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RESEARCH ARTICLE

Hungry or angry? Experimental evidence for the effects of food availability on two measures of stress in developing wild raptor nestlings

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ABSTRACT

Food shortage challenges the development of nestlings; yet, to cope with this stressor, nestlings can induce stress responses to adjust metabolism or behaviour. Food shortage also enhances the antagonism between siblings, but it remains unclear whether the stress response induced by food shortage operates via the individual nutritional state or via the social environment experienced. In addition, the understanding of these processes is hindered by the fact that effects of food availability often co-vary with other environmental factors. We used a food supplementation experiment to test the effect of food availability on two complementary stress measures, feather corticosterone (CORTf) and heterophil/lymphocyte ratio (H/L) in developing red kite (*Milvus milvus*) nestlings, a species with competitive brood hierarchy. By statistically controlling for the effect of food supplementation on the nestlings' body condition, we disentangled the effects of food and ambient temperature on nestlings during development. Experimental food supplementation increased body condition, and both CORTf and H/L were reduced in nestlings of high body condition. Additionally, CORTf decreased with age in non-supplemented nestlings. H/L decreased with age in all nestlings and was lower in supplemented last-hatched nestlings compared with non-supplemented ones. Ambient temperature showed a negative effect on H/L. Our results indicate that food shortage increases the nestlings' stress levels through a reduced food intake affecting both their nutritional state and their social environment. Thus, food availability in conjunction with ambient temperature shapes between- and within-nest differences in stress load, which may have carry-over effects on behaviour and performance in further life-history stages.

KEY WORDS: Food supplementation, Development, Feather corticosterone, H/L ratio, Hatching rank

INTRODUCTION

External stimuli such as adverse environmental conditions can challenge an individual's homeostasis (Blas, 2015), and the associated physiological responses are at the core of plastic interactions of organisms with their environment. Birds respond to stressors through a hormonal cascade following the hypothalamus–pituitary–adrenal axis (HPA axis), resulting in the release of the glucocorticoid hormone corticosterone (CORT) (Siegel, 1980). These hormones then trigger behavioural or metabolic changes that help to overcome stressful situations and restore homeostasis by redirecting energy from non-essential to essential activities (Sapolsky et al., 2000; Romero, 2004). However, strong and sustained stress responses might come at high costs, both in adult (Boonstra et al., 2001; Lupien et al., 2009) and in developing (Kitaysky et al., 2003; Lupien et al., 2009) organisms. In the last two decades, much attention has been focused on whether and how such costs carry over from early development to later life-history stages (Boogert et al., 2014; Naguib and Gil, 2005; Eyck et al., 2019), but it remains unclear how the stress response changes across developmental stages, and how food availability, one of the most important environmental factors, affects the upregulation of the stress response.

Physiological processes such as stress responses are governed by trade-offs between costs and benefits (Romero et al., 2009; Pelster and Burggren, 2018). Such trade-offs are continuously changing in relation to the individual's physical and physiological status. In altricial birds ('Species whose chicks remain in the nest for much or all of their development': Starck and Ricklefs, 1998), the developmental hypothesis predicts that the stress response will gradually increase with age, allowing nestlings to respond to perturbations according to their age-specific metabolic and behavioural abilities (Sims and Holberton, 2000; Blas et al., 2006; Jenni-Eiermann et al., 2022). While adult birds may respond to nutritional stress with increased foraging, altricial nestlings cannot, and may develop a stronger stress response only as they reach independence from their parents (Sims and Holberton, 2000). However, the support for this pattern is equivocal. While some studies documented a reduced stress response during the early nestling phase in altricial species (Bebus et al., 2020; Blas et al., 2006; Müller et al., 2010), others reported a decrease (Dehnhard et al., 2011a,b; López-Jiménez et al., 2016) or non-linear relationship of the stress response during development (Walker et al., 2005).

Most studies investigating the ontogeny of stress responses in altricial birds used handling stress to induce the response (Walker et al., 2005; Jones et al., 2021). As the ability of nestlings to defend themselves or escape from predators develops only late in the nestling period, an increase of the response to handling with age is expected. However, other forms of stressful events such as nutritional or climatic stress may require a stress response throughout the nestling phase, and in some altricial species, behaviours that might enhance food intake (e.g. begging, aggressive behaviour towards siblings) or thermoregulation are

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also important during early development (Groothuis and Ros, 2005; Wright and Leonard, 2007; Lynn and Kern, 2014). Thus, the inconsistent patterns reported in studies investigating the ontogeny of the stress response in altricial birds may be due to the specific stressor investigated. In older nestlings, CORT regulates both begging behaviour (Quillfeldt et al., 2006; Kitaysky et al., 2001) and physiological responses to challenging situations such as prolonged exposure to cold ambient temperatures (Crino et al., 2020) and nutritional stress (Remage-Healey and Romero, 2001; Zaytsoff et al., 2019; Kitaysky et al., 2003). Nutritional stress is not expected to affect all siblings equally (Mock, 2004; Herring et al., 2011), particularly in species with dyadic forms of competition between nestlings. This is because younger siblings are often marginalised through physical competition or large size differences and, as such, receive less food than their older siblings (Rodríguez-Gironés et al., 2001). Nutritional stress can, therefore, also be modulated by the social environment experienced in the nest. Though it has been widely documented that frequent elicitation of the stress response in nestlings affects behaviour and performance later in life, it remains open how the interaction between food shortage and the social structure within the nest shapes the reactivity of the HPA axis of nestlings during their development.

In this study, we investigated the effects of food availability, a key environmental factor, on two stress measures in wild red kite, *Milvus milvus* (Linnaeus 1758), nestlings. To consider short-term and long-term indicators of stress, we measured the ratio between heterophils and lymphocytes in the peripheral blood vessels (H/L ratio, short-term: Gross and Siegel, 1983; McFarlane and Curtis, 1989; Moreno et al., 2002; Davis et al., 2008) and CORT in feathers (CORTf, long-term: Bortolotti et al., 2008; Jenni-Eiermann et al., 2015). CORTf has been ubiquitously used as an index of physiological stress (Bortolotti et al., 2008). CORTf is deposited during feather growth through the blood quill and, thus, provides a retrospective measure of an individual's cumulative CORT release during the feather growth period (Jenni-Eiermann et al., 2015). Further, CORT is known to alter the H/L ratio in the peripheral blood vessels, with increased CORT determining the recruitment of lymphocytes from the blood stream to the glands and, conversely, an increase of circulating heterophils (Gross and Siegel, 1983; McFarlane and Curtis, 1989; Moreno et al., 2002). Thus, increased CORT can determine an increase in the H/L ratio. This ratio is a well-established indicator of physiological stress in birds (Koutsos and Klasing, 2014; Lentfer et al., 2015) and is less susceptible to handling than circulating CORT (Davis, 2005; Gross and Siegel, 1983; Hõrak et al., 2002; Romero and Reed, 2005; Müller et al., 2011).

First, we aimed to disentangle the effect of food availability from the effects of a co-varying environmental factor, ambient temperature, on the two stress measures by using a food supplementation experiment. This is important because weather conditions can affect foraging behaviour and the foraging success of the parents (Garcia-Heras et al., 2017; Jenni-Eiermann et al., 2008) and, thereby, influence food provisioning (Öberg et al., 2015). Thus, correlative studies often fail to properly distinguish between the effects of food availability and other environmental factors (López-Jiménez et al., 2016; Nägeli et al., 2021). Moreover, recent research provided evidence that temperature is a driver of nestling development and survival in red kites, particularly in last-hatched nestlings (Nägeli et al., 2021). In addition, the effect of food shortage is often pronounced in last-hatched nestlings, suggesting a rank-dependent stress response (Skagen, 1988). We, therefore, expect that the effects of feeding treatment and temperature on the two stress measures vary with rank.

Second, we investigated whether increased food provisioning affects the stress measures only by improving the individual's nutritional state or also by affecting the social environment in the nest (White et al., 2010). We, therefore, included body condition as a proxy for nutritional state, and rank as a proxy for social environment in the analyses and recorded the two stress measures across the nestling period to investigate age dependence in stress levels. If food availability affects stress levels solely through the nutritional state, we would expect an effect of body condition, but not a separate, additional effect of food supplementation. If the impact of food availability on the social environment further affects the stress levels, we would expect an additional decrease of the stress measures in food-supplemented nestlings. As food demands increase (Barba et al., 2009; Bertram et al., 1996) and competition pressure decreases among siblings in the nest with nestling age (Scherler et al., in prep; Viñuela, 1999), an age-dependent increase or decrease in stress levels can be expected.

MATERIALS AND METHODS
Study species and study area
Our study area extends 387 km^2 across the Swiss cantons of Fribourg and Bern (N: 46°47′60″, E: 7°15′00″) and covers an elevation gradient ranging from 482 to 1763 m.a.s.l. (detailed descriptions of the study area can be found in Welti et al., 2020 and Nägeli et al., 2021). Red kites are tree-breeding, opportunistic scavengers, feeding mostly on small vertebrates such as rodents, but also on carrion and various anthropogenic food sources (Cramp and Simmons, 1980; Cereghetti et al., 2019; Andereggen, 2020). Between the end of March and the beginning of May, they lay 1–4 eggs, and the eggs are incubated for ca. 32 days. However, in this study, 3 eggs was the maximum clutch size observed. The nestlings stay in the nest for a period of 50–60 days, during which strong sibling aggression can often be observed in the first weeks (Scherler et al., in prep.).

Food supplementation
Between 2016 and 2018, we placed 78 feeding platforms (60×60 cm on poles 2 m high) at 20–200 m from known nests. Platforms were distributed along the altitudinal gradient of the study area using a stratified sampling design. The feeding experiment started with the provision of one-day-old dead chicken chicks on the feeding platforms every second day, and acceptance was monitored through observation and video recording. The amount of food was then regulated based on the information gathered (i.e. whether the target individuals accepted the food, whether and how many other individuals, belonging to which species, were also feeding on the platform). During incubation, 10 chicks were provided so that each targeted adult would have five chicks available every 2 days. After hatching, we added five chicks per nestling every 2 days, and after 10 days of age, we doubled the amount to 10 chicks per nestling. The degree of platform acceptance varied between years: 12 out of 29 platforms were accepted in 2016, 33 out of 37 in 2017, and 11 out of 12 in 2018. Across the three years, 13 broods failed before the nest could be climbed, leaving us with 82 nestlings from 43 broods that were food supplemented. An additional 423 nestlings from 244 non-supplemented broods from 2016 to 2019 were assigned to a control group.

Data collection and derived variables
To age the nestlings adequately, we monitored nests with different methods: observation via binoculars and scopes, webcams (Microsoft LifeCam Cinema HD), camera trap systems (Reconyx PC900

Hyperfire camera, RECONYX Inc., Holmen, WI, USA) and surveillance cameras (CCTV Sony Effio-E 700TVL). Nest trees were climbed twice to obtain morphometric measures (mean±s.d. age at first sampling 22.52±5.34 days; second sampling 39.74±6.14 days). To minimise disturbance during climbing events, blood was collected at the first sampling only for a subset of individuals (*n*=50). Similarly, feathers were primarily collected at the second sampling, unless a significant proportion of feather had already grown outside the feather shaft at the first climbing event (*n*=56). We collected two neck feathers from the birds in the nest (minimum age 22 days) and ~50 µl of blood from the brachial vein, using a sterile needle (0.5×16 mm) and heparinised micro-haematocrit capillaries. Body feathers in red kites are fully grown at the age of ca. 36 days (Aebischer and Scherler, 2021), meaning that we sampled both fully grown and developing feathers. The blood was smeared on microscopy slides (two per individual). Blood samples were taken on average within 30 min of beginning the climb (s.d. 15.25 min, range 12–68 min), a time frame within which no effect of disturbance on H/L ratio was detected (for details, see Supplementary Materials and Methods). Feathers were stored at −20°C until analysis. In a subsample of individuals, two extra feathers were sampled to assess the repeatability of CORTf measurements (*n*=30) and to investigate CORT deposition patterns in different segments of the feathers (*n*=11). This research was performed under the Swiss national animal experimentation licence 2017_29_FR.

We defined body condition as the residual of a linear mixed model (LMM; package lme4; Bates et al., 2015) with body mass as response and age as explanatory variable, while controlling for sex-specific differences in the growth curve by including sex in the model as covariate. Bird ID was included as a random effect to account for repeated measurements from the same individual (see Table S1 and Fig. S1). Details about age estimation can be found in Nägeli et al. (2021). As rank measure, we used the last-hatched categorisation (being last-hatched or not), because in our study population the hatching interval is consistently larger between the last-hatched and its elder sibling regardless of the brood size (P. Scherler et al., unpublished). Individuals were assigned to either the former or latter category at the first climbing event based on their wing length. Nestlings were ringed at a minimal wing length of 80 mm, allowing their identification at the following climbing events. Even though last-hatched individuals can catch up considerably with their older siblings under favourable circumstances, a significant size difference is usually maintained throughout the growth period.

CORTf extraction and assay

We used one feather per individual per climbing event for the CORTf measurements. The feathers were weighed, and the length was measured to the closest millimetre (mean±s.d. mass 19.59±5.88 mg; feather length 53.86±11.57 mm). After removing the calamus, the feather was placed in a 2 ml polypropylene microcentrifuge tube, cut into small pieces and ground into powder with a RETSCH mixer mill MM400 at 30 Hz for 6 min with 10, 3 mm grinding metal balls. Then, 1.5 ml of methanol was added to each tube, and tubes were placed in a sonicating bath for 30 min at room temperature and subsequently incubated overnight at 50°C in a water bath. The methanol with feather powder was pipetted into a new microcentrifuge tube, centrifuged at 14,000 rpm for 10 min and 1 ml of the supernatant was transferred into a soda-lime glass centrifuge tube. The methanol was evaporated with nitrogen gas at 50°C and the extracts stored in the freezer at −20°C until measurement. The extracted CORT was measured through enzyme immunoassay (EIA; for details, see Supplementary Materials and Methods). A pool of red kite feathers was used as an internal control (average intra- and inter-assay variation, respectively: 11.34±5.74%, 12.05±5.70%). A subsample of feathers (*n*=11) was cut in half and CORTf was measured separately in each segment to validate the age-specific CORT deposition.

H/L ratio

Blood smears were air-dried and stored in the dark at ambient temperature (<25°C). They were stained with a Diff-Quick set (Medion Diagnostics, Düdingen, Switzerland). Five types of leucocytes were counted in each sample by the same observer (B.C.) with a light microscope (Olympus BHA) at 1000× magnification: heterophils, lymphocytes, monocytes, eosinophils and basophils. The same observer counted 100 leucocytes per slide and calculated the H/L ratio. Both methods (CORTf and H/L) were tested for repeatability (see Supplementary Materials and Methods).

Weather data

For each brood, we extracted average daily temperature (°C) from the weather station of Fribourg-Posieux in the study area (MeteoSwiss, http://www.meteoswiss.admin.ch). For the analysis of CORTf measurements, we averaged the daily means of ambient temperature over the period of neck feather growth, starting from nestling day 12 (hatching day counted as day 1) (Aebischer and Scherler, 2021) until the day of sampling. For the analysis of H/L, we averaged the daily means of ambient temperature over the week before sampling, as commonly done in experimental settings when testing environmental effects (McFarlane and Curtis, 1989). Because ambient temperature was averaged over a period, its correlation with nestling age at sampling was only moderate (for CORTf: Pearson's correlation: 0.45, *P*<0.01, for H/L: Pearson's correlation: 0.50, *P*<0.01).

Statistical analysis

All analyses were performed in R (version 3.13). To quantify the uncertainty for model inference, we used the 95% credible intervals (CrI) of the Bayesian posterior distributions using the package 'arm' (https://CRAN.R-project.org/package=arm). We followed a full model approach: all the explanatory variables were kept in the final model, but *a priori* defined interactions were excluded when the 95% CrI overlapped zero.

To investigate the effects of food supplementation and temperature on body condition, we fitted a linear mixed model (LMM; package lme4; Bates et al., 2015) with Gaussian data distribution. Explanatory variables included food supplementation (binary covariate), hatching rank (being last-hatched or not), their interaction, temperature (used for H/L) and brood size (factor with levels 1, 2 and 3 nestlings), as well as brood identity as the random intercept.

To evaluate the effect of environmental and individual factors on CORTf, we fitted a linear mixed model (LMM; package lme4; Bates et al., 2015) with CORTf (pg mm^{-1}) as the response variable. CORTf was log-transformed to achieve normal distribution of residuals. Our focus variables were food supplementation (binary covariate), body condition (g), hatching rank (being last-hatched or not), ambient temperature (°C) and age (days). To investigate changes in CORTf with increasing nestling age and differences of effects between nestlings in the nest, we included two-way interactions between age and last-hatched, and between these two and food supplementation, body condition and temperature, respectively (age×last-hatched, age×food supplementation, age×body condition, age×temperature, last-hatched×food supplementation,

Table 1. Summary of the linear mixed model investigating factors affecting nestling body condition

Fixed effects	Estimate	95% CrI
(Intercept)	−0.43	−7.96–7.10
Food supplementation	**13.19**	**3.49–22.89**
Brood size [2]	5.63	−4.04–15.30
Brood size [3]	1.17	−9.19–11.53
Last-hatched	**−14.12**	**−21.96– −6.29**
Temperature	−2.03	−5.49–1.43
Random effect	s.d.	
Brood ID	3.471	

Nestling body condition was defined as residuals of a regression of body mass on age across all data. Effects with 95% credible interval (CrI) excluding zero are shown in bold. Square brackets refer to the level of the categorical variable evaluated in the model.

last-hatched×body condition and last-hatched×temperature). Further, we included sampling year to control for inter-annual variability, and brood size at sampling to control for variability in sibling competition among broods. All continuous variables were scaled to s.d.=1 and centred prior to modelling. Brood ID and plate ID were included as two separate random intercepts to account for among-brood and among-plate (from the EIA) variation in CORTf.

For H/L, we fitted a generalised linear mixed model (GLMM; package lme4; Bates et al., 2015) with binomial data distribution. The response variable H/L was taken into the model as a two-part vector of the number of heterophils (H) and lymphocytes (L), and subsequently back-transformed into H/L for graphical purposes. We built the model with the same focus variables, interactions and control variables as for the CORTf model. Brood ID was included as a random intercept. Bird ID was not added as a random effect in either model because of the considerably smaller subset of individuals with multiple measurements (ca. 50 out of 500), which caused convergence issues.

RESULTS

Over the 4 years of our study, we analysed 551 CORTf samples from 475 red kite nestlings in 280 broods, and 497 blood smears (H/L) from 385 nestlings in 220 broods.

Environmental effects: food and ambient temperature

Body condition of last-hatched nestlings was reduced compared with that of non-last-hatched nestlings (Table 1). While food supplementation increased nestling body condition (Table 1), this effect was not conditional on the nestling's hatching rank (food supplementation×last-hatched: effect size 6.40; 95% CrI −13.29–26.10).

CORTf showed a moderate negative association with body condition across all nestlings (Table 2). After controlling statistically for body condition, food-supplemented nestlings had reduced CORTf compared with non-food-supplemented ones at a young age (food supplementation×age: Table 2, Fig. 1). H/L ratio decreased with increasing body condition across all nestlings (Table 2, Fig. 2A). After controlling statistically for body condition, food-supplemented last-hatched nestlings had a reduced H/L ratio compared with non-food-supplemented last-hatched individuals (food supplementation×last-hatched: Table 2, Fig. 3).

Low ambient temperature tended to be associated with increased CORTf (Table 2) and the same effect was found for H/L (Table 2, Fig. 2B). However, body condition was not related to ambient temperature (Table 1). The effect of temperature was independent of age (CORTf analysis: temperature×age estimate −0.02, 95% CrI: −0.12–0.08; H/L analysis: temperature×age estimate 0.008, 95% CrI: −0.01–0.03).

Age and brood size

While CORTf decreased with nestling age in non-supplemented nestlings (food supplementation×age: Table 2, Fig. 1), H/L ratio did so across all nestlings (Table 2, Fig. 2C). This negative age effect was not due to an initial selective mortality of individuals with high stress measures, as the effect remained even in a separate analysis restricted to individuals that survived throughout the entire nestling period (n=45 nestlings from 22 broods, estimate −0.16, CrI −0.33– −0.03). A separate analysis of feather segments revealed a significantly higher amount of CORTf in distal compared with proximal segments (two-sided paired Wilcoxon signed-rank test, P<0.001; Fig. S2), confirming that more CORT was deposited in the feathers during the early compared with the late nestling phase (see Fig. S3 and

Table 2. Summary of the linear mixed model and generalised linear mixed model investigating factors affecting feather corticosterone (CORTf) and heterophil/lymphocyte (H/L) ratio, respectively

	CORTf		H/L	
	Estimate	95% CrI	Estimate	95% CrI
Fixed effects				
(Intercept)	**1.61**	**1.37–1.80**	**0.31**	**0.21–0.42**
Body condition	**−0.05**	**−0.11– −0.01**	**−0.04**	**−0.07– −0.02**
Food supplementation	−0.06	−0.22–0.09	−0.10	−0.22– −0.01
Year [2017]	−0.06	−0.35–0.20	0.10	−0.02–0.21
Year [2018]	**−1.32**	**−1.59– −1.07**	−0.04	−0.19–0.08
Year [2019]	**−0.80**	**−1.09– −0.56**	**0.13**	**0.00–0.25**
Temperature	−0.04	−0.15–0.04	**−0.07**	**−0.12– −0.03**
Age	**−0.20**	**−0.29– −0.11**	**−0.09**	**−0.13– −0.06**
Last-hatched	**0.19**	**0.09–0.27**	**0.16**	**0.11–0.21**
Brood size [2]	0.08	−0.06–0.20	**−0.12**	**−0.23– −0.04**
Brood size [3]	**0.29**	**0.11–0.43**	−0.05	−0.18–0.06
Food supplementation×last-hatched	–	–	**−0.14**	**−0.26– −0.04**
Food supplementation×age	**0.19**	**0.00–0.36**	–	–
Random effects	s.d.		s.d.	
Brood ID	0.31		0.25	
Plate ID	0.21		–	

Effects with CrI excluding zero are shown in bold. Dashes in the fixed effects columns indicate interaction terms that were excluded from the model because their 95% CrI included 0.
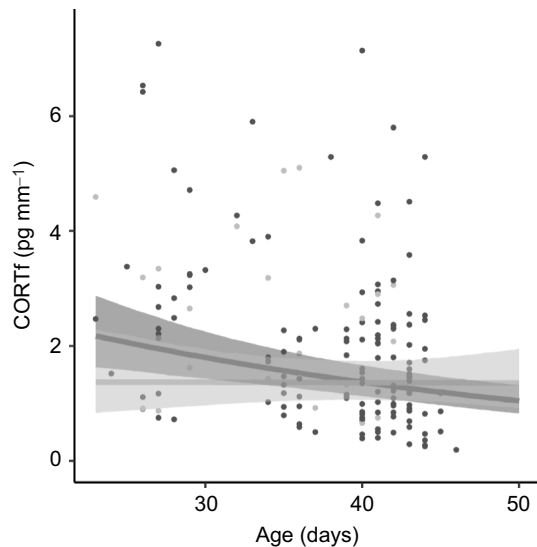
Fig. 1. Feather corticosterone (CORTf) in relation to age for non-food-supplemented and food-supplemented red kite nestlings. Shown are model predictions (lines), 95% credible intervals (CrI; shaded) and raw data points from 2018 (dots) for food-supplemented (light grey; *n*=29) and non-food-supplemented (dark grey; *n*=141) nestlings.

Supplementary Materials and Methods for details on the analysis of feather segments).

CORTf was elevated in large broods, particularly in broods of three nestlings (Table 2). H/L ratio was reduced in broods of two nestlings compared with that in broods with singletons and broods with three nestlings (Table 2).

DISCUSSION

Our experimental study investigating the effects of environmental factors on two stress measures of altricial red kite nestlings revealed three general results. First, food supplementation increased the body condition of nestlings and stress levels were reduced in nestlings of increased body condition. Thus, it appears that food supplementation affected the stress measures through a change in body condition. Second, food supplementation affected stress measures beyond augmenting body condition and showed

additional rank- and age-dependent effects. Thus, the food-driven social dynamics in the nest probably represent a separate driver of stress levels, supporting the existence of an additional mechanism through which food shortage affects stress levels. Sensitivity of stress to the competitive environment experienced in the early nestling phase is supported by the decline in stress levels over the nestling period and by the increased CORTf in large broods. Third, sub-optimal food availability and cool temperatures represent independent stressors in altricial nestlings. Increased H/L ratio and the analogous trend in CORTf consistently pointed towards an increase of stress levels at low temperatures. We, therefore, provide experimental evidence that food availability and adverse environmental conditions have independent, additive effects on stress levels in developing red kite nestlings. The results provide insights into the pathways of environmental effects influencing changes in stress levels during the nestling period, resulting in within- and between-nest differences in stress load.

Food supplementation enhanced the body condition of nestlings, which resulted in changes in CORTf and H/L ratio. Both measures decreased with increasing body condition across all nestlings. Thus, the feeding experiment provided two basic insights. First, it confirmed the well-known positive effect of food availability on growth and body condition of nestlings (Perrig et al., 2014; Nägeli et al., 2021). Second, it strongly supported the idea that nestling stress levels are lowered in optimal food conditions (Kitaysky et al., 2001, 2005; Müller et al., 2011). We, therefore, suggest that spatio-temporal variation in food availability will result in variation in nestling stress load, a possible mechanism for how food availability during the nestling period can carry over to later life-history stages.

We also found that food supplementation reduced CORTf in young nestlings and H/L ratio in last-hatched nestlings (Fig. 3). These effects occurred independent of the effect of experimentally enhanced body condition, suggesting an additional mechanism through which food can affect stress levels. Food availability has often been addressed as a key factor regulating sibling antagonism in the nest (Creighton and Schnell, 1996; Morandini and Ferrer, 2015), and red kites are known to be a facultatively siblicidal species (Scherler et al., in prep.). In red kite nestlings, aggressive behaviour peaks before 25 days of age (Scherler et al., in prep.). A higher food availability during the early nestling phase and for last-hatched nestlings, therefore, not only affects food intake but also might
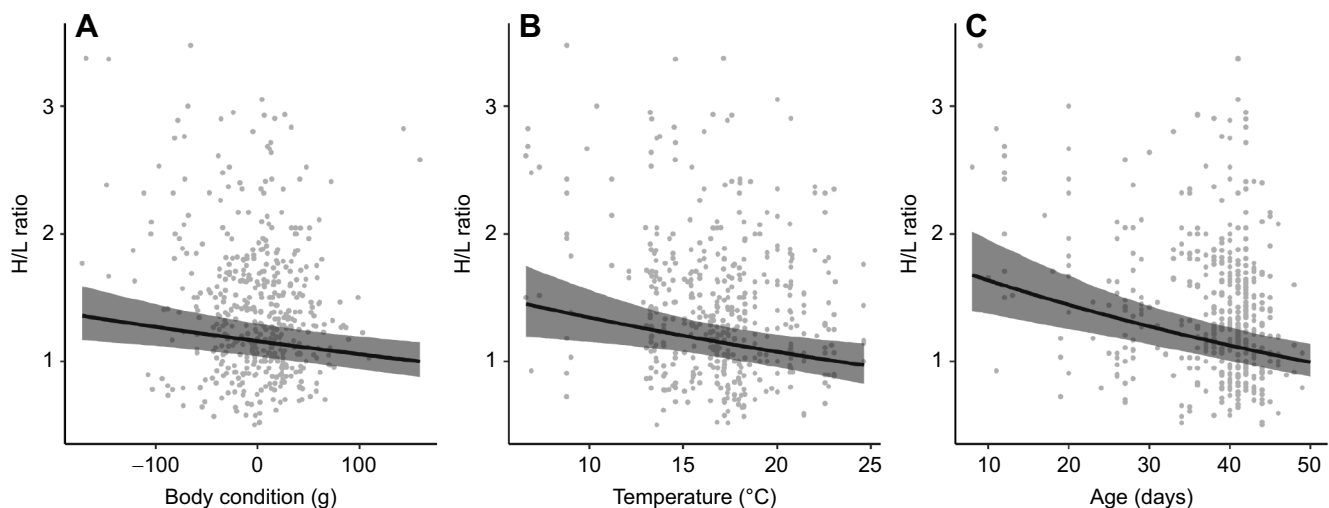
Fig. 2. Heterophil/lymphocyte (H/L) ratio of red kite nestlings in relation to body condition, ambient temperature and age. Shown are model predictions (lines), 95% CrI (shaded) and raw data points (dots) for H/L ratio versus (A) body condition, (B) temperature and (C) age (*n*=497).
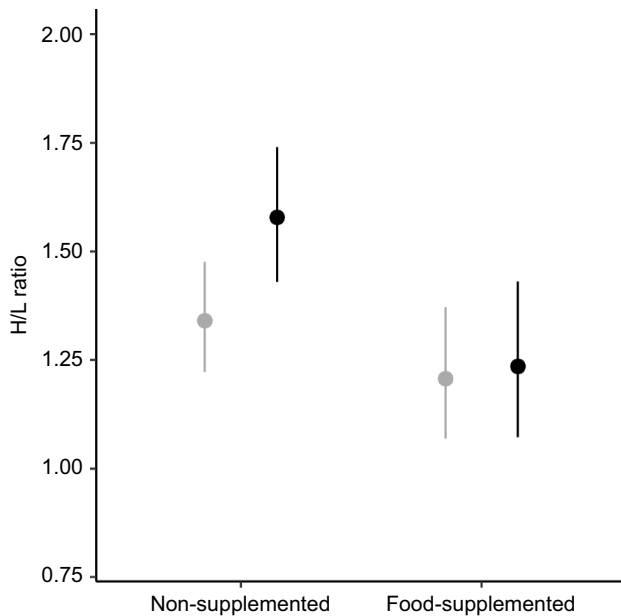
Fig. 3. H/L ratio in relation to food supplementation for last-hatched and non-last-hatched red kite nestlings. Shown are model predictions (dots) and 95% CrI (error bars) for non-food-supplemented non-last-hatched (light grey; *n*=255), non-food-supplemented last-hatched (dark grey; *n*=145), food-supplemented non-last-hatched (light grey; *n*=64) and food-supplemented last-hatched (dark grey; *n*=33) nestlings.

strongly decrease the competition between siblings over food, thereby reducing stress load (Morandini and Ferrer, 2015). This is supported by the age- and rank-dependent effects of food supplementation beyond the pure nutritional effect. The results, therefore, suggest that food shortage also affects stress levels by modifying the social environment of the nestlings, and reinforcing within-nest differences in stress load. Reduced food availability within the parental home range is, therefore, expected to increase both the stress load of fledglings and its between-sibling variation.

Under natural (control) conditions, both measures of stress decreased with age. Two mechanisms have been proposed to explain this pattern: negative selection (negative trend as a result of a progressive dropout of weak nestlings from the sampled population) and social challenge (decrease of intensive social competition with age) (López-Jiménez et al., 2016). In our study, the age effect persisted even when the analysis was restricted to broods where no mortality occurred; young nestlings deposited higher amounts of CORTf than old nestlings. The increased CORTf deposition in the distal segments further suggests that red kite nestlings need a strong physiological response capacity in the first part of development rather than towards fledging. Moreover, the higher CORTf in large broods is in line with increased food competition in the nest environment (Kitaysky, 2001; Loiseau et al., 2008). Thus, our results clearly support the social challenge hypothesis and, therefore, are in line with the idea that food shortage can drive changes to the HPA axis reactivity through an alteration of the nestlings' social environment.

The age effects in our study contrast with recent studies on avian development, which showed an increase in the stress response over the nestling period (Blas et al., 2006; Müller et al., 2010; Sims and Holberton, 2000). We see two ways to explain these contrasting results, both connected to the fact that existing studies mostly focused on stress-induced plasma CORT levels, whereas we used an integrative measure of CORT (CORTf) and a secondary stress measure (H/L ratio). First, stress-induced levels of CORT are inevitably always measured following a capture event that resembles a predation threat and, thus, age-specific patterns in stress-induced CORT may relate only to this type of stressor, and not to others such as environmental stressors (but see Lynn and Kern, 2018). Second, while plasma CORT proxies the intensity of a stress-induced response (Blas et al., 2005, 2006; Jimeno et al., 2018; Jones et al., 2021), CORTf is only interpretable in the light of both intensity and frequency of the response. Thus, the age-dependent decrease in CORTf in our study and the broadly reported age-dependent increase in CORT are not mutually exclusive: if the adrenal response of red kite nestlings is repeatedly triggered during the early developmental phase, their CORTf will sum up to high levels at a young age, even if the adrenal response to an acute stressor itself is weaker than it would be close to fledging.

We still have limited knowledge about the H/L ratio during development in birds (but see Quillfeldt et al., 2008). While heterophils are the major phagocytising cells belonging to the innate immune system, lymphocytes are part of the acquired immune system and act much more specifically against viruses, pathogens and ectoparasites, and their production is more costly and slower compared with that of heterophils (Klasing and Leschinsky, 1999; Lochmiller and Deerenberg, 2000; Lee, 2006). For this reason, a decrease in H/L ratio with age has been attributed to leucocyte ontogeny itself (Dehnhard et al., 2011b). Yet, other studies found no (Masello et al., 2009; Quillfeldt et al., 2009; Dehnhard et al., 2011a) or opposite (Quillfeldt et al., 2008) effects of age on H/L ratio. Hence, it has been proposed that H/L ratio in nestlings reflects a synergistic combination of the ontogeny of the immune system and developmental stress (Dehnhard et al., 2011b). Because in our study a high H/L ratio was associated with poor body condition, we propose that the negative relationship with age reflects the stress experienced rather than the ontogeny of the immune system. This is further supported by the analogous age effect in CORTf.

We found an effect of temperature on H/L ratio, but no effect of temperature on body condition, suggesting that temperature operates directly and not through changing food availability. This effect might reflect a trade-off between energy allocation to thermoregulation and proliferation of lymphocytes. Under cold stress, it is adaptive to increase the number of heterophils in the blood vessels, which provide non-specific protection against a broad array of pathogens (Skomsø, 2013; Skwarska, 2018). We found a similar but only weak relationship between temperature and CORTf. Thus, H/L ratio was a more sensitive measure than CORTf of the influence of short-term changes of environmental stressors, such as cold spells or food availability.

Conclusions
Our study aimed at experimentally investigating the effects of food availability on two stress measures. We provide evidence that the social environment in the nest can drive changes in stress levels, reinforcing within-nest differences in stress load resulting from rank-dependent nutritional stress. This is a critical step forward in understanding the mechanisms through which poor food conditions during early life may have fitness consequences in later life-history stages (Freeman et al., 2021). The physiological consequences of reduced food availability in large broods may, therefore, be part of the quality–quantity trade-off in altricial birds. The increased stress load due to poor environmental conditions such as cold temperatures or food shortage is expected to shape future behaviour and performance.

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