_S = -0.09, n = 22, P = 0.70; flycatchers r_S = 0.18, n = 7, P = 0.70; Fig 1B,C). The number of hours between collection and blood processing had no effect on the BKA of fresh plasma either when considering all samples pooled ($F_{1,36}$ = 0.65, P = 0.42) or when we considered bluebirds only ($F_{1,21}$ = 0.65, P = 0.43).

Discussion

We found that using frozen plasma samples in the BKA assay did not produce comparable results to using fresh samples. Interestingly, not all frozen samples lost their killing ability. In chickens, the killing ability of plasma dropped close to zero after being frozen. This matches the results reported by Liebl and Martin (2009) in house sparrows (*Passer domesticus*). However, while bluebird and flycatcher plasma did not produce repeatable results after freezing, in both these species, the plasma retained some ability to kill bacteria. The frozen plasma samples still produced seemingly viable results, but differences in the BKA of individuals could not be reliably inferred from the frozen samples. Given that many studies using the BKA seek to quantify individual differences in immunity, this presents a serious problem. Unless researchers explicitly test whether frozen plasma produces similar results to fresh plasma, they could falsely assume that frozen plasma gives valid results simply based on its continued ability to kill bacteria. Other studies in which researchers have used frozen plasma for the BKA assay have produced what appear to be viable results (Morrison et al., 2009; Merrill et al., 2014). However,

given that BKA values for frozen plasma may not drop to zero, simply noting that frozen plasma still retained some killing ability is not enough. Our results suggest that further caution is needed, and that values obtained with frozen plasma should be compared to values from fresh plasma on a species-by-species basis.

The temperature at which samples were stored may explain why our frozen plasma samples retained some killing ability while those used by Liebl and Martin (2009) did not. Liebl and Martin (2009) stored their samples at -40 degrees Celsius, whereas we kept ours at -80 degrees. Certain proteins in the blood may be preserved below -70 degrees, but not at warmer temperatures (Petrakis, 1985). However, all our samples were stored in the same freezer at the same temperature. It is therefore unclear why in some species in our study, the plasma retained its bactericidal abilities after freezing, while in other species this ability disappeared.

Since this assay used plasma and not whole blood, there were no phagocytes present in the mixture to kill the bacteria, and killing ability probably relies primarily on non-cellular components such as complement and natural antibodies (Millet et al., 2007). Loss of bactericidal activity presumably results from the destruction of these proteins during freezing. However, while complement is typically inactivated by heat (Noguchi and Bronfenbrenner, 1911), it can be remarkably unaffected by freezing, and even by occasional thawing and refreezing (O'Shaughnessy et al., 2012). Likewise, antibodies in serum remain viable when frozen below -70 degrees Celsius, provided they are not thawed and refrozen (Petrakis, 1985).

Studies that have used heat to inactivate complement have observed that complement seems to be responsible for most of the bactericidal abilities of the plasma (Matson et al., 2006). However, other factors in the blood and plasma may play a role in activating complement, including Mannan-binding lectins (Laursen and Nielsen, 2000). It is possible that these proteins

were degraded by freezing rather than the complement itself. If there are species-specific differences in the typical pathways used to activate complement, this could explain why in some species, the ability of plasma to kill bacteria disappears completely following freezing, while in other species it does not. Future studies comparing the proteins present in fresh and frozen serum may be helpful in answering these questions.

In a few instances, the average BKA for an individual's frozen plasma was higher than the average obtained using fresh plasma. We are not sure why such a difference occurred in these cases. It is possible that contamination in the fresh plasma samples resulted in the appearance of reduced killing ability, although we did not detect such contamination in our negative controls. It is also important to note that because we used only plasma for this assay, we may not have obtained a complete picture of individual bactericidal ability. While this strain of *E. coli* can be killed primarily by non-cellular mechanisms, phagocytosis may be necessary to kill other microbes (Millet et al., 2007). If researchers are required to use only fresh samples (as our results suggest), then it may be better to use whole blood for the assay rather than plasma alone.

For this study, we followed the protocols laid out in Liebl and Martin (2009). In their initial description of the Nanodrop version of the BKA, Liebl and Martin (2009) specified that plasma and bacteria mixtures should be incubated for 30 minutes at 37 degrees Celsius to allow the proteins within the plasma to kill the bacteria. However, many other studies on birds that utilized the plate method also used an incubation temperature of 41 degrees Celsius for this step (Matson et al., 2006; Forsman et al., 2008). Given that birds tend to have a higher body temperature than mammals (around 40 degrees Celsius; Gill, 2007), it may be more biologically relevant to run the incubation step of the assay at a higher temperature. However, given that we kept the incubation temperature consistent across all samples, we find it unlikely that the lower

temperature why we obtained different results from different species. These differences may relate to other aspects of the assay, such as the dilution factor used for the plasma. We used the same dilution for each species; however, different dilutions of plasma may alter the results in some species, including chickens (Matson et al., 2006). In our assay, most of the BKA results fell within a middle range of killing ability (i.e. most samples killed some, but not all, of the bacteria), indicating that this dilution worked reasonably well. However, researchers may need test different dilutions to optimize the assay on a species-by-species basis.

Other work testing for repeatability of the BKA assay have found that results are repeatable within an individual (Tieleman et al., 2010). Tieleman et al. (2010) took multiple blood samples from stonechats (*Saxicola torquata*) and found that the results for *E. coli* killing were highly repeatable for individuals. While this study used the plate method described in Millet et al. (2007) rather than the Nanodrop method from Liebl and Martin (2009), the results suggest that individual BKA values collected using fresh blood are repeatable. Likewise, within a single sample, our replicates showed repeatable results. Thus we are inclined to attribute the lack of correlation between fresh and frozen plasma in our study to the freezing process, rather than the assay itself.

In conclusion, we found that while frozen plasma of some species retained its ability to kill bacteria, the BKA values from frozen samples were not a good indicator of the killing ability of fresh plasma. Researchers using frozen plasma samples should first validate that these samples give comparable results to freshly collected plasma before drawing conclusions from their results.

Acknowledgments

263 We would like to thank C. Hathcock for assistance in the field and S. Loftin for giving us access
264 to his chickens. We thank Y. Shou, K. McCabe, and the Bradbury laboratory for assistance with
265 the immune assays. Training for ACJ was funded by a travel grant from the Research
266 Coordination Network in Ecoimmunology. This project was funded by Los Alamos National
267 Security, LLC, operator of the Los Alamos National Laboratory under Contract No. DE-AC52-
268 06NA25396 with the US Department of Energy.

References

Fair, J.M., Myers, O.B., Ricklefs, R.E., 2003. Immune and growth response of western bluebirds and ash-throated flycatchers to soil contaminants. Ecol. Appl. 13, 1817-1829.

Fair, J.M., Paul, E., Jones, J., Clark, A.B., Davie, C., Kaiser, G., 2010. Guidelines to the Use of Wild Birds in Research. The Ornithological Council, Washington D.C.

Forsman, A.M., Vogel, L.A., Sakaluk, S.K., Grindstaff, J.L., Thompson, C.F., 2008. Immune-challenged house wren broods differ in the relative strengths of their responses among different axes of the immune system." J. Evol. Biol. 21, 873-878.

French, S.S., Moore, M.C, Demas, G.E., 2009. Ecological immunology: the organism in context. Integr. Comp. Biol. 49, 246-253.

Gervasi, S.S., Hunt, E.G., Lowry, M., Blaustein, A.R. 2013. Temporal patterns in immunity, infection load and disease susceptibility: understanding the drivers of host responses in the amphibian-chytrid fungus system. Funct. Ecol. 28, 569-578.

Gill, F.B., 2007. Ornithology, 3rd ed. W.H. Freeman and Company, New York, New York.

Jacobs, A.C., Reader, L.L., Fair, J.M., 2013. What determines the rates of double brooding in the western bluebird? Condor 115, 386-393.

Jacobs, A.C., Fair, J.M., Zuk, M., 2015. Coloration, paternity, and assortative mating in western bluebirds. Ethology 121, 176-186.

Keusch, G.T., Douglas, S.D., Ugurbil, K., 1975. Intracellular bactericidal activity of leukocytes in whole blood for the diagnosis of chronic granulomatous disease of childhood. J. Infect. Dis. 131, 584-587.

Laursen, S.B., Nielsen, O.L., 2000. Mannan-binding lectin (MBL) in chickens: molecular and functional aspects. Dev. Comp. Immunol. 24, 85-101.

Lessells, C.M., Boag, P.T., 1987. Unrepeatable repeatabilities: a common mistake. Auk 104, 116-121.

Liebl, A.L., Martin, L.B., 2009. Simple quantification of blood and plasma antimicrobial capacity using spectrophotometry. Funct. Ecol. 23, 1091-1096.

Matson, K.D., Tieleman, B.I., Klasing, K.C., 2006. Capture stress and the bactericidal competence of blood and plasma in five species of tropical birds. Physiol. Biochem. Zool. 79, 556-564.

Merrill, L., Levinson, S.D, O'Loghlen, A.L, Wingfield, J.C, Rothstein, S.I., 2014. Bacteria-killing ability is negatively linked to epaulet size, but positively linked to baseline corticosterone, in male Red-Winged Blackbirds (*Agelaius phoeniceus*). Auk 131, 3-11.

Millet, S., Bennett, J., Lee, K.A, Hau, M., Klasing, K.C., 2007. Quantifying and comparing constitutive immunity across avian species. Dev. Comp. Immunol. 31, 188-201.

Morrison, E.S., Ardia, D.R., Clotfelter, E.D., 2009. Cross-fostering reveals sources of variation in innate immunity and hematocrit in nestling tree swallows *Tachycineta bicolor*. J. Avian Biol. 40, 573-578.

Noguchi, H., Bronfenbrenner, J., 1911. Effects of mechanical agitation and of temperature upon complement. J. Exp. Med. 13, 229–233.

Norris, K., Evans M.R., 2000. Ecological immunology: life history trade-offs and immune defense in birds. Behav. Ecol. 11, 19-26.

O'Shaughnessy, C.M., Cunningham, A.F., MacLennan, C.A., 2012. The stability of complement-mediated bactericidal activity in human serum against *Salmonella*. PLoS ONE 7, e49147.

Petrakis, N.L., 1985. Biological banking in cohort studies, with special reference to blood. Natl. Cancer Inst. Monogr. 67, 193-198.

Salvante K.G., 2006. Techniques for studying integrated immune function in birds. Auk 123, 575-586.

SAS Institute, 2008. SAS/STAT User's Guide, Version 9, 2nd ed. SAS Institute Inc., Cary, NC.

Schneeberger, K., Czirják, G.Á., Voigt, C.C., 2013. Measures of the constitutive immune system are linked to diet and roosting habits of neotropical bats. PLoS ONE 8, e54023.

Sheldon B.C., Verhulst, S., 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol. Evol. 11, 321.

Tieleman, B.I, Croese, E., Helm, B., Versteegh, M.A. 2010. Repeatability and individual correlates of microbicidal capacity of bird blood. Comp. Biochem. Physiol. A 156, 537-540.

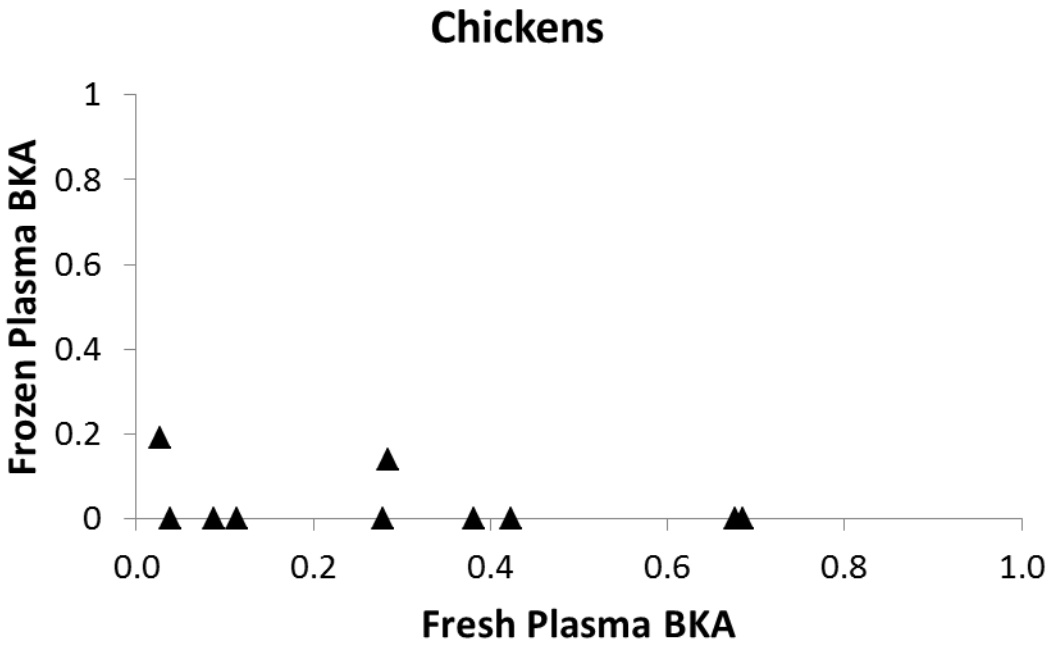
Venesky, M.D., Wilcoxen, T.E., Rensel, M.A., Rollins-Smith, L., Kerby, J.L., Parris, M.J., 2012. Dietary protein restriction impairs growth, immunity, and disease resistance in southern leopard frog tadpoles. Oecologia 169, 23-31.

Figure Legend

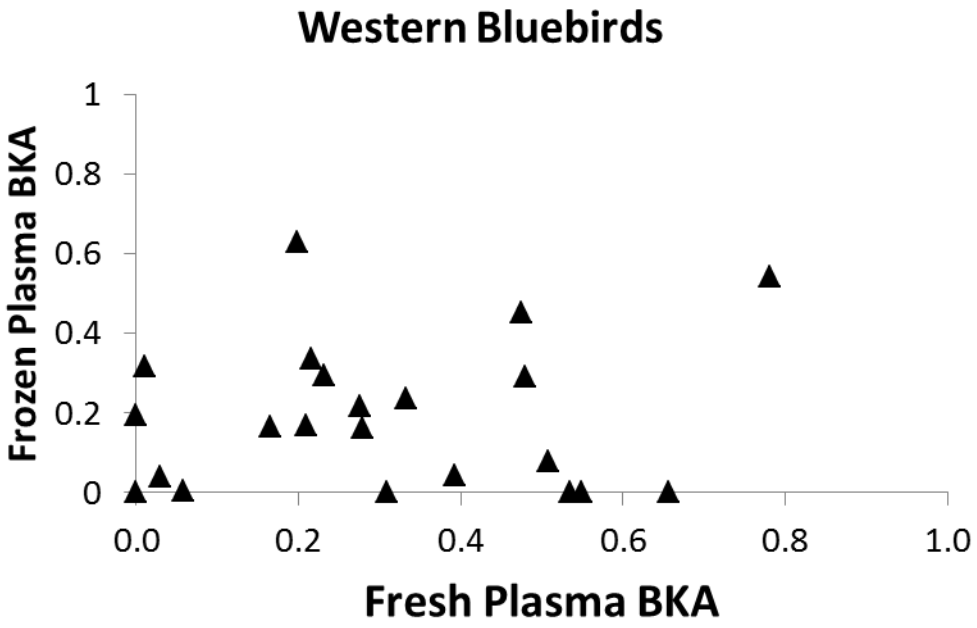
Figure 1: The bacteria killing ability of fresh and frozen plasma samples for a) chickens b) western bluebirds, and c) ash-throated flycatchers.

FIGURE 1

A.


Chickens
Frozen Plasma BKA
1
0.8
0.6
0.4
0.2
0
0.0
0.2
0.4
0.6
0.8
1.0
Fresh Plasma BKA

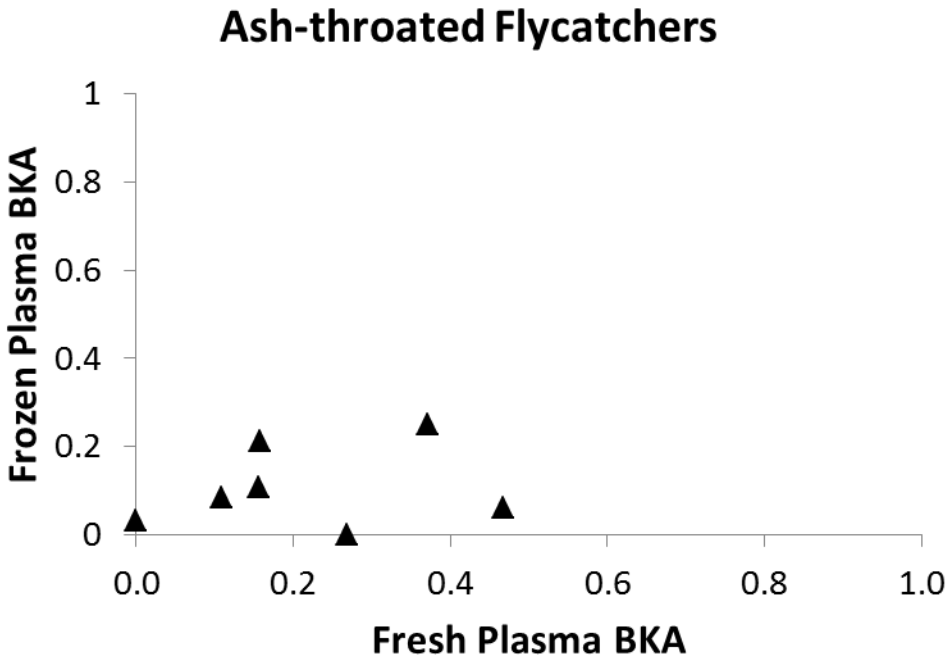
B.


Western Bluebirds
Frozen Plasma BKA
1
0.8
0.6
0.4
0.2
0
0.0
0.2
0.4
0.6
0.8
1.0
Fresh Plasma BKA

340

341

342 C.



343