Jessica Gutierrez
Savaloja Grant 2020 Final Report

Project Title: Effect of within-brood genetic diversity on nest parasite abundance in eastern bluebirds

Savaloja Grant Award Amount: $1,496

Personnel:

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Overview: Hosts have evolved defense mechanisms to deal with their parasites. Populations with lower genetic diversity often have similar susceptibility to parasite feeding, resulting in a rapid infestation, while increased genetic diversity can enhance resistance (Altermatt and Ebert 2008). Parasite abundance may vary among nests within and between host populations (reviewed in Grab et al. 2019). One potential explanation for this variation is that the nestlings' genetic composition within a nest influences parasite resistance. Many female passerines engage in extrapair opportunities with males outside of their bonded pair (Hasselquist 2001) and a single nest can contain young from different males (extra-pair young). Consequently, females that mate with more extra-pair males, especially those with high parasite resistance, may increase the chances of their young inheriting resistance genes (Hamilton and Zuk 1982).

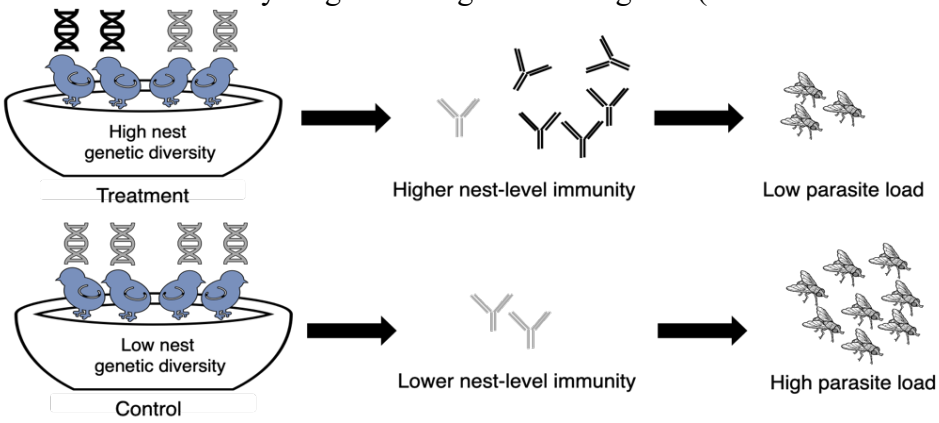


Figure 1. Predicted outcomes of nest-level immunity and parasite load of nests with partial cross-fostered and non-cross-fostered nests.

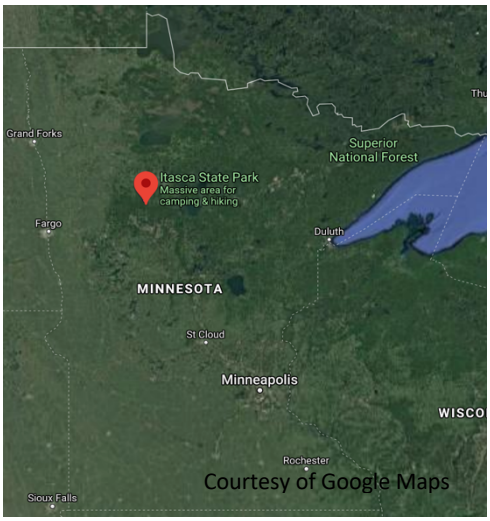
Objectives: The goal of this study was to determine if host genetic diversity within a nest affects overall resistance to the parasitic nest fly (*Protocalliphora sialia)* of eastern bluebirds (*Sialia sialis*). Eastern bluebirds have low resistance to *P. sialia*

(Grab et al. 2019, Knutie 2020) and their prevalence of extra-pair young in the brood is relatively low when compared to other passerines (Stewart et al. 2010). In contrast, other passerine species, such as tree swallows (*Tachycineta bicolor*), that are more resistant to the parasitic nest flies (Grab et al. 2019) also have higher incidence of extra-pair young (Kempenaers et al. 2001). The question of why bluebirds are less resistant to *P. sialia* than other host species also remains unanswered. One hypothesis to explain this pattern is that the low genetic diversity within bluebird nests influences *P. sialia* abundance.

To determine the effects of genetic diversity on parasite resistance in nestling bluebirds, I used an experimental manipulation of brood composition of bluebirds' young (partial cross-fostering) and quantified their specific IgY antibody (immune) response to *P. sialia* and the resulting quantity the parasitic nest fly (abundance and density).

1. I predicted that partial cross-fostered nests, will have increased genetic diversity when compare to nests in which all nestlings are maternally related (Figure 1).
2. Consequently, I predicted that increasing genetic diversity within a nest via partial cross-fostering will decrease parasite load when compared to non-cross-fostered nests with lower genetic diversity (Figure 1).

This project will enhance our understanding of how parasites can shape host population genetics and defenses by exploring the evolutionary benefits of extrapair mating opportunities in wild birds.



Study Location: This project took place near the University of Minnesota Itasca Biological Station and Laboratories (IBSL) in Clearwater and Hubbard county, Minnesota (47°13'33" N, -95°11'42" W). This region is ecologically relevant to my project because it falls within the eastern bluebird breeding range and a 200 nest box system has been established here by my academic advisor, Dr. Sarah Knutie, since 2014.

Methods Completed:

Partial cross-fostering experiment

Nest boxes were monitored weekly for bluebird egg laying. Once all eggs were laid (determined by a cease in egg-laying after 24 hours), the bluebird mother incubated the eggs for 14 days undisturbed. After the14th incubation day, nests were checked for hatching once every day. After the first day of hatching (hatch day = 0), the first two nests (synchronized) were randomly assigned a group (control or treatment); the following paired nests were assigned in alternating order. Nests were considered synchronized if hatching of eggs occurred 0-24 hrs apart from each other. When all nestlings from two or more synchronized nests hatched (1-2 days old), 2-3 nestlings within a nest were cross-fostered with another nest (treatment) or not (control). Early cross-fostering is necessary to avoid environmental and parental biases that are associated with this method (Winney et al. 2015).

Prior to cross foster, all nestlings were weighed to the nearest 0.1 g using the Brifit Digital Mini Scale. The nestlings were then numerically ranked from lightest to heaviest in mass. The heaviest nestlings from both nests were randomly assigned to stay in the original nest or leave and the following nestlings were assigned in alternate order. This was done to avoid size

differences within a nest; see Giordano et al. (2014) for cross-fostering details. After nestlings were weighed, the natal down feathers were partially trimmed into unique cuts to identify nestlings at day 10; the patterns of these trims were as followed: 1) no cut, 2) right or left shoulder, 3) right or left head, and 4) top or bottom back. To prevent artificial parasite introduction to the nests, nestlings were checked thoroughly and attached parasites were removed and returned to the original nest. To avoid stress during the transfer, nestlings were kept in a padded box with a Multi-purpose jumbo 72-hour Uniheat Heat Pack. Time out of the nests did not exceed 35 minutes. Control nestlings were handled in the exact same manner but returned to their original nests.

Quantifying nestling growth and survival

On day 10, nestlings received a Fisheries and Wildlife numbered metal band and a unique color band combination for individual identification after fledging. Nestlings were weighed (to the nearest 0.1g) with a Ohaus CS200-100 portable compact scale balance and measured (bill length (mm), right tarsus length (mm), and first primary feather (mm)) using calipers. Since nestling sex can affect feeding preferences of the parasite (O'Brien and Dawson 2013), nestlings were sexed on day 13 using visual differences in feather pigmentation (O' Brien and Dawson 2009). Starting on day 15, nests were checked every other day from a distance to determine the age of fledging (success) or death (failure).

Eastern bluebird nestling banded at day 10.

Nestling and adult tissue collection

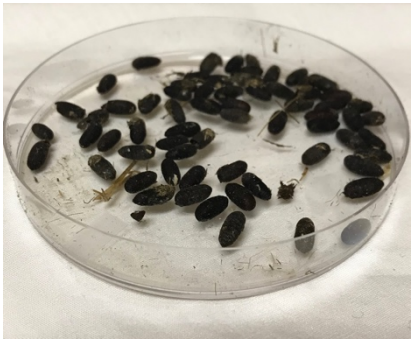
During cross-fostering (day 1-2), nestling buccal cells were collected by orally inserting and swirling a sterile foam swab on the buccae. Buccal swabs are a minimally invasive way to collect DNA from nestlings that are too small in mass for blood collection. Although buccal swabs provide less genetic material than blood samples, collecting buccal swabs will prevent a sample loss if the birds naturally die before 10 days old (when we collect blood samples). Buccal swabs were placed in a 0.6 mL tube in 300 uL of 70% ethanol, kept in a cooler with ice packs in the field, and stored in a -80°C freezer.

During day 10, a small blood sample (<20 uL) was collected from the brachial vein of the nestlings using a 30-gauge needle and a 70 uL heparinized capillary tube. Whole blood was placed in a 0.6 mL snap top tube and then in a cooler with ice packs in the field until we returned to the lab. The tubes were centrifuged at 6,000 XG for 3 minutes to separate the red blood cells from the plasma. The plasma was placed into 0.6 mL tubes and stored in -80°C. The blood cells were removed from the tube and preserved on a Whattman™ FTA Card at room temperature to dry for 24 hours before being transferred to a -20°C freezer. At the end of the season, all samples were shipped on dry ice to the University of Connecticut; then plasma and buccal swabs were stored in a -80°C freezer until used in immunoassay or DNA extractions, respectively. Blood cards were stored at -20°C until used in the DNA extractions.

Adults bluebirds were captured opportunistically from nest boxes during systematic nest-box monitoring and when nestlings were 5-7 days old. To capture adults, a trap door was created using Hygloss Overhead Projector Sheets cut into a 3.5 X 6.5 cm rectangle and attached

vertically to the inside of the nest box with 2 push pins placed at the top. The trap film was placed 1.5 cm above the box hole and overlapped the other sides of the hole by 2 mm. Once an adult bluebird entered the nest box, a black extra-large hoodie was placed over the box, the door was half way opened, and we carefully reached inside to handle the adult bluebird. If parents were captured, buccal swabs were collected using the same method as the nestlings and received a Fisheries and Wildlife numbered metal band and a unique color band combination for individual identification and measured (mass (g), wing length (mm), bill length (mm), and right tarsus (mm))

Quantifying parasite abundance and size



Once the nestlings fledged or died (15-21 days), nests were collected and dissected for *P. sialia* larvae (1st, 2nd, or 3rd instar), pupae, and pupa cases to quantify total parasite abundance. If the nest had parasites with pupae, we measured 0-5 pupae and measured the length and center width (mm) with plastic dial calipers. The parasites were separated by life stage and were stored in a 50 mL falcon tube with original nest material and closed with a mesh cap. During nest collection 5-10 3rd instars were randomly selected from every other nest and placed in a 2 mL screw cap tube then stored at -80°C. After 1-3 days of collection, a subset of the pupated larvae was measured for a total of 10 parasites (i.e 5 pupae at collection and 5 larvae that pupated after collection) and returned to their tubes. If the nest had no pupae at collection then a subset of 10 pupated larvae were measured. Finally, after 10-14 days of the collection date, 2-3 flies were collected in 2 mL screw cap tubes filled with 90% EtOH; the remaining were released.

Blood DNA extractions

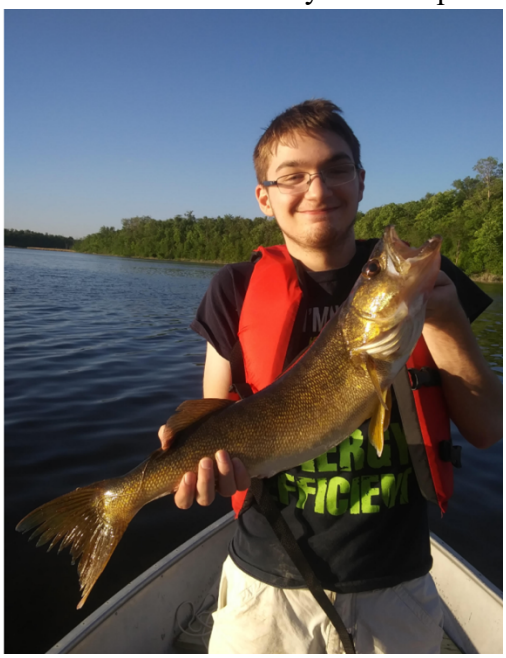
Blood DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (April 2016 protocol) with some modifications. Blood DNA were first released from the Whattman™ FTA Cards. During part 1 of the DNA extraction protocol, we hole-punched blood cards and placed the fragments into1.5 mL microcentrifuge tubes and added 30 µL of proteinase K and 190 µL 1X PBS. After the Buffer AL addition step, the incubation time was increased to 35 minutes and samples were vortexed for 15 s every 5 minutes. After incubation, the sample mix was pipetted into the spin column while leaving the filter paper behind. The rest of the protocol remained constant.

Quantifying the parasite-binding antibody response

Enzyme-linked immunosorbent assays (ELISA) were used to detect *P. sialia*-binding antibody (IgY) levels in nestlings using the Grab et al. (2019) and DeSimone et al. (2018) protocol. Ninety-six well plates were coated with 100 µl/well of *P. sialia* protein extract (capture antigen) and was 1:100 diluted in carbonate coating buffer (0.05 M, pH 9.6) and incubated for 1 hr on an orbital table. Plates were then washed with a plate washer, coated with 200 µl/well of bovine serum albumin (BSA) blocking buffer, and incubated at 4°C overnight. Between each of the following steps, plates were washed using a plate reader and Tris-buffered saline wash solution, loaded as described, and incubated for 1 hr on an orbital table at room temperature. Plasma was 1:100 diluted with Sample Buffer, which was made up of BSA blocking buffer and Tween 20. Wells were loaded with 100 µl/well of individual diluted host plasma in triplicate. Plates were then loaded with 100 µl/well of Goat-αBird-IgG-Heavy and Light Chain HRP

(diluted 1:50,000; A140-110P; Bethyl Laboratories). Plates were loaded with 100 µl/well of peroxidase substrate (tetrameth-ylbenzidine, TMB: Bethyl Laboratories) and incubated for exactly 30 min. Finally, the reaction ceased using 100 µl/well of stop solution (Bethyl Laboratories).

Optical density (OD) was measured with a spectrophotometer (PowerWave HT; 450 nm filter; BioTek). A higher OD value corresponded to higher IgY concentration. On each plate, a positive control of pooled plasma from naturally parasitized nestlings and adults were used in triplicate to correct for inter-plate variation. We corrected for inter-plate variation by first dividing the mean OD value for the positive controls for each plate by the highest OD value among all plates then by multiplying the mean for each sample by this correction factor. In addition, each plate contained two nonspecific binding (NSB1 and NSB2) samples. The NSB1 control contained capture antigen but excluded plasma. While NSB2 contained plasma but no capture antigen. Finally, each plate included a blank sample in which only the detection antibody was added, but plasma and capture antigen were excluded. The highest NSB values (NSB1 or NSB2) were subtracted from the mean OD value of each sample to account background binding of the detection antibody to the capture antigen.



Outreach, Diversity, Inclusion and Equity:
Unfortunately, due to COVID-19 regulations and personal safety my team and I could not engage with property owners as much as we would have liked but occasionally property owners were still able to watch us work and ask questions from a safe distance. The 2020 Science in Nature public engagement event that was supposed to be held jointly with Itasca State Park and IBSL was canceled. However, I was able to mentor and work with our technician, Suzanna Tupy a recent graduate from the University of Minnesota. I was also able to virtually mentor Logan Stech, a recent graduate of Waubun Highschool (the awardee of the White Earth Itasca Internship). Logan was able to partake in our research by observing behavioral data, engaging in professional development, and shadowing my work for a day).

Project Progress:
The molecular work is still in progress. The buccal DNA extraction protocol has been optimized and are in the process of being extracted. The polymerase chain reaction (PCR) protocol using the Type-it Microsatellite PCR Kit (December 2009 protocol) has been tested. There was only one modification: The total volume per reaction was 12.5 µL: 6.25 µL of 2x Type-it Multiplex PCR Master Mix, 4 µL RNase-free water, 1.25 µL of 10x primer mix, and 1 µL of DNA template. The final concentrations for 2x Type-it Multiplex PCR Master Mix,10x primer mix, the DNA template were 1X, 2µM, and 10 ng, respectively. However, I am still optimizing the thermocycler settings to produce quality resolution of amplicons. I am also simultaneously in the process of analyzing field and nestling immune response results and drafting the manuscript.

Thank you for your time and contribution of making my project possible!

Budget Reconciliation:
MOU Savaloja Grant: $**1,496**
Waubun Intenship: $5,000
UConn EEB Grant: $896
UConn EEB Stipend: $3,000
Total Funding: $10, 392

Laboratory Supplies		
Qiagen DNeasy Blood and Tissue Kit		*Generously Covered by:*
o $172/kit*2 + $50 Shipping	**$392**	MOU Savaloja Grant
PCR primers for parentage analysis		
o $7/primer set*16	**$112**	MOU Savaloja Grant
Electrophoresis systems for parentage analysis		
o $4/sample*~150 samples	**$750**	MOU Savaloja Grant and Dr. Knutie
ELISA for plasma IgY antibodies		
o Detection antibodies	**$152**	MOU Savaloja Grant
o Blocking buffer	**$42**	MOU Savaloja Grant
o TMB	**$48**	MOU Savaloja Grant
o Stop solution	$35	Dr. Sarah Knutie
Local Field Site Travel		
$0.20*432miles	$86	Dr. Sarah Knutie
Travel to and from field site (CT to MN)		
o $0.58/mile*1548 miles	$897	Waubun Itasca Internship Program
Expenses Covered by Other Grants		
Housing for 10 weeks at Field Site		
o $50/day*83 days	$4,150	Waubun Itasca Internship Program
Field station Fees		
o $5/day *83 days	$415	Waubun Itasca Internship Program
Field Site Laboratory Space		
$20/week*12 weeks	$240	Waubun Itasca Internship Program
Grand Total Expenses	**$**7,324	
Remaining	$68	