VARIATION IN PLASMA OXIDATIVE STATUS AND TESTOSTERONE LEVEL IN RELATION TO EGG-EVICTION EFFORT AND AGE OF BROOD-PARASITIC COMMON CUCKOO NESTLINGS

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Abstract. To avoid competition for parental care, brood-parasitic Common Cuckoo (*Cuculus canorus*) nestlings evict all of the host's eggs and nestlings within a few days after hatching. Little is known about the physiological effects of eviction behavior on the cuckoo nestling's oxidative balance or about age-related variation in plasma oxidative status and testosterone level of developing birds. We examined whether the cuckoo nestling's plasma oxidative status was related to prior effort in eviction and quantified variation in the level of reactive oxygen metabolites, of nonenzymatic antioxidant capacity, and of testosterone concentration in plasma at various phases of the cuckoo's development. Levels of both reactive oxygen metabolites and antioxidant capacity were greater in older than in younger nestlings, suggesting that younger nestlings effectively counterbalance their increased production of free radicals, whereas, near fledging, levels of reactive oxygen metabolites increase despite improved antioxidant capacity. Possibly, overall energy expenditure increases with age and elevates the production of reactive oxygen species to a rate higher than what the antioxidant system could eliminate. Plasma testosterone level was the highest at nestlings' intermediate phase of growth. High levels of testosterone may be required during the period of fastest growth, and when the growth rate levels off near fledging, testosterone levels may also decline. Cuckoo chicks that evicted more host eggs from steeper nests had higher plasma levels of reactive oxygen metabolites shortly after the eviction period, suggesting that eviction is costly in terms of an increased level of oxidative stress.

Key words: antioxidant capacity, brood parasitism, eviction behavior, oxidative stress, plasma testosterone.

Variación en el Estatus Oxidativo del Plasma y Niveles de Testosterona en Relación al Esfuerzo de Desalojo de Huevos y Edad de los Pichones Parásitos de Nidada de *Cuculus canorus*

Resumen. Para evitar la competencia por el cuidado parental, los polluelos parásitos de nidada de *Cuculus canorus* desalojan todos los huevos y los polluelos del hospedador a los pocos días después de la eclosión. Se sabe poco sobre los efectos fisiológicos del comportamiento de desalojo en el balance oxidativo de los polluelos de *C. canorus* o sobre la variación en el estatus oxidativo del plasma y el nivel de testosterona relacionado con la edad de las aves en desarrollo. Examinamos si el estatus oxidativo del plasma de los polluelos de *C. canorus* se relacionaba con un esfuerzo previo de desalojo y cuantificamos la variación en el nivel de metabolitos reactivos de oxígeno, la capacidad antioxidante no enzimática y la concentración de testosterona en el plasma en varias fases del desarrollo de *C. canorus*. Tanto los niveles de metabolitos reactivos de oxígeno como la capacidad antioxidante fueron superiores en los polluelos de mayor edad que en los más jóvenes, lo que sugiere que los polluelos de menor edad contrarrestan eficazmente el aumento de la producción de radicales libres, mientras que, cuando se aproximan al abandono del nido, los niveles de metabolitos reactivos de oxígeno aumentan a pesar de una mejora en la capacidad antioxidante. Posiblemente, el gasto total de energía se incrementa con la edad, elevándose la producción de formas reactivas de oxígeno a una tasa mayor de la que el sistema antioxidante puede eliminar. El nivel de testosterona en el plasma fue máximo en la fase intermedia del crecimiento de los polluelos. Pueden requerirse altos niveles de testosterona durante el período de mayor crecimiento

y, cuando la tasa de crecimiento se estabiliza cerca del abandono del nido, los niveles de testosterona también podrían disminuir. Los polluelos de *C. canorus* que desalojaron más huevos del hospedador en nidos con una estructura más empinada tuvieron niveles de plasma de metabolitos reactivos de oxígeno en plasma más altos poco después del período de desalojo, sugiriendo que el desalojo es costoso en términos de un incremento en el nivel de estrés oxidativo.

INTRODUCTION

Avian interspecific brood parasites, including the Common Cuckoo (*Cuculus canorus*), lay their eggs in the nests of other bird species, and the foster parents incubate the parasitic egg and provision the foreign young at the cost of all or some of their own reproductive success (Wyllie 1981, Davies 2000). For the cuckoo, hatching earlier than the host's young is advantageous as it gives the parasite a competitive advantage (Davies 2000). Cuckoos' rate of embryonic development is generally greater than that of the host's young (Honza et al. 2001), although this brood parasite's advanced hatching may also be the result of internal incubation before laying (Birkhead et al. 2011). Cuckoo nestlings are able to evict the host's eggs or hatchlings over the rim of the nest while they are still blind and relatively small, typically between 1 and 3 days of age (Wyllie 1981, Davies 2000, Honza et al. 2007). The eviction behavior of the cuckoo nestling is considered to be a strategy to eliminate future competitors from the nest (Kilner 2005, Grim et al. 2009a, Hauber and Moskát 2008, Moskát and Hauber 2010). Consequently, cuckoo chicks can receive more care from their foster parents and are able to grow and fledge faster and survive better (Martín-Gálvez et al. 2005, Hauber and Moskát 2008, Grim et al. 2009b, Geltsch et al. 2012).

However, eviction behavior is presumed to entail a high energetic cost for the cuckoo nestling, as it reduces its rate of mass growth during and immediately after the eviction phase (Anderson et al. 2009, Grim et al. 2009b), although the energetic costs of this behavior have not been measured directly. There may also be a trade-off between eviction and other activities of the cuckoo chick, namely, begging and receiving feedings, which could result in starvation of the evictor nestling (Soler 2002, Grim et al. 2009b). Nevertheless, evictor cuckoo nestlings increase their growth rate after the eviction period and by fledging reach a mass similar to that of experimentally manipulated solitary cuckoo nestlings, as predicted by the compensatory-growth hypothesis (Anderson et al. 2009, Grim et al. 2009b).

There could also be other, previously undocumented physiological costs associated with eviction behavior. As evicting several eggs out of a deep nest is presumably an energetically costly and time-consuming physical activity (Anderson et al. 2009, Grim et al. 2009b, 2011), it may impose an oxidative challenge to the cuckoo. Severe physical exercise increases oxygen consumption and may elevate mitochondrial generation of reactive oxygen species (ROS) because of the need for greater ATP production (Ji 1999, Leeuwenburgh and Heinecke 2001, Jackson 2008). The exercise-induced elevated production of ROS may disturb the delicate balance between antioxidants and prooxidants (Ji 1999, Leeuwenburgh and Heinecke 2001, Chevion et al. 2003, Costantini et al. 2008). Overproduction of ROS could cause oxidative damage to such important macromolecules as lipids, proteins, and nucleic acids (Finkel and Holbrook 2000, Halliwell and Gutteridge 2007), and the peroxidation of biological macromolecules leads to the production of reactive oxygen metabolites (ROMs; Beckman and Ames 1998). These compounds could accumulate if the antioxidant system is not capable of controlling their generation by neutralizing the ROS produced or of removing them from the body through antioxidant enzymatic activity (Halliwell and Gutteridge 2007). Animals cope with ROS by means of endogenously produced and dietary antioxidants (Surai 2002, Halliwell and Gutteridge 2007), and the antioxidant defenses could be upregulated in response to a more intense production of ROS (Monaghan et al. 2009, Costantini 2010). Oxidative stress results from the imbalance between the production of ROS and the capacity of the antioxidant defenses in favor of the former, consequently increasing the rate of generation of oxidative damage (Sies 1991, Halliwell and Gutteridge 2007, Costantini and Verhulst 2009).

The cuckoo nestling's intensive begging behavior (Davies et al. 1998, Kilner et al. 1999, Butchart et al. 2003) and rapid growth after the eviction period (Anderson et al. 2009, Grim et al. 2009b) could be stimulated by the steroid hormone testosterone. Birds start to produce testosterone shortly after or even before hatching (Tanabe et al. 1986, Williams et al. 1987, Adkins-Regan et al. 1990, Goymann et al. 2005). The level of testosterone in the plasma of nestlings can be associated with begging behavior (Goodship and Buchanan 2006, Quillfeldt et al. 2006), level of aggression (Groothuis and Ros 2005), and competitive abilities (Sasvári et al. 1999, Kozlowski and Ricklefs 2011). Testosterone maternally deposited in the yolk increases the rate of postnatal growth (Schwabl 1996, Eising et al. 2001, Gil 2008), although the results for the association between plasma testosterone levels and nestlings' growth rate are ambiguous (Goodship and Buchanan 2006, Quillfeldt et al. 2006, Buchanan et al. 2007, Fargallo et al. 2007). In turn, a high level of circulating testosterone could have detrimental effects as well, as it may increase metabolic rate (Hänssler and Prinzinger 1979, Buchanan et al. 2001), increase production of ROS, and potentially generate oxidative stress (von Schantz et al. 1999, Alonso-Alvarez et al. 2007). Additional adverse effects include androgen-induced immunosuppression (Folstad and Karter 1992, Naguib et al. 2004, Fargallo et al. 2007). However, some recent studies also suggest that testosterone increases the bioavailability of some dietary antioxidants (Blas et al. 2006, McGraw et al. 2006, Noguera et al. 2011) and may reduce the level of oxidative damage (Noguera et al. 2011).

In this study, we tested for the first time whether the eviction effort of cuckoo nestlings is related to the oxidative status of their plasma. Cuckoo nestlings start to evict the host's eggs or chicks 1–2 days after hatching (Honza et al. 2007, Grim et al. 2009b; pers. obs.), and the eviction instinct disappears when the cuckoo is 4–5 days old (Wyllie 1981, Davies 2000, Anderson et al. 2009, Grim et al. 2009b). Therefore, we analyzed plasma levels of compounds representing the intermediate step of oxidative damage (ROMs) and nonenzymatic antioxidant capacity of plasma by the assay of the ferric reducing ability of plasma (FRAP, hereafter "plasma antioxidant capacity") shortly after the eviction period (days 5–6) in relation to prior eviction effort. We expected that cuckoo nestlings that made a greater effort in eviction should experience a higher level of oxidative stress than nestlings that made less effort. Alternatively, cuckoo nestlings may be able to cope with the elevated production of ROS associated with the greater eviction effort because the antioxidant defense systems of evictor brood parasites could have evolved to improve quickly in response to an acute increase in physical activity.

We also quantified variation in the plasma level of ROMs, plasma antioxidant capacity, and plasma testosterone concentration in three phases of the development of cuckoo nestlings: (1) shortly after the eviction period (days 5–6), (2) during the intensive, linear phase of growth (days 8–9), and (3) when the growth rate slows down and reaches a plateau near fledging (days 14–16; Kleven et al. 1999, Grim 2006, Anderson et al. 2009). Data on age-specific variation in oxidative status and plasma testosterone level could provide insights into how the various phases of nestling development are related to nestlings' physiological ontogeny. Data on these aspects of physiological measures during nestling development are relatively scarce in wild birds (Williams et al. 1987, Costantini et al. 2006, Kozlowski and Hahn 2010, Noguera et al. 2011). During the intensive, linear phase of mass growth, we expected the level of oxidative stress to be high because of the presumed relationship between the high metabolic rate required for fast growth (Vleck and Vleck 1980, Dietz and Drent 1997, Vézina et al. 2009) and elevated production of ROS accompanying a high metabolic rate (Monaghan et al. 2009, Metcalfe and Alonso-Alvarez 2010; but see Barja 2007, Hulbert et al. 2007). Moreover, we expected antioxidant capacity to increase with age as a result of the maturation of the antioxidant system (Blount et al. 2003) and the upregulation of antioxidant defenses because of the high rate of production of ROS (Radak et al. 2008, Monaghan et al. 2009, Costantini 2010). In addition, nestlings may use more antioxidants when prooxidants are more abundant, which may also result in lower plasma antioxidant capacity in younger nestlings. Finally, we expected higher levels of plasma testosterone during the period of intensive growth to stimulate begging behavior and increase growth rate, but as the maintenance of a high level of plasma testosterone is presumably costly, we expected its circulating level to decline by the end of the period of rapid growth.

METHODS

FIELD PROCEDURES

We studied cuckoo nestlings that hatched in the nests of Great Reed Warblers (*Acrocephalus arundinaceus*) around Apaj and Kiskunlacháza (47° 07′ N, 19° 06′ E), central Hungary, in 2009. The Great Reed Warbler is a well-known, relatively large cuckoo host, and cuckoo nestlings develop faster and reach a greater mass at fledging in nests of this species than in nests of smaller hosts (Kleven et al. 1999, Butchart et al. 2003). The eggs of the cuckoo and Great Reed Warbler differ in shape and shell thickness (Hargitai et al. 2010, Bán et al. 2011, Igic et al. 2011), but their volumes are similar (Honza et al. 2001, Hargitai et al. 2010). Cuckoos generally evict Great Reed Warbler eggs successfully: Molnár (1939) recorded only few cases of cuckoo hatchlings being unsuccessful in their attempts to evict eggs from the nests of this host, and those cuckoos typically became exhausted during the attempt and died after a few days. In our study area, Great Reed Warblers breed in reed beds 2–4 m wide along small channels, and typically more than 50% of the nests are parasitized by cuckoos (Moskát and Honza 2002).

We measured the depth of the nest cup with a ruler (to the nearest 5 mm) in the middle of the nest. We took two measurements of nest cup's diameter perpendicular to each other at the rim of the nest (to the nearest 5 mm), then averaged them. Following Grim et al. (2009b), we calculated the steepness of the nest cup as the ratio between the cup's depth (range 50–85 mm) and average diameter (range 80–115 mm). Thus higher values indicate steeper nest cups, which reduce the success of eviction behavior (Grim et al. 2009b, 2011). We quantified the eviction effort of a cuckoo nestling by multiplying the number of evicted host eggs (range 2–5) and the nest cup's steepness index. Most cuckoos completed the displacement of the host's eggs or chicks at an age of 1–3 days, usually within one day. Cuckoos generally fledged at the age of 18–22 days.

When nestlings were 5–6, 8–9, and 14–16 days old, we took a blood sample from the leg or wing vein (one cuckoo nestling was bled at 7 days, and another at 12 days of age, instead of 8–9 and 14–16, respectively) with heparinized capillary tubes (see justification of age categories in the Introduction; assigning the data point of the nestling bled at 12 days of age to the nestling-age category of 8–9 days did not change our conclusions). The samples were kept cool (0–5 °C) until centrifugation, which was completed within 7 hr at 2720×g for 20 min. Plasma was frozen at −20° C for a few days in the field, then stored at −50° C for 6 months until laboratory analyses; levels of ROMs and plasma antioxidant capacity have

been shown to be stable under these storage conditions (Cavalleri et al. 2004, Cohen et al. 2007, van de Crommenacker et al. 2011). We collected samples from 21 cuckoo chicks, but sample sizes for different blood measures and age categories vary because of the limited amount of plasma collected and the loss of subjects due to the predation of some nestlings before they reached the age of 14–16 days. The Middle Danube Valley Inspectorate for Environmental Protection, Nature Conservation, and Water Management kindly provided permission for field work (permission no. 31873-2/2009).

MEASUREMENT OF REACTIVE OXYGEN METABOLITES

We measured the plasma level of ROMs (primarily hydroperoxides) by the d-ROM assay (Diacron International, Grosseto, Italy) according to protocols from previous studies (Costantini et al. 2006, van de Crommenacker et al. 2010). ROMs are compounds reflecting an intermediate step of oxidative damage and are generated by the peroxidation of biomolecules such as lipids, proteins, and nucleic acids, in the early phase of the oxidative cascade (Beckman and Ames 1998, Alberti et al. 2000, Halliwell and Gutteridge 2007). For example, some studies of birds have found that the level of ROMs increases as a consequence of immune challenge (Costantini and Dell'Omo 2006, van de Crommenacker et al. 2010) and are related positively to oxygen consumption (van de Crommenacker et al. 2010).

We added 10 µL of plasma to the wells and diluted it with 200 µL of a solution of a chromogenic mixture (aromatic alkyl-amine) and acetate buffer (pH 4.8). The plate was incubated for 75 min at 37 °C. After incubation, we read the absorbance with a microplate spectrophotometer (Thermo Multiskan Spectrum, Thermo Fisher Scientific, Inc.) at 505 nm. We calculated the level of ROMs in plasma from a calibration curve obtained by measuring the absorbance of a serial dilution of a standard solution (0–5.64 mM H_2O_2 equivalents). We omitted some samples of plasma from the analysis as they were turbid. The mean intraplate coefficient of variation (CV) was 5.2% ($n = 4$ duplicates), while the mean interplate CV was 7.4% ($n = 5$ duplicates).

FRAP TEST

The FRAP test estimates the ferric-reducing ability of plasma, providing a measure of the antioxidant potential of circulating free-radical scavengers (Benzie and Strain 1996, Vassale et al. 2004). At low pH, when the ferric-tripyridyl-triazine (Fe^{+3}-TPTZ) complex is reduced to the ferrous form (Fe^{2+}), a blue color develops. The color's intensity is positively proportional to the antioxidant capacity of the plasma sample. On the basis of samples of human plasma, uric acid, ascorbic acid, proteins and α-tocopherol are estimated to contribute 60%, 15%, 10%, and 5% to the total FRAP value, respectively (Benzie and Strain 1996). However, the FRAP assay does not measure enzymatic antioxidants, and the contributions of glutathione and protein thiols are estimated to be relatively low (Benzie and Strain 1996, Janaszewska and Bartosz 2002).

The FRAP reagent and the standard solution were prepared on the day of analysis. The FRAP reagent is composed of a Fe^{3+} solution (20 mM ferric chloride), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine), and acetate buffer (pH 3.6) in a volume ratio of 1:1:10. For the standard calibration curve, we dissolved 0.0834 g of ferrous sulfate heptahydrate in 100 mL distilled water to yield a 3-mM Fe^{2+} standard stock solution and diluted it with distilled water to make a series of standards (1.5, 0.75, 0.5, 0.25, 0.1 mM). We diluted plasma samples 1:1 with distilled water and added 5 µL plasma in duplicate to the wells of a 96-well microplate. Then, we pipetted 15 µL distilled water and 150 µL FRAP reagent to each well. Plates were incubated for 20 min at room temperature, and absorbance was read at 593 nm with a microplate spectrophotometer (see above). We express the measurements as mM of Fe^{2+} equivalents in reference to the standard curve. On the basis of the duplicates of all samples, the mean intraplate CV was 3.7%.

PLASMA URIC ACID ASSAY

Uric acid is birds' main nitrogenous waste product (Wright 1995), but it also has antioxidant properties (Machín et al. 2004, Stinefelt et al. 2005). Therefore, the interpretation of a high level of uric acid is difficult, as it could indicate elevated amino acid and purine catabolism (Hollmén et al. 2001) as well as enhanced antioxidant protection.

We estimated the plasma concentration of uric acid with a Uric Acid Assay Kit (BioVision Research Products, Mountain View, CA). To each well, we added 5 µL plasma, 45 µL buffer, and 50 µL reaction mix (46 µL uric acid assay buffer, 2 µL uric acid probe, 2 µL uric acid enzyme mix). Plates were incubated for 30 min at 37 °C. We read absorbance at 570 nm with a microplate spectrophotometer (see above) and calculated uric acid concentration from the calibration curve of the serial dilution of the standard. Mean intraplate CV was 2.1% ($n = 15$ duplicates).

PLASMA TESTOSTERONE ASSAY

Plasma samples were extracted by a liquid-extraction protocol with diethyl ether (95% extraction recovery) and assayed in a Cayman Testosterone EIA Kit in duplicate. The standard curve provided a linear region (1.95–500 pg mL^{-1}) where the readings were taken. We checked the linearity of the EIA reaction by means of a dilution series derived from a mix of pooled samples. On the basis of duplicates of all samples mean intraplate CV was 16.5%, while the mean interplate CV was 11.5%.

STATISTICAL TESTS

As plasma uric acid concentration strongly positively correlated with FRAP value in all age categories ($F_{1,28.76} = 643.60$, $P < 0.001$, estimate = 0.33; age interaction: $P = 0.22$), we

included uric acid as a covariate in the analyses of FRAP in order to control for its effect. Controlling for uric acid concentration is important for distinguishing FRAP from the effects of protein and purine catabolism, which are not related to antioxidant capacity but may affect uric acid concentration (Hollmén et al. 2001, Cohen et al. 2007, Costantini 2011). Therefore we used FRAP values as indicators of nonenzymatic antioxidant capacity resulting from circulating compounds other than uric acid (e.g., ascorbic acid, proteins, tocopherols; see Benzie and Strain 1996).

We applied general linear mixed models (GLMM) with the cuckoo's identity as a random factor, using maximum-likelihood estimation, which is considered more suitable for mixed models than is restricted maximum-likelihood estimation (Singer and Willett 2003). In the analysis of eviction effort, we present the parameter estimate for the fixed effect (eviction effort) of the mixed model, which can be interpreted in the same way as the regression coefficient of a traditional linear regression. We categorized age into three classes, 5–6 days old, 8–9 days old, and 14–16 days old, and included it as a fixed factor in the models. Because sample sizes were relatively small, we could not fit random slopes in the model (Grueber et al. 2011), only random intercepts. We initially included date of hatching and time of bleeding in these models, but neither had a significant effect on blood variables (hatch date: all $P > 0.08$; time of bleeding: all $P > 0.14$), so we omitted them from the final analyses. For all tests we used SPSS 17.0 (SPSS, Inc., Chicago) and prepared the figures in Statistica 5.0 (StatSoft, Inc., Tulsa, OK).

RESULTS

We found that those cuckoo chicks that removed more host eggs from steeper nests had at 5–6 days of age levels of ROMs that were higher than those of similar-aged cuckoos that made less effort ($F_{1,8} = 38.47$, $P < 0.001$, estimate: 0.22; Fig. 1). The plasma antioxidant capacity of 5- to 6-day-old cuckoo chicks showed no significant relationship with prior eviction effort ($F_{1,8} = 0.08$, $P = 0.79$, estimate: −0.03; Fig. 2).

Cuckoo nestlings' plasma antioxidant capacity and level of ROMs increased from ages 5–6 days to 14–16 days (FRAP: $F_{2,23.22} = 7.05$, $P = 0.004$; ROM: $F_{2,16.87} = 9.26$, $P = 0.002$; Figs. 3a,b). Pairwise comparisons of GLMM (least-significant-difference test) revealed that neither variable differed statistically between days 5–6 and 8–9 (all $P > 0.19$) but those for the last age category differed significantly from the earlier ones (all $P < 0.003$). That is, 14- to 16-day-old cuckoo chicks had plasma antioxidant capacity and levels of ROMs greater than those of younger cuckoo chicks. Plasma testosterone concentration varied significantly with the age of nestling cuckoos ($F_{1,17} = 4.27$, $P = 0.03$; Fig. 3c). Pairwise comparisons of GLMM (least-significant-difference test) revealed that testosterone level was the highest in the intermediate period of nestling development and lower at ages
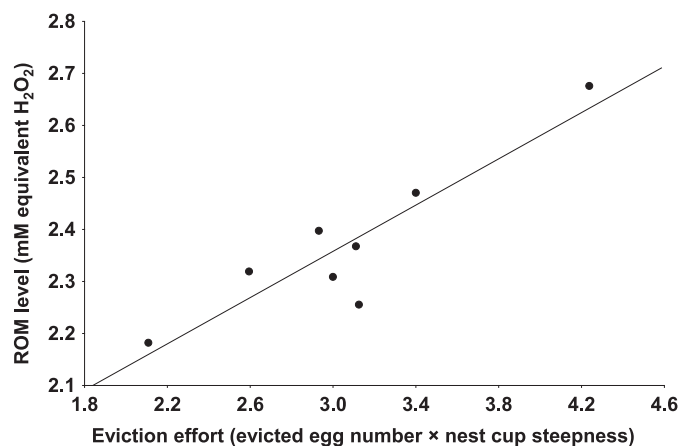

FIGURE 1.   Relationship between level of ROMs (reactive oxygen metabolites) and eviction effort of 5- to 6-day-old Common Cuckoo nestlings. Eviction effort was calculated as the product of the number of host eggs removed and the index of the nest cup's steepness (depth/diameter).

5–6 days and 14–16 days (pairwise comparisons, 5–6 and 8–9 days: $P = 0.01$; 5–6 and 14–16 days: $P = 0.61$, 8–9 and 14–16 days: $P = 0.02$).

DISCUSSION

In this study, we found that those Common Cuckoo nestlings that engaged in greater eviction effort (i.e., evicted more Great Reed Warbler eggs from steeper nests) had higher levels of ROMs shortly after the eviction period than did cuckoo nestlings making a lesser effort. However, the plasma antioxidant
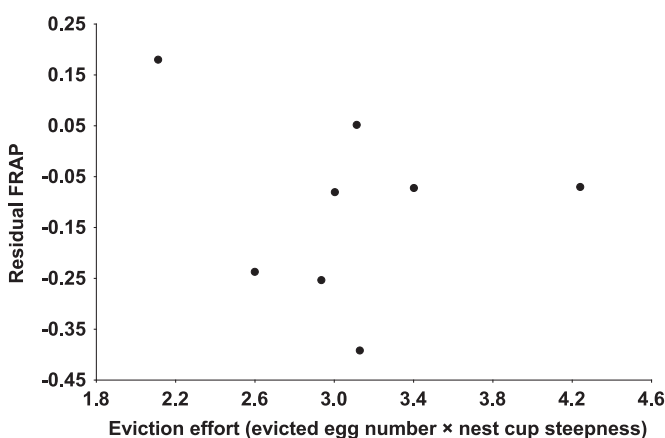

FIGURE 2.   Relationship between FRAP (plasma nonenzymatic antioxidant capacity) and eviction effort of 5- to 6-day-old Common Cuckoo nestlings. Eviction effort was calculated as the product of the number of host eggs removed and the index of the nest cup's steepness (depth/diameter). FRAP was controlled for covariation with uric acid concentration by residuals of a linear regression.
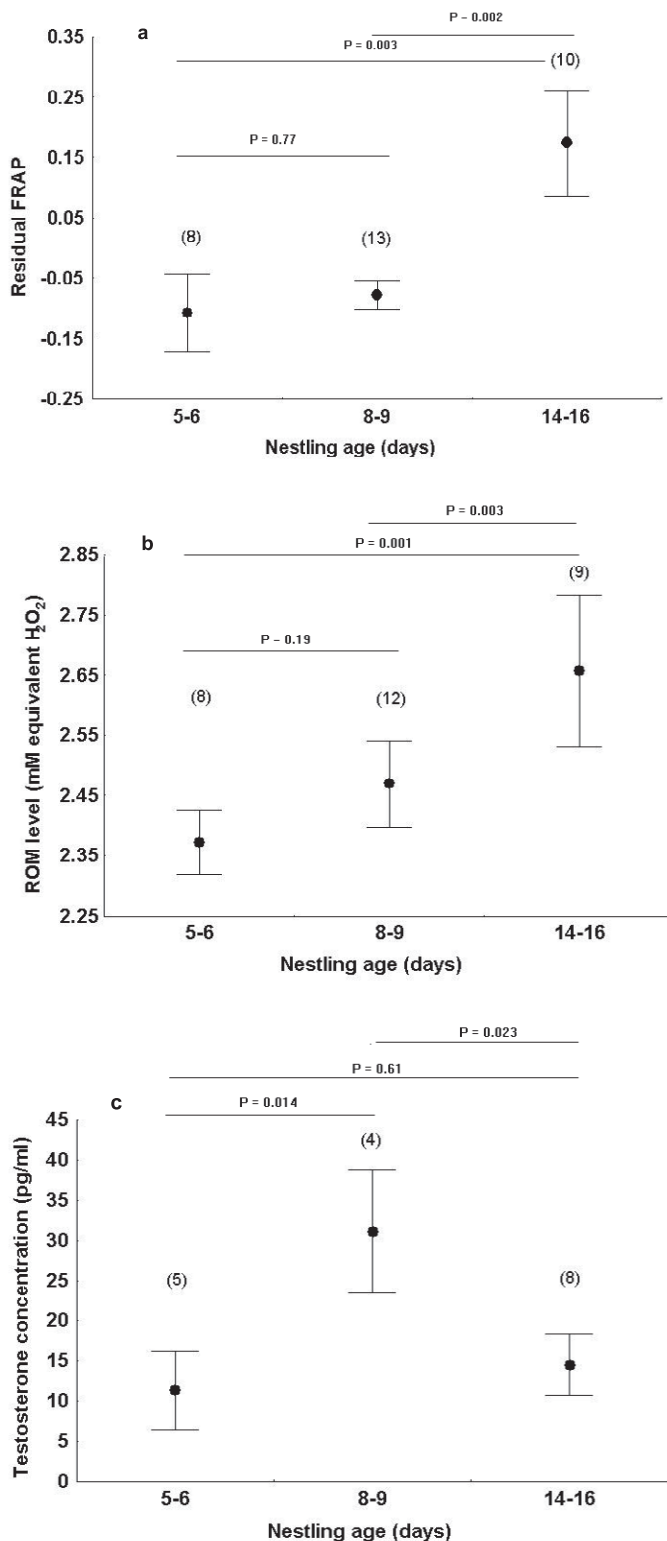
FIGURE 3.   Mean ± SE plasma (a) residual FRAP value (plasma non-enzymatic antioxidant capacity), (b) level of ROMs (reactive oxygen metabolites), and (c) testosterone concentration in brood-parasitic Common Cuckoo nestlings of three age categories. FRAP was controlled for covariation with uric acid concentration by residuals of a linear regression. Numbers of nestlings sampled are specified in parentheses.

capacity of cuckoo nestlings was not related to prior eviction effort. As evicting several eggs from a steep nest is presumed to be a physically demanding exercise, it likely increases aerobic metabolism and may be a potential source of oxidative stress for the cuckoo nestling (Ji 1999, Leeuwenburgh and Heinecke 2001, Chevion et al. 2003, Costantini et al. 2008). Our results suggest that the antioxidant system of the cuckoo chick is not capable of completely counterbalancing the elevated production of ROS related to increased physical exercise, and greater eviction effort can disturb the oxidative balance of the parasitic nestling. In addition to temporarily constraining mass growth (Anderson et al. 2009, Grim et al. 2009b), these correlative data suggest that eviction behavior has a physiological cost in terms of an increase in the level of oxidative stress of young brood-parasitic nestlings.

Younger cuckoo nestlings (days 5–6 and 8–9) had levels of circulating ROMs and plasma antioxidant capacity lower than those of older cuckoo nestlings (days 14–16), despite our expectation that a period of intensive linear growth should lead to more oxidative damage (Finkel and Holbrook 2000, Monaghan et al. 2009, Metcalfe and Alonso-Alvarez 2010). It is possible that young cuckoo nestlings are able to keep oxidative damage at a relatively low level by using circulating antioxidants, which in turn reduce their plasma antioxidant capacity. Egg yolk antioxidants, which are present in higher concentration in cuckoo eggs than in the hosts' eggs (Hargitai et al. 2010), may also improve the nestling's ability to use dietary antioxidants efficiently (Koutsos et al. 2003).

At the plateau phase of growth near fledging, cuckoo nestlings showed increased plasma antioxidant capacity, which could be the consequence of upregulated antioxidant defenses (Costantini 2008, Radak et al. 2008, Monaghan et al. 2009), the maturation of the antioxidant system (Blount et al. 2003), or higher availability of dietary antioxidants (Cohen et al. 2007). However, plasma level of ROM molecules was also higher in older nestlings, which could be the consequence of either overproduction of prooxidants or a noncompensating antioxidant system (Beckman and Ames 1998, Halliwell and Gutteridge 2007). During the linear, most rapid phase of nestling growth, energy is required mainly for tissue synthesis, while in the plateau phase of growth, energy is predominantly consumed by tissues functioning at high intensity (e.g., heart, kidney, liver; Vézina et al. 2009). Moreover, older cuckoo nestlings beg more intensely (Butchart et al. 2003), and begging is presumed to be an antioxidant-demanding activity (Helfenstein et al. 2008, Noguera et al. 2010, Boncoraglio et al. 2012). Thus it is possible that the overall energy expenditure of cuckoo nestlings increases with age, which could elevate production of ROS to a rate higher than what their antioxidant system can eliminate. This may lead to an increasing level of ROMs and so elevated oxidative stress with age despite the improved plasma antioxidant capacity of older nestlings.

Nevertheless, care is needed in interpreting our results, as our assay of antioxidant capacity does not measure enzymatic antioxidants, which also play an important role in maintaining

the redox balance (Godin and Garnett 1992, Berglund et al. 2007). In addition, different assays provide information on different components of the antioxidant system (Prior and Cao 1999, Del Rio et al. 2002, Cohen and McGraw 2009). Moreover, lower levels of some nonenzymatic antioxidants may be associated with higher levels of antioxidant enzymes and vice versa because of down- or upregulation of different components of the antioxidant machinery (Selman et al. 2006, Costantini et al. 2011). Therefore, we cannot rule out that the lower level of ROMs in younger cuckoo nestlings is due to elevated activity of antioxidant enzymes during the period of rapid development.

Our results imply that cuckoo nestlings produce more testosterone during the intermediate part of the linear phase of growth than at younger or older ages. High concentrations of plasma testosterone may improve begging behavior (Goodship and Buchanan 2006, Quillfeldt et al. 2006), which Butchart et al. (2003) showed to intensify with the age of cuckoo nestlings. At 14–16 days of age, when the growth rate slows down (Kleven et al. 1999, Anderson et al. 2009) and the level of oxidative stress is relatively high (this study), plasma testosterone level was lower. We hypothesize that maintaining a high level of testosterone throughout nestling development could be counterselected, as it may compromise immunocompetence (Naguib et al. 2004, Fargallo et al. 2007) and elevate oxidative stress (von Schantz et al. 1999, Alonso-Alvarez et al. 2007; but see Blas et al. 2006, McGraw et al. 2006). This may result in that a higher level of testosterone is required in the period of intensive growth, but when growth rate levels off near fledging, testosterone level declines.

In summary, we found that cuckoo nestlings that engaged in a greater eviction effort suffered from a higher level of oxidative stress shortly after the eviction period than did cuckoo nestlings making a lesser effort. Moreover, plasma oxidative status and testosterone level varied significantly through the cuckoo's nestling period. As our study was correlative and our sample sizes were relatively small, we suggest further experimental studies in which eviction effort is manipulated, which could provide more information on the possible physiological costs of the timing and mechanism of eviction behavior. It remains to be also elucidated whether the eviction behavior of other brood-parasitic species is costly in terms of an increase in oxidative stress.

ACKNOWLEDGMENTS

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