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## Experimental facilitation of heat loss affects work rate and innate immune function in a breeding passerine bird

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**RESEARCH ARTICLE**

# Experimental facilitation of heat loss affects work rate and innate immune function in a breeding passerine bird

Fredrik Andreasson[1,*], Arne Hegemann[1], Andreas Nord[1,2] and Jan-Åke Nilsson[1]

## ABSTRACT

The capacity to get rid of excess heat produced during hard work is a possible constraint on parental effort during reproduction [heat dissipation limit (HDL) theory]. We released hard-working blue tits (*Cyanistes caeruleus*) from this constraint by experimentally removing ventral plumage. We then assessed whether this changed their reproductive effort (feeding rate and nestling size) and levels of self-maintenance (change in body mass and innate immune function). Feather-clipped females reduced the number of feeding visits and increased levels of constitutive innate immunity compared with unclipped females but did not fledge smaller nestlings. Thus, they increased self-maintenance without compromising current reproductive output. In contrast, feather clipping did not affect the number of feeding visits or innate immune function in males, despite increased heat loss rate. Our results show that analyses of physiological parameters, such as constitutive innate immune function, can be important when trying to understand sources of variation in investment in self-maintenance versus reproductive effort and that risk of overheating can influence innate immune function during reproduction.

KEY WORDS: Body temperature, Heat dissipation, Immunology, Life-history theory, Parental effort, Thermoregulation

## INTRODUCTION

In species with bi-parental care, parents have to work hard to provide for their young during reproduction. Traditionally, several limitations to the capacity for work have been suggested, including environmental resource supply (Stearns, 1992; McNab, 2002), limitations in energy absorption (central limitation; Drent and Daan, 1980), the capacity of nutrient/energy delivery to the tissues (metabolic theory of ecology; West et al., 1999; Brown et al., 2004) and a limitation in work capacity of peripheral tissues (peripheral limitation; Hammond et al., 1994; Hammond et al., 1996). However, recent studies have shown that limits to maximum energy expenditure can also be set by the capacity to dissipate heat [heat dissipation limit (HDL) theory; Speakman and Król, 2010], under the assumption that sustained hyperthermia resulting from insufficient heat dissipation may be costly, e.g. as a result of oxidative stress (Lin et al., 2006; Jimenez and Williams, 2014), enzyme malfunction (Daniel et al., 2010) and protein degradation (Del Vesco et al., 2015). Most studies on the HDL theory have used

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captive rodents as model species, where facilitated heat dissipation (through removal of fur) results in increased energy turnover rate and increased reproductive effort (Król et al., 2007; Simons et al., 2011; Sadowska et al., 2016). Some correlative studies suggest that free-living animals are also constrained by the risk of overheating. For example, small mammals and birds decrease activity at high ambient temperature ($T_a$) (Zub et al., 2013; Wiley and Ridley, 2016; Powers et al., 2017) or when their body temperature ($T_b$) is high (Guillemette et al., 2016). Recently, Nord and Nilsson (2019) tested the HDL theory experimentally by facilitating heat dissipation in adult blue tits [*Cyanistes caeruleus* (Linnaeus 1758)] during the most active period of nestling rearing by removing the feathers covering the pectoral muscles and abdomen. In support of the HDL theory, clipped birds produced larger and sometimes heavier nestlings. These birds also maintained lower $T_b$ and lost less body mass during the experimental period, which could indicate simultaneous benefits for self-maintenance.

The immune system, which consists of an adaptive and an innate arm, is an integral part of self-maintenance and crucial for survival through preventing disease and fighting infections. The innate immune system is the first line of defense. It detects antigens that are present on a wide array of pathogens and to prevent these pathogens from establishing themselves in the body it immediately launches an acute phase response – an energetically costly suite of behaviors and physiological responses aimed at clearing infection (reviewed in Owen-Ashley and Wingfield, 2007). Innate immune function is costly (Råberg et al., 2002) and, accordingly, experimentally increased reproductive effort has been shown to decrease levels of innate immune function in several bird species (e.g. Hegemann et al., 2013a; Neggazi et al., 2016). This pattern is less clear for adaptive immune function (Williams et al., 1999; Bowers et al., 2012). Thus, innate immune function seems to be compromised when reproductive effort is increased. Hence, maintaining sufficient levels of constitutive innate immunity (i.e. the circulating baseline defense) could be especially important during breeding if this allows an animal to avoid the energetically costly acute phase response and related sickness behaviors, such as reduced activity and lethargy (Burness et al., 2010; Sköld-Chiriac et al., 2014).

A logical next step to increase our understanding of the potential benefits to self-maintenance following reduced constraints on heat dissipation rate (Nord and Nilsson, 2019) would be to study whether such manipulations increase investment in constitutive innate immune function. To test this, we experimentally increased heat loss rate by removing the plumage covering the abdomen and pectoral muscles on free-living blue tits during the most active part of breeding; the brood-rearing stage. Facilitation of increased heat loss rate may allow individuals to increase work rate, and thereby heat production, without incurring costs associated with hyperthermia. To quantify current reproductive effort and output, we recorded the effect on parental feeding rates and nestling size. To

measure self-maintenance, we recorded subcutaneous temperature ($T_s$) continuously with implanted PIT-tags (in males), and measured four parameters of constitutive innate immune function in all adult birds. If reduced constraints on heat dissipation rate allow for increased investment in reproductive effort and/or self-maintenance, we predicted that clipped birds would increase feeding rate and/or improve constitutive innate immune function.

## MATERIALS AND METHODS

### Ethics

All applicable institutional and national guidelines for the care and use of animals were followed. Experimental protocols were approved by Lund/Malmö Animal Care committee (permit no. M67-16). Catching and ringing of birds was performed under the permission of the Swedish Ringing Centre (license no. 475).

### Study population

Fieldwork was conducted during May–June 2017 in a nest-box population of blue tits around Lake Krankesjön (55°70′N, 13°48′E) in the south of Sweden, where roughly 500 wooden nest-boxes scattered over 64 km² have been monitored since 1983. The area mainly consists of cattle-grazed pastures with interspersed forest patches. For this study, we also included an additional population with approximately 150 identical nest-boxes that have been monitored yearly since 2005, located in a managed beech (*Fagus sylvatica*) forest in Öved (55°71′N, 13°61′E) ~10 km away from the main site. In our population, blue tits start nest building in late March and the first egg is laid mid- to late-April. The female usually lays one egg a day and incubation starts when the clutch is complete (Perrins, 1979), with the female incubating the eggs for 12–14 days (Nilsson, 2000). During the first week of the nestling phase, nestlings are brooded intermittently by the female and she also roosts at night in the nest-box together with the nestlings (Perrins, 1979). Nestlings are fed by both parents until they fledge ~3 weeks after hatching, and fledglings are then fed and tended to by the parents for an additional 2 weeks.

To determine the start of breeding (defined as laying of the first egg, back-calculated assuming one egg was laid per day) and clutch size, we visited nest-boxes at least once a week starting in mid-April. When egg laying was completed and females had incubated the eggs for 12 days, we checked nest-boxes daily for hatching (defined as nestling day 0). We wanted to evaluate the effect of facilitated heat dissipation in birds that were working close to or at the maximum capacity and hence when body temperature is highest (Nilsson and Nord, 2018). Therefore, we decided to enlarge all broods of our study; a treatment that pushes birds to maximum working rates (Nur, 1984; García-Navas and Sanz, 2010). Thus, on nestling day 6 (1 day before we feather clipped the parents) we performed brood-size manipulation of broods with an original clutch size of 10–12 eggs (mean±s.d. clutch size in 2015–2017 was 10.4±1.7, *n*=531). We weighed the nestlings to the nearest 0.1 g using a Pesola spring scale (Pesola AG, Baar, Switzerland) and ringed them with a ring from the Swedish Bird Ringing Centre. All broods were enlarged to achieve a brood size that was 5 nestlings larger than the original clutch size, by moving nestlings of the same age from a donor nest. Thus, all manipulated broods had 15–17 nestlings (+42–50%) at the start of the experiment, which was still within the natural range in our population (≤18). We measured $T_a$ ±0.0625°C, every 30 min with a temperature logger (iButton DS1922-L, Sunnyvale, CA, USA; accuracy ±0.5°C) placed in the shade, 1.5 m above ground, at Stensoffa field station in the center of the main study area.

### Feather clipping and PIT tagging

When nestlings were 7 days old, each nest-box was randomly assigned (coin-toss) to either a feather-clipping treatment (to facilitate dry heat transfer; see Nord and Nilsson, 2019) or an unclipped group. We then caught, ringed, weighed (±0.1 g) and measured (wing: ±0.5 mm, tarsus: ±0.1 mm) the parents, aged them based on plumage characteristics (Svensson, 1992), and sexed them based on the absence/presence of a brood patch. We chose to start the feather clipping at day 7 because nestlings have their highest growth rate around this time (Andreasson et al., 2016; Andreasson et al., 2018) and any experimental manipulation of parental effort would thus have the strongest effect on nestling development. To measure $T_s$, males were implanted with a temperature-sensitive passive integrated transponder tag (PIT-tag; BioTherm13, Biomark, Boise, ID, USA; height: 13.0 mm; diameter: 2.1 mm) subcutaneously in the neck under antiseptic conditions (following Nord et al., 2013; Sköld-Chiriac et al., 2015). The incision was sealed using cyanoacrylate (Loctite Power Easy Gel, Henkel AG & Company, Düsseldorf, Germany). We did not implant PIT-tags in the females, as parents usually stay away from the nest for some period of time after implantation (as they also do after routine procedures like blood sampling) and we did not want to increase the risk of nest abandonment which could happen if both parents stayed away for an extended period of time and returned to cold and unresponsive nestlings. Instead, females were equipped with a PIT-tag taped to two color-rings on the left tarsus. Both parents in the clipped group were also feather clipped at this time following the procedure in Nord and Nilsson (2019), whereby plumage was removed over ca. 20% of the birds' ventral surface. This increases heat loss rate from the ventral area by ca. 50% under steady-state conditions (Nord and Nilsson, 2019). Birds in the unclipped group received the same treatment except for the clipping.

We returned to each nest on day 14 to measure body mass, and tarsus and wing length of all nestlings. We also caught and weighed both parents, and validated our measurements of $T_s$ against $T_b$ by measuring the two temperatures simultaneously in 17 birds (see Nord et al., 2013; Andreasson et al., 2016). Cloacal $T_b$ correlated well with simultaneously recorded $T_s$ (linear regression using lm function in base R: $F_{1,15}$=23.3, $T_s$=−9.38+1.21$T_b$, $P$<0.001, $R^2$=0.61) and $T_s$ never deviated more than 0.8°C from $T_b$. Afterwards, we collected a 100 µl blood sample from the jugular vein (within 15 min of capture). We kept blood samples on ice in an Eppendorf tube for ≤5 h until centrifuging for 10 min at 4000 rpm. Samples for relevant immunological assays can be stored on ice for 12 h without negative effects (Hoye, 2012). Samples were then immediately frozen, first at −50°C and later at −80°C, until subsequent laboratory analyses.

### Recording feeding rate and $T_s$

A PIT-tag reader (Biomark HPR Plus, Biomark) connected to a circular antenna (diameter 18 cm) placed directly underneath a nest-box was deployed when nestlings were 10 days old. The antenna recorded all nest visits and measured $T_s$ via the subcutaneous PIT-tags for 24 h (mean±s.d. duration of recording: 23.8±1.2 h, range: 20.6–26.5 h).

$T_s$ was recorded from PIT-tags as soon as a bird was within the reading range of the antenna (≤20 cm), i.e. when the bird entered the nest-box. PIT-tag readers were set to record each individual PIT-tag, but with a refractory period of 1 min. Thus, a new recording of $T_s$ could only be obtained after 60 s from the initial reading (if the bird was still within reading range, the next reading usually occurred after 60–62 s). This way of recording feeding visits

could create two potential sources of error (see Iserbyt et al., 2018): (1) a bird might stay inside the nest-box >1 min, which would create false double/multiple readings; (2) if a bird was able to feed twice within 1 min of the initial recording, we would miss one true recording. We deemed scenario 2 to be very unlikely as feeding visits usually take between 10 and 30 s (F.A., personal observation) and the bird would have to make two of these visits in addition to the foraging trip in between, all within 60 s. We thus filtered data to remove false double readings.

For males, we adopted a simple approach where we first listed all readings that occurred 60–62 s after one another, i.e. potential double readings and not true unique feeding visits. If there were more than two such readings directly after one another, all subsequent readings were removed. For example, if a male was recorded on four occasions, all within 60–62 s of one another, the last three of these were removed.

Females, more often than males, regularly turned up on the recordings in 60–62 s intervals. We made use of the leg-mounted temperature-sensitive PIT-tags that the females were carrying to filter false positives. Firstly, we listed all readings that were recorded within 60–62 s of one another. The lowest possible reading for these tags was 23.3°C, which normally occurred when flying and entering the nest-box for a feeding visit. Of these listed readings, we removed all readings where tarsus temperature was above 30°C, most likely indicating an event where the female had stayed in the nest-box for several minutes (allowing tarsus temperature to rise). This could happen if a female stayed in the nest-box for a prolonged period of time, such as during the night.

All night-time readings (for females) were manually removed. The start of night for males was defined as when the male had stopped feeding and for females as when the female had been in the nest-box for >5 min (these five readings were also removed); the end of night for males was defined as when the male made the first feeding visit and for females as when the first reading with a temperature <30°C was recorded (i.e. the first feeding visit). On average (±s.d.), 2.7±2.6% of male and 11.7±7.9% of female recordings were removed (excluding night-time readings).

## Immune function

Analyzing variation in immune function is not always straightforward. Measuring a single immune parameter will not encompass total immunocompetence (Adamo, 2004; Martin et al., 2006a; Matson et al., 2006) and the correlation between different immune indices and an individual's resistance to disease can be pathogen specific (Adamo, 2004). In addition, different parts of the immune system could trade off against each other (Martin et al., 2006b). To address this complexity, we measured four innate immune indices that are indicative of protection against a broad array of targets that are present at all times (Fig. S1). By summarizing these in a principal component analysis (PCA; see below for details), we effectively avoided issues with covariance between immune indices, so enabling us to describe how the experiment affected the level of constitutive innate immunity in a meaningful way (Adamo, 2004; Buehler et al., 2011). Specifically, we used a hemolysis–hemagglutination assay to quantify titers of (1) lytic enzymes of the complement system (lysis) and (2) non-specific natural antibodies (agglutination) (Matson et al., 2005). Scans of individual samples were randomized among all plates and scored blindly with respect to treatment (by A.H.). Complement and natural antibodies are both components of the constitutive innate immune system (Schmid-Hempel and Ebert, 2003; Matson et al., 2005). Natural antibodies, in contrast to antibodies of the adaptive

immune system, do not require previous exposure to an antigen (although they can be affected by exogenous antigens; see Holodick et al., 2017, for a proposed definition of what a natural antibody is). One of their functions is the initiation of the complement cascade, resulting in cell lysis of invading pathogens (Ochsenbein and Zinkernagel, 2000). We used a commercially available colorimetric assay kit (TP801, Tri-Delta Diagnostics, Maynooth, County Kildare, Ireland) to quantify (3) haptoglobin, or any functional equivalent (i.e. PIT54; see Wicher and Fries, 2006), concentration in plasma samples (Hegemann et al., 2012; Matson et al., 2012). Haptoglobin is an acute phase protein that is normally present at low concentrations in plasma but is released from the liver during a pathogenic challenge (Thomas, 2000; Cray et al., 2009; Matson et al., 2012). It binds free hemoglobin that otherwise could harm cell membranes and produce reactive oxygen species (ROS) and it also serves as a general antioxidant (Gutteridge, 1987; Quaye, 2008). (4) We quantified total bacteria killing capacity (BKA) against *Escherichia coli* following the method described by French and Neuman-Lee (2012) with a few modifications (see Eikenaar and Hegemann, 2016). Specifically, we used a dilution of 3 µl plasma mixed in 4 µl of 105 *E. coli* solution (based on validation tests) and measured bacteria growth at 600 nm using a microplate reader. BKA of plasma is an integrative measure of innate immune function that includes both natural antibodies and phagocytosis as well as complement activation (French and Neuman-Lee, 2012).

## Data analysis

In total, 41 nest-boxes were included in the experiment. The upper limit to sample size was set by the number of available PIT-tag readers ($n$=4), i.e. we could not include more than four boxes per day. Twelve of these (unclipped: $n$=4, clipped: $n$=8) were either partially predated or abandoned before feeding rate measurements at day 10 and were not included in the final dataset. We also excluded one nest-box with unclipped parents where nestlings were provisioned by two males, making assessment of feeding effort, $T_s$, biometrics and immune function difficult. Thus, the final dataset included 28 nest-boxes (clipped: $n$=14, unclipped: $n$=14). One female lost her PIT-tag before day 10 and, accordingly, we could not record feeding rate for this individual. In the analyses of body mass change from day 7 to day 14, nestling biometrics and immune function, we also excluded data from five nest-boxes (4 unclipped and 1 clipped) where only one parent was feeding at day 14. We could not run the hemolysis–hemagglutination assay for five individuals (clipped: $n$=4, unclipped: $n$=1) because of insufficient plasma volume. Immune data from these individuals were consequently not included in the PCA.

All analyses were performed in R v.3.5.1 (http://www.R-project. org/). We used PCA (prcomp function in base R) to avoid possible covariance issues and to reduce the number of immune variables. We retained principal components with an eigenvalue >1 (Kaiser, 1960).

Firstly, we analyzed mean male $T_s$ from all feeding visits using linear models (lm function in R base) with experimental treatment category (clipped/unclipped) and age ('young'=second calendar year; 'old'=third calendar year or older) as fixed factors and feeding rate, Julian date, $T_a$ and body condition as covariates. We also added the two-way interactions treatment category×$T_a$, treatment category×age and treatment category×feeding rate. $T_a$ was defined as the mean $T_a$ during the active feeding period between day 10 and 11 (grand mean±s.d.: 17.4±2.4°C). Body condition was calculated as the scaled body mass index *sensu* Peig and Green (2009) using adult body mass at day 7.

We then proceeded to analyze feeding rate, defined as the number of visits per hour between the first feeding visit after the deployment of the reader and the last feeding visit before the reader was recollected (excluding the nightly hours when there were no feeding events) using linear mixed models (lmer function in the lme4 package; Bates et al., 2015). We included experimental treatment category (clipped/unclipped), sex and age (second calendar year/ third calendar year or older) as fixed factors and Julian date, $T_a$ and body condition as covariates. We also included the two-way interactions treatment category×sex, treatment category×age and treatment category×$T_a$.

The change in adult body mass from day 7 to day 14 was analyzed in a mixed effects model that was identical to the feeding rate model except that the $T_a$ variable was calculated as the mean of daytime $T_a$ (sunrise to sunset) from day 7 to day 14.

PCA scores were analyzed using lmer models, using treatment category, sex and age as fixed factors, Julian date and body condition (based on body mass at day 14) as covariates and nest-box as a random factor. Again, we also included the two-way interactions treatment category×sex, treatment category×age and also the interaction between sex×age. Plasma redness (absorbance at 405 nm) did not have any effect on haptoglobin concentration (linear regression using lm function in base R: $F_{1,44}=2.57$, $P=0.12$, $R^2=0.06$) and we therefore did not consider it further in the PCA.

We analyzed nestling biometrics (body mass, and tarsus and wing length) at day 14 with treatment category, female age and male age (and the two-way interactions between these three factors) as fixed factors, body mass at day 6 and Julian date as covariates and nest-box as a random factor.

Degrees of freedom and P-values for mixed models were calculated (Satterthwaite approximation) using the lmerTest package (Kuznetsova et al., 2017). All lmer models were fitted using restricted maximum likelihood and non-significant interactions ($P>0.05$) were removed. However, all fixed factors and covariates were included in final models. Estimates for factors (±s.e.m.) presented in text and tables are predicted marginal means (emmeans package; https://CRAN.R-project.org/package=emmeans) unless otherwise stated and P-values for pairwise comparisons on these marginal means were adjusted using the Holm method (Holm, 1979) implemented in the emmeans package. Figures are based on raw data.

## RESULTS
### $T_s$
Mean male $T_s$ varied between 41.3 and 42.5°C (grand mean: 41.9±0.1°C), but there was no effect of clipping on $T_s$ (clipped: 41.9±0.1°C, unclipped: 42.0±0.1°C, $P=0.75$; Table 1). $T_s$ was not significantly affected by male age, feeding rate, $T_a$, Julian date or body condition (Table 1).

### Feeding rate
Feather clipping had a sex-dependent effect on feeding effort (treatment×sex; $P=0.021$, Table 1, Figs 1 and 2), such that clipped females visited the nest-box to feed nestlings five times (or ca. 19%) less each hour (21.6±1.2) compared with unclipped females (26.8±1.4, $P=0.012$), while there was no difference in the number of visits between clipped and unclipped males (23.2±1.3 versus 22.5±1.3, $P=0.71$). Over the length of the working day (ca. 16.5 h from sunrise to sunset), this meant that clipped females made, on average, 86 fewer visits to the nest compared with unclipped ones. Julian date was a strong predictor of feeding rate ($P<0.001$; Table 1), with feeding rate decreasing by 0.6±0.1 visits h⁻¹ day⁻¹ since the

**Table 1. Estimates, test statistics and significance values from models on subcutaneous temperature ($T_s$), feeding rate and body mass in adult blue tits in which plumage of the ventral surface was either clipped to increase heat loss rate or left unclipped**

| Variable | Estimate (s.e.m.) | d.f. | $F/\chi^2$ | P |
|---|---|---|---|---|
| $T_s$ (°C) | | | | |
| Treatment | | 1, 21 | 0.1 | 0.75 |
| Age | | 1, 21 | 1.7 | 0.21 |
| Feeding rate | −0.01 (0.02) | 1, 21 | 0.7 | 0.40 |
| $T_a$ | 0.04 (0.03) | 1, 21 | 2.3 | 0.14 |
| Julian date | 0.01 (0.02) | 1, 21 | 0.1 | 0.71 |
| Body condition (scaled mass index) | 0.05 (0.17) | 1, 21 | 0.1 | 0.76 |
| Feeding rate (feeding visits h⁻¹) | | | | |
| Treatment | | 1, 47 | 3.2 | 0.081‡ |
| Sex | | 1, 47 | 0.8 | 0.37 |
| Treatment×sex | | 1, 47 | 5.7 | 0.021* |
|   Unclipped males | 22.52 (1.29) | | | |
|   Unclipped females | 26.82 (1.42) | | | |
|   Clipped males | 23.18 (1.33) | | | |
|   Clipped females | 21.55 (1.23) | | | |
| Age=young | 24.79 (1.01) | 1, 47 | 3.7 | 0.062‡ |
| Age=old | 22.24 (0.82) | | | |
| $T_a$ | −0.38 (0.27) | 1, 47 | 2.0 | 0.17 |
| Julian date | −0.63 (0.14) | 1, 47 | 19.4 | <0.001* |
| Body condition (scaled mass index) | −0.81 (1.54) | 1, 47 | 0.3 | 0.60 |
| Nest-box (random) | | 1 | 0.0 | 1 |
| Body mass change (g) | | | | |
| Treatment | | 1, 17.7 | 0.5 | 0.48 |
| Sex=male | 0.18 (0.06) | 1, 22.7 | 25.2 | <0.001* |
| Sex=female | −0.27 (0.06) | | | |
| Age | | 1, 38.8 | 2.0 | 0.17 |
| $T_a$ | −0.05 (0.09) | 1, 16.2 | 0.3 | 0.58 |
| Julian date | −0.03 (0.01) | 1, 16.6 | 4.5 | 0.049* |
| Body condition (scaled mass index) | −0.07 (0.09) | 1, 32.5 | 0.7 | 0.42 |
| Nest-box (random) | | 1 | 0.5 | 0.46 |

*$P<0.05$; ‡$0.05<P<0.1$.

start of the experiment. $T_a$ and body condition did not significantly affect feeding rate (Table 1).

### Nestling biometrics on day 14
We found no effect of feather clipping of parents on nestling biometrics (body mass, and wing and tarsus length: all $P≥0.16$; Table S1). Body mass at day 6 was a strong predictor of all nestling biometrics at day 14 ($P<0.001$; Table S1). Hatching date and age of the parents did not have any effect on nestling biometrics (Table S1).

### Immune function
The PCA showed two principal components with eigenvalues >1. PC1 explained 42.3% of the variation in the immune parameters and was positively correlated with all four immune indices (Fig. 3), thereby representing an overall measure of constitutive innate immunity. PC2 explained 28.5% of the variation and correlated positively with haptoglobin and agglutination and negatively with BKA and lysis (Fig. 3). PC1 showed a sex-dependent treatment effect (treatment×sex interaction: $P=0.011$; Table 2) such that clipped females had a mean positive score for PC1, while clipped males and unclipped birds all had mean negative scores for PC1. Thus, clipped females had higher levels of constitutive innate immunity than unclipped females ($P=0.003$), while there was no difference between clipped and unclipped males ($P=0.95$). There was no sex-dependent effect of clipping on PC2 (treatment×sex interaction: $P=0.83$) or any main effects of clipping ($P=0.80$) or sex
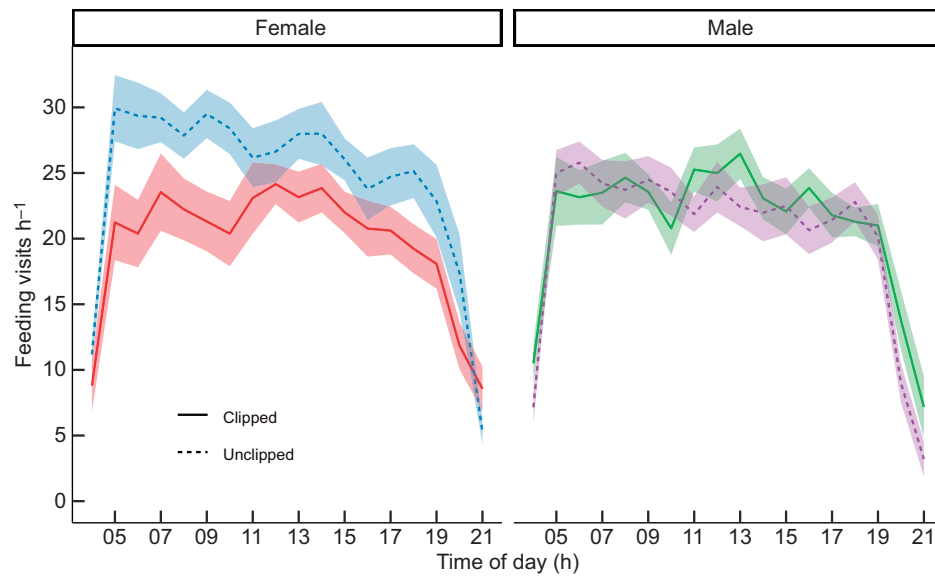
**Fig. 1. Feeding visits made by clipped and unclipped blue tits over 24 h.** Mean (±s.e.m.) number of feeding visits per hour in clipped and unclipped blue tits as a function of time of day. Sample sizes for clipped and unclipped birds of each sex during each hour are reported in Table S2.

($P$=0.93; Table 2). Neither PC1 nor PC2 was influenced by age, Julian date or body condition (Table 2).

## DISCUSSION

Clipped females, with an increased heat loss rate, fed their offspring less frequently (Figs 1 and 2), and had higher innate immune function (Table 2, Fig. 3), than unclipped females. Males fed their offspring at similar rates regardless of treatment (Figs 1 and 2), and their immune function indices were unaffected by the experiment (Fig. 3). Despite reduced effort and increased investment in self-maintenance in females, body mass and size were not compromised in nestlings of clipped parents (Table S1). Thus, reduced constraints of overheating allowed for increased constitutive innate immune function, but only in females. A relatively higher level of constitutive innate immune function is supposed to be beneficial for survival. For example, levels of complement activity have been shown to correlate positively with survival (Hegemann et al., 2015), and high BKA correlated positively with survival probability upon an epidemic outbreak (Wilcoxen et al., 2010).

These benefits possibly occur because higher constitutive immune function could increase the likelihood of clearing pathogens before they start replicating and establishing inside a host which, in turn, could reduce behavioral and physiological costs associated with an acute phase response.

Hence, clipped females in our study might have invested resources saved on heat dissipation and reduced feeding rate into improved defense against pathogens. Yet, high values of innate immune indices and suppressed activity can also be a consequence



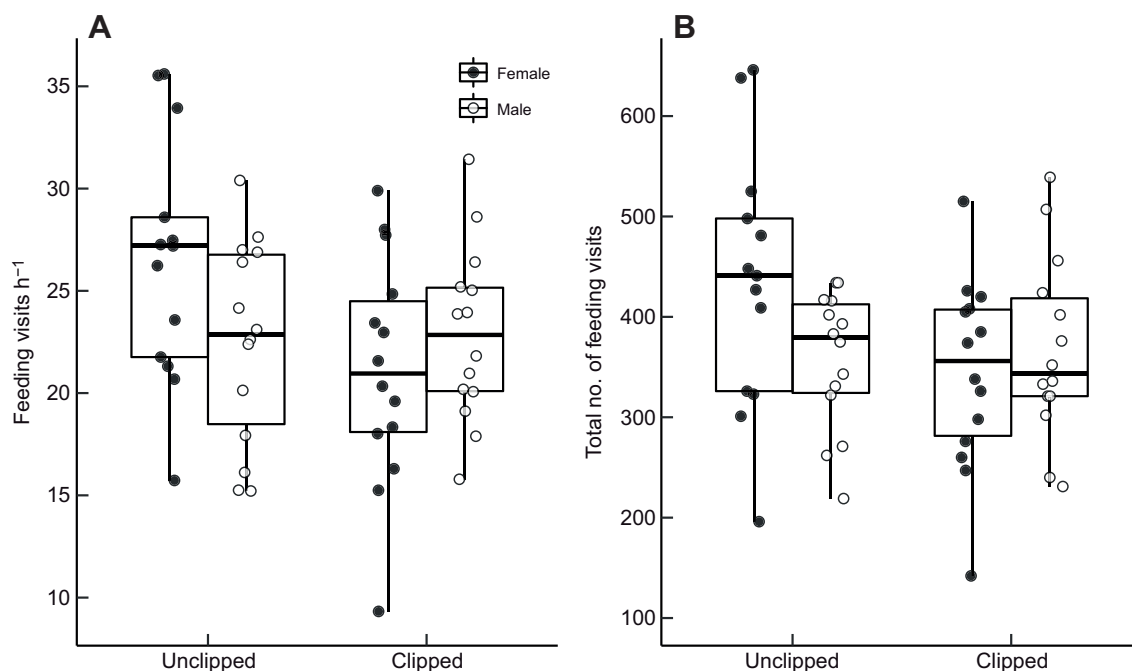**Fig. 2. Hourly and total number of blue tit feeding visits at day 10–11 of nestling development.** (A) Number of feeding visits per hour. (B) Total number of feeding visits during the ~24 h of measurements in clipped and unclipped blue tits. Box plots indicate the median (line), first and third quartile (box limits) and whiskers extend to minimum and maximum values. Statistics are reported in Table 1.
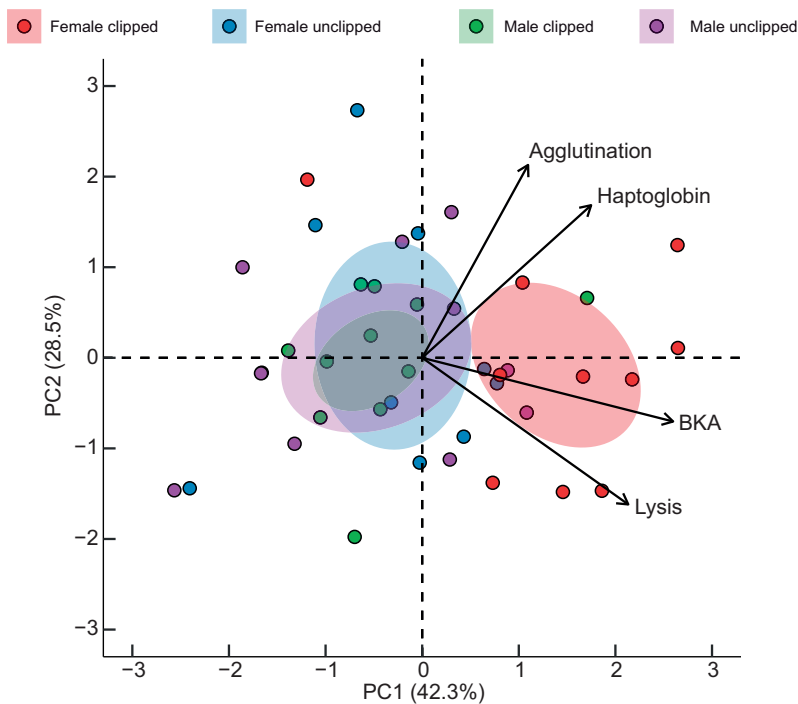
**Fig. 3. Principal components analysis (PCA) on four measures of innate immune function.** Individual scores (circles) and loadings of variables (vectors) in relation to the first two principal components (PCs) from a PCA on four innate immune indices: bacteria killing activity (BKA), haptoglobin, lysis and agglutination titer. The length of vectors indicates the strength of the relationship between the variable and the principal component and the angle between vectors indicates the degree of correlation (adjacent vectors are positively correlated, vectors that are orthogonal are uncorrelated and vectors that are 180 deg from each other are negatively correlated). Individual dots and confidence ellipses (95%) are colored according to sex and treatment category. Values for each immune index are displayed in Fig. S1 and statistics are reported in Table 2.

of disease (Hegemann et al., 2013b; Sköld-Chiriac et al., 2014). However, we do not believe that these responses in clipped females were caused by an ongoing sickness response related to the experimental treatment. If that was the case, we would have expected a corresponding, or larger, effect in males where the effect of feather clipping is greater than in females (because females already have a brood patch; Nord and Nilsson, 2019) and where we also pierced the skin to inject the temperature-sensitive PIT-tag. We do not know whether clipped birds changed other parts of their

physiology or behavior following our handling (e.g. stress). However, we consider it unlikely that our manipulation was causing a prolonged stress response as the same experimental feather-clipping technique has allowed parents to invest more in self-maintenance (Nord and Nilsson, 2019), nestling size (Nord and Nilsson, 2019) and feeding effort (Tapper et al., 2019 preprint) without any sign of sickness (Nord and Nilsson, 2019; Tapper et al., 2019 preprint) in previous studies.

Assuming constant feeding rates during the 16.5 h of daylight, nestlings fed by clipped parents received about 600 fewer food deliveries per nest-box during the 7 day experimental period. Even so, they did not grow smaller or lighter than nestlings fed by unclipped parents. This could be explained if clipped females were able to increase their foraging effort in terms of prey size or quality to compensate for reduced feeding rate (cf. Wright et al., 1998; García-Navas and Sanz, 2010). This could be achieved by foraging further away from the box where prey can be assumed to be less depleted versus close to the box and are most likely of higher quality, or by bringing more food to the nest on each visit. We do not know why clipped males did not also reduce feeding rate while increasing immune function. One possible explanation could be that females have a higher need to invest in immune function as they spend more time in the nest-box, especially when roosting at night (25 of 28 females, but no males, roosted with the nestlings between day 10 and 11). This probably increases their exposure to parasites, as nest-boxes often contain a large amount of pathogens and ectoparasites (Tomás et al., 2008; Castaño-Vázquez et al., 2018). Another, not mutually exclusive, explanation could be that males had to keep up feeding rate to compensate for reduced female investment in order to safeguard nestling growth. If so, the similar $T_s$ among treatments may indicate that clipped males were able to increase their foraging efficiency enough to fully compensate for the loss of female provisioning without incurring higher $T_b$, which often follows increased workload (Nilsson and Nord, 2018), or without increasing feeding rate. We only implanted the males with PIT-tags, but as the risk of injury or mortality of subcutaneous implantation has been shown to be minimal in small passerines (Oswald et al.,

**Table 2. Estimates, test statistics and significance values from models on the two first principal components from PCA on four measures of innate immune function in adult blue tits in which plumage of the ventral surface was either clipped to increase heat loss rate or left unclipped**

| Variable | Estimate (s.e.m.) | d.f. | $F/\chi^2$ | $P$ |
|---|---|---|---|---|
| PC1 | | | | |
| Treatment | | 1, 34 | 6.7 | 0.014* |
| Sex | | 1, 34 | 5.8 | 0.021* |
| Treatment×sex | | 1, 34 | 7.2 | 0.011* |
| Unclipped males | −0.43 (0.34) | | | |
| Unclipped females | −0.50 (0.39) | | | |
| Clipped males | −0.46 (0.31) | | | |
| Clipped females | 1.33 (0.34) | | | |
| Age | | 1, 34 | 1.3 | 0.26 |
| Julian date | 0.07 (0.04) | 1, 34 | 2.5 | 0.12 |
| Body condition (scaled mass index) | 0.44 (0.36) | 1, 34 | 1.5 | 0.23 |
| Nest-box (random) | | 1 | 0.0 | 1 |
| PC2 | | | | |
| Treatment | | 1, 19.5 | 0.1 | 0.80 |
| Sex | | 1, 20.5 | 0.0 | 0.93 |
| Age | | 1, 35.0 | 0.3 | 0.62 |
| Julian date | 0.03 (0.05) | 1, 24.7 | 0.6 | 0.46 |
| Body condition (scaled mass index) | 0.14 (0.37) | 1, 34.2 | 0.1 | 0.71 |
| Nest-box (random) | | 1 | 0.0 | 0.93 |

PCA, principal components analysis. *$P$<0.05.

2018), we do not think that the observed sex-specific patterns in feeding frequency and innate immune function were caused by the PIT-tag implantation of males.

Nord and Nilsson (2019) found that clipped parents had lower work-induced $T_b$, lost less body mass over the experimental period and raised larger nestlings than did unclipped parents, indicating that they were able to invest in both current reproduction and self-maintenance despite similar feeding rates across treatments. Given those results, it was unexpected that we did not see any effects on either $T_s$ or body mass loss using the same experimental treatment. However, there are many reasons why the physiological costs of parental care may differ between years and individuals (Williams, 2018). Nord and Nilsson (2019) conducted their experiment during the breeding season of 2013. Although mean (±s.d.) daily $T_a$ (measured 18 km from the main study area) during the experimental period was similar between years (2013: 13.8±2.3°C, 2017: 14.8±1.9°C), early spring was colder in 2013 than in 2017 (mean±s.d. daily $T_a$ in March, 2013: −1.8±2.5°C, 2017: 4.0±2.5°C) (Swedish Meteorological and Hydrological Institute, 2019; https://www.smhi.se/data). The colder spring in 2013 was also reflected in breeding phenology, with median first egg date being almost a week later in 2013 than in 2017 (2013: 30 April, 2017: 24 April) and with the breeding season lasting for almost twice as long in 2017 (range of first egg date; 2013: from 19 April to 9 May; 2017: 6 April to 14 May). In addition, birds were, on average (±s.d.), heavier before experimental manipulation in 2013 (11.7±0.5 g) compared with 2017 (11.4±0.5 g) ($t_{123}$=3.73, P<0.001). This might have left more room for mass loss among hard-working parents in 2013 than in 2017. Whether differences between studies were a consequence of variation in early spring temperature or breeding phenology and/or potential differences in peak food availability, is hard to ascertain but merits further investigation (*sensu* Williams, 2018). Importantly, both studies show that release of constraints on parental work load resulted in increased self-maintenance, even if the pathways involved might have differed between years.

## Conclusions

We found that experimentally increased heat loss rate in hard-working blue tits caused females to reduce work rate and increase innate immune function, a measure of self-maintenance, without compromising current reproductive output (i.e. nestling size). Surprisingly, we did not find any analogous effects of feather clipping on feeding effort, $T_s$ or innate immune function in males. This could reflect that the need to increase immune function is not as urgent in males, or that males had to keep up work rate to compensate reduced female effort. Including immune function as a measure of self-maintenance within the HDL framework can inform on why reproductive investment may vary between years, which is especially important in the light of current climate change predictions. Future studies of the trade-off between working capacity and heat dissipation should investigate how relieved constraints on heat dissipation affect other measures of nestling quality, e.g. immune function, and ideally also aim to measure future reproductive effort and survival to investigate whether increased female investment in self-maintenance has long-term fitness consequences.

## Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.219790.supplemental

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# Supplementary Information

**Fig. S1** A) bacteria killing capacity, B) haptoglobin concentration, C) lysis and D) agglutination in clipped- and unclipped blue tits.
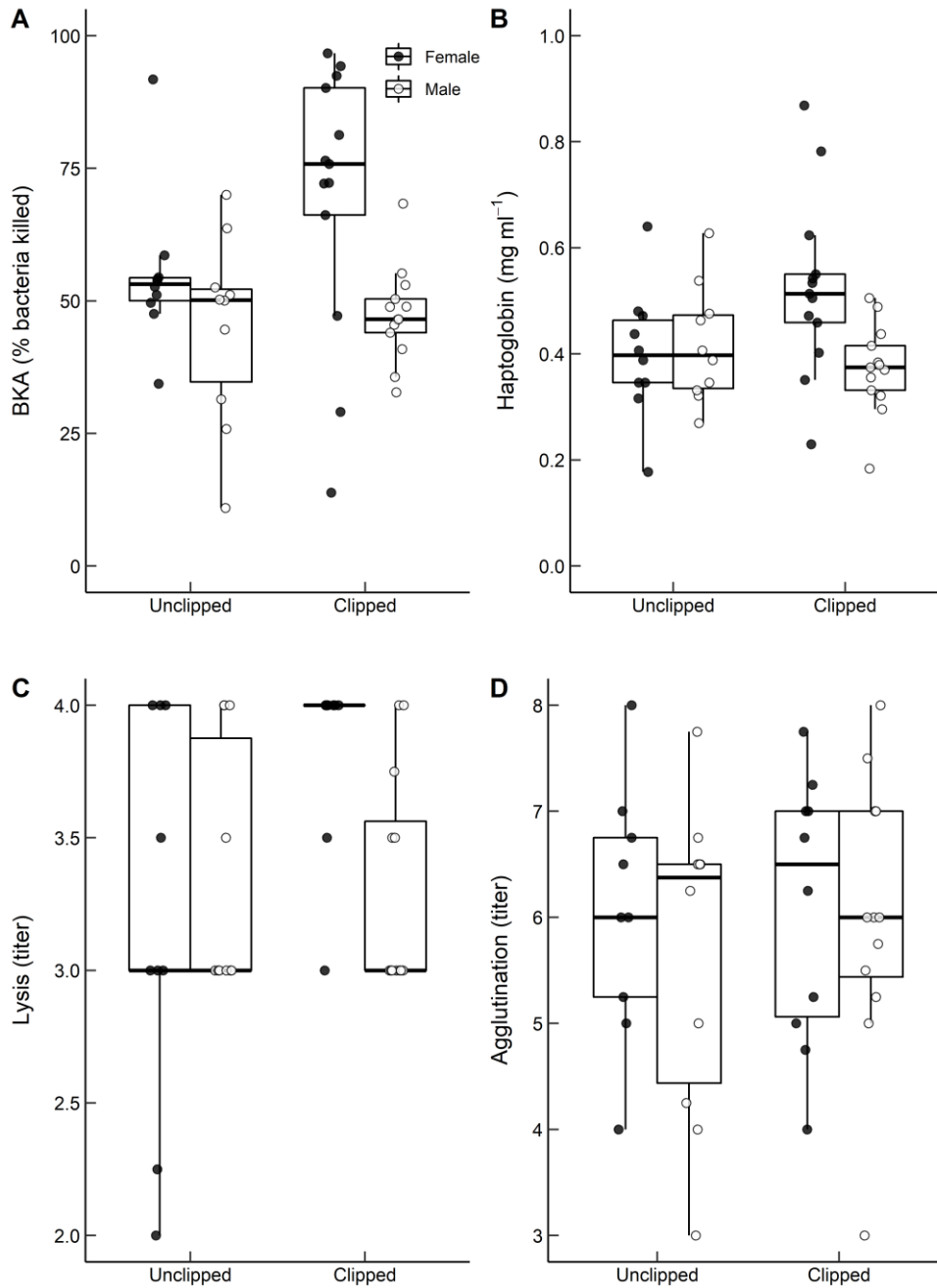
**Table S1**. Estimates, test statistics and significance values from models on biometrics on day 14 of blue tit nestlings whose parents were either feather-clipped to increase heat loss rate or were left unclipped. Significance values where p<0.05 are given in bold.

| Variable | Estimate (s.e.m.) | d.f. | $F/\chi^2$ | $P$ |
|---|---|---|---|---|
| **Body mass day 14 (g)** | | | | |
| Treatment | | 1, 17.9 | 2.2 | 0.16 |
| Body mass day 6 | 0.72 (0.05) | 1, 308.7 | 207.3 | **<0.001** |
| Julian date | -0.03 (0.05) | 1, 18.2 | 0.3 | 0.57 |
| Female age | | 1, 17.9 | 1.4 | 0.25 |
| Male age | | 1, 18.0 | 0.4 | 0.55 |
| Nest-box (random) | | 1 | 236.9 | **<0.001** |
| **Wing length day 14 (mm)** | | | | |
| Treatment | | 1, 17.9 | 0.3 | 0.57 |
| Body mass day 6 | 2.67 (0.11) | 1, 306.2 | 611.6 | **<0.001** |
| Julian date | 0.15 (0.12) | 1, 18.1 | 1.4 | 0.25 |
| Female age | | 1, 17.9 | 0.9 | 0.35 |
| Male age | | 1, 18.0 | 0.8 | 0.40 |
| Nest-box (random) | | 1 | 305.9 | **<0.001** |
| **Tarsus length day 14 (mm)** | | | | |
| Treatment | | 1, 17.8 | 0.0 | 0.87 |
| Body mass day 6 | 0.42 (0.04) | 1, 316.7 | 128.6 | **<0.001** |
| Julian date | 0.00 (0.02) | 1, 18.5 | 0.0 | 0.88 |
| Female age | | 1, 17.7 | 1.7 | 0.20 |
| Male age | | 1, 17.9 | 2.9 | 0.11 |
| Nest-box (random) | | 1 | 103.3 | **<0.001** |

**Table S2**. Sample size and summary statistics for number of feeding visits made during each hour of the day by female and male blue tits in the clipped and unclipped group.

| Hour | Sex | Treatment | Sample size | Mean no. feeding visits | s.e.m. |
|---|---|---|---|---|---|
| 04 | Female | Clipped | 10 | 8.80 | 1.97 |
| 04 | Female | Unclipped | 11 | 11.18 | 0.90 |
| 04 | Male | Clipped | 10 | 10.50 | 1.56 |
| 04 | Male | Unclipped | 13 | 7.15 | 1.16 |
| 05 | Female | Clipped | 13 | 21.23 | 2.87 |
| 05 | Female | Unclipped | 14 | 29.93 | 2.53 |
| 05 | Male | Clipped | 13 | 23.62 | 2.64 |
| 05 | Male | Unclipped | 14 | 25.00 | 1.74 |
| 06 | Female | Clipped | 13 | 20.38 | 2.56 |
| 06 | Female | Unclipped | 14 | 29.36 | 2.53 |
| 06 | Male | Clipped | 13 | 23.15 | 2.09 |
| 06 | Male | Unclipped | 14 | 25.79 | 1.62 |
| 07 | Female | Clipped | 13 | 23.54 | 2.94 |
| 07 | Female | Unclipped | 14 | 29.21 | 1.86 |
| 07 | Male | Clipped | 14 | 23.50 | 2.43 |
| 07 | Male | Unclipped | 14 | 24.21 | 1.79 |
| 08 | Female | Clipped | 13 | 22.23 | 2.36 |
| 08 | Female | Unclipped | 14 | 27.86 | 1.77 |
| 08 | Male | Clipped | 14 | 24.64 | 1.90 |
| 08 | Male | Unclipped | 14 | 23.71 | 2.18 |
| 09 | Female | Clipped | 13 | 21.31 | 2.27 |
| 09 | Female | Unclipped | 14 | 29.50 | 1.84 |
| 09 | Male | Clipped | 14 | 23.57 | 1.36 |
| 09 | Male | Unclipped | 14 | 24.50 | 1.78 |
| 10 | Female | Clipped | 13 | 20.38 | 2.48 |
| 10 | Female | Unclipped | 14 | 28.41 | 1.96 |
| 10 | Male | Clipped | 14 | 20.79 | 2.03 |
| 10 | Male | Unclipped | 14 | 23.56 | 1.84 |
| 11 | Female | Clipped | 13 | 23.10 | 2.72 |
| 11 | Female | Unclipped | 13 | 26.16 | 2.25 |
| 11 | Male | Clipped | 13 | 25.26 | 1.69 |
| 11 | Male | Unclipped | 13 | 21.87 | 1.36 |
| 12 | Female | Clipped | 11 | 24.15 | 1.52 |
| 12 | Female | Unclipped | 9 | 26.63 | 2.37 |
| 12 | Male | Clipped | 12 | 24.99 | 2.18 |
| 12 | Male | Unclipped | 10 | 23.94 | 1.95 |
| 13 | Female | Clipped | 13 | 23.16 | 1.94 |
| 13 | Female | Unclipped | 12 | 27.98 | 1.90 |
| 13 | Male | Clipped | 14 | 26.47 | 1.91 |

| 13 | Male | Unclipped | 12 | 22.42 | 1.47 |
|----|------|-----------|----|-------|------|
| 14 | Female | Clipped | 13 | 23.85 | 1.85 |
| 14 | Female | Unclipped | 13 | 28.00 | 2.42 |
| 14 | Male | Clipped | 14 | 23.07 | 1.62 |
| 14 | Male | Unclipped | 14 | 21.97 | 2.18 |
| 15 | Female | Clipped | 13 | 22.00 | 1.58 |
| 15 | Female | Unclipped | 14 | 26.04 | 1.59 |
| 15 | Male | Clipped | 14 | 22.07 | 1.76 |
| 15 | Male | Unclipped | 14 | 22.50 | 2.17 |
| 16 | Female | Clipped | 13 | 20.77 | 2.14 |
| 16 | Female | Unclipped | 14 | 23.79 | 2.40 |
| 16 | Male | Clipped | 14 | 23.86 | 1.50 |
| **16** | Male | Unclipped | 14 | 20.64 | 1.83 |
| 17 | Female | Clipped | 13 | 20.62 | 1.82 |
| 17 | Female | Unclipped | 14 | 24.71 | 2.18 |
| 17 | Male | Clipped | 14 | 21.79 | 1.70 |
| 17 | Male | Unclipped | 14 | 21.43 | 1.73 |
| 18 | Female | Clipped | 13 | 19.23 | 1.87 |
| 18 | Female | Unclipped | 14 | 25.14 | 2.05 |
| 18 | Male | Clipped | 14 | 21.29 | 1.10 |
| 18 | Male | Unclipped | 14 | 22.79 | 1.51 |
| 19 | Female | Clipped | 13 | 18.08 | 1.89 |
| **19** | Female | Unclipped | 14 | 22.86 | 2.77 |
| 19 | Male | Clipped | 14 | 21.00 | 1.63 |
| 19 | Male | Unclipped | 14 | 20.14 | 1.71 |
| 20 | Female | Clipped | 13 | 11.85 | 1.79 |
| 20 | Female | Unclipped | 13 | 17.38 | 2.94 |
| 20 | Male | Clipped | 14 | 13.79 | 2.26 |
| 20 | Male | Unclipped | 13 | 9.00 | 1.48 |
| 21 | Female | Clipped | 9 | 8.56 | 1.71 |
| 21 | Female | Unclipped | 10 | 5.30 | 0.94 |
| 21 | Male | Clipped | 6 | 7.17 | 2.34 |
| 21 | Male | Unclipped | 5 | 3.20 | 1.32 |