AGE-RELATED CHANGES IN THE ACTIVITY OF BONE ALKALINE PHOSPHATASE AND ITS APPLICATION AS A MARKER OF PREFLEDGING MATURITY OF NESTLINGS IN WILD PASSERINES

Vallo Tilgar,[1] Priit Kilgas, Marko Mägi, and Raivo Mänd

Department of Zoology, Institute of Ecology and Earth Sciences, University of Tartu, Vanemuise 46, 51014, Tartu, Estonia

Abstract.—Recent studies have suggested that bone alkaline phosphatase (ALP) may be a valuable indicator of skeletal development in wild birds. However, the information about age-related dynamics of ALP isoforms in passerines is very scanty. We examined age-related changes in the activity of bone ALP and liver ALP and tested the applicability of these isoenzymes as indicators of chick maturity in randomly selected nestlings of a small passerine bird, the Great Tit (*Parus major*). Bone ALP activity was elevated in the middle of the nestling period (day 8), when skeletal growth is assumed to be most rapid, and declined significantly during the prefledging stage (day 15 posthatch). Bone ALP activity at this age was positively and highly significantly related to the overall duration of nestling period and negatively and less significantly related to wing length and body mass. All three morphological traits of the 15-day-old nestlings were nearly significantly negatively correlated with the duration of the nestling period. Liver ALP activity neither changed with nestling age nor was related to nestling morphology. We suggest that prefledging activity of bone ALP is a more reliable indicator of nestling maturity than traditionally used morphological measurements. *Received 9 January 2007, accepted 22 August 2007.*

Key words: alkaline phosphatase, Great Tit, growth, maturity, *Parus major.*

Cambios Relacionados con la Edad en la Actividad de la Fosfatasa Alcalina del Hueso y su Aplicación como Marcador de Madurez Previa al Emplumamiento de los Pichones en Paserinos Silvestres

Resumen.—Estudios recientes han sugerido que la fosfatasa alcalina (FA) ósea puede ser un indicador eficaz del desarrollo del esqueleto en las aves silvestres. Sin embargo, la información sobre la dinámica relacionada con la edad de las isoformas de FA en los paserinos es muy limitada. Examinamos cambios relacionados con la edad en la actividad de la FA ósea y la FA hepática, y evaluamos la aplicabilidad de estas isoenzimas como indicadoras de la madurez de los pichones en individuos seleccionados al azar de un ave paserina pequeña, *Parus major.* La actividad de la FA ósea fue elevada durante la mitad del período de anidación (día 8), cuando se supone que el crecimiento del esqueleto es más rápido, y disminuyó significativamente durante el estadío previo al emplumamiento (día 15 luego de la eclosión). La actividad de la FA ósea a esta edad estuvo positiva y muy significativamente relacionada con la duración total del período de pichones, y negativa y menos significativamente relacionada con la longitud del ala y el peso del cuerpo. Los tres caracteres morfológicos de los pichones de 15 días de edad estuvieron correlacionados negativa y casi significativamente con la duración del período de pichones. La actividad de la FA hepática no cambió con la edad del pichón y tampoco estuvo relacionada con la morfología del pichón. Sugerimos que la actividad de la FA ósea previa al emplumamiento es un indicador más confiable de la madurez del pichón que las medidas morfológicas tradicionalmente usadas.

It is widely known that newly hatched birds with initial size differences and different growth trajectories often achieve similar body sizes before fledging (Smith and Wettermark 1995, Gebhardt-Henrich and Richner 1998, Badyaev and Martin 2000, Metcalfe and Monaghan 2001). However, a question remains whether catch-up growth in certain body traits (e.g., in body mass or tarsus length) reflects compensation in all developmental aspects. For example, brood members may be indistinguishable in overall skeletal size but may exhibit developmental differences in plumage and in soft, flexible leg bones (Tilgar et al. 2004a, b). Hence, achieving full skeletal size is not, in itself, a reliable indication of developmental stage (Ricklefs 1975, Graveland and van Gijzen 1994, Dobado-Berrios and Ferrer 1997). Therefore, assessing nestling quality shortly before fledging merely on the basis of morphological traits may entail considerable error. One can even speculate that a depressed growth rate and delayed ossification may result

in a prolonged nestling period, extending the time during which the young are vulnerable to predators (e.g., Perrins 1979) and parasites (Møller 1990, Gebhardt-Henrich and Richner 1998). Alternatively, if the fledging date of less-developed nestlings is not postponed, such nestlings may have a higher risk of mortality immediately after fledging because of their weaker flying abilities (soft bones, shorter wings; Barclay 1994, McCarty 2001). Hence, ascertaining the developmental stage of a nestling before it leaves the nest would provide valuable information on its possible future performance.

Because it is usually difficult to discriminate among fledglings at different developmental stages solely on the basis of morphology, there is a need for additional indicators. A potentially suitable indicator of nestling developmental maturity is the enzyme serum alkaline phosphatase (ALP), because its level varies with age (e.g., Viñuela et al. 1991, Hoffmann et al. 1994). Serum ALP activity is the sum of ALP activities from different organs: bone, liver, and intestine. The two most relevant isoforms of this enzyme in the blood are liver ALP and bone ALP, which are products of the same gene but differ as a result of post-translational glycosylation (Hoffmann et al. 1994, Romagnoli et al. 1998). Liver ALP is synthesized by hepatocytes (Sherlock and Dooley 2002). It reflects the function of ATPase (Ohkubo et al. 1974) and participates actively in the down-regulation of secretory activity of the intrahepatic biliary epithelium (Alvaro et al. 2000). Increased activity of liver ALP in serum is typically associated with abnormalities in liver functions (Sherlock and Dooley 2002), but its level can also change with diet (Watkins et al. 2000) and with hormonal treatment (Solter et al. 1994). The bone isoform (bone ALP), a membrane-bound protein that is synthesized by osteoblasts in the bone tissue (de Behr et al. 2003), may be a sensitive indicator of skeletal development in both mammals and birds (Price 1993, Hoffmann et al. 1999, Allen et al. 2000, de Behr et al. 2003, Tilgar et al. 2004b, Smits et al. 2007).

In wild birds, ALP activity has been associated with bone growth in various bird species. Studies with raptors (Viñuela et al. 1991, Viñuela and Ferrer 1997, Bailey et al. 1998, Dobado-Berrios et al. 1998, Villegas et al. 2002), the Pigeon Guillemot (*Cepphus columba*; Seiser et al. 2000), and the White Stork (*Ciconia ciconia*; Smits et al. 2007) have shown that blood levels of ALP tend to be at their maximum near the end of the nestling period, when the growth of long bones is practically finished. Note that the above-mentioned studies, except Smits et al. (2007), were based on total ALP and provided no information about the bone ALP isoenzyme. In passerine birds, ALP activity has been associated with nestling development in only a few studies; total ALP was measured by Ormerod et al. (1991) and bone ALP by Tilgar et al. (2004a, b). There is some evidence that the rate of bone ossification (Bilby and Widdowson 1971, Ricklefs 1975) as well as bone ALP level (Tilgar et al. 2004b) of small passerines, in contrast to large birds, decline gradually before fledging. However, we still lack reliable information about the age-related dynamics of bone ALP in passerine birds.

The main objectives of the present study were (1) to investigate age-related changes in the activities of ALP isoenzymes in the blood of growing nestlings and (2) to assess the suitability of bone ALP as a marker of general nestling development. We assumed that bone ALP activity predicts the rate of bone formation that, in turn, should be reflective of overall nestling maturity. Although liver ALP is not indicative of bone mineralization, its level was also measured as potentially reflective of some other aspects of age-specific metabolism (e.g., Hoffmann et al. 1994, Alvaro et al. 2000 and references therein, Watkins et al. 2000). For these purposes, the nestlings of a small cavity-nesting passerine bird, the Great Tit (*Parus major*), were sampled at two distinct age points, in the middle (day 8) and near the end (day 15) of the nestling period. Specifically, we hypothesized that during the peak growth period (in the middle of the nestling period; Tilgar and Mänd 2006), the bone ALP level should be significantly higher than at the prefledging stage. Prefledging activity of bone ALP was expected to correlate with the duration of the nestling period and with wing length, a nestling trait known to increase steadily near and beyond fledging time (e.g., Kunz and Ekman 2000). On the other hand, we expected prefledging tarsus length and body mass to be weakly related to bone ALP activity, because these traits are fully developed well before nestlings leave the nest (Ricklefs 1975, Kunz and Ekman 2000, McCarty 2001).

Methods

The study was conducted near Kilingi-Nõmme (58°7′N, 25°5′E), southwest Estonia, in 2004. The study area (~50 km²) contained both deciduous and coniferous forest plots. Great Tits bred in nest boxes mounted on tree trunks at a height of 1.5–2.0 m. The internal size of nest boxes was $11 \times 11 \times 30$ cm, and the diameter of the entrance hole was 3.5–4.0 cm. Old nest material was removed in March, before the start of nest building. The nest boxes were checked daily by the end of the incubation period (11–14 days), until all nestlings were hatched. In each brood, one synchronously hatched nestling was chosen randomly for measurements. On day 8 posthatch, it was weighed using a Pesola spring balance to the nearest 0.1 g, tarsus length was measured with a sliding caliper to the nearest 0.1 mm, and a blood sample (~100 μL) was collected into a heparinized capillary tube from the brachial vein. These procedures were performed again on day 15 posthatch (before the usual fledging time, which occurs between 17 and 19 days). In addition, wing length of the 15-day-old nestlings was measured with a sliding caliper to the nearest 1 mm. All samples were collected between 1000 and 1600 hours to minimize any possible variation in blood chemistry caused by the birds' daily rhythm (Ferrer 1990 and references therein). All broods were checked daily to determine the duration of the nestling period. Only successful nests (where at least one nestling fledged) were included in analyses.

Plasma was separated from blood cells by 10-min centrifugation at 10,000 rpm and stored at −20°C until analyzed. Total ALP activity was assessed as U/L by regular colorimetric test. The activities of bone ALP and liver ALP were estimated with a standard agarose gel electrophoresis with an REP system (commercial REP ALP isoenzyme kit; Helena Laboratories, Beaumont, Texas). This procedure is based on physicochemical and electrophoretic properties of isoenzymes. After electrophoresis, gels were incubated with REP ALP isoenzyme chromagen and then densitometrically scanned at a wavelength of 595 nm. The control (gel ALP isoenzyme control, Helena Laboratories) was used to aid in the identification of ALP isoenzymes by agarose electrophoresis. It was prepared from pooled human serum and contained a liver and bone isoenzyme band. The control was a stabilized liquid. The repeatability of ALP measurements was very high (bone ALP: $r = 0.88$, $F = 16.1$, df = 2 and 5, $P = 0.005$; liver ALP: $r = 0.95$, $F = 38.4$, df = 2 and 5, $P < 0.001$;

TABLE 1. Activities (U/L) of different isoforms of plasma alkaline phosphatase (ALP) in the blood of Great Tit nestlings measured at days 8 and 15 posthatch ($n = 17$ pairs).

	Day 8 (mean ± SD)	Day 15 (mean ± SD)	Paired t-test	
			t	P
Bone ALP	170.5 ± 64.0	129.3 ± 43.1	2.82	0.012
Liver ALP	858.9 ± 222.9	751.9 ± 108.8	1.82	0.09

total ALP: $r = 0.96$, $F = 54.0$, df = 2 and 6, $P < 0.0001$; calculated as intraclass correlation coefficients from one-way analysis of variance [ANOVA] according to Lessells and Boag [1987]).

We used STATISTICA, version 7.1 (Statsoft, Tulsa, Oklahoma), for data analyses. Pairwise t-tests and Pearson correlations (r) were computed for normally distributed data; otherwise, Spearmans correlations (r_s) were used. Because no habitat-related differences were found in nestling body mass and tarsus length ($P > 0.16$) or in ALP values ($P > 0.5$), combined data over habitats were used. All significance levels are for two-tailed tests.

RESULTS

Age-related dynamics of ALP.—Bone ALP activity declined significantly between days 8 and 15 posthatch. Only a weak tendency to decline was seen in liver ALP (Table 1).

Neither bone ALP nor liver ALP activity measured at day 8 predicted its level at day 15 significantly (bone ALP: $r = 0.42$, $P = 0.09$, $n = 17$; liver ALP: $r = 0.24$, $P = 0.3$, $n = 17$). Bone ALP was positively correlated with liver isoform at day 8 ($r = 0.55$, $P = 0.014$, $n = 19$), but this relationship was not significant at day 15 ($r = 0.31$, $P = 0.3$, $n = 17$).

ALP, nestling morphology, and duration of nestling period.—Bone ALP measured at day 15 was strongly positively correlated with the duration of the nestling period ($r_s = 0.63$, $P = 0.006$, $n = 16$; Fig. 1), which was not the case for liver ALP ($r_s = 0.02$, $P = 0.9$,

TABLE 2. Correlations among three traits in Great Tit nestlings measured at days 8 and 15 posthatch ($n = 17$ at day 8, $n = 16$ at day 15).

	Wing length		Tarsus length		Body mass	
	r	P	r	P	r	P
Day 8						
Bone ALP			0.30	0.24	0.34	0.18
Liver ALP			0.08	0.8	0.08	0.8
Tarsus length					0.88	<0.001
Day 15						
Bone ALP	−0.51	0.043	−0.43	0.095	−0.51	0.044
Liver ALP	−0.44	0.085	−0.12	0.6	−0.06	0.8
Wing length			0.63	0.007	0.85	<0.001
Tarsus length					0.81	<0.001

ALP = alkaline phosphatase.

$n = 16$). Neither of these isoenzymes measured at day 8 predicted the duration of the nestling period (bone ALP: $r_s = −0.20$, $P = 0.4$, $n = 17$; liver ALP: $r_s = −0.24$, $P = 0.35$, $n = 17$). All three morphological traits of the 15-day-old nestlings were (or nearly were) significantly and negatively correlated with the length of the nestling period (wing length: $r_s = −0.49$, $P = 0.047$; tarsus length: $r_s = −0.47$, $P = 0.058$; body mass: $r_s = −0.48$, $P = 0.051$; $n = 16$ for each trait). None of the morphological traits measured at day 8 predicted the duration of the nestling period (tarsus length: $r_s = −0.32$, $P = 0.2$, $n = 17$; body mass: $r_s = −0.38$, $P = 0.13$, $n = 17$; wing length was not measured at this age).

Neither bone ALP nor liver ALP level was correlated significantly with morphological traits of nestlings at day 8 (Table 2). At day 15, bone ALP activity was inversely related to wing length and body mass, but not to tarsus length (Table 2). None of these body traits was correlated significantly with liver ALP at that age (Table 2).

DISCUSSION

Bone formation and ALP dynamics.—Bone ALP activity measured in nestlings in the middle of the nestling period was higher than that measured a week later, shortly before fledging. Given that bone ALP is produced by bone-forming osteoblast cells, the changes in its activity directly reflect bone formation (Cubo et al. 2000, Hamade et al. 2003, Tilgar et al. 2008). Therefore, declining values of bone ALP observed in full-grown nestlings probably indicate the decelerating phase of skeletal ossification. This result is also consistent with our previous findings that bone ALP level tends to decline before fledging, and the onset of this decline was postponed in the nestlings with reduced growth rates (Tilgar et al. 2004a, b). However, the dynamics of bone ALP throughout the growth period can be different in birds with large body size. For example, in the White Stork, bone ALP tended to reach a maximum level shortly before fledging (Smits et al. 2007). Hence, intensive ossification may overlap with the active phase of somatic growth in small birds (e.g., in Great Tits, the maximum mass gain occurs at day 6; Tilgar and Mänd 2006), whereas these two processes appear to be more separated temporally in large birds. This indicates that maximum mineralization in the latter species is predominantly related to appositional bone
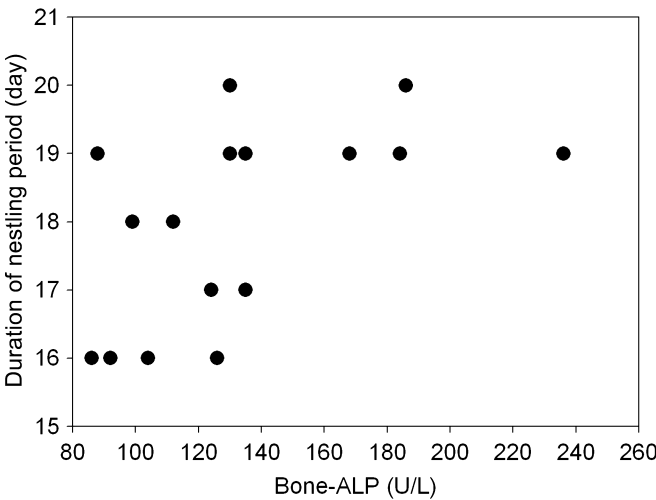

FIG. 1. The relationship between bone alkaline phosphatase (ALP) activity of nestlings at the prefledging stage (day 15 posthatch) and duration of the nestling period.

growth. However, further investigations are needed to clarify these observed differences among bird groups.

Another important finding of the present study is that age-related changes in liver ALP activity were less evident than the respective changes in bone ALP dynamics. This suggests that liver ALP cannot be used as a sensitive indicator of maturity. It is still noteworthy that both bone and liver isoenzymes were positively correlated with each other in the middle of the nestling period, whereas this association was much weaker at the prefledging stage. Although liver ALP activity is not related to skeletal development, this isoenzyme is susceptible to hormonal control (Solter et al. 1994) and dietary treatment (Watkins et al. 2000), thus potentially reflecting the overall intensity of metabolism. Hence, one can speculate that the intensity of metabolic processes is correlated with the rate of bone ossification at day 8, but not at the prefledging stage.

Bone ALP as an indicator of nestling maturity.—Our previous studies indicated that ALP activity measured shortly before fledging can be used to reveal subtle developmental differences between nestlings of similar size (Tilgar et al. 2004a, b). We proposed that young with faster growth rates may complete the rapid phase of ossification earlier than those with slower growth rates, and respective differences between developmental stages can be ascertained by different values of bone ALP measured at the prefledging stage. We also suggested that measuring ALP in nestlings may help to predict the fledging date. Given that broods with faster growth rates usually fledge earlier than those with slower growth rates (Keller and van Noordwijk 1994, Viñuela and Ferrer 1997), we expected that the duration of the nestling period is closely linked to the prefledging maturity of nestlings. Our results demonstrated that bone ALP level measured at day 15 was positively correlated with the duration of the nestling period. Moreover, we found that the prefledging activity of bone ALP was inversely related to all three morphological measurements. This was expected in the case of wing length, because feather growth steadily increases up to, and even after, fledging in tits (*Parus* spp. and *Poecile* spp.; e.g., Nilsson and Svensson 1996, Kunz and Ekman 2000, Oddie 2000). On the other hand, body mass and tarsus length of nestlings usually stabilize several days before fledging in most passerine birds (Ricklefs 1975, Kunz and Ekman 2000, Oddie 2000, McCarty 2001, Tilgar and Mänd 2006). We emphasize that prefledging measurements were taken several days before the usual fledging time for Great Tits, when growth and development probably were not complete. However, although fledging date was also correlated with the morphological traits we measured in 15-day-old nestlings, these correlations were only marginally significant and were considerably weaker than the correlation between fledging date and bone ALP. In conclusion, we suggest that the bone ALP level is a reliable indicator of nestling maturity at the prefledging stage, potentially facilitating the prediction of offspring postfledging survival.

Literature Cited

Allen, L. C. V., M. J. Allen, G. J. Breur, W. E. Hoffmann, and D. C. Richardson. 2000. A comparison of two techniques for the determination of serum bone-specific alkaline phosphatase activity in dogs. Research in Veterinary Science 68:231–235.

Alvaro, D., A. Benedetti, L. Marucci, M. D. Monache, R. Monterubbianesi, E. Di Cosimo, L. Perego, G. Macarri, S. Glaser, G. Le Sage, and G. Alpini. 2000. The function of alkaline phosphatase in the liver: Regulation of intrahepatic biliary epithelium secretory activities in the rat. Hepatology 32:174–184.

Badyaev, A. V., and T. E. Martin. 2000. Individual variation in growth trajectories: Phenotypic and genetic correlations in ontogeny of the House Finch (*Carpodacus mexicanus*). Journal of Evolutionary Biology 13:290–301.

Bailey, T. A., U. Wernery, J. Howlett, J. Naldo, and J. H. Samour. 1998. Age-related plasma chemistry findings in the Buff-crested Bustard (*Eupodotis ruficrista gindiana*). Journal of Veterinary Medicine B 45:635–640.

Barclay, R. M. R. 1994. Constraints on reproduction by flying vertebrates: Energy and calcium. American Naturalist 144:1021–1031.

Bilby, L. W., and E. M. Widdowson. 1971. Chemical composition of growth in nestling blackbirds and thrushes. British Journal of Nutrition 25:127–134.

Cubo, J., V. Fouces, M. Gonzáles-Martín, V. Pedrocchi, and X. Ruiz. 2000. Nonheterochronic developmental changes underlie morphological heterochrony in the evolution of the Ardeidae. Journal of Evolutionary Biology 13:269–276.

de Behr, V., D. Daron, A. Gabriel, B. Remy, I. Dufrasne, D. Serteyn, and L. Istasse. 2003. The course of some bone remodelling plasma metabolites in healthy horses and in horses offered a calcium-deficient diet. Journal of Animal Physiology and Animal Nutrition 87:149–159.

Dobado-Berrios, P. M., and M. Ferrer. 1997. Age-related changes of plasma alkaline phosphatase and inorganic phosphorus, and late ossification of the cranial roof in the Spanish Imperial Eagle (*Aquila adalberti* C. L. Brehm, 1861). Physiological Zoology 70:421–427.

Dobado-Berrios, P. M., J. L. Tella, O. Ceballos, and J. A. Donázar. 1998. Effects of age and captivity on plasma chemistry values of the Egyptian Vulture. Condor 100:719–725.

Ferrer, M. 1990. Hematological studies in birds. Condor 92:1085–1086.

Gebhardt-Henrich, S., and H. Richner. 1998. Causes of growth variation and its consequences for fitness. Pages 324–339 *in* Avian Growth and Development: Evolution within the Altricial-Precocial Spectrum (J. M. Starck and R. E. Ricklefs, Eds.). Oxford University Press, Oxford, United Kingdom.

Graveland, J., and T. van Gijzen. 1994. Arthropods and seeds are not sufficient as calcium sources for shell formation and skeletal growth in passerines. Ardea 82:299–314.

Hamade, E., G. Azzar, J. Radisson, R. Buchet, and B. Roux. 2003. Chick embryo anchored alkaline phosphatase and mineralization process *in vitro*: Influence of Ca^{2+} and nature of substrates. European Journal of Biochemistry 270:2082–2090.

HOFFMANN, W. E., N. EVERDS, M. PIGNATELLO, AND P. F. SOLTER. 1994. Automated and semiautomated analysis of rat alkaline phosphatase isoenzymes. Toxicologic Pathology 22:633–638.

HOFFMANN, W. E., P. F. SOLTER, AND B. W. WILSON. 1999. Clinical enzymology. Pages 399–454 in The Clinical Chemistry of Laboratory Animals, 2nd ed. (W. F. Loeb and F. W. Quimby, Eds.). Taylor and Francis, Philadelphia.

KELLER, L. F., AND A. J. VAN NOORDWIJK. 1994. Effects of local environmental conditions on nestling growth in the Great Tit Parus major L. Ardea 82:349–362.

KUNZ, C., AND J. EKMAN. 2000. Genetic and environmental components of growth in nestling Blue Tits (Parus caeruleus). Journal of Evolutionary Biology 13:199–212.

LESSELLS, C. M., AND P. T. BOAG. 1987. Unrepeatable repeatabilities: A common mistake. Auk 104:116–121.

McCARTY, J. P. 2001. Variation in growth of nestling Tree Swallows across multiple temporal and spatial scales. Auk 118:176–190.

METCALFE, N. B., AND P. MONAGHAN. 2001. Compensation for a bad start: Grow now, pay later? Trends in Ecology and Evolution 16:254–260.

MØLLER, A. P. 1990. Effects of parasitism by a haematophagous mite on reproduction in the Barn Swallow. Ecology 71:2345–2357.

NILSSON, J.-Å., AND M. SVENSSON. 1996. Sibling competition affects nestling growth strategies in Marsh Tits. Journal of Animal Ecology 65:825–836.

ODDIE, K. R. 2000. Size matters: Competition between male and female Great Tit offspring. Journal of Animal Ecology 69:903–912.

OHKUBO, A., N. LANGERMAN, AND M. M. KAPLAN. 1974. Rat liver alkaline phosphatase: Purification and properties. Journal of Biological Chemistry 249:7174–7180.

ORMEROD, S. J., J. O'HALLORAN, S. D. GRIBBIN, AND S. J. TYLER. 1991. The ecology of Dippers Cinclus cinclus in relation to stream acidity in upland Wales: Breeding performance, calcium physiology and nestling growth. Journal of Applied Ecology 28:419–433.

PERRINS, C. M. 1979. British Tits. Collins, Glasgow, United Kingdom.

PRICE, C. P. 1993. Multiple forms of human serum alkaline phosphatase: Detection and quantification. Annals of Clinical Biochemistry 30:355–372.

RICKLEFS, R. E. 1975. Patterns of growth in birds. III. Growth and development of the Cactus Wren. Condor 77:34–45.

ROMAGNOLI, E., G. MINISOLA, V. CARNEVALE, A. SCILLITANI, V. FRUSCIANTE, G. ALIBERTI, AND S. MINISOLA. 1998. Assessment of serum total and bone alkaline phosphatase measurement in clinical practice. Clinical Chemistry and Laboratory Medicine 36:163–168.

SEISER, P. E., L. K. DUFFY, A. D. McGUIRE, D. D. ROBY, G. H. GOLET, AND M. A. LITZOW. 2000. Comparison of Pigeon Guillemot, Cepphus columba, blood parameters from oiled and unoiled areas of Alaska eight years after the Exxon Valdez oil spill. Marine Pollution Bulletin 40:152–164.

SHERLOCK, S., AND J. DOOLEY. 2002. Diseases of the Liver and Biliary System, 11th ed. Blackwell Science, Malden, Massachusetts.

SMITH, H. G., AND K.-J. WETTERMARK. 1995. Heritability of nestling growth in cross-fostered European Starlings Sturnus vulgaris. Genetics 141:657–665.

SMITS, J. E., G. R. BORTOLOTTI, R. BAOS, R. JOVANI, J. L. TELLA, AND W. E. HOFFMANN. 2007. Disrupted bone metabolism in contaminant-exposed White Storks (Ciconia ciconia) in southwestern Spain. Environmental Pollution 145:538–544.

SOLTER, P. F., W. E. HOFFMANN, M. D. CHAMBERS, D. J. SCHAEFFER, AND M. S. KUHLENSCHMIDT. 1994. Hepatic total 3α-hydroxy bile-acids concentration and enzyme activities in prednisone-treated dogs. American Journal of Veterinary Research 55:1086–1092.

TILGAR, V., P. KILGAS, A. VIITAK, AND S. J. REYNOLDS. 2008. The rate of bone mineralization in birds is directly related to alkaline phosphatase activity. Physiological and Biochemical Zoology 81:106–111.

TILGAR, V., AND R. MÄND. 2006. Sibling growth patterns in Great Tits: Does increased selection on last-hatched chicks favour an asynchronous hatching strategy? Evolutionary Ecology 20:217–234.

TILGAR, V., R. MÄND, I. OTS, M. MÄGI, P. KILGAS, AND S. J. REYNOLDS. 2004a. Calcium availability affects bone growth in nestlings of free-living Great Tits (Parus major), as detected by plasma alkaline phosphatase. Journal of Zoology (London) 263:269–274.

TILGAR, V., I. OTS, AND R. MÄND. 2004b. Bone alkaline phosphatase as a sensitive indicator of skeletal development in birds: A study of the Great Tit nestlings. Physiological and Biochemical Zoology 77:530–535.

VILLEGAS, A., J. M. SÁNCHEZ, E. COSTILLO, AND C. CORBACHO. 2002. Blood chemistry and haematocrit of the Black Vulture (Aegypius monachus). Comparative Biochemistry and Physiology A 132:489–497.

VIÑUELA, J., AND M. FERRER. 1997. Regulation of growth in Red Kites and Imperial Eagles. Wilson Bulletin 109:92–101.

VIÑUELA, J., M. FERRER, AND F. RECIO. 1991. Age-related variations in plasma levels of alkaline phosphatase, calcium and inorganic phosphorus in chicks of two species of raptors. Comparative Biochemistry and Physiology A 99:49–54.

WATKINS, B. A., Y. LI, K. G. D. ALLEN, W. E. HOFFMANN, AND M. F. SEIFERT. 2000. Dietary ratio of (n-6)/(n-3) polyunsaturated fatty acids alters the fatty acid composition of bone compartments and biomarkers of bone formation in rats. Journal of Nutrition 130:2274–2284.