

The effect of a carotenoid-rich diet on immunocompetence and behavioural performances in Moorhen chicks

S. FENOGLIO ¹, M. CUCCO and G. MALACARNE

Dipartimento di Scienze e Tecnologie Avanzate, Università degli Studi del Piemonte Orientale, Via Cavour 84, 15100 Alessandria, Italy

Received 17 August 2001, accepted 13 June 2002

Carotenoids have attracted the attention of behavioural ecologists because of their modulatory effects on the immune system, colour expression and several physiological functions affecting body conditions. To evaluate whether carotenoid abundance influences growth, behavioural performance, immunocompetence and bill colour, we raised two groups of Moorhen chicks for 1 month with different food intakes (control diet versus carotenoid-rich diet). The two groups did not show significant differences in growth rate, nor in the red or yellow colouration of the bill, although there was a greater immune response in the carotenoid-rich group. Locomotor activity, tested in an open-field apparatus, did not differ between the two groups, but carotenoid-fed chicks showed more intense pecking at a model mimicking the parents. In this experiment, we demonstrated that carotenoid abundance can influence both immune and behavioural responses, with a greater availability of carotenoids being reflected in improved behavioural performance and immunocompetence.

KEY WORDS: carotenoids, immunocompetence, moorhen, development, behaviour.

INTRODUCTION

Recently carotenoids have attracted the attention of behavioural ecologists because of their modulatory effect on the immune system (BURTON & INGOLD 1984, BENDICH 1989, MØLLER et al. 2000) and also their role in red-yellow colour expression (HILL 1990, 1996, 2000; HILL et al. 1994). Carotenoids have other important physiological functions: they are involved, as lipid antioxidants (BURTON 1989), in the development of neurosensory networks, in which lipids are abundant (BENDICH 1989), while some carotenoids act as precursors for the synthesis of vitamin A (OLSON 1989).

¹ Corresponding author (E-mail: fenoglio@unipmn.it).

Carotenoids are terpenic pigments, produced mostly in plants, algae and in some fungi and bacteria. They cannot be synthesized by animals and must be assimilated with food. Thus they represent a limited resource that animals could allocate optimally between physiological-immunocompetence functions and pigmentation. Several studies on brightly coloured species of birds (HILL 1990, FIGUEROLA et al. 1999, SAINO et al. 2000) and fishes (KODRIC-BROWN 1983, MILINSKI & BAKKER 1990, HOUDE & TORIO 1992) found that, in adults, carotenoid-based colouration is related to health status or parasite infection. In birds, it is known that chromatic features can play a role in parent-offspring interactions and it is evident that chick begging in some species is related to certain colour patterns (it was first demonstrated by TINBERGEN & PERDECK 1950, that newly hatched herring gulls peck at the red dot on the bill of their parents).

The carotenoid effects in young have received far less attention. Chicks receive carotenoids from the egg contents. Carotenoids in the yolk of the newly laid egg depend to a large extent on the type and quantity of carotenoids present in the mother's diet and on her metabolic efficiency (SURAI & SPEAKE 1998). There is some evidence that the eggs of poultry supplemented with carotenoid-enriched food produce chicks with increased immunocompetence (HAQ et al. 1995, 1996).

In chickens, β -carotene significantly increases disease protection (TENGERDY et al. 1990) and the development of the visual system (BENDICH 1993). In white stork nestlings *Ciconia ciconia*, NEGRO et al. (2000) found a direct relationship between food and carotenoid expression: carotenoid plasma levels and skin pigmentation intensity were higher in a population with a diet rich in red swamp crayfish. However, the effect of carotenoids in the chick diet is still poorly known in wild birds.

Our study model, the moorhen *Gallinula chloropus*, is a waterbird with a red-yellow bill, evident in sexually mature birds and in newborn chicks. We have hypothesized that the bill colour in adults could signal individual quality, since we found that yellow intensity is correlated with some health parameters, e.g. fat abundance and heterophil/lymphocyte ratio (FENOGLIO et al. 2002), and the immune response to the PHA test (unpublished data). In chicks, a red and yellow bill colouration is temporarily present in the first 3 weeks after hatching and decreases with increasing self-sufficiency. These colourations were first believed to work as signals to confuse predators (HINGSTON 1933) or to help parents find their chicks (PYCRAFT 1934). However, the currently accepted idea (STEINBACHER 1930, BOYD & ALLEY 1948, pers. obs.) is that head colouration of rail chicks is used to stimulate parental care, like the coloured gape and palate in passerine birds. Interestingly, in another rail, the American coot *Fulica americana*, parents preferentially feed more ornamented chicks (LYON et al. 1994).

In this study, we raised two groups of moorhen chicks: one was fed with a carotenoid-enriched diet and the other with a control diet. We measured several indexes of body and health condition, as well as the bill and shield colours, and we examined the behavioural responses of chicks in two different tests. The aim of the study was to test whether carotenoid supplementation increases body condition, behavioural performance, colour expression and immunocompetence.

METHODS

Moorhen clutches were collected in mid-May 2000 from eight natural nests in the open-visitors area of the Stork Centre of Racconigi, NW Italy. We are confident that our sam-

pling did not cause additional damage to the pairs breeding in this area of the Centre, since in late May a large number of schools visit the Centre and the disturbance often causes parental nest desertion. The collected eggs were put in an incubator until hatching. After hatching, we randomly assigned chicks to two groups (control and carotenoid-rich): each group was composed of eight individuals, one from each of the sampled nests.

The two groups were raised with different food: the control chicks were fed with a basic diet of cereal pellets and earthworms, while the carotenoid-rich chicks received the same food plus 0.07 mg/day of the canthaxantin carotenoid. All chicks were raised together in a heated aviary, so they had the same environmental conditions and stresses. Each chick was marked with a coloured ring for individual recognition and it was fed apart from the others until satiated 4 times a day. The experimental rearing period lasted 32 days, after which the chicks were set free.

Biometry, bill colour, haematic assays and immunological test

Every 2 days the chicks were weighed, and the wing and tarsus lengths were measured. Every 5 days, we measured the colour intensity of the yellow and red parts of the bill using a Pantone code, and we later converted them to colour ranks by evaluating their coordinates in the L*a*b CIE method (FIGUEROLA et al. 1999). The red ranks ranged from 1-6 and the yellow from 1-5, from dullest to brightest.

Blood was taken from the brachial vein into a 7.5 cm long heparinized capillary at 21 days age. The capillaries were placed vertically for 4 hr in a refrigerator at 4 °C, and then the sedimentation rate was measured as the ratio between the length of the capillary tube with blood cells and the total length (SAINO et al. 1997). We also made blood smears, which were air dried, fixed with absolute ethanol and stained with the May-Grünwald Giemsa method. Smears were scanned at 630× magnification until 100 leukocytes were counted. Heterophils, eosinophils, basophils, lymphocytes and monocytes were differentiated and erythrocytes were counted. Leukocyte abundances were expressed in relation to 10,000 erythrocytes, according to SAINO et al. (1997).

We conducted the first immunological test on chicks at 12 days of age: to measure immunocompetence by means of the T-lymphocyte response (LOCHMILLER et al. 1993), we injected 0.25 mg of PHA (Sigma L-8754) diluted in 0.05 PBS ml in the right wing web area. Subcutaneous injection with PHA produces a local inflammation, and its relative thickness (wing-web index) is directly related to immunological conditions (MERINO et al. 1999). The thickness of the right wing web was measured with a spessimeter (Alpha spa, Milan, code SM112) with an accuracy of 0.01 mm. At 32 days, we performed the second PHA test.

Behavioural tests

When the chicks were 12 and 32 days old, they were subjected to two different behavioural tests measuring two indexes of activity.

- Open-field movement index: each chick was placed separately in an enclosed fenced chequered area. A hidden observer and a videocamera followed the path (run) of the chick: the movement index was calculated as the number of squares visited in 10 min (mean = 116.6; SD = 91.3). All observations were made in the morning, from 8:00 to 9:00 a.m.

- Pecking index: we measured the response to a red stimulus by moving a decoy with a red frontal shield toward each chick in a standard way and then counting the number of pecks the chick delivered to it in 1 min. Red was chosen because naive hatchlings show a pecking response and a colour preference for red or yellow rather than green, blue-green or blue (KEAR 1965, WEIDMANN 1965). Observations were made in the morning, on 12-day old chicks; they were not performed at 32 days because chicks usually do not peck after about 18-20 days of age.

RESULTS

Growth

The carotenoid-rich diet had no effect on growth (Fig. 1). The two groups of chicks had similar weights at all ages (repeated measures ANOVA, $F_{3,42} = 1.01$; $P = \text{NS}$; mean final weights: control = $135.1 \text{ g} \pm 4.2 \text{ SE}$, carotenoid-rich = $146.3 \text{ g} \pm 6.1 \text{ SE}$) and there were no significant differences in wing and tarsus lengths at all ages (Wing: repeated measures ANOVA, $F_{3,42} = 0.82$; $P = \text{NS}$; mean final lengths: control = $49.1 \text{ mm} \pm 2.5 \text{ SE}$, carotenoid-rich = $48.9 \text{ mm} \pm 1.4 \text{ SE}$. Tarsus: repeated measures ANOVA, $F_{3,42} = 1.12$; $P = \text{NS}$; mean final lengths: control = $54.4 \text{ mm} \pm 0.8 \text{ SE}$, carotenoid-rich = $54.0 \text{ mm} \pm 1.0 \text{ SE}$).

Bill colours

Chicks fed with the carotenoid-rich and control diets did not differ in bill colouration, either in the yellow or red parts, at all ages (Mann-Whitney tests, all $P = \text{NS}$; mean final red rank: control = $3.3 \pm 0.5 \text{ SE}$, carotenoid-rich = $3.6 \pm 0.3 \text{ SE}$; mean final yellow rank: control = $1.4 \pm 0.35 \text{ SE}$, carotenoid-rich = $1.1 \pm 0.13 \text{ SE}$).

Blood and immunological analyses

A comparison of the abundance of leukocyte families and the ES rate is reported in Table 1. No statistics were calculated for monocytes and basophils because they were rare or absent in all the smears. There was no influence of diet on the blood variables, the values being similar in the two groups of chicks.

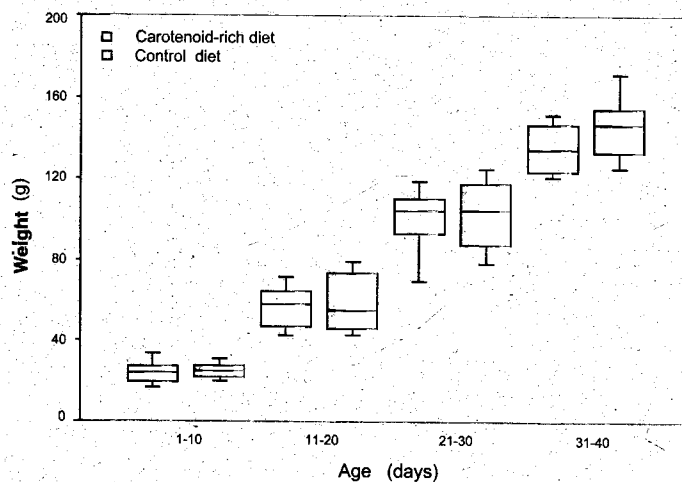


Fig. 1. — Growth rate in chicks fed with control (white) and carotenoid-rich (gray) diets. Box plot shows the 10th, 25th, 50th (median), 75th and 90th percentiles.

Reaction to the PHA injection differed between chicks raised with the carotenoid-rich diet and control chicks, both in the first (12 days old: Mann-Whitney test $U = 2$, $P < 0.002$; mean thickness: control = $0.4 \text{ mm} \pm 0.05 \text{ SE}$, carotenoid-rich = $0.9 \text{ mm} \pm 0.1 \text{ SE}$) and second test (32 days old: $U = 4.5$, $P < 0.004$; control = $2.5 \text{ mm} \pm 0.3 \text{ SE}$, carotenoid-rich = $5.2 \text{ mm} \pm 0.7 \text{ SE}$). The immunocompetence was higher in carotenoid-rich chicks in both tests (Fig. 2).

Behaviour

The open-field movement index showed no significant difference between the two groups of chicks (12 days old: $U = 22$, $P = \text{NS}$; mean values: control = $102.2 \pm 20.1 \text{ SE}$, carotenoid-rich = $163.9 \pm 42.2 \text{ SE}$; 32 days old: $U = 12$, $P = \text{NS}$; control = $30.1 \pm 62.8 \text{ SE}$, carotenoid-rich = $53.5 \pm 26.5 \text{ SE}$).

Table 1.

Differences between the carotenoid-rich and control groups at 21 days old (Mann-Whitney U tests).

Haematological parameters	Control mean \pm SD	Carotenoid-rich mean \pm SD	U	P
Eosinophils ¹	3.1 \pm 4.3	1.5 \pm 2.0	35	0.37 NS
Heterophils ¹	32.5 \pm 40.0	10.8 \pm 8.3	39	0.20 NS
Lymphocytes ¹	59.8 \pm 43.5	52.3 \pm 27.8	20	0.99 NS
Erythrocyte sedimentation rate	0.37 \pm 0.03	0.30 \pm 0.16	26	0.38 NS

¹ Eosinophils, heterophils, and lymphocytes abundances reported as N/10000 red blood cells.

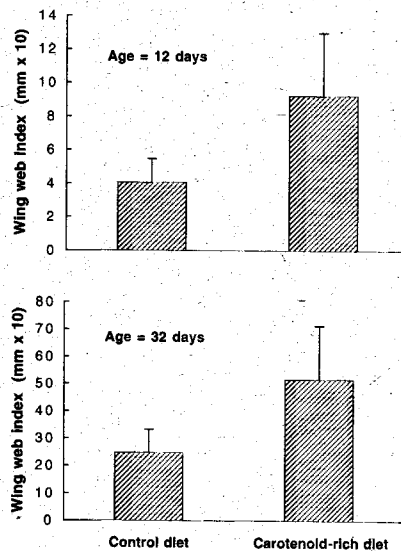


Fig. 2. — Immunocompetence (wing-web response to PHA injection) in chicks fed with the control and carotenoid-rich diets.

However, the chicks fed with a carotenoid-rich diet showed greater pecking activity, since they gave a larger number of pecks to the red decoy (12 days old: $U = 4$, $P < 0.009$; carotenoid-rich = 23.6 ± 3.7 SE, control = 8.4 ± 1.8 SE).

DISCUSSION

In this study, we have shown that moorhen chicks fed with a carotenoid-rich diet had a greater immune function and were more reactive in a behavioural test (pecking a bill model simulating the parents), while bill colouration, locomotor activity and growth did not differ from that of control chicks.

Carotenoids are a group of more than 600 different biochemicals. In this study we utilized canthaxanthin, one of the two major carotenoids found in nature, the second being beta-carotene. Birds preferentially accumulate lutein, canthaxanthin, and zeaxanthin (MØLLER et al. 2000). Canthaxanthin has been extensively considered in studies concerning immunocompetence (PALOZZA & KRINSKY 1992, BAILEY & CHINNAH 1995, HAQ et al. 1996, CARPENTER et al. 1997) and, as a scavenger of hydroxyl radicals, canthaxanthin is more effective than β -carotene (ROUSSEAU et al. 1992).

The absence of an effect of carotenoid administration on growth rate agrees with previous studies on domestic chicks (HAQ et al. 1996). However, the lack of an effect on bill colouration contrasts with a study on white stork nestlings, in which there was increased skin pigmentation in individuals fed with more carotenoids (NEGRO et al. 2000), and with one on great tit nestlings, in which plumage colours were more intense following carotenoid supplementation (TSCHIRREN et al. 2000). Furthermore, some studies have demonstrated that carotenoid administration in adult birds can also enhance colour intensity (HILL et al. 1994). The lack of an effect on bill coloration could be due to different reasons. Among these, (a) there is only indirect evidence (FAIVRE et al. 2001) that structural colouration of the bill depends on carotenoids; the metabolic pathway for the absorption of canthaxanthin and its accumulation in the moorhen bill was not investigated in this study, and it deserves to be verified; (b) in a life history perspective, it could be hypothesized that a threshold for adequate bill colouration has to be ensured first in moorhen chicks, and this fact should minimize the difference between experimental groups. Indeed, we believe that in the early phases of the chick's life, when sibling competition can be very high, the communicative function of carotenoid-supported colouration is a priority (SAINO et al. 2000). In rails, the chick's ornamental traits communicate its quality to the parents and the parents feed ornamented chicks in preference to non-ornamented chicks (LYON et al. 1994).

In our study, leukocyte abundances did not differ between the two groups. This could be due to slow maturation in primary and secondary lymphoid organs. Indeed, it has been shown in poultry that chicks cannot synthesize their own antibodies until several days post-hatching (BLOUNT et al. 2000).

In our moorhen chicks, a direct immune response challenge (PHA test) showed that carotenoid enrichment enhances immunocompetence. This agrees with the findings of TENGEDY et al. (1990) of enhanced humoral immunity in chickens receiving a carotenoid supplement.

The chicks with a carotenoid-rich diet showed a higher response to a stimulus: they pecked at a model bill faster and more intensely than control chicks. Since the open-field test showed no differences between the two groups, it can be

supposed that the effect of carotenoids is specific to neurosensory development, while there is little or no effect on general and aspecific locomotor activity (carotenoids are involved in the development of visual system implementation and light perception; LATSCHA 1990, BENDICH 1993). Our results on the positive effect of carotenoids on pecking activity and immune response can be useful in understanding the ecological effects involving these substances. As suggested by BLOUNT et al. (2000), their influence might be greater than has been realized previously.

ACKNOWLEDGEMENTS

We thank Beatrice Guasco, Viviana Cocito, Bruno and Gabriella Vaschetti for their help during the fieldwork, and Camilla Acquarone and three anonymous referees for their constructive comments on the manuscript. Financial support was provided by Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica 40 and 60% grants.

REFERENCES

- BAILEY C.A. & CHINNAH A.D. 1995. Neonatal immune response and growth performance of chicks hatched from SCWL breeders fed diets supplemented with β -carotene, canthaxanthin, or lutein. *Poultry Sciences* 74: 844-851.
- BENDICH A. 1989. Carotenoids and the immune response. *Journal of Nutrition* 119: 112-115.
- BENDICH A. 1993. Biological functions of dietary carotenoids. *Annals New York Academy of Sciences* 691: 61-67.
- BLOUNT J.D., HOUSTON D.C. & MØLLER A.P. 2000. Why egg yolk is yellow. *Trends in Ecology & Evolution* 15: 47-49.
- BOYD H.J. & ALLEY R. 1948. The function of the head coloration of the nestling coot and other nestling rallidae. *Ibis* 90: 582-593.
- BURTON G.W. & INGOLD K.U. 1984. Betacarotene: an unusual type of lipid antioxidant. *Science* 224: 569-573.
- BURTON G.W. 1989. Antioxidant actions of carotenoids. *Journal of Nutrition* 119: 109-111.
- CARPENTER K.L.H., VAN DER VEEN C., HIRD R., DENNIS I.F., DING T., MITCHINSON M.J. 1997. The carotenoids beta-carotene, canthaxanthin and zeaxanthin inhibit macrophage-mediated LDL oxidation. *FEBS Letters* 401: 262-266.
- FAIVRE B., PRÉAULT M., THÉRY M., SECONDI J., PATRIS B., CÉZILLY F. 2001. Breeding strategy and morphological characters in an urban population of blackbirds, *Turdus merula*. *Animal Behaviour* 61: 969-974.
- FENOGLIO S., CUCCO M. & MALACARNE G. 2002. Bill colour and body condition in the Moorhen (*Gallinula chloropus*). *Bird Study* 49: 89-92.
- FIGUEROLA J., MUÑOZ E., GUTIÉRREZ R. & FERRER D. 1999. Blood parasites, leukocytes and plumage brightness in the ciril bunting *Emberiza cirilus*. *Functional Ecology* 13: 594-601.
- HAO A., BAILEY C.A. & CHINNAH A. 1995. Neonatal immune response and growth performance of chicks hatched from single comb white leghorn breeders fed diet supplemented with β -carotene, canthaxanthin, or lutein. *Poultry Science* 74: 844-851.
- HAO A., BAILEY C.A. & CHINNAH A. 1996. Effect of β -carotene, canthaxanthin, lutein and vitamin E on neonatal immunity of chicks when supplemented in the broiler breeder diets. *Poultry Science* 75: 1092-1097.
- HILL G.E. 1990. Female house finches prefer colourful males: sexual selection for a condition dependent trait. *Animal Behaviour* 40: 563-572.
- HILL G.E. 1996. Redness as a measure of the production cost of ornamental coloration. *Ethology Ecology & Evolution* 8: 157-175.

- HILL G.E. 2000. Energetic constraints on expression of carotenoid-based plumage coloration. *Journal of Avian Biology* 31: 559-566.
- HILL G.E., MONTGOMERIE R., INOUE C.Y. & DALE J. 1994. Influence of dietary carotenoids on plasma and plumage colour in the house finch: intra- and intersexual variation. *Functional Ecology* 8: 343-350.
- HINGSTON R.G.W. 1933. The meaning of animal colour and adornment. *London*.
- HOUDE A.E. & TORIO A.J. 1992. Effect of parasitic infection on male colour pattern and female choice in guppies. *Behavioural Ecology* 3: 346-351.
- KEAR J. 1965. The pecking response of young coots *Fulica atra* and moorhens *Gallinula chloropus*. *Ibis* 108: 118-122.
- KODRIC-BROWN A. 1983. Determinants of male reproductive success in pupfish (Cyprinodontidae: *Cyprinodon*). *Animal Behaviour* 31: 128-137.
- LATSCHA T. 1990. Carotenoids – their nature and significance in animal feeds. *F. Hoffmann-LaRoche*.
- LOCHMILLER R., VETSEY M.R. & BOREN J.C. 1993. Relationship between protein nutritional status and immunocompetence in Northern Bobwhite chicks. *Auk* 110: 503-510.
- LYON B.E., EADIE J.M. & HAMILTON L.D. 1994. Parental choice selects for ornamental plumage in American coot chicks. *Nature* 371: 240-243.
- MERINO S., MARTINEZ J., MØLLER A.P., SANABRIA L., DE LOPE F., PÉREZ J. & RODRIGUEZ-CAABEIRO F. 1999. Phytohaemoagglutinin injection assay and physiological stress in nestling house martins. *Animal Behaviour* 58: 219-222.
- MILINSKI M. & BAKKER T.C.M. 1990. Female sticklebacks use male colouration in mate choice and hence avoid parasitised males. *Nature* 344: 330-333.
- MØLLER A.P., BIARD C., BLOUNT J.D., HOUSTON D.C., NINNI P., SAINO N. & SURAI P.F. 2000. Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? *Avian and Poultry Biology Reviews* 11: 137-159.
- NEGRO J.J., TELLA J.L., BLANCO G., FORERO M.G. & GARRIDO-FERNANDEZ J. 2000. Diet explains interpopulation variation of plasma carotenoids and skin pigmentation in nestling white storks. *Physiological Biochemical Zoology* 73: 97-101.
- OLSON J.A. 1989. Biological actions of carotenoids. *Journal of Nutrition* 119: 94-95.
- PALOZZA P. & KRINSKY N.I. 1992. Astaxanthin and canthaxanthin are potent antioxidants in a membrane model. *Archives of Biochemistry and Biophysics* 297: 291-295.
- PYCRAFT W.P. 1934. Birds of Great Britain and their natural history. *London*.
- ROUSSEAU E.J., DAVISON A.J. & DUNN B. 1992. Protection by beta-carotene and related compounds against oxygen-mediated cytotoxicity and genotoxicity: implications for carcinogenesis and anticarcinogenesis. *Free Radical Biology and Medicine* 4: 407-433.
- SAINO N., CALZA S. & MØLLER A.P. 1997. Immunocompetence of nestling barn swallows in relation to brood size and parental effort. *Journal of Animal Ecology* 66: 827-836.
- SAINO N., NINNI S., CALZA S., MARTINELLI F., DE BERNARDI F. & MØLLER A.P. 2000. Better red than dead: carotenoid-based mouth coloration reveals infection in barn swallow nestlings. *Proceeding of the Royal Society London (B)* 267: 57-61.
- STEINBACHER G. 1930. Entwicklung und Bau der roten Stirnpapillen bei Dünenjungen von *Fulica atra*. *Journal für Ornithologie* 78: 53-58.
- SURAI P.F. & SPEAKE B.K. 1998. Distribution of carotenoids from the yolk to the tissues of the chick embryo. *Journal of Nutritional Biochemistry* 9: 654-651.
- TENGERDY R.P., LACETERA N.G. & NOCKELS C.F. 1990. Effect of beta carotene on disease protection and humoral immunity in chickens. *Avian Diseases* 34: 848-854.
- TINBERGEN N. & PERDECK A.C. 1950. On the stimulus situations releasing the begging response in the newly hatched herring gulls (*Larus argentatus* Pont.). *Behaviour* 3: 1-39.
- TSCHIRREN B., FITZE P. & RICHNER H. 2000. Carotenoids, condition and bright birds - an experimental study on the mechanisms of variation in plumage coloration in Great tit nestlings (*Parus major*). *Proceedings of the 8th International ISBE Congress, Zürich*, p. 200.
- WEIDMANN U. 1965. "Colour preference" and pecking response in young moorhens *Gallinula chloropus* and coots *Fulica atra*. *Ibis* 107: 108-110.