The Journal of Experimental Biology 207, 475-482
Published by The Company of Biologists 2004
doi:10.1242/jeb.00787

Effect of aestivation on long bone mechanical properties in the green-striped burrowing frog, *Cyclorana alboguttata*

Nicholas J. Hudson[1], Michael B. Bennett[2] and Craig E. Franklin[1,*]

[1]*School of Life Sciences* and [2]*School of Biomedical Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia*

*Author for correspondence (e-mail: cfranklin@zen.uq.edu.au)

Accepted 7 November 2003

Summary

The green-striped burrowing frog, *Cyclorana alboguttata*, survives extended drought periods by burrowing underground and aestivating. These frogs remain immobile within cocoons of shed skin and mucus during aestivation and emerge from their burrows upon heavy rains to feed and reproduce. Extended periods of immobilisation in mammals typically result in bone remodelling and a decrease in bone strength. We examined the effect of aestivation and, hence, prolonged immobilisation on cross-sectional area, histology and bending strength in the femur and tibiofibula of *C. alboguttata*. Frogs were aestivated in soil for three and nine months and were compared with control animals that remained active, were fed and had a continual supply of water. Compared with the controls, long bone size, anatomy and bending strength remained unchanged, indicating an absence of disuse osteoporosis. This preservation of bone tissue properties enables *C. alboguttata* to compress the active portions of their life history into unpredictable windows of opportunity, whenever heavy rains occur.

Key words: osteoporosis, disuse, immobilisation, anuran, aestivation, *Cyclorana alboguttata*.

Introduction

Bone tissue is phenotypically plastic and is continually remodelled in mammals and birds to maintain a form appropriate to its mechanical function (Biewener and Bertram, 1993). Increases in bone usage (i.e. loads acting on the bone *via* activity or gravity) can lead to increases in size and overall strength of the bone (Umemura et al., 1995; Biewener and Bertram, 1994). Conversely, disuse following limb immobilisation, cage constraint, hindlimb suspension and space travel can elicit one or more of several responses, including demineralisation, decrease in size and corresponding loss of bone strength (Bagi and Miller, 1994; Globus et al., 1984; Whedon and Heaney, 1993; Turner, 2000). For example, in a clinical setting, Hanssen et al. (1975) described a 2% spinal bone loss per week in humans during prolonged bed rest after scoliosis surgery. Furthermore, 12 weeks of space travel led to an 8% decrease in bone density in human astronauts (Holick, 1998). These effects are directly attributable to disuse and are over and above the homogeneous crystallisation of calcium carbonate induced by microgravity (Liu, 2001).

In addition to skeletal disuse resulting from clinical or otherwise unnatural circumstances, many animals experience extended periods of immobilisation in nature that could also affect bone properties or size. Two prominent examples of natural immobilisation are hibernation and aestivation. Hibernation in endotherms is a dormancy strategy characterised by a reduction in body temperature near to ambient temperature, a markedly reduced metabolic rate and spontaneous arousals by activation of major heat-producing mechanisms (Bartholomew and Hudson, 1960; Kayser, 1961; Lyman et al., 1982). Similarly, aestivation is a state of reduced metabolism seen most commonly in organisms, such as anurans, inhabiting periodically dry habitats (Pinder et al., 1992). The green-striped burrowing frog, *Cyclorana alboguttata*, survives droughts through the excavation of an underground chamber, the formation of a waterproof cocoon and the storage of water in the bladder (Flanigan et al., 1993; Withers, 1993). The inactivity of hibernation is known to be correlated with decreases in bone size and strength (Doty and Nunez, 1985; Whalen et al., 1971) although the response varies between species. However, whether aestivation also induces such changes in bone properties is unknown.

Limb immobilisation related to aestivation can be extreme. In such a capacity, the long bones of the hindlimbs can be effectively immobilised for several years at a time. *C. alboguttata* induced to aestivate on top of pressure-sensitive piezoelectric film in glass jars showed no limb movements in a 12-week experimental period (N. Hudson and C. Franklin, unpublished data). However, it has become apparent that the morphology of the semimembranosus capillary beds is unaffected by aestivation (Hudson and Franklin, 2003). Moreover, following nine months of aestivation in a laboratory setting, *C. alboguttata* have the immediate capability to resume

muscle performance as measured by burst swimming and *in vitro* gastrocnemius force production (Hudson and Franklin, 2002a).

Taken together, these data strongly suggest that *C. alboguttata* emerge from aestivation in the field with a fully competent locomotor system, and therefore within hours of surfacing can transmit forces through the long bones that are representative of an active specimen. This contrasts with the situation in hibernating mammals where there is some impairment in muscle performance. For example, Harlow et al. (2001) found a 23% deficit in the contractile performance of the tibialis anterior in the overwintering black bear *Ursus americanus*. Although overall locomotor performance has never been measured in a hibernator, it is likely to parallel the deficit in isolated muscle performance and thereby reduce the demands on the skeletal system until the muscle regains its pre-hibernation performance. Hibernation also induces loss of bone in bats (Whalen et al., 1971), hamsters (Kayser and Frank 1963) and squirrels (Mayer and Bernick, 1959). In light of the empirically determined difference in muscle performance between emerging hibernators and aestivators, it seems possible that, in contrast to hibernators, aestivating frogs may preserve long bone size and strength in order to withstand the forces associated with jumping upon emergence.

To assess the impact of three and nine months of aestivation on the skeletal system of *C. alboguttata*, we tested the *ex vivo* mechanical properties of the femur and tibiofibula using three-point bending and compared these results with those of frogs kept active in the laboratory. The femur and tibiofibula are the main skeletal elements in the hind limbs and are typically sensitive to usage-dependent remodelling in vertebrates. Although bending has never been experimentally demonstrated in frogs (Calow and Alexander, 1973), it is predicted on the grounds that the long bones (particularly the femur) display some curvature and are subject to compression and because the predominant direction of muscle and reaction forces rarely coincides with the bones' longitudinal axes (Biewener, 1983).

We estimated an appropriate *in vivo* loading rate to apply to the long bones of *C. alboguttata* using force plate records of ground reaction forces developed during jumping. The mechanical data were supplemented by histological analysis to assess osteocyte activity, as it is known that in hibernating bats osteocytic osteolysis accounts for much of the associated bone loss (Whalen et al., 1971).

Materials and methods

Animal capture and husbandry

Adult green-striped burrowing frogs (*C. alboguttata* Günther 1867; body mass 7–30 g) were captured from flooded roadsides following summer rains in southeast and mid-eastern Queensland. Animals were transported back to the laboratory in individual plastic bags (containing water and plant matter) within 24 h. They were randomly assigned to one of three treatment groups (controls, 3-month aestivators and 9-month aestivators) with animals matched as closely as possible for sex and mass between the three groups. Control frogs were housed in individual plastic boxes (27 cm×14 cm×12 cm) containing wet paper towelling and fed *ad libitum* with grasshoppers and cockroach nymphs. To induce aestivation, frogs were placed into individual plastic containers filled with wet clay (obtained from the locality of animal capture) that was allowed to dry naturally over a period of several weeks. Most frogs burrowed almost immediately and did not resurface. When the aestivation period was complete, frogs were extracted by breaking open the soil block across the burrow line. Euthanasia was achieved by double pithing.

Estimation of in vivo *bone loading rate during jumping*

To determine a biologically relevant loading rate for bending tests, we used force platform records of jumps to evaluate an *in vivo* rate of bone loading. Ground reaction forces represent one of the major forces experienced by the long bones during locomotion; therefore, the time to peak force was judged to be the most biologically relevant duration of loading to apply during tests of bone mechanical properties. Control frogs (N=35) of various masses were encouraged to leap off a custom-built force platform at 25°C (platform based on design of Katz and Gosline, 1993; see Wilson and Franklin, 2000 for details) that simultaneously measured the vertical, horizontal and lateral ground reaction forces exerted during a single jump. Net ground reaction force was calculated as the vector sum of the three force components. Changes in force in the three dimensions were detected with 5-mm aluminium foil strain gauges attached to the outer side of each spring blade. Each strain gauge, which corresponded to a separate dimension, formed a quarter of a bridge circuit that fed a signal directly into a Maclab bridge amplifier. Data were collected by a Maclab 4e (AD Instruments, Castle Hill, Australia) analog-to-digital data acquisition system that sampled at 1000 Hz. Data were recorded and analysed using Chart 3.5 software. As it has previously been shown in swimming *C. alboguttata* that locomotor performance after nine months aestivation is indistinguishable from control levels (Hudson and Franklin, 2002a), it was assumed that the same would be true for jumping; thus, force traces from post-aestivating frogs were not obtained. For each individual, the longest of three jumps was considered to represent a maximal performance. However, we cannot exclude the possibility that variations in takeoff angle could confound the results, as Marsh (1994) has previously pointed out.

Ex vivo *bone bending*

The femur and tibiofibula of control (N=8; mass=19.75±3.86 g; snout–vent length=5.60±0.22 cm, mean ± S.E.M.), 3-month-aestivating (N=11; mass=23.70±4.94 g; snout–vent length=5.53±0.24 cm, data collected immediately post aestivation for both aestivation treatment groups) and 9-month-aestivating frogs (N=6; mass=19.2±5.06 g; snout–vent length=5.38±0.35 cm) were removed by disarticulation at the hip joint. The variation in sample size was a simple function of the availability of animals. The overlying soft tissues were

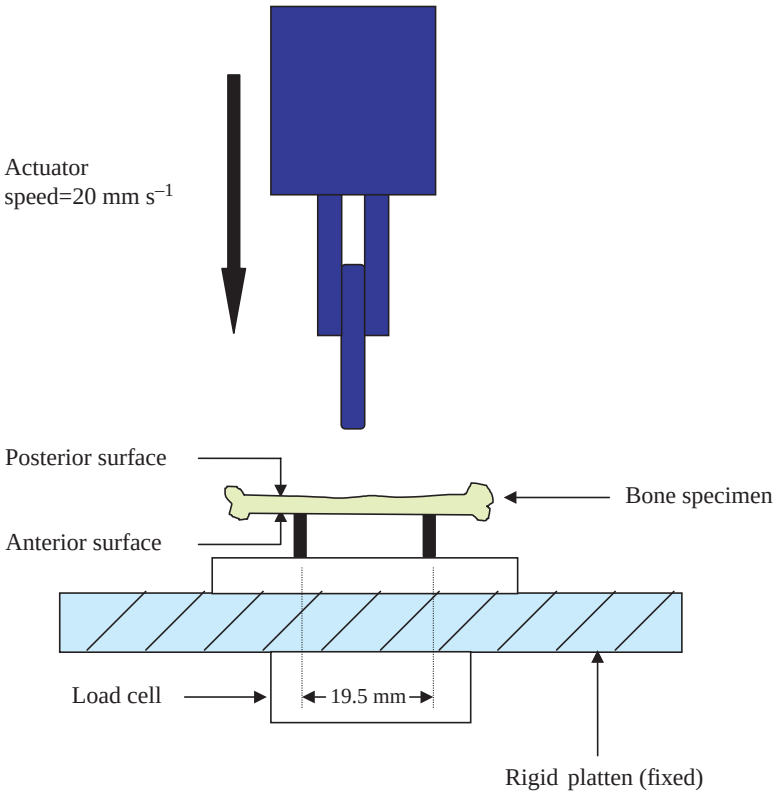

Fig. 1. Illustration of the Instron materials testing apparatus used to measure femoral and tibiofibular bending strength in the green-striped burrowing frog (*Cyclorana alboguttata*). The diagram shows the direction of actuator travel with respect to bone orientation such that the posterior surface was loaded in compression.

left intact. The samples were wrapped in 0.9% saline-moistened tissue, placed in plastic vials within sealed polystyrene bags and stored frozen at –20°C. Prior to mechanical testing, the bones were thawed to room temperature (22°C), cleared of surrounding soft tissue and measured.

Each bone was rested (metaphysis to metaphysis) on rigid brass supports 19.5 mm apart and broken by three-point bending using an Instron 8722 servo-hydraulic materials testing machine (Instron, High Wycombe, UK). Three-point bending was achieved using a hydraulically driven actuator with a single point of load application equidistant between the supports. All bones were loaded at approximately mid-shaft with the posterior surface experiencing compressive loading (Fig. 1). This loading regime was chosen for practical reasons as the bones were stable in this orientation throughout the whole loading event. *In vivo*, the tibiofibula is most probably loaded in this way, with the femur experiencing anterior compressive loading primarily by the cruralis and gluteus magnus, coupled with a bending moment caused by the resultant ground reaction force. However, the femur is relatively cylindrical with little variation in cortical thickness; as such, the second moment of area is not influenced by the bone's orientation, assuming a neutral axis passing through the centre of the bone's cross-section. Consequently, the effect of orientation on femoral bending strength was considered minimal; either way, the orientation was the same in all tests. An actuator speed of 20 mm s^{-1}, used as a preliminary experiment, showed that it led to bone fracture after approximately 50 ms, comparable with the time-to-maximum force recorded for *in vivo* jumping of *C. alboguttata*. This loading rate is somewhat faster than has been used on organisms of comparable size. Correspondingly, values for stress and modulus of elasticity will be higher than those generated at less realistic loading rates (Currey, 1975).

The load cell output was pre-amplified (QUANTEC, Brisbane, Australia) and subsequently collected at 1000 Hz on a Maclab data acquisition system interfaced with a Macintosh 4e computer running Chart software (version 3.5). Load-displacement records were re-plotted using Sigmaplot 5.0 software (SPSS, Chicago, IL, USA). Values for the load and displacement at the yield and failure points were determined from these plots. The point of yield was defined as the intersection of tangents drawn to the linear elastic and plastic deformation portions of each curve. Areas under the curves, indicative of the energies of yield and of failure, were also determined from the plots.

Following mechanical testing, each bone was sectioned transversely approximately 1 mm distal to the site of fracture with a diamond saw microtome (Leitz 1600, Oberkochen, Germany). Each bone was then mounted under a dissection microscope (Leica M26, Witzlar, Germany) and the cross-section photographed (Panasonic KR-222 digital camera, Osaka, Japan) at known magnification. Images were rotated to the same orientation (Corel Draw 7.0; Corel Corporation) and then printed. The endosteal and periosteal perimeters were traced on these printouts using a digitising tablet attached to a Macintosh computer running NIH image analysis software. The cross-sectional area and second moment of area about the assumed bending axis were calculated using a custom-written macro. These data were used in conjunction with the force data to calculate femoral and tibiofibular elastic moduli (a measure of stiffness) and ultimate strengths in accordance with the following formulae (Carrier and Leon, 1990):

$$E = \frac{\mathbf{F}_Y L^3}{48ID} \quad (1)$$

and

$$\sigma_{ULT} = \frac{\mathbf{F}_B LY}{8I}, \quad (2)$$

where E is the modulus of elasticity (GPa), σ_{ULT} is the ultimate strength (MPa), $\mathbf{F}_Y$ is the yield force (N), L is the inter-support

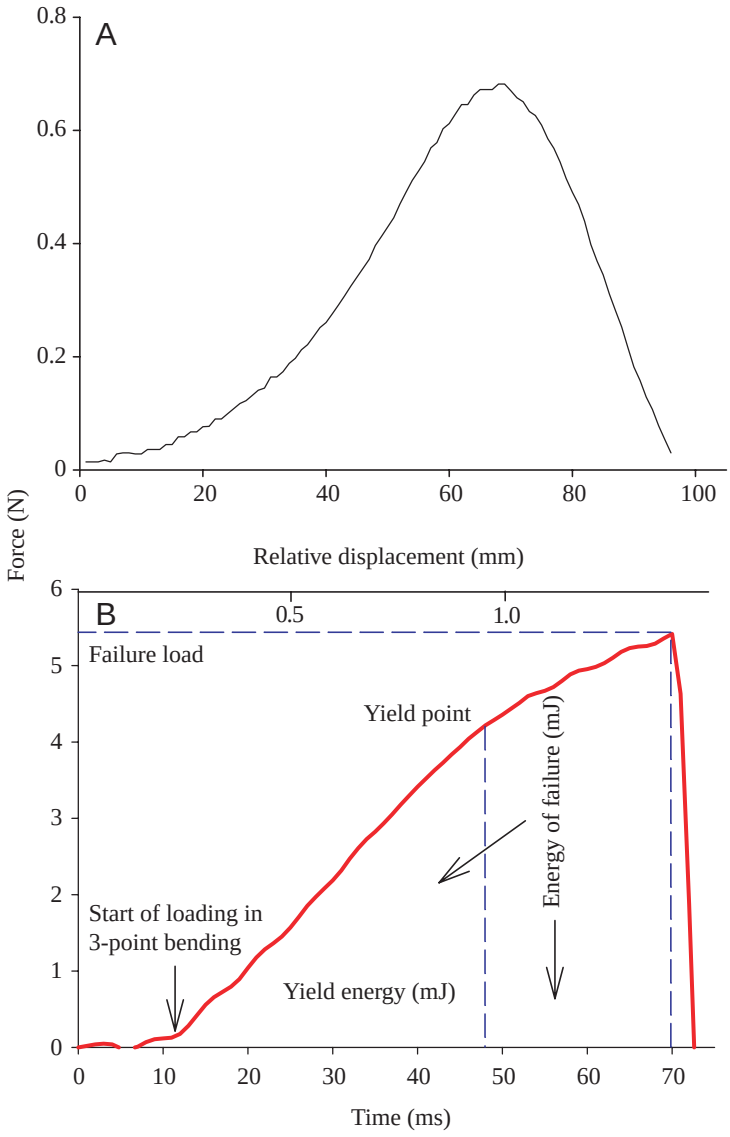

Fig. 2. (A) Change in resultant ground reaction force magnitude during a typical maximal jump for an adult *C. alboguttata* at 25°C recorded with a force platform sampling at 1000 Hz. (B) Typical load-displacement curve of a *C. alboguttata* long bone broken in three-point bending, sampled at 1000 Hz.

distance (m), I is the second moment of area at the site of failure (m^4; Vose and Kubala, 1959), D is the deflection distance of the bone (m), $\mathbf{F}_B$ is the breaking force (N) and Y is the external depth of the specimen at load point (m).

The cross-sectional areas of the femur and tibiofibula were normalised to snout–vent length (cross-section/snout–vent length; $mm^2\ cm^{-1}$) for the tabulated data to determine if there were any gross changes in tissue size with aestivation.

Histology

The tibiofibula and femur from two control frogs and two 9-month aestivators were fixed in 10% neutral buffered formalin, embedded in methyl methacrylate and mounted on glass slides. Serial sections (65 μm thick) were cut from the undecalcified midpoints of bones using a Leitz 1600 diamond saw microtome. For each bone, a representative section was qualitatively examined and photographed using an Olympus DP10 digital camera attached to an Olympus BX60 microscope.

Statistical analysis

Data are presented as means ± S.E.M. of N measurements. The comparisons of cross-sectional area were made using analysis of covariance (ANCOVA) on the original (non-normalised) data with snout–vent length as the covariate. Comparisons of ultimate strength, moduli, second moment of area, energy of yield, energy of failure and yield displacement between the treatment groups were made using 1-way analysis of variance (ANOVA). Data were log transformed if they initially failed to meet the assumption of normality. All analyses were performed using Sigmastat and SPSS software. $P<0.05$ was considered significant.

Results

Estimation of in vivo *bone loading rate*

The maximum jumping performance of 35 *C. alboguttata* weighing between 7.13 g and 30.84 g was determined at 25°C. A typical jump involved a rapid development of force resulting in a peak ground reaction force between 43 ms and 80 ms (mean ± S.E.M., 62±1.61 ms) after force development commenced (Fig. 2A). Time to peak ground reaction force scaled with body mass ($r^2=0.13$, $P<0.05$, $N=35$) but was independent of snout–vent length ($r^2=0.09$, $P=0.081$, $N=35$) within this size range, but it is probably inappropriate to attribute much difference between the two relationships given that the r^2 values are low for both comparisons (Fig. 3).

Ex vivo *bone bending*

During a typical load-displacement on a control femur and tibiofibula, strain initially varied with stress linearly (Fig. 2B). When the bones yielded (after 53±3 ms for the femur and 54±1 ms for the tibiofibula), increments in strain led to smaller increases in stress. Soon thereafter (64±3 ms for the femur and 66±2 ms for the tibiofibula) the bone would fracture and stress dropped abruptly.

The ultimate strength and moduli of the tibiofibula and femur of *C. alboguttata* after 3-months and 9-months aestivation were compared with those of active frogs. Aestivation had no significant effect on these bending mechanical properties in the long bones (Table 1), with 9-month-aestivating frogs displaying the same resistance to bending forces as the controls (femoral ultimate strength, $P=0.45$; femoral modulus, $P=0.32$; tibiofibular ultimate strength, $P=0.09$; tibiofibular modulus, $P=0.21$). Energy of yield (femoral, $P=0.91$; tibiofibular, $P=0.42$) and failure

Table 1. *The effect of 3-months and 9-months aestivation on long bone moduli, ultimate strength, second moment of area, yield/failure energy and cross-sectional area in* C. alboguttata

Bone	Property	Control	3 month	9 month	Significance
Femur	Elastic modulus (GPa)	12.51±2.07	9.45±2.60	10.27±1.87	NS
	Ultimate strength (MPa)	328.2±45.9	265.6±25.4	297.5±47.1	NS
	Yield energy (mJ)	5.35±0.79	4.98±0.38	4.94±1.11	NS
	Failure energy (mJ)	8.37±1.34	7.76±0.75	8.97±1.36	NS
	Yield displacement (m)	$1.07\times10^{-3}\pm6.65\times10^{-5}$	$1.12\times10^{-3}\pm4.51\times10^{-5}$	$1.10\times10^{-3}\pm5.98\times10^{-5}$	NS
	Second moment of area (m^4)	$1.54\times10^{-13}\pm1.12\times10^{-14}$	$1.89\times10^{-13}\pm2.33\times10^{-14}$	$1.97\times10^{-13}\pm3.56\times10^{-14}$	NS
	Normalised cross-sectional area ($mm^2\ cm^{-1}$)	0.153±0.007	0.164±0.006	0.181±0.011	NS
Tibia	Elastic modulus (GPa)	8.81±0.46	10.56±0.86	9.19±0.37	NS
	Ultimate strength (MPa)	253.8±15.4	303.6±19.0	256.5±11.7	NS
	Yield energy (mJ)	4.24±0.55	4.96±0.25	4.50±0.45	NS
	Failure energy (mJ)	7.57±1.15	8.80±0.74	7.40±1.32	NS
	Yield displacement (m)	$1.10\times10^{-3}\pm3.22\times10^{-5}$	$1.09\times10^{-3}\pm2.82\times10^{-5}$	$1.10\times10^{-3}\pm6.93\times10^{-5}$	NS
	Second moment of area (m^4)	$1.81\times10^{-13}\pm1.05\times10^{-14}$	$1.80\times10^{-13}\pm1.60\times10^{-14}$	$2.02\times10^{-13}\pm2.31\times10^{-14}$	NS
	Normalised cross-sectional area ($mm^2\ cm^{-1}$)	0.188±0.009	0.167±0.007	0.201±0.014	NS

NS, no significant difference between treatment groups.

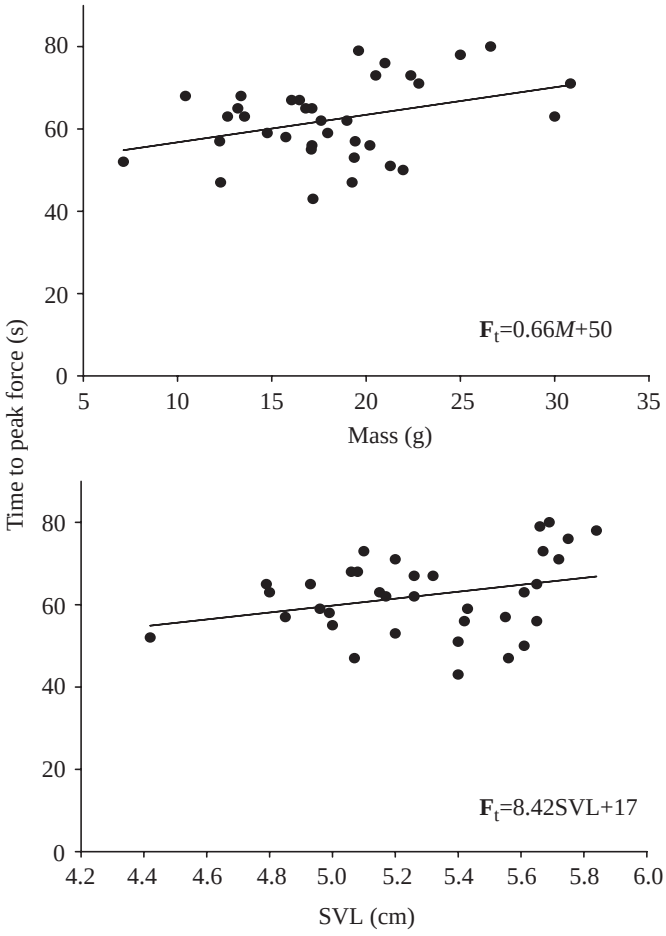

Fig. 3. Relationship between *C. alboguttata* body mass (A) and body length (B) and time to peak ground reaction force during a maximal jump recorded with a force platform sampling at 1000 Hz. SVL, snout–vent length.

(femoral, P=0.73; tibiofibular, P=0.59) and yield displacement (femoral, P=0.80; tibiofibular, P=0.96) were also compared, and again aestivation had no significant effect on these parameters (Table 1). Admittedly, the data do approach significance at the 0.05 level in some of the comparisons, and a higher sample size may have produced a significant difference. Having said this, previous studies in which differences following dormancy were found (e.g. Krook et al., 1977) used smaller sample sizes than the present study.

Bone size

Second moment of area was compared, and aestivation had no significant effect (femoral, P=0.53; tibiofibular, P=0.68). Neither femoral nor tibiofibular cross-sectional area (normalised to snout–vent length) changed following the disuse associated with either 3- or 9-months aestivation (femoral, P=0.108; tibiofibular, P=0.063; Table 1).

Histology

Qualitative examination of the histological parameters indicated the absence of disuse osteoporosis despite 9 months of limb immobilisation. For example, the bone margins of aestivators had no ruffle border, remaining relatively smooth and bearing no evidence of osteoclastic activity (Fig. 4). Osteocytes were distributed similarly through the cortex in both controls and aestivators and there was no intracortical remodelling in any bone. Finally, the osteocytes in the aestivators were not enlarged compared with those of the controls and did not possess either basophilia or metachromasia.

Of further note was the presence of arrest/growth lines running parallel to the endosteal perimeter in the middle of the cortex in some of the specimens. This feature was evident in

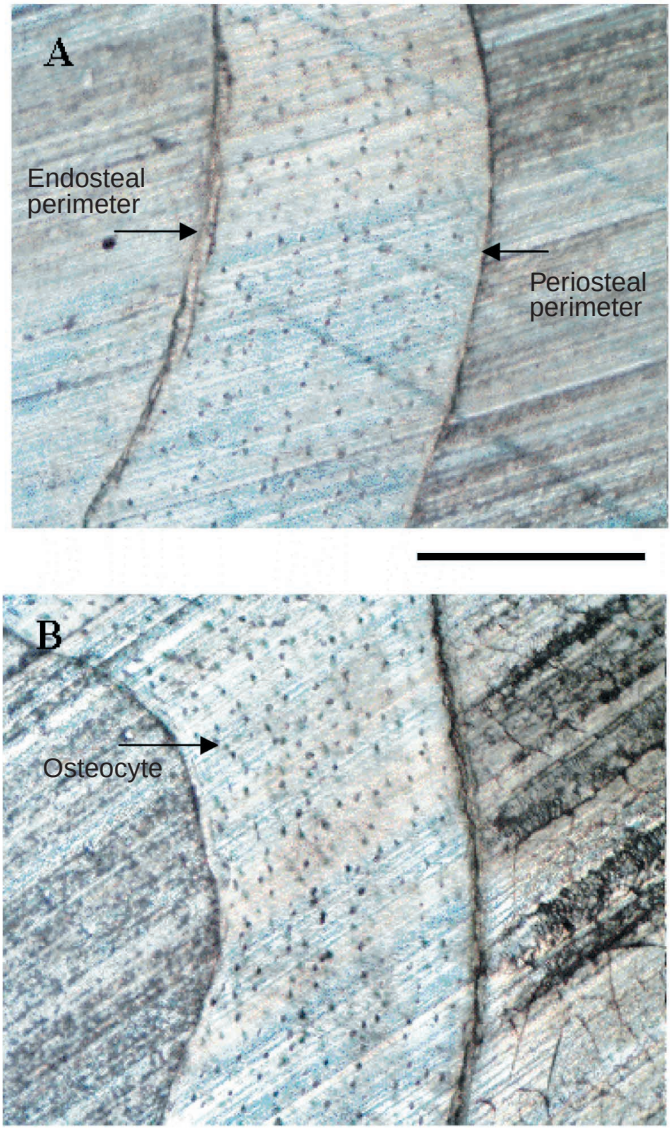

Fig. 4. Transverse mineralised sections of the tibiofibula in (A) control and (B) 9-month-aestivating *C. alboguttata*. Scale bar, 0.2 mm.

both the tibiofibula and femur of one of the aestivators and in the femur of one of the controls.

Discussion

The biomechanical properties of a structure depend on both the materials in question and the way the material is distributed. The chronic bone unloading associated with 9-months aestivation had no measurable effect on *C. alboguttata* long bone morphology and mechanical properties. These data support the hypothesis that bone mechanical competence and morphology are preserved during aestivation in this species. This contrasts with both clinical systems of unloading and, more intriguingly, with the other natural disuse systems examined to date (hibernating mammals) in which bone remodelling is evident. For example, bone loss has been described in hibernating bats (*Myotis lucifugus*; Whalen et al., 1971), ground squirrels (*Spermophilus undulatus*; Mayer and Bernick, 1959) and hamsters (*Cricetus cricetus*; Kayser and Frank, 1963). In bats, much of the bone loss has been attributed to enhanced osteolysis (Whalen et al., 1971). However, the distribution and morphology of osteocytes in aestivating frogs indicate no osteocytic osteolysis. Given that the cross-sectional area of the bones was constant and the overall bending properties of the frog bones were unchanged, it is likely that only negligible changes occurred in the material properties of the frog bone tissue. The occasional presence of arrest lines in *C. alboguttata* femora and tibiofibula may indicate that the long bones undergo cyclical phases of growth and arrest similar to those found in the dentine bone of hibernating microchiroptera such as *Nyctalus noctula* (Klevezal and Kleinenberg, 1969). If so, it is not clear whether the changes in bone histology are due to seasonal effects, aestivation or some internal physiological cycle (e.g. linked to reproduction).

The major findings of this study are consistent with the preservation of other components of the locomotor system in *C. alboguttata*, namely hind limb muscle masses (Hudson and Franklin, 2002a), capillary tortuosity (Hudson and Franklin 2003), *in vitro* contractile properties of the gastrocnemius and burst swimming performance (Hudson and Franklin, 2002a).

Mechanical properties

The present study presents a comprehensive analysis of bone mechanical properties in an amphibian. The ultimate strength of the femur and tibiofibula in *C. alboguttata* (328 MPa and 253 MPa in the controls) corresponds favourably with values calculated for small mammals. For example, Biewener (1982) found that the chipmunk (*Tamais striatus*) femur and tibia had ultimate strengths of 263 MPa and 303 MPa, respectively, while the rat (*Rattus norvegicus*) femur and tibia showed values of 253 MPa and 233 MPa. Equally, the bending strengths of whole bones in birds are also broadly comparable, although in these cases there seems to be a greater disparity between femoral and tibiofibular data. Analysis of three-point bending in the painted quail (*Excalfatoria chinensis*) gave values of 311 MPa and 170 MPa for the femur and tibia, respectively, while those of the bobwhite quail (*Colinus virginianus*) were 193 MPa and 294 MPa (Biewener, 1982).

In the present study, no unequivocal pattern emerges when comparing the strength of the femur with respect to the tibiofibula, which is consistent with some prior data on small mammals and birds (Biewener, 1982). However, greater femoral strength has previously been noted for alligators, iguana (Blob and Biewener, 1999) and horses (Currey, 1984). In these cases, one possible explanation was given in terms of the bone's position. The more distal the bone, the greater contribution it makes to the leg's moment of inertia and the more energy is required to move it (Currey, 1984). As such, in some cases the energy saved by the animal in having a lighter tibia (which is distal to the femur) might offset the 'riskier' safety factor that follows the reduction in strength. This general pattern may be evident in the elongated metatarsals of frogs

that are distal to the tibiofibula but were not examined in this study of *C. alboguttata*. Alternatively, the advantages of reducing distal limb segments may scale with mass and the lack of a pattern in these frogs may be a simple function of small body size.

Blob and Biewener (1999) have shown that magnitudes of peak ground reaction force acting on the limbs, relative to body weight, are lower in green iguanas (*Iguana iguana*) and American alligators (*Alligator mississippiensis*) than in mammals and birds. Force platform recordings indicate that peak ground reaction force magnitudes acting on a single limb range between 1.3× and 2.4× body weight for quadrupedal mammals but only 1.1× for iguanas and alligators. The peak ground reaction force during jumping for *C. alboguttata* is about 1.4× body weight (each limb supports half the 0.49 N force exerted during a jump; i.e. 0.49/2=0.245 N, the mass of the frog=0.018 kg or 0.17 N; thus, 0.245/0.17=1.4), falling well into the range for the ectotherms but very much at the lower end of the scale for the endotherms. Fundamentally, the ground reaction force data for *C. alboguttata* are in line with those found in the American alligator and green iguana, species that have high safety factors (Blob and Biewener, 2001). Moreover, a relatively high safety factor was shown experimentally by Calow and Alexander (1973) for a different species of anuran, the European common frog *Rana temporaria*. Accordingly, it seems probable that *C. alboguttata* have a high safety factor, although one cannot be accurately calculated from the present data without detailed strain gauge data or frog bone strains *in vivo*. The fact that bone strength could presumably be lost in these frogs without undue risk of fracture, but is not, points to a simple, inherent preservation mechanism.

Mechanism underpinning the preservation of bone size and strength

Bradymetabolic organisms are relatively quiescent, which means prior to immobilisation their bones have only a moderate loading history. Assuming that the rate of remodelling depends on the extent of unloading (*sensu* Jee and Ma, 1999), bone tissue of relatively inactive organisms (such as amphibians) should be intrinsically more resistant to disuse osteoporosis than more active, tachymetabolic organisms (such as mammals) because the change in stimulus is that much weaker. In a sense, it could be argued that burrowing frogs are predisposed to withstand lengthy periods of disuse without losing bone mass. We have previously invoked this argument to partly explain the preservation of skeletal muscle structure in aestivating *C. alboguttata* (Hudson and Franklin, 2002b). In light of this predisposition, the pertinence of *C. alboguttata* as a model system for biomedical studies of disuse osteoporosis is reduced. This logic points clearly to the ideal biomedical system: a tachymetabolic mammal that suffers no disuse osteoporosis during natural disuse.

Nevertheless, in mammals, a period of disuse produces a fairly rapid response involving the loss of bone and there may be compensatory cellular mechanisms in operation in aestivating frogs to prevent bone loss. In hibernating mammals, parafollicular cells in the thyroid gland are enlarged and active compared with those in non-hibernators (Whalen et al., 1971). Parafollicular cells secrete calcitonin, an inhibitor of bone resorption. In hibernating bats, the parafollicular cells show striking seasonal changes in structure. During the first half of hibernation, the amount of granular endoplasmic reticulum is reduced and they lose their solid dense core, suggesting that calcitonin may be functionally inactive during hibernation (Whalen et al., 1971). This is consistent with the bone resorption observed in these animals. Intriguingly, Krook et al. (1977) showed that injections of calcitonin could prevent this bone loss in hibernating bats and argued that this was reflected in smaller femoral osteocytes compared with those in untreated hibernators. Unfortunately, the endocrinology of aestivating frogs has been neglected and its study may provide us with a better understanding of the effects of aestivation.

Ecological implications

Aestivating *C. alboguttata* can emerge within 24 h of the first heavy summer rainfall (N.J.H., personal observation). Indeed, the emergence process itself is presumably demanding on the skeletal system as it involves digging through wet clay. The frogs immediately engage in predator avoidance strategies that require a fully competent locomotor system such as burst swims and powerful jumps. The latter transmits considerable forces through the long bones of the hindlimbs during the leg extension phase. Given that post-aestivation locomotor performance in swimming matches control levels (Hudson and Franklin, 2002a) and that frogs may actually gain mass during aestivation (N.J.H., unpublished data), the transmission of forces through the hindlimbs in frogs after dormancy is almost certainly equivalent to that in control frogs. The maintenance of bone mechanical properties throughout aestivation facilitates performance of these activities by ensuring that an adequate capacity for load bearing is maintained.

We thank Mark Forwood for advice and support in bone morphometry. C.E.F. wishes to acknowledge the ARC for a large research grant. N.J.H. would like to acknowledge the Northcote trust from which he received a generous postgraduate scholarship and Peter Kraft for technical advice.

References

Bagi, C. and Miller, S. (1994). Comparison of osteopenic changes in cancellous bone induced by ovariectomy and/or immobilisation in adult rats. *Anat. Rec.* **239**, 243-254.

Bartholomew, G. and Hudson J. (1960). Aestivation in the mohave ground squirrel (*Citellus mohavensis*). *Bull. Mus. Comp. Zool.* **124**, 193-208.

Biewener, A. A. (1982). Bone strength in small mammals and bipedal birds: do safety factors change with body size? *J. Exp. Biol.* **98**, 289-301.

Biewener, A. A. (1983). Bone stress in the horse forelimb during locomotion at different gaits: a comparison of two experimental methods. *J. Biomech.* **16**, 565-576.

Biewener, A. A. and Bertram, J. E. A. (1993). Mechanical loading and bone growth *in vivo*. In *Bone Growth B*, vol. 7 (ed. B. K. Hall), pp. 1-36. Nova Scotia, Canada: CRC Press.

Biewener, A. A. and Bertram, J. E. A. (1994). Structural response of growing bone to exercise and disuse. *J. Appl. Physiol.* **76**, 946-955.

Blob, R. W. and Biewener, A. A. (1999). *In vivo* locomotor strain in the hindlimb bones of *Alligator mississipiensis* and *Iguana iguana*: implications for the evolution of limb bone safety factor and non-sprawling limb posture. *J. Exp. Biol.* **202**, 1023-1046.

Blob, R. W. and Biewener. A. A. (2001). Mechanics of limb bone loading during terrestrial locomotion in the green iguana (*Iguana iguana*) and american alligator (*Alligator mississippiensis*). *J. Exp. Biol.* **204**, 1099-1122.

Calow, L. J. and Alexander, R. McN. (1973). A mechanical analysis of a hind leg of a frog (*Rana temporaria*). *J. Zool. Lond.* **171**, 293-321.

Carrier, D. and Leon, L. R. (1990). Skeletal growth and function in the California gull (*Larus californicus*). *J. Zool. Lond.* **222**, 375-389.

Currey, J. D. (1975). The effects of strain rate, reconstruction and mineral content of bovine bone. *J. Biomech.* **8**, 81-86.

Currey, J. D. (1984). *The Mechanical Adaptations of Bones.* Princeton, NJ: Princeton University Press.

Doty, S. and Nunez, E. (1985). Activation of osteoclasts and the repopulation of bone surfaces following hibernation in the bat, *Myotis lucifugus*. *Anat. Rec.* **213**, 481-495.

Flanigan, J., Withers, P., Fuery, C. and Guppy, M. (1993). Metabolic depression and Na^+/K^- gradients in the aestivating Australian goldfields frog *Neobatrachus wilsmorei*. *J. Comp. Physiol. B* **163**, 587-593.

Globus, R., Bikle, D. and Morey-Holton, E. (1984). Effects of simulated weightlessness on bone mineral metabolism. *Endocrinology* **114**, 2274-2280.

Hanssen, T., Roos, B. and Nachemson, A. (1975). Development of osteopenia in the fourth lumbar vertebra during prolonged bed rest after operation for scoliosis. *Acta Orthop. Scand.* **46**, 621-630.

Harlow, H., Lohuis, T., Beck, T. and Iaizzo, P. (2001). Muscle strength in overwintering bears. *Nature* **409**, 997.

Holick, M. (1998). Perspective on the impact of weightlessness on calcium and bone metabolism. *Bone* **22**, 105S-111S.

Hudson, N. J. and Franklin, C. E. (2002a). Effect of aestivation on muscle characteristics and locomotor performance in the green striped burrowing frog, *Cyclorana alboguttata*. *J. Comp. Physiol.* **172**, 177-182.

Hudson, N. J. and Franklin, C. E. (2002b). Maintaining muscle mass during extended disuse: aestivating frogs as a model species. *J. Exp. Biol.* **205**, 2297-2303.

Hudson, N. J. and Franklin, C. E. (2003). Preservation of 3-dimensional capillary structure in frog muscle during aestivation. *J. Anat.* **202**, 471-474.

Jee, W. and Ma, Y. (1999). Animal models of immobilisation osteopenia. *Morphologie* **83**, 25-34.

Katz, S. and Gosline, J. (1993). Ontogenetic scaling of jump performance in the African desert locust (*Schistocerca gregaria*). *J. Exp. Biol.* **177**, 81-111.

Kayser, C. (1961). *The Physiology of Natural Hibernation*. Oxford: Pergamon Press.

Kayser, C. and Frank, R. (1963). Comportement des tissues calcifies du hamster d'Europe *Cricetus cricetus* au cours du l'hibernation. *Arch. Oral Biol.* **8**, 703-713.

Klevezal, G. and Kleinenberg, S. (1969). Age determination of mammals from annular layers in teeth and bones. *Israel Prog. Sci. Trans. Jerusalem* **TT 69-55033**, 1-128.

Krook, L., Wimsatt, W., Whalen, J., MacIntyre, I. and Nunez, E. (1977). Calcitonin and hibernation bone loss in the bat, *Myotis lucifugus*. *Cornell Vet.* **67**, 265-271.

Liu, X. (2001). Effect of microgravity on Ca mineral crystallisation and implications for osteoporosis in space. *Appl. Phys. Lett.* **79**, 3539-3541.

Lyman, C., Willis, J., Malan A. and Wang, L. (1982). *Hibernation and Torpor in Mammals and Birds*. New York: Academic Press.

Marsh, R. (1994). Jumping ability of anuran amphibians. *Adv. Vet. Sci. Comp. Med.* **38B**, 51-111.

Mayer, W. and Bernick, S. (1959). Dental caries in hibernating arctic ground squirrels, *Spermophilus undulatus*. *Anat. Rec.* **134**, 606-607.

Pinder, A., Storey, K. and Ultsch, G. (1992). Estivation and hibernation. In *Environmental Physiology of the Amphibia* (ed. M. E. Feder and W. W. Burggren), pp. 250-258. Chicago: University of Chicago Press.

Turner, R. (2000). What do we know about the effects of spaceflight on bone. *J. Appl. Physiol.* **89**, 840-847.

Umemura, Y., Ishiko, T., Tsujimoto, H., Miura, H., Mokushi, N. and Suzuki, H. (1995). Effect of jump training on bone hypertrophy in young and old rats. *Int. J. Sports Med.* **16**, 364-367.

Vose, G. and Kubala, A. (1959). Bone strength-its relationship to X-ray determined ash content. *Hum. Biol.* **31**, 262-270.

Whalen, J., Krook, L. and Nunez, E. (1971). A radiographic and histologic study of bone in the active and hibernating bat, *Myotis lucifugus*. *Anat. Rec.* **172**, 97-108.

Whedon, G. and Heaney, R. (1993). Effects of physical inactivity, paralysis and weightlessness on bone growth. In *Bone Growth B*, vol. 7 (ed. B. K. Hall), pp. 57-77. Nova Scotia, Canada: CRC Press.

Wilson, R. and Franklin, C. (2000). Allometric scaling relationships of jumping performance in the striped marsh frog *Limnodynastes peronii*. *J. Exp. Biol.* **203**, 1937-1946.

Withers, P. (1993). Metabolic depression during aestivation in the Australian frogs, *Neobatrachus* and *Cyclorana*. *Aust. J. Zool.* **41**, 467-473.