The relation of hematocrit levels and local environment:

Inter- and intra-population differences in altricial nestlings of a bird species breeding in climatic extremes

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Abstract

Hematocrit is commonly used as a proxy of body fitness to indicate nestling health. Temperature, habitat quality and body size are known to influence nestling hematocrit. Hematocrit is influenced by several factors, but temperature is generally agreed as the key driver. However, it is unclear at what point in nestling development is temperature most important for determining nestling hematocrit. We investigated nestling hematocrit in thorn-tailed rayadito nestlings (*Aphrastura spinicauda*) alongside body size, habitat quality and temperature in one warm and one cold location in Chile, South America. This study aims to identify when in nestling development can temperature determine nestling hematocrit before fledging. We find that the ambient temperature around the day of hatching is important in determining nestling hematocrit close to fledging, which may have consequences in post-fledging survival. We also confirmed that temperature is the best predictor of nestling hematocrit in a strong negative relationship. Our results suggest that high temperatures on the day of hatching will result in lower nestling hematocrit and poorer health in fledglings. As atmospheric temperatures continue to increase from global warming, nestling hematocrit and nestling health are at risk. This is a threat to many bird species, especially those in locations vulnerable to climate breakdown.

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Introduction

Hematocrit is the percentage of red blood cells found in the blood stream in all vertebrates and has several applications in ecology. Avian hematocrit can be useful for assessing nestling health, which is commonly measured alongside other fitness-related traits when assessing overall fitness (Swanson, 1990; Muchacka *et al.,* 2012; Kilgas, 2014; Farag and Alagawany, 2018). Hematocrit is a fitness-related trait and a useful proxy of nestling health because nestlings with lower hematocrit suffer from anemia, which complicates oxygen transport and metabolic rates (Booth and Elliot, 2002; Williams *et al.,* 2004). On the other hand, nestlings with abnormally high hematocrit levels suffer from hypoxia and dehydration which causes similar metabolic complications (Burton *et al.,* 1969; Jaeger and McGrath, 1974). Avian hematocrit is a well-studied area because it is simple and cost-effective to measure (Owen, 2011).

Early work by Natt and Herrick (1955) used hematocrit as an indicator of health in observations on adult broiler chickens, which found that chickens kept in coops with higher temperatures had lower hematocrit and were of poorer health. Further experiments on broiler chickens found that as ambient temperature was increased, hematocrit would decrease up to certain point, but at very high temperatures (above 35 degrees Celsius), hematocrit was abnormally high, indicating dehydration (Deaton, Reece and Tarver, 1969; Kubena *et al.,* 1972; Vo, Boone and Johnston, 1978; Yahav *et al.,* 1997). This is significant because this revealed a quadratic relationship, suggesting optimum hematocrit levels required optimum temperatures. As studies were extended into field observations on wild birds it was generally agreed that hematocrit is phenotypically plastic, meaning it is influenced by environmental variables over genetics and heritability (Hunter and Powers, 1980; Potti *et al.,* 1999; Simon *et al.,* 2005; Potti, 2007; Markowski *et al.,* 2015). Field based

studies on closely related species to the thorn-tailed rayadito (*Aphrastura spinicauda*) such as great tits (*Parsus major)*, blue tits (*Cyanistes caeruleus*) and European starlings (*Sturnus vulgaris*) subsequently identified similar trends between ambient temperature and nestling hematocrit; as ambient temperatures increased, nestling hematocrit decreased (Norte *et al.,* 2008; Serra *et al.,* 2012; Markowski *et al.,* 2015). The same linear trend has even been found in the nestlings of distantly related species such as; goshawks (*Accipiter gentilis*), coopers hawks (*Accipiter cooperii*), red-tailed hawks (*Buteo jamaicensis*) and American kestrels (*Falco sparverius*) (Hunter and Powers, 1980; Dawson and Bortolotti, 1997b). Literature rarely finds a quadratic relationship between nestling hematocrit and ambient temperature in field-studies, simply because temperatures rarely exceed those to which species are naturally evolved to survive in. This demonstrates how widely accessible nestling hematocrit is across different bird species as a fitness-related trait. Some studies took this knowledge a step further and found that nestlings with low hematocrit levels suffered low survival rates and reduced longevity as adults (Naef-Daenzer, Widmer and Nuber, 2001; Bowers *et al.,* 2014). Nestling hematocrit is a useful tool for measuring the impact of global warming on nestling health, owing to the strong link identified between ambient temperatures, nestling hematocrit and nestling fitness. However, what remains unclear is at what point in nestling development is ambient temperature most critical for determining hematocrit levels in nestlings close to fledging? Although temperature has widely been identified as the main driver of nestling hematocrit in wild birds, other factors like body size and habitat quality have also been found to affect nestling hematocrit.

Traits such as body mass, tarsus and culmen length are commonly measured alongside hematocrit as combined proxies of nestling fitness (Fair, Whitaker and Pearson, 2007; Lill *et al.,* 2013). Many studies correlate nestling body mass and hematocrit to suggest that larger

bodied individuals have higher hematocrit levels (Dawson and Bortolotti, 1997a; Christe *et al.,* 2002; Masello and Quillfeldt, 2004). Whilst this may be true, more recent studies highlight that overarching factors like parasite loads or food abundance cause the two to follow the same pattern (Christe *et al.,* 2002; Granthon and Williams, 2017). However, occasionally proxies of nestling health can cause changes in other proxies; for example, Kilgas (2014) found that higher hematocrit in laughing doves (*Spilopelia senegalensis)* subsequently caused a higher basal metabolic rate (BMR) when manipulated through stress responses. It is useful to measure body size of nestlings when collecting hematocrit to bridge these correlations which link them to overarching predictor variables like temperature, food availability or habitat quality.

Studies which investigate habitat quality as a predictor of nestling hematocrit commonly use proxies like diameter breast height of trees (DBH) (Beyer Jr., 1996 and Amininasab *et al.,* 2016), food abundance (Kloskowski *et al.,* 2017) forest type (Kilgas *et al.,* 2006), tree density (Sánchez, Javier Cuervo and Moreno, 2007) and sometimes a combination of these factors (Chalfoun and Martin, 2007; Sumasgutner *et al.,* 2019). Proxies like forest types, tree densities and diameter breast height (DBH) of trees are important as these are commonly collinear with insect abundance; for example, higher tree densities result in more insects which results in healthier nestlings and optimum hematocrit levels (Kloskowski *et al.,* 2017; Sumastgutner *et al.,* 2019). One study by Sánchez, Cuervo and Moreno (2007) in great tits (*Parus major*) found nestling hematocrit to positively correlate with the DBH and the number of trees around nests. Other studies have found that nestling hematocrit correlates with specific tree species and forest structures (Mazerolle and Hobson, 2002) which is linked to insect abundance (Hogstad, 2005; Veen *et al.,* 2010; Kaliński *et al.,* 2015). In these studies, food availability is a broad proxy which measures food availability

of whole areas, not per nest, which creates uncertainty about the reliability per nest. A good example from studies in agricultural ecology combine insect abundance and vegetation characteristics of different farmland areas to assess this effect on nestling health per nest (Kleijn and Van Langevelde, 2006; Grüebler *et al.,* 2018). This is an ideal index which gives values of habitat quality per nest (including food availability) instead of whole areas.

Food availability is central to nestling health. The abundance of insects in areas as repeated measurements over a season are typically used as a proxy of insect availability, depending on the primary food source of the species (Visser, Holleman and Gienapp, 2006; Pérez *et al.,* 2016). Studies by Acquarone et al. (2002) & Kalinski et al. (2015) on hooded crows (*Corvus cornix*) and pied flycatchers (*Ficedula hypoleuca*) found strong evidence that as insect availability decreases, nestling body size and hematocrit decrease simultaneously. This simultaneous decline makes it difficult to separate if body mass or hematocrit declines first. The decline in insect availability occurs as the season progresses in these studies, which subsequently correlates with fluctuating temperatures. The negatgive effect of lowered insect availability on nestling health is clear (Cucco *et al.,* 2002; Sabat *et al.,* 2004). This is useful as there is a strong link between lowered food availability, lowered nestling body mass and lowered nestling hematocrit.

One important confounding factor in some studies is brood size, which also has the potential to effect nestling hematocrit (Gladalski *et al.,* 2015; Wascher *et al.,* 2017). One study on nestling tree swallows (*Tachycineta bicolor*) conducted a cross fostering experiment in manipulating brood size between warm locations and found a positive trend with nestlings in larger brood sizes having lower hematocrit (Morrison, Ardia and Clotfelter, 2009). However, in colder climates we see the opposite effect; Krause *et al.* (2016) found

that as brood size increased in the Alaskan white-crowned sparrow (*Zonotrichia leucophrys*) nestling hematocrit also increased. Brood size may be an important confounding factor for determining nestling hematocrit, but it must be acknowledged that birds with larger brood sizes tend to favor colder climates, so may have higher hematocrit levels for reasons unrelated to brood size (Sandercock, Martin and Hannon, 2005). Colder climates cause higher hematocrit levels, and the environmental context of brood sizes should be considered before making strong conclusions from correlations (Potti, 2007).

The first aim of this study was to identify differences in hematocrit of nestlings between two extreme cold and warm climates. Our second aim was to find if ambient temperature affected pre-fledging nestling hematocrit during nestling development.

Methods

Study sites and species

The thorn-tailed rayadito (*Aphrastura spinicauda)* is a small insectivorous passerine bird found across Argentina and Chile in South America. Their range varies from temperate-tropical rainforest to sub-Antarctic forests with breeding seasons ranging from September to February (Remsen, 2003). The unique range of this species provides an opportunity to investigate the potential effects of climate breakdown on nestling health, by studying fitness-related traits like hematocrit. Our observations took place at two different sites in Chile: one on Navarino Island (54.9325°S, 67.6059° W) and the other in Pucon (39.2723° S, 71.9776° W). The Navarino site is a sub-Antarctic deciduous forest, with low temperatures averaging 7.7^{O}C across the breeding season (INIA, 2020) and snow every month of the year. Our cold site only has 6 tree species, which are predominantly made up

of deciduous species such as *Nothofagus antarctica, Nothofagus betuloides* and *Nothofagus pumilio*. The understory is predominantly *Berberis mycrophylla* across all nest box sites. Whilst Pucon is a temperate tropical forest on the outskirts of Patagonia with high rainfall and relatively higher temperatures averaging 13.7^{O}C over the breeding season (Direccion meterogoligica de Chile, 2020). Our nest boxes in Pucon were predominantly surrounded by evergreen species such as *Aextoxicon punctatum, Drimys winteri, Eucryphia cordifolia, Gevuina avellana* and *Persea lingue* with deciduous species such as *Nothofagus* obliqua and Nothofagus *dombeyi*. The understory consists of mainly bamboo species such as *Rhaphithamnus spinosus* and different *Azara* tree sapling species across all sites. One main difference between our sites is that nest boxes in Pucon are subjected to predation pressures from colocolo opossums (*Dromicipiops gliroides*), whilst Navarino is predator-free. Data was collected in two seasons from September 2018 – February 2019 and from September 2019 – February 2020, which will be referred to as 2018 and 2019 season, respectively.

Figure… Shows our two study sites; the red star indicates the temperature tropical forest (warm location) and the blue star indicates the sub-Antarctic forest (cold location).

Body condition and physiology

There are 200 nest boxes at each site which were monitored throughout the season, allowing us to record the nest building phase all the way to nestling fledging. After hatching, the brood size was recorded until fledging, noting when/if nestlings died prematurely. Day 0 was standardized as the day of hatching. On day 0 the culmen (mm) was measured to 0.1mm accuracy and mass (g) to 0.1g accuracy with digital calipers and scales. The toenails were then clipped with small scissors in different orders to track individual growth until they could be ringed. These same measurements were taken on day 4, 8, 12 and 16. Then on day 8, nestlings were ringed, and we measured the tarsus length (mm) using

digital calipers to 0.1mm accuracy. Nestling hematocrit was extracted 16 days after hatching (D16). Nestlings were not always 16 days old on “Day 16” because egg hatching is asynchronous. Blood was taken from the renal vein underneath the wing in the nestlings armpit by using a 25G disposable needle to prick the vein and then used 75µl capillaries to collect. Blood samples were then transported in a cool box to slow coagulation and transported to a field station to be centrifuged, we recorded the transport time between collection and centrifugation. Blood samples were centrifuged at 8000x spins per minute for 3 minutes. When the plasma and red blood cells fully separated, the total amount of liquid was measured (mm) in the capillary using digital calipers (to 2 decimal places). Hematocrit was then calculated with the equation (*red blood cells / total blood sample * 100 = % hematocrit level).*

Habitat quality and temperature

Insects were collected once every 6 days on average. In the cold and warm site, there were 4 fixed trees visited every 6 days. This was conducted by using a 3-sided sheet (**fig..)** which was held up underneath a branch and the branch was struck 10x with a stick to dislodge insects and spiders clinging to the branches. After striking the branch, the sheet was lowered, and the insects and spiders were counted for total insect abundance, giving a weekly average from all 4 trees.

Fig… Shows our insect collecting apparatus made of a 3-sided sheet (80cmx80cmx80cm) attached to 3 poles.

Temperature (degrees Celsius) data was taken from meteorological stations in Pucon which were ~5-10km from our nest boxes (Direccion meterogoligica de Chile, 2020*)* and Navarino which were ~5km away from our nest boxes (INIA, 2020*).* This included the daily average temperature from September to February in both years, which was at least 30 days of temperature data before the first hematocrit measurement of each season. Habitat quality data was collected in 2019 only in both locations which included; the diameter of trees at breast height of all trees within an 11.2m radius of the nest box (Amininasab *et al.,* 2016) and the total number of trees in the 11.2m radius around a nest box (Jones, Harris and Siefferman, 2014). The radius of 11.2m was used based on previous studies which assessed habitat quality in this species around the same artificial nest boxes (Altamirano, 2014). These measurements are not in the territory of a nesting pair as the territory is far too large (Cornelius, 2008). These trees were then classified as either deciduous or

evergreen trees as deciduous trees are important for caterpillar abundance, which is the rayadito's main food source (Reyes-Arriagada, Jiménez and Rozzi, 2015).

Statistics

All statistics were carried out in RStudio 3.5.3. statistical software. Graphs were made with ggplot2. Graphs used two datasets; the first data set included all hematocrit measurements from both locations, and the second dataset included the hematocrit measurements from both locations in the 2019 season only.

Prior to model building, collinearity was checked between related predictor variables e.g. (body mass, culmen and tarsus length) & (DBH_AV, total number of trees, % of deciduous trees and insect abundance). If collinearity was found, then only one predictor would be selected, based on simplicity and to avoid excess noise in model building (Bedeian, 2014). Insect abundance was measured per location, per week and total number of trees was measured per nest box. To relate insect abundance per nest box, an index was made of habitat quality = (insect abundance * total number of trees around nest box). Insect abundance was related to nestling hematocrit by using weekly insect abundance over when nestling hematocrit was collected. Insect abundance and tree density, which are both on different scales, form a habitat quality index per nest box. We transformed the factor 'date of measurement' into Julian dates as an integer (Bishop, Meyers and McNeley, 2000) with the start date as 1st September in both seasons.

Our first research question was to determine if ambient temperature was the best predictor of nestling hematocrit. To answer this, we used a type of generalized linear model

('glmmTMB') appropriate for beta distributions with a random effect. The model of best fit was determined using the 'buildglmmTMB' function which combines all possible combinations of predictors and gives the best model *via* AIC scoring. The model of best fit was followed up with a post-hoc tukey test, using the 'lsmeans' function to identify differences in nestling hematocrit between groups (locations*seasons) in our model of best fit. Model residuals were checked for model fit and outliers, if any were found, models were re-run with excluded outliers to see if results changed, if not, outliers were included.

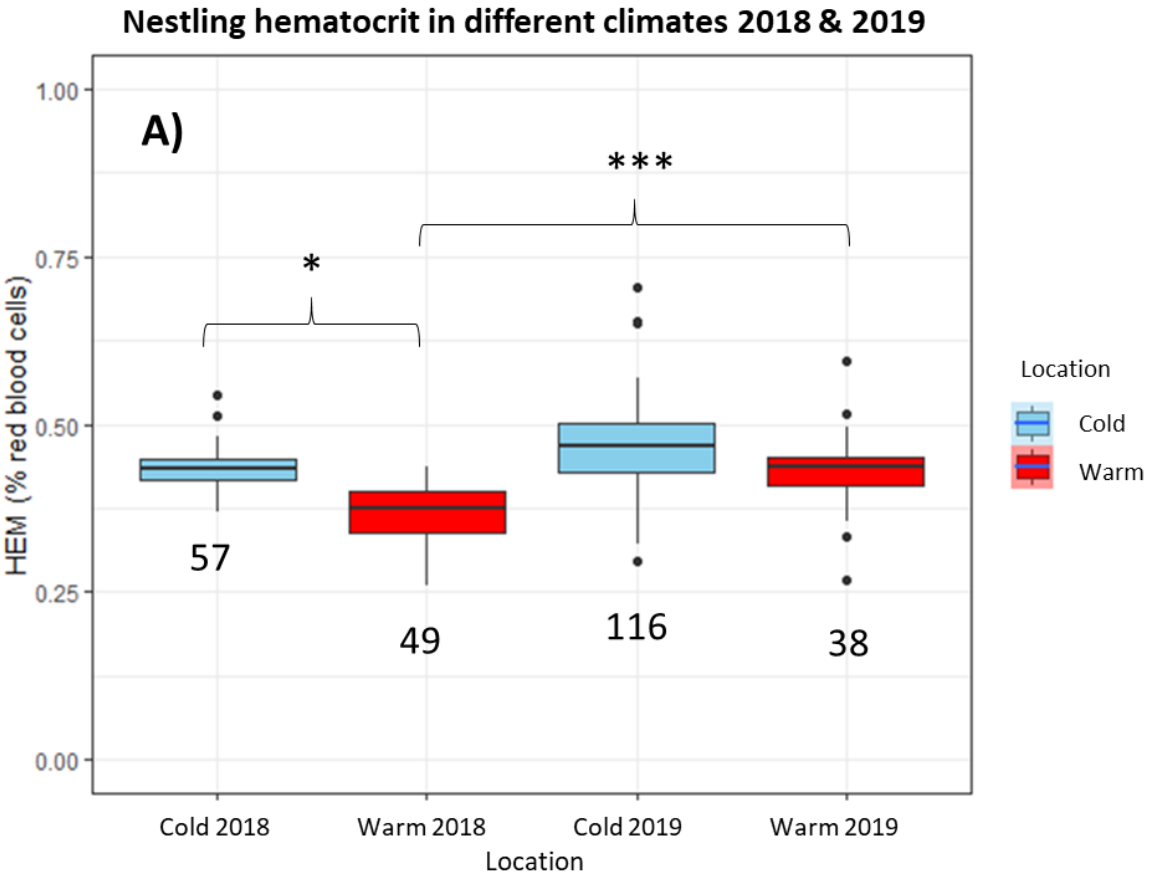
Our second research question was to find if ambient temperature was important during nestling development in determining nestling hematocrit close to fledging. To answer this, we used the 'climwin' package in RStudio (Bailey, and Van de Pol, 2016). The 'slidingwin' function in this package was used on the hematocrit (%) data and the ambient temperature data (^{O}C) to identify the best correlative window between temperature and hematocrit. In this section, groups were separated by both season and location. Our range to assess temperature was 30 to 0 days before hematocrit was measured. This allows climwin to assess all possible climate windows with hematocrit which covers the days of egg laying, incubation and hatching, until the day hematocrit was measured. We also looked for the mean, minimum and maximum temperature in all these windows correlated with hematocrit. We selected both 'linear' and 'quadratic' distribution functions to examine the relationship between temperature and hematocrit. The model with the lowest AIC score was selected as the best model which provides a climate range window where temperature was most influential on hematocrit measurements. After this has been determined, a table of coefficients will be used to summarize the relationship between temperature and hematocrit within our given time frame. To assess the validity and significance of our findings, we will then run 1000 random models with the same parameters to determine that our model of

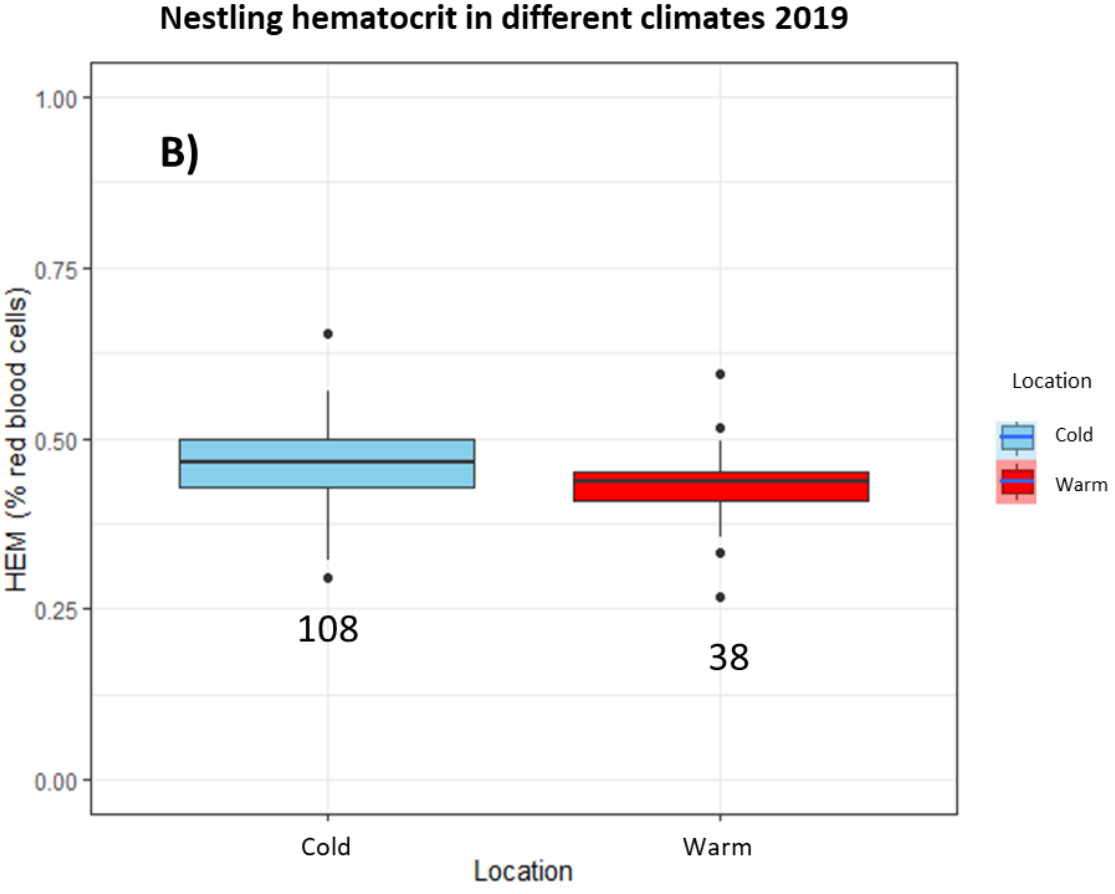
best fit is significantly different from random. The outcomes can then be plotted as visual aids which also show the significance of the climate window identified by the package.

Results

We collected hematocrit from 265 nestlings in total from 2 locations in 2 seasons. In 2018 in the cold location (n= 57 nestlings) and (n= 51 nestlings) in the warm location. In 2019 in the cold location (n= 117 nestlings) and (n= 40 nestlings) in the warm.

Our collinearity matrices revealed that total number of trees and insect abundance were collinear (P= 0.68***), and body mass and tarsus were also collinear (P= 0.67***). We therefore chose tarsus length as a proxy of nestling size instead of body mass which is dependent on the time of day as parental feeding frequencies fluctuate (Reyes-Arriagada, Jiménez and Rozzi, 2015).


Nestling hematocrit in different climates 2018 & 2019
A)
***
*
HEM (% red blood cells)
1.00
0.75
0.50
0.25
0.00
57
49
116
38
Cold 2018
Warm 2018
Cold 2019
Warm 2019
Location
Location
Cold
Warm


Nestling hematocrit in different climates 2019
B)
HEM (% red blood cells)
1.00
0.75
0.50
0.25
0.00
108
38
Cold
Warm
Location
Location
Cold
Warm

Figure… *Boxplot **(A)** shows the hematocrit levels of all nestlings over two seasons (2018 & 2019) in both cold (Navarino) and warm (Pucon) locations. Boxplot **(B)** shows the hematocrit of nestlings from cold (Navarino) and warm (Pucon) locations from 2019 only. Each boxplot uses slightly different sample sizes because of NA exclusions.*

Within locations

The effect of temperature on nestling hematocrit was assessed within locations which mostly confirmed ambient temperature as the key driver of nestling hematocrit (**tab..**).

Table 2 – generalized linear mixed model of temperature and hematocrit within locations.

Group	Estimate	Standard error	Degrees of freedom	P-value
Cold 2018	- 0.0002	0.0092	-	0.98
Cold 2019	-0.0307	0.0123	-	0.01*
Warm 2018	-0.0206	0.0096	-	0.03*
Warm 2019	-0.0361	0.0143	-	0.01*

Between locations

The boxplots (**fig**) show a trend, that nestlings in colder climates have higher hematocrit than those in warm climates and hematocrit is slightly higher both climates in the 2019

season, although only some were significant. Our models found that overall, there was no difference in nestling hematocrit between the two locations overall (P = 0.12). Our models including all nestling hematocrit in both locations from both seasons used the predictors, season, location, brood size, nestling age, daily average temperature, date of measurement and tarsus length, with nest box ID as a random factor. It was found that the model of best fit with the lowest AIC included group (season*location) and daily average temperature, with nest box ID as a random factor. The daily average temperature had a strong negative relationship with nestling hematocrit (P = <0.0001***) and groups (season*location) were also significantly different (P = <.0001***). However, this model tested significant for outliers (P = .04*) so we excluded these and re-ran the model. The results did not change when outliers were excluded, so the original model, including outliers was used. Following this an 'lsmeans' post-hoc tukey test was used to pinpoint where significant differences occurred between the groups.

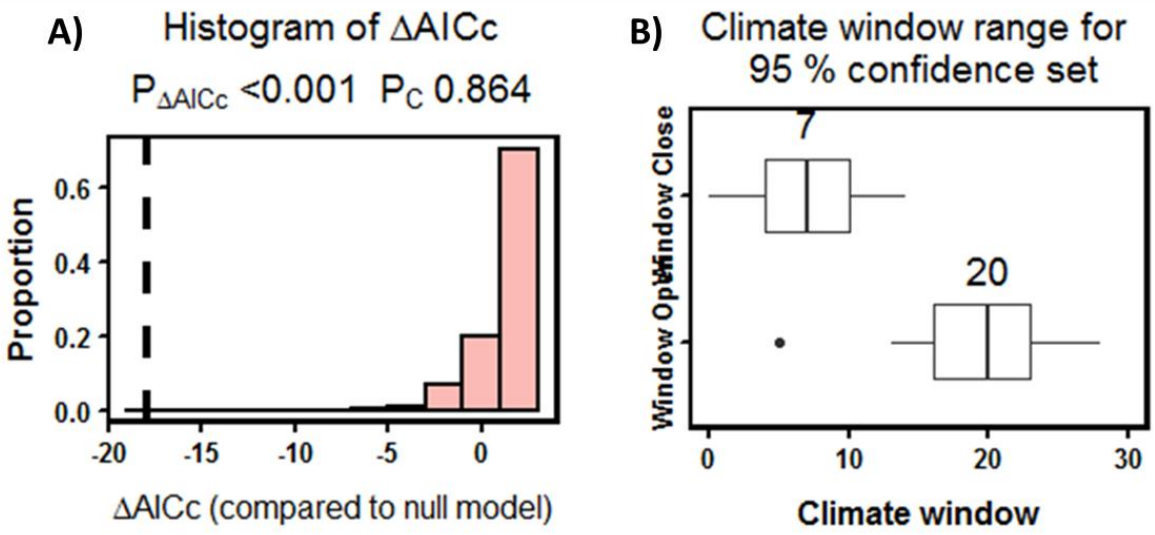
When analyzing the second dataset from 2019, we used the predictors; location, brood size, age, daily average temperature, date, tarsus length, diameter breast height of trees and habitat quality, with nest box ID as a random factor. An interaction between all predictors and location was included to differentiate between the sets of predictor values (ie. location * tarsus length). It was found that the model of best fit excluded all predictors except daily average temperature and location. Models were checked for outliers, but none were found. Our model of best fit showed that daily average temperature had a strong negative relationship with hematocrit (P = .0004***) and there was no difference in nestling hematocrit between locations in 2019 (P = 0.36).

Table 1 – The post-hoc tukey test results of all nestling hematocrit data including both seasons (2018 & 2019) and both locations (cold & warm).

Group	Estimate	Standard error	Degrees of freedom	P-value
Cold 2018 - Warm 2018	0.17	0.06	253	0.01*
Cold 2018 - Cold 2019	-0.04	0.04	253	0.74
Warm 2018 - Warm 2019	-0.26	0.05	253	<.0001***
Cold 2019 - Warm 2019	-0.04	0.06	253	0.89

Temperature and hematocrit

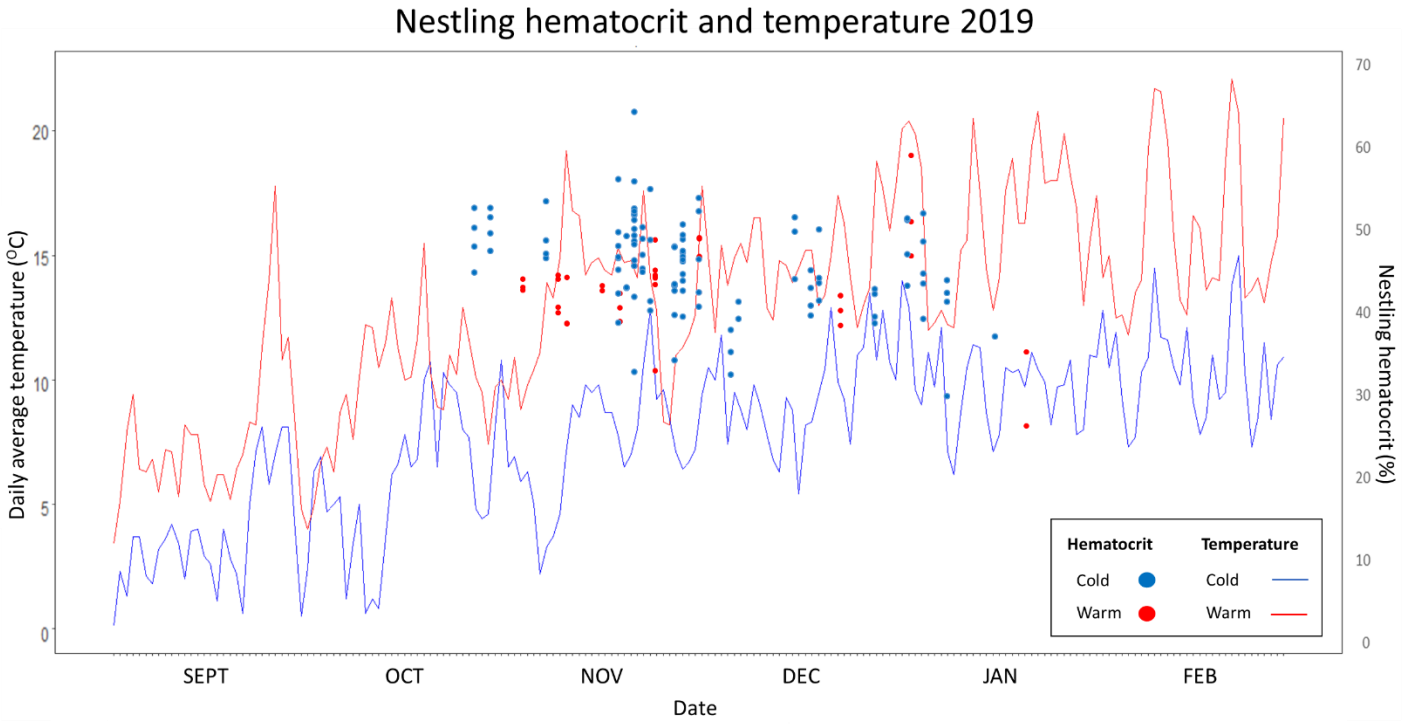
The climwin models found that both linear and quadratic functions with mean temperature were the best models within 0.04 AIC points of each other. Both these models were checked and in a linear function, climate had a strong negative relationship with hematocrit (P= <.0001***) whilst climate and hematocrit in a quadratic relationship had a positive but non-significant relationship with hematocrit (P = .8765). The model shows that the temperature 14 days before hematocrit is collected (*known from here as* 2 days after hatching) had the strongest relationship with nestling hematocrit (P = <.0001***).



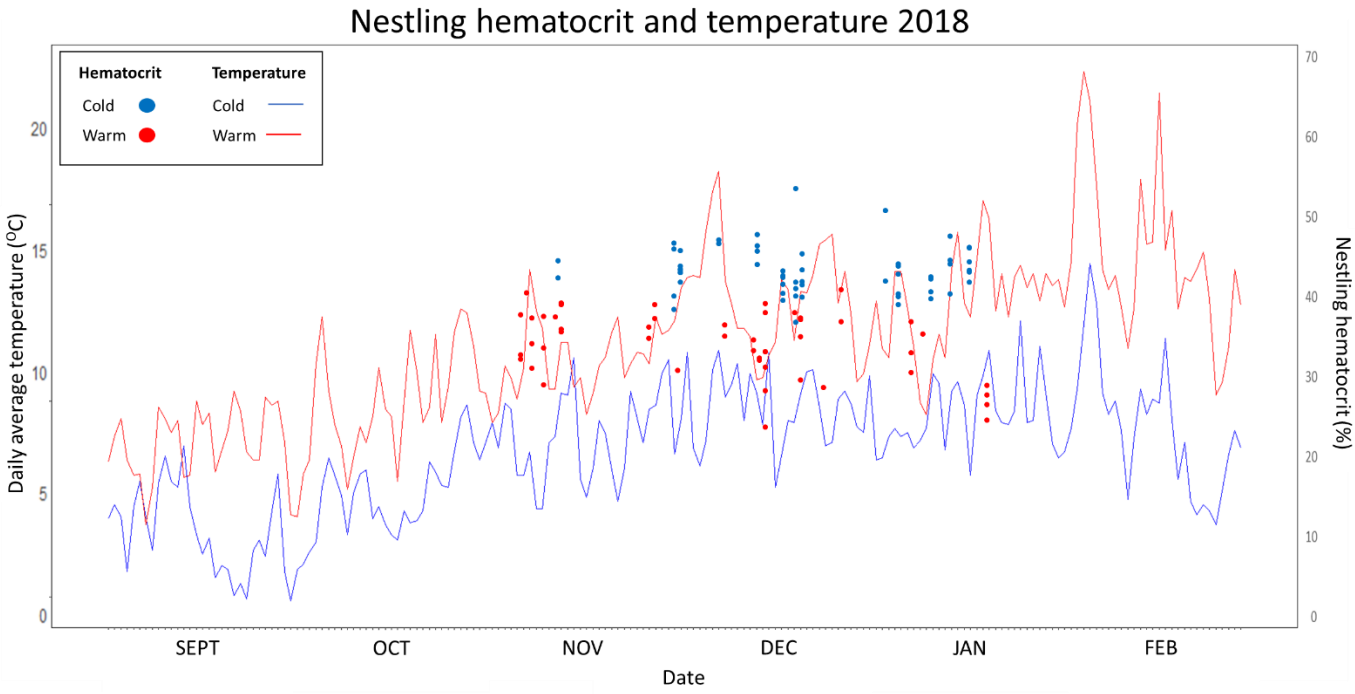
Figure… – *Shows part of the output from the climwin package. The first graph (**a**) is a histogram of the distribution of our model, compared to the 1000 random models. The second model (**b**) is a boxplot showing when the climate window opens and closes to a 95% confidence interval.*

From **fig…** graph (A) gives the AIC of our model compared to 1000 random models shows that our model is significantly different from random (P = <.001**). The dashed line in graph (A) is our model and the others are the random models. Because we used 1000 random models we can trust the PΔAICc in the histogram chart, whilst the Pc-value is the relevant P-value when less than a 1000 simulations are used (P = <0.5) (Bailey and Van de Pol, 2016). The boxplot in graph (B) shows that our climate window range is significant between 7 and 20 days before the measurement was taken, meaning that we can be 95% sure that the ambient temperature 7-20 days before hematocrit is measured is the best predictor of pre-fledging hematocrit. We also investigated the differences in temperatures on important days between years in the warm location. The temperature at 2 days after hatching were very similar in the warm location 2019 and 2018 (13.98^{O}C & 13.93^{O}C, respectively). Whilst the ambient temperature was lower on the day of nestling hatching in the warm location in

2019 than in 2018 (13.25 ^{O}C & 13.71 ^{O}C, respectively).



Figure… *The daily average temperature in 2019 (1st y-axis) from Sept-Feb (x-axis) and each individual hematocrit measurement (2nd y-axis). Data has been aligned so hematocrit measurements match the temperature 14 days before they were measured.*



Figure… *The daily average temperature in 2018 (1st y-axis) from Sept-Feb (x-axis) and each individual hematocrit measurement (2nd y-axis). Data has been aligned so hematocrit measurements match the temperature 14 days before hematocrit was measured.*

Discussion

Could high predation have caused the high hematocrit levels in 2019 in Pucon?

We found that nestling hematocrit was higher in the colder location in 2018 but there was no difference between locations in 2019. This resulted in no overall difference in nestling hematocrit between cold and warm locations overall. But the observed differences were clearly due to a temperature effect. We found a strong negative relationship between ambient temperature and nestling hematocrit, i.e. as temperature increased, nestling

hematocrit decreased. We also find evidence that the ambient temperature around hatching is important for pre-fledging nestling hematocrit.

Within locations

We find that temperature is the main predictor of nestling hematocrit and has a negative effect on nestlings within our locations, except in the cold location in 2018. There are several explanations for the apparent lack of effect on nestlings in the cold location in 2018. Firstly, habitat quality was not measured in 2018. Because of this, habitat quality may have explained nestling hematocrit in 2018 in the cold location, as observed elsewhere (Studds and Marra, 2005; Busch *et al.,* 2011; Kaliński *et al.,* 2015). Two, our proxy of ambient temperature in our models is the average temperature 2 days after hatching (provided by the climwin package). Extreme temperatures outside of this date could have influenced nestling hematocrit and overshadowed the temperature 2 days after hatching. Our climwin package did not provide the best climate window for all 4 datasets separately, it analysed all data together to give one climate window for all nestling hematocrit measurements. The datasets from the cold location in 2018 should be analyzed separately to determine this. Lastly, adults may have adapted their parental behaviors to changes in temperatures. Mueller et al. (2019) found that adults show a trade-off between incubation and feeding when temperatures fluctuate consistently throughout a season. Adult birds may have invested more time into feeding and less time into incubation when temperatures were high and the opposite when temperatures were low, to counterbalance the negative effect on nestling health. This is the most likely scenario but why this is not the case in other groups is unclear. Specific circumstances must have arisen in the cold location in 2018 to cause the apparent lack of temperature effect at 2 days after hatching. But as Mueller et al. (2019)

stated, this trade-off only occurs when temperatures are extreme for extended periods of time. In other words, some years may have had consistent temperatures but had extreme fluctuations on the days which were most important for nestling hematocrit (like the day of hatching for example), which was not enough time for adults to adapt their parental behavior accordingly. Whether temperature in the cold location in 2018 was consistent and gave adults time to adapt their parental behaviors or were simply favorable on the day of hatching is unclear. To determine this it would be prudent to conduct the models again, using the temperature on the day of hatching as a predictor, instead of 2 days after hatching.

Between locations

We found that although there was no overall difference in nestling hematocrit between cold and warm locations, our post-hoc tests revealed some differences between certain groups. Nestling hematocrit was higher in cold locations in 2018 but was similar between the two in 2019. Our data also shows that nestling hematocrit was much higher in 2019 than 2018 in our warm locations **(fig…boxplot**).

The spike in hematocrit in 2019 in the warm location caused no differences in nestling hematocrit between cold and warm locations in 2019, and as a result, between locations overall. Our models confirm this is a temperature related effect which therefore suggests temperature was lower in 2019 than 2018 in our warm location, which caused an increase in nestling hematocrit in 2019. There are several explanations for these results; firstly, a drop in temperature in 2019 could have caused this difference, but as we found, temperature was similar in the warm location in 2019 and 2018 (2 days after hatching).

However, the temperature was on average lower on the day of hatching for nestlings in the warm location in 2019 compared to 2018. Assuming that nestling hematocrit is higher when ambient temperatures are lower, this may offer an explanation. The differences in temperatures are small (13.71 ^{O}C in 2018 & 13.25 ^{O}C in 2019 on the day of hatching, respectively) but we must consider the impacts of small increases in temperatures on ecological and physiological scales (Hansen *et al.,* 2006; Kraaijenbrink *et al.,* 2017). Secondly, nestling hematocrit may have returned to "normal" in 2019 and was at a trough last year. Because we do not have any nestling hematocrit data before 2018, we cannot place our current results in a historic ecological context. Nestling hematocrit may historically have been very different between our locations but ongoing climate breakdown with rising temperatures may have already altered nestling hematocrit to be similar between locations some years ago. Unfortunately, data on nestling hematocrit in these locations before 2018 does not exist, so we could not know when nestling hematocrit was different between locations. But what we can assume is that nestling hematocrit was higher in sub-Antarctic locations when temperatures were much lower compared to the warm location. Another explanation is the high levels of predation in the warm location in 2019, elevations in nestling stress and corticosterone levels may have responded in raised nestling hematocrit (Vasquez, 2014; Sallah-Hudin *et al.,* 2017). Or a final explanation is that rainfall had a significant effect on nestling hematocrit. The warm location is a temperate tropical rainforest, where rainfall frequently varies, occasionally independent of ambient temperature. One study by Busch et al. (2011) conducted in a tropical rainforest on song wrens (*Cyphorhinus phaeocephalus*) found that as rainfall decreased across a geographical gradient, nestling hematocrit also decreased. Interestingly, temperature and rainfall were not collinear in this study as tropical rainforest can maintain consistent temperatures but vary in rainfall. Although, this was at the mercy of other biotic and abiotic variables related

to habitat quality, it provides evidence that rainfall can influence nestling hematocrit independently of temperature in tropical ecosystems. Other studies also highlight rainfall as an important predictor of nestling hematocrit and agree that in specific circumstances rainfall can act independently of temperature (Bańbura *et al.,* 2011; Bowers *et al.,* 2014 & Kalinski *et al.,* 2016). Although, no studies investigate rainfall patterns in early nestling development on post-fledging fitness, this may help explain the difference in nestling hematocrit between the two years in our warm location. It seems most likely that the high predation levels in Pucon in 2019 caused an elevated stress response, which in-turn increased nestling hematocrit. Although, rainfall patterns and nestling hematocrit are an understudied area which deserves more attention when investigating variations in nestling hematocrit and physiology. What we can assume from this study is that unless climate breakdown comes to a halt, nestling hematocrit will become sub-optimum in both locations and nestling health as a result will go into decline, if they have not already. This may impact post-fledging survival rates and longevity in the future as seen in other Passerines (Bowers *et al.,* 2014; Facey *et al.,* 2020). As trends in other studies use other bird species as examples (Markowski *et al.,* 2015; Grantham and Williams, 2017; Mueller *et al.,* 2019) and follow the same patterns as our study, we can extend these findings to make assumptions about other bird species, which is what makes these results so important.

Temperature and hematocrit

A linear relationship was selected over the quadratic one between nestling hematocrit and ambient temperature because, temperature and hematocrit were better distributed over a linear curve, despite literature mostly suggesting that hematocrit and temperature have a quadratic relationship (Özkan *et al.,* 2007; Mohammadalipour *et al.,* 2017). Literature

identifying a quadratic relationship typically involves lab-controlled manipulations of temperature, whilst the temperatures observed in the field are typically not extreme enough to show a true quadratic relationship. Our model shows that the temperature 2 days after hatching can successfully predict nestling hematocrit 16 days after hatching. This is unlikely to be true, as 2 days after hatching seems too specific. However, we can be 95% sure that the most important climate window is between 7 and 20 days before nestling hematocrit is collected (Bailey and Van de Pol, 2016). Our results suggest that ambient temperature has a delayed effect on nestling hematocrit and that nestlings are physiologically most vulnerable around hatching. To our knowledge, no literature manipulates temperature at different points in nestling development and measures nestling hematocrit. Studies which observe temperatures at different times in nestling development and record hematocrit simultaneously are very rare, as are studies which look at the impact of lowered hematocrit on nestling survival or post-fledging health.

There are many studies that consider the effect of ambient temperature in early nestling development on post-fledging fitness and survival, which use different proxies of nestling health. For example, Greño *et al.* (2008) used post-fledging survival as a proxy of individual fitness using 12 years of blue tit data. They observed that nests with higher than average temperatures, produced nestlings with lower survival rates, and thus lower fitness. This is relatable to our study as when temperatures increase, hematocrit decreases and therefore nestling health too. This study comes to the same conclusion through a different method. Although this study uses different proxies of nestling health compared to our study, are still comparable, as they still follow the same trends with temperature. Using different proxies of nestling health can be useful when making comparisons between study species. It reduces the probability that the results were obtained by chance, if studies consistently reach the

same conclusion by using different proxies of nestling health like body mass, survival or hematocrit. In addition, many studies agree that hematocrit and body are both good indicators of nestling health (Markowski *et al.,* 2015; Krause *et al.,* 2016; Farag and Alagawany, 2018). Another similar example by Facey *et al.* (2020) conducted observations on barn swallows (*Hirundo rustica*) and found that ambient temperature, rainfall and wind speed during nestling development, affects nestling body mass (and therefore health) just before fledging. This follows the same trend as our data but again does not identify specific days of which these predictor variables mattered most in nestling development. These types of studies are the closest to the edge in the gap in current literature. To our knowledge, our study is the first of its kind in identifying the effect of climate windows on fitness-related traits like hematocrit. Opposing literature finds evidence that ambient temperature during nestling development does not predict hematocrit but nestling hematocrit is predicted by the time of day the hematocrit is measured (Smith and Barber, 2012). This is true to some extent, but this study collected hematocrit between 0900-1800h in Nova Scotia, Canada. It is likely that 1800h was far too late in the day to sample nestling hematocrit, as adults are more likely to incubate nestlings at these times, which is disrupted by collecting hematocrit. Other studies which also find sharp changes in hematocrit unrelated to ambient temperature, instead find that parasitism is responsible for changes in hematocrit (Heylen and Matthysen, 2011). Although, the aforementioned studies combine correlational data on the effect of temperature on nestling health, none address the specific days in nestling development which are most important for determining fitness-related traits.

Based on our findings we propose an experiment on nestlings take place; manipulating the temperature of wild nest boxes during the egg incubation period, nestling hatching day (day 0), day 2 (14 days before hematocrit collection), day 9 (7 days before hematocrit collection)

and on the day of hematocrit collection. Some investigations have already taken steps towards this in egg incubation but not in nestlings; one study by Black and Burggren (2004) manipulated the incubation temperature of broiler chicken eggs and tested nestling hematocrit 13 days after hatching and found that broilers incubated in colder temperatures had reduced hematocrit. Later studies by Ardia *et al.* (2010) & Mueller et al. (2019) find that this same trend occurred when cooling eggs in field studies between body mass and incubation temperatures; as incubation temperatures decreased, nestling body mass decreased. To our knowledge no studies exist which manipulate the temperature of nests on the day of hatching and record nestling health throughout development. These studies show strong evidence that the temperature in the incubation period may determine nestling hematocrit and nestling health, which warrants an investigation into manipulating incubation temperatures and recording pre-fledging hematocrit. One obstacle of this experiment highlighted by Mueller et al. (2019) who recorded both ambient and nest box temperatures and found a difference in the effect on nestling health. They posit that parents exhibit a trade-off between incubation and feeding when internal and external temperatures fluctuate. This makes it difficult to differentiate between the effects of nest temperature and ambient temperature, as their impacts on nestling hematocrit are different. This highlights the point that nest temperature during incubation in these studies are kept in a constant state of manipulation, which allows the parents to adjust to incubating conditions. However, if temperatures were not manipulated and then increased/decreased dramatically on one day only during incubation/nestling development, this will identify on which days eggs/nestling physiology are most vulnerable. Mueller et al. (2019) also mentions the potential impact of confounding factors like habitat quality which may complicate the interpretation of results further. This study did not record hematocrit but shows lower temperatures in early stage development negatively affects nestling health at fledging. To

bridge the gap between studies, the proposed experiments should take place by manipulating nest temperatures in wild populations of birds during nestling development (as well as incubation) and record fitness-related traits like hematocrit and body mass.

Some studies argue that nestling hematocrit alone is not sufficient to act as a proxy of overall nestling fitness, but is a good indicator of nestling health (Dawson and Bortolotti, 1997a; Lill *et al.,* 2013; Petit, Clavijo-Baquet and Vézina, 2017). Field studies which posit that hematocrit is an inaccurate predictor of fitness record many environmental and physiological variables which makes more noise in their data, which may ultimately blur their results. What is certain is that nestling hematocrit is an indicator of nestling health but combining nestling hematocrit and body mass as an index is useful (Hatch and Smith, 2010) but generally, many studies agree that nestling hematocrit is a useful and reliable proxy of nestling health (Fair, Whitaker and Pearson, 2007; Markowski *et al.,* 2015; Mohammadalipour *et al.,* 2017; Farag and Alagawany, 2018). What can be concluded based on our results is that as ambient temperatures continue to rise, nestling hematocrit will continue to decrease. This may lead to reduced fitness through complications in oxygen transport and metabolic rates. Regardless of our correlative assessments in this study, causation cannot be inferred until the suggested experiments are carried out.

Habitat quality and physiology

Our models excluded habitat quality and body condition from our models as predictors of nestling hematocrit. We expected that temperature would be a better predictor of nestling hematocrit but for habitat quality to play no role in predicting nestling hematocrit was unexpected. Our index of habitat quality was a combination of insect abundance and tree

density. Ambient temperatures facilitate insect abundance throughout the breeding season by dictating favorable breeding and feeding conditions (Volney and Fleming, 2000). As ambient temperature and insect abundance are collinear it is possible that these could not be separated statistically, which is why our model of best fit excluded habitat quality. Literature frequently concludes that lower food availability can decrease nestling hematocrit (Cucco *et al.,* 2002; Simon *et al.,* 2005; Kaliński *et al.,* 2015; Criscuolo et al., 2019). These studies do not take habitat quality and ambient temperature into consideration simultaneously. One study which does by Busch *et al.* (2011) did not analyze insect abundance and temperature together for the same reason of collinearity. Instead, they analyze these separately and find that both have an effect on nestling hematocrit separately. A next step in this investigation would be to conduct a PCA analysis on all of our predictors on nestling hematocrit to see how close insect abundance and ambient temperature operate in predicting nestling hematocrit.

Concluding remarks

We found that nestling hematocrit in the field is significantly affected by ambient temperature and the ambient temperature around the time of nestling hatching is important in determining pre-fledging hematocrit. The effect of hematocrit on nestlings post-fledging or whether it is important in our area remains unknown. Other studies have found an effect of temperature on post-fledging fitness, survival, longevity and nestling hematocrit which agree that nestling hematocrit is a useful indicator of body condition. But further proxies may be needed to make stronger conclusions about overall fitness. We propose that low nestling hematocrit caused by rising ambient temperatures in our study will affect post-fledging fitness and survival, and have proposed an experiment to confirm this as causality.

If this is confirmed, the consequences of climate warming (excluding factors like habitat fragmentation, human disturbance and pollution) on this species' survival is at serious risk. These results indicate that in a warming climate, nestling health will deteriorate (indicated by declining hematocrit) and will reduce post-fledging survival, as suggested in other studies. The rapid progression of climate breakdown threatens bird species essential to their ecosystems around the globe, of which the consequences only grow more dire.

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Extra parts

Hematocrit and temperature in birds - intro

Temperature is generally agreed as the main driver of hematocrit in both adult birds and nestlings (González and Hiraldo, 1991; Hatch and Smith, 2010; Grantham and Williams, 2017; Farag and Alagawany, 2018).

In addition to this, the time of hatching is important for hematocrit; studies on egrets (*Egretta garzetta)*, European starlings (*Sturnus vulgaris*) and gray catbirds (*Dumetella carolinensis)* have found that chicks raised later in the breeding season (when temperatures are higher) have lower hematocrit levels (Hatch and Smith, 2010; Serra *et al.,* 2012; Neb, Hammouda and Selmi, 2019).

Body mass and hematocrit

Organisms were split up into 5 different groups; ´Beetles´, ´Caterpillars´, ´Flies´, ´Spiders´ and ´Other´. These were then classified as small (<0.1mm), medium (0.1-0.5mm) or large (>0.5mm). Didn't use, so shouldn't mention?

Hematocrit and body mass are effected by temperature and habitat quality but hematocrit and body mass are not correlated. This suggests that although body mass and hematocrit may be affected by temperature and habitat quality, they are effected in different ways.

Hematocrit alone is cannot determine fitness (Dawson and Bortolotti, 1997) but it is a useful indicator of fitness, body condition and environment quality.

Other physiological factors

Traits like hemoglobin, glucose levels and basal metabolic rate (BMR) correlate with body mass and hematocrit but are not causal of fluctuations in body mass or hematocrit (Kilgas, 2014; Gladalski *et al.,* 2015).

In a study on Rayaditos in Chile, baseline cortisone hormone levels were measured as a stress response indicator between different populations from Sub-Antarctic to tropical. It

was found that populations in colder climates had lower baseline cortisone concentrations, supporting the hypothesis that environmental productivity is a key driver of baseline cortisone stress (Quirici *et al.,* 2014). *Environmental productivity is higher in the south; based on martone aridity index; insect abundance, temperature and monthly rainfall.*

Corticosterone increased with supplementary feeding in house sparrows (Salleh-Hudin *et al.,* 2017) whereas hematocrit did not increase with food supplementation in other studies but corticosterone and hematocrit have been found to correlate before in other studies. Climate and habitat quality together cause the biggest effects on hematocrit, in isolation these effects are less pronounced.

According to Simon et al. 2005 hematocrit is a phenotypically plastic trait and is based on adaptation to environmental conditions other than genetics. (Does this explain my variation). Caterpillar abundance in this study explained significant differences in nestling body mass but explained no differences in hematocrit between two different populations of Blue Tits. Although parasitism did explain differences in hematocrit which is an emerging factor in our study areas but was not documented during this study.

One meta-analyses showed that birds living in colder climates had a higher maximum thermogenic capacity than those living in warmer climates, supporting the pace of life hypothesis and explanation for differences in hematocrit (Stager *et al.,* 2016).

Habitat quality

We must also consider the potential biases posed by using nest boxes versus natural cavities in our study as research has been extensive on the biases this could cause (Lambrechts *et al.,* 2010). Potential bias: Increased depredation risk (bigger entrances), More weather exposure (less secure, box position, more gaps, more easily damaged), Bigger nests required (cavities can be quite small). Need to read deeper.

The same effects have also been found in Great Tits, with the addition of assessing habitat quality, with fitter nestlings, higher hematocrit and higher parasite loads being found in mature forests, and the opposite in young forests (Sánchez Javier-Cuervo and Moreno,

2007).

Rayadito's prefer forest edges but need habitat connectivity to promote higher insect abundances. In fragmented populations, it creates more niches for Rayadito's, but the insect abundance is much lower (Vergara *et al.,* 2010).

According to Botero-Delgadillo et al 2017; Rayadito's spent less time building nests as the breeding season progressed, which saw a decrease in nest weight and depth. But is this linked to chick survival or fitness? Paper not downloaded or checked.

Findings from Careau, Buttemer and Buchanan, (2014) on zebra finches supports our hypothesis as stress at the altricial stage caused higher hematocrit levels. Based on this study it is also likely that nestlings in our area had higher basal metabolic rates than those at higher latitudes.

Food abundance

Has already been seen in Blue Tits that when populations face different food abundances and environmental conditions that nestling weight and hematocrit are effected (Simon *et al.,* 2005). But in kestrels, adult hematocrit did not vary with prey abundance (Dawson, 1980).

Passerines with lower protein diets have been known to have lower survival (Winn, 2002) which is why insect quality is important. The number of Caterpillars between two sites will be important as they're a high protein insect which according to Winn (2002) when insectivores are fed a low protein diet it reduces survival and immune-competence, which has been connected to hematocrit (refs…).

Should be noted that food supplementation does not increase hematocrit levels (**refs**) but in some cases food availability does (Hoi-Leitner *et al.,* 2001). This suggests that adults do not take more food than they need or something..?

Concluding remarks

No single factor could contribute a majority explanation for hematocrit correlation which shows variation between species; correlating with age, sex, geographical elevation, energy expenditure, parasitism, nutrition and genetics (Fair, Whittaker and Pearson, 2007).

Consider if needed...

Rainfall - Something we did not consider which is not necessarily linked to temperature is rainfall. Our warm location is temperate rainforest so rainfall can vary dramatically. One study by Busch et al. (2011) found that as rainfall decreased across a geographical gradient, nestling hematocrit also decreased and temperature was similar as it was across tropical rainforest. Although this was at the mercy of other biotic and abiotic variables, it gives evidence that rainfall can determine nestling hematocrit and is independent of temperature in tropical ecosystems. Whilst other studies also highlight rainfall as important in determining nestling hematocrit (Bańbura *et al.,* 2011; Bowers *et al.,* 2014 & Kalinski *et al.,* 2015). These studies demonstrate that rainfall can independently affect nestling fitness and in tropical systems, works independently of temperature. This may help explain the variation in hematocrit between seasons which did not consider rainfall.

Some birds (how many) were displaced during nestling or incubation period by Chilean Swallows *Tachycineta meyeni* (Botero-Delgadillo *et al.,* 2015) or House Wrens *Troglodytes aedon (**ref**). Added stress? Some were injured.*

<u>Mainly about the adults, not nestlings.</u>

Age of bird was not taken into account as a recent long term study on Rayadito's has found that chronological age, nor terminal reproductive output explains variations in reproductive success (Quirici *et al.,* 2019). Hematocrit has a complex relationship in predicting reproductive success, thus caution should be made when interpreting these correlations (Fowler *et al.,* 2018). Intermediate hematocrit levels in House Wrens had the highest recruitment rates and longest time as an active breeder (Bowers *et al.,* 2014).

A study on hematocrit in European Starlings found that adult females during egg laying have reduced hematocrit pre and post laying and lower compared to males (Williams *et al.,* 2004). Can be interpreted that we are unsure if hematocrit in Rayadito is inherited from

male or female parents or if this influences the fledgling hematocrit in Rayadito's.