The quality of protein sources for egg production in Tawny Owls (*Strix aluco*) and Eurasian Sparrowhawks (*Accipiter nisus*)

A qualidade das fontes de proteína para a produção de ovos em coruja-do-mato (*Strix aluco*) e gavião (*Accipiter nisus*)

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ABSTRACT

The Netherlands is second after the USA in the export of agricultural and dairy products, which comes at a high price for the Dutch environment and biodiversity. In order to document the effects of nitrogen deposition and soil acidification therefrom on forest fauna, we study several bird species – e.g. Great Tit (*Parus major*), Eurasian Sparrowhawk (*Accipiter nisus*), Tawny Owl (*Strix aluco*) – on the Veluwe, a large forested area in the Netherlands. Effects of forest degradation include large-scale tree mortality of common oak (*Quercus robur*), lowered protein synthesis in trees, shifts in the composition of free amino-acids in trees, severe calcium deficiency in Great Tits, and amino-acid deficiencies in reproducing Eurasian Sparrowhawks. Raptor species have strongly declined, but, surprisingly, Tawny Owls have not. We hypothesized that this was due to amino-acid producing cecal bacteria present in (Tawny) owls, which lack in diurnal birds of prey. In contrast, Eurasian Sparrowhawks break down breast muscle tissue to complement dietary amino-acid deficiencies. I compared amino-acid compositions of owl eggs, Wood Mice (*Apodemus sylvaticus*), owl cecal sacs, owl breast muscles, and Eurasian Sparrowhawk eggs and breast muscles to determine whether protein amino-acid composition varies with protein source, and to compare egg amino-acid requirements between Eurasian Sparrowhawks and Tawny Owls. Amino-acid measurements were done commercially by a certified food-quality research facility (TNO). Results show that wood mice and breast muscle tissue of both bird species were short in several essential amino-acids, up to -56% for cysteine, compared to egg requirements. The amino acid content of the cecal sacs was also low in cysteine but only -16%. In addition, cecal sac amino acids are renewed continuously, whereas breast muscle protein is a limited source of amino-acids. As Eurasian Sparrowhawks lack cecal sacs, they are more vulnerable to dietary amino acid-shortages in degenerated forests than Tawny Owls.

Keywords: *Accipiter nisus*, amino-acids, egg production, protein source, *Strix aluco*

RESUMO

A Holanda ocupa o segundo lugar, após os EUA, na exportação de produtos agrícolas e laticínios, o que tem consequências para o ambiente e a biodiversidade holandeses. A fim de documentar os efeitos da deposição de azoto e da acidificação do solo na fauna florestal, estudámos várias espécies de aves – por exemplo, chapim-real (*Parus major*), gavião (*Accipiter nisus*), coruja-do-mato (*Strix aluco*) – em Veluwe, uma grande área florestal na Holanda. Os efeitos da degradação florestal incluem mortalidade em larga escala de carvalho comum (*Quercus robur*), baixa síntese proteica em árvores, mudanças na composição de aminoácidos livres em árvores, deficiência severa de cálcio em chapim-real e deficiências de aminoácidos em gavião durante a reprodução. As espécies de aves de rapina apresentaram grandes declínios, mas, surpreendentemente, a coruja-do-mato não. A nossa hipótese defende que essa resistência se deve a bactérias cecais produtoras de aminoácidos presentes na coruja-do-mato, que não ocorrem em aves de rapina diurnas. Por outro lado, o gavião consome músculo peitoral para complementar as deficiências dietéticas de aminoácidos. Neste estudo comparei a composição em aminoácidos dos ovos de coruja, no rato-do-campo (*Apodemus sylvaticus*), em cecos de coruja, e em músculo peitoral e ovos de gavião, para determinar se a composição em aminoácidos varia com a fonte de proteína e comparar os requisitos de aminoácidos do ovo entre o gavião e a coruja-do-mato. As medições de aminoácidos foram efetuadas por um laboratório certificado de análise da qualidade alimentar (TNO). Os resultados mostram que os ratos e músculo peitoral de ambas as espécies de aves apresentavam quantidades reduzidas de vários aminoácidos essenciais, até -56% para cisteína, em comparação com as exigências dos ovos. O conteúdo em aminoácidos dos cecos também apresentava reduzida cisteína, mas apenas -16%. Adicionalmente, os aminoácidos dos cecos são renovados continuamente, enquanto a proteína do músculo peitoral constitui uma fonte limitada de aminoácidos. Como os gaviões não possuem cecos, são mais vulneráveis à falta de aminoácidos na dieta em florestas degeneradas do que as corujas-do-mato.

Palavras-chave: : *Accipiter nisus,* aminoácidos, fonte de proteína, produção de ovos, *Strix aluco*

Introduction

Studying nature in the Netherlands cannot be done without taking into account possible effects of high nitrogen deposition levels. In my study area on the Southwest-Veluwe nitrogen deposition mounts up to 4500 mol per hectare per year (data RIVM[1]). According to the World Trade Organisation (WTO, on the website of the Dutch Central Bureau of Statistics[2]) the Netherlands is the second-largest exporter of agricultural products in the world after the USA, and followed by Germany, Brazil, and France (in declining order). As the Netherlands only comprises 0,4% of the land surface area of the USA and 11.6% of Germany, the magnitude and efficiency of its agricultural sector are astonishing. Regrettably this economic 'success' comes at a high price for the quality of the Dutch environment and biodiversity. According to PBL (a Dutch governmental agency for the environment) 2014, the Netherlands has lost 85% of its biodiversity during the last century[3]. This is mainly caused by (semi-) natural habitats being converted into intensive farmlands which are extremely poor in biodiversity (e.g. only 3% of plant species-rich grasslands is remaining[4]). Additionally, the Dutch agricultural system makes ample use of neonicotinoids (Hallmann et al. 2014), glyphosate, and many other pesticides (e.g. Toumi et al. 2016).

The use of artificial fertilizer and the import of cattle feed from abroad (like soy-protein from tropical South America) represents a flow of nitrogen flow towards the Netherlands, which is only partially compensated by the export of meat and dairy products. Ammonia evaporating from fields and stables reacts in the air to ammonium, which is not only deposited in close proximity of intensive cattle farms, but nation-wide. In nature areas on dry sandy soils, this causes soil acidification (De Vries et al. 2017), low soil mineral and plant nutrient levels (Bergsma et al. 2016, Van den Burg 2017a), and excessive nitrogen availability. Mineral nutrient deficiencies in degraded forest ecosystems also affect fauna, as they cause, among others, poor egg shell formation and broken bones in nestling songbirds, such as Great Tits (*Parus major*; Van den Burg 2017[a]). The combination of low mineral availability and excessive nitrogen results in a cascade of changes in forest and heathland ecosystems and a further loss of biodiversity (Van den Burg & Vogels 2017). Currently, forests of Common Oak (*Quercus robur*) are dying at a large scale and soil acidification and excess nitrogen availability have also been implicated in causing this process (Lucassen et al. 2014[a,b]). This forest decay equals the local destruction of the entire oak forest ecosystem. Regrettably, nitrogen deposition is not only a problem of the Netherlands, but as wide-spread as intensive livestock farming (Duce et al. 2008).

Another route of detrimental effects of nitrogen deposition on biodiversity is through altered plant physiology (Van den Burg & Vogels 2017). In plants which are not nitrogen but mineral nutrient (e.g. K, Mn, or Ca) limited, amino-acid and protein production are reduced. Also, the composition of free amino acids is shifted towards types that contain more than a single nitrogen atom, such as arginine – studied in Scots Pine (*Pinus sylvestris*; Perez-Soba 1995). The overall reduction of amino-acids is propelled up the food chain, affecting the reproduction of raptors such as Eurasian Sparrowhawks (*Accipiter nisus*, Siepel et al. 2009).

To compensate shortages of particular amino-acids due to feeding on poor quality protein, birds can metabolize their breast muscles (especially the greater flight muscles, *pectoralis major*) to enable egg production (Houston et al 1995, Selman & Houston 1996). Birds adopt this strategy if the amino acid make-up of the diet does not match the requirements for egg production, but not to compensate a general deficiency of protein, i.e. food shortage. Of special importance in this respect are the so-called essential amino-acids, that the avian body cannot synthesize itself, but must be derived from dietary intake, or, as explained below, from bacterial sources.

We demonstrated the use of breast muscle by Eurasian Sparrowhawks in order to produce eggs, next to other indicators of amino-acid shortages in this species (Van den Burg 2009). Also, Eurasian Sparrowhawks (and other bird of prey species) have strongly declined in forest habitats during the nineteen nineties where they used to be abundant (Van den Burg 2009). This coincided with the peak-period of nitrogen deposition. In contrast, Tawny Owl populations have remained stable over time, and I hypothesized that this may be due to physiological differences between diurnal raptors and owls (Van den Burg 2009). Owls have large intestinal blind sacs (ceca) which lack in Eurasian Sparrowhawks. In owls, the ceca are used to recycle uric acid, as bacteria within utilize the nitrogen from uric acid to synthesize their own amino-acids and proteins (Denbow 2000). Uric acid is excreted as waste material by the kidney, but is transported anti-peristaltically from the cloaca to the ceca (Clench & Mathias 1995). Owls harvest amino-acids from the ceca by excreting proteases and absorbing the free amino-acids from digested bacterial protein. If the amino-acid supply from the ceca complements dietary intake, this may explain why owls are not amino-acid limited for egg production whereas birds of prey living in the same forest are. As such, this may also explain why both bird groups differ in population trend despite their similar diets, consisting mainly of small mammals and birds.

For this paper, I compared amino-acid compositions of Tawny Owl eggs, breast muscles, cecal sacs, Wood Mice (*Apodemus sylvaticus*), and Eurasian Sparrowhawk eggs and breast muscles to determine whether protein amino-acid composition varies with protein source, and to compare egg amino-acid requirements between Eurasian Sparrowhawks and Tawny Owls.

Material and methods

Sample origins and handling

Tawny Owl and Eurasian Sparrowhawk carcasses (N=3 for each species) were road casualties provided by members of the Dutch public, mostly in winter and spring. Carcasses came from the central and eastern parts of the Netherlands. Fresh Tawny Owl eggs (N=3) were from nests in the Gelderse Vallei region, that had been deserted in extreme cold weather and got frozen in the breeding cavity (or nest box). Land use in this area is predominantly intensive agriculture, interspersed with small patches of old woodland. Dead woodmice (N=3) were collected from Tawny Owl nest boxes on the Southwest-Veluwe, a forested area centrally in the Netherlands. Stored prey items in the late egg and early chick stage were removed from the nest and replaced by day-old poultry chicks. Three specimens were randomly selected for amino acid analyses. Eurasian Sparrowhawk eggs (N=30) were collected fresh during the egg-laying period from nests on the Southwest-Veluwe for an earlier study into environmental impacts on egg quality in this species (e.g. Van den Burg 2006). All samples originated from the time period 2002-2009.

Samples were stored at -20 ^{0}C before further sample preparation. Egg samples were homogenized prior to amino-acid analysis. Breast muscles (*pectoralis major*) were dissected from adult owl and hawk carcasses. In owls, the intestine was spread out to reveal the cecal sacs. These were dissected from the intestine and emptied by gently pressing. Soft tissues from dead mice were stripped from the bone, and offered for amino acid analyses. Also the fur was excluded, as most of the hairy material is regurgitated into pellets. Amino-acid measurements were done commercially by a certified food-quality research facility (TNO). The methodology used included acid digestion of all protein. To reduce costs, only a selection of amino-acids was determined (Glycine (Gly), Alanine (Ala), Glutamic acid (Glu), Aspartic acid (Asp), Lysine (Lys), Arginine (Arg), Methionine (Met), Isoleucine (Iso), Leucine (Leu), Cysteine (Cys), Threonine (Thr), Valine (Val), Serine (Ser), Tryptophan (Trp). The choice for this selection was based upon the inclusion of the Sulphur-containing amino-acids (Met, Cys). As these are also crucial in feather-formation, birds have a relatively high need for these compounds. Trp was measured separately, as acid digestion of protein destroys this amino acid. As it is one of the rarest essential amino acids and crucial in energy metabolism (also as precursor of Niacin, vitamin B3), I opted to include this amino acid in the analyses.

Statistics

All amino-acids measurements were analyzed using relative data, i.e. only focusing on the protein make up and not concentration. Clearly, protein concentration in solid tissues (muscle) is higher than watery samples such as eggs and cecal content. A Principal Component Analysis (PCA) was performed in Genstat to see variance and similarity among samples. Using the same data I also studied the balance of each source of amino-acids relative to the amino-acid levels found in the eggs of both species. This method gives better insight into the magnitude of amino-acid limitations for egg production. For each amino-acid I determined the amount (in gram) per 100 gram egg protein and each protein source. From this I calculated for each amino-acid the amount of source-protein required to produce 100 gram egg protein. So, for example, if it takes 200 gram breast muscle protein to free enough Cys to produce 100 gram egg protein, breast muscle protein has a -50% Cys unbalance compared to egg production requirements.

Results

PCA yielded clear separation of protein amino acid content of most sample types (Fig. 1; explained variance x-axis 80.4%,y-axis 12.5%). Muscle tissues were similar in amino-acid composition in both species. As the mouse samples comprised a greater variety of tissue types, it is not surprising to find them slightly different in composition (variation on the y-axis only) from the birds muscle proteins. The egg samples had a completely different composition, and also differed between Tawny Owls and Eurasian Sparrowhawks. The latter could be expected, as egg proteins have in the past been used to clarify avian phylogeny (Sibley 1960). Eggs were particularly rich in Ser (Fig. 2), but this is of minor importance in terms of food quality, as Ser can be synthesized by the birds themselves. Of the essential amino-acids, Thr, Val, Cys, and Leu have higher levels in eggs compared to food (mouse) and muscle protein sources. The amino-acid composition of the cecal sacs content was different from both egg and tissue samples, most likely due to its bacterial origin. The cecal sacs were intermediate on the x-axis between tissue samples and egg samples.

Of the amino-acids that are in short supply for egg production in animal tissue samples, Cys was the most limiting (Eurasian Sparrowhawk, Fig 3; Tawny Owl, Fig. 4). As the relative Cys availability in muscle and mouse protein is about 50% lower than required for egg production, meat consumption (either from food or body reserves) to make a single egg should be twice the amount of total egg protein content (e.g. 40 grams meat protein is required to produce 20 grams of egg protein). Cys is in fact semi-essential as it can also be produced by birds from Met, but not from other amino-acids, because only Met and Cys

Figure 1 - Clustering of avian samples and mice with respect to their amino-acid composition after principal component analysis (PCA) (explained variance x-axis 80.4%,y-axis 12.5%). Muscle tissue of both bird species clusters together, and, on the x-axis, ground mice is also very similar in amino acid composition. Quite opposite on the x-axis (right hand side) is the amino-acid composition required for egg production, i.e. tissue samples clearly differ from eggs. Intermediate are the cecal proteins, which are for a great extent of bacterial origin and show most variation along the y-axis.

Figura 1 - Agrupamento das amostras de aves e de mamíferos, relativamente à composição em aminoácidos, em análise de componentes principais (PCA) (variância explicada: eixo horizontal 80,4%, eixo vertical 12,5%). O tecido muscular das aves surge agrupado e, no eixo horizontal, a composição em aminoácidos do rato é também muito semelhante. Em oposição, a composição em aminoácidos necessária para a produção de ovos surge no lado direito do eixo horizontal, i.e. as amostras de tecido muscular diferem claramente das amostras de ovo. As proteínas presentes nos cecos ocupam uma posição intermédia, sendo maioritariamente de origem bacteriana e apresentando a maior variação ao longo do eixo vertical

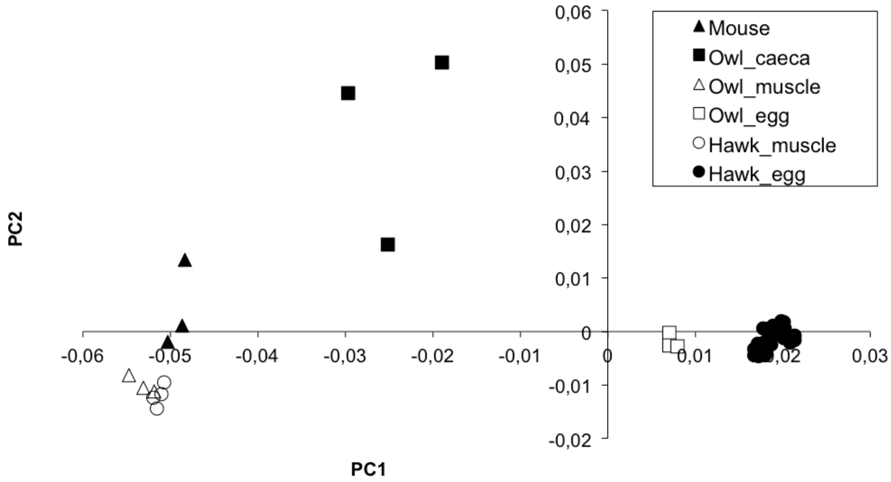


Figure 2 - End-points of vector loadings after principal component analysis (PCA) on the amino-acid composition of animal samples (samples and axes identical to Fig. 1). Essential amino-acids which cannot be synthesized by birds are indicated separately. Eggs are particularly rich in Ser, but of the amino-acids that are food-derived Thr, Val, Cys and Leu have higher levels in eggs compared to other animal samples.

Figura 2 - Terminação dos vetores dos "loadings" da análise de componentes principais (PCA) relativamente à composição de aminoácidos de amostras de animais (amostras e eixos idênticos à Fig. 1). Os aminoácidos essenciais que não são sintetizados pelas aves são indicados separadamente. Os ovos são particularmente ricos em Ser mas, dos aminoácidos derivados de alimentos, a Thr, a Val, a Cys e a Leu apresentam níveis mais elevados nos ovos, em comparação com outras amostras de animais.

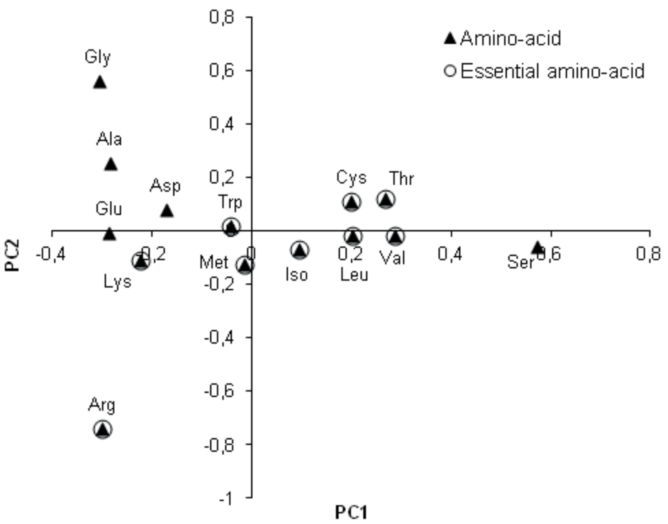


Figure 3 - Average surpluses and deficiencies of amino-acids of animal samples relative to what is required for egg production (zero on the y-axis depicts the average amino-acid level inside an egg) in Eurasian Sparrowhawks. In both food and the hen's breast muscle, Cys is the amino that is most limited for egg production; for example a Eurasian Sparrowhawk should consume about 40 gram of breast muscle or mouse protein to free sufficient Cys to produce 20 gram of egg protein.

Figura 3 - Valores médios dos excedentes e das deficiências de aminoácidos em amostras de animais, relativamente às quantidades necessárias para a produção de ovos em gavião (zero no eixo vertical representa o nível médio de aminoácidos dentro de um ovo). Tanto no alimento quanto no músculo peitoral da galinha, a Cys é o aminoácido mais limitante para a produção de ovos; por exemplo, um gavião deve consumir cerca de 40 gramas de músculo peitoral ou proteína do rato para libertar Cys suficiente para produzir 20 gramas de proteína do ovo.

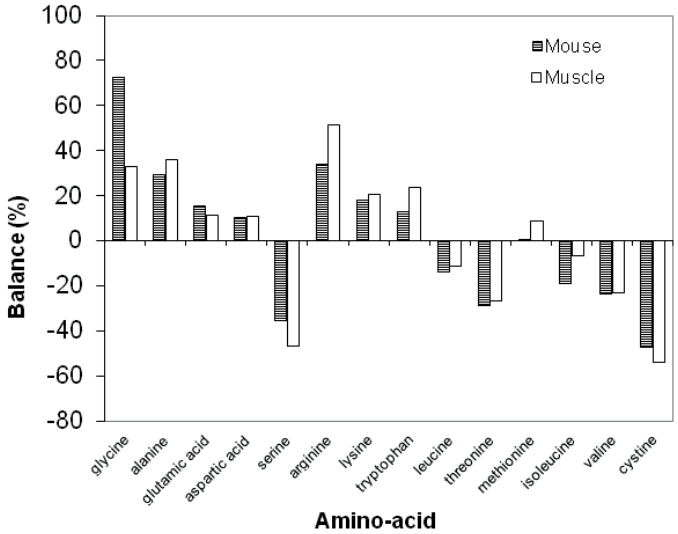
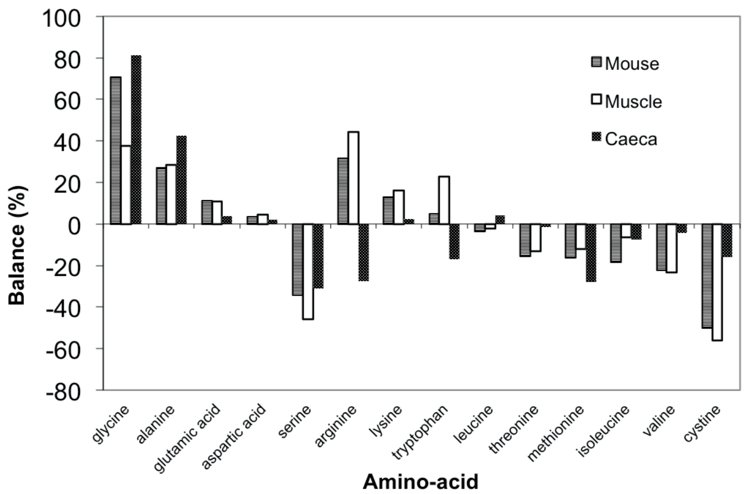


Figure 4 - Average surpluses and deficiencies of amino-acids of animal samples relative to what is required for egg production (zero on the y-axis depicts the average amino acid level inside an egg) in Tawny Owls. In both food and the hen's breast muscle, Cys is the amino that is most limited for egg production; for example a Tawny Owl should consume about 42 gram of breast muscle or mouse protein to free sufficient Cys to produce 20 gram of egg protein. However, it requires only 24 grams of cecal protein for 20 grams egg protein.

Figura 4 - Valores médios dos excedentes e das deficiências de aminoácidos em amostras de animais, relativamente à quantidade necessária para a produção de ovos em coruja-do-mato (zero no eixo vertical representa o nível médio de aminoácidos dentro de um ovo). Tanto no alimento quanto no músculo peitoral da galinha, a Cys é o aminoácido mais limitante para a produção de ovos; por exemplo, uma coruja deve consumir cerca de 42 gramas de músculo peitoral ou proteína do rato para libertar Cys suficiente para produzir 20 gramas de proteína do ovo. No entanto, requer apenas 24 gramas de proteína dos cecos para 20 gramas de proteína do ovo.



contain Sulphur-groups. As Met is also in too short supply, this transition would not ease the amino-acid limitation: a reduction in the deficiency of one amino-acid would increase the deficiency of another. As Tawny owls have cecal sacs, they carry another protein source, which is much better for Cys. It only requires 24 grams of cecal protein to synthesize 20 grams egg protein. Cecal sac protein was, however, short in Arg and to a lesser extent Trp.

Discussion

Egg proteins serve very different functions than proteins in animal tissues, as many egg proteins are involved in making eggs resistant to bacterial invasion, and only some (especially vitellogenin) have the prime function of supplying the young embryo the amino-acids it needs to grow (e.g. Van den Burg 2017[b]). Hence the amino-acid build-up of egg protein is much different from animal tissues, and it is the natural condition that some amino acids are relatively short in the diet. So to produce a single egg, birds need to consume more protein than the total protein content of that egg, as to compensate for the rarer amino acids in the diet compared to egg requirements. Due to nitrogen deposition, lowered plant protein production therefrom, and shifts in amino acid content, such as excess Arg in free plant amino-acids (Perez-Soba, 1995),the chances of particular amino-acids shortages in birds may increase, making it more difficult to produce eggs. We observed Eurasian Sparrowhawks to increase the use of their breast muscles to facilitate egg production, but not owls (Van den Burg 2009).

The present data indicate that owls may benefit from cecal sac proteins during egg formation, as the cecal sacs provide some amino-acids proportionally better compared to prey or muscle proteins. So, adverse effects of nitrogen deposition that result in lowered amino-acid availability can be, at least in part, mitigated by bacterial proteins from the cecal sacs. The magnitude at which egg amino-acids in owls are indeed derived from cecal sacs is thus far unknown. However, as these amino acids enter the blood stream similarly as dietary amino acids, it is unlikely that they will be treated differently during egg production. Therefore, it is likely that amino acids from the ceca are indeed used to build proteins for the purpose of egg formation.

The ceca are small compared to egg volume in Tawny Owls, so to be of significance during egg production, the rate of transport of uric acid to the ceca and amino-acid absorption therefrom should be large enough to keep up with egg laying. Regrettably the magnitude of the uric acid and amino-acid fluxes into and from the ceca, appear unknown. Interestingly, whereas Barn Owls invest less lipid into their eggs compared to Eurasian Sparrowhawk eggs of the same size, they allocate more protein for egg production (Van den Burg 2002). Perhaps, this is a reflection of cecal bacteria activity in the owls, which lacks in Eurasian Sparrowhawks.

It is highly unlikely that the cecal sacs in meat-eaters originated (or did not regress) during evolution in order to cope with adverse dietary amino-acid compositions. The current situation regarding nitrogen deposition has never occurred before in history, so adaptive behavior or physiology could not have developed in the past. Instead, the ceca have most likely evolved or been maintained in owls as a source of energy, in which case the amino-acids are used as fuel.

As the ceca themselves also provide proteins that are short in some amino-acids for egg production, dietary protein intake is still important to obtain a suitable mixture of essential amino acids for egg production. Surely, without dietary amino-acid intake no eggs would be produced in neither of the studied species. In poor food years there may be a trade-off in the use of cecal sac protein between egg quality and energy availability for egg production. As a result of deteriorated dietary amino-acid composition, owls may, more frequently compared to several decades ago, be forced not to lay at all in poor food years. As such an effect will be very difficult to discriminate from general food shortage, it will most likely go unnoticed to human observers.

Acknowledgements

I would like to thank Dr. Wouter Vaes and his team for the amino-acid analyses at TNO. Funding for this work has come from various sources over time. Parts were funded by Technology Foundation NWO-STW (whilst working at the Netherlands Institute of Ecology, NIOO), Town council of Ede (which also permitted forest access), and Bargerveen Foundation. I wish to thank Prof. João E. Rabaça, Ines M. F. Roque and an anonymous reviewer for their valuable comments on the manuscript of this publication.

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