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## Research article

### The delayed effect of climatic conditions on pre-fledging nestling haematocrit in a suboscine species in Patagonia

Joseph E. Churchill<sup>1</sup>✉, Martje L. M. Birker<sup>1,2</sup>, Maaïke Versteegh<sup>1</sup>, Tomás A. Altamirano<sup>3,4</sup>, Rodrigo A. Vasquez<sup>2,4</sup> and Jan Komdeur<sup>1</sup>

<sup>1</sup>Behavioural and Physiological Ecology, Groningen Institute for Evolutionary Life Sciences (GELIFES), Faculty of Science and Engineering, University of Groningen, Groningen, the Netherlands

<sup>2</sup>Instituto de Ecología y Biodiversidad (IEB), Facultad de Ciencias, Universidad de Chile, Santiago, Chile

<sup>3</sup>Department of Forest and Conservation Science, University of British Columbia, Vancouver, BC, Canada

<sup>4</sup>CHIC Cape Horn International Center, Parque Etnobotánico Omora, Universidad de Magallanes, Puerto Williams, Chile

Correspondence: Joseph E. Churchill ([j.churchill.eco@gmail.com](mailto:j.churchill.eco@gmail.com))

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Haematocrit, the proportion of blood comprising erythrocytes, is often used as a proxy of individual condition. Nestling haematocrit is influenced by several factors but ambient temperature is generally agreed as a key driver. It is unclear which day(s) in embryonic or nestling development are most influential in determining pre-fledgling haematocrit. This is important, because if we are able to identify what day(s) nestlings are physiologically most vulnerable to climatic conditions, this may inform future conservation management and help mitigate the effects. We investigated the effect of ambient temperature, precipitation, body size, brood size, age, food abundance and habitat on nestling haematocrit in *Aphrastura spinicauda* (thorn-tailed rayaditos). We collected this data from two climatically different locations in Chile: northern Patagonia (Pucón) and Sub-Antarctic Patagonia (Navarino Island), located ~ 1800 km further south. We aimed to identify the key drivers of nestling haematocrit and find when nestlings are physiologically most vulnerable to climatic conditions. We confirm that ambient temperature is the key driver of nestling haematocrit, with increasing ambient temperatures nestling haematocrit decreased. However, precipitation also affected haematocrit in the late incubation stage. At low and high precipitation, nestling haematocrit was low and is optimum in light rainfall. Our results show a delayed effect of ambient temperature and precipitation in late incubation and on the day of hatching which can determine pre-fledgling haematocrit, which may cause lower post-fledgling fitness. We found that climatic conditions on the day haematocrit was collected was not important in determining haematocrit. We found that haematocrit was higher in the sub-Antarctic area and that higher temperatures and precipitation on the day of hatching will result in lower nestling haematocrit and poorer pre-fledgling condition. As atmospheric temperatures rise, nestling fitness is at risk. This is a threat to many bird species, especially those in locations vulnerable to climate breakdown.

Keywords: climate breakdown, environmental change, haematocrit, nestlings, precipitation, temperature



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## Introduction

Haematocrit is the percentage of red blood cells found in the bloodstream in all vertebrates and has several applications in biological studies. Studies have shown that avian haematocrit is useful for assessing nestling fitness and condition (Swanson 1990, Muchacka et al. 2012, Kilgas 2014, Farag and Alagawany 2018). Nestlings with low haematocrit suffer from anaemia, which complicates oxygen transport and metabolic rates (Booth and Elliot 2002, Williams et al. 2004). On the other hand, nestlings with high haematocrit levels suffer from hypoxia and dehydration which causes similar metabolic complications (Burton et al. 1969, Jaeger and McGrath 1974). The optimum haematocrit in passerines is around 50% red blood cells, depending on the species (Minias 2020). Early work by Winter (1935) found that haemoglobin decreased in adult *Gallus gallus domesticus* (broiler chickens) with increasing ambient temperatures. Further experiments found that very high ambient temperatures (above 35°C) caused dehydration and other physiological complications (Deaton et al. 1969, Kubena et al. 1972, Vo et al. 1978, Yahav et al. 1997). These experiments all monitored individual quality through indicators such as protein plasma, heart weight, body weight, feeding efficiency, water consumption, red blood cell count and mortality. They repeatedly found a quadratic relationship between temperature and haematocrit thereby showing that optimum haematocrit levels require intermediate ambient temperatures, within a range of 10–20°. Studies have shown that haematocrit is phenotypically plastic, meaning it is influenced by environmental conditions over genetic ones (Hunter and Powers 1980, Potti et al. 1999, Simon et al. 2005, Potti 2007, Markowski et al. 2015). Field studies found the same correlations, when ambient temperature increases, nestling haematocrit decreases across a variety of bird species such as *Parus major* (great tits), *Cyanistes caeruleus* (blue tits), *Sturnus vulgaris* (European starlings), *Accipiter gentilis* (goshawks), *Accipiter cooperii* (Cooper hawks), *Buteo jamaicensis* (red-tailed hawks) and *Falco sparverius* (American kestrels) (Hunter and Powers 1980, Dawson and Bortolotti 1997, Norte et al. 2008, Serra et al. 2012, Markowski et al. 2015). However, none of these studies provided support for the exact timing of influence of ambient temperatures over haematocrit levels.

It is important to identify key drivers of nestling haematocrit and what day(s) during development individuals are most physiologically vulnerable. This is important, because climate breakdown is altering environmental conditions such as precipitation and ambient temperatures which is causing seasonal changes in weather conditions. These seasonal changes impact nestling physiology and therefore body condition, and by extension post-fledging fitness. If we are able to identify what day(s) during nestling development environmental factors are most important, this will help to inform future conservation management plans. Key environmental factors which have been identified as key drivers previously are ambient temperature and precipitation. This varies in importance in different studies. For example, one study by

Arct et al. (2022) found that nestling weight and the environmental temperature just after hatching could predict their pre-fledging weight. Another study by Capilla-Lasheras et al. (2021) found that high rainfall in the first ten days after hatching lowers the number of successful fledglings. Another study conducted in tropical rainforests on *Cyphorhinus phaeocephalus* (song wrens) found that as precipitation decreased across a geographical gradient, nestling haematocrit decreased (Busch et al. 2011). Interestingly, temperature and precipitation were not collinear in this study as tropical rainforests can maintain consistent temperatures but vary in precipitation. Precipitation was more influential on nestling haematocrit over ambient temperature, because in heavy precipitation, adults could not forage as much and therefore provided less food for nestlings which resulted in lower body weight and haematocrit (Capilla-Lasheras et al. 2021, Arct et al. 2022). The aforementioned studies show that the timing of climatic conditions are crucial in producing the best offspring and ensuring the best chances for post-fledging survival. However, to our knowledge, there is no study investigating the specific period that weather patterns in embryonic or nestling development can affect late stage nestling quality (indicated by haematocrit). This is why investigating how and when nestling conditions are impacted by climatic conditions at different life stages is crucial to understanding physiological responses to climate breakdown. This may help to predict evolutionary responses to climate breakdown if we have a better understanding of physiological responses.

Traits such as body mass, tarsus and culmen length are commonly measured alongside haematocrit as combined proxies of nestling condition (Fair et al. 2007, Lill et al. 2013). Many studies have correlated nestling body mass and haematocrit to suggest that larger bodied individuals naturally have higher haematocrit levels (Dawson and Bortolotti 1997, Christe et al. 2002, Masello and Quillfeldt 2004). Whilst this may be true, overarching factors like parasite loads or food abundance can cause collinearity (Christe et al. 2002, Granthon and Williams 2017). Sometimes indices of nestling condition just correlate with other indices. For example, Kilgas (2014) found that higher haematocrit in adult *Spilopelia senegalensis* (laughing doves) correlated with a higher basal metabolic rate (BMR). Thus, it is useful to measure numerous predictors when collecting haematocrit to assess collinearity and detangle predictors that are linked to other predictors, which are not necessarily caused by the same factor(s).

Insect abundance or food availability are typically measured to assess the impacts on co-indices of body condition, individual quality and survival in nestlings such as body weight, haemoglobin and haematocrit (Visser et al. 2006, Kaliński et al. 2015, Arct et al. 2022). We therefore expect that food abundance will be important in predicting pre-fledging nestling haematocrit. Studies by Kaliński et al. (2015) and Brown et al. (2021) on *Ficedula hypoleuca* (pie flycatchers) and *Acrocephalus sechellensis* (Seychelles warblers), respectively, found correlations between insect availability, nestling body mass and haematocrit, all decreasing. Repeated correlation between body mass and haematocrit makes them

difficult to separate as it is unclear which one causes the other to change, if either. The decline in insect availability occurs as the year progresses, which subsequently correlates with a changing climate, further complicating interactions because, statistically, it makes sense to remove correlating predictors as these give multicollinearity and make results difficult to explain. In any case, there are clear negative effects of lowered food availability on nestling haematocrit, condition and fitness (Cucco et al. 2002, Sabat et al. 2004). Many studies commonly use food availability as a proxy of habitat quality (Kloskowski et al. 2017). Although other proxies are used too such as, tree diameter at breast height (DBH) (Beyer et al. 1996, Amininasab et al. 2016), forest type (Kilgas et al. 2006), tree density (Sánchez et al. 2007) or a combination of these factors (Chalfoun and Martin 2007, Sumasgutner et al. 2019), they are important as they are commonly collinear with food abundance. For example, higher tree density results in more insects which result in better feeding opportunities and therefore better body condition in birds (Kloskowski et al. 2017, Sumasgutner et al. 2019), usually measured through weight and haematocrit.

Brood size is known to sometimes affect nestling haematocrit (Gladalski et al. 2015, Wascher et al. 2017). One study by Morrison et al. (2009) did a cross-fostering experiment on *Tachycineta bicolor* (tree swallow) nestlings in Amherst, USA, where the average ambient temperature is as high as 25°C. They experimentally manipulated brood size and found that larger broods caused sub-optimum nestling haematocrit and smaller broods to have optimum haematocrit, i.e., it was a temperature based effect. However, in colder climatic conditions the opposite effect is observed. For example, Krause et al. (2016) found that larger brood sizes in *Zonotrichia leucophrys* (Alaskan white-crowned sparrow) resulted in optimum nestling haematocrit and smaller brood sizes had sub-optimum haematocrit. The effect of brood size on nestling haematocrit is therefore mainly climate dependent. In colder climatic conditions, larger broods increase the nest temperature to compensate for the cold ambient temperatures. This benefits the nestlings by reducing the cost of thermoregulation. Whilst the opposite is observed in warm climatic conditions where more nestlings cause high nest temperatures and result in nestling dehydration and very high haematocrit levels, which damages their chances of survival. This mechanism is part of what makes this research so important in discovering how and when climatic conditions impact nestlings. As we have two different climates in our study, this effect may be relevant in a less extreme version.

*Aphrastura spinicauda* (hereafter just rayadito) is a small insectivorous passerine bird found across Argentina and Chile in South America (Fig. 1; Moreno et al. 2005, 2007). The species varies from temperate rainforest to sub-Antarctic forests with breeding seasons ranging from October to December (Remsen 2003). Rayaditos are secondary cavity nesting species so take readily to nest boxes. Breeding pairs produce clutch sizes varying from 2 to 7 eggs and produce 1–3 clutches per season (Moreno et al. 2005, Altamirano et al. 2015). The unique range of this species provides an opportunity to



Figure 1. Locations of our two study sites; the yellow star in the north indicates the temperate rainforest (Pucón) and the yellow star in the south indicates the sub-Antarctic forest (Navarino Island). The green coverage shows the distribution of the thorn-tailed rayadito.

investigate the potential effects of varying climatic conditions on nestling haematocrit. In this study we used two climatically contrasting locations, Pucón (39.272°S, 71.977°W) and Navarino Island (54.932°S, 67.605°W), aiming to: 1) identify differences in nestling haematocrit between locations, 2) find the key driver(s) of nestling haematocrit and 3) identify the specific day(s) in which our key driver(s) affected nestlings most that determined their pre-fledging haematocrit levels. We expect haematocrit to be higher in Pucón than Navarino, due to higher ambient temperatures in the former. Based on other field studies (Mainwaring and Hartley 2016, Arct et al. 2022), we expect ambient temperature to be the key driver of nestling haematocrit in rayadito nestlings. Furthermore, we expect that haematocrit levels will be determined either on the day haematocrit is collected (16 days after the first hatching [or D16]) or around hatching when nestlings are physiologically more sensitive. Climatic conditions are defined as the prevailing atmospheric conditions which occur in an area over a long period of time. As we are looking at two locations in which the average conditions are different over a long period of time, we will use the term climatic conditions and ambient temperature going forward.

## Material and methods

### Study sites and species

Data were collected from two climatically contrasting sites in Chile: one on Navarino Island (54.932°S, 67.605°W) which was ~ 5 km in radius, and other it is in Pucón (39.272°S, 71.977°W) which was ~ 10 km in radius and ~ 1800 km north of the other population. Data were collected in two years from September 2018–February 2019 and from September 2019–February 2020, during the rayadito breeding season. Data will be referred to from here as the 2018–2019 and 2019–2020 years, respectively. Navarino Island is a sub-Antarctic deciduous forest, with low ambient temperatures averaging 7.7°C across the breeding season (INIA 2020) and snow every month of the year. Nest boxes in Navarino were surrounded by six tree species, which are predominantly deciduous species such as *Nothofagus antarctica*, *N. betuloides* and *N. pumilio*. The understory was predominantly *Berberis mycrophylla* (evergreen shrubs) and are similar at all sites at this location. On the other hand, Pucón is a temperate rainforest on the outskirts of northern Patagonia with high precipitation and higher ambient temperatures averaging 13.7°C over the breeding season (Dirección meteorológica de Chile 2020). Of the six sites in Pucón, four were dominated by broadleaf species such as *Lophozonia obliqua*, *Nothofagus dombeyi* and *Laurelia sempervirens*, whilst the other two sites were conifer-broadleaf mixed forests dominated by *Saxegothaea conspicua*, *Laureliopsis philippiana*, and *N. dombeyi*. The understory composition at all six Pucón sites were dominated by *Chusquea* spp. (bamboo) *Rhaphithamnus spinosus* (evergreen shrub) *Azara* spp. (flowering plants) and tree saplings.

### Nestling condition

There were 200 nest boxes in Navarino and 240 nest boxes in Pucón, which were at least 10 m apart each. These were checked every day or every other day of the field season to monitor from nest building through to fledging (Moreno et al. 2005, Altamirano et al. 2015). After hatching, the brood size was recorded until fledging, noting when/if nestlings died prematurely. Day 0 was standardised as the day of hatching. On day 0 the culmen (mm) was measured to 0.1 mm accuracy and mass (g) to 0.1 g accuracy with digital callipers and scales. The toenails were clipped in different orders to keep track of individual growth until they could be ringed. The same measurements were taken at 4, 8, 12 and 16 days old. On day 8, nestlings were ringed, and we began tarsus length (mm) measurements using digital callipers to 0.1 mm accuracy. Nestling haematocrit was measured 16 days after the first hatching (D16). Nestlings were not always 16 days old on 'day 16' because egg hatching is asynchronous (Altamirano et al. 2015). We tested for collinearity between explanatory variables using correlation matrices through the 'rcorr' and 'chart.Correlation' functions. Tarsus, culmen length and body mass were all collinear ( $n = 146$ ,  $R^2 = 0.66$ ,

0.50,  $p < 0.001$ ), so we only used the tarsus length on day 16 during analyses. Body mass can vary diurnally due to feeding patterns (Reyes-Arriagada et al. 2015) and culmen length had very little variation.

We measured nestling haematocrit in two years in 2018–2019 and 2019–2020. We sampled 265 nestlings in total ( $n = 174$  in Navarino,  $n = 91$  in Pucón) from 86 nests ( $n = 56$  in Navarino,  $n = 30$  in Pucón). In 2018–2019 the first haematocrit measurement was taken in Navarino on 7 November 2018 and the last on 10 January 2019. In 2018–2019 in Pucón, the first haematocrit measurement was taken on 13 November 2018 and the last on 1 February 2019. In 2019–2020 the first haematocrit measurement was taken in Navarino on 12 November 2019 and the last on 16 January 2020. In 2019–2020 in Pucón the first haematocrit measurement was taken on 20 November 2019 and the last on 16 January 2020. Blood samples were processed following standard procedure (Owen 2011), we used a 21 gauge 25 mm disposable needle to prick the renal vein and used a 75  $\mu$ l capillary to collect blood. Samples were then transported in a cool box to slow coagulation to a field station to be centrifuged. We recorded the transport time between collection and centrifugation (average = 138 min, SD = 90 min,  $n = 265$ ). Blood samples were centrifuged at 8000 spins per minute for three minutes. When the plasma and red blood cells fully separated, the total amount of liquid was measured (mm) in the capillary using digital callipers (to 2 decimal places). Haematocrit was then calculated (red blood cells/total blood sample  $\times 100 = \%$  haematocrit). We sampled each nestling once and did not take repeat measurements. Other studies have shown high repeatability of haematocrit measurements, so we consider one sample per individual a reliable value (Hatch and Smith 2010, Brown et al. 2021).

Insect counts took place once every six days in both years. In 2018–2019 in Pucón, insect counts began 23 September 2018 and finished on 22 January 2019, and in 2019–2020 began 25 September 2019 and finished on 3 January 2020. Insect counts on Navarino in 2018–2019 began 25 September 2018 and finished on 10 January 2019, and in 2019–2020 began 14 October 2019 and finished on 9 January 2020. The start/end dates were deliberate to coincide with the first incidence of egg laying and the last fledgling leaving the nest. In both years, we used four fixed trees in each location (eight trees in total) to sample insect abundance every six days. This was conducted by using a three-sided sheet (80  $\times$  80  $\times$  80 cm) attached to three poles, which was held up underneath a branch and the branch was struck 10 times with a stick to dislodge anything 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 trees in different areas (Leather 2008). In 2019–2020 we used the same trees as the previous year. These were both averaged for each area. We then assigned the insect abundance to nest boxes based on which fixed tree was the closest. Each sample tree was selected based on centrality to clusters of nest boxes, species and size, which were all estimated to be similar.

Ambient temperature (°C) and precipitation (accumulated ml per day) data were taken from meteorological stations in both sites. In Pucón the station was located a maximum of 7 km from the nest boxes (Dirección Meteorológica de Chile 2020) and in Navarino the station was a maximum of 5 km away from the nest boxes (INIA 2020). The data included the daily average temperature and total daily precipitation from the first recorded egg laying to the last day haematocrit was taken in both years, which was at least 30 days before the first haematocrit measurement of each year. We took the ambient temperature which was recorded every hour for 12 h (6:00–18:00 h) and we then calculated a daytime average (excluding night temperatures) and used the accumulated precipitation for the full 24 h.

Habitat data were collected in both locations in 2019–2020 only (due to logistical limitations) which included the total number of trees within 11.2 m radius around a nest box (Jones et al. 2014, below). We only sampled nest boxes for habitat data which were more than 10 m apart to avoid overlap, as some nest boxes were exactly 10 m apart. The radius of 11.2 m was used based on previous studies in cavity nesting birds by Martin and Eadie (1999) who developed a consistent way to measure the microhabitat of cavity nesting birds. This was then adapted to fit the Rayaditos specific habitat (Altamirano 2014, Altamirano et al. 2015). These measurements do not cover the entire territory but do not overlap with other nesting pairs, as their territory is much larger (Cornelius 2008). Habitat quality was a relatively stable variable throughout years as it relies on tree density. This has been measured in Pucón previously (Altamirano 2014, Altamirano et al. 2015) so a subsample was measured during our study to test repeatability. This had never been conducted on Navarino before, so our study is the first attempt to measure habitat quality. We corrected insect abundance for tree density at nest boxes to make a habitat quality factor which was calculated by (insect abundance  $\times$  total number of trees/1000).

## Statistical analyses

All statistics were carried out using R in RStudio ver. 3.5.3 ([www.r-project.org](http://www.r-project.org)) statistical software. Graphs used the dataset which included all haematocrit measurements from both locations for both years combined with accompanying climatic data taken from meteorological stations. We corrected insect abundance for vegetation density at nest boxes. This was done by calculating the average insect abundance over the rearing period (hatching to fledging) and then combined with vegetation density (total number of trees  $\times$  insect abundance/1000).

We wanted to know the specific days in which ambient temperature and precipitation can determine pre-fledgling haematocrit. We did this by using the 'climwin' package. This package tests the effect of different climate windows on haematocrit, and evaluates them based on the Akaike Information Criterion corrected for sample size (AICc) (Bailey and Van De Pol 2016). We used haematocrit as our

response variable, with daily average temperature and daily accumulated precipitation as our explanatory variables with location and year as our baseline variables (Bailey and Van De Pol 2016).

The 'slidingwin' function in the climwin package was used on nestling haematocrit (%), the ambient temperature data (°C) and total accumulated precipitation per day (ml) in two separate models to identify the time-window where the climatic factors explained nestling haematocrit best. This works by using the average daily temperature and accumulated precipitation every day from the day haematocrit was collected (day 0) and 30 days before, which covers part of the incubation period, egg laying, hatching and brooding periods up to the day haematocrit was collected. This then observes correlations each day (0–30 days) with every haematocrit measurement we took and gives the day on which our climatic factors correlated strongest with nestling haematocrit. In this analysis, groups were separated by location. We selected both 'linear' and 'quadratic' distribution functions to examine the relationship between climatic factors and nestling haematocrit. If a quadratic function was found to be the best, we include our explanatory variable squared to account for this. To assess the validity and significance of the best window, we ran 1000 random models with the same data to determine that our best window is significantly different from random, this was done by using the 'RandWin' function in the climwin package. The outcomes can then be plotted as visual aids which show the significance of the correlations identified by the models (Fig. 4).

We want to know if there are differences in nestling haematocrit between two climatically different locations and to find the key driver of nestling haematocrit in this species. To do this we used the 'glmmTMB' package, with a beta error structure with a log link function. We built a glmmTMB with haematocrit as the response variable, and location, ambient temperature, precipitation, precipitation<sup>2</sup>, tarsus length at 16 days old, nestling age, brood size, year and location as explanatory variables with Nest ID as a random factor (a combination of nest box number and location). We then conducted backwards elimination of our explanatory variables, recording the outputs just before elimination. Habitat quality (insect abundance and tree count) was analysed separately because of collinearity with ambient temperature and precipitation. We found using 'rcorr', 'chart.Correlation' and linear models that collinearity occurred between habitat quality and ambient temperature ( $n=259$ , estimate =  $-0.16$ ,  $p < 0.01$ ) and precipitation ( $n=259$ , estimate =  $-0.05$ ,  $p < 0.01$ ). Precipitation and ambient temperature were not collinear ( $n=259$ , estimate =  $0.03$ ,  $p > 0.05$ ) neither were precipitation<sup>2</sup> and ambient temperature ( $n=259$ , estimate =  $<0.01$ ,  $p > 0.05$ ). We then ran our model of best fit with habitat quality separately from precipitation and ambient temperature. Ambient temperature and precipitation were extracted from the windows identified by climwin modelling (below). Model residuals were checked for fit and outliers using the 'simulateResiduals' and 'testResiduals' functions. We found outliers in our analysis but kept these in our final analyses,

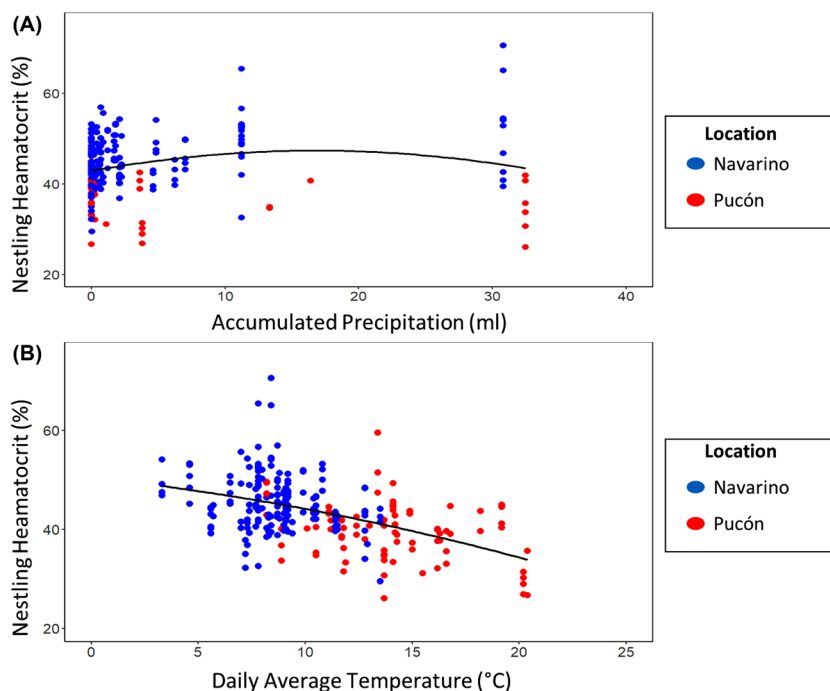


Figure 2. This shows the effect of location, ambient temperature and daily accumulated precipitation on nestling haematocrit. Nestlings from Navarino (blue dots) have higher haematocrit levels compared with those from Pucón (red dots). The data show the climate windows identified by the climwin model of best fit. The statistics for these figures are shown in Table 1. (A) Our climwin analysis indicates the precipitation on the day of hatching of each nestling best predicts haematocrit levels taken at 16 days old. Precipitation has a quadratic effect on haematocrit, suggesting an ‘optimum’ precipitation level in the field (black line). (B) Our climwin analysis shows the temperature at two days old best predicted nestling haematocrit taken when nestlings were 16 days old. Higher temperatures give lower haematocrit levels in the field. This shows a clear linear relationship (black line).

because we analysed our data both with and without outliers and the results did not change.

## Results

### The drivers of nestling haematocrit

The climwin model with daily average ambient temperature (Fig. 4), showed a linear function was the model of better fit over a quadratic one (Fig. 2b). We found that ambient temperature 14 days before haematocrit was collected (known from here as two days old) explained pre-fledgling nestling haematocrit best. As temperature increases, nestling haematocrit subsequently decreases (Table 1, Fig. 2b). The ambient temperature on the day haematocrit was collected did not fall within the range and did not drive nestling haematocrit. Our analysis found that 32% of simulated models fall within our 95% confidence interval which creates doubt as to which model is true as 32% of our randomly generated models could also be the true model (Fig. 4). Overall our analysis presents a range of 7–20 days before haematocrit is collected. This means that we can be 95% sure that the ambient temperature 7–20 days before haematocrit is collected is the best predictor of pre-fledgling haematocrit. This is interesting as our best model shows 14 days before haematocrit is measured

is the most important, suggesting that pre-fledgling haematocrit can be determined by ambient temperature in the late incubation stage, at hatching and/or early nestling development in the field. It is clear from our results that weather conditions have a delayed effect on pre-fledgling nestling haematocrit.

We then carried out the same analysis on accumulated precipitation using climwin. This model found a strong quadratic correlation between precipitation and haematocrit (Fig. 2a). With lower haematocrit at low precipitation, optimum haematocrit levels at average precipitation levels, and lower haematocrit levels at higher precipitation (Fig. 2a). Accumulated precipitation per day seems similar between locations (Table 2) but Navarino is consistently dry, whilst Pucón has days of extreme precipitation followed by dry days, hence the similar values. This model was checked for outliers which were included in future analyses (as significance of the explanatory variables did not qualitatively change) and the data is skewed to the left (Fig. 2a) and the data is skewed to the left (Fig. 2a). Overall our analysis showed that 18–24 days before haematocrit was collected was the window in which precipitation was important in determining pre-fledgling haematocrit with a 95% confidence interval (Fig. 4). Climwin also recognises 16 days before haematocrit was measured as having the strongest correlation with precipitation, which is the day of hatching. The reason our model of best fit falls outside the

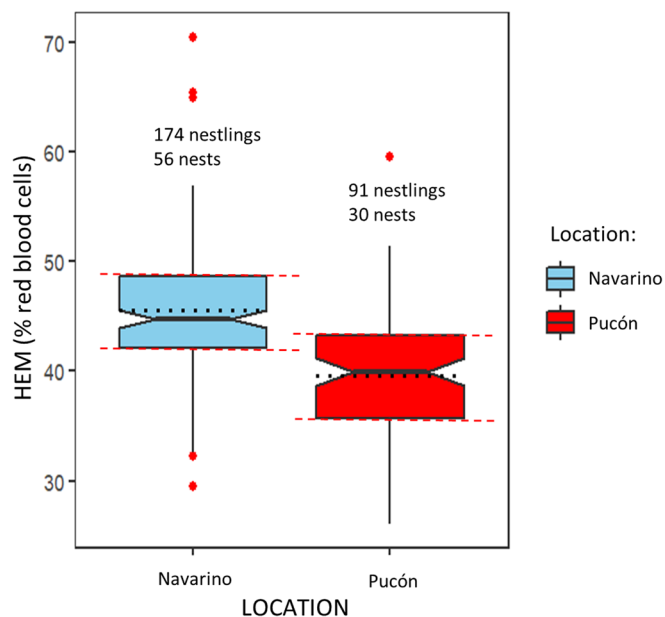


Figure 3. Haematocrit in rayadito nestlings collected from Navarino Island and Pucón. Haematocrit was significantly higher in Navarino than in Pucón overall as expected (Table 1). The black dotted lines show the means of each group and the notches show the median. The red dashed lines show 0.25 and 0.75 quantile ranges. The red dots are potential outliers which were included throughout our analysis.

best window, is that these are both probabilistically important, one at 16 days before and one at 18–24 days before haematocrit is collected. This gives two possibilities of the specific time in which precipitation predicts nestling haematocrit just before fledging, but both are set at late incubation

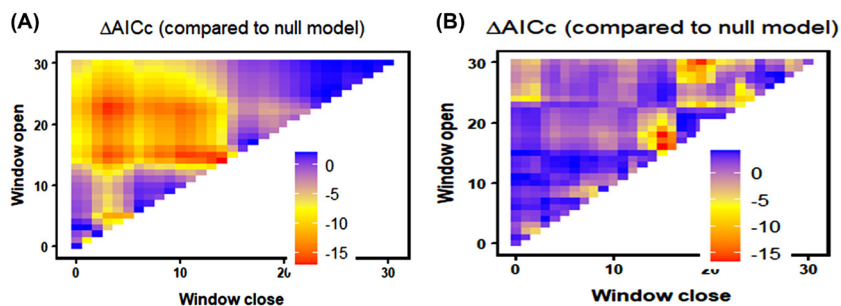


Figure 4. These heat maps are the key outputs from the climwin analysis. We ran 1000 random models alongside our models of best fit to see how likely it is that our model is random. The axis (x) and (y) show the climate window ranges, which ranges from 0–30 days. Zero days is the day haematocrit was collected and 30 days before haematocrit was collected, which is the incubation stage. Each pixel represents a random model and the darker red spots show a strong relationship with nestling haematocrit and ambient temperature or precipitation. Our models are the darkest red pixels. (A) The output from the daily average temperature climwin results. Our best model is placed at the darkest red spot at 14 days open and 14 days close on the axis. Although other points of interest show in our range of 7–20 days before haematocrit is measured, as can be seen from the darkest red spots on the map. Our outputs found that 32% of these models fall within the 95% confidence interval, so we cannot be certain that our model is not random. But this does show that the temperature around hatching or in late incubation is important for pre-fledgling haematocrit. (B) This heat map is the output from the climwin analysis for accumulated precipitation. Our model of best fit showed the climate window to be 15–16 days before haematocrit was measured, as seen on the darkest red spot of our map. We found that only 5% of these models fall within the 95% confidence interval, so we can be fairly certain that our model is the true model. This shows that the precipitation on the day of hatching is the most important for pre-fledgling haematocrit, similar to temperature.

and at hatching, which makes this interesting in either case. Although precipitation on the day of hatching falls within a 95% confidence interval, some dates before hatching in the incubation period do as well. In this case, 5% of models fall within the 95% confidence interval which means we can be fairly certain that the model showing precipitation on the day of hatching as the most important is very likely to be the true model, but we believe two things can be true at the same time. It is likely that precipitation is important both in the late incubation and on the day of hatching, finding the most important may not be relevant if nestling condition is degraded by both or either anyway.

Our best model included ambient temperature, location and year as the most significant factors (Table 1). The best model excluded precipitation, precipitation<sup>2</sup>, tarsus length, age, and brood size. When tested independently, precipitation in the late incubation period can drive pre-fledgling nestling haematocrit, however, when included with other explanatory variables, this effect is not significant. Part of the variation in haematocrit is explained by both precipitation and ambient temperature. Haematocrit was higher in Navarino than in Pucón (Fig. 3) and was lower as the year progressed. When we analysed habitat quality separately to ambient temperature and precipitation by replacing the latter with habitat quality in our best model, habitat quality was not important in predicting nestling haematocrit (estimate = 0.043, standard error = 0.025,  $p > 0.05$ ).

## Discussion

We found overall that nestling haematocrit was higher in the sub-Antarctic location than in Pucón (northern

Table 1. The results of our 'GlmTMB' modelling. This shows the effects of all of our explanatory variables on nestling haematocrit including; estimates, SE and p-values. This includes results of backward elimination on explanatory variables, with statistics for non-significant variables at the moment they were removed from the model.

Key drivers of nestling haematocrit			
Explanatory variables	Estimate	SE	p-values
Ambient temperature (°C)	-0.023	0.007	<0.01
Location (Pucon)	-0.101	0.058	0.05
Year	0.167	0.033	<0.01
Accumulated precipitation (per day ml)	0.003	0.002	0.14
Accumulated precipitation (per day ml) <sup>2</sup>	<0.001	<0.001	0.43
Tarsus length (cm)	-0.009	0.017	0.58
Age	-0.022	0.031	0.47
Brood size (n)	-0.010	0.017	0.55

Patagonia). The observed differences were temperature and location dependent. We found that as ambient temperature increases and the season progresses, nestling haematocrit decreases. Our results show that climatic conditions have a delayed effect on nestling haematocrit and that nestlings are physiologically most vulnerable to ambient temperature and precipitation at the late incubation stage and on the day of hatching. We find evidence that the ambient temperature during late incubation and at hatching (7–20 days before haematocrit is measured) is most important in determining pre-fledgling nestling haematocrit. We found that precipitation at late incubation (18–24 days before haematocrit is measured) is important in determining pre-fledgling haematocrit. Our hypothesis is therefore that weather conditions (temperature and precipitation) throughout the breeding season can determine pre-fledgling nestling haematocrit, but the mechanism by which this works varies largely. Sometimes it is from lower food abundance later in the year or that temperature/rainfall patterns are different later in the year (Nord and Giroud 2020, Sauve et al. 2021). We found that predictors such as brood size, age and tarsus length do not drive haematocrit levels. In contrast, we found that climatic conditions in the late incubation stage and at hatching are essential and drive pre-fledgling haematocrit levels in late stage nestlings. The mechanism by which this works is likely that poor conditions at these critical developmental stages stunt the nestling's growth which biologically suppresses growth in later stages and reduces individual quality. This idea is certainly worthy of being experimentally tested further in the field. The potential

effects on post-fledging fitness remains unknown which is worthy of investigation. Other studies have found evidence of nest temperature in the nestling phase affecting post-fledging fitness, survival, longevity and nestling haematocrit in other Passerines (Greño et al. 2008, Facey et al. 2020 and Brown et al. 2021) but specific days are never given in these studies and this is the first study of its kind on this species, which is what makes our study so important in advancing this branch of research.

### Temperature and precipitation dependent effects on nestling haematocrit

We found that as ambient temperature increases, nestling haematocrit decreases. Lab controlled experiments consistently find a quadratic relationship between ambient temperature and nestling haematocrit in chickens (Vo et al. 1978, Yahav et al. 1997). However, the un-manipulated temperatures we observed were not high enough to show a true quadratic relationship in the natural population. Our model shows that the ambient temperature two days after hatching successfully predicts nestling haematocrit 16 days after hatching. We are cautious of this, as two days after hatching seems too specific to be true in reality. However, we can be 95% certain that the most important climate window is between 7 and 20 days before nestling haematocrit is collected, so we are sure that early nestling and late embryonic development is crucial. In a broader context, our results show that ambient temperature has a delayed effect on nestling haematocrit and that nestlings are physiologically most vulnerable at hatching and/or the late incubation stage. To our knowledge, no other published literature manipulates external temperatures at different points in nestling development and measures nestling haematocrit in the field to pinpoint crucial developmental days. Studies which observe temperatures at different stages in nestling development and record haematocrit simultaneously are rare. Only one study to our knowledge has looked at the impact of lowered haematocrit on post-fledging survival in long lived *A. sechellensis* (Seychelles warblers) (Brown et al. 2021). This study found that younger individuals' (<1.5 years) survival was linked to lowered haematocrit levels, but not in older individuals. Which gives reason to believe this may occur at our study sites too, so is worthy of being investigated in the future.

There are studies that consider the effect of ambient temperature in early nestling development on post-fledging fitness and survival, which use different proxies of nestling

Table 2. Shows the climatic information collected from meteorological stations at both locations in both seasons during the nesting season September–January.

Factor	Ambient temperature and precipitation of both locations				
	Season	Navarino	SD	Pucón	SD
Ambient temperature (season average)	2018–2019	8.04°C	3.18	13.71°C	3.67
	2019–2020	8.51°C	3.25	13.84°C	4.14
Accumulated precipitation (average per day)	2018–2019	1.31 ml	2.88	9.08 ml	17.15
	2019–2020	1.96 ml	2.58	5.79 ml	12.07

condition. For example, [Greño et al. \(2008\)](#) looked at post-fledging survival in an individuals' first year after fledging, using 12 years of *C. caeruleus* (blue tit) data, and they observed that nests with high temperatures and nestlings with lower body masses had lower survival rates in the first year of fledging. This is relatable to our study as when ambient temperatures increase, haematocrit decreases and therefore nestling condition too, which suggests haematocrit could be related to survival. However, we cannot be sure of this until haematocrit is tested as being directly linked to survival. Although, an important distinction here is that this study uses internal nest temperatures and we use ambient temperatures. Nest temperatures and ambient temperatures may not follow similar trends because parents may adjust incubation behaviour based on external temperatures. The main distinction between our study and the [Greño et al. \(2008\)](#) study was that we used haematocrit and they used individual survival. In any case, we predict that haematocrit will only ever be able to explain a part of post-fledging survival rates as other factors such as food abundance, population dynamics and genetics may also contribute to this. The accessibility and cost-effectiveness of obtaining haematocrit makes it a desirable explanatory variable. If we can discover to what extent nestling haematocrit is able to explain and predict short- and long-term survival, reproductive rates and recruitment, this may help in answering difficult questions which often require lengthy fieldwork and expensive equipment regarding species' survival and fitness.

Another similar example by [Facey et al. \(2020\)](#) conducted observations on *Hirundo rustica* (barn swallows) and found that ambient temperature, rainfall and wind speeds during nestling development, affects nestling body mass just before fledging. 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 condition-related traits like haematocrit. Some literature finds evidence that ambient temperature during nestling development does not predict haematocrit but nestling haematocrit is predicted by the time of day that haematocrit is measured ([Smith and Barber 2012](#)). This is true, but this specific study collected haematocrit between 09:00 and 18:00 h in Nova Scotia, Canada. It is likely that 18:00 h was far too late in the day to sample nestling haematocrit, as adults are more likely to incubate nestlings at these times, which is disrupted by collecting haematocrit. We collected our haematocrit samples at peak feeding times between 9:00 and 15:00 h, and hence we do not see this as a problem.

Precipitation was not the main driver of nestling haematocrit when compared with other factors but it did have a significant effect on nestling haematocrit in isolation. We found that precipitation during late incubation can predict nestling haematocrit just before fledging. The relationship identified showed that nestlings have low haematocrit at low precipitation levels and optimum haematocrit with light precipitation, becoming low again at high precipitation. This shows that there is an optimum level of precipitation for optimum

nestling haematocrit. This finding is supported by a study from [Mainwaring and Hartley \(2016\)](#) who found that no precipitation and high precipitation caused poorer nestling body condition (measured through weight, tarsus, bill and wing length) in *C. caeruleus* (blue tits). Light precipitation increased caterpillar numbers and movement which created more food for parents to forage, this in turn produced fitter nestlings because they had access to more food. This is likely to be the case in our field sites too where invertebrates are subject to the same weather limitations. Our findings on precipitation are also supported by [Capilla-Lasheras et al. \(2021\)](#) who found that high precipitation in the first 10 days of nestling development correlated with less nestlings successfully fledging. That study uses precipitation averages from the first 10 days of nestling development whilst our study is more specific, giving exact days when this is most important. We believe our study is the first to investigate the effects of precipitation on nestling development, which can pinpoint specific day(s) in which precipitation has the strongest impact on pre-fledging conditions. This is certainly the first of its kind on the thorn-tailed Rayadito, and our results show that too high or too low precipitation during late egg incubation can drive sub-optimum haematocrit levels in pre-fledging nestlings. It has been predicted that sub-Antarctic regions will become much warmer and drier over the next 60–70 years ([Pendlebury and Barnes-Keoghan 2007](#)) as will northern Patagonia ([Natalia et al. 2020](#)), which as we have shown, can cause sub-optimum haematocrit levels in nestlings. Unless climate breakdown comes to a halt, nestling haematocrit will likely become sub-optimum in both locations and as a result nestling fitness will suffer. This may also impact post-fledging survival rates and longevity in the future as has been observed in other passerines ([Facey et al. 2020](#), [Brown et al. 2021](#), [Capilla-Lasheras et al. 2021](#)).

Habitat quality had a complicated relationship with nestling haematocrit. Our index of habitat quality was a combination of insect abundance and tree density. In our study, habitat quality was collinear with ambient temperature and precipitation, so we resolved this by testing habitat quality separately from temperature and precipitation. Habitat quality did not predict nesting haematocrit. Previous literature frequently concludes that lower food availability and poorer quality habitats decreases nestling haematocrit ([Cucco et al. 2002](#), [Simon et al. 2005](#), [Kaliński et al. 2015](#), [Crisuolo et al. 2019](#)). We believe this may have happened at our site but climatic conditions simply have a greater impact on nestling haematocrit than habitat quality or insect abundance. [Busch et al. \(2011\)](#) did not analyse insect abundance and temperature together for the reason of collinearity. Instead, they analyse these separately and find that both had an effect on nestling haematocrit separately, but similarly to our study maintains that temperature was a better predictor as temperatures are likely to predict insect counts, not the reverse. None of the predictors we investigated work in the field in isolation and undoubtedly the temperature and precipitation of a region will influence habitat quality and insect abundances.

## Conclusions and recommendations for future research

Our study shows that nestling haematocrit and therefore nestling condition can be predicted by ambient temperature and precipitation in the late incubation stage and at hatching. Based on our findings, we propose an experiment on nestlings take place; manipulating the external temperature of nest boxes during the egg incubation period, nestling hatching day (day 0), day 2 (14 days before haematocrit collection), day 9 (7 days before haematocrit collection) and on the day of haematocrit collection in the field. Some investigations have already taken steps towards this in egg incubation but not in early stage nestlings and only on internal nest temperatures. Studies by [Ardia et al. \(2010\)](#) and [Mueller et al. \(2019\)](#) found that cooling eggs in field studies caused body mass to decrease in nestling development compared to control groups, which may provide some insight. We must also recognise confounding factors that may influence nestling haematocrit too, like parental investment ([Storey et al. 2017](#)) parent quality ([Dupont et al. 2018](#)) and wind speeds ([Facey et al. 2020](#)) which are all worthy of consideration in similar studies in the future.

Our study shows that climatic conditions in the incubation period and at hatching may determine nestling haematocrit and nestling condition, which warrants further investigation into monitoring post-fledging survival of individuals for whom we have collected haematocrit samples from as nestlings. What can be concluded based on our results is that as climatic conditions continue to change, nestling haematocrit will continue to become sub-optimum, which will reduce individual condition through complications in oxygen transport and metabolic rates which will have numerous knock-on effects for individual fitness and survival. The consequences of climate breakdown on this species' survival is at risk. These results show that in a warming climate, nestling condition will deteriorate (indicated by sub-optimum haematocrit) and will reduce post-fledging survival and population persistence, as found in other studies ([Facey et al. 2020](#), [Brown et al. 2021](#), [Arct et al. 2022](#)). The rapid progression of climate breakdown threatens bird species essential to their ecosystems around the globe, of which the consequences grow dire.

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## Author contributions

**Joseph E. Churchill:** Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (equal); Resources (equal); Software (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing – original draft (lead); Writing – review and editing (lead). **Martje L. M. Birker:** Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Project administration (equal); Resources (equal); Software (equal); Supervision (equal); Validation (equal); Visualization (equal); Writing – original draft (supporting); Writing – review and editing (supporting). **Maaïke Versteegh:** Conceptualization (supporting); Data curation (equal); Formal analysis (equal); Methodology (supporting); Software (supporting); Visualization (supporting); Writing – review and editing (supporting). **Tomás A. Altamirano:** Conceptualization (supporting); Investigation (supporting); Methodology (equal); Resources (supporting); Supervision (supporting); Writing – review and editing (supporting). **Rodrigo A. Vasquez:** Conceptualization (supporting); Formal analysis (supporting); Funding acquisition (lead); Methodology (supporting); Project administration (supporting); Resources (lead); Software (supporting); Supervision (lead); Validation (supporting); Visualization (supporting); Writing – review and editing (supporting). **Jan Komdeur:** Conceptualization (supporting); Funding acquisition (lead); Methodology (supporting); Project administration (supporting); Resources (supporting); Supervision (lead); Writing – review and editing (lead).

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## Data availability statement

Data are available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.j6q573nk7> ([Churchill et al. 2023](#)).

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