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Concentrations of plasma metabolites as predictors of nestling condition in the Great Cormorant (*Phalacrocorax carbo sinensis*)

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We investigated the reliability of basic plasma metabolite concentrations as predictors of body condition in the nestlings of the Great Cormorant (*Phalacrocorax carbo sinensis*), a species with marked hatching asynchrony and well-established intra-brood hierarchies. For this purpose we analyzed relationships between the levels of plasma proteins, triglycerides and glucose measured at the age of ca. 3 weeks with body mass, mass-size residuals, and growth rates of 185 Great Cormorant chicks from the colony in central Poland. Concentrations of all studied plasma metabolites correlated with at least one of body condition indices. We found concentration of plasma glucose to be the most robust predictor of body condition, as it was positively related with size-adjusted and unadjusted body mass, as well as with chick growth rates. The level of plasma proteins was found to be of limited practical applicability (positive relationship only with mass-tarsus residuals), while the usefulness of plasma triglycerides as an index of body condition was intermediate (positive relationships with mass-size residuals and growth rates). Predictive power of all significant relationships was low.

1. Introduction

Body condition is usually defined as the quantity of accumulated nutrient reserves that exceeds the daily nutritional demands of a bird (Schulte-Hostedde *et al.* 2005). One of the simplest indices of body condition that are used in avian studies is individual body mass. However, this parameter depends on the structural size of birds, which is likely to mask at least part of variation in condition among individuals. Therefore, size-adjusted body mass has been suggested as a more reliable measure of body condition in birds (Schulte-Hostedde *et al.* 2005). Scaling body mass by morphological characters may follow two alternative approaches, i.e., calculating ratios or residuals of body mass against the measure of structural size. In various taxa, size-adjusted and unadjusted body mass have been linked to the fitness components of life-histories, such as productivity and survival (Dobson 1992, Litzgus *et al.* 2008).

Mass-related indices of body condition may not be sufficiently sensitive to detect variation in body condition of birds from populations with high food availability, where even the short-term periods of starvation are infrequent. Similarly, the applicability of mass-related indices could be limited in taxa or during the annual stages, when birds do not accumulate fat stores that are directly reflected by mass differences. In such situations con-

centrations of various plasma metabolites have been suggested as alternative indicators of body condition (e.g., Jenni-Eiermann & Jenni 1998).

Numerous studies have revealed quantitative relationships between levels of certain plasma metabolites and food intake (e.g., Ferrer *et al.* 1987, Totzke *et al.* 1999). Blood parameters, such as the concentration of plasma triglycerides, have been suggested to reliably indicate short periods of food deprivation (Jenni-Eiermann & Jenni 1997). The concentrations of plasma proteins and glucose have also been found to correlate with the nutritional state of birds. The positive relationships of protein and glucose levels with body mass have been recorded, among others, in birds of prey (González & Hiraldo 1991, Ferrer & Dobado-Berrios 1998) and wading birds (de le Court *et al.* 1995).

On the other hand, many studies have not managed to confirm the relationships between levels of plasma metabolites and body condition (e.g., Migliorini *et al.* 1973, Jeffrey *et al.* 1985). Some studies have also demonstrated increased concentrations of plasma metabolites, such as glucose, during the periods of food deprivation (García-Rodríguez *et al.* 1987). Inconsistencies in these findings have been attributed to interspecific differences in the effectiveness of metabolic pathways (Ferrer *et al.* 1987).

As a consequence, appropriate validation of biochemical indices of avian body condition should be a necessary step before they may be applied in ecological studies. Furthermore, it would be of great practical value to determine robust biochemical indices of physical state in birds, which could be used across a range of taxa and for individuals at different stages of development or annual cycle. For this reason, it is of importance to verify the applicability of different biochemical parameters in avian groups for which no empirical data exist.

The objective of this study was to establish a reliable biochemical predictor of body condition in nestlings of the Great Cormorant (*Phalacrocorax carbo sinensis)*, a species with marked hatching asynchrony and well-established intrabrood hierarchies. For this purpose we examined the relationships between body mass, its size-adjusted residuals and growth rate of chicks with the concentrations of three plasma metabolites related to the metabolism of protein (total protein level), fat (triglycerides), and carbohydrates (glucose). We assumed that a robust biochemical indicator of body condition should correlate well with different mass-related parameters and growth rates of nestlings. Although numerous studies have verified the reliability of plasma metabolite concentrations to reflect body condition in free-living adult birds (e.g., Ferrer *et al.* 1987, de le Court *et al.* 1995), similar studies in nestlings are scarce (Dawson & Bortolotti 1997a, Geens *et al.* 2009).

Great Cormorant nestlings may be considered an appropriate model for this kind of validation, as they exhibit high variation in body condition during the entire pre-fledging period. Variation in physical condition of chicks is mainly attributed to asynchronous hatching, which produces asymmetries in competitive ability within broods. Nestlings hatched more recently in the hatching sequence have lower access to food delivered by parents and are frequently subjected to starvation, which leads to significant reductions in body mass and slower growth rates in comparison to older siblings. We are aware of no previous studies that have verified the applicability of plasma metabolite levels as predictors of body condition in Phalacrocoracidae species.

2. Material and methods

2.1. Study site and field data collection

Our study was conducted at Jeziorsko reservoir (51°73' N, 18°63' E), central Poland, during April–May 2010–2011. Great Cormorant nests were located on willow shrubs dominated by white (*Salix alba*) and grey willow (*S. cinerea*). Each year, we randomly selected 30 out of ca. 150 nests at the time of hatching to study. To establish hatching dates, we monitored all nests at 2–3 day intervals. We took blood samples for molecular sexing and measurements for establishing hatching ranks from all nestlings (N = 226) within two days of hatching of the last chick in a given nest. We took blood (ca. 10 μL) from the ulnar vein with a 0.5-mm needle, and suspended the sample immediately in 96% ethanol, stored for subsequent analysis. We took the following measurements: wing length (± 1 mm), culmen length and tarsus length (both ±

0.1 mm). We also weighed the chicks (± 1 g) using an electronic scale. We tagged all chicks on the tarsus with flexible Velcro™ strips of different colours. We adjusted these strips according to the size of chicks during successive visits. At the age of 13 days we marked all chicks with individually-numbered metal rings and removed the Velcro™ strips.

We repeated biometrical measurements of chicks in 3–5 day intervals, until the oldest chick in the brood reached the age of 22 ± 1 days (at least 5 measurements of each trait per chick). This was the latest possible moment allowing a successful collection of data, since chicks older than 25 days may jump out of the nests if humans approach (Platteeuw *et al.* 1995, authors' pers. obs.). During the last visit we also collected blood samples for biochemical analyses from all alive nestlings (N = 185). The mortality of chicks before the time of sampling was 18.1% (N = 226), mainly due to nest collapses and brood reductions. We took approximately 2 ml of blood from a brachial vein of each chick into EDTA tubes, which we stored in a cooler. We collected all blood samples between 9:00 AM and 8:00 PM. We centrifuged the samples at 3,000 rpm for 15 min within eight hours of collection. Plasma was separated from blood cells and kept at –20°C until analysis.

2.2. Hatching order

To determine hatching order, body measurements collected within two days of hatching (culmen length, tarsus length, wing length, and body mass) were reduced to the first principal component (PC1) of the Principal Component Analysis (PCA). We standardized all variables entered into the PCA to equal unit variances (z scores) prior to the analysis. The PC1 accounted for 96.1% of the variability in all chosen variables. All body measurements had similar contributions to PC1 (from 0.251 to 0.256). Hatching ranks were established based on size ranks assigned to each chick from PC1 values. Mean PC1 of chicks differed significantly among the first four hatching ranks (ANOVA, $F_{3, 220} = 44.77$, $P < 0.001$; Tukey *post hoc* test $P < 0.002$ for all pair-wise comparisons). PC1 of chicks having the fifth and the sixth hatching rank were not included in this analysis due to small sample sizes. A similar method of establishing hatching order has been used previously with different species of water birds that exhibit marked hatching asynchrony (e.g., Cash & Evans, 1986), including several species of cormorants (Williams & Cooper 1983, Shaw 1985, Stockland & Amundsen 1988).

2.3. Molecular sexing

We performed molecular sexing on DNA extracted with the Blood Mini kit (A&A Biotechnology, Gdynia, Poland) from the blood, after evaporation of alcohol. The chromohelicase-DNA binding protein (CHD) gene was amplified with the primer pair 2550F and 2718R (Fridolfsson & Ellegren 1999), according to the protocol described by Griffiths *et al.* (1998). We used a 50°C annealing temperature for PCR reaction. We separated PCR products (ca. 200 bp) by electrophoresis in a 2% agarose gel until differences in size were clearly visible.

2.4. Indices of body condition

We used nestling body mass, measured when the oldest chick in the brood reached 22 ± 1 days of age, as one indicator of body condition. Additionally, we used two size-adjusted measures of body mass to assess body condition. The first approach to scale body mass for structural size was to calculate mass residuals against the multivariate measure of structural size (PC1 of PCA). We calculated PC1 for culmen, tarsus, and wing length, all standardized to equal unit variances (z scores) prior to the analysis. PC1 accounted for 79.4% of variation in all chosen variables. All body measurements had similar contributions to PC1 (from 0.29 to 0.37). We also calculated residuals of body mass against a univariate biometric measure. For this purpose, we chose tarsus length, a measurement which is commonly used to calculate mass-size residuals in avian studies (Green 2001). Mass-tarsus residuals may explain more variation in nutritional stores than residuals calculated based on multivariate measures of body size in birds (Ardia 2005).

Apart from applying standard indicators of body condition, we also estimated growth rates of all nestlings. For this purpose we fitted logistic curves

Table 1. Characteristics of the Great Cormorant nestlings reared in the colony at the Jeziorsko reservoir, Poland. Within-brood consistency of brood characteristics measured by within-brood repeatability (R). Number of broods (*N*), adjusted mean brood size (n_0), ANOVA *F* statistics with 51 and 133 degrees of freedom are also given.

Variable	Mean ± SE	R	*N* (n_0)	*F*	*P*
Plasma protein (g/l)	32.35 ± 0.30	0.24	56 (3.30)	2.07	< 0.001
Plasma triglycerides (mg/dl)	125.94 ± 3.98	0.32	56 (3.30)	2.54	< 0.001
Plasma glucose (mg/dl)	381.63 ± 3.01	0.26	56 (3.30)	2.17	< 0.001
Body mass (g)	1,629.32 ± 19.99	0.19	56 (3.30)	1.78	0.004

$$y = A / [1 + B \times \exp(-KT)] \quad (1)$$

to the collected measurements of chick body mass, where y refers to the body measurement at age T, A is an asymptotic value, B is a constant of integration, and K is the growth-rate constant (Richner 1989). We used parameter K from fitted curves as an indicator of chick growth rate. Because we stopped collecting measurements at 22 ± 1 day of age of the oldest chick within a brood, i.e., when nestlings had not yet reached asymptotic values of their body mass, the parameter A of the equation was constrained with the expected mean fledgling body mass of male (2,379 g) or female (1,946 g) chicks (Liordos & Goutner 2008).

2.5. Biochemical analyses

We analyzed concentrations of the following plasma metabolites (methods in parentheses): total protein (biuret reaction), triglycerides (glycerol phosphate oxidase/peroxidase), and glucose (glucose oxidase/peroxidase). All parameters were analyzed using commercial kits and reagents (BioSystems Reagents & Instruments, Barcelona, Spain) with a spectrophotometer (BTS-330, BioSystems Reagents & Instruments, Barcelona, Spain). The applied biochemical methods followed the standard methodology used in avian studies (e.g., Artacho *et al.* 2007).

2.6. Statistical analyses

To evaluate within- and between-brood variation in plasma metabolite concentrations and body mass of nestlings, we used variance components from ANOVA with broods representing the compared groups. Based on these results, we calculated intra-class correlations, which are a statistical measure of within-brood repeatability and estimate the percentage of variation in the dependent variable attributed to differences between broods (Lessells & Boag 1987, Nadolski *et al.* 2006).

To investigate relationships between the concentrations of plasma metabolites and different indices of body condition, we applied General Linear Mixed-effects Models (GLMMs). Because different measures of body condition were highly correlated (Pearson correlation: $0.40 < r < 0.69$) we ran separate GLMMs for each pair of plasma metabolites and condition indices to avoid multicollinearity of independent variables (a total of 12 models). In each model we also entered the following confounding variables: sex, year, sampling hour, and hatching position.

We included the effect of brood identity as a random factor to account for pseudoreplication (Hurlbert 1984) and nested it within year. We used a stepwise backward model procedure by deleting non-significant terms until only significant terms remained in the model. We used β coefficients of regression to assess the nature and strength of significant relationships. We evaluated the percent of variance in the dependent variable explained by particular independent variables with partial eta-squared η^2 (Cohen 1973). All statistical analyses followed Zar (1996) and were performed with STATISTICA 9.0 (Statsoft Inc.).

3. Results

Mean values of all studied plasma metabolite concentrations and body mass are shown in Table 1. As indicated by low values of intra-brood repeatability, the high proportions of the total variation

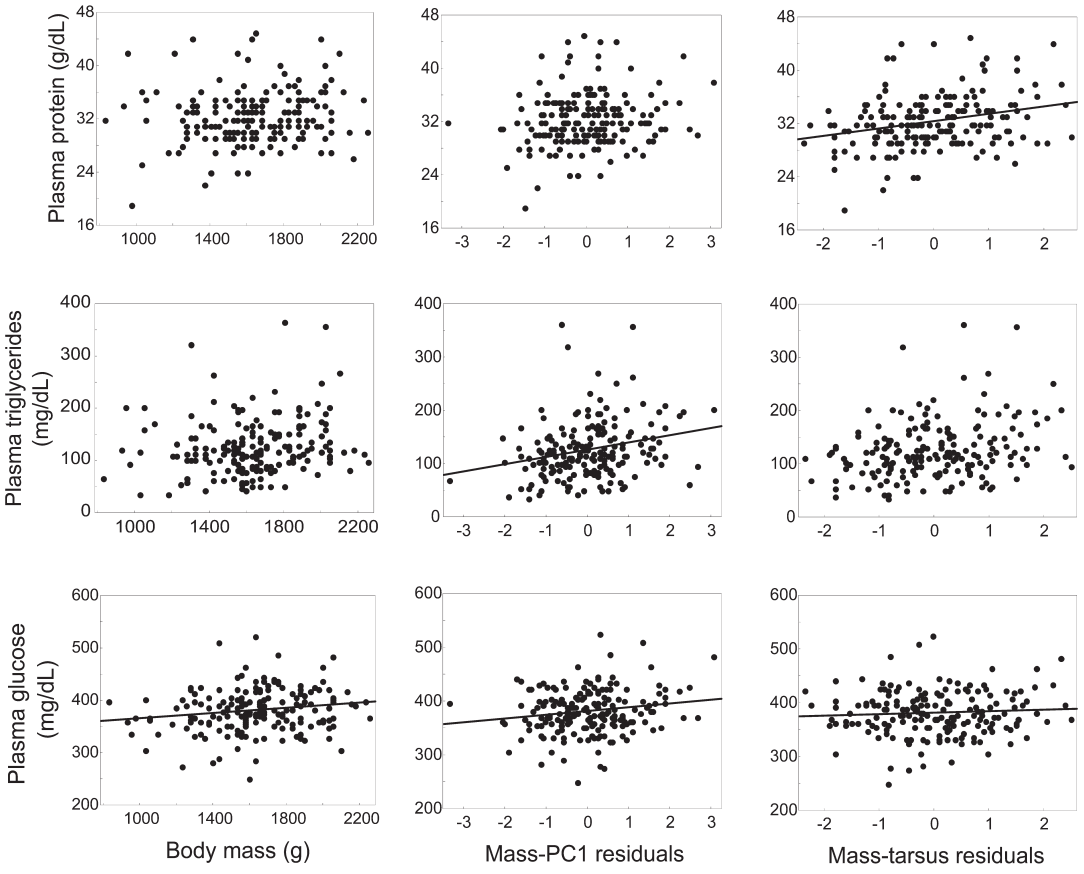

Fig. 1. Relationships between the concentrations of basic plasma metabolites (proteins, triglycerides, and glucose) and mass-related indices of body condition (body mass, mass-PC1 residuals, and mass-tarsus residuals) of Great Cormorant nestlings at the Jeziorsko reservoir, Poland.

in the concentrations of plasma metabolites were attributed to differences within broods (68.2%–75.5%), which corresponded with high within-brood variation in body mass. We found that 80.8% of variation in chick body mass was attributed to differences within broods, whereas only 19.2% of the variation was explained by differences among broods. Within-brood differences in body mass were associated with marked hatching asynchrony of nestlings, with 52.4% of variation in body mass of chicks explained by hatching order ($F_{1,127} = 134.71$, $P < 0.001$).

We found no significant diurnal or between-seasonal variation in the concentrations of the analyzed plasma metabolites ($P > 0.05$). Significant differences between male and female chicks occurred only for plasma protein level, with males having lower protein concentrations than females (32.02 ± 0.38 vs. 32.74 ± 0.47 g/l, $F_{1,127} = 5.17$, $P = 0.025$).

Concentrations of all analyzed plasma metabolites correlated with at least one mass-related indicator of body condition (Fig. 1). Plasma protein appeared to be of lowest usefulness as a predictor of body condition. After accounting for nestling sex, protein concentration was related only with mass-tarsus residuals ($F_{1,126} = 5.22$, $P = 0.022$; $\eta^2 = 0.040$). The relationship was positive, as chicks with higher mass-tarsus residuals had higher levels of plasma protein ($\beta = 0.004 \pm 0.002$). There was no significant relationship between the level of plasma proteins and body mass ($F_{1,125} = 0.55$, $P = 0.460$) or mass-PC1 residuals ($F_{1,125} = 1.54$, $P = 0.220$). Similarly, protein concentration was not significantly related with growth rates of chicks ($F_{1,125} = 1.15$, $P = 0.290$). The effect of hatching

position was excluded from all the models as being non-significant.

The concentration of plasma triglycerides was significantly related with mass-PC1 residuals ($F_{1,127} = 5.28$, $P = 0.023$; $\eta^2 = 0.040$), i.e., chicks with higher mass-PC1 residuals had higher levels of plasma triglycerides ($\beta = 10.15 \pm 4.42$). We also found a positive relationship between the concentration of plasma triglycerides and nestling growth rate ($F_{1,127} = 8.10$, $P = 0.005$; $\eta^2 = 0.059$; $\beta = 439.99 \pm 154.60$). We found no significant relationship between the concentration of plasma triglycerides and body mass ($F_{1,126} = 0.92$, $P = 0.34$) or mass-tarsus residuals ($F_{1,127} = 3.35$, $P = 0.07$). The effect of hatching position was excluded from all the models as being non-significant.

Among the analyzed plasma metabolites, glucose concentration was the most robust indicator of body condition, as it correlated with all applied mass-related indices of physical state. All relationships were positive, indicating higher concentrations of plasma glucose in chicks with better body condition (body mass: $F_{1,127} = 11.50$, $P < 0.001$, $\eta^2 = 0.083$, $\beta = 0.040 \pm 0.011$; mass-PC1 residuals: $F_{1,126} = 13.38$, $P < 0.001$, $\eta^2 = 0.096$, $\beta = 11.77 \pm 3.22$; mass-tarsus residuals: $F_{1,127} = 9.21$, $P = 0.003$, $\eta^2 = 0.068$, $\beta = 0.058 \pm 0.019$). Glucose level was also positively associated with chick growth rate ($F_{1,127} = 4.41$, $P = 0.038$; $\eta^2 = 0.033$, $\beta = 248.79 \pm 118.47$). The effect of hatching position was significant when entered in the model along with mass-PC1 residuals ($F_{1,126} = 5.07$, $P = 0.026$; $\eta^2 = 0.034$), suggesting that chicks hatched later in the hatching sequence had lower plasma glucose concentration ($\beta = -5.42 \pm 2.41$). The effect of hatching position explained considerably less variation in plasma glucose level as compared with mass-PC1 residuals ($\eta^2 = 0.039$ vs. $\eta^2 = 0.096$).

4. Discussion

We found considerable within-brood variation in the concentrations of all analyzed plasma metabolites in the Great Cormorant nestlings. As much as 68%–76% of variation in the concentrations was attributed to differences between siblings. Such a relationship is likely associated with the marked hatching asynchrony in the Great Cormorant (usually 1–2 chicks hatching each day), resulting in substantial differences in the physiological state between siblings. In fact, we confirmed significant differences in plasma glucose concentration between nestlings of different positions in hatching sequence.

The level of within-brood variation in blood parameters is generally lower in species with synchronous hatching, such as passerines. Only 13–55% of variation in various blood parameters in the Great Tit (*Parus major*) nestlings was attributed to differences between siblings (Nadolski *et al.* 2006). Similar results have been obtained for the Pied Flycatcher (*Ficedula hypoleuca*) nestlings, with 30% of variation in haematocrit being attributed to differences within broods (Potti *et al.* 1999).

Our results indicated that the concentrations of all analyzed plasma metabolites correlated with at least one body-condition index in the Great Cormorant nestlings. The concentration of plasma proteins appeared to be a limited predictor of body condition, as it correlated only with body mass adjusted against tarsus length. In birds, plasma protein concentration has been suggested as an index of total protein reserves (Allison 1955). Total plasma protein has been shown to increase due to elevated intake of dietary proteins (Leveille & Sauberlich 1961) and to decrease as a consequence of dietary inadequacies or malnutrition (Smith & Bush 1978). Total plasma protein concentration increases shortly after feeding of fasted birds of prey (Ferrer *et al.* 1987) and decreases continuously during fasting in Herring Gulls (*Larus argentatus*; Totzke *et al.* 1999). Correlations between total plasma protein levels and physical condition have been found in both birds and mammals (e.g., Messier *et al.* 1987), but the strength of these relationships is usually low, resulting in a low predictive power of such models (de le Court *et al.* 1995, Dawson & Bortolotti 1997b). Not all studies have reported significant correlations between plasma protein level and body condition (Dawson & Bortolotti 1997a). Our results led to a similar conclusion, suggesting that plasma protein level may not necessarily be a reliable indicator of body condition in birds, or that it may work reliably only under specific conditions. Low applicability of this parameter could be explained by physiological effects of dehydration and infec-

tions, which may elevate plasma protein levels and simultaneously reduce mass-related condition (Lumeij 1987, Tomás *et al.* 2005).

We also found a positive correlation between plasma triglyceride concentrations and size-adjusted body mass. Plasma triglyceride levels are widely recognized as a proxy of the nutritional state in birds (Jenni-Eiermann & Jenni 1997). High concentrations of plasma triglycerides indicate a resorptive state where fat is deposited in adipose tissue (Hõrak *et al.* 2002). Because most plasma triglycerides originate from the diet, their levels have been suggested to be proportional to the amount of food absorbed several hours before blood sampling (Jenni-Eiermann & Jenni 1994). In several species of migrating passerines, levels of triglycerides decrease rapidly in response to an interruption in food intake (Jenni-Eiermann & Jenni 1994), and they have been proposed as a reliable predictor of future mass-gain rate in both passerines (Jenni & Schwilch 2001, Cerasale & Guglielmo 2006) and waders (Williams *et al.* 1999). Plasma triglycerides have also been confirmed to indicate the total body fat content in poultry (Bacon *et al.* 1989) as well as in wild birds (Dabbert *et al.* 1997, Masello & Quillfeldt 2004, Sarasola *et al.* 2004).

Although concentrations of plasma triglycerides have been suggested to reflect only short-term metabolic processes, we found that this parameter may be treated as a more robust indicator of body condition. The level of plasma triglycerides measured in chicks at the age of ca. three weeks correlated well with their growth rate over most of the pre-fledging period. We obtained similar results for plasma glucose. In fact, the concentration of the latter was the most reliable indicator of body condition of the Great Cormorant nestlings among all analyzed plasma metabolites. Apart from its positive relationship with chick growth rate, plasma glucose level positively correlated with both size-adjusted and unadjusted body mass. Although glucose plasma concentrations usually remain within a narrow range, a reduced plasma glucose turnover has been recorded during fasting in birds (Groscolas & Rodriguez 1981). Low concentrations of plasma glucose under natural starvation have been recorded in several avian taxa (González & Hiraldo 1991, Jenni-Eiermann & Jenni 1997, Ferrer & Dobado-Berrios 1998). However, other studies have failed to find significant relationships between glucose concentrations and body condition (e.g., Migliorini *et al.* 1973, Jeffrey *et al.* 1985, de le Court *et al.* 1995).

Moreover, García-Rodríguez *et al.* (1987) found that plasma glucose levels *increased* in response to starvation in the Common Buzzard (*Buteo buteo*). Inter-specific differences in effectiveness of gluconeogenesis pathway may be responsible for such different responses of plasma glucose levels during food deprivation (Ferrer *et al.* 1987). For these reasons, the practical applicability of plasma glucose concentration as a predictor of body condition should be tested in other avian taxa. It must also be noted that the proxies of body condition, used here, explained only small parts of variation in all analyzed plasma metabolite concentrations, rendering the predictive power of such relationships low.

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Plasmametaboliittien pitoisuudet merimetson pesäpoikasten kunnon ennustajana

Selvitimme plasman perusmetaboliittien käyttökelpoisuutta merimetson (*Phalacrocorax carbo sinensis*) pesäpoikasten kunnon ennustajina. Lajilla tavataan poikueen sisällä huomattavaa kuoriutumisen eriaikaisuutta ja selkeä arvojärjestys. Tutkimme noin kolmen viikon ikäisten poikasten plasmaproteiinien, triglyseriidien ja glukoosin pitoisuuksien yhteyttä lintujen massaan, massan-koon residuaaleihin, sekä kasvukertoimeen. Aineisto perustui 185 merimetson poikasen mittauksiin yhdessä Keski-Puolassa sijaitsevasta koloniassa.

Kaikki mitatut plasmametaboliittien pitoisuudet korreloivat ainakin yhden kuntoa kuvaavan in-

deksin kanssa. Kuntoa ennusti parhaiten plasmaglukoosin pitoisuus, jolla oli positiivinen suhde niin massaan, massan-koon residuaaleihin, kuin poikasten kasvukertoimeen. Plasmaproteiinien käyttökelpoisuus oli vähäinen (positiivinen suhde ainoastaan massan-nilkan pituuden residuaaleihin), kun taas plasman triglyseriidien kyky ennakoida ruumiin kuntoa oli keskitasoa (positiivinen suhde massan-koon residuaaleihin ja kasvukertoimeen). Kaikkien merkitsevienkin riippuvuussuhteiden ennustusvoima oli alhainen.

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