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EFFECT OF CALCIUM SUPPLEMENTATION ON REPRODUCTIVE PERFORMANCE OF THE PIED FLYCATCHER *FICEDULA HYPOLEUCA* AND THE GREAT TIT *PARUS MAJOR*, BREEDING IN NORTHERN TEMPERATE FORESTS

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Dissertation is accepted for the commencement of the degree of Doctor of Philosophy in animal ecology at the University of Tartu on December 13, 2001 by the Council of the Faculty of Biology and Geography, University of Tartu

Opponent: Dr S. James Reynolds, University of Memphis, USA

Commencement: room 301, Vanemuise St. 46, Tartu,
on February 22, 2002 at 9.15 a.m.

The publication of this dissertation is granted by the University of Tartu

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is a summary of the following papers which are referred to in the text by the Roman numerals:

I Tilgar, V., Mänd, R. & Leivits, A. 1999. Effect of calcium availability and habitat quality on reproduction in Pied Flycatcher *Ficedula hypoleuca* and Great Tit *Parus major.* — *Journal of Avian Biology* 30: 383–391.

II Mänd, R., Tilgar, V. & Leivits, A. 2000. Reproductive response of Great Tits, *Parus major*, in a naturally base-poor forest habitat to calcium supplementation. — *Canadian Journal of Zoology* 78: 689–695.

III Mänd, R., Tilgar, V. & Leivits, A. 2000. Calcium, snails, and birds: a case study. — *Web Ecology* 1: 63–69.

IV Tilgar, V., Mänd, R. & Mägi, M. Calcium shortage as a constraint on reproduction in Great Tits *Parus major*: a field experiment (submitted manuscript)

V Mänd, R. & Tilgar, V. 2002. Does supplementary calcium reduce the cost of reproduction in the Pied Flycatcher *Ficedula hypoleuca*? — *Ibis* (in press)

VI Ots, I., Tilgar, V., Mänd, R., Mägi, M. & Kilgas, P. Maternal Ca availability affects bone growth in great tit (*Parus major*) nestlings as detected by alkaline phosphatase (submitted manuscript)

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1. INTRODUCTION

In life-history models, as well as in conservation biology, lack of proper knowledge about ecological constraints is a recurring problem with crucial importance. To understand what limits bird populations within the habitats they occupy, it is helpful to distinguish between the environmental factors (e.g. resource availability, predation pressure, or temperature) that influence populations. Particular populations may be affected by different factors but often one factor emerges as of overriding importance at any one time. Food is one of the most important extrinsic factors influencing life-history traits in birds (Lack 1968, Stearns 1992, Newton 1998). To raise young successfully birds are generally held to adjust their breeding decisions ultimately to food availability which may limit reproduction of birds through its effects on the timing of egg laying (Källander 1974, Svensson & Nilsson 1995, Perrins 1996, Nager *et al.* 1997, Ramsay & Houston 1997), egg production (Arcese & Smith 1988, Riddington & Gosler 1995) and nestling growth (Arcese & Smith 1988, Richner 1992, Styrsky *et al.* 2000). The production of eggs and caring for young are nutritionally demanding processes particularly for small passerine birds laying a clutch weighing more than the female's own body weight. Reproductive traits of passerines often vary in years (Lundberg & Alatalo 1992, Nager & van Noordwijk 1995, Winkel & Hudde 1997), habitats or territories (van Balen 1973, Gezelius *et al.* 1984, Riddington & Gosler 1995, Sanz 1997), presumably due to different levels of food supply (Perrins 1965, Lundberg *et al.* 1981, Newton 1998). However, several experiments providing supplementary food to breeding birds have found only moderate or no effect at all on reproductive performance (references in Nager *et al.* 1997). It has been suggested that differences in the protein and energy content of the supplementary food may explain some of the inconsistencies in the results (Arcese & Smith 1988). Hence, besides the energy content of the diet, the specific nutrients and microelements are essential for reproduction, but the availability of these food components is still poorly understood (Carey 1996).

Recently, a number of studies have estimated the role of food in avian reproduction from the perspective of proteins (Bolton *et al.* 1992, Nager *et al.* 1997, Ramsay & Houston 1998) and micronutrients (Triyuwanta & Nys 1992, Graveland *et al.* 1994, Graveland & van Gijzen 1994, Reynolds 1997, Boorman & Gunaratne 2001). Availability of calcium-rich food items has turned out to be crucial for many bird species as indicated by several experimental and comparative studies (Glooschenko *et al.* 1986, Ormerod *et al.* 1988, 1991, Graveland *et al.* 1994, Eeva 1996, Beintema *et al.* 1997, Pahl *et al.* 1997, Scheuhammer *et al.* 1997, Weimer & Schmidt 1998, Bureš & Weidinger 2000, 2001, Dhondt & Hochachka 2001). The reason is that the normal food of most birds contains insufficient calcium for eggshell formation (Graveland & van Gijzen 1994, Graveland & Berends 1997, Reynolds 1997, 2001) and for the skeleton growth in nestlings (St. Louis & Breebaart 1991, Barclay 1994, Johnson & Lombardo 2000, Bureš & Weidinger 2000, 2001). Therefore, birds need additional calcium-rich material such as, for example, snail shells and calcareous grit during these critical periods (see Graveland 1996 for references). Some birds (e.g. chickens, pigeons, knots) store calcium in the interior of their leg bones prior to egg laying using the skeleton as a temporary store of endogenous calcium for eggshell formation (Peakall 1970, Piersma *et al.* 1996). In small passerines, however, the capacity to use skeletal calcium for eggshell formation is quite limited, therefore they search for calcareous material on a daily basis during egg production (Graveland & van Gijzen 1994, Graveland & Berends 1997, Houston *et al.* 1995, Perrins 1996, Reynolds 1997, 2001).

Due to the special importance of dietary calcium, passerines are very susceptible to acidification of soil or water that reduces the availability of calcium-rich food (Ormerod *et al.* 1988, Ormerod & Rundle 1998, Drent & Woldendorp 1989, Graveland *et al.* 1994, Weimer & Schmidt 1998, Bureš & Weidinger 2001). Graveland *et al.* (1994) provided experimental evidence that production of eggs with defective shells in Great Tits in the Netherlands was causally related to a decline in snail abundance on acidified soils. Molluscs need calcium for their calcareous shells, but acidification and base cation leaching may lead to decreased calcium content in litter and the surface soil layer (Wäreborn 1969, 1970, 1992).

So far, such data had almost exclusively been collected in acidified areas. However, Graveland & Drent (1997) have suggested that calcium limitation may be a widespread phenomenon also in non-acidified calcium-poor areas. There are large regions in the world with acidic rock or soils where snail shells or other calcium sources are scarce. For example, a substantial part of the territory of Estonia is covered with pine forests on poor, naturally acidic soils (Taimre 1989) with low snail abundance (**III**). Due to the low density of human population, calcium deficiency in such habitats cannot be compensated for as easily as in the Netherlands by using anthropogenic calcium sources (Graveland 1996, Graveland & Drent 1997).

The key problem in estimating the true cost of shell formation in naturally base-poor areas is that the time required for formation of specific adaptations may have been sufficient and adaptive response might be expected (Graveland & Drent 1997, Bureš & Weidinger 2001). For example, passerine birds may respond to decreased calcium supply by increasing their search effort for calcium (Turner 1982, Graveland & Berends 1997, Bureš & Weidinger 2001). As a result, birds might lay smaller or fewer eggs with good shells in calcium-poor areas due to increased energy expenditure (Graveland & Drent 1997). By using observational data, it is hard to distinguish such calcium-specific behavioural adaptation from possible parallel adaptation to low availability of other nutrients. To eliminate the influence of confounding effects, an experimental approach is needed.

The main purpose of my thesis was to test experimentally whether natural calcium deficiency may significantly constrain reproduction of forest passerines breeding in non-acidified base-poor areas. In addition, the following specific hypotheses were tested:

First, the effect of calcium deficiency on breeding performance may vary with season and habitat type, presumably depending on climatic and nutritional conditions.

Second, there may exist certain species-specific differences in exact response to calcium shortage because life-history traits and feeding behaviour are remarkably different in different species.

Third, susceptibility of birds to calcium deficiency may also vary within species, depending, e.g., on the age of parents (breeding experience) or current parental investment.

To address the questions mentioned above, calcium supplementation experiments were carried out during several years with Great Tits breeding both in deciduous and coniferous forest habitats, and with Pied Flycatchers breeding in the same coniferous habitats. Simultaneously, the abundance of land snails (potential natural calcium source) was estimated in different habitats of the study area.

2. MATERIAL AND METHODS

2.1. Studied species

The Great Tit is a small (18–19 g) resident and monogamous passerine bird inhabiting the whole Eurasian continent (Perrins 1979). Great Tits forage mainly in the tree canopy during breeding time, feeding on a variety of insects, especially on Lepidoptera and sawfly larvae, and spiders (Rootsmäe & Veroman 1974, Gosler 1993, Cramp & Perrins 1993). In Estonia, Great Tits usually start egg laying at the end of April or in early May, producing approximately 9–12 eggs per clutch (Hõrak *et al.* 1995). The egg volume is about 1.60–1.70 cm^3 in our study area (**I**). Both sexes feed the nestlings. The number of fledglings per nest is usually 7–9, and the young leave the nest normally on the 17th day after hatching. In our study area, about 40–60 per cent of females lay second clutches during the same breeding season.

The Pied Flycatcher is an even smaller (12–13 g) migratory passerine bird ranging over most of northern and eastern Europe (Lundberg & Alatalo 1992). The species winters in tropical West Africa and Mediterranean countries (Lundberg & Alatalo 1992). Pied Flycatchers obtain food from trees or on the ground, and the diet mainly consists of various arthropods (Rootsmäe & Veroman 1974, Lundberg & Alatalo 1992). In Estonia, the first Pied Flycatchers arrive at breeding areas in early May, and the autumn migration to wintering areas begins in the middle of August (Leibak *et al.* 1994). Flycatchers in our area usually start egg-laying in late May, producing normally six to seven eggs per clutch. The egg volume (about 1.60–1.65 cm^3) is nearly the same as for Great Tit, although Great Tit females are much heavier than Pied Flycatcher females just before the laying season (18 g and 14 g respectively, Ojanen 1983). Both sexes feed the nestlings. The number of fledglings per nest is about 5–7 in our study area. The young leave the nest normally on the 15–16th day after hatching. A proportion of males are polygynous, and such males mostly feed their primary brood (Lundberg & Alatalo 1992).

Great Tits and Pied Flycatchers breed in tree holes but also readily accept nest-boxes. The breeding biology of these birds has been intensively studied over the whole of Europe during the past 50 years (Lundberg & Alatalo 1992, Gosler 1993). Both species are short-lived, and more than half of the individuals breed only once during their life-time (Perrins 1979, Lundberg & Alatalo 1992). However, the breeding time, foraging habits and reproductive traits are remarkably different for these species. For example, the number of eggs produced per clutch is much greater for tits as compared to flycatchers. On the other hand, the weight of the egg in relation to the size of a female bird is remarkably larger for Pied Flycatcher than for Great Tit (11.4% versus 9.4% of their body weight, proportions calculated from data in Ojanen 1983). Hence, there is reason to believe that Great Tits and Pied Flycatchers may also respond to calcium deficiency quite differently.

2.2. Study area

The study was conducted in the surroundings of Kilingi-Nõmme (58° 7′ N, 25° 5′ E), south-western Estonia, in 1995–1997 and 1999–2000 in the case of the Great Tit, and in 1995–1997 and 1999 in the case of the Pied Flycatcher. The area is situated in the transition zone from a base-rich moraine plateau to base-poor sedimentary sand dunes. Various forest habitats in the area can be divided into two contrasting types — deciduous and coniferous habitats.

The deciduous woodlands occur mainly as isolated patches between cultivated fields or as galleries along stream valleys. Grey Alder *Alnus incana* and Silver Birch *Betula pendula* are the predominant tree species with rich deciduous understorey. The coniferous woodlands are located on nutrient-poor sandy and (in the lower parts) peat soils. The dominant tree species is Scots Pine *Pinus sylvestris*, which sometimes forms mixed stands with Norway Spruce *Picea abies* (on sandy soil) and Downy Birch *Betula pubescens* (on peat soil). In the field layer, dwarf shrubs of *Vaccinium myrtillus* and *Rhodococcum vitis-idaea* are the most frequent species.

Studies of land molluscs carried out in our study area in 1995 and 1999 revealed that the density and species richness of snails (a potential calcium source for forest birds) were significantly lower in coniferous than in deciduous habitat (**I**, **III**). In 1999, the density of live snails plus empty shells was almost three times smaller in coniferous habitat than in deciduous, while also the mean size of the snails was smaller in coniferous habitat (**III**).

Our study plots contained nest-box lines and were distributed over an area of approximately 50 km^2 (see **I** for the scheme of study area).

2.3. Sampling and measurements

Great Tits and Pied Flycatchers bred in nest-boxes mounted on tree trunks at a height of about 1.5–2.0 m. The internal size of nest-boxes was approximately 11×11×30 cm and the diameter of the entrance was 3.5–4.0 cm. The distance between the boxes was approximately 50–60 m. Old nest material was removed every year.

Nest-boxes were checked regularly to record laying date for the first egg, clutch size, hatching date, brood size, and number of fledglings. One egg from each clutch (2 eggs in the case of Great Tit in 1995) was collected randomly in 1995–1996 to measure the thickness of the eggshell. The clutches were photographed for estimating egg dimensions and volume as described in Mänd *et al.* (1986). Mean egg volume per clutch was used in the analysis.

The nestlings were weighed with a precision of 0.1 g using a Pesola spring balance and their tarsi were measured to the nearest 0.1 mm with a sliding caliper on 13th day (Pied Flycatcher) or 15th day (Great Tit) after hatching.

Adults were captured during the second half of the nestling period and their sex and age of non-ringed individuals were determined after Svensson (1984). They were also weighed and their tarsal lengths were measured.

Only first clutches of the Great Tit were taken into account, except in 1999 when both first and second clutches were studied separately. Predated or deserted nests were omitted from analysis when calculating number of fledglings per nest. Breeding was considered successful when at least one chick fledged from the nest, and unsuccessful when no chicks fledged.

During the breeding season of 2000, blood samples were collected from Great Tit nestlings to compare the catalytic activity of alkaline phosphatase (ALP) between calcium-supplemented and control groups. The level of this enzyme, especially the bone isoform, is a highly specific marker for bone formation rate (Price 1993). The aim of this biochemical analysis was to test whether calcium-fed nestlings complete their skeleton ossification earlier than controls. Nestlings were sampled only once on 15th day after hatching (see **VI** for detailed methods), when their skeleton growth is nearly completed.

2.4. Calcium-provisioning experiment

The nest-boxes in our study plots were arranged in lines. The exact number of the nest-box lines where the experiment was carried out varied annually depending on available manpower (in five consecutive study years the number of lines considered was 7, 10, 11, 12 and 12 in coniferous habitat and 4, 4, 5, 8 and 8 in deciduous habitat). Each nest-box line, consisting usually of some tens of nest-boxes, ran in markedly homogeneous (either coniferous or deciduous) habitat. Each nest-box line was divided into several alternating experimental and control blocks consisting of approximately five consecutive nest-boxes. The number of blocks per line varied from 2 to 20 (on average 11 in coniferous and 9 in deciduous habitat), depending on the total number of nest-boxes in the line. Each year the first block of a nest-box line was randomly assigned as a control or supplemented block. Hence, the blocks were switched between treatment and reference allocation in different years. No more than one nest-box per block was occupied by the studied species, while some blocks remained unoccupied at all.

In the first study years, breeding data were also collected from some lines containing only control nest-boxes (from deciduous habitat in 1995–97 and coniferous habitat in 1995). As no significant statistical difference was detected in breeding parameters between the nests from such 'pure' control lines and control nests from experimental lines, both types of control nests were pooled in mentioned years.

Small metal feeders were mounted on nest-boxes. The feeders on experimental nest-boxes were filled with small fragments of domestic chicken (*Gallus domesticus*) eggshells about two weeks before the start of nest building in tits, and exceptionally during nest building in deciduous habitat in 1995. In 1995–1996 crushed snail shells were also provided. The feeders at occupied nest-boxes were regularly checked and refilled, so that the birds of the experimental group were always provided with a surplus of calcium-rich material. The feeders on the control nest-boxes were empty. Visual observations as well as nest material analysis confirmed that birds did consume eggshell fragments in feeders (**I**). Filled feeders might have also been used by birds from adjacent nest-boxes where supplementary calcium was absent (Graveland & Drent 1997). Therefore data from control birds nesting within 100 m of an experimental block were excluded from analyses.

In addition, another experiment (referred to as ‘calcium-exchange treatment’) was performed with Great Tits in 2000. From a certain number of nests provided with calcium before egg laying, calcium was removed after completing the clutch. Another group of nests, not supplemented during egg laying, were provided with calcium before hatching of nestlings. Blood samples were taken from nestlings on 15th day after hatching and activity of ALP was compared between these manipulated groups (**VI**).

3. RESULTS

3.1. Laying date

In the Pied Flycatcher, the provisioning of extra calcium had no effect on laying date in any but one year (**III**, **V**). This was the year with the latest start of breeding in flycatchers when calcium-fed pairs started egg production significantly earlier than controls (**III**). In the Great Tit, calcium supplementation advanced egg laying in base-poor coniferous habitat (**II**, **III**). The ANOVA results for all five study years for the Great Tit in coniferous habitat are as follows: calcium: $F_{1,\ 223}=9.58$, $P=0.002$; year: $F_{4,\ 223}=102.45$, $P<0.001$; interaction n.s.). Such effect was not found in deciduous habitat (calcium: $F_{1,\ 188}=0.05$, $P=0.829$; year: $F_{3,\ 188}=55.92$, $P<0.001$; interaction n.s.). It is noteworthy that the laying of calcium-fed tits in coniferous habitat was more advanced relative to the controls the later breeding began in a particular year (Fig. 1, **II**, **III**).

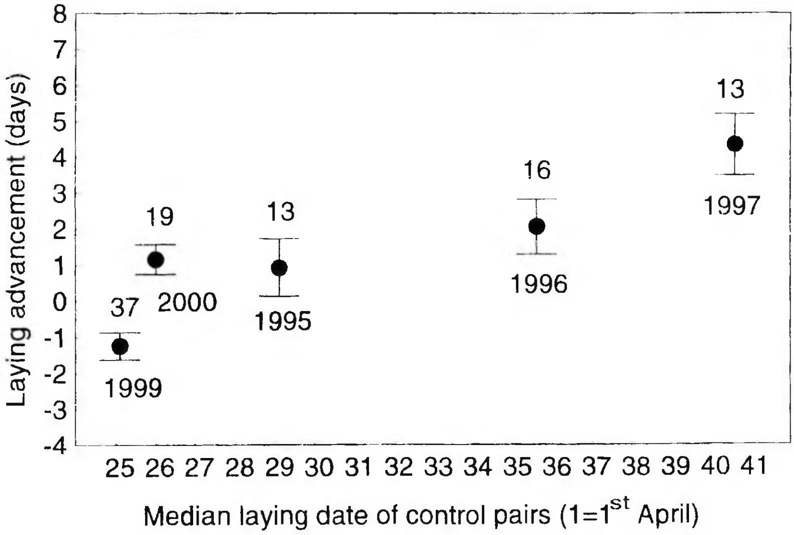

Figure 1. Advancement of egg laying in calcium-supplemented Great Tits in relation to median laying date of controls in 1995–1997, 1999 and 2000. In all figures, whiskers = s.e., numbers above whiskers denote sample sizes. Numbers below whiskers here are the breeding years.

3.2. Egg production

Egg volume (Fig. 2, **I**, **V**) and eggshell thickness (Fig. 3, **I**) were positively affected by calcium supplementation in the Pied Flycatcher. No significant effect of calcium supplementation on egg size or eggshell thickness was revealed in the case of the Great Tit (**I**, **II**).

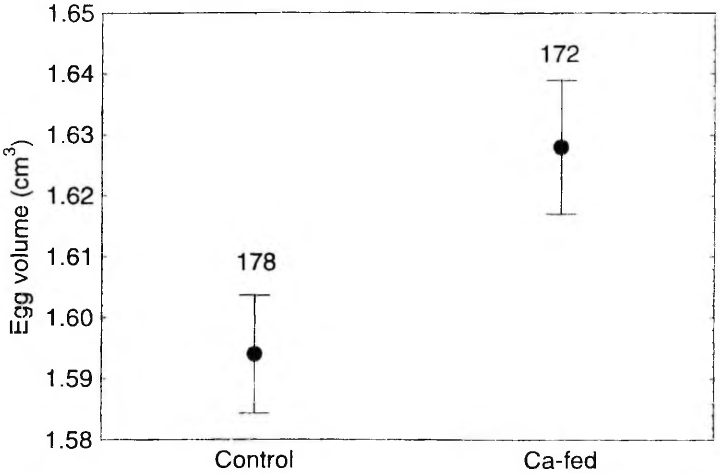

Figure 2. Egg volume (cm^3) in control and calcium-supplemented groups for the Pied Flycatcher (1995–1997 and 1999). Henceforth, group means in the figures are represented as average of yearly means.

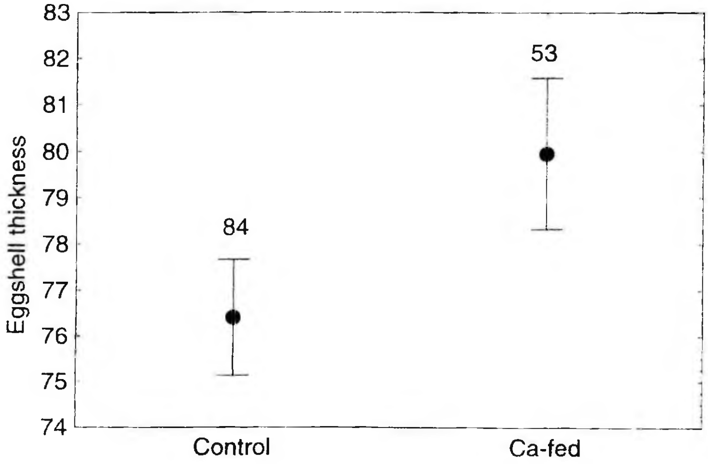

Figure 3. Eggshell thickness (µm) in control and calcium-supplemented groups for the Pied Flycatcher (1995–1996).

Clutch size was not affected by supplementation in the Pied Flycatcher (**I, V**). In the Great Tit, number of eggs laid was higher in calcium-supplemented than in control nests, while no interaction between supplementation and habitat was detected (Fig. 4, **IV**).

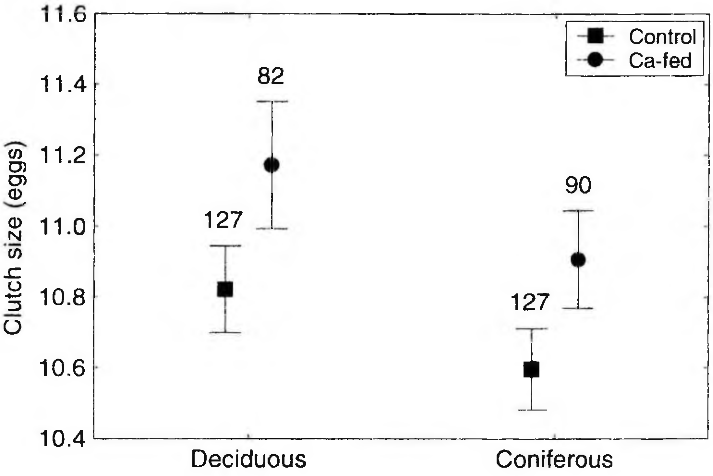

Figure 4. Clutch size in control and calcium-supplemented groups for the Great Tit (1995–1997, 1999 and 2000).

3.3. Hatching success and production of fledglings

Calcium supplementation did not improve hatching success in either species (**I, II**).

In the first two study years (1995 and 1996), 1–2 eggs were collected from each clutch of both species to study eggshell thickness (see details in **I**). In neither species was a significant effect of extra calcium on the production of fledglings found in these years (**I**). Probably, necessary parental effort was reduced to such an extent that parents could easily compensate for the differences in calcium availability in compared groups. For that reason, the effect of supplemental calcium on the number of fledglings was tested in the years when no eggs were removed from the nests.

No difference was found in the fledgling numbers between supplemented and control nests in the Pied Flycatcher (**V**). However, in the Great Tit, significantly more young hatched and fledged from supplemented nests compared to control nests, while no interaction between supplementation and habitat was detected (Fig. 5, **IV**). When clutch size was included as a covariate in the model, the effect of calcium provision on the number of fledglings was no more significant (**IV**). Thus it is not clear whether the larger number of fledglings in the experimental nests results merely from the larger initial number of eggs laid by the supplemented females, or whether calcium provision has some additional effect on hatching and/or fledging success.

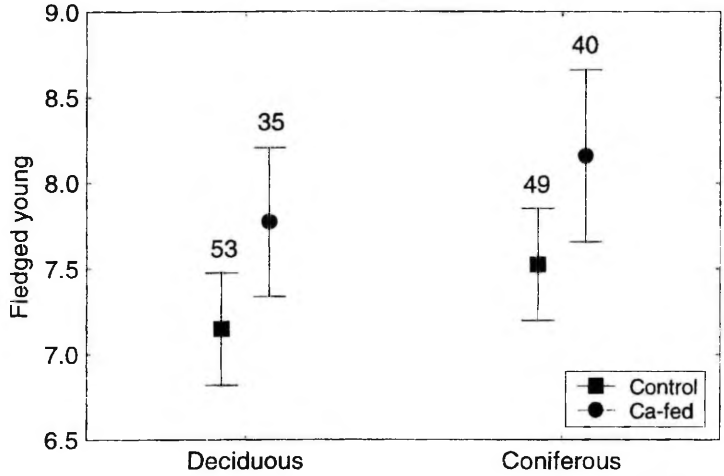

Figure 5. Number of fledglings per nest in control and calcium-supplemented groups for the Great Tit (successful nests in 1997, 1999 and 2000).

3.4. Characteristics of fledglings

In the Pied Flycatcher, calcium supplementation increased tarsus length of fledglings, but no effect of calcium on weight of fledglings was detected (**V**). In the Great Tit, no significant effect of extra calcium on tarsus length and weight of fledglings were found in the years when 1–2 eggs were removed from the nests (**I**). However, calcium supplementation increased tarsus length of fledglings in the Great Tit in the years when no eggs were removed from the nests (**IV**).

Furthermore, in 2000, activity of bone alkaline phosphatase (ALP — a highly specific marker of bone growth rate) was measured in the blood plasma of the Great Tit nestlings on 15th day after hatching (see Material and methods). Irrespective of the habitat type, bone-ALP values were lower in calcium-provided than in control fledglings (Fig. 6, **VI**). This indicates that supplemented nestlings had more developed bones on 15th day after hatching than controls.

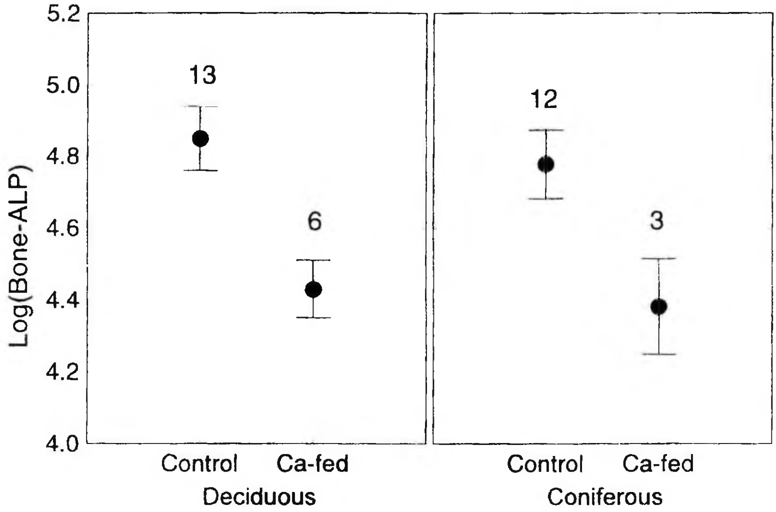

Figure 6. Bone-ALP activity in fledglings of the Great Tit in control and calcium-supplemented groups in 2000.

In addition, another experiment with calcium-exchange (see Material and methods) was performed to compare the relative impact of calcium availability at embryonic and post-hatching stages on the skeletal development of nestlings. The results revealed that fledglings of Great Tits reared in base-poor coniferous forest had lower bone-ALP activity in nests provided with calcium only during egg production as compared with nests where calcium was supplemented only after hatching of the young (**VI**). This difference was not detected in deciduous habitat (**VI**).

3.5. Characteristics of adults

In the Pied Flycatcher, female weight in the nestling phase was not significantly affected by calcium supplementation, but a highly significant interaction term between calcium and clutch size class became evident (**V**). Post-hoc analysis revealed that supplemented females laying larger than average clutches were significantly heavier in the nestling phase than controls laying large clutches (Fig. 7a, **V**). Moreover, when female age was included in the model, the significant calcium and female age interaction term emerged, indicating the positive effect of extra calcium on yearling's weight (Fig. 7b, **V**). In the Great Tit, calcium supplementation did not affect female weight in the nestling phase (**I**, **II**), and no significant interaction was found in relation to habitat, clutch size or female age (unpublished data).

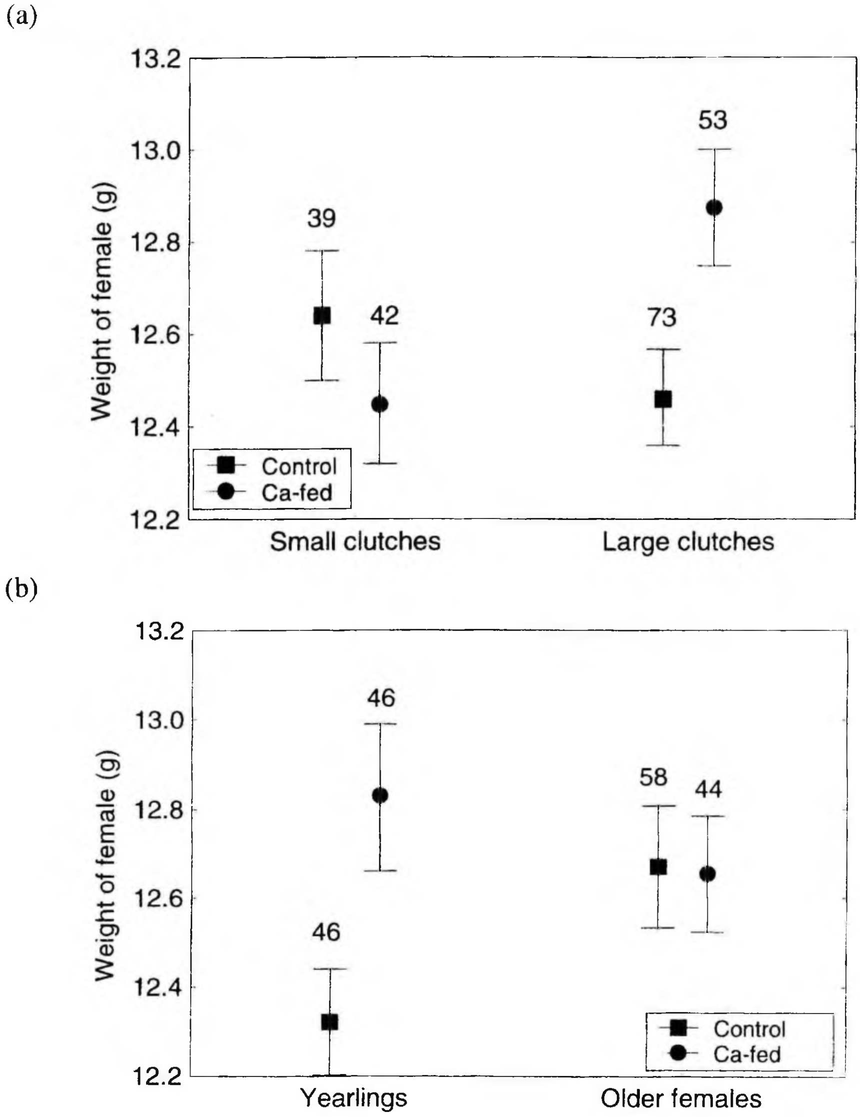

Figure 7. Female weight in control and calcium-supplemented groups in relation to (a) clutch size and (b) female age for the Pied Flycatcher (1995–1997 and 1999).

There was no relationship between calcium supplementation and female tarsus length in either species (**I**, **II**, **V**), and no significant interaction was detected in relation to habitat, clutch size or female age (**V**).

Male weight and tarsus length did not differ between groups in either species (**I**, **II**, **V**).

3.6. Second broods

The effect of calcium availability on second clutches of the Great Tit was studied in 1999. Calcium supplementation affected positively clutch size and number of fledged young during second breeding (**IV**).

No significant effect of extra calcium on other studied traits was found in the case of second clutches in 1999. Also, the probability of the double brooding did not depend on calcium supplementation during the first brood period (unpublished data).

4. DISCUSSION

4.1. Calcium shortage as a reproductive constraint in non-acidified habitat

This thesis provides the first experimental evidence that specific calcium limitation occurs in forest passerines breeding in non-acidified base-poor areas. Several breeding parameters of both studied species were improved by calcium provisioning, and no one parameter was negatively affected.

In the Great Tit, calcium supplementation advanced egg laying and increased clutch size and number and tarsus length of fledglings (**II**, **III**, **IV**). Moreover, indirect evidence was found that calcium deficiency might also contribute to delayed skeletal ossification of the nestlings (**VI**).

In the Pied Flycatcher, extra calcium increased the egg volume, eggshell thickness and tarsus length of fledglings (**I**, **V**). A more detailed study revealed that female weight in the nestling phase was positively affected by calcium supplementation in the case of females who laid larger than average clutches (**V**).

There may be two different mechanisms mediating the effect of calcium availability on the breeding performance of birds. First, eggshells and nestling skeletons, which require calcium in large quantities during their formation, may be directly dependent on the amount of available calcium (Graveland & van Gijzen 1994, Pahl *et al.* 1997). Second, an indirect mechanism, based on a trade-off between the time spent searching for calcium-rich items and time spent foraging for other nutrients (Graveland & Berends 1997) may be responsible for the positive effect of additional calcium on certain breeding parameters in calcium-poor environments (e.g., egg and clutch size or fledging success).

Graveland & Drent (1997) have hypothesized that in non-acidified, naturally calcium-poor areas there may have lapsed sufficient time for the development of specific adaptations in reproduction or feeding behaviour, in contrast to acidified areas where calcium deficiency is a recent phenomenon. Perhaps the most conspicuous example is that of the Blue Jay *Cyanocitta cristata* hoarding calcium in the autumn for next breeding period (Dhondt & Hochachka 2001). The cowbirds may supplement their diet with calcium from eggs removed from hosts' nests (Sealey 1994 cited in Dhondt & Hochachka 2001). It seems relatively common that passerine birds respond to a decreased calcium supply by increasing their searching effort for calcium-rich material (Turner 1982, Graveland & Berends 1997, Bureš & Weidinger 2001). For example, egg-laying females of the Great Tit on a calcium-deficient diet spent 43% of the daylight searching for calcium, which is almost twice that of females with sufficient calcium (Graveland & Berends 1997).

Besides increasing searching time for natural calcium-rich items, some species may learn to use anthropogenic calcium sources. For example, Great

Tits nesting close to picnic sites in the Netherlands used chicken eggshells (Graveland 1996). Furthermore, Meadow Pipits *Anthus pratensis* and Water Pipits *A. spinoletta* selected and brought supplemented calcium-rich items to their nestlings more often in acidified areas than in limestone areas (Bureš & Weidinger 2001). Also, birds may postpone breeding onset until they find an adequate source of calcium or until their body condition and rate of food intake reach a critical threshold for producing viable eggs and young (**II, III**). Indeed, it appeared that low calcium availability caused laying postponement in Great Tits in coniferous habitat, while no effect of calcium on egg volume or shell quality was detected (**II, III**). Therefore, it was assumed, in an earlier paper (**II**), that postponement of laying in tits may serve as an adaptive behaviour sufficient to prevent harmful consequences of calcium deficiency. However, later it became evident that calcium shortage affected not only traits indirectly related to breeding success but also nestling growth and reproductive output in terms of production of the young (**IV**). These findings support the conclusions about the constraining effect of low calcium availability on the development of nestling skeleton (Beintema *et al.* 1997, Pahl *et al.* 1997) and productivity of some bird species (Ormerod & Tyler 1993, Dekhuijzen & Schuijl 1996, Beintema *et al.* 1997, Weimer & Schmidt 1998).

In the Pied Flycatcher, calcium supplementation affected positively egg traits and tarsus length in fledglings, but hatching success and number of fledglings were not affected. Supposedly the females reduced the amount of resources invested per egg just within the limits of reaction norm, without a significant effect on reproductive output. Also, other authors, e.g., Johnson and Barclay (1996), have suggested that calcium shortage in habitats less affected by acid precipitation may be too weak to act as a significant constraint for reproduction. On the other hand, experimental provision of extra calcium had a positive effect on weight and condition of females tending large clutches (**V**). This finding indicates that low natural calcium availability may increase the cost of reproduction for individuals caring for large broods, since a poor nutritional condition of females may indicate physiological stress (Ricklefs 1974, Bryant 1979, Drent & Daan 1980, Nur 1984, Hillström 1995). There is even some evidence that lowered adult body mass decreases the probability of survival to the next breeding season (Askenmo 1977, Coulson *et al.* 1983, Nur 1984, Slagsvold 1984, Gustafsson & Sutherland 1988, Cichon *et al.* 1998). Moreno *et al.* (2001) suggest that a trade-off between daily energy expenditure (DEE) and immunity could underlie the cost of reproduction in female Pied Flycatchers. Hence, the fact that flycatchers laying large clutches were particularly vulnerable to low calcium indicates that calcium shortage may indirectly act as a selection factor against high fecundity.

Although the effect of calcium shortage in a naturally base-poor area of Estonia did not lead to such dramatic consequences as, for example, in the acidified area in the Netherlands (Graveland *et al.* 1994), the assumptions about adaptation of forest passerines to low calcium availability in non-acidified habitats must be treated with caution. As already assumed by Graveland & Drent (1997), even in such habitats calcium shortage appears to be a specific ecological constraint for birds, resulting either in lowered reproductive output or increased cost of reproduction. Thus birds breeding in naturally base-poor habitats may be vulnerable to possible increases in acid deposition. The results of the above experiments confirm the idea that the cost of egg formation and raising of nestlings of passerines in calcium-poor areas involves much more than the traditional estimate based on the protein and energy demand for eggs or nestlings.

4.2. Variation in response to calcium deficiency

There is a large annual variation in climate conditions and food availability in the temperate regions. Late start of egg laying in birds usually correlates with unfavourable weather conditions during the pre-breeding period (Lundberg & Alatalo 1992, Nager & van Noordwijk 1995), which limits the supply of energy and nutrients needed by the female for egg production (Perrins 1970, 1996). In this study, calcium supplementation in coniferous woods significantly advanced egg laying in both species in the years when egg laying started very late (**II**, **III**). Controlling for hatching date, there was also a positive effect of extra calcium on tarsus length and body mass of fledglings of the Great Tit in the year when breeding onset was the latest (**II**). It seems that food shortage during a particularly harsh breeding season amplifies the negative effect of calcium deficiency. This can be most plausibly explained by the above-mentioned trade-off between feeding time and time spent on calcium searching.

The results of experiments in a particular year often differed for the two studied species (**III**). For example, the latest breeding in the Pied Flycatcher was in 1999, when an extremely severe cold wave befell the country in mid-May, just before the normal time of egg laying for this species. In contrast, this was the year with the earliest breeding for the Great Tit. The reason for this is that the time interval between the starts of breeding of the studied species is quite long. Due to unstable climatic conditions in the study area, it may happen that the same year is favourable for one species but unfavourable for the other species, or *vice versa* (**III**). Hence, the extent of calcium limitation on reproduction of birds varies in different years and long-term studies are necessary to make reliable conclusions about its effect on a particular species in a particular area.

Further, calcium availability for breeding birds may change during the reproductive season. For example, birds in base-poor areas may have limited access to various calcium sources, and after depletion of a certain source it might be difficult to find another source. Knowledge about seasonal dynamics of calcium availability is important, since there are several peaks in calcium demand during breeding. The first peak takes place during egg formation before laying, due to high calcium content of eggshell, and the second peak is in the nestlings period, during rapid growth of nestling skeletons (Graveland & van Gijzen 1994). Moreover, the results of the calcium-exchange experiment in coniferous habitat (see section 3.4 in Results) indicate that for normal nestling development, the initial calcium deficiency during egg formation cannot be very easily compensated for by increased calcium consumption during the nestling stage (**VI**). The possible reason for this is that calcium availability affects nestling growth in different developmental stages: maternal effect via egg quality may be important for embryonic growth, and differences in consumption of calcium-rich items by nestlings may cause additional variation in nestling skeletal growth rate. Therefore, calcium availability during egg production may act like a double bottleneck both for laying eggs with normal shells and for producing young with well-developed skeletons.

The calcium-provision experiment with second broods of Great Tits showed that specific calcium limitation in the studied area exists not only early in spring but also in mid-summer. Extra calcium increased both clutch size and number of fledglings during second breeding (**IV**). Such a strong effect of extra calcium on second clutches within one particular year raises the question that calcium limitation during second breeding may be even more pronounced than during first breeding. This would be expected if food and/or calcium availability during second breeding were lower than during first breeding. Unfortunately there are no adequate data on seasonal dynamics of food and calcium availability in studied area. There exists also an additional factor which may play a significant role during second breeding and amplify the effect of calcium shortage: in this period tits must allocate part of their resources to moult (Eeva *et al.* 2000), which may amplify the effect of calcium shortage. Anyway, the finding that calcium availability in base-poor habitats may constrain reproduction of Great Tits not only in spring, when food is relatively scarce, but also later, is important as the second broods may contribute significantly to the overall annual reproductive output in this species.

In the beginning of the studies, the reproductive response of studied species to calcium supplementation was expected to be also habitat-dependent. In the first papers (**I** and **III**), it was presumed that calcium limitation is the case only in base-poor coniferous habitat and not in base-rich deciduous habitat. This presumption was based on the fact that land snails were significantly more abundant in deciduous as compared to coniferous habitat in our study area (**I**, **III**), while the snail density in coniferous forests was not remarkably higher than that in strongly acidified habitats in the Netherlands (**III**). However, the only breeding trait in the Great Tit, which was differentially affected by extra calcium in different habitats, was laying date. Calcium supplementation advanced the start of laying in coniferous but not in deciduous habitat. During the egg-forming phase, tits breeding in coniferous habitat presumably suffer stronger calcium limitation than conspecifics breeding in deciduous habitat where natural calcium sources are more available. Remind that calcium supplementation at egg-laying stage (maternal effect on embryonic growth) affected nestlings' bone formation in coniferous habitat but not in deciduous habitat (**VI**).

However, calcium-provision improved the reproductive output of Great Tits in both habitats, and no significant interaction term was detected between the experimental treatment and the habitat (**IV**). Hence, in spite of between-habitat differences in snail abundance, obtaining sufficient calcium requires much time and energy from tits even in deciduous habitat. Thus our results support the conclusion of Graveland & Berends (1997) that obtaining of sufficient calcium might be time-consuming even in relatively calcium-rich environments. Besides this, food resources may become exhausted faster in deciduous woodland patches than in large coniferous forests in our study area. Although Great Tits start laying earlier and lay more and larger eggs in deciduous than in coniferous habitat (**I**), in the latter they raise more and heavier fledglings (unpublished data). Unfortunately no data were collected about the effect of calcium-provision on Pied Flycatchers in deciduous habitat.

Comparison of data from Pied Flycatchers with those from Great Tits, collected from the same study area and from the same study period, revealed differences between the two species (Table 1).

Table 1. Effects of supplemental calcium on reproductive parameters of the Great Tit and the Pied Flycatcher in the same base-poor area. + — significant increase due to calcium supplements, ns — no significant effect of supplemental calcium

Breeding parameter	Great Tit[1]	Pied Fly-catcher[2]	References
Start of egg-laying*	+	ns	[1]**II, III** , [2]**V**
Eggshell thickness	ns	+	[1,2]**I**
Egg size	ns	+	[1]**I, II,** [2]**I, V**
Clutch size	+	ns	[1]**IV,** [2]**V**
Fledgling numbers	+	ns	[1]**IV,** [2]**V**
Fledgling tarsus length	+	+	[1]**IV,** [2]**V**
Female weight	ns	+	[1]**II,** [2]**V**

* — positive effect means that laying started earlier

In the Pied Flycatcher, calcium supplementation increased eggshell thickness and tarsus length of fledglings, the traits that are thought to depend directly on the amount of calcium in the diet. In the Great Tit, tarsus length and ossification of the skeleton in nestlings were also positively affected by supplementation (**IV, VI**). However, clearer discrepancies between other traits in the two species indicate that Great Tits and Pied Flycatchers in our study area may respond to calcium deficiency quite differently. For Great Tits, calcium availability significantly affected reproductive traits that are closely related to current reproductive success (laying date, clutch size and fledgling numbers). For Pied Flycatchers, body weight and egg size of the most productive females were affected, both of these parameters probably being related to female condition (**V**). Thus, Pied Flycatchers breeding in calcium-poor habitats seem to sacrifice their body condition for their current reproductive success, while Great Tits tend to sacrifice current reproductive output, thereby safeguarding their body condition. The finding of Pahl *et al.* (1997) that when exogenous calcium availability was low, Great Tits relax their reproductive effort in preference to depleting calcium deposits in their skeletons, supports this conclusion.

Such a difference between the two species can be explained by the fact that 40–60 per cent of Great Tits in our study area are the so-called multiple breeders, reproducing twice per season, while all Pied Flycatchers breed only once a year. Therefore, under unfavourable conditions, tits would benefit by not investing too heavily in the first brood, but by producing the second brood in the same season.

Experiments with the Pied Flycatcher revealed that the susceptibility of birds to calcium deficiency may also vary within species or populations. Flycatchers that laid larger clutches appeared to be more susceptible to calcium shortage than those that laid smaller clutches (**V**), possibly because of the relatively higher investment in reproduction (Charnov & Krebs 1974, Slagsvold & Lifjeld 1988, Lundberg & Alatalo 1992, Sanz 1995). Furthermore, extra calcium improved female weight in yearlings, but not in older birds (**V**). Hence, yearling females may be more vulnerable to reproductive stress as a result of calcium shortage than older and more experienced females.

In summary, the detailed reproductive response of passerines to low calcium availability has no uniform character, but varies annually and seasonally, showing also species/population specific and habitat specific differences. Although calcium shortage seems to be particularly pronounced in harsh breeding seasons and among those individuals investing relatively more in current reproduction, calcium limitation still exists even in midsummer, and in relatively base-rich deciduous habitat.

SUMMARY

Increasing numbers of studies have shown that several bird species have serious difficulties in obtaining sufficient calcium for their reproduction in acidified areas. It has been hypothesised that a specific calcium deficiency may constrain reproduction also in birds inhabiting non-acidified, naturally base-poor environments. However, no clear support for this assumption had been found before this study.

To test this assumption, calcium supplementation experiments with two hole-nesting passerines were carried out in northern temperate forests in Estonia during several years. So far, this thesis provides the strongest experimental evidence that a specific calcium limitation occurs in forest passerines breeding in naturally base-poor areas.

In the Great Tit, calcium supplementation advanced start of egg laying and increased clutch size and number and tarsus length of fledged young. Moreover, indirect evidence was found, using a biochemical marker, that calcium deficiency may contribute to delayed skeletal ossification of the nestlings, and that the initial calcium deficiency during egg formation cannot be easily compensated for by increased calcium consumption during the nestling stage.

In the Pied Flycatcher, extra calcium increased egg volume, eggshell thickness and fledgling tarsus length. Also, female weight (and body condition) in the nestling phase was positively affected by calcium supplementation in the case of yearlings and those females who lay larger than average clutches. The latter result indicates that calcium deficiency may increase cost of reproduction for this species and act as selective factor against large clutches.

A calcium-provision experiment with second broods of the Great Tit showed that calcium limitation exists not only early in spring, but also in mid-summer. Unexpectedly, no substantial differences were detected between base-rich deciduous and base-poor coniferous habitats as regards the effect of extra calcium on reproduction of the Great Tit. The only exception was start of egg laying, which was advanced by supplementation only in coniferous habitat. Also, certain evidence was found that calcium limitation is greater in those years when egg laying starts later than usually.

These findings imply that susceptibility of birds to calcium deficiency is not uniform, but varies between species as well as within species, depending also on weather conditions in the particular breeding season. Calcium limitation in non-acidified northern temperate forests is not so severe as in heavily acidified areas, but it still appears to be a specific ecological constraint for birds, resulting either in lowered current reproductive output or increased cost of reproduction.

SUMMARY IN ESTONIAN

Täiendava kaltsiumirikka toidu mõju põhja-parasvöötme metsades pesitsevate must-kärbsenäpi *Ficedula hypoleuca* ja rasvatihase *Parus major* sigimisnäitajaile

Üha rohkem uurijaid on leidnud, et hapestunud regioonides pesitsevatel lindudel on suuri raskusi munade moodustamiseks ja poegade luustiku kasvuks vajamineva kaltsiumi hankimisel. On oletatud, et kaltsiumipuudus võib piirata lindude sigimisedukust ka mittehapestunud, looduslikult kaltsiumivaestes elupaikades. Seni ei ole siiski leitud piisavalt tõendeid, mis seda oletust usaldusväärselt kinnitaks.

Hüpoteesi eksperimentaalseks kontrollimiseks söödeti parasvöötme mittehapestunud piirkonna metsades (Edela-Eestis) pesitsevale kahe linnuliigi esindajatele mitme aasta vältel lisakaltsiumi ja võrreldi nende sigimisnäitajaid kontrollisendite omadega. Uurimiste tulemused näitavad, et selles piirkonnas pesitsevate metsavärvuliste jaoks on kaltsiuminappus tõepoolest spetsiifiline pesitsust limiteeriv tegur.

Kaltsiumiga söödetud rasvatihased alustasid munemist varem, munesid suuremaid kurni ning kasvatasid üles rohkem ja pikemate jooksmetega poegi kui kontrollisendid. Biokeemilise markeri abil leiti kaudseid tõendeid, et kaltsiumidefitsiit võib aeglustada ka rasvatihase poegade skeleti luustumisprotsessi ning kaltsiumipuudust lootestaadiumis (munades) tundub olevat hiljem raske kompenseerida kaltsiumirikka toidu lisamisega poegadele.

Must-kärbsenäpil suurendas kaltsiumi lisamine munade mõõtmeid, munakoore paksust ja poegade jooksme pikkust. Samuti tõstis kaltsiumi lisamine üheaastastel ja keskmisest suuremaid kurni munevatel emalindudel poegade toitmise aegset kehakaalu ja kehalist konditsiooni. See tulemus näitab, et kaltsiumipuudus võib must-kärbsenäpil suurendada sigimise hinda, olles potentsiaalselt kurna suurust piiravaks valikuteguriks.

Kaltsiumi lisasöötmine rasvatihase teise pesitsuskorra ajal näitas, et kaltsiumivähesus võib olla metsavärvuliste pesitsust limiteerivaks teguriks mitte ainult kevadel, vaid ka südasuvel. Vastupidi algsetele eeldustele avaldas kaltsiumi lisasöötmine samalaadset mõju nii lehtmetsas kui ka okasmetsas pesitsevate rasvatihaste sigimisnäitajatele. Ainsaks selgeks erandiks oli munemise algus, mis sõltus kaltsiumi lisamisest üksnes okasmetsas (kaltsiumiga söödetud isendid alustasid munemist varem). Samuti leiti tõendeid, et kaltsiumipuudus mõjutab pesitsust rohkem neil pesitsusaastatel, mil munemist alustati tavapärasest hiljem.

Uurimistöö tulemused näitavad, et kaltsiumipuuduse mõju lindudele varieerub liigiti ja ka liigisiseselt, sõltudes samuti pesitsusaasta ilmastikutingimustest. Põhja-parasvöötme looduslikult kaltsiumivaestel muldadel paiknevates elupaikades pesitsevatel lindudel ei ole kaltsiumipuuduse limiteeriv mõju küll nii drastiline kui hapestunud aladel, ent seda tuleb siiski pidada arvestatavaks ökoloogiliseks teguriks, mis pärsib sigimisedukust või suurendab sigimise hinda.

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ACKNOWLEDGEMENTS

First of all, I owe a special debt to my supervisor Raivo Mänd, who has read and commented critically on the thesis in draft. I have benefited greatly over the years from discussions with him, and much that I have learned has been incorporated in this thesis. He also guided and assisted me in fieldwork and data processing.

I am grateful to the staff of the Nigula Nature Reserve, especially to Agu Leivits, for all-round help.

I would like to thank Agu Leivits, Marko Mägi, Ülle Mustonen and Priit Kilgas for their assistance in fieldwork. Special thanks also to Piret Kiristaja and Annelie Veegen who identified and analysed the snails. Indrek Ots kindly analysed blood samples.

In addition, I am grateful to Indrikis Krams and Oscar Merne for checking the manuscript.

Finally, I thank my parents for every kind of support and encouragement during my studies.

The study was conducted at the Institute of Zoology and Hydrobiology, Tartu University and (during the initial stage) at the Institute of Zoology and Botany, Estonian Agricultural University. The study was financially supported by the Estonian Science Foundation (research grants no. 2217 and 3905 to Raivo Mänd) and by the Estonian Ministery of Education (research grants no. 0180817s99 to Raivo Mänd and DBGZH0627 to Vallo Tilgar).

PUBLICATIONS

Tilgar, V., Mänd, R. & Leivits, A. 1999.
Effect of calcium availability and habitat quality on reproduction in Pied
Flycatcher *Ficedula hypoleuca* and Great Tit *Parus major.* —
Journal of Avian Biology 30: 383–391.

JOURNAL OF AVIAN BIOLOGY 30: 383–391. Copenhagen 1999

Effect of calcium availability and habitat quality on reproduction in Pied Flycatcher *Ficedula hypoleuca* and Great Tit *Parus major*

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Tilgar, V., Mänd, R. and Leivits, A. 1999. Effect of calcium availability and habitat quality on reproduction in Pied Flycatcher *Ficedula hypoleuca* and Great Tit *Parus major*. – J. Avian Biol. 30: 383–391.

Recent studies have demonstrated that wild birds breeding in acidified areas may have difficulties in obtaining sufficient calcium for their eggshells, and that a shortage of land snails may be the cause. Here we present experimental evidence that calcium deficiency may affect reproductive traits also in birds breeding in non-acidified but naturally base-poor forest habitats. The study was conducted in Estonia in 1995 and 1996. We compared egg characteristics and other life-history traits of the Pied Flycatcher *Ficedula hypoleuca* and the Great Tit *Parus major* in two habitats differing in natural availability of calcium (base-poor pine forests and base-rich deciduous forests). In both species, egg volume was smaller in the poor than the rich habitat. Eggshell thickness also tended to be less in the poor habitat in 1996, but the difference was significant only in the Pied Flycatcher. An experimental supplementation of calcium was performed in the habitats to determine to what extent differences between rich and poor habitats might be caused by differences in calcium availability. Egg volume and shell thickness were positively affected by calcium supplementation in the Pied Flycatcher. The same tendency was observed in the Great Tit in 1996, although not significant. However, there was no effect of calcium provisioning on hatching success, nest desertion rate and number or quality of fledglings.
Our results show that calcium limitation may occur in wild birds on naturally calcium-poor soils. Birds breeding in such habitats may be vulnerable to possible increases in acid deposition.

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In life-history models of wild animals, lack of proper knowledge about ecological constraints is a recurring problem of crucial importance. In birds, several breeding parameters, such as laying date, clutch size and number of fledglings, depend on habitat quality (van Balen 1973, Gezelius et al. 1984, Eeva et al. 1989, Hõrak et al. 1995, Sanz 1995, Stauss and Glück 1995) and in certain habitats breeding may be constrained by insufficient food availability (e.g., Lundberg and Alatalo 1992, Riddington and Gosler 1996). Recently, new evidence has accumulated that not only the energy content of regular food but also the availability of specific food components, for example calcium, may play an important role in the breeding success of passerine birds (Graveland 1990, Graveland et al. 1994, Graveland 1996a, b, Graveland and Van Gijzen 1994, Graveland and van der Wal 1996, Graveland and Berends 1997, Graveland and Drent 1997).

The importance of calcium availability for birds has been highlighted by several observations indicating that certain species have trouble obtaining sufficient calcium for their eggs (Drent and Woldendorp 1989, Graveland 1990, 1996a, b, Perrins 1996, Beintema et al. 1997). Moreover, some studies have shown an increase in the proportions of birds laying either abnormally thin-shelled eggs or no eggs at all, and authors have suggested that increasing atmospheric pollution may be the cause (Nyholm and Myhrberg 1977, Nyholm 1981, Scheuhammer 1991, Eeva and Lehikoinen 1995). Also, acidification of the environment may reduce the amount of calcium available to laying females. In aquatic ecosystems, Dippers *Cinclus cinclus* breeding on acidified streams with low pH laid eggs with thinner shells compared with eggs laid at streams with high pH. This effect was obviously caused by the dramatic decline of Ca-rich food items in acidic streams (Ormerod et al. 1988). In terrestrial ecosystems, Graveland et al. (1994) provided experimental evidence that defective eggshells in forest passerines were related to a decline in snail abundance on acidified soils. Molluscs need calcium for their calcareous shells, but continuing acidification and base cation leaching results in decreased calcium content in litter and the surface soil layer (Wäreborn 1969, 1970, 1992).

The evidence presented above suggests that obtaining sufficient calcium may be problematic for many bird species. However, such data have almost exclusively been collected in acidified areas. Graveland and Drent (1997) have suggested that calcium limitation may well be a widespread phenomenon also in non-acidified, calcium-poor areas. If this is true, the cost of egg formation in base-poor areas might be much higher than has been estimated on the basis of the energy and protein content of eggs. The problem is that in such areas there may have been sufficient time for the development of specific adaptations, in contrast to acidified areas, where calcium shortage is a recent phenomenon (Graveland and Drent 1997). The extent of calcium limitation in non-acidified areas is hard to derive from observational data, because certain adaptations, like the production of fewer and smaller eggs, or increasing time devoted to collection of extra calcium, may have evolved in parallel to adaptations to the low availability of other nutrients. To eliminate the influence of confounding effects an experimental approach is needed.

Although industrial acidification does not yet pose a serious problem in Estonia (Frey 1988, Sepp and Asi 1994), a substantial portion of the country's territory (about 16%, and 40% of all woodlands) is covered with pine forests on poor, naturally acidic soils (Taimre 1989). Here we report the results of a calcium supplementation experiment on the reproductive parameters of two passerine species breeding in both rich and poor forest habitats in Estonia.

Material and methods

Study area

Our study was conducted in the surroundings of Kilingi-Nõmme (58° 7′ N, 25° 5′ E), south-west Estonia, in 1995 and 1996. The area is situated in the transition zone from a base-rich moraine plateau to base-poor sand dunes. Different habitats in the area can be divided into two contrasting types in terms of both vegetation richness and abundance of land snails (potential source of calcium for birds). These two types of habitat will be referred to as the rich and the poor habitat, respectively.

The woodlands of the rich habitat occurred mainly as isolated patches between cultivated fields, or as galleries along stream valleys. *Alnus incana* and *Betula pendula* were the predominant tree species, and there was a rich deciduous understorey. The woodlands of the poor habitat were located on nutrient-poor sandy and (in lower parts) peat soils. The dominant tree species was *Pinus sylvestris*, which sometimes formed mixed stands with *Picea abies* (on sandy soil) and *Betula pubescens* (on peat soil). In the field layer, the dwarf shrubs *Vaccinium myrtillus* and *Rhodococcum vitis-idaea* were the most prevalent species.

Our study plots contained nestbox-lines and were distributed over an area of approximately 50 km^2 (Fig. 1).

In order to estimate the abundance of land snails in the two habitats, ten 25 × 25 cm litter samples were collected from different patches of both habitat types all over the area. The material was carefully hand-sorted by experienced snail specialists, who counted and identified all the snails.

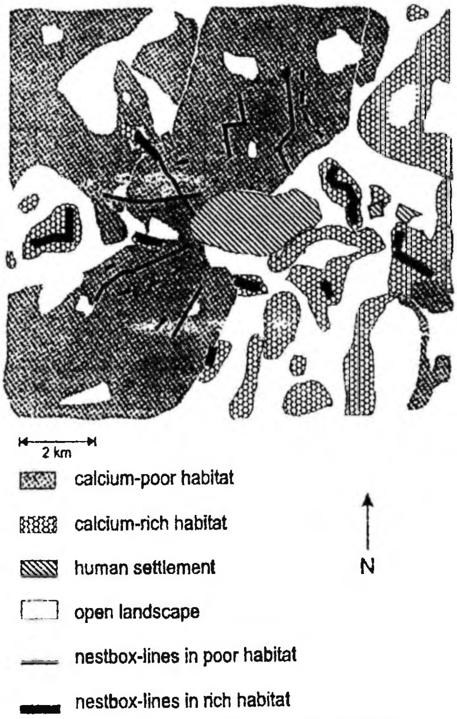

Fig. 1. Study sites in the surroundings of Kilingi-Nõmme.

Sampling and measurements

Wooden board nestboxes with an entrance of 3.5–4.0 cm in diameter and a nest cavity with approximate dimensions 11 × 11 × 30 cm were mounted on tree trunks 1.5–2 m above ground level to attract Great Tits *Parus major* and Pied Flycatchers *Ficedula hypoleuca*. The distance between neighbouring boxes was approximately 50 m. Old nest material was removed each year.

The nestboxes were checked regularly to record: (1) date of the first egg, (2) clutch size, (3) hatching success and (4) number of fledged young. Only first clutches were taken into account. Depredated or deserted nests were omitted when calculating fledging success. We also calculated the desertion rate of clutches in both habitats.

One egg from each clutch (2 eggs in the case of Great Tit in 1995) was collected at random to measure the thickness of the eggshell. In the Pied Flycatcher, the egg was removed on the 3rd or 4th day after the beginning of laying, in the Great Tit, on the 6th (± 1) day of incubation. The eggs were photographed. A graphics digitizer was used for the input of egg contours from photographs, and egg volume was estimated using the method described in Mänd et al. (1986). In 1996, whole clutches of the Great Tit were photographed in the field, and mean egg volume per clutch was used in the analysis (see Hõrak et al. 1997 for more details).

The collected eggs were cut into two halves using a razor-blade, their content was removed and the eggshell carefully washed. The eggshells were dried in a desiccator and shell thickness was measured using a digital micrometer with an accuracy of 10 µm. The mean of three measurements from the equatorial zone of an egg was used in the analysis.

The nestlings were weighed with a Pesola spring balance with a precision of 0.1 g and their tarsi were measured with a sliding caliper to the nearest 0.1 mm on the 13th day (Pied Flycatcher) and 15th day after hatching (Great Tit).

Adults were captured during the second half of the nestling period and their age and sex were determined from plumage characteristics (Svensson 1984). They were also weighed and their tarsal and wing lengths were measured.

Calcium-provisioning experiment

Calcium supplementation was carried out in the poor habitat. In 1995, part of the Great Tits nesting in rich habitat were fed with extra calcium as well. Feeders filled with small snail shell and chicken egg-shell fragments were mounted on nestboxes about two weeks before the tits' start of nest building in 1996, and during their nest building (about 3–4 weeks before the nest building of flycatchers) in 1995. The feeders were regularly checked and refilled, so that there was always a surplus of calcium-rich material. We also attached empty feeders to control nestboxes to check for the possible influence of the feeders *per se* on breeding results. Experimental and control nestboxes were arranged in separate five-box blocks. Since there existed a certain probability that filled feeders were used also by birds from adjacent nestboxes without calcium (Graveland and Drent 1997), control nestboxes next to experimental boxes were omitted from analysis. The distance between control and experimental blocks was at least 100 m (see methods in Graveland and Drent 1997). As a rule, no more than one nestbox per block was occupied by either species.

Both visual observations and nest material analysis confirmed that the birds had consumed snail shell and egg shell fragments in the feeders. Chicken eggshell fragments were found mostly in the Ca-supplemented nests of the Great Tit and only in the Ca-supplemented nests of the Pied Flycatcher. Consumption of artificially supplied calcium-rich material by adult birds has also been observed by Eeva (1996) in the Pied Flycatcher, Graveland et al. (1994), Graveland (1996a) and Graveland and Drent (1997) in the Great Tit and Johnson and Barclay (1996) in the House Wren *Troglodytes aedon*.

Statistical procedures

Most of the comparisons were made using unbalanced two-way ANOVA (Sokal and Rohlf 1995), with year and group (R – rich habitat control, RCa – rich habitat Ca-fed, P – poor habitat control, and PCa – poor habitat Ca-fed) as factors. Laying date was not included in the model, since this variable had no significant effect on egg traits. Whenever comparisons across treatments were significant, we examined significant pairwise differences with Tukey's HSD test for unequal sample sizes (Spjøtvoll/Stoline test). In case of the Great Tit, two-way ANOVA was performed without RCa due to missing cells (we had data for RCa only from 1995). To estimate the effect of RCa, we performed a separate analysis in 1995. Where the condition of normality was violated, non-parametric methods like Kruskal-Wallis test, Mann-Whitney U-test and chi-square test were used. All significance levels apply to two-tailed tests. When pooled data were used in analysis, values of the traits were first standardised by subtracting the yearly mean value from the observed value and dividing the result by the standard deviation for that year and trait. Laying dates were standardised by subtracting the median laying date of that particular year from the observed laying date.

Table 1. Comparison of snail numbers and species richness in litter samples collected from rich and poor habitat.

	Rich habitat		Poor habitat		Mann-Whitney U-test	
Parameter	Mean ± SE	(n)	mean ± SE	(n)	Z	p
Individuals per 100 cm^2	0.92 ± 0.27	(10)	0.13 ± 0.03	(10)	−2.42	0.010
Species per square of 25 × 25 cm	4.70 ± 0.90	(10)	1.00 ± 0.20	(10)	−2.72	0.005

Results

Snail abundance

Table 1 shows that the density of snails as well as the mean number of species per sample was significantly smaller in the poor than in the rich habitat. This agreed with our visual impressions during the whole study period.

Laying date and clutch size

There was a significant difference in laying dates across groups in both species (Kruskal-Wallis test: $H(2, n = 218) = 14.04$, $p = 0.001$ for the Pied Flycatcher and $H(2, n = 226) = 22.24$, $p < 0.001$ for the Great Tit). In the Pied Flycatcher, both control and Ca-fed birds in the poor habitat started egg-laying significantly later than birds in the rich habitat (Mann-Whitney U-test, between R and P: $Z_{92,74} = -2.02$, $p = 0.042$, between R and PCa: $Z_{92,53} = -3.70$, $p = 0.0002$). In the Great Tit, the difference in laying dates was significant only between the control groups of both habitats (R and P: $Z_{125,72} = -4.57$, $p < 0.0001$, later laying in the poor habitat). There were no significant differences in laying dates between the Ca-fed and the control group in the poor habitat (Tables 2 and 3; Mann-Whitney U-test; Pied Flycatcher: $Z_{53,74} = -1.70$, $p = 0.09$; Great Tit: $Z_{29,72} = -1.81$, $p = 0.07$), although in the case of the Great Tit Ca-fed birds tended to start laying slightly earlier in both years. When we calculated differences in laying dates for the Great Tit in 1995, experimental nests of the rich habitat were excluded from analysis because the feeders were attached to boxes during nest-building.

Mean clutch size did not vary significantly across groups in either species (Tables 2 and 3; Kruskal-Wallis test; Pied Flycatcher: $H(2, n = 227) = 0.38$, $p = 0.83$; Great Tit: $H(2, n = 233) = 2.45$, $p = 0.29$). Neither was there any effect of additional calcium on the clutch size of the Great Tit in the rich habitat in 1995 (Kruskal-Wallis test: $H(3, n = 146) = 2.54$, $p = 0.47$).

Egg volume and eggshell thickness

Egg volume of both species varied significantly across groups (two-way ANOVA, Pied Flycatcher: group $F = 5.50$, $p = 0.0047$, year $F = 13.29$, $p = 0.0003$, interaction $F = 1.33$, $p = 0.27$, Great Tit: group $F = 8.57$, $p = 0.0003$, year $F = 1.71$, $p = 0.19$, interaction $F = 0.43$, $p = 0.65$; see Fig. 2 for sample sizes). In the Pied Flycatcher, differences in egg volume were significant both between the control groups of the rich and poor habitats (Fig. 2a; Tukey's HSD for unequal sample sizes: $p = 0.024$, larger eggs in the rich habitat) and between the control and the Ca-fed group of the poor habitat ($p = 0.011$, larger eggs in the Ca-fed group). In the Great Tit, a significant difference was detected only between the control groups of the two habitats (Fig. 2b; $p = 0.002$, larger eggs in the rich habitat). Also, Ca-provisioning in the rich habitat in 1995 caused no increase in mean egg size in the Great Tit (Fig. 2b, pairwise comparisons were not significant).

Eggshell thickness of the Pied Flycatcher differed significantly across groups, and the group × year interaction term was also highly significant (Fig. 3a; two-way ANOVA; group $F = 10.75$, $p < 0.001$, year $F = 28.56$, $p < 0.001$, interaction $F = 7.78$, $p = 0.001$). Pairwise differences were significant both between the control groups of the rich and the poor habitat (Fig. 3a; Tukey's HSD, $p = 0.004$, thicker shells in the rich habitat) and between the control and the Ca-fed group of the poor habitat ($p = 0.0001$, thicker shells in the Ca-fed group). In the Great Tit, shell thickness did not vary significantly across groups (Fig. 3b; two-way ANOVA; group: $F = 1.70$, $p = 0.19$, year: $F = 4.96$, $p = 0.027$, interaction: $F = 2.45$, $p = 0.09$). There was, however, a "borderline" significant difference between R and P in 1996 (Tukey's HSD, $p = 0.060$, slightly thicker shells in the rich habitat). Calcium supplementation in the rich habitat in 1995 caused no significant increase in mean shell thickness (Fig. 3b, pairwise tests were not significant).

There was no significant correlation between egg volume and shell thickness in either species (Pied Flycatcher: $r = 0.03$, n.s.; Great Tit: $r = 0.13$, n.s.).

Nest desertion and hatching success

In the Pied Flycatcher, more females nesting in the poor compared with the rich habitat deserted their clutches (7 nests out of 79 versus 1 out of 95, corrected Chi-square = 5.96, df = 1, $p = 0.015$). However, there was no significant difference in desertion frequency between the Ca-fed and the control group in the poor habitat (4 nests out of 50 versus 7 out of 79, corrected chi-square = 0.03, df = 1, p = 0.865). In the Great Tit, significant differences in clutch desertion were observed neither between the rich and the poor habitat (8 nests out of 135 versus 5 out of 73, corrected chi-square = 0.07, df = 1, p = 0.793) nor between the Ca-fed and the control group in the poor habitat (1 nest out of 29 versus 5 out of 73, corrected chi-square = 0.43, df = 1, p = 0.512).

We found no significant differences in hatching success (percentage of eggs hatched in non-deserted and non-depredated nests) across groups either in the Pied Flycatcher (Table 2; Kruskal-Wallis test, 1995: H(2, n = 56) = 3.64, p = 0.16; 1996: H(2, n = 126) = 0.40, p = 0.82) or in the Great Tit (Table 3; 1995: H(3, n = 69) = 6.25, p = 0.10; 1996: H(2, n = 71) = 3.31, p = 0.19).

Fledglings' characteristics and number

No significant variation in fledglings' weight or tarsus length across groups was found in either the Pied Flycatcher (Table 4) or in the Great Tit (Table 5). Calcium supplementation in the rich habitat in 1995 caused no increase in fledglings' weight or tarsus length either (Table 5, pairwise tests were non-significant).

Neither was there any significant difference in the average number of fledglings across groups (Kruskal-Wallis test, Pied Flycatcher: H(2, n = 176) = 1.41, p = 0.49; Great Tit: H(2, n = 137) = 1.56, p = 0.46).

Discussion

Calcium limitation in non-acidified base-poor areas

In our study area, certain reproductive traits (laying date, egg size, eggshell thickness, nest desertion) of one or both of the studied passerines varied significantly between contrasting habitats which differed in land snail abundance as well as in other characteristics. Birds in poor coniferous forests tended to start laying later, lay smaller eggs with thinner shells and desert clutches more often than birds in rich deciduous forests.

The results of our calcium manipulation experiments show that some of these differences may indeed, at least partly, be caused by calcium limitation in the base-poor habitat. In the Pied Flycatcher, both egg volume and eggshell thickness in the poor pine wood were on average higher in those birds that had received extra calcium during the egg-laying period. In the Great Tit, the same tendency, though not significant, was revealed in 1996 but not in 1995. No other effects of calcium manipulation were revealed. However, this is the first experimental evidence for calcium shortage in a non-acidified area.

So far only two other studies have been published where the hypothesis that calcium availability may constrain the reproductive output of wild birds in natural habitats not subjected to heavy anthropogenic acidification has been tested experimentally. Johnson and Barclay (1996) did not detect any effect of calcium supplementation on egg size in the House Wren in Wyoming, USA. The most likely reason was that their study area was comparatively base-rich and birds had sufficient access to various natural sources of calcium, like snail shells (Johnson and Barclay 1996).

Table 2. Laying date, clutch size and hatching success of the Pied Flycatcher in the rich and the poor habitat.*

	1995			1996		
	Median	Quartile range	n	Median	Quartile range	n
Lay-date (May)						
R	22	4	29	18	3	62
P	24	4	48	18.5	9	26
PCa	24	2	20	21	5	33
	Mean	SD	n	Mean	SD	n
Clutch size						
R	6.68	0.78	22	6.69	0.76	80
P	6.83	0.67	41	6.85	0.71	33
PCa	6.67	0.77	18	6.61	0.79	33
% hatched young						
R	80.0	9.5	16	79.3	12.3	68
P	82.2	7.8	26	79.8	8.8	30
PCa	80.2	6.8	14	80.9	7.2	28

* In all tables and figures: R – control in rich habitat, RCa – Ca-fed group in rich habitat, P – control in poor habitat, PCa – Ca-fed group in poor habitat.

Table 3. Laying date, clutch size and hatching success of the Great Tit in the rich and the poor habitat.

	1995			1996		
	Median	Quartile range	n	Median	Quartile range	n
Lay-date (April)						
R	27	2	65	33	5	60
RCa	26	3	11			
P	29	6	50	35.5	4	22
PCa	27	4	13	34	2	16
	Mean	SD	n	Mean	SD	n
Clutch size						
R	11.07	1.43	76	11.02	1.07	59
RCa	11.55	0.82	11			
P	11.15	1.01	46	10.61	1.03	23
PCa	11.46	1.51	13	11.13	1.20	16
% hatched young						
R	92.1	13.3	31	90.3	12.3	43
RCa	100	0	7			
P	92.6	13.8	21	93.5	7.7	18
PCa	93.4	17.2	10	97.3	4.3	10

In the Pied Flycatcher, nesting in a comparatively base-poor habitat in SE Finland, the eggs tended to be a little larger in calcium-supplemented nests as compared with control nests (Eeva 1996). Although the difference was not significant (perhaps due to small sample sizes), the data of Eeva provide some support for our conclusion that smaller egg size of the Pied Flycatcher in base-poor forests may be caused by calcium deficiency.

Calcium limitation and egg size

The effect of calcium on the size of an egg may seem paradoxical. In small passerines the period of follicle growth lasts a little more than 4 days, whereas eggshell deposition takes place during the last 24 hours before egg-laying (Ojanen 1983). Thus variation in egg size is mainly determined by follicle growth before shell deposition, which in turn is influenced by general food availability during this period. Indeed, several studies have demonstrated the effect of food abundance on egg size (Lundberg and Alatalo 1992, Föger and Pegoraro 1996, Riddington and Gosler 1996). However, calcium availability may also affect egg size via food. Observations of the behaviour of wild birds as well as experiments with captive birds have shown that eggshell formation requires accurate timing of the calcium intake and that obtaining a sufficient amount of calcium is time-consuming even in Ca-rich environments (Graveland 1996a, Graveland and van der Wal 1996, Graveland and Berends 1997). There exists a trade-off between feeding time and time spent searching for calcium. For example, egg-laying Great Tit females on a calcium-deficient diet spent 43% of the daylight hours searching for Ca-rich items, which is almost twice that of females with sufficient calcium (Graveland and Berends 1997). As a result, Ca-fed birds may receive more protein and other nutrients, and lay larger eggs with good shells. Graveland and Berends (1997) and

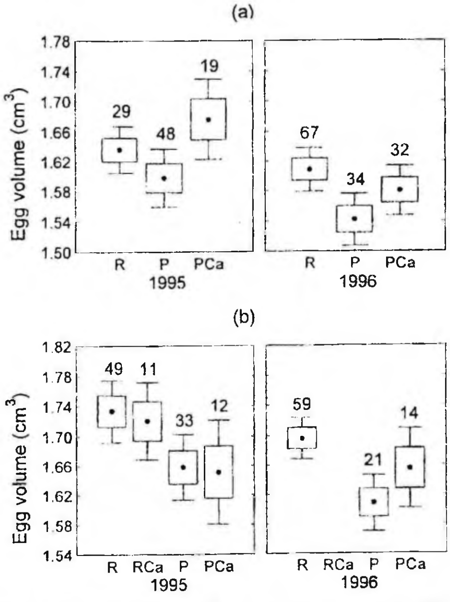

Fig. 2. Egg volume (cm³) in Ca-fed and control groups in rich and poor habitat for (a) the Pied Flycatcher and (b) the Great Tit. Rectangles denote ± SE and vertical lines 95% confidence intervals. Numbers are sample sizes.

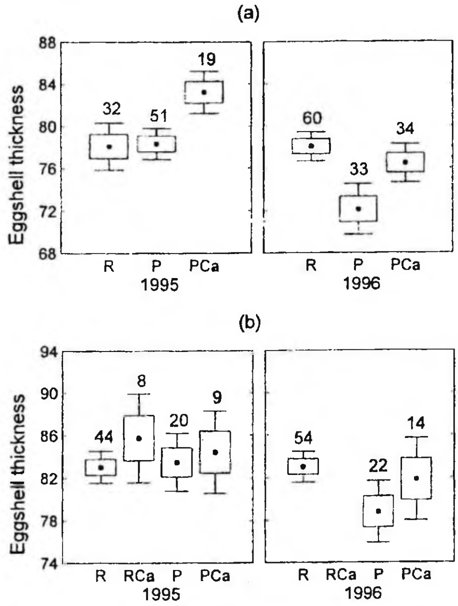

Fig. 3. Eggshell thickness (μm) in Ca-fed and control groups in rich and poor habitat for (a) the Pied Flycatcher and (b) the Great Tit.

Graveland and Drent (1997) suggest that poor calcium availability may therefore affect not only eggshell quality but also egg size and other reproductive parameters. Indeed, egg weight in Dippers correlated with Ca content in their food and the pH of water in the stream (Ormerod et al. 1988).

Table 4. Tarsus length (mm) and body weight (g) of 13 days old nestlings of Pied Flycatchers breeding in the rich and the poor habitat. Brood means were used.

	1995	1996
	Mean ± SD (n)	Mean ± SD (n)
Tarsus length (mm)		
R	17.47 ± 0.50 (3)	17.41 ± 0.44 (62)
P	17.08 ± 0.34 (10)	17.19 ± 0.36 (28)
PCa	16.97 ± 0.38 (8)	17.25 ± 0.37 (26)
Weight (g)		
R	14.54 ± 1.09 (3)	14.00 ± 0.70 (62)
P	13.81 ± 0.56 (10)	13.95 ± 0.86 (28)
PCa	13.70 ± 0.64 (8)	14.09 ± 0.62 (26)

ANOVA models (group factor involves habitats and treatment). Tarsus length, group: $F = 2.85$, $p = 0.062$, year: $F = 1.04$, $p = 0.31$; weight, group: $F = 1.37$, $p = 0.26$, year: $F < 0.01$, $p = 0.98$.

Table 5. Tarsus length (mm) and body weight (g) of 15 days old nestlings of Great Tit breeding in the rich and the poor habitat. Brood means were used.

	1995	1996
	Mean ± SD (n)	Mean ± SD (n)
Tarsus length (mm)		
R	19.98 ± 0.37 (26)	19.65 ± 0.53 (56)
RCa	19.70 ± 0.35 (6)	
P	20.04 ± 0.28 (17)	19.69 ± 0.40 (17)
PCa	20.10 ± 0.32 (13)	19.76 ± 0.38 (13)
Weight (g)		
R	17.98 ± 1.20 (26)	17.30 ± 1.35 (56)
RCa	17.37 ± 0.45 (6)	
P	18.45 ± 0.82 (17)	17.65 ± 0.99 (17)
PCa	17.94 ± 0.69 (13)	17.84 ± 1.55 (13)

ANOVA models (group factor involves habitats and treatment). Tarsus length, group: $F = 0.65$, $p = 0.52$; year: $F = 16.75$, $p < 0.001$, pairwise comparisons in 1995 were non-significant; weight, group: $F = 1.45$, $p = 0.24$; year: $F = 5.29$, $p = 0.023$, pairwise comparisons in 1995 were non-significant.

Calcium limitation and eggshell thickness

Far more evidence is available for the negative effect of calcium deficiency on the quality of eggshell. Pheasants *Phasianus colchicus* and domestic fowl on a Ca-poor diet started laying thin-shelled eggs (see Graveland and Drent 1997 for references). Eggshells of Eastern Kingbirds *Tyrannus tyrannus* were more permeable in acidic compared with circumneutral wetlands presumably due to a limited calcium supply (Glooschenko et al. 1986). Recall also the results of Ormerod et al. and Graveland et al., given in the Introduction. However, the results given in this paper represent the first experimental evidence of the influence of calcium deficiency on eggshell thickness in a naturally base-poor habitat.

There existed a significant effect of year on egg parameters. In the base-poor habitat, both egg volume and shell thickness tended to be smaller in 1996 than in 1995. Interestingly there appeared to be no difference in eggshell thickness between the rich and the poor habitat in 1995. It seems that strongly fluctuating weather conditions in 1996 had some kind of negative effect on egg traits, which was stronger in the low quality habitat.

Laying date and clutch size

Calcium availability did not obviously contribute to any earlier start of laying in the rich habitat in either species, since we found no significant effect of calcium manipulation on the laying date. This is consistent with the results of Graveland and Van Gijzen (1994), Graveland (1996b) and Graveland and Berends (1997), who showed that females of passerines store little or no calcium in their skeleton during the pre-laying period and that increased calcium consumption starts just 1–2 days before laying the first egg. Therefore, laying date is affected by the availability of ordinary food rather than calcium. The positive effect of food provisioning on the laying date has been found by several authors (e.g. Källander 1974, Svensson and Nilsson 1995).

Graveland and Drent (1997) speculate that one possible adaptation in birds breeding in calcium-poor habitats might be the production of fewer eggs. Indeed, Ormerod and Tyler (1993) have shown that this is the case in Dippers. In Finland, Ca-fed females of the Pied Flycatcher tended to produce slightly smaller clutches than control birds, but the difference was not significant (Eeva 1996). In our case, there was no significant variation in clutch size between contrasting habitats. Neither did we find any effect of calcium manipulation on clutch size. The last result is not surprising if one recalls that females of small passerines have to collect enough calcium each day in the laying phase. So decreasing the number of eggs laid in conditions of calcium deficiency would not help much.

Nest desertion and hatching success

Graveland and his colleagues (Graveland et al. 1994, Graveland and Drent 1997) found that extra calcium in the Great Tit reduced nest desertion rate and the proportion of unhatched eggs in an acidified area in the Netherlands. Also, Dippers breeding on acidic streams in Wales deserted their clutches more often than those breeding on circumneutral streams (Ormerod et al. 1991). In our area, despite the effect of calcium limitation on egg parameters, no differences were detected in hatching success or nest desertion rate between the Ca-fed and control groups of either species. It is possible that statistically detectable differences in egg parameters between our contrasting habitats may still be within the limits of the reaction norm and have no fitness consequence for embryos. Several authors have demonstrated high tolerance of embryos to variation of eggshell parameters (Simkiss 1980, Carey 1986, Ormerod et al. 1988). Both shell thickness and the volume of unhatched eggs in the Great Tit and the Pied Flycatcher tended to be remarkably smaller in Finland than the mean values in our base-poor habitat (Eeva and Lehikoinen 1995). Also, captive tits kept on a calcium-deficient diet in the Netherlands laid eggs with far thinner shells than did tits in our poor habitat (Graveland and Berends 1997).

Fledglings' characteristics and number

Calcium is also required for the skeleton of the young. Indeed, both studied species have been found to feed their young with extra calcium (Graveland et al. 1994, Eeva and Lehikoinen 1995). Likewise, we found dropped snail shell fragments more frequently in the nest material of Ca-supplemented than control birds in the Pied Flycatcher (Chi-square = 6.95, df = 1, p = 0.008). However, there were no differences in the number and morphology of fledglings between habitats, or between Ca-fed and control birds in the poor habitat. This is in disagreement with the results of Eeva (1996) who found in the Pied Flycatcher that extra calcium most strongly affected the development of nestlings' legs and wings. Perhaps calcium availability is not constraining nestlings' growth in our base-poor habitat. Another possibility is that removal of 1–2 eggs per clutch reduced necessary parental effort to such an extent that parents could easily compensate for the local calcium deficiency by increasing the care of the young.

Conclusion

Calcium deficiency does not seem to bring about the same dramatic consequences for the reproductive output of forest passerines in Estonia as in highly acidified areas in Western Europe. However, birds breeding in poor pine forests, which constitute a substantial part of the available breeding habitats for forest birds in our region, seem to be closer to a critical level in terms of calcium availability than birds breeding in rich habitats. We do not rule out that in particularly "bad" years reproductive performance in poor habitats may be significantly impaired. Birds in such habitats may also be vulnerable to possible future increases in acidification.

Our results provide experimental evidence that calcium limitation in wild birds may occur also in a non-acidified area and support the assumptions made by Graveland and Drent (1997) that the cost of egg formation of passerines in calcium-poor areas may involve much more than the traditional estimate based on the protein and energy content of eggs.

Acknowledgements – We are grateful to Endla Pruks for laboratory assistance and to the Director and the staff of the Nigula Nature Reserve for all-round help. We thank Piret Kiristaja and Annelie Veegen for analysing snail samples as well as Staffan Ulfstrand, Peeter Hõrak, Steve Ormerod and an anonymous reviewer for valuable comments on the manuscript. The study was partly supported by Estonian Science Foundation grants Nos 2217 and 1631.

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(*Received 13 August 1997, revised 14 August 1998, accepted 17 September 1998.*)

II

Mänd, R., Tilgar, V. & Leivits, A. 2000.
Reproductive response of Great Tits, *Parus major*,
in a naturally base-poor forest habitat to calcium supplementation. —
Canadian Journal of Zoology 78: 689–695.

Reproductive response of Great Tits, *Parus major*, in a naturally base-poor forest habitat to calcium supplementation

Raivo Mänd, Vallo Tilgar, and Agu Leivits

Abstract: Recent studies have revealed that some forest passerines have difficulty obtaining sufficient calcium (Ca) for their eggshells in heavily acidified areas. However, the effect of Ca limitation on breeding success in non-acidified but naturally base-poor breeding habitats is not yet clear. The issue itself is important, insofar as the cost of egg formation in a certain habitat depends on the availability not only of energy and proteins but also of shell-formation material. We studied the effect of experimental supplementation with extra Ca on the reproductive output of Great Tits, *Parus major*, breeding in base-poor pine forests in Estonia. Pairs provided with Ca started egg laying earlier than control pairs, and clutch volume was larger in Ca-supplemented nests. There was also a positive effect of Ca supplementation on fledglings' tarsus length and body mass in the year when breeding conditions were the most unfavorable. However, no significant differences were found between the experimental and control groups in hatching success or number of fledglings. We suggest that Ca deficiency is a less limiting factor in naturally base-poor forests than in highly acidified areas, and that birds are able to adjust their breeding efforts to conditions of Ca deficiency. Great Tits, for instance, may respond to Ca shortage by postponing egg laying until their body condition and rate of food intake reach a critical threshold for producing viable eggs and young.

Résumé : Des études récentes ont démontré que certains passereaux des forêts ont de la difficulté à trouver suffisamment de calcium pour les coquilles de leurs oeufs dans les milieux très acidifiés. Cependant, l'effet d'une carence en calcium sur le succès de la reproduction en milieu non acidifié mais naturellement pauvre en bases n'est pas clair. Il s'agit là d'un problème important car l'estimation du coût de la formation des oeufs dans un habitat donné ne dépend pas seulement de la disponibilité d'énergie et de protéines, mais dépend aussi de la disponibilité des matériaux de la coquille. Nous avons étudié l'effet de l'administration de suppléments de calcium sur la reproduction de la Mésange charbonnière, *Parus major*, dans des pinèdes à sol pauvre en bases en Estonie. Les couples qui ont reçu des suppléments de calcium ont commencé à pondre plus tôt que les couples témoins. Le nombre d'oeufs était plus élevé dans les nids des couples nourris de calcium. Nous avons également enregistré un effet positif des suppléments de calcium sur la taille du tarse et sur la masse des oisillons emplumés l'année où les conditions de la reproduction ont été le plus mauvaises. Cependant, il n'y avait pas de différence significative entre le groupe expérimental et le groupe témoin quant au succès de l'éclosion ou au nombre d'oisillons parvenus à l'envol. Nous croyons donc qu'une carence en calcium est un facteur moins limitant dans les forêts à sol pauvre en bases que dans les zones très acidifiées et il semble que les oiseaux soient capables d'ajuster leur effort reproducteur aux conditions de carence en calcium. Les Mésanges charbonnière, par exemple, peuvent réagir à une carence en calcium en retardant leur ponte jusqu'à ce que leur condition physique et leur consommation alimentaire atteignent un seuil critique nécessaire à la production d'oeufs et d'oisillons viables.

[Traduit par la Rédaction]

Introduction

Calcium is an important "building material" for wild birds during egg laying and during skeleton formation in nestlings, since eggshell contains approximately 35% Ca and the nestling's skeleton contains 22% Ca (Graveland and Van Gijzen 1994; Carey 1996). Many species are known to look particularly for external Ca sources during these critical periods. Pied Flycatchers *(Ficedula hypoleuca)*, Great Tits (*Parus major*), and Blue Tits (*Parus caeruleus*) consume snail shells (Graveland 1990; Eeva 1996; Perrins 1996), Dunlins (*Calidris alpina*) collect the bones and teeth of lemmings (MacLean 1974), while House Sparrows (*Passer domesticus*), Queleas (*Quelea quelea*), corvids, and American Pipits (*Anthus rubescens*) pick grit during the laying stage (Jones 1976; Soler et al. 1993; Verbeek 1994; Gionfriddo and Best 1995), hummingbirds have been recorded feeding on ash (Des Lauriers 1994), and terns eat mollusc shells during egg formation to restore their Ca balance (Brenninkmeijer et al. 1997; Nisbet 1997). Some birds

Received April 8, 1999. Accepted November 23, 1999.

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(e.g., chickens, pigeons, and knots) store Ca in the interior of their leg bones prior to egg laying (Peakall 1970; Piersma et al. 1996). In small passerines, however, the capacity to use skeletal Ca for eggshell formation is quite limited, therefore they search for calcareous material on a daily basis during egg production (Graveland and Van Gijzen 1994; Houston et al. 1995).

Considering the special role of Ca metabolism, birds appear very susceptible to several environmental changes, such as acidification or chemical poisoning of soil or water, that reduce the availability of Ca-rich foods (Nyholm and Myhrberg 1977; Nyholm 1981; Ormerod et al. 1988; Drent and Woldendrop 1989; Graveland et al. 1994). In the Netherlands, a decline in the abundance of land snails had a severe negative effect on reproduction in Great Tits (Drent and Woldendrop 1989; Graveland 1996*b*; Graveland and Van der Wal 1996).

So far, evidence of the problems facing birds under conditions of Ca shortage has been obtained almost exclusively in acidified areas. However, Ca limitation may well be a widespread phenomenon in non-acidified, Ca-poor areas also (Graveland and Drent 1997). Indeed, we have recently shown, using an experimental approach, that Ca limitation affects the breeding performance of Pied Flycatchers in a naturally base-poor habitat (Tilgar et al. 1999). Therefore, the cost of egg formation in base-poor areas might be much higher than has been estimated from the energy and protein contents of eggs.

The key problem in estimating the true cost of shell formation is that in naturally base-poor areas the time required for developing specific adaptations may have been sufficient, whereas in acidified areas Ca shortage is a recent phenomenon (Graveland and Drent 1997). Consequently, the extent of Ca limitation in non-acidified areas is hard to determine from observational data, because certain adaptations may have developed in parallel with adaptations to low availability of other nutrients. To eliminate the influence of confounding effects, an experimental approach is needed.

Industrial acidification does not yet pose a significant problem in Estonia (Frey 1988; Sepp and Asi 1994). At the same time, a substantial portion of the country's territory (about 16%; 40% of all woodlands) is covered with pine forests on poor, naturally acidic soils (Taimre 1989) with a low-density human population.

In this paper we report the results of a Ca-supplementation experiment on reproductive parameters of Great Tits breeding in naturally base-poor pine forests.

Material and methods

Study area

The study was conducted in pine forests near Kilingi-Nõmme (58°08′N, 24°56′E), southwest Estonia, from 1995 to 1997. The woodlands in the study area were situated on nutrient-poor sandy and (in lower areas) peat soils. The most common tree species was *Pinus sylvestris*, which sometimes formed mixed stands with *Picea abies* (on sandy soil) and *Betula pubescens* (on peat soil). In the understory, the dwarf shrubs *Vaccinium myrtillus* and *Rhodococcum vitis-idaea* were the most prevalent species.

The numbers and species richness of land snails (a potential Ca source for forest birds) in the forests of our study area were low. During a pilot study, 13 snails per square metre were found, on average (Tilgar et al. 1999), which is similar to or lower than the corresponding numbers for base-poor forests in the Netherlands before severe acidification brought about dramatic consequences for Great Tits (Graveland et al. 1994). The density and species richness of land snails were much higher in neighboring deciduous forests (preferable habitat for forest passerines) than in pine forests (Tilgar et al. 1999).

Our study plots contained lines of nest boxes and were distributed over an area of approximately 30 km^2.

Sampling and measurements

The Great Tits studied bred in nest boxes mounted on tree trunks 1.5–2 m above ground level. The internal dimensions of the nest boxes were approximately 11 × 11 × 30 cm. The approximate distance between neighboring boxes was 50 m. Old nest material was removed every year.

The nest boxes were checked regularly to record the laying date for the first egg, clutch size, hatching date, brood size, and number of fledglings. Predated or deserted nests were omitted when calculating fledging success. Only first clutches were taken into account.

Whole clutches were photographed using a special portable stand. A graphics digitizer was used for inputting egg contours from photographs, and egg volume was estimated using the method described in Mänd et al. (1986). When calculating mean egg volume in the clutch, one egg per clutch, the one most dissimilar to the others in size, shape, or appearance, was excluded, in order to minimize the influence of a few aberrant eggs on clutch mean values. Rejection was assessed on the basis of the researcher's immediate impression, not on calculated egg parameters (for details see Hõrak et al. 1997). However, clutch volume was calculated by summing the volumes of all eggs in the clutch (including the most aberrant egg).

To measure the mass of eggshell material, two eggs from each clutch were collected at random in 1995 and one egg was collected in 1996. The eggs were removed on day 6 (±1) of incubation. No egg was removed in 1997. Collected eggs were cut into two halves with a razor blade, their contents were removed, and the eggshells were carefully washed. Then the eggshells were dried to constant mass in a desiccator and weighed to the nearest 0.1 mg. Shell material per clutch was estimated by multiplying the mass of a single eggshell by the clutch size.

Nestlings were weighed using a Pesola spring balance with a precision of 0.1 g and their tarsi were measured with a sliding caliper to the nearest 0.1 mm on the 15th day after hatching. Adults were captured during the second half of the nestling period and their age and sex were determined using plumage characteristics (Svensson 1984). They were also weighed and their tarsus and wing lengths were measured.

Calcium-supplementation experiment

Feeders with small fragments of snail shells and chicken eggshell were mounted on the nest boxes about 2 weeks before the start of nest building. The feeders attached to occupied nests were regularly checked and refilled, so there was always a surplus of Ca-rich material for experimental birds. We also attached empty feeders to control nest boxes to check for any influence of the feeders per se on breeding performance. All nest-box lines were divided into alternating experimental and control blocks, each consisting of five consecutive nest boxes. Since no more than one nest box per block was occupied by Great Tits, our results concern one nest box per block. Since there was a certain probability that filled feeders were also used by birds from adjacent nest boxes that were devoid of Ca, the data from control birds nesting within 100 m of an experimental block were rejected from analyses (as in Graveland and Drent 1997). Henceforth, Ca-supplemented pairs are referred to as the Ca-fed group and control pairs as the control group.

Both visual observations and nest-material analyses (Tilgar et al. 1999) confirmed that the birds did consume fragments of snail shells and eggshell in the feeders.

Statistical procedures

Where conditions of normal distribution were not violated, unbalanced two-way ANOVA (Sokal and Rohlf 1995) was used for comparisons, with year and group (Ca-fed or control) as factors. When the laying or hatching date had a significant effect on the other reproductive parameters, we included it in the model as a covariate. A χ^2 test was used to compare nest-box occupancy between groups. The values of the other traits were standardized yearly before Mann–Whitney U tests were used on pooled data. Clutch size and number of fledged young were standardized by subtracting the yearly mean value from the observed value and dividing the result by the standard deviation for that year and trait. Laying and hatching dates were standardized by subtracting the median date in that particular year from the observed date (Nager and Van Noordwijk 1995). All significance levels apply to the two-tailed test.

Results

Nest-box occupancy and characteristics of adult birds

Despite the alternating pattern of experimental and control nest blocks (see Methods), we could not exclude the possibility that the nests of one group would occasionally be in preferable territories or would be occupied by individuals of better quality than others. To test this possibility, we compared nest-box occupancy and adults' morphological characteristics for Ca-fed and control groups.

No significant difference in nest-box occupancy was found between the groups. In 1995, 10.2% of 127 Ca-supplemented and 13% of 391 control nest boxes were occupied by Great Tits ($\chi^2 = 0.45$, df = 1, $P = 0.50$). In 1996, the corresponding figures were 7.1% of 311 Ca-supplemented and 9.1% of 254 control nest boxes ($\chi^2 = 0.50$, df = 1, $P = 0.48$), and in 1997, occupancy was 6.7 and 6.3% ($\chi^2 = 0.05$, df = 1, $P = 0.83$; sample size was the same as in 1996).

Differences between the groups with respect to body parameters were not significant for either females (wing length ($F_{[1,86]} = 0.01$, $P = 0.9$; year: $F_{[2,86]} = 0.47$, $P = 0.63$), tarsus length ($F_{[1,87]} = 0.20$, $P = 0.66$; year: $F_{[2,87]} = 2.74$, $P = 0.07$), body mass ($F_{[1,82]} = 0.44$, $P = 0.51$; year: $F_{[2,82]} = 0.61$, $P = 0.55$; Table 1) or males (wing length ($F_{[1,49]} = 0.01$, $P = 0.9$; year: $F_{[2,49]} = 2.47$, $P = 0.09$); tarsus length ($F_{[1,50]} = 0.15$, $P = 0.7$; year: $F_{[2,50]} = 0.82$, $P = 0.44$); body mass ($F_{[1,45]} = 0.28$, $P = 0.6$; year: $F_{[2,45]} = 7.3$, $P = 0.002$; Table 1).

In the light of the results obtained there is little reason to assume that differences in reproductive parameters between the experimental and control groups, presented below, were caused by some pre-existing factors other than Ca supplementation.

Laying and hatching dates

The Ca-fed pairs tended to start egg laying significantly earlier than the control pairs (Table 2). Laying in the Ca-fed pairs was more advanced relative to the controls the later breeding began in control pairs in a particular year (Fig. 1). This difference persisted at least until the hatching period, because the young hatched significantly earlier in Ca-supplemented nests than in control nests (Table 2). Like egg laying, hatching in Ca-fed pairs was more advanced relative to the controls the later hatching occurred in control nests in a particular year (Table 2).

Table 1. Characteristics of Ca-fed and control groups of adult Great Tits (data are pooled over years).

	Ca-fed			Control		
	Mean	SD	*N*	Mean	SD	*N*
Wing length (mm)						
Females	74.84	1.49	31	74.39	1.57	61
Males	78.32	2.04	17	78.07	1.66	38
Tarsus length (mm)						
Females	19.49	0.52	31	19.55	0.66	62
Males	20.29	0.56	18	20.13	0.49	38
Body mass (g)						
Females	17.97	0.82	31	18.00	0.85	57
Males	18.38	0.98	16	18.55	0.74	35

Note: All differences are statistically nonsignificant.

Egg and shell production

The Ca-fed pairs tended to lay slightly larger (by 0.3–0.5 eggs) clutches each year than control pairs, although the difference was "borderline" significant (Table 3). Calcium supplementation did not affect mean egg volume (Table 3). Clutch volume, however, was significantly larger in Ca-supplemented nests than in controls (Table 3). Although there was remarkable annual variation in clutch volume, the lack of a significant group–year interaction indicated that the difference between the two groups remained similar over years. Note that "clutch volume" is not a simple product of "clutch size" and "egg volume," because one aberrant egg per clutch was excluded when mean egg volume in the clutch was calculated (see Methods).

The amount of eggshell material per clutch was also higher in Ca-supplemented nests than in control nests (treatment: $F = 4.62$, $P = 0.035$; year: $F = 1.22$, ns; interaction: $F = 0.35$, ns). However, this effect disappeared when clutch volume was included in the model. Hence, the shells of individual eggs of Ca-fed birds were not significantly heavier than those of control birds.

Laying date was not included in models, since it had no significant effect on the variation in any of the above-mentioned parameters.

Hatching success and fledgling parameters and number

In no year was a significant effect of Ca supplementation on hatching success found. In 1995 (when clutches were reduced by two eggs), Ca-fed females hatched 93.4% and control females 92.6% of eggs ($Z_{[10,21]} = -1.10$, $P = 0.27$; subscripts refer to sample sizes). The respective figures were 97.3 and 93.5% ($Z_{[10,18]} = -1.39$, $P = 0.16$) in 1996 (when clutches were reduced by one egg) and 91.8 and 94.5% ($Z_{[9,9]} = -0.93$, $P = 0.35$) in 1997 (when no eggs were removed).

Similarly, we did not detect a significant effect of Ca supplementation on the number of fledglings or their body mass and tarsus length (Table 4). However, in 1997, in contrast to other years, hatching date had a strong effect on

Table 2. Onset of egg laying and hatching in Ca-fed and control groups of Great Tits.

		Ca-fed			Control			Difference	
	Year	Median	Quartile range	*N*	Median	Quartile range	*N*	*Z*	*P*
Laying time	1995	27	4	13	29	6	50	–2.85	0.004
	1996	34	2	16	35.5	4	22		
	1997	36	4	13	40.5	6.5	12		
Hatching time	1995	21.5	4.5	12	23	5	37	–2.06	0.039
	1996	27	3	15	29	4	21		
	1997	30	4	10	34	8	12		

Note: Laying time is given as the number of days from 1 April (e.g., a median time of 34 denotes 4 May) and hatching time is given as the number of days from 1 May. Comparisons between groups were made using pooled data of yearly standardized values.

fledgling parameters (Table 4). When hatching date was included in the model as a covariate, a significant positive effect of Ca supplementation on fledgling tarsus length and body mass was revealed (Table 4). Note that weather conditions during the breeding season in 1997 were more unfavorable for Great Tits than in previous years. This is also clearly reflected by the latest start of breeding and the smallest clutch size occurring in 1997 (Tables 2 and 3). Recall too that we did not remove any eggs from clutches in 1997 and hence did not diminish the necessary breeding effort of the parents, whereas in previous years the original clutch size was reduced.

Discussion

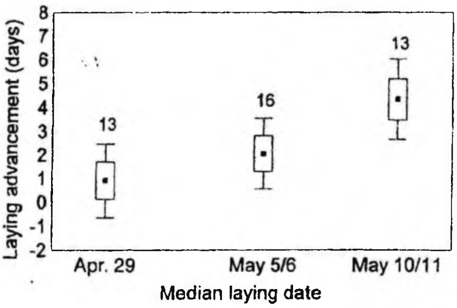
Calcium level and time of breeding

In our study area, Ca supplementation advanced the start of laying in Great Tits by 2–4 days. Several other studies indicate that Ca deficiency may indeed affect breeding time. Dippers (*Cinclus cinclus*) in Wales laid their eggs later on acidic than on circumneutral streams (Ormerod et al. 1991). The authors suggested that the delay on acidic waters might have been due to insufficient nutritional reserves for breeding. However, it has also been proposed that Dippers have problems obtaining enough Ca as a result of the absence of Ca-rich food items in acidic streams (Scheuhammer et al. 1997). Black Terns (*Chlidonias niger*) nesting in acidified bogs also tended to lay later than normal (Beintema et al. 1997).

The presence of Ca is not so important for female birds during the period of follicular growth, but it proves to be a key factor prior to shell secretion (Reynolds 1997). Female passerines store little or no Ca in their skeleton during the prelaying period, and normally, elevated Ca consumption starts immediately before shell secretion (Graveland and Berends 1997). Thus, in conditions of Ca deficiency, birds may have to postpone egg laying until they find an adequate source of Ca on their territory or even outside it. However, we consider it even more plausible that Ca shortage affects laying time indirectly via food.

It has been hypothesized that the onset of laying is constrained by a limited supply of energy and nutrients needed by the female for egg production (Perrins 1970, 1996). The results of several experimental studies in which supplemental feeding has advanced laying by a few days have supported this hypothesis (Källander 1974; Svensson and Nilsson 1995; Perrins 1996; Nager et al. 1997; Ramsay and Houston 1997). Similarly, flycatchers (Sanz 1995) and tits (Tilgar et al. 1999) breeding in deciduous forests with a more abundant food supply have been shown to start laying earlier than the same species in coniferous forests with poorer feeding conditions. Eggshell formation requires accurate timing of Ca intake, and obtaining an adequate amount of Ca is time-consuming even in Ca-rich environments (Graveland 1996*a*; Graveland and Van der Wal 1996; Graveland and Berends 1997). There is a trade-off between time spent feeding and time spent searching for Ca. Graveland and Berends (1997) have shown that egg-laying female Great Tits on a Ca-deficient diet spent 43% of daylight hours searching for Ca-rich items, almost twice as much time as females on a Ca-rich diet. Graveland and Berends (1997), and Graveland and Drent (1997) suggest that low Ca availability may therefore affect not only eggshell quality but also other reproduction parameters. Thus, Ca-fed birds may, for instance, receive more protein and other nutrients and be able, energetically, to start laying earlier than control birds.

Fig. 1. Advancement of laying in Ca-fed Great Tits in relation to median laying date of control pairs in 1995, 1996, and 1997 (ANOVA, year: $F = 4.39$, $P = 0.019$).



Our finding that advancement of laying was positively correlated with the annual median laying date of the control pairs also supports the hypothesis of an indirect rather than a direct effect of Ca. It is well documented that late breeding is often related to low spring temperature (Lundberg and Alatalo 1992; Nager and Van Noordwijk 1995; Sanz 1995;

Table 3. Clutch and egg parameters for Ca-fed and control groups of Great Tits.

	Ca-fed			Control			ANOVA		
	Mean	SD	N	Mean	SD	N	Source	F	P
Egg volume (mL)									
1995	1.65	0.12	12	1.65	0.13	34	Treatment	0.50	0.48
1996	1.66	0.10	14	1.61	0.09	21	Year	0.46	0.63
1997	1.63	0.11	10	1.62	0.11	12	Interaction	0.46	0.64
Clutch volume (mL)									
1995	19.42	2.53	12	18.37	2.48	34	Treatment	5.61	0.020
1996	18.38	2.56	14	16.98	1.72	19	Year	5.13	0.008
1997	17.55	1.69	10	16.62	1.75	12	Interaction	0.1	0.92
Clutch size[a]									
1995	11.46	1.51	13	11.15	1.01	46	Treatment	3.28	0.072
1996	11.13	1.20	16	10.61	1.03	23	Year	3.95	0.021
1997	10.63	1.12	11	10.33	0.89	12	Interaction	0.07	0.94

[a] Clutch size was normalized before analysis: $(\text{observed value}/\text{overall mean})^2$.

Table 4. Fledgling parameters for Ca-fed and control groups of Great Tits (two eggs were removed from each clutch in 1995 and one egg was removed in 1996).

	Ca-fed			Control			Statistical significance	
Fledgling characteristic and year	Mean	SD	N	Mean	SD	N	Statistic	P
Number fledged								
1995	7.82	2.27	11	8.00	1.67	21	$Z = -0.06$	0.95
1996	8.90	1.10	10	8.56	1.34	18	$Z = -1.02$	0.31
1997	7.75	1.16	8	7.00	1.80	9	$Z = -0.10$	0.27
Tarsus length (mm)								
1995	20.15	0.32	11	20.05	0.28	16	$F = 0.81$	0.38
1996	19.75	0.39	12	19.69	0.42	15	$F = 0.14$	0.71
1997	19.05	0.52	9	18.78	0.57	9	$F = 1.05$	0.32
Body mass (g)								
1995	18.03	0.69	11	18.49	0.83	16	$F = 2.30$	0.14
1996	17.87	1.61	12	17.60	1.01	15	$F = 0.28$	0.60
1997	16.88	0.90	9	16.09	1.74	9	$F = 1.48$	0.24

Note: Controlling for hatching date in 1997; tarsus length: treatment, $F = 4.65$ $P = 0.048$; hatching date, $F = 11.55$, $P = 0.004$; body mass: treatment, $F = 8.02$, $P = 0.013$; hatching date, $F = 18.81$, $P = 0.0006$.

Winkel and Hudde 1997) and is most probably mediated by the general state of vegetation and available food supply (Perrins 1965; Lack 1966). This was perhaps the case also in our study area in 1997, where poor feeding conditions exacerbated the trade-off between the time spent on searching for Ca and time spent on searching for food.

In contrast to our results, as well as those of the above-mentioned comparative studies on Dippers and Black Terns, Graveland and Drent (1997) found no effect of extra Ca on laying date in Great Tits in the Netherlands. Evidently, food was less limited there than in our study area. We suggest that Ca shortage affects laying time only in low-quality habitats where food is really scarce. There may exist a certain "saturation point" for food availability, above which the laying date is not energetically constrained but is determined by other factors (Svensson and Nilsson 1995).

Calcium level and egg production

Since an increase in time spent searching for Ca-rich items under conditions of Ca deficiency decreases foraging time considerably, Graveland and Drent (1997) hypothesize that birds breeding in base-poor habitats may lay smaller or fewer eggs than those in base-rich areas. Indeed, we recently showed that extra Ca increased mean egg size in Pied Flycatchers in a naturally base-poor habitat (Tilgar et al. 1999). Also, Ormerod and Tyler (1993) have shown that Dippers breeding on acidified streams laid smaller clutches than those on circumneutral waters.

We found no effect of Ca supplementation on either the mean volume or hatchability of Great Tit eggs in our study area, but Ca-fed females tended to lay slightly larger clutches with significantly larger overall volumes (and also more eggshell material) than control females. Since mean egg volume was calculated after the most different-looking egg in each clutch was rejected (see Methods), the smaller clutch volume for control birds may have resulted from the higher frequency of unusually small eggs in their clutches compared with those of Ca-fed birds.

A probable explanation for the discrepancies between the results of different studies is that birds do not always respond similarly to Ca deficiency. Furthermore, different species may use alternative tactics under different breeding conditions.

Is postponement of breeding adaptive under conditions of Ca shortage?

Several studies on different species of birds have demonstrated that postfledging survival declines with hatching date, and that breeding time per se, but not any other factor correlated with it, appears the causal factor (Nilsson 1989, 1990; Smith et al. 1989; Hochachka 1990; Lindén et al. 1992). Our study showed that although Ca-fed pairs laid clutches with larger overall volume and hatched their young earlier than control pairs, no significant difference in fledglings number or quality between the groups in 1995 and 1996 was revealed. In 1997, there was a positive correlation between hatching date and fledgling parameters, which is at odds with the above results. When hatching date was included in the model as a covariate, a positive effect of Ca supplementation on nestlings' tarsus length and body mass in 1997 was revealed.

As we have already mentioned in the Results, there were two distinct differences between 1997 and the two previous years. First, weather conditions were extremely severe in 1997, which resulted in later laying and smaller clutches than in the other years. Second, by removing one or two eggs from clutches in 1995 and 1996, the required parental effort was artificially reduced. It is probable that comparatively bad breeding conditions and high parental workload were the reasons why the effects of Ca supplementation on the start of laying and growth of the young were most pronounced in 1997.

However, owing to the above-mentioned positive correlation between hatching date and fledgling parameters, there was no significant difference in the quality of young between the Ca-fed and control groups. Although it might seem rather speculative to draw conclusions from results obtained in only one year, a possible explanation for this fact is that postponement of laying in conditions of Ca (and food) shortage is adaptive, since it helps to synchronize hatching of the young with maximal food abundance. In this case Ca-fed pairs were tricked into starting to breed too early in relation to the optimal time for rearing their young. In several other studies, supplemental feeding led to advancement of laying by several days but had no positive effect on breeding success (Svensson and Nilsson 1995; Nager et al. 1997). Thus, there is also a cost of early breeding, and Ca supplementation before egg laying does not contribute to breeding success. If this is the case, adding Ca only during the growth of nestlings (but not before egg laying) would have better consequences for fledgling quality, and this should be tested in future.

Conclusion

The results of this study support Graveland and Drent's (1997) idea that birds breeding in non-acidified, naturally base-poor habitats have adapted to these conditions. Therefore, Ca deficiency is a less limiting factor in naturally base-poor pine forests than in highly acidified areas. We suggest that Great Tits respond to Ca shortage by postponing egg laying until their body condition and rate of food intake reach a critical threshold for producing viable eggs and young. We also suggest that an exact response to Ca shortage depends on the bird species and on the characteristics of their current breeding environment. Possibly the effects of Ca deficiency (and corresponding adaptations) are most clearly expressed in "marginal" habitats in particularly "bad" years.

Acknowledgements

We are grateful to the staff of the Nigula Nature Reserve for all-round help during fieldwork. The study was supported by the Estonian Science Foundation (grant No. 2217 and No. 3905).

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Calcium, snails, and birds: a case study

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Recent studies have shown that wild birds breeding in acidified areas have difficulties with obtaining sufficient calcium for their eggshells, and that the cause of it is the shortage of land snails. Many birds have to search for Ca-rich snail shells on a daily basis during egg production. Molluscs depend on litter calcium, which has decreased due to acidification of the environment. Calcium limitation may be a widespread phenomenon also in non-acidified, naturally Ca-poor areas. The problem is that while in the latter areas the time for development of specific adaptations may have been sufficient, then in acidified areas, on the contrary, calcium shortage is a recent phenomenon. Therefore, since the extent of calcium limitation in non-acidified areas is hard to derive from observational data, experimental approach is needed. We provide experimental evidence that specific calcium deficit does affect reproductive traits also in the birds breeding in naturally base-poor habitats. Our study was conducted in a heterogeneous woodland area in Estonia containing deciduous forest patches as well as base-poor pine forest with low snail abundance. Ca supplementation, using snail shell and chicken eggshell fragments, was carried out for pied flycatchers and great tits. Extra calcium affected positively several reproductive traits like egg volume and eggshell thickness, start of breeding, and fledglings' parameters. The negative relationship between calcium availability and lay-date suggests that birds adjust their breeding tactics to conditions of Ca deficiency, for example, by postponing laying.

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The importance of calcium for living organisms is a well-known fact. It is widely understood that some animals, like crustaceans, molluscs and birds, require calcium in unusual quantities during certain stages of their life. For example, egg-laying birds need 10–15 times as much calcium per day as reptiles and mammals with developing eggs or embryos (Simkiss 1967). The reason is that ca 95% of eggshell's dry weight is calcium carbonate. Due to high calcium demand and a restricted ability to store this element in the skeleton, most insectivorous and granivorous birds must collect calcium on a daily basis (Graveland and van Gijzen 1994, Perrins 1996, Reynolds 1997, Graveland and Berends 1997, Pahl et al. 1997). At the same time the calcium content of their normal food is usually insufficient for shell formation (Graveland and van Gijzen 1994). Several observations show that some birds collect additional calcium-rich material, like snail shells or calcareous grit, during egg-laying (see Perrins 1996 for examples).

Despite all these facts, the subject itself has remained poorly studied, and the position of calcium availability among the ecological factors affecting avian breeding performance has been underestimated. However, the subject has been highlighted in recent years, particularly by Graveland and his colleagues (Graveland 1990, 1996a, b, Grave-

Accepted 3 March 2000

ISSN 1399-1183

land and van Gijzen 1994, Graveland et al. 1994, Graveland and van der Wal 1996, Graveland and Berends 1997, Graveland and Drent 1997). They paid attention to several observations that certain species of birds appeared to have trouble acquiring sufficient calcium for their eggs in some heavily acidified regions (Drent and Woldendorp 1989, Graveland 1990). They showed experimentally that defective eggshells in great tits in the Netherlands were related to decline in snail abundance in acidified soils. Snail shells are claimed to be the main calcium source for eggshell formation in many forest bird species (Schifferli 1973, Graveland et al. 1994, Perrins 1996). Molluscs need calcium for their calcareous shells, but continuing acidification and base cation leaching may lead to decreased calcium content in litter and the surface soil layer (Wäreborn 1969, 1970, 1992). Indeed, a remarkable decline in land snail populations has recently been revealed not only in the Netherlands (Graveland et al. 1994, Graveland and van der Wal 1996) but also in southern Sweden (Wäreborn 1992, Gärdenfors et al. 1995).

Graveland and Drent (1997) have hypothesised that calcium limitation may, in fact, occur not only in acidified areas but also in non-acidified, calcium-poor areas. There are large regions in the world with acidic rock or soils where snail shells or other calcium sources are scarce. As Graveland and Berends (1997) have demonstrated, obtaining sufficient calcium is time-consuming even in calcium-rich environments. Therefore the real cost of egg formation in base-poor areas might be much higher than has been estimated on the basis of the energy and protein content of eggs. However, in naturally base-poor areas the time for development of specific adaptations may have been sufficient, in contrast to acidified areas where calcium shortage is a recent phenomenon (Graveland and Drent 1997). Certain adaptations may have developed in parallel to adaptations to low availability of other nutrients. Consequently, since the extent of calcium limitation in non-acidified areas cannot be derived from observational data, an experimental approach is needed.

Industrial acidification does not yet pose a grave problem in Estonia (Frey 1988, Sepp and Asi 1994). At the same time, a substantial part of the country's territory is covered with pine forests on poor, naturally acidic soils (Taimre 1989). Due to the rather low density of human population, calcium deficiency in such habitats cannot be compensated for as easily as in the Netherlands by using anthropogenic calcium sources (Graveland 1996a, Graveland and Drent 1997).

In 1995–1997 and 1999, we carried out a series of calcium-supplementation experiments in great tits *Parus major* L. and pied flycatchers *Ficedula hypoleuca* Pall. in SW Estonia. Simultaneously, we collected data on the abundance of land snails in our study area as well as in other forests all over the country. In this paper we 1) show that the density of snails in many Estonian forests is low, and 2) provide experimental evidence that birds in such forests do suffer specific calcium shortage.

Material and methods

Study area

Our study was conducted in a heterogeneous woodland area in southwestern Estonia (58°7'N, 25°5'E) containing deciduous forest patches as well as coniferous forests. The area is situated in the transition zone from a base-rich moraine plateau to base-poor sand dunes. In deciduous forests, *Alnus incana* (L.) Moench and *Betula pendula* Roth were the predominant tree species, and there was a rich understorey. In coniferous forests, the dominant tree species was *Pinus sylvestris* L., which sometimes formed mixed stands with *Picea abies* (L.) H. Karst (on sandy soil) and *Betula pubescens* Ehrh (on peat soil). In the field layer, the dwarf shrubs *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L. were the most prevalent species. Our study plots contained nest-box lines and were distributed over an area of ca 50 km^2.

Bird study

Wooden board nest-boxes with an entrance of 3.5–4.0 cm in diameter and a nest cavity with approximate dimensions 11 × 11 × 30 cm were mounted on tree trunks 1.5–2 m above ground level. The distance between neighboring boxes was ca 50 m. Old nest material was removed each year. The boxes were checked regularly to record: 1) date of the first egg, 2) clutch size, 3) hatching success and 4) number of fledged young. Only first clutches were taken into account. Depredated or deserted nests were omitted when calculating fledging success. In 1995–1996, one egg from each clutch (with an exception of 2 eggs in great tit in 1995) was collected randomly to measure the thickness of the eggshell. In the pied flycatcher, the egg was removed on the 3rd or 4th day after the start of laying and, in the great tit, on the 6th (1) day of incubation. The eggs were photographed. A graphics digitizer was used for the input of egg contours from photographs, and egg volume was estimated using the method described in Mänd et al. (1986). In 1996, whole clutches of the great tit were photographed in the field, and mean egg volume per clutch was used in analysis (see Hõrak et al. 1997 for more details). The collected eggs were cut into halves using a razor-blade, their content was removed and the eggshell carefully washed. The eggshells were dried in a desiccator and shell thickness was measured using a digital micrometer. The mean of three measurements from the equatorial zone of an egg was used in analysis. Nestlings were weighed with a Pesola spring balance, and their tarsi were measured with a sliding caliper on the 13th day (pied flycatcher) and 15th day (great tit) after hatching. Adults were captured during the second half of the nestling period, and their age and sex were determined from plumage characteristics (Svensson 1984). They were also weighed, and their tarsal and wing lengths were measured.

Calcium supplementation was carried out in pine forests in 1995–1997 and both in pine and deciduous forests in 1999. In 1995, part of the great tits nesting in the deciduous habitat were also fed with extra calcium. Feeders filled with small snail shell and chicken egg shell fragments were mounted on nest-boxes. The feeders were regularly checked and refilled, so that there was always a surplus of calcium-rich material. We also attached empty feeders to control nest-boxes to check for the possible influence of the feeders per se on breeding results. Experimental and control nest-boxes were arranged in separate five-box blocks. Since there existed a certain probability that filled feeders were used also by birds from adjacent nest-boxes without calcium (Graveland and Drent 1997), control nest-boxes next to experimental boxes were omitted from analysis. The distance between control and experimental blocks was at least 100 m (see methods in Graveland and Drent 1997). As a rule, no more than one nest-box per block was occupied by either species.

Snail study

In 1995, a quantitative study of land snails was carried out in a number of forests all over Estonia (Mänd et al. 1997). Ten litter samples were collected from each study plot using 25 × 25 cm square quadrates with a distance of 10 m from each other. All samples were collected in dry weather during a short mid-summer period between 22 July and 8 August. The samples contained herbs, lichens, mosses and the litter layer plus the top 1–2 cm of the mineral soil. The material was air-dried and carefully hand-sorted by experienced snail specialists in laboratory conditions, using a magnifying glass. Searching time per sample was 0.5–1.5 h depending on sample volume. All snails (alive individuals and empty shells separately) were counted and identified, and their maximum diameter was measured.

In 1995 and 1999, we counted snails also in the area of our calcium-supplementation experiments. Ten litter samples were taken from random sites in pine forests and ten samples from deciduous forests. The samples were collected on 20 May in 1995 and 3 May in 1999.

Results

Snails

Available data on snail density in several Estonian deciduous and pine forests are summarized in Table 1 (together with data from the Netherlands). As expected, more snails live in deciduous than in pine forests. Hundreds of snail

Table 1. Density of land snails (alive + empty shells) in the forests of the Netherlands and Estonia.

Country	Description of the forest	Year	Season	Snail shells m^{-2}	Source
	Forests on nutrient-rich soils				
Netherlands	Three ash forests on nutrient-rich clay or loam soils	1973	September	35–304	Graveland and van der Wal 1996
"	Same forests	1992	"	72–206	"
"	Four oak forests on nutrient-rich clay or loam soils	1992	Late April – early June	58–1581	"
Estonia	Three deciduous/mixed forests on nutrient-rich soils, W Estonia	1995	Late July – early August	146–427	Mänd et al. 1997
"	Three deciduous/mixed forests on nutrient-rich loam soils, S Estonia	1995	Late July – early August	69–219	"
"	Deciduous forest on nutrient-rich loam soil, SW Estonia	1995, 1999	May	92–214	Tilgar et al. 1999, this study
	Forests on nutrient-poor soils				
Netherlands	Two pine forests on nutrient-poor sand soils	1970	November	34–81	Graveland and van der Wal 1996
"	Same forests	1992	"	7–39	"
"	Ten mixed coniferous-deciduous forests on nutrient-poor sand soils	1992	Late April – early June	0–826	"
Estonia	Three pine forests on nutrient-poor sand soils, W Estonia	1995	Late July – early August	10–48	Mänd et al. 1997
"	Three pine forests on nutrient-poor sand soils, S Estonia	1995	Late July – early August	0–2	"
"	Pine forest on poor sand or peat soils, SW Estonia	1995, 1999	May	13–75	Tilgar et al. 1999, this study

shells can be counted from a square metre in deciduous forests, while in pine forests the density of snails does not exceed some tens of individuals per square metre. There exists also remarkable contrast between western Estonia (situated on Silurian limestone bedrock) and southern Estonia (Devonian sandstone bedrock) within each type of forest. Extremely few snails were found in extensive homogeneous pine forests of S. Estonia where most litter samples did not contain snail shells at all.

Table 2 presents data on snail abundance and species richness in the two contrasting habitats of our experimental area during the breeding season of 1999. The density of snails in the deciduous habitat was more than two times and the species richness four times higher than in pine forests. As regards to a possible calcium source for egg-laying birds, the mass of the snail shells per unit surface area would have served as the best indicator. Regrettably, we did not weigh the shells. Still, "shell index" in the last row of the table yields a rough estimate of this parameter. Since it is more than five times larger in deciduous than in coniferous forests, it means that not only the density is lower but also the average size of snails is smaller in the latter habitat.

Birds

Both visual observations and analysis of nest material confirmed that birds had consumed snail shell and egg shell fragments in the feeders. Chicken eggshell fragments were found mostly in the Ca-supplemented nests of the great tit and only in the Ca-supplemented nests of the pied flycatcher.

Ca-fed females of the great tit tended to start egg-laying earlier than did control birds in three of four years studied (Table 3). There exists a significant positive relationship between annual median laying date and advancement of laying in Ca-fed pairs (Fig. 1, $R^2 = 0.37$, $F_{1,77} = 46.1$, $p < 0.001$). When data for the year 1999 (the earliest start of laying) was omitted from analysis, the effect of calcium supplementation on lay-date became significant ($p < 0.01$). In the pied flycatcher, there was no effect of extra calcium on laying date in any but one year, 1999. This was the year with the latest start of breeding in flycatchers. In the same year Ca-fed females of the pied flycatcher tended to start egg-laying earlier than did control birds both in the coniferous and deciduous habitats, although the difference was significant only for pine forest (coniferous habitat: $Z_{82,73} = -2.42$, $p = 0.016$; deciduous habitat: $Z_{38,40} = -1.0$, $p = 0.32$).

Data on egg volume are available for the years 1995–1997 and on eggshell thickness for 1995–1996. In the pied flycatcher, both these parameters tended to have higher values in Ca-fed females than in the control group in all the years studied (Table 4, egg volume: $p < 0.01$; eggshell thickness: $p < 0.001$). In the great tit we observed no significant effect of calcium supplementation on egg parameters, although the mean egg volume of Ca-fed females was slightly larger than in control birds in 1996–1997, the two years with the latest start of breeding. There was also a significant positive effect of extra calcium on clutch volume (sum of the volumes of all laid eggs) in the great tit.

We detected no regular effect of calcium supplementation on the clutch size and production of fledglings when data were pooled over years. However, in the pied flycatcher, clutch size in Ca-fed females was slightly larger than in controls both in the deciduous (6.9 vs 6.6 eggs) and coniferous habitats (6.8 vs 6.6 eggs) in 1999, the year with the latest start of breeding. The difference became significant only when data were pooled over habitats (t-test, $t_{121,106} = 2.18$, $p = 0.03$, square root transformation of data).

In the pied flycatchers breeding in the coniferous habitat, the tarsus of fledglings was longer in Ca-fed than in control nests ($F_{1,158} = 4.6$, $p = 0.03$). Tarsus length and body weight of great tit fledglings in Ca-fed nests were significantly larger than in controls only in 1997, the year with the latest start of breeding, when the results were controlled for hatching date (tarsus: $p < 0.05$, body weight: $p = 0.05$).

Table 2. Abundance* and species richness of land snails in different habitats of the study area in May 1999.

	Pine forest n = 10		Deciduous n = 10		p**
	Mean	SD	Mean	SD	
Alive individuals	2.6	4.7	6.7	6.3	0.019
Alive + empty shells	4.7	8.0	13.4	12.3	0.019
Number of species	1.4	1.8	5.8	3.2	0.002
Shell index***	10.2	16.6	52.3	50.2	0.012

* all means are calculated per 25 × 25 cm litter sample

** Mann-Whitney U-test

*** calculated as the sum of the maximum diameters of all snail shells (alive + empty) per 25 × 25 cm litter sample

Table 3. Start of egg-laying in Ca-fed and control groups of great tits in the coniferous habitat.

Year	Ca-fed			Control		
	Median	Quartile range	N	Median	Quartile range	N
1995	27	4	13	29	6	50
1996	34	2	16	35.5	4	22
1997	36	4	13	40.5	6.5	12
1999	26	3	37	25	3	37

Note. Laying time is given in days from 1 April (e.g., median time 34 in the table means 4 May). Mann-Whitney U test for pooled data: $p = 0.082$. When data for the year 1999 (the earliest start of laying) was omitted from analysis, the effect of calcium became significant ($p < 0.01$).

Discussion

Snails

Comparison of snail abundance in different geographical or ecological regions is often problematic, since different investigators have used different methods, and snail samples have been collected in different periods of the year. We used the same method as Graveland and colleagues (Graveland and van der Wal 1996), while both they and we collected part of the material in spring. There exists no large difference in snail numbers between forests in the acidified area of the Netherlands and the non-acidified area of Estonia. In pine forests of southern Estonia, situated on the Devonian sandstone bedrock, the density of snails is extremely low. Data for snails from our main study area in 1999 as well as the results of an earlier study (Tilgar et al. 1999) suggest that there is a clear contrast in snail abundance between our deciduous and coniferous breeding habitats.

Pine forests on poor, naturally acidic soil constitute a substantial part (ca 16%) of Estonia's territory (40% of all woodlands) (Taimre 1989). Thus, if snail abundance is indeed a reliable indicator of calcium accessibility for birds in their breeding habitats, then passerines in many base-poor forests of Estonia might probably face similar problems as their conspecifics in acidified areas.

Birds

The results of our experiments support the above assumption. Both studied passerine species consumed the calcium-rich material in the feeders. Several breeding parameters revealed regularly better values in Ca-fed females than in controls, and no one parameter behaved the other way round. It is noteworthy that the effect of extra calcium seemed to be the more pronounced, the later occurred the start of breeding in that particular year. Some differences proved to be significant only in the years with the latest breeding, or when data for the year with the earliest start of egg-laying was excluded from analysis. Late start of egg-laying in birds usually correlates with unfavourable weather conditions during the pre-breeding period (Lundberg and Alatalo 1992, Nager and van Noordwijk 1995), which limits the supply of energy and nutrients

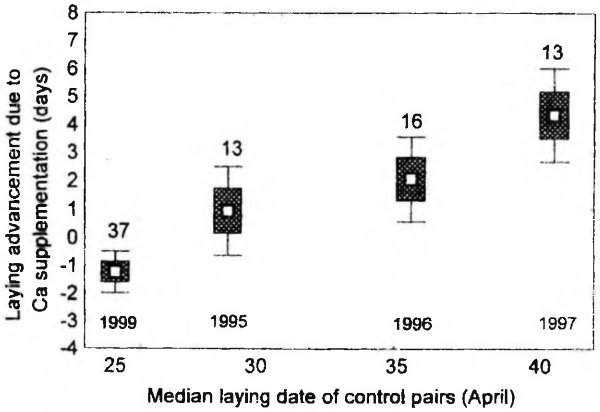

Fig. 1. Laying advancement in Ca-fed great tits in relation to median laying date in control pairs in 1995–1997 and 1999. Rectangles denote SE and vertical lines 95% confidence intervals. Numbers are sample sizes.

Table 4. Egg volume and eggshell thickness in Ca-fed and control groups of the pied flycatcher in the coniferous habitat. After Tilgar et al. 1999.

Egg parameter	Year	Ca-fed			Control		
		Mean	SD	N	Mean	SD	N
Volume (cm^3)	1995	1.68	0.12	19	1.61	0.15	48
	1996	1.58	0.10	32	1.53	0.11	34
	1997	1.60	0.15	57	1.57	0.12	52
Shell thickness (µm)	1995	83.2	4.5	19	78.5	5.3	51
	1996	76.5	5.4	34	73.5	7.7	33

ANOVA; the effect of calcium on egg volume: $p < 0.01$, on eggshell thickness: $p < 0.001$; the effect of year on both characters was highly significant.

needed by the female for egg production (Perrins 1970, 1996). Indeed, the latest breeding year in the great tit was 1997, when the spring was cold and the snow stood unusually long. Similarly, the latest breeding in the pied flycatcher was in 1999, when an extremely severe cold wave, accompanied with a snowstorm, befell the country in mid-May, just prior to the normal time of egg-laying for this species. It seems, therefore, that food shortage during a particularly harsh breeding season amplifies the negative effect of calcium deficiency. This can be explained by a trade-off between feeding time and the time spent on calcium searching (Graveland and Berends 1997, Graveland and Drent 1997). The same trade-off may also help to understand why calcium supplementation affected not only eggshell thickness and fledglings' tarsus, but also other traits, revealing no direct relationship with calcium availability.

Note that the results of our experiments in a particular year often differed for the two bird species. The reason of this is that the time interval between the median laying dates of the two species is quite long. Due to unstable climatic conditions in our study area, it may happen that one and the same year is very favourable for tits but very unfavourable for flycatchers, or vice versa. Moreover, even for one species, conditions in a particular year may be unfavourable during egg-laying but favourable during the nestling period (as was the case with, e.g. flycatchers in 1999).

Regrettably, most of our experiments were carried out only in the coniferous habitat. Therefore we do not know if calcium deficiency is a more limiting factor for birds in the coniferous than in the deciduous habitat. However, our finding that the effects of calcium supplementation on laying date and clutch size of the pied flycatcher coincided in both habitats in 1999, suggests that flycatchers suffer calcium shortage also in our relatively snail-rich deciduous forests, at least in the most unfavourable years.

Our results also supported the assumption that birds in naturally base-poor regions may have been adaptated to calcium shortage (Graveland and Drent 1997). We detected positive effects of extra calcium on the onset of laying in all but the earliest breeding season in case of the great tit and the most unfavourable season in case of the pied flycatcher. Advancement of laying in the great tit was positively correlated with the annual median laying date. These findings suggest that birds postpone breeding in conditions where low calcium availability is accompanied with scanty food supply. However, we found no evidence that production of fledglings in such birds was lower than in calcium-fed birds.

Acknowledgements – The study was supported by Estonian Science Foundation grants No. 1631, 2217 and 3905. We are grateful to Annelie Ehlvest, Tiina Talvi and Piret Kiristaja for analysis of snail samples.

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IV

Tilgar, V., Mänd, R. & Mägi, M.
Calcium shortage as a constraint on reproduction in Great Tits *Parus major*:
a field experiment (submitted manuscript).

Calcium shortage as a constraint on reproduction in Great Tits *Parus major*: a field experiment

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ABSTRACT

Several bird species breeding in acidified habitats face serious problems caused by lowered availability of calcium-rich foods. Moreover, some recent studies have revealed that calcium shortage causes phenotypic variation in certain reproductive parameters of passerines even in non-acidified habitats. However, to date no clear evidence has been found of the limiting effect of calcium deficiency on the reproductive output of birds breeding in such habitats. In this paper, we present experimental evidence from a long-term study that calcium shortage may indeed limit reproductive output of passerines breeding in non-acidified habitats. We carried out calcium-supplementation experiments with Great Tits (*Parus major*) breeding in coniferous and deciduous woodlands in Estonia. Calcium-supplemented birds laid more eggs, tended larger broods and raised more fledglings with longer tarsi compared with control birds. Calcium shortage constrained reproduction of tits not only in spring, but also in mid-summer, during second breeding attempts. Moreover, no significant differences were detected between deciduous and coniferous habitats with respect to the effect of calcium supplements on reproductive success.

INTRODUCTION

In the last decade, a number of studies have addressed a previously overlooked reproductive constraint, namely calcium availability on the breeding territories of birds. Most passerine birds lay relatively large clutches containing much more calcium than is stored in the female skeleton prior to laying and they have to rely predominantly on external sources of calcium such as calcium-rich invertebrates and snail shells (Graveland *et al.* 1994, Graveland and van Gijzen 1994, Perrins 1996, Pahl *et al.* 1997, Reynolds 1997, 2001, Bureš and Weidinger 2000, Dhondt and Hochachka 2001). Dietary calcium is also important for nestlings because of the physiological demand for calcium for bone growth (St. Louis and Breebaart 1991, Barclay 1994, Johnson and Lombardo 2000).

In acidified areas of Europe and America, calcium availability has declined and birds face difficulties in obtaining sufficient calcium for eggshell formation (Drent and Woldendorp 1989, Carlsson *et al.* 1991, Graveland *et al.* 1994, Graveland 1996, Graveland and van der Wal 1996, Graveland and Drent 1997, Weimer and Schmidt 1998, Zang 1998). Graveland and Drent (1997) have hypothesised that calcium limitation may also be a common phenomenon in non-acidified, naturally calcium-poor areas, e.g., in many parts of northern Europe or eastern Canada. Indeed, some experimental studies carried out in such areas have revealed weak positive effects of supplementary calcium on several reproductive parameters of different bird species, e.g. laying date (Mänd *et al.* 2000a,b in the Great Tit *Parus major*), egg volume and eggshell thickness (Tilgar *et al.* 1999a,b in the Pied Flycatcher *Ficedula hypoleuca*), and fledglings' characteristics (Johnson and Barclay 1996 in the House Wren *Troglodytes aedon*, Eeva 1996 and Mänd and Tilgar, in press, in the Pied Flycatcher). However, some other experiments failed to reveal any effect of supplementary calcium either on the number and quality of the eggs laid (Ramsay and Houston 1999 in the Blue Tits *Parus caeruleus*) or on the growth of nestlings (Poulin and Brigham 2001 in the Purple Martin *Progne subis*).

In spite of indirect evidence of the limiting effect of calcium availability in non-acidified habitat on reproduction of some forest passerines, no unequivocal proof exists of its effects on production of the young (number of laid eggs and/or fledged young). It has previously been suggested that calcium shortage in habitats less affected by acid precipitation may be too weak to act as a significant constraint on reproductive output (Johnson and Barclay 1996, Tilgar *et al.* 1999a), or that birds breeding in such habitats may be adapted to such calcium shortage (Graveland and Drent 1997, Mänd *et al.* 2000a,b). However, earlier studies are problematic. First, calcium shortage may affect reproduction of birds only in years when food availability is critically low (Tilgar *et al.* 1999b, Mänd *et al.* 2000a,b). Hence a longer study period is necessary to include more seasons of different food availabilities. Second, Tilgar *et al.* (1999a) removed 1–2 eggs from each clutch in the first two study years and this presumably reduced the parental effort to such an extent that parents could easily compensate for differences in calcium availability between the compared groups. Third, to date studies have only examined the first clutches laid. However, double-brooding is common for the Great Tit in Estonia (in our study area 40–60 per cent of females usually lay a second clutch). Hence, calcium shortage may influence the success of second, as well as first, broods. To address these problems, we performed a calcium-supplementation experiment for five years and in the last three years of the study we removed no eggs from the nests. In 1999, second broods were also subjected to the experiment.

This paper provides the first experimental evidence that both the number of eggs laid and the number of fledglings of the Great Tit are limited by calcium availability, even in a non-acidified area. In addition, we demonstrate that calcium limitation also exists during the second breeding attempt, and that there seems to be no substantial difference between coniferous and deciduous habitats with respect to the effects of supplementary calcium on reproduction in birds.

MATERIAL AND METHODS

Study area

Our study was conducted in the surroundings of Kilingi-Nõmme (58° 7′ N 25° 5′ E), south-western Estonia, in 1995–1997 and 1999–2000. The area is situated in the transition zone from a base-rich moraine plateau to base-poor sedimentary sand dunes. Various forest habitats in the area can be divided into two contrasting types — deciduous and coniferous. The woodlands of the deciduous habitat occur mainly as isolated patches between cultivated fields or as galleries along stream valleys. Grey Alder *Alnus incana* and Silver Birch *Betula pendula* are the predominant tree species, and there is a rich deciduous understorey. The woodlands of the poor habitat are on nutrient-poor sandy and (in the lower parts) peat soils. The dominant tree species is Scots Pine *Pinus sylvestris*, which sometimes forms mixed stands with Norway Spruce *Picea abies* (on sandy soil) and Downy Birch *Betula pubescens* (on peat soil). In the field layer, dwarf shrubs of Bilberry *Vaccinium myrtillus* and Cowberry *Vaccinium vitis-idaea* are the most frequent species.

Industrial acidification does not pose a significant problem in Estonia (Frey 1988, Sepp and Asi 1994). Our study area is far from industrial centres. From previous studies of land molluscs carried out there in 1995 and 1999, density and species richness of snails (a potential calcium source for forest birds) were significantly lower in coniferous than in deciduous habitat (Tilgar *et al.* 1999a, Mänd *et al.* 2000b).

Sampling and measurements

Great Tits bred in nestboxes mounted on tree trunks at a height of about 1.5–2.0 m. The internal size of nestboxes was approximately 11×11×30 cm and the diameter of the entrance hole was 3.5–4.0 cm. Old nest material was removed every year. The nestboxes were checked regularly to record laying date for the first egg, clutch size, hatching date and number of hatchlings and fledglings. The nestlings were weighed to the nearest 0.1 g using a Pesola spring balance and tarsus length was measured to the nearest 0.1 mm with a sliding caliper on day 15 post-hatching. Only first clutches with a known laying date were considered. In 1999, both first and second clutches were studied. Depredated or deserted nests were omitted from analysis when calculating fledgling production per nest. Moreover, there was a number of nests each year where we failed to record the number of hatchlings or fledglings, or where fledglings were not measured. Therefore, the samples are not fully identical with respect to different breeding traits. Breeding was considered successful when at least one chick fledged from the nest, and unsuccessful when no chicks fledged.

Calcium-provisioning experiment

The nestboxes in our study plots were arranged in lines which were distributed over an area of approximately 50 km^2 (see Tilgar *et al.* 1999a for the plan of the study area). The exact number of the nestbox lines where the experiment was carried out varied annually depending on available manpower (in five consecutive study years the number of lines considered was 7, 10, 11, 12 and 12 in coniferous habitat and 4, 4, 5, 8 and 8 in deciduous habitat). Each nestbox line, consisting usually of some tens of nestboxes, ran in a markedly homogeneous (either coniferous or deciduous) habitat. The distance between the nestboxes in a line was approximately 50–60 m. Each nestbox line was divided into several alternating experimental and control blocks consisting of approximately five consecutive nestboxes. The number of blocks per line varied from 2 to 20 (on average 11 in coniferous and 9 in deciduous habitat), depending on the total number of nestboxes in the line. Each year the first block of a nest-box line was randomly assigned as a control or a supplemented block. Hence, the blocks were switched between treatment and reference allocation in different years. No more than one nestbox per block was occupied by Great Tits in a season, while some blocks remained unoccupied altogether.

Small metal feeders were mounted on the nestboxes. The feeders on experimental nestboxes were filled with small fragments of domestic chicken (*Gallus domesticus*) eggshell about two weeks before the start of nest-building, and exceptionally during nest-building in 1995. In 1995–1996 crushed snail shell was also provided. The feeders at occupied nestboxes were regularly checked and refilled, so that birds were always provided with *ad libitum* calcium-rich material. Feeders on control nestboxes were empty. Our visual observations as well as the analysis of nest material confirmed that birds consumed provided calcium-rich items from the feeders. In a pilot study, chicken eggshell fragments were found mostly in calcium-supplemented nests (Tilgar *et al.* 1999a). Consumption of artificially supplied calcium-rich items by adult birds in a number of passerine species has been observed also by other authors (e.g., Graveland *et al.* 1994 and Graveland and Drent 1997 in the Great Tit, Eeva 1996 and Tilgar *et al.* 1999a in the Pied Flycatcher, Johnson and Barclay 1996 in the House Wren and Bureš and Weidinger 2001 in the Meadow Pipit *Anthus pratensis* and in the Water Pipit *A. spinoletta*.). Filled feeders might have also been used by birds from adjacent nestboxes where supplementary calcium was absent (Graveland and Drent 1997). Therefore data from control birds nesting within 100 m of an experimental block were excluded from analyses.

In 2000, calcium was removed, after completing the clutch, from a number of nests provided with calcium during egg-laying (for another experiment). Therefore, in the case of fledglings, the treatment group was considerably smaller than the reference group for that year.

Statistical procedures

For comparisons across years and habitats, we used the unbalanced ANOVA or ANCOVA (when the effect of a covariate was significant). Comparisons within years and habitats were performed with the t-test or the non-parametric Mann-Whitney U test. In one case, where unsuccessful nests were included in the analysis, the number of fledglings per nest was standardised by subtracting yearly habitat mean values from the observed values and dividing the result by the standard deviation for that year and habitat (after Statsoft, Inc. 1999), before the Mann-Whitney U test was applied to the pooled data. All significance levels are for two-tailed tests.

RESULTS

First broods

The number of eggs laid was higher in calcium-supplemented than in control birds, while the effect of year was also significant (Table 1; ANOVA; calcium $F_{1,406}$=5.93, P=0.015; year $F_{4,406}$=9.29, P<0.001; habitat $F_{1,406}$=3.27, P=0.071; all interactions n.s.). The effect of extra calcium on clutch size remained significant when laying date was included in the model as a covariate (ANCOVA; calcium $F_{1,405}$=5.08, P=0.025; year $F_{4,405}$=10.40, P<0.001; habitat $F_{1,405}$=2.88, P=0.090; lay-date: beta=–0.13, $F_{1,405}$=6.96, P=0.009; all interactions n.s.). Within separate years, the effect of calcium supplementation on clutch size was usually not significant (Table 1). The only exception was 1999 for the deciduous habitat.

To test for effects of calcium supplementation on the number of hatched and fledged young, we used only data from the years 1997, 1999 and 2000, because in 1995 and 1996 we reduced the original clutch size of tits, thereby reducing parental effort (see Tilgar *et al.* 1999a for details). More young hatched in the nests where extra calcium was provided compared with the control nests (Table 2; ANOVA; calcium $F_{1,202}$=5.10, P=0.025; year $F_{2,202}$=2.96, P=0.054; habitat $F_{1,202}$=0.14, P=0.071; all interactions n.s.). The number of fledglings was also larger in the calcium-supplemented nests than in the control nests, although the difference was only 'borderline' significant (Table 3; Mann-Whitney U test for the pooled data; $Z_{115,81}$= 1.95, P= 0.051). However, when nests from which no chicks fledged were omitted, it was possible to perform an ANOVA which revealed a significant positive effect of provided calcium on the number of fledglings in successful nests (ANOVA; calcium $F_{1,168}=4.82$, $P=0.030$; year $F_{2,168}=11.79$, $P<0.001$; habitat $F_{1,168}=4.23$, $P=0.041$, more fledglings per nest in coniferous habitat; all interactions n.s). Within separate years, the effect of calcium supplementation on the number of fledglings was not significant (Table 3). The same held true for the hatchlings' number, except for 1999 when significantly more young hatched from the supplemented nests than from the control nests (Table 2).

Table 1. Clutch size in control and calcium-fed nests of Great Tits in first broods.

Year	Habitat*	Control nests			Calcium-fed nests			Difference	
		Mean	SD	N	Mean	SD	N	t-value	P
1995	Dec	10.92	1.38	13	11.54	0.82	11	−1.31	0.20
	Con	11.11	0.97	27	11.46	1.51	13	−0.89	0.38
1996	Dec	11.36	0.93	14	11.60	1.14	5	−0.47	0.64
	Con	10.48	0.93	21	11.13	1.20	16	−1.85	0.072
1997	Dec	10.31	1.08	16	10.60	1.14	5	−0.51	0.61
	Con	10.33	0.89	12	10.80	1.03	10	−1.14	0.27
1999	Dec	10.19	1.19	47	10.90	1.14	29	−2.54	**0.013**
	Con	10.41	1.01	32	10.10	1.28	33	1.10	0.28
2000	Dec	11.32	1.08	37	11.22	1.26	32	0.37	0.71
	Con	10.66	1.33	35	11.06	1.30	18	−1.04	0.30

* In all tables, Dec = deciduous habitat, Con = coniferous habitat

Table 2. Hatchlings number in control and calcium-fed nests of Great Tits in first broods.

Year	Habitat	Control nests			Calcium-fed nests			Difference	
		Mean	SD	N	Mean	SD	N	t-value	P
1997	Dec	8.22	1.56	9	9.75	2.06	4	−1.48	0.17
	Con	9.78	1.39	9	10.00	1.32	9	−0.35	0.73
1999	Dec	9.13	1.45	40	10.00	1.49	28	−2.42	**0.018**
	Con	9.58	1.39	26	9.18	1.61	28	0.97	0.34
2000	Dec	10.06	0.93	16	10.41	1.50	22	−0.82	0.42
	Con	9.50	2.27	10	10.00	2.58	10	−0.46	0.65

Table 3. Fledglings number in control and calcium-fed nests of Great Tits in first broods.

Year	Habitat	Control nests			Calcium-fed nests			Difference	
		Mean	SD	N	Mean	SD	N	Z-value	P
1997	Dec	4.23	3.54	13	7.20	0.45	5	–1.56	0.12
	Con	6.30	2.79	10	6.89	2.80	9	–0.88	0.38
1999	Dec	5.38	2.62	32	6.04	2.76	25	–1.34	0.18
	Con	6.63	3.46	24	7.47	2.61	30	–0.56	0.57
2000	Dec	8.65	2.71	17	8.67	2.96	9	–0.03	0.98
	Con	8.00	2.62	19	9.00	1.73	3	–0.73	0.46

When clutch size was included as a covariate in the ANCOVA model, the effect of calcium provision on the number of fledglings was no more significant (ANCOVA; calcium $F_{1,163}=1.78$, P=0.18; year $F_{2,163}=6.38$, P=0.002; habitat $F_{1,163}=2.00$, P=0.16; clutch size: beta=0.30, $F_{1,163}=16.67$, P<0.001; all interactions n.s.). Thus it is not clear whether the larger number of fledglings in the experimental nests results merely from the larger initial number of eggs laid by the supplemented females, or whether calcium provision has some additional effect on hatching and/or fledging success.

The fledglings of the calcium-supplemented group had longer tarsi compared with those of the control group (Table 4; ANOVA; tarsus length: calcium $F_{1,158}=4.59$, P=0.034; year $F_{2,158}=6.14$, P=0.003; habitat $F_{1,158}=0.27$, P=0.60; habitat*year $F_{2,158}=4.12$, P=0.018; all other interactions n.s.). No significant effect of calcium-provision was found in the case of fledgling weight (ANOVA; calcium $F_{1,158}=2.49$, P=0.12; year $F_{2,158}=1.01$, P=0.37; habitat $F_{1,158}=3.82$, P=0.052; interactions n.s.).

Table 4. Fledgling tarsus length (mm) in control and calcium-fed nests of Great Tits in first broods.

		Control nests			Calcium-fed nests			Difference	
Year	Habitat	Mean	SD	N	Mean	SD	N	t-value	P
1997	Dec	18.97	0.55	8	19.44	0.61	5	–1.44	0.18
	Con	18.78	0.57	9	19.05	0.52	9	–1.03	0.32
1999	Dec	19.23	0.81	26	19.28	0.49	20	–0.26	0.80
	Con	19.66	0.47	19	19.64	0.38	27	0.17	0.86
2000	Dec	19.45	0.56	16	19.55	0.55	9	–0.45	0.66
	Con	19.31	0.68	19	19.84	0.20	3	–1.32	0.20

Second broods

Calcium-supplementation during the second breeding attempt in 1999 significantly increased both clutch size (Table 5; ANOVA; calcium $F_{1,24}$=4.75, P=0.039; habitat $F_{1,24}$=0.27, P=0.61; interaction n.s.,) and number of fledglings per brood (Table 5; ANOVA; calcium $F_{1,24}$=4.67, P=0.041; habitat $F_{1,24}$=0.57, P=0.46; interaction n.s.). As regards hatchlings' number, the effect of calcium provision was nearly significant (Table 5; ANOVA; calcium $F_{1,23}$=4.23, P=0.051; habitat $F_{1,23}$=0.01, P=0.94; interaction n.s.).

Table 5. Clutch size and number of hatchlings and fledglings in control and calcium-fed nests of Great Tits in second broods in 1999.

Breeding parameter	Habitat	Control nests			Calcium-fed nests			Difference	
		Mean	SD	N	Mean	SD	N	t-value	P
Clutch size	Dec	8.40	1.14	5	9.83	1.33	6	−1.90	0.091
	Con	9.13	1.13	8	9.56	0.88	9	−0.88	0.39
Number of hatchlings	Dec	7.40	1.14	5	8.57	1.90	7	−1.22	0.25
	Con	6.86	3.29	7	9.00	0.93	8	−1.77	0.10
Number of fledglings	Dec	6.60	1.52	5	8.14	1.86	7	−1.52	0.16
	Con	5.50	2.10	8	7.88	3.21	8	−1.75	0.10

DISCUSSION

Three new findings emerged from this study. First, calcium limitation in a non-acidified habitat appeared to affect not only laying date in the Great Tits (as it was demonstrated by Mänd *et al.* 2000a,b), but also reproductive output itself in terms of production of the young. We reject suggestions that the effects of calcium shortage may be too weak to act as significant constraints on reproductive output in non-acidified areas (Johnson and Barclay 1996, Tilgar *et al.* 1999a), or that birds breeding in naturally base-poor habitats may be adapted to calcium shortage (Graveland and Drent 1997, Mänd *et al.* 2000a,b).

We suggest that both direct and indirect effects of calcium limitation may result in impaired reproductive success in passerines. For example, in the former case, insufficient dietary calcium can result in abnormal eggshell formation and impaired skeletal development in chicks (Glooschenko *et al.* 1986, Ormerod *et al.* 1988, Graveland and van Gijzen 1994, Houston *et al.* 1995, Eeva 1996, Beintema *et al.* 1997, Graveland and Berends 1997, Pahl *et al.* 1997, Weimer and Schmidt 1998, Tilgar *et al.* 1999a). In the latter case, detrimental effects of limited calcium on reproduction may be mediated through reductions in foraging time. For example, egg-laying Great Tit females on a calcium-deficient diet spent 43% of the daylight hours searching for calcium, almost double the time spent by females with sufficient calcium (Graveland and Berends 1997). Turner (1982) calculated that Swallows *Hirundo rustica* require four to six times more foraging time to meet calcium demands than to satisfy their energy requirements. Thus, obtaining sufficient calcium may involve significant temporal and energetic expenditure. Therefore, birds breeding in habitats where calcium is limited may obtain less protein and other nutrients required for successful reproduction. This probably accounts for the smaller egg and clutch sizes and the lower number and quality of fledglings produced on habitats where calcium availability is low (Ormerod and Tyler 1993, Dekhuijzen and Schuijl 1996, Beintema *et al.* 1997, Zang 1998, Ormerod *et al.* 1988, Weimer and Schmidt 1998, Tilgar *et al.* 1999a, Mänd and Tilgar in press).

Some authors have found only very weak effects of provided calcium on the reproductive parameters of the studied passerines (Johnson and Barclay 1996, Eeva 1996), or have failed to detect any effect at all (Ramsay and Houston 1999, Poulin and Brigham 2001), even in an area affected most severely by acid rain (Ramsay and Houston 1999). The negative results of calcium-supplementation experiments can be attributed to various reasons. For instance, birds may have access to some unknown sources of calcium-rich material. Great Tits in the Netherlands exploited anthropogenic calcium sources close to breeding territories, while data for other species indicate that birds may obtain calcium-rich items even several kilometres from their nest site (Graveland and Drent 1997). In this context it is remarkable that a study by Ramsay and Houston (1999) of the gizzard contents of a Blue Tit's laying female revealed a large number of small snails, although the estimated density of snails in the litter of this heavily acidified area was extremely low (only 0.36 snails per m^2!). Another possible factor which may conceal the limiting effect of calcium deficiency is a relatively favourable nutritional situation in the study area. There is some evidence that the limiting effect of calcium shortage may be the stronger, the lower is food availability in a given season (Tilgar *et al.* 1999b, Mänd *et al.* 2000a,b). Recall that in our study, too, the effect of calcium supplementation was usually not significant within separate years. Moreover, Ormerod and Rundle (1998) warn about ungrounded presumptions about calcium shortage in acidified habitats, because they found no differences in the calcium content of invertebrate prey between artificially acidified or limed and reference habitat plots. Finally, there may exist species-specific differences between birds in response to calcium deficiency, as suggested by Mänd and Tilgar (in press).

Our second new finding was that calcium limitation affected not only first broods but also second broods of Great Tits. In 1999, calcium-fed pairs laid larger second clutches and fledged more young during the second breeding attempt than did control pairs. Although we have no data of second broods from other years, we can still conclude that calcium limitation in Great Tits may occur not only early in spring but also in mid-summer. Unfortunately, we have little data on seasonal dynamics of calcium availability in our study area. However, Phillipson and Abel (1983) found that in Wytham Woods near Oxford, UK, the number of snails decreased significantly towards mid-summer. Hence, the negative consequences of calcium shortage during a first breeding attempt cannot be compensated for during a second breeding attempt, at least not every year.

The third important finding of this study was that calcium shortage occurred in deciduous as well as in coniferous habitats. Food is usually more plentiful in deciduous than in coniferous habitats (van Balen 1973, Paquin and Coderre 1997, Eeva *et al.* 2000) and, more specifically, land snails are significantly more abundant in deciduous compared with coniferous habitats (Tilgar *et al.* 1999a, Mänd *et al.* 2000b). However, calcium-supplementation improved the reproductive output of Great Tits in both habitats, and no significant interaction term (treatment*habitat) was found suggesting that despite differences in snail abundance between habitats, even tits in deciduous habitats expend much time and energy obtaining sufficient calcium for reproduction. Thus, our results support the conclusion of Graveland and Berends (1997) that calcium-specific foraging involves considerable investment even in relatively calcium-rich environments. Furthermore, food resources may become exhausted faster in deciduous woodland patches than in large coniferous forests of our study area. Although Great Tits start laying earlier and lay more and larger eggs in deciduous than in coniferous habitat (Tilgar *et al.* 1999a), in the latter they raise more and heavier fledglings (Mänd *et al.* unpublished data). This agrees with Lens and Dhondt (1994) and Hinsley *et al.* (1999) who found that nestlings of different tit species had lower body masses in small habitat fragments than in larger forest stands.

In conclusion, our results clearly provide evidence that calcium shortage represents a constraint for forest passerines even in non-acidified and relatively base-rich habitats. We agree with Carey (1996) that availability of a certain micronutrient, like calcium, may prove to be even more limiting than energy for some species in the wild. Calcium limitation deserves much more attention than it has received to date.

ACKNOWLEDGEMENTS

We thank Agu Leivits, Ülle Mustonen and Priit Kilgas for assistance in field as well as Steve Ormerod and Jim Reynolds for valuable comments on the manuscript. The study was supported by an Estonian Science Foundation's research grant to R. Mänd.

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Does supplementary calcium reduce the cost of reproduction in the Pied Flycatcher *Ficedula hypoleuca*? — *Ibis* (in press)

Does supplementary calcium reduce the cost of reproduction in the Pied Flycatcher *Ficedula hypoleuca*?

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ABSTRACT

Studies in acidified as well as in naturally base-poor areas have recently revealed that availability of extra calcium-rich food items is an important component of habitat quality affecting breeding performance in several bird species. However, these mostly short-term studies have provided equivocal results concerning the exact consequences of calcium shortage on different species in different regions. We studied the effect of calcium availability on reproduction of the Pied Flycatcher *Ficedula hypoleuca* breeding in pine forests in Estonia, NE Europe, during four years. Experimental pairs were provided with supplementary calcium-rich material when breeding, while control pairs were left unsupplemented. Experimental females laid larger eggs and their nestlings had longer tarsi than those of controls. Moreover, the mass and condition of females tending larger than average clutches were increased by calcium-supplementation. Our results provide the first experimental evidence that calcium availability may affect the overall cost of reproduction in free-living passerines. We compared these results with similar data for the Great Tit *Parus major*, collected from the same area during the same study period. Great Tits responded to low calcium availability mainly by restrained reproductive behaviour and reduced breeding success, while Pied Flycatchers invested significantly more in current reproductive effort despite the increased cost of reproduction. Thus, the effects of calcium deficiency on birds seem to be species-specific or population-specific. This partly explains discrepancies between the results of earlier studies.

INTRODUCTION

Several experimental and comparative studies in acidified as well as in naturally base-poor areas have recently shown that availability of calcium-rich food items is an important component of habitat quality affecting breeding performance in several bird species (Glooschenko *et al.* 1986, Ormerod *et al.* 1988, 1991, Graveland *et al.* 1994, Eeva 1996, Graveland 1996ab, Graveland & van der Wal 1996, Beintema *et al.* 1997, Graveland & Drent 1997, Pahl *et al.* 1997, Scheuhammer *et al.* 1997, Zang 1998, Weimer & Schmidt 1998, Tilgar *et al.* 1999ab, Mänd *et al.* 2000ab, Bureš & Weidinger 2000). According to Carey (1996), this issue deserves attention because energy may prove to be less limiting for some species in the wild than availability of certain micronutrients such as calcium. Breeding birds need calcium for eggshell production and for the skeletal growth of nestlings (Simkiss 1967, Graveland & van Gijzen 1994, Graveland 1996a, Krementz & Ankney 1995, Graveland & Berends 1997, Reynolds 1997, 2001, Bureš & Weidinger 2000, Blum *et al.* 2001). Since the capacity for using endogenous reserves is quite limited in small passerines laying relatively large clutches (Houston *et al.* 1995, Krementz & Ankney 1995, Pahl *et al.* 1997, Reynolds 1997, 2001), and the calcium content of many foods is low (Graveland & van Gijzen 1994), many species have to rely on additional calcium-rich material, like mollusc shells, bones, eggshells and calcareous grit, during egg-laying and when feeding young (see, e.g., Perrins 1996, Graveland 1996a, Tilgar *et al.* 1999a, Mänd *et al.* 2000a and Bureš & Weidinger 2000 for references).

However, there are inconsistencies in the results from different studies. Although most studies report a positive effect of extra calcium on certain reproductive parameters of birds, some experimental studies have failed to detect any significant effect, for example: Blue Tits *Parus caeruleus* in a heavily acidified area in Scotland (Ramsay & Houston 1999) and House Wrens *Troglodytes aedon* in a non-acidified area of Wyoming, U.S.A. (Johnson & Barclay 1996). Calcium-supplementation improved the quality of eggshell as well as hatching success in Great Tits *Parus major* breeding in an acidified area in The Netherlands (Graveland *et al.* 1994, Graveland 1996a, Graveland & van der Wal 1996, Graveland & Drent 1997). No similar effects were detected in Great Tits breeding in a naturally base-poor coniferous habitat in Estonia (Tilgar *et al.* 1999a, Mänd *et al.* 2000b). However, in Estonia tits responded to calcium deficiency by delaying egg-laying and reducing the clutch-size (Mänd *et al.* 2000a). Extra calcium positively affected egg parameters in Pied Flycatchers *Ficedula hypoleuca* in Estonia (Tilgar *et al.* 1999ab). However, no calcium limitation was revealed in the same species in The Netherlands (Graveland 1996b).

There may be a number of explanations for inconsistencies between different studies. First, different species might respond to calcium shortage in different ways (Mänd *et al.* 2000a). Second, in non-acidified but naturally calcium-poor areas, sufficient time might have elapsed for the development of specific adaptations in reproduction or feeding behaviour, in contrast to acidified areas where calcium deficiency is a recent phenomenon (Graveland & Drent 1997). Third, our earlier studies have provided some evidence that calcium shortage affects reproduction of birds mainly in bad breeding years when food availability is critically low (Tilgar *et al.* 1999b, Mänd *et al.* 2000ab). Thus, in the case of short-term studies results for a given species might be applicable to the year(s) of study. Fourth, the results may depend on the absolute abundance of snails or availability of other calcium sources in the study area. Both for theoretical and conservation purposes, it is important to distinguish between species-specific responses and other possible effects. Long-term studies on different species in the same area would be helpful in this respect.

In this paper, we present the results of a four-year experimental study where Pied Flycatchers, breeding in a base-poor coniferous forest habitat, were provided with supplementary calcium-rich material. The results will be compared with those of a similar study on Great Tits in the same area.

We also considered other factors, which may modify the effect of calcium shortage. For example, the specific effect of harsh years may reveal that a bird's susceptibility to calcium deficiency may be related to its condition and/or invested parental effort. If this is the case, birds that are younger and/or in poor body condition, or that are investing heavily in their breeding attempt (the so-called 'maximal investors') should be relatively more susceptible to calcium deficiency than birds with more experience, in better condition or investing less in reproduction. We therefore considered clutch size, female age and female body condition in the analysis of our results.

MATERIAL AND METHODS

Study area

The study was conducted in pine forests near Kilingi-Nõmme (58° 7′ N, 25° 5′ E), Estonia, in 1995–1997 and 1999. The woodlands of the study area were located on base-poor sandy and (in lower parts) peat soils. The dominating tree species was Scots Pine, *Pinus sylvestris* L., but Norway Spruce, *Picea abies* (L.) (on sandy soils) and Downy Birch, *Betula pubescens* Ehrh (on peat soils) were also present. In the field layer, the dwarf shrubs *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L. were most prevalent. The abundance and diversity of land snails (as a potential natural source of calcium for forest birds) was much lower in the pine forests of our study area than in neighbouring deciduous forests (Tilgar *et al.* 1999a, Mänd *et al.* 2000b). There is no significant difference in snail numbers between forests in an acidified area of The Netherlands and pine forests located on base-poor soils in a non-acidified area of Estonia (Graveland & van der Wal 1996, Mänd *et al.* 2000b).

Our study plots contained lines of nestboxes and were distributed over an area of approximately 30 km^2.

Sampling and measurements

Pied Flycatchers bred in nestboxes mounted on tree trunks at a height of about 1.5–2.0 m. The internal dimensions of the nestboxes were approximately 11 × 11 × 30 cm and the diameter of the entrance was 3.5–4.0 cm. Old nest material was removed each year. The distance between the boxes was usually 50–60 m.

All nestboxes in our area were checked three times during egg-laying, with intervals of approximately one week. Nestboxes where nest building was found to be in progress were checked more often. The laying date of the first egg was estimated on the basis of the number of eggs in the incomplete clutch, assuming that Pied Flycatchers lay one egg per day early in the morning. Clutch size, hatching date, brood size and number of fledglings were also recorded. Predated or deserted nests were omitted when calculating the mean number of fledglings. In 1995 and 1996, one egg from each clutch was collected at random on the 3rd or 4th day of laying to photograph the egg and to measure eggshell thickness. In 1997 and 1999, no eggs were removed from clutches, and whole clutches were photographed in the field using a special portable stand. A graphics digitizer was used to input egg contours to a computer from photographs, and egg volume was estimated with the method described in Mänd *et al.* (1986). Mean egg volume per clutch was used in analysis (see Hõrak *et al.* 1997 for more details of the method).

The nestlings were weighed using a Pesola spring balance to the nearest 0.1 g and their tarsi were measured with a sliding caliper to the nearest 0.1 mm on day 13 after hatching (assuming hatch day = day 0). All nestlings were ringed. Adults were captured during the second half of the nestling period and their sex and age were determined using plumage characteristics (Svensson 1984). They were also weighed and their tarsi were measured.

The Pied Flycatcher shows facultative polygyny. Secondary females have significantly poorer breeding success than simultaneously-laying monogamous females (Alatalo & Lundberg 1984, Lundberg & Alatalo 1992). To control for the effect of partial polygyny, the nests where we did not detect a male during nest visits were omitted from analysis (see also Siikamäki *et al.* 1997).

Calcium-provisioning

Small metal feeders mounted on the nestboxes were filled with small snail shell and chicken eggshell fragments before the arrival of flycatchers on the breeding grounds. The feeders attached to occupied nests were checked regularly and refilled, so that the supplemented birds were always provided with a surplus of calcium-rich material. Empty feeders were also attached to control nestboxes to check for the possible influence of the feeders *per se* on breeding. Each nestbox line was divided into many alternating experimental and control blocks consisting of five (with some exceptions) consecutive nestboxes. No significant difference was detected in nestbox occupancy between experimental and control groups. As a rule, no more than one nestbox per block was occupied by Pied Flycatchers. Hence our results usually concern one nestbox per block. Since it was possible that calcium supplements were also used by flycatchers from neighboring nestboxes, data from control birds nesting within 100 m of an experimental block were excluded from analysis. Human population density is low in our study area, and only a few houses (a possible calcium source) are scattered randomly in the vicinity of both experimental and control blocks. Both visual observation and nest material analysis confirmed that the birds did consume the fragments of snail shell and eggshell from the feeders. In a pilot study, we found dropped snail shell fragments significantly more frequently in the nest material of calcium-supplemented Pied Flycatchers than of control birds, whereas eggshell fragments were found only in the calcium-supplemented nests (Tilgar *et al.* 1999a, Mänd *et al.* 2000b). Consumption of artificially supplied calcium-rich material by Pied Flycatchers has also been observed by Eeva (1996).

Analysis

We used an unbalanced ANOVA design (Statistica '99, release 5.5, StatSoft, Inc.) for most comparisons. For *post hoc* analysis, a Tukey HSD test for unequal samples was used. Laying dates were standardized by year [(observed value-yearly mean)/yearly SD)] before a Mann-Whitney U test was applied. All significance levels are for two-tailed tests.

The residual mass of fledglings or adults (considered an index of condition) was calculated as the residual from the regression line between mass and the cube of tarsus length.

In accordance with our hypothesis that calcium shortage would affect flycatchers more strongly if laying larger clutches, we divided the nests into two classes on the basis of average clutch size (overall mean = 6.57 and median = 7 eggs). Clutches with fewer than seven eggs were referred to as 'small clutches', while those with seven eggs or more were referred to as 'large clutches'. We used two age classes for adults termed as 'yearlings' and 'older birds'.

RESULTS

Clutches

Experimental and control groups did not differ significantly in either laying date (Table 1) or clutch size (Table 1; calcium: $F_{1,395}=0.40$, $P=0.53$; year: $F_{3,395}=11.40$, $P<0.001$). Inclusion of laying date (calcium: $F_{1,394}=0.20$, $P=0.66$; year: $F_{3,394}=11.01$, $P<0.001$; laying date: $F_{1,394}=5.24$, $P=0.023$; larger clutches were started earlier) or female age (calcium: $F_{1,253}=0.50$, $P=0.48$; year: $F_{3,253}=10.12$, $P<0.001$; female age: $F_{1,253}=7.55$, $P=0.006$; older females laid larger clutches than yearlings) in the model did not change the main result.

However, egg volume was affected positively by calcium supplementation (Table 1; calcium: $F_{1,340}=4.29$, $P=0.039$; year: $F_{3,340}=18.18$, $P<0.001$; clutch class: $F_{1,340}=1.25$, $P=0.26$). Again, the main result was unchanged by including laying date in the model (calcium: $F_{1,333}=5.01$, $P=0.026$; year: $F_{3,333}=5.01$, $P=0.026$; clutch class: $F_{1,333}=1.34$, $P=0.25$; laying date: $F_{1,333}=4.12$, $P=0.043$). *Post hoc* analysis revealed that eggs from the large clutches of the control group were significantly smaller than those of the calcium-fed group in both small and large clutches (Fig. 1; $P=0.038$ and $P=0.037$, respectively). When we included female age in the model, the positive effect of calcium was no longer significant (calcium: $F_{1,225}=1.66$, $P=0.20$; year: $F_{3,225}=12.31$, $P<0.001$; clutch class $F_{1,225}=0.51$, $P=0.48$; female age: $F_{1,225}=0.02$, $P=0.90$). However, this was probably due to reduced sample size because not all females were trapped.

The young

Supplemented pairs did not raise more young than control pairs (Table 2; calcium: $F_{1,189}=0.08$, $P=0.77$; year: $F_{3,189}=15.74$, $P<0.001$; clutch class: $F_{1,189}=61.70$, $P<0.001$). There were no significant interactions between calcium supplementation and other main effects, and inclusion of female age in the model did not change the main result (calcium: $F_{1,163}=0.37$, $P=0.54$; year: $F_{3,163}=14.15$, $P<0.001$; clutch class: $F_{1,163}=70.39$, $P<0.001$; female age: $F_{1,163}=0.05$, $P=0.83$).

We detected no significant effect of calcium on nestling mass (Table 2; calcium: $F_{1,163}=1.01$, $P=0.32$; year: $F_{3,163}=0.23$, $P=0.88$; clutch class: $F_{1,163}=6.57$, $P=0.011$; lighter young in large clutches). Again, there were no significant interactions between calcium supplementation and the other main effects, and inclusion of female age in the model did not change the main result (calcium: $F_{1,140}=0.14$, $P=0.71$; year: $F_{3,140}=0.09$, $P=0.96$; clutch class: $F_{1,140}=4.31$, $P=0.040$; female age: $F_{1,140}=2.25$, $P=0.136$). When we used nestling residual mass instead of mass, in the model, the result remained unchanged (calcium: $F_{1,163}=0.004$, $P=0.94$; year: $F_{3,163}=0.65$, $P=0.58$; clutch class: $F_{1,163}=1.67$, $P=0.20$).

However, nestlings of supplemented pairs had significantly longer tarsi than those of the control pairs (Table 2; calcium: $F_{1,160}$= 5.44, P=0.021; year: $F_{3,160}$=6.64, P=0.0002; clutch class: $F_{1,160}$=0.50, P=0.48). There were no significant interactions between calcium supplementation and the other main effects. When female age was included in the model, the effect of calcium-supplementation on nestling tarsus was no longer significant (calcium: $F_{1,140}$=2.36, P=0.126; year: $F_{3,140}$=4.76, P<0.001; clutch class: $F_{1,140}$=1.71, P=0.193; female age: $F_{1,140}$=0.87, P=0.35), perhaps due to missing cells in the model.

Nestling tarsus length also correlated positively with egg volume (r=0.15, P=0.045, n=178), female tarsus length (r=0.26, P<0.001, n=177) and male tarsus length (r=0.37, P<0.001, n=196). The correlation coefficients did not differ significantly between chicks from calcium-fed and control nests.

Adults

Female mass in the nestling phase was not affected significantly by supplementation, but there was a highly significant interaction between calcium and clutch-size class (Table 3; calcium: $F_{1,197}$=0.98, P=0.32; year: $F_{3,197}$=2.17, P=0.092; clutch class: $F_{1,197}$=0.89, P=0.35; calcium*clutch class: $F_{1,197}$=7.40, P=0.007). *Post hoc* analysis revealed that supplemented females laying large clutches were significantly heavier than controls laying large clutches (P=0.038). When female age was included in the model, the positive effect of extra calcium on female mass became significant (calcium: $F_{1,179}$=4.48, P=0.035; year: $F_{3,179}$=4.00, P=0.009; clutch class: $F_{1,179}$=0.89, P=0.35; female age: $F_{1,179}$=0.23, P=0.63, calcium*clutch class: $F_{1,179}$=10.84, P=0.0012; calcium*female age: $F_{1,179}$=4.90, P=0.028). Moreover, a significant calcium*female age interaction term also emerged. *Post hoc* analysis revealed that extra calcium had a significant positive effect on the mass of females laying large clutches (P<0.001), whereby the effect was stronger in the case of yearlings (P<0.001) (Fig. 2). When we used the female's residual mass rather than the mass itself, the main result was the same (calcium: $F_{1,196}$=1.13, P=0.29; year: $F_{3,196}$=2.87, P=0.037; clutch class: $F_{1,196}$=0.82, P=0.37; calcium*clutch class: $F_{1,196}$=6.60, P=0.012). *Post hoc* analysis revealed that supplemented females laying large clutches were significantly heavier than were controls laying large clutches (P=0.035).

There was also a significant positive correlation between female mass and egg volume in the group laying large clutches (r=0.25, P=0.004, n=132) but not for those laying small clutches (r=0.04, P=0.70, n=85).

There was no difference in tarsus length between the supplemented and control females (Table 3; calcium: $F_{1,204}$= 1.82, P=0.18; year: $F_{3,204}$= 6.01, P=0.001; clutch class: $F_{1,204}$=0.67, P=0.41), irrespective of the female's age (calcium: $F_{1,190}$=2.51, P=0.12; year: $F_{3,190}$= 6.70, P<0.001; clutch class: $F_{1,190}$=

0.36, $P=0.55$; female age: $F_{1,\ 190}=0.25$, $P=0.62$). There were no significant interactions between calcium supplementation and the other main effects.

In males, neither calcium availability nor clutch class had a significant effect on body mass (Table 3; calcium: $F_{1,221}=2.31$, $P=0.13$; year: $F_{3,221}=4.44$, $P=0.005$; clutch class: $F_{1,221}=0.001$, $P=0.95$), residual mass (calcium: $F_{1,221}=1.59$, $P=0.21$; year: $F_{1,221}=3.33$, $P=0.020$; clutch class: $F_{1,221}<0.01$, $P=0.99$) or tarsus length (calcium: $F_{1,224}=0.49$, $P=0.49$; year: $F_{3,224}=2.33$, $P=0.075$; clutch class: $F_{1,224}=0.03$, $P=0.86$). There were no significant interactions between clutch size class and calcium provision. Nor did *post hoc* tests reveal any significant differences.

DISCUSSION

Calcium availability affects eggs, nestlings and adults

Summarising the results of this study and an earlier study carried out in the same non-acidified area (Tilgar *et al.* 1999a), four significant effects of calcium supplementation on Pied Flycatchers become evident. First, Tilgar *et al.* (1999a), showed that extra calcium increases the thickness of eggshell. Second, in the same study it was revealed that calcium-fed females lay larger eggs on average than control females. The present study showed that egg size is particularly affected by calcium supplementation in females laying larger than average clutches. In other words, while control females appear to show a trade-off between egg size and clutch size, this is not the case in supplemented females. Third, the tarsi of the nestlings of supplemented pairs tended to be longer than those of control pairs. This result became evident only after a longer study period; no such effect was observed in the first study year (Tilgar *et al.* 1999a). Fourth, in the nestling phase, the mass and residual mass of females laying large clutches, were increased by calcium-supplementation.

Two different mechanisms might mediate the effect of calcium availability on the breeding performance of birds. First, eggshell and the nestling's skeleton, which require large quantities of calcium during formation, may depend directly on the amount of calcium available. Second, an indirect mechanism operating through a trade-off between the time spent searching for calcium-rich items and time spent foraging for other nutrients (Graveland & Berends 1997) may result in the positive effect of additional calcium on certain breeding parameters in calcium-poor environments.

A direct result of elevated calcium availability is an increase in eggshell thickness (Tilgar *et al.* 1999a). Calcium availability is reported to exert a direct effect also on nestling skeletal growth (Pahl *et al.* 1997). Indeed, several authors have reported negative effects of calcium deficiency on the skeletal development of young birds (Eeva 1996 for Pied Flycatchers, Beintema *et al.* 1997 for Black Terns *Chlidonias niger*). However, in the case of the nestling skeleton, calcium supplements may also have indirect effects. It has been shown, for example, that tarsus growth in Pied Flycatchers is strongly influenced by food provisioning by adults (Lifjeld & Slagsvold 1989, Lundberg & Alatalo 1992). Increasing local calcium availability may reduce the energy expenditure in calcium-specific foraging, leaving parents more time to feed their nestlings with other food (Turner 1982). If so, we should also expect to see a positive effect of extra calcium on nestling mass. However, we detected no significant differences in nestling body mass (or residual mass) between the calcium-supplemented and control nests. Thus the effects of increased calcium availability appear to be acting directly on nestling tarsus. Differences in the tarsus length of nestlings may partly also have been a maternal effect expressed through egg size. There is some evidence that, on average, larger chicks hatch from larger eggs (Williams 1994). Potti and Merino (1994) found a significant positive relationship between egg size and fledgling tarsus length in the Pied Flycatcher. A positive effect of egg mass on tarsus length in nestlings was recently revealed for the House Wren (Styrsky *et al.* 2000). In our study too, nestlings with longer tarsi developed from clutches with larger eggs. This indicates that larger eggs in the calcium-fed group could contribute, at least partly, to the between-treatment difference in nestling tarsus length.

Does supplementary calcium reduce the cost of reproduction in Pied Flycatchers?

The effect of increased calcium availability on egg size and parent mass can be explained most plausibly through the indirect effects discussed above. It is noteworthy that flycatchers laying larger clutches appear to be more susceptible to calcium shortage than those that lay smaller clutches. Clutch size may reflect the female's investment in the current breeding attempt (Slagsvold & Lifjeld 1988, Lundberg & Alatalo 1992, Sanz 1995) and is hence proportional to her reproductive effort (Charnov & Krebs 1974). Many observations provide either direct or indirect support for this assumption. For example, there is some evidence of increased mortality in birds caring for large broods (see references in Bryant 1979, Hõrak 1995). In Pied Flycatcher females, loss of mass increases with brood size (Askenmo 1977, Hillström 1995). Sanz (1995) found that female Pied Flycatchers breeding in 'good habitat' laid larger clutches and were markedly heavier during the incubation period than those breeding in 'marginal habitat'. However, the habitat-related difference in female mass decreased during the nestling period, indicating a greater loss of mass in the most productive birds. Boyce and Perrins (1987) established that Great Tit pairs laying large clutches in poor years experienced very low nesting success compared with pairs laying smaller clutches. Ots and Hõrak (1996) found a positive correlation between total pre-fledging brood mass and *Haemoproteus* infection intensity in adult Great Tits, indicating that high reproductive effort can result in immuno-suppression. Siikamäki (1995) and Thompson *et al.* (1998) obtained evidence that larger clutches are energetically more costly to incubate than smaller clutches and therefore their incubation is prolonged.

A relatively large decline in parent body mass during breeding may indicate physiological stress (Ricklefs 1974, Bryant 1979, Drent & Daan 1980, Nur 1984, Hillström 1995), implying low energy reserves and low buffering capacity (Norberg 1981, Nur 1984, Bryant 1987, Hillström 1995). There is some direct, as well as indirect, evidence that reduced adult body mass may decrease the probability of survival to the next breeding season (Askenmo 1977, Coulson *et al.* 1983, Nur 1984, Slagsvold 1984, Gustafsson & Sutherland 1988, Dijkstra *et al.* 1990, Hillström 1995, Cichon *et al.* 1998). Hence, in our study, female Pied Flycatchers that lay large clutches seem to have chosen to invest in the current breeding attempt at the expense of their own condition. Experimental provision of calcium did not affect the number or mass of the young but had a positive effect on the mass of females laying large clutches. Moreover, the positive effect of calcium on egg size indicates that calcium availability may influence maternal body condition during egg-laying; there is abundant evidence of a close relationship between egg size and different aspects of female quality (Ojanen *et al.* 1979, Järvinen & Väisänen 1983, Houston *et al.* 1983, Mänd 1988, Reid & Boersma 1990, Smith *et al.* 1993, Hõrak *et al.* 1995, 1997). In our study also, for females laying large clutches, body mass during the nestling phase was positively correlated with the size of her eggs. Although we have no data on the body mass of egg-laying females, we can still hypothesize that supplemented birds laid larger eggs because they were in better condition than controls. This difference in body condition may have been maintained throughout the breeding season. This seems likely given the so-called 'incubatory mass constancy strategy', which has been demonstrated in a number of altricial bird species, including Pied Flycatchers (Moreno 1989, Hillström 1995). The disparity in body condition between supplemented and non-supplemented birds may have increased further during the nestling phase, when calcium requirements increased once more. The most rapid decrease in female body mass takes place shortly after eggs hatch, when females feed and brood the young (Moreno 1989, Hillström 1995).

Furthermore, the exact relationship between clutch size and reproductive effort may depend on other factors, e.g. parental age. Laying of large clutches and/or tending of large broods may result in a relatively higher energy expenditure in younger than in older and more experienced females. In our study, extra calcium had a significant positive effect on female mass in yearlings, but not in older birds. De Steven (1980) also found that Tree Swallow *Iridoprocne bicolor* yearlings lost more mass when breeding than older females. It is possible that inexperienced yearlings are more likely to overestimate their ability to care for large broods than are older birds. Hence, yearling females raising large broods may be particularly vulnerable to reproductive stress as a result of calcium shortage.

Our results provide the first experimental evidence that calcium deficiency may increase the cost of reproduction in wild birds. The most productive flycatchers, and younger flycatchers, appear to be more susceptible to calcium shortage than less productive birds, possibly because of the significant energetic investment in reproduction. This is consistent with our earlier observations (Tilgar *et al.* 1999b, Mänd *et al.* 2000ab) that calcium shortage tends to be more acute in years when food availability is critically low and consequently reproductive effort is relatively high.

Pied Flycatchers and Great Tits in calcium-poor environments

Comparison of data from Pied Flycatchers with those from Great Tits, collected from the same study area and from the same study period (Table 4) revealed differences between the two species. In Pied Flycatchers, calcium supplementation increased eggshell thickness and nestling tarsus length, the traits that are thought to depend directly on the amount of calcium in the diet. Although for Great Tits this effect was not statistically significant, perhaps due to relatively small samples, the tendency was the same in all studied seasons (Tilgar *et al.* 1999, Mänd *et al.* 2000a). However, much clearer discrepancies in other traits indicate that Great Tits and Pied Flycatchers in our study area may respond quite differently to calcium deficiency. For Great Tits, calcium availability significantly affected reproductive traits that are closely related to current reproductive success (laying date, clutch size and fledgling numbers). For Pied Flycatchers, body mass and egg size of the most productive females were affected, both of these parameters probably being related to female condition. Thus, Pied Flycatchers breeding in calcium-poor habitats seem to sacrifice their body condition for their current reproductive success, while Great Tits tend to sacrifice current reproductive output, thereby safeguarding their body condition. Pahl *et al.* (1997) found that when exogenous calcium availability was low, Great Tits relax their reproductive effort in preference to depleting calcium deposits in their skeletons.

Forty to sixty per cent of Great Tits in our study area are multiple breeders, reproducing twice per season, while all Pied Flycatchers breed only once in a given year. Therefore, under unfavourable conditions, tits would benefit by not investing too heavily in the current brood, but by conserving resources that might be invested in the next brood in the same season. Since flycatchers do not use such a bet-hedging strategy, it should be more rewarding for them to increase their current parental effort in response to poor breeding conditions, even at the expense of their own body condition. Järvinen (1991) also suggested that Pied Flycatchers seem to have a rather inflexible breeding strategy compared with Great Tits.

In conclusion, the results of our study provide evidence that some effects of calcium deficiency on birds are species-specific or population-specific. While

Great Tits in our study area respond to low availability of calcium mainly with restrained reproductive effort and reduced breeding success, Pied Flycatchers in the same environment seem to invest in the current breeding attempt despite an increased cost of reproduction.

ACKNOWLEDGEMENTS

We thank Agu Leivits, Marko Mägi and Ülle Mustonen for assistance in the field as well as S. James Reynolds and Stanislav Bureš for valuable comments on an earlier version of the manuscript. The study was funded by an Estonian Science Foundation grant to Raivo Mänd.

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Table 1. Start of laying, egg volume and clutch size in calcium-fed and control groups of Pied Flycatchers.

Breeding	Clutch	Calcium-fed			Control			*P*-value*
parameter	size class	Mean	s.e.	n	Mean	s.e.	n	
Lay-date (1 = 1 May)	Large	22.84	0.42	110	22.25	0.34	132	0.19
	Small	23.72	0.52	78	23.97	0.46	83	0.87
	Total	23.21	0.33	188	22.51	0.27	215	0.38
Egg volume (cm^3)	Large	1.63	0.01	100	1.58	0.01	101	**0.037**
	Small	1.64	0.01	72	1.62	0.02	77	0.78
	Total	1.63	0.01	172	1.59	0.01	178	**0.039**
Clutch size	Large	7.18	0.05	110	7.12	0.04	132	0.82
	Small	5.74	0.07	78	5.83	0.06	83	0.60
	Total	6.56	0.07	188	6.62	0.06	215	0.53

* Mann-Whitney U test (in the case of lay-date) or ANOVA (other parameters) was applied. In the latter case, P-values for separate clutch size classes were found with post hoc test.

Table 2. Fledgling numbers and body parameters at day 13 in calcium-fed and control groups of Pied Flycatchers.

Fledgling	Clutch	Calcium-fed			Control			*P*-value*
breeding parameter	size class	Mean	s.e.	n	Mean	s.e.	n	
Number	Large	6.08	0.15	43	5.99	0.11	68	0.96
	Small	4.90	0.14	43	5.08	0.14	42	0.77
	Total	5.47	0.11	86	5.63	0.09	110	0.77
Weight (g)	Large	13.97	0.15	44	13.75	0.13	51	0.60
	Small	14.05	0.17	37	14.11	0.17	38	0.98
	Total	13.99	0.11	81	13.89	0.10	89	0.32
Residual weight **	Large	–0.09	0.12	44	–0.17	0.11	51	0.69
	Small	0.15	0.13	37	0.22	0.13	38	0.87
	Total	–0.06	0.09	81	0.02	0.09	89	0.76
Tarsus length (mm)	Large	17.26	0.05	44	17.18	0.06	51	0.62
	Small	17.36	0.07	37	17.18	0.07	38	0.064
	Total	17.31	0.04	81	17.18	0.04	89	**0.021**

* for separate clutch size classes found with post hoc test

** calculated as the residual from the regression line between weight and the cube of tarsus length

Table 3. Parental body parameters during the nestling phase in calcium-fed and control groups of Pied Flycatchers.

Parental breeding parameter	Clutch size class	Calcium-fed			Control			*P*-value*
		Mean	s.e.	n	Mean	s.e.	n	
Female								
Weight (g)	Large	12.87	0.12	53	12.46	0.10	73	**0.038**
	Small	12.45	0.13	42	12.64	0.14	39	0.72
	Total	12.66	0.08	95	12.53	0.09	112	0.32
Tarsus length (mm)	Large	17.37	0.07	57	17.28	0.06	74	0.71
	Small	17.28	0.08	42	17.18	0.09	38	0.78
	Total	17.33	0.04	99	17.27	0.05	112	0.18
Residual weight**	Large	0.28	0.12	53	–0.12	0.09	73	**0.035**
	Small	-0.11	0.13	42	0.01	0.14	38	0.89
	Total	0.08	0.08	95	–0.07	0.07	111	0.29
Male								
Weight (g)	Large	12.05	0.11	59	12.20	0.09	82	0.67
	Small	11.94	0.12	48	12.17	0.12	45	0.42
	Total	12.01	0.08	108	12.19	0.08	127	0.13
Tarsus length (mm)	Large	17.26	0.09	60	17.26	0.07	80	0.99
	Small	17.22	0.10	48	17.38	0.10	46	0.58
	Total	17.25	0.06	108	17.29	0.05	126	0.49
Residual weight**	Large	–0.11	0.14	59	–0.02	0.12	80	0.84
	Small	–0.11	0.16	48	0.12	0.16	45	0.55
	Total	–0.11	0.11	107	0.04	0.10	125	0.21

* for separate clutch size classes found with *post hoc* test

** calculated as the residual from the regression line between weight and the cube of tarsus length

Table 4. Effects of supplemental calcium on reproductive parameters of Great Tits and Pied Flycatchers in the same base-poor area. + – significant increase due to calcium supplements, ns — no significant effect of supplemental calcium

Breeding parameter	Great Tit[1]	Pied Fly-catcher[2]	References
Start of egg-laying*	+	ns	[1]Mänd et al. 2000ab, [2]Tilgar et al. 1999a
Eggshell thickness	ns	+	[1,2]Tilgar et al. 1999a
Egg size	ns	+	[1,2]Tilgar et al. 1999a, [1]Mänd et al. 2000a
Clutch size	+	ns	[2]Tilgar et al. 1999a [1]unpublished data
Fledgling numbers	+	ns	[2]Tilgar et al. 1999a, [1]unpublished data
Fledgling tarsus length	ns	+	[1]Mänd et al. 2000a, [2]this study
Female weight	ns	+	[1]Mänd et al. 2000a, [2]this study

* — positive effect means that laying started earlier

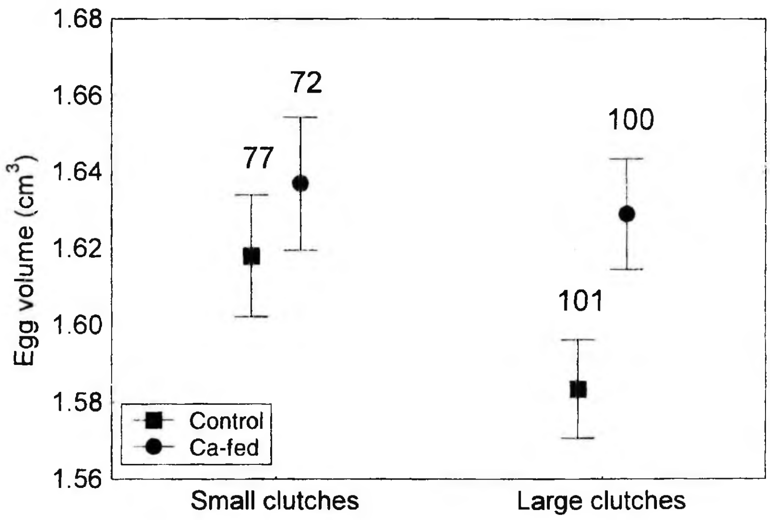

Fig. 1. Volume of eggs of control and supplemented females of Pied Flycatchers. Whiskers = s.e. Numbers above whiskers represent sample sizes.

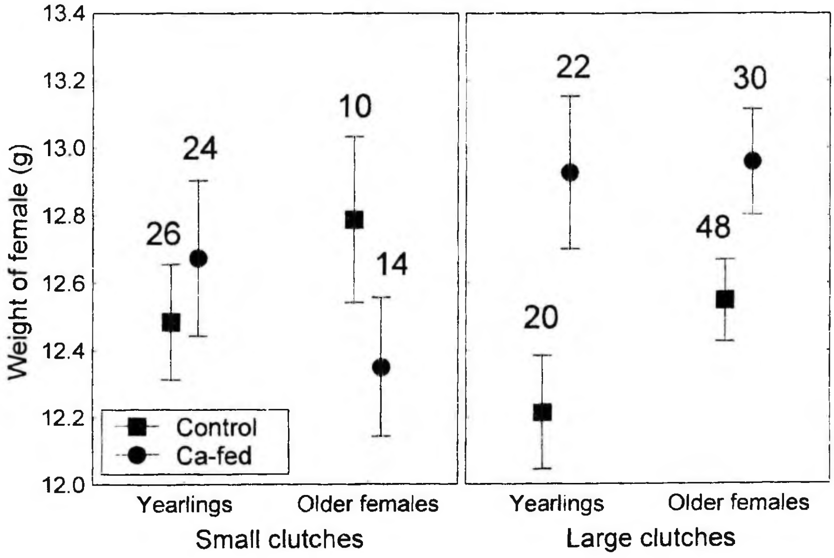

Fig. 2. Female weight for control and supplemented Pied Flycatchers. Whiskers = s.e. Numbers above whiskers represent sample sizes.

VI

Ots, I., Tilgar, V., Mänd, R., Mägi, M. & Kilgas, P.
Maternal Ca availability affects bone growth in great tit (*Parus major*)
nestlings as detected by alkaline phosphatase (submitted manuscript).

Maternal Ca availability affects bone growth in great tit (*Parus major*) nestlings as detected by alkaline phosphatase

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ABSTRACT

In order to detect differences in bone formation processes between habitats and the effect of Ca-supplementation, we measured the activity of different alkaline phosphatase (ALP) isoforms in the blood plasma and morphological characteristics of great tit fledglings on the 15th day after hatching. Bone-ALP activity correlated negatively with fledglings' tarsus length. Evidently smaller fledglings have higher bone-ALP activity due to more intense osteoblastic activity and incomplete ossification of bones compared to fledglings with longer tarsi. Irrespective of the habitat type, bone-ALP values were lower in Ca-provided than in control fledglings. This indicates that Ca-fed fledglings had more developed bones than controls. Fledglings reared in base-poor pine forest had lower bone-ALP activity in nests provided with calcium only during egg production as compared with nests where calcium was supplemented only after hatching of the young. We suggest that initial Ca-deficiency in base-poor habitat during egg-formation cannot be compensated for by accelerated growth due to Ca-provision during the nestling stage. We did not detect any effect of experiments to liver-ALP activity and total ALP activity. Fledglings' morphological parameters did not differ significantly between the treatment groups. Hence, we suggest that bone-ALP activity is more sensitive marker for bone formation than morphological measurements.

INTRODUCTION

Evidence from a number of studies suggests that daily ingested calcium is essential for eggshell formation in passerine birds, since endogenous reserves provide only a minor fraction of the required calcium (Houston et al. 1995, Krementz and Ankney 1995, Pahl et al. 1997, Reynolds 1997). Birds consume calcium-rich materials during peak demand of egg production (Graveland and van Gijzen 1994, Graveland 1996, Brenninkmeijer et al. 1997, Tilgar et al. 1999a, Bureš and Weidinger 2000). Moreover, dietary calcium is also important for nestlings because of the physiological demand of calcium for bone growth (St. Louis and Breebaart 1991, Barclay 1994, Johnson and Lombardo 2000).

Due to the special role of calcium metabolism, birds should be very susceptible to certain environmental changes reducing Ca availability, such as acidification or chemical contamination (Nyholm and Myhrberg 1977, Ormerod et al. 1988, Drent and Woldendorp 1989, Graveland et al. 1994, Eeva and Lehikoinen 1995, Graveland 1996, Graveland and van der Wal 1996). Recent experimental studies have revealed that Ca limitation may be a common phenomenon also in non-acidified, naturally calcium-poor areas (Tilgar et al. 1999ab, Mänd et al. 2000ab).

Studies on various bird species have established that nestlings reared in Ca-poor environments frequently suffered broken and deformed leg-bones, whereas nestlings in Ca-supplemented nests had no defects and fledged normally (Eeva 1996, Beintema et al. 1997, Pahl et al. 1997). Moreover, reduced availability of calcium-rich items may contribute to slow growth of nestlings (Blancher and McNicol 1988, Ormerod et al. 1991). In this context, Ca-provided nestlings may grow faster and mature at a higher rate than control nestlings.

This presumption can be tested by biochemical markers which show earlier changes in bone development than other methods (Dobado-Berrios and Ferrer 1997). In particular, bone isoform of alkaline phosphatase (bone-ALP) is a highly specific marker for skeleton growth (Kubota et al. 1981, Price 1993). Active bone growth is accompanied by increased activity of bone-ALP in the plasma, and after completion of the skeleton development, the level of bone-ALP decreases quickly (Price 1993). Hence, we can expect that in the case of Ca-deficiency, Ca-supplemented nestlings will grow faster than control nestlings, which will be accompanied by lower bone-ALP activity in Ca-supplemented fledglings due to earlier maturation.

Most previous studies have measured the activity of total alkaline phosphatase (ALP) in the serum of birds, which consists actually mainly of two different isoforms: liver- and bone-ALP (e.g. Genge et al. 1988). The activity of total ALP is shown to be associated with bone differentiation and calcification processes, but this is mainly due to the bone isoform of ALP (Price 1993).

Increase in ALP levels in the first week after hatching has been described in American kestrels (*Falco sparverius*) (Hoffmann et al. 1985), red kites (*Milvus milvus*) and black kites (*Milvus migrans*) (Viñuela et al. 1991). Once birds have completed their skeleton formation, ALP-activity falls rapidly to a remarkably lower level compared with its level in juveniles (Costa et al. 1993, Bailey et al. 1998). The activity of ALP is shown to be only weakly related to the quantitative composition of diet (Al-Bustany et al. 1998), and the only factor affecting the activity of ALP during skeletal growth seems to be the level of calcium intake (Ogura 1981).

In this paper, we present the results of the effect of supplementary calcium on alkaline phosphatase activity in the serum of great tit fledglings. We assumed that Ca-supplementation has a different effect on the growth rate of nestlings raised in deciduous and in coniferous wood due to differences in the level of natural calcium sources in these habitats (Tilgar et al. 1999a, Mänd et al. 2000b). We hypothesized that Ca-supplemented nestlings grow faster and complete bone formation earlier than controls, which can be traced by different activity of bone-ALP in reference groups. Furthermore, to study the effect of embryonic Ca availability on the nestlings' growth rate, we performed another experiment, where some nests, provided with extra calcium during egg production, were deprived of it during the nestling stage, while other nests which did not receive calcium initially, were supplemented with it after hatching. We expected that Ca-fed females transfer more Ca into the egg than controls, and this so-called 'maternal effect' influences the nestlings' development.

MATERIAL AND METHODS

Study area and field observations

The study was conducted in 2000 in the surroundings of Kilingi-Nõmme (58° 7′ N 25° 5′ E), Southwest Estonia. Various forest habitats in the area can be divided into two contrasting types — deciduous and coniferous habitats. Deciduous habitat occurred mainly as isolated patches between cultivated fields or as galleries along stream valleys. Coniferous habitats were located on nutrient-poor sandy and peat soils. The density and species richness of snails (a potential Ca source for forest birds) were significantly lower in the coniferous than in the deciduous habitat (Tilgar et al. 1999a, Mänd et al. 2000b).

Our study plots contained nestbox lines and were distributed over an area of approximately 50 km^2 (see Tilgar et al. 1999a for the scheme of the study area).

The nestboxes were checked regularly (with 4–6 day interval) to record laying date for the first egg and clutch size. At the expected hatching time (13 days after laying the last egg) nests were checked daily until hatching. Egg volume was estimated applying the method described in Mänd et al. (1986). The nestlings were weighed using a Pesola spring balance with a precision of 0.1 g and their tarsi were measured with a sliding calliper to the nearest 0.1 mm on the 15th day after hatching.

Experimental design

We mounted small metal feeders on the nestboxes. Feeders on experimental nestboxes were filled with small chicken egg-shell fragments about two weeks before the start of nest building. The feeders attached to occupied nests were regularly checked and refilled, so that the birds of the experimental group were always provided with a surplus of calcium-rich material. Feeders on the nestboxes of the control group were left empty. Our pilot study (Tilgar et al. 1999a) as well as a study of Graveland et al. (1994) showed that birds indeed consumed eggshell fragments in the feeders. All nestbox-lines were divided into alternating experimental and control blocks, each consisting of approximately five consecutive nestboxes. Since no more than one nestbox per block was occupied by great tits, our results concern one replicate per block.

In addition to this experiment, we performed another experiment (referred to as 'exchange treatment') for testing the possible maternal effect, exerting via egg quality, on nestlings' growth. From a certain number of occupied nestboxes provided with calcium before egg-laying, calcium was removed after completing the clutch. At the same time, an equal number of nestboxes, devoid of calcium during egg-laying, were filled with calcium before the hatching of nestlings. Note that in the first case also all nestboxes within 100 m were cleaned from calcium-rich material.

Blood collecting procedures and biochemical analysis

All fledglings were sampled on 15th day after hatching (just before leaving the nest). Fledglings' plasma samples and morphological measurements of fledglings were obtained from the same individuals. Blood samples (ca. 100 µl) from the tarsal or brachial vein were collected into heparinized capillary tubes. Plasma was separated from blood cells after 10 min centrifugation at 10,000 rpm and stored at — 20°C until analyzed. Standard agarose gel electrophoresis with REP system (Helena Laboratories) was used for detection of bone- and liver-ALP. After electrophoresis, gels were incubated with REP ALP Isoenzyme Chromagen and were densitometrically scanned at a wavelength of 595 nm. Total ALP activity was assessed as U/l by regular colorimetric test.

Statistical analysis

A two-way ANOVA was used to compare independent data groups after previous normality tests had been passed (with the STATISTICA 5.3 package by Statsoft, Inc.). The variable of bone-ALP activity was log-transformed to satisfy the condition of normality before comparisons were made. Significant interaction terms were analyzed with Newman-Keuls' post-hoc tests.

Pearson's product-moment correlation was used to examine the linear relationship of two variables if they met normality assumptions, otherwise the Spearman rank-order correlation was used. Fledgling's residual mass was calculated as the residual from the regression line between mass and cubed tarsus length. When more than one (max. two) fledgling from one brood was sampled, the mean values of these individuals were used in analysis.

RESULTS

Egg volume, clutch size, fledglings' number, tarsus length or residual mass did not vary significantly for different treatments and habitats, except for fledglings' residual mass which was significantly larger in coniferous compared with deciduous wood in the exchange experiment (habitat $F_{1,29}$=10.58 P=0.003, calcium $F_{1,29}$=0.02 P=0.90, interaction n.s.).

Fledglings' bone-ALP activity and tarsus length were negatively correlated (Fig. 1, R=–0.28 P=0.033 N=58), but no such relationship was found for liver-ALP (R=–0.10 P=0.47 N=58). The relationship between fledglings' tarsus length and total ALP was nearly significant (R=–0.24 P=0.07 N=58). There occurred no significant correlation between bone-ALP, liver-ALP or total ALP and fledglings' residual mass (bone-ALP R=–0.02 P=0.83 N=58, liver-ALP R=–0.22 P=0.10 N=58, total ALP R=–0.21 P=0.11 N=58).

A two-way ANOVA revealed that bone-ALP level was significantly lower for Ca-fed fledglings compared with controls (Fig. 2, calcium F=11.03 P=0.002, habitat F=0.24 P=0.63, interaction n.s.), but no such difference was detected in the case of liver-ALP (calcium: $F_{1,30}$=0.01 P=0.93, habitat: $F_{1,30}$=0.07 P=0.80, interaction n.s.) or total ALP (calcium $F_{1,30}$=2.00 P=0.17, habitat $F_{1,30}$=0.06 P=0.81, interaction n.s.).

In the experiment with exchange treatments, the effect of Ca-provision on activity of fledglings' bone-ALP depended on the habitat type (Fig. 3; calcium F=1.05 P=0.31; habitat F=1.41 P=0.24, interaction F=5.46 P=0.026). In coniferous forest, fledglings raised in nests supplied with Ca during egg-laying but deprived of Ca after egg production had significantly reduced bone-ALP activity compared with fledglings raised as controls during egg-laying but supplemented with Ca during the nestling stage (post-hoc test: P=0.039). No significant variation was detected among exchange treatments in deciduous habitat (post-hoc test: P=0.67), but fledglings provided with Ca only in the nestling stage had almost significantly lower bone-ALP activity in deciduous compared with coniferous habitat (Fig. 3, post-hoc test: p=0.052).

Liver-ALP did not vary significantly among exchange treatments, but it tended to be higher in deciduous than in coniferous habitat (calcium $F_{1,30}$=0.03 P=0.87, habitat $F_{1,30}$=3.37 P=0.08, interaction n.s.). The activity of total ALP did not differ among the exchange treatments or among the habitats (calcium $F_{1,30}$=0.11 P=0.75, habitat $F_{1,30}$=1.12 P=0.30, interaction n.s.)

DISCUSSION

Bone-ALP activity is considered to be directly related to bone formation (e.g. Price 1993). In our study, fledglings with shorter tarsi had higher bone-ALP activity than fledglings with longer tarsi. We suggest that smaller fledglings had higher bone-ALP activity due to incomplete ossification of bones on the 15th day after hatching, which was manifested by a higher rate of osteoblastic activity. Once growth has ceased, the coupled activity of osteoblasts and osteoclasts continues, but at a much lower rate (Price 1993).

Interestingly, although Ca-fed fledglings showed remarkably lower bone-ALP values than control birds, no significant difference was detected in tarsus length between the treatments. Dobado-Berrios and Ferrer (1997) suggest that after the end of somatic growth, complete ossification may be still somewhat delayed, which may be reflected by activity of ALP. Pahl et al. (1997) demonstrated that great tit nestlings raised in Ca-rich conditions had substantially thicker bones than those raised in Ca-deprived conditions. In the pied flycatcher, no difference was found in fledgling's body weight between the Ca-treated and control nests in a contaminated area in Finland, but significantly more nestlings in control nests had defective legs (Eeva 1996). These studies support indirectly our finding that the Ca-fed young grow faster and they have a better developed skeleton on the 15th day after hatching compared with controls. Proceeding from bone-ALP activity, we can suggest that prolonged bone ossification results from Ca-deficiency.

We propose that calcium availability may affect nestlings' growth rate in different developmental stages: maternal effect, exerting via egg quality, may be important for embryonic growth, and differences in consumption of Ca-rich materials by nestlings may cause additional variation in skeleton growth rate.

Increase in dietary calcium increased significantly plasma Ca in laying hens (Reichmann and Connor 1977, Luck and Scanes 1979) as well as in humans (Tsuboi et al. 2000). Serum calcium was also significantly lower in female dippers at acidic, calcium-poor sites than at circumneutral sites in the pre-breeding period (Ormerod et al. 1991). Maternal calcium supplementation affected positively fetal bone mineralization in humans with low dietary calcium intake (Koo et al. 1999). This kind of maternal effect may also occur in birds, but it is exerted via eggs. Hence, we suggest that serum Ca in females is transferred into the egg yolk, and Ca-fed females with elevated serum Ca level also lay eggs containing more calcium in the yolk compared with control females.

During development, the chick embryo mobilizes necessary calcium from two extraembryonic sources, first from the yolk and then from the eggshell (Cheville and Coignoul 1984, Tuan and Ono 1986, Ono and Tuan 1991). Approximately 80% of the calcium needed by the embryo for skeleton formation is derived from the shell, while the reminder is largely derived from the yolk (Carey 1996). Presumably, Ca translocation from the eggshell is not so limiting for embryonic development because of presence of excessive calcium reserves in the shell. Consequently, Ca deficiency may limit development of embryos mainly in the first phase when the yolk is the only source of calcium (Sahoo et al. 1998, Cheville and Coignoul 1984, Ono and Tuan 1991). In this case, bone growth of embryos may start more intensively in the eggs of Ca-fed females compared with unmanipulated females.

In addition to maternal factors, Ca-provision in the nestling stage may affect also the rate of bone formation and different activity of ALP (Ogura 1981, Aletor and Aturamu 1990). Chickens growing faster and maturing earlier had higher activities of ALP during skeleton development (Singh et al. 1983). This is in accordance with our conclusion that ossification was more complete in Ca-fed fledglings than in controls on the 15th day after hatching.

According to our finding from exchange treatment, Ca-supplementation in base-poor habitat during the egg-formation period seems to be more important for nestling's skeleton growth than Ca-provision during the posthatching period (Fig. 3). At the same time, the activity of bone-ALP in deciduous habitat did not differ significantly among the treatments. At first glance, there seems to be a disagreement with the results of the first experiment where in both habitats fledglings' bone-ALP activity was significantly lower in nests provided with supplementary calcium during the whole breeding period (Fig. 2). Explanation for such discrepancy might be that, unlike the coniferous habitat, deciduous habitat is fragmented and with relatively high density of breeding birds, leading probably to strong food competition. This explanation is in accordance with studies of Lens and Dhondt (1994) and Hinsley et al. (1999) who found that nestlings of different tit species had a lower body mass in habitat fragments than in continuous forest. Indeed, although in our study area great tits start laying earlier and lay more and larger eggs in the deciduous than in the coniferous habitat (Tilgar et al. 1999a, Mänd et al. 2000a), the fledglings' number and weight is larger in coniferous habitat (our unpublished data).

Thus, during the egg-forming phase, tits breeding in pine forest presumably suffer stronger Ca-limitation than tits breeding in deciduous woodland where natural calcium sources are more available (Tilgar et al. 1999a, Mänd et al. 2000b). Later, in the nestling period, the above mentioned food competition may enhance the limiting effect of calcium shortage in deciduous habitat, although the shortage of this mineral is initially not so severe there.

To our knowledge, this is the first study where different isoforms (liver/bone) of ALP are used as biochemical markers for bone growth in wild birds. Our results indicate that changes in bone-ALP isoform activity are strongly dependent on bone formation. At the same time, when using total ALP as an indicator for bone growth, we were unable to detect differences between the treatments. Since calcium availability seems to influence the growth rate of nestlings, it is possible that the Ca-supplemented young mature at a higher rate than the young in Ca-poor conditions. Furthermore, in certain conditions, maternal effect, exerted via egg quality may have even greater effect on nestling's development than posthatching Ca-supplementation. We propose that initial Ca-deficiency in base-poor habitat during egg-laying cannot be compensated for by accelerated growth due to Ca-provision in the nestling stage.

ACKNOWLEDGEMENTS

We are grateful to Ülle Mustonen for the assistance in blood sampling. We also thank Agu Leivits for all kind of help in the field. Peeter Hõrak made valuable comments on the manuscript. United Laboratories, Clinicum of the Tartu University, kindly provided facilities for plasma analyses. This study was funded by Estonian Science Foundation.

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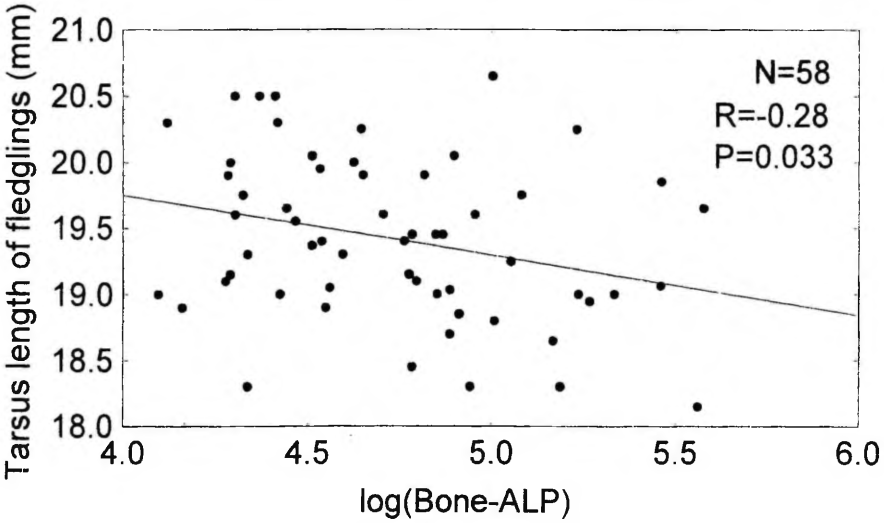

Fig. 1. Relationship between bone-ALP activity and fledglings' tarsus length at 15th day after hatching.

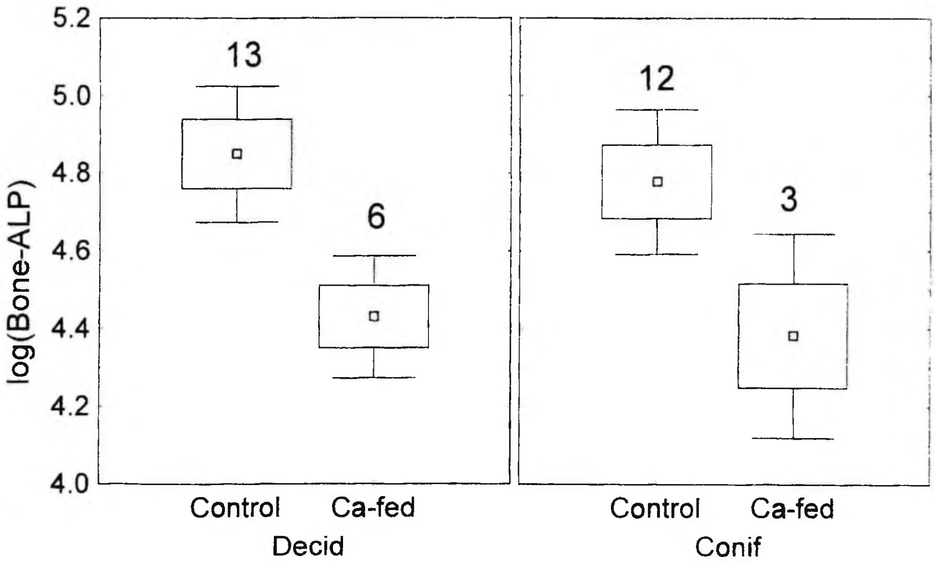

Fig. 2. Fledglings' bone-ALP activity in control and Ca-provided groups in different habitats (Decid — deciduous wood, Conif — coniferous wood). Rectangles denote ±SE and vertical lines 95% confidence intervals.

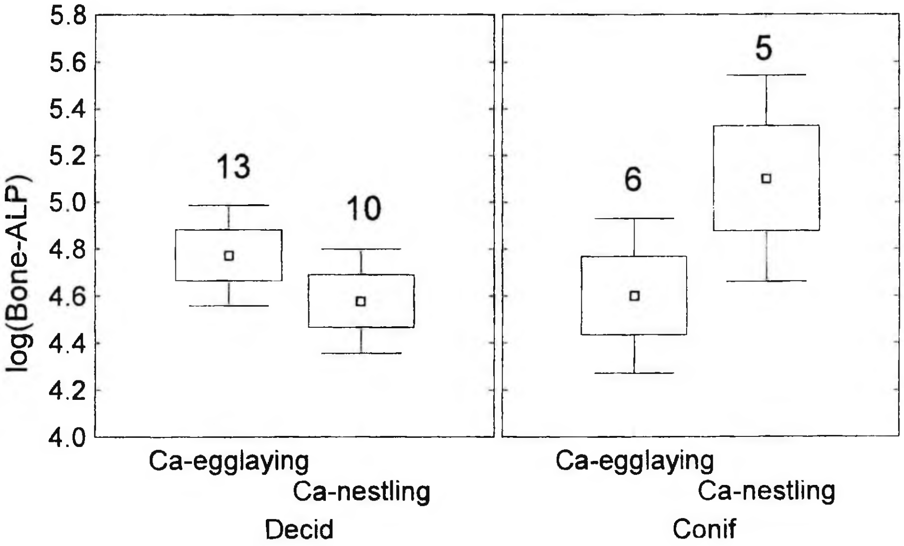

Fig. 3. Fledglings' bone-ALP activity in exchange treatments (Ca-egglaying — nests provided with Ca during egg-laying, Ca-nestling — nests provided with Ca during nestling period). Legend as in Fig. 2.

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1998: Scholarship of Uppsala University Intenational Summer Session
1998–2001: University of Tartu, scholarship no. DBGZH0627 for doctoral students

Publications

1. Tilgar, V., Mänd, R. ja Leivits, A. 1999. Effect of calcium availability and habitat quality on reproduction in Pied Flycatcher *Ficedula hypoleuca* and Great Tit *Parus major*. *Journal of Avian Biology* 30: 383–391.
2. Mänd, R., Tilgar, V. ja Leivits, A. 2000. Reproductive response of Great Tits *Parus major* to calcium supplementation in a naturally base-poor forest habitat. *Canadian Journal of Zoology* 78: 689–695.
3. Tilgar, V., Mänd, R. ja Leivits, A. 1999. Breeding in calcium-poor habitats: are there any extra costs? *Acta Ornithologica* 34: 215–218
4. Mänd, R., Tilgar, V. ja Leivits, A. 2000. Calcium, snails, and birds: a case study. *Web Ecology* 1: 63–69.
5. Mänd, R. ja Tilgar, V. 2002. Does supplementary calcium reduce the cost of reproduction in the Pied Flycatcher *Ficedula hypoleuca*? *Ibis* (in press)

Presentation of work

1. Mänd, R., Tilgar, V. ja Leivits, A. 1997. Calcium deficiency as an ecological constraint for Passerines in oligotrophic forests of Estonia. First meeting of the European Ornithological Union, Bologna, Italy 28–30 August 1997.
2. Tilgar, V., Mänd, R. ja Leivits, A. 1997. Calcium deficiency in forest passerines breeding in calcium-poor habitats. The IXth Finnish-Estonian Ornithological Congress. Lammi, 27–29 September.
3. Mänd, R., Tilgar, V. ja Leivits, A. 1999. Breeding in calcium-poor habitats: are there some extra costs? 2th Meeting of the European Ornithologists Union. Gdansk, Poland, 15–18 September.
4. Mänd, R., Tilgar, V. ja Leivits, A. 1999. Calcium, snails, and birds: a case study. VIII European Ecological Congress. Halkidiki, Greece, 18–23 September.
5. Tilgar, V., Mänd, R., Leivits, A. ja Mägi, M. 2001. All that glitters is not gold: fragments of rich habitat function like "ecological trap" for Great Tits *Parus major*. 3rd Meeting of the European Ornithologists Union. Groningen, The Netherlands, 21–25 August.

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Eesti Ornitoloogiaühingu liige

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1998: *International Summer Session* stipendium õppetööks Uppsala Ülikoolis
1998–2001: Tartu Ülikooli doktorandi teadustööstipendium nr DBGZH0627

Teaduspublikatsioonid

1. Tilgar, V., Mänd, R. ja Leivits, A. 1999. Effect of calcium availability and habitat quality on reproduction in Pied Flycatcher *Ficedula hypoleuca* and Great Tit *Parus major*. *Journal of Avian Biology* 30: 383–391.

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Konverentside teesid

1. Mänd, R., Tilgar, V. ja Leivits, A. 1997. Calcium deficiency as an ecological constraint for Passerines in oligotrophic forests of Estonia. First meeting of the European Ornithological Union, Bologna, Italy 28–30 August 1997.
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ISSN 1024–6479
ISBN 9985–56–628–9