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Toe-bud clipping of juvenile small marsupials for ecological field research: No detectable negative effects on growth or survival

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Abstract Toe clipping is widely used to permanently mark many species of small vertebrates including marsupials, particularly didelphids and dasyurids. Small marsupials are marked as juveniles, by removing the tip of developing toe buds. It has recently been shown that survival and/or recapture probability decreases with increasing number of toes clipped in frogs. Because of this and other animal welfare concerns, toe clipping of adult vertebrates is increasingly being discouraged. The short- and long-term effects of toe-bud clipping have not been evaluated in marsupials. We used an experiment to test if marking more toes results in slower growth or higher mortality in the brown antechinus (*Antechinus stuartii*, Dasyuridae) in the short or long term. We found no harmful effects of toe-bud clipping. There were no infections associated with clipping, marking more toes did not reduce growth in young or adults, and did not affect survival of young in captivity, survival of independent animals in the wild, or recapture probability. Toe-bud clipping is done at an extremely immature stage, when the area cut is tiny and perception and memory of pain is unlikely to be a problem. We suggest that toe-bud clipping is a humane and benign method of permanently marking antechinuses, and probably also the young of other morphologically similar small marsupials.

Key words: *Antechinus*, growth, mark–recapture, marking methods, survival, toe clipping.

INTRODUCTION

Ecological field studies often require unique, permanent marking of dependent juveniles. Many species of lizards, frogs and small mammals have young that are too small to be microchipped or to have anything attached to extremities. These can be marked with either a mutilation method (e.g. toe clipping or ear notching) or injection of pigment, such as a tattoo (Soderquist & Dickman 1988; Lindner & Fuelling 2002). Not only are marsupial young tiny (e.g. antechinuses are around 4 mm long at birth), but they are also born at a near-embryonic stage of development (Lee & Cockburn 1985). Tattooing or otherwise marking the extremely thin and delicate body of pouch young is likely to result in damage, which might cause infection or affect their ability to obtain oxygen; an important function of the moist skin in some marsupial species (Mortola *et al.* 1999). Another problem with marking the skin is that antechinus mothers constantly clean young attached to their teats, and can seriously harm the young by trying to remove the mark (A. Cockburn, pers. comm., 2004). Ear tattooing or ear nicks can be used to mark young of a few species that have large enough ears, such as pygmy possums and bandicoots (Soderquist & Dickman 1988; Pestell & Petit 2007). However, toe-bud clipping is considered to be the only way to safely mark dependent offspring of many small marsupials such as dasyurids (e.g. antechinuses; Fisher *et al.* 2006) and didelphids (e.g. mouse opossums; Gentile *et al.* 2004).

Toe clipping of adult vertebrates has been criticized for two reasons: (i) wounds might become infected (McCarthy & Parris 2004), or normal locomotion disrupted (Bloch & Irschick 2005), increasing mortality and decreasing growth and breeding success, and (ii) toe removal is assumed to cause pain and stress that is unacceptable to some authors (Henshaw 1981; National Health & Medical Research Council Animal Welfare Committee 2007). Studies on frogs support the first of these criticisms. Inflammation of toe-clipping wounds has been noted in several studies of frogs. A recent cross-species meta-analysis has shown that toe clipping reduces recaptures, and apparent mortality increases in proportion to the number of toes removed (Parris & McCarthy 2001; McCarthy & Parris 2004; May 2004).

There are no published studies on the effect of toe clipping on marsupials, but investigations of small

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reptiles and rodents during the last 35 years have failed to show any detrimental effects on return rate, survival or growth in adults or young (Ambrose 1972; Fairley 1982; Pavone & Boonstra 1985; Korn 1987; Wood & Slade 1990; Jennings *et al.* 1991; Braude & Ciszek 1998; Paulissen & Meyer 2000). Studies of small mammals have typically had large sample sizes (e.g. Pavone & Boonstra 1985 used 280 voles to compare two experimental groups of equivalent age, sex and size; Korn 1987 assessed timing of returns in 435 toe-clipped voles and mice; Wood and Slade 1990 used 650 voles to compare recapture rate and growth between two experimental groups). The only negative finding in small mammals has been that recapture rate is temporarily reduced 1–2 days after clipping in adult wood mice and prairie voles (Fairley 1982; Wood & Slade 1990), but this result has been explained as an artefact of non-resident captures on the trapping grid (Korn 1987). There have been no studies investigating how growth and survival might change according to the number of toes marked in small vertebrates other than frogs.

Pain and stress are difficult to assess in wild animals. Large vertebrates such as coyotes show obvious pain and distress when they lose a toe (Henshaw 1981), but small animals are unlikely to draw attention to themselves when injured or distressed (Langkilde & Shine 2006). One way to reliably quantify physiological stress in small animals is to measure blood glucocorticoid levels. To our knowledge, the effect of toe clipping has not been measured using glucocorticoids in mammals. However, in adult water skinks (small lizards, *Eulamprus* spp.), recent experiments have shown that toe clipping does not increase levels of these stress hormones, and running speed and behaviour are normal immediately afterwards (Borges-Landaez & Shine 2003; Langkilde & Shine 2006). This was not because the experimental design was inadequate to detect hormonal effects of marking; microchipping did cause physiological stress in water skinks (Langkilde & Shine 2006). Kinkead *et al.* (2006) found no difference between stress hormone levels or behaviour associated with toe clipping and other marking methods in adult salamanders, either with or without anaesthesia.

Brown antechinuses (*Antechinus stuartii*) are small (~25 g) insectivorous marsupials that are common in south eastern Australian forests. They forage both on the ground and in trees, and frequently climb tree trunks, as they nest inside hollows (Lazenby-Cohen 1991). If loss of toes or toenails affects climbing efficiency, foraging success or predator evasion could be hindered by toe clipping, reducing survival and growth. Antechinuses are popular model organisms in evolutionary ecology, and recent work shows that fast growth is crucial for male reproductive success (Fisher *et al.* 2006; Holleley *et al.* 2006). Body mass is strongly correlated with male reproductive success because it affects survival during the mating season, sperm competition success, and dominance, which affects access to females and female mate choice (Fisher & Cockburn 2006; Holleley *et al.* 2006). Therefore, if toe clipping reduced growth, we would predict additional negative effects on males due to impaired reproduction.

Young brown antechinuses are born as early stage embryos (mass ~0.016 g) synchronously in late August or early September. They fuse obligatorily to a teat in the pouch for 35–40 days before detaching (Fig. 1a). A full pouch consists of eight or nine neonates, one on each teat. Young are weaned after ~90 days (Fisher 2005). Brown antechinuses reach maturity in June/July. All males and most females mature at around 10.5 months old and only live for one breeding season; males live for 11.5 months and females usually for

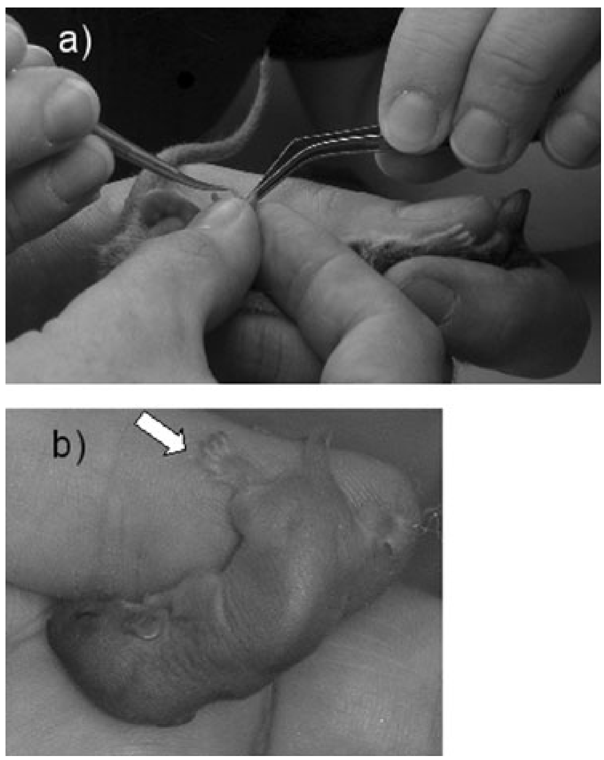

Fig. 1. (a) Toe-bud clipping of a brown antechinus pouch young. This procedure requires two people. The mother is held on her back by one person, who also lifts the hindquarters of the young to be marked. The second person holds the foot gently with blunt forceps, lifts the end of the toe using the tip of the eye surgery scissors, and removes it, while observing through a magnifier from above. (b) A brown antechinus nestling at 50 days old showing a permanent mark on right second toe (arrow, clip right back two). This toe is around two-thirds the length of the other toes, and has no nail. Toe one (the thumb) naturally lacks a nail, and is never clipped.

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16–20 months (Lee & Cockburn 1985). The brown antechinus and its similar close relative, the agile antechinus (*Antechinus agilis*) are two of the most frequently studied mammals in Australia. Many field studies have relied on toe-bud clipping of juveniles, but the effect of marking young on subsequent growth and survival is not known. The aim of this study was to test if the number of toes clipped in dependent young (46 days old) affects growth or survival between the end of pouch life and maturity.

METHODS

Trapping and maintenance

We trapped 48 adult female and 24 male antechinuses in coastal forest at Kioloa, southern NSW (35°32′S, 150°22′E) in late July and early August 2004, shortly prior to the predictable onset of the mating season (see Fisher 2005) for details of the study site and vegetation). Around 40% of these adults had been bred in captivity in 2003, and released as dependent young as part of an earlier experiment.

The antechinuses were captured in Elliott traps, waterproofed with plastic bags and placed in lines, 10 m apart, with lines set 25 m apart. Trap locations were permanently marked and numbered. Traps contained Dacron fibre for bedding, and were baited with peanut butter and oats. We set traps shortly before dusk, and checked them at dawn. Each captured animal was microchipped (Trovan, ID-100 transponder, 11 mm × 2.2 mm). Animals were transferred to Canberra and maintained in captivity in 30-l plastic containers (45 cm × 35 cm, 20 cm high, clear polyurethane) with wire mesh lids and a wooden nest box, and wood shavings and shredded paper as bedding. Mouse running wheels were provided for exercise. Their diet consisted of minced meat and dog chow with infant vitamins (Pentavite liquid) and mineral (calcium carbonate powder), supplemented by mealworms, crickets and thawed, frozen mouse pups. Water was always available via an inverted drip bottle (see Fisher *et al.* 2006) for full details of animal husbandry). We mated the antechinuses in captivity (Fisher *et al.* 2006). Pregnant females were maintained individually. All females were given the same food mixture, divided equally so that each animal had some food left over after feeding on most days. Females were checked once a day for young in the pouch, beginning 27 days (the minimum gestation period) after the date of first mating. Thirty-eight females gave birth to a total of 243 young. Seven young died before marking because their mother died, so 236 were marked.

Offspring marking and measurements

We compared growth and survival of young marked with one, two or three clips. We could not include an unclipped control group, because there is no alternative permanent marking method for juvenile antechinuses for comparison. Measurements of pouch young began when young were 3 days old. In order to see if there was a reduction in growth rate associated with marking, we used calipers to measure the crown–rump length of each young in the pouch on every third day at around 16.00 hours, until the young were 35 days old. Measurements were to the nearest 0.1 mm. We found this to be the most appropriate level of precision, as young were only around 6 mm long initially. Small pouch young did not move or flex, and were exposed by holding the female on her back, so that the pouch was flat (Fig. 1a). Mean crown–rump length was calculated for each litter at each sampling time. Young brown antechinuses detach from the teat at around 6 weeks old. We allowed them to make the transition between pouch and nest without disturbance, and did not measure young between 36 and 46 days old.

At 46 days old (at a head-body length of 11–14 mm, when they were still blind and naked), we sexed the young, and removed the tip of one to three toe buds using small eye surgery scissors (~3-mm blade) under a magnifier (Fig. 1a). Removal of the tiny (<1 mm) toe-bud tips was quick, and caused very little or no bleeding. We did not clip toe one (the thumb) on front or back feet, because this toe has no nail, and it is assumed to be important for climbing. Our marking scheme avoided marking three toes from the same foot, and used the sex of the young as part of the code, so that each mark could be used twice (i.e. 32 individuals were marked on one toe). Toe-bud clipping of late pouch young/early nestlings results in the toe developing normally, but without a nail, and usually with the marked toe shorter than the others (Fig. 1b).

As each litter reached the age of 46 days, marks were assigned using a printed sheet. Each diagram on a sheet had a different code (e.g. left front 5, right back 5), and the toes to be clipped were shaded. We marked young in the order of diagrams on the sheet, which began with the simplest combinations of each toe, so that marks including three toes were not allocated until 90 offspring of each sex had been marked. As each offspring was marked, we wrote the mother's identity on the relevant diagram. There was a separate copy of the sheet for males and females. This scheme resulted in 22 of the 38 litters being marked on varying numbers of toes (six litters only contained young with two toes clipped, and 10 only contained young with three toes clipped). Young were typically suckling in the nest box at 16.00 hours, so we first detached the mother, and put her and her litter in separate cloth bags. We chose the order of young to mark and

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measure by putting a hand in the bag and picking up one at a time haphazardly (not by sight). Because marks were allocated in the order on the sheet, the appearance of young was unrelated to the mark. We placed newly marked young in the bag with the mother, before returning the family to their container.

We continued to measure crown–rump length so that we could plot growth from 3 to 66 days old using the same litters. Body mass is a more precise measurement of growth in older young (because they can flex), but can only be obtained once young detach from the teat. We weighed each nestling to the nearest 0.01 g, by placing it directly on a digital balance. All nestlings were measured and weighed every third day at around 16.00 hours, until they were 66 days old. The same person carried out all measurements.

Offspring survival was monitored daily from birth until 85 days of age, whereupon families were returned to the wild. We used a soft-release method in which the same wooden nest boxes used in captivity (22 cm^3 with 3 cm diameter entrance holes and removable lids) were attached to a tree at head height, using nylon packing tape. Boxes containing the mothers and young were placed where the mother had been originally captured.

Mark–recapture experiment

Survival monitoring of independent animals in the field began when the young were 105 days old, and available to be trapped outside the nest (Fisher 2005). We comprehensively trapped the site every fourth week from December 2004 to June 2005. Marked animals were identified and released where they were caught, and unmarked animals were microchipped and released. We compared growth and survival in the wild of young born in 2004 and marked with one, two or three toe-clips.

Statistics

We analysed growth and survival of young before weaning in R 2.6.0 (R Development Core Team 2007). To test for an effect of the number of toes clipped on offspring growth, we transformed the data so that they better approximated a normal distribution, then fitted a linear mixed model using REML to estimate parameters using the nlme package (Pinheiro & Bates 2000). Log(10) body mass of nestlings was the response variable, clip number (one, two or three toes) and age were fixed factors, and litter identity was treated as a random factor. We fitted models with and without a random effect for individual, in order to test for individual variance within litters. Because some young had incomplete data, $n = 229$ for growth comparisons. We used a mixed effects Cox Proportional Hazards model in R to test for differences in pre-weaning survival between offspring marked with one, two or three toe clips, with litter identity (mother) again treated as a random factor. We analysed survival rates of weaned antechinuses (independent animals in the wild) using capture–mark–recapture data in MARK (White & Burnham 1997). There were 16 capture periods over two consecutive years. To model survival rates in the wild, we only included animals that were trapped at least once ($n = 107$). We did not include young that were bred in captivity and died before trapping began, or young only ever encountered in nest boxes. Finally, we compared the proportion of individuals clipped on one, two and three toes recaptured after their first release into the field, using a three-sample χ^2 test for equality of proportions in R.

RESULTS

Number of toes marked versus growth

Growth in crown–rump length was approximately linear in both pouch young and nestlings, and much faster in nestlings (Fig. 2). There was an inflection point at around 40 days when growth rate increased strongly, but no obvious reduction in growth rate immediately following marking at 46 days (Fig. 2). Growth in (logged) mass (+1) was also approximately linear (Fig. 3). The among individual (within litter) variance in growth rate was essentially zero. According to both the more complex model with litter and

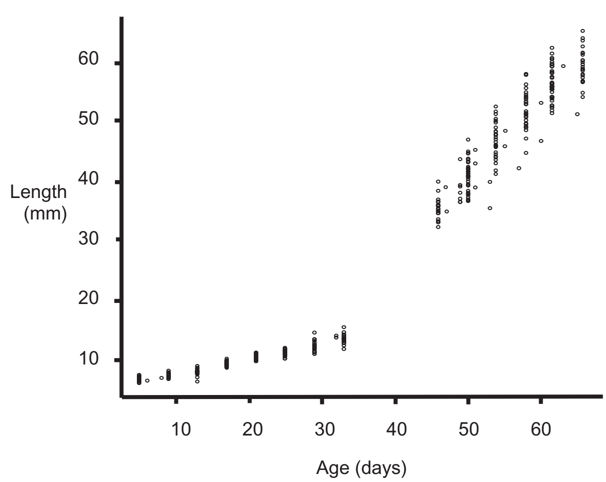

Fig. 2. Growth of newborn brown antechinuses in crown–rump length. Each data point is the mean length of all young in a litter for each mother ($n = 38$ mothers, each mother had 2–9 young). Pouch young detached from the teat and commenced the nestling stage between 35 and 46 days of age. We did not measure them during this interval.

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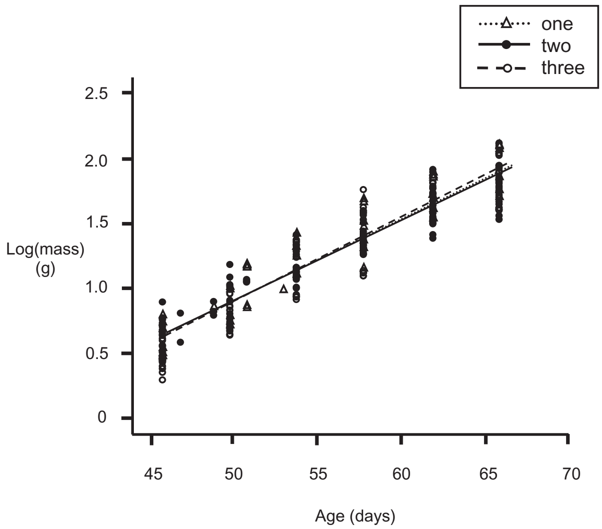

Fig. 3. Growth in body mass of nestling brown antechinuses between 46 and 66 days of age, for young with one, two or three toe tips removed. Data points are shown for individual young ($n = 229$). Lines are linear regressions, fitted separately for each group.

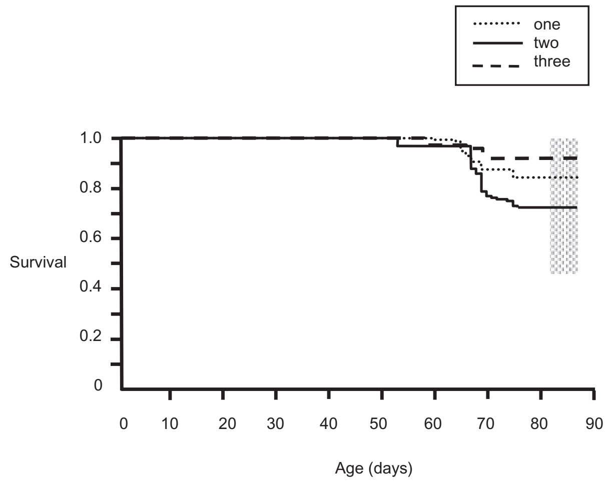

Fig. 4. Survival of newborn brown antechinuses until 85 days of age, for young with one, two or three toe tips removed. Lines are survival curves fitted using a Cox proportional hazard model, comparing the three groups. There is no significant difference among the three curves: the hatched area shows the 95% confidence interval for mean survival at 85 days.

individual random effects (not presented), and the reduced model without individual effects, there was no difference in growth rate between young with one versus two toe-bud clips, but young with two clips grew at a significantly slower rate than young with three clips ($t_{221} = -3.3$, $P = 0.001$). The mean final mass (at 66 days) of nestling young with three toes clipped was 6.3 ± 0.18 g, 8% greater than the mean final mass of young with two toes clipped (5.8 ± 0.08 g), and 3% greater than the mean final mass of young with one toe clipped (6.1 ± 0.21 g). There was no difference in body mass of mature females in the wild that had been marked with one (17.3 ± 0.75 g), two (15.0 ± 1.2 g) or three (15.8 ± 0.28 g) clips (ANOVA of logged data, $F_{1,14} = 0.91$, $P = 0.36$). Only two males marked as nestlings were caught in June 2005; they both had three toes clipped.

Number of toes marked versus survival

Except for the death of one mother and her pouch young (data not included in the analysis), there were no deaths of pouch young, and very few nestlings died until 60–75 days of age, more than 2 weeks after marking (Fig. 4). All clips appeared to be completely healed on day 50 (the next measurement after clipping on day 46), and there were no infections of clipping wounds. According to our model, survival and capture probability did not differ among antechinuses marked with one, two or three clips during the nestling stage (Fig. 4, $z = 0.02$, $P = 0.98$). After they were released into the wild, we found no evidence of any difference

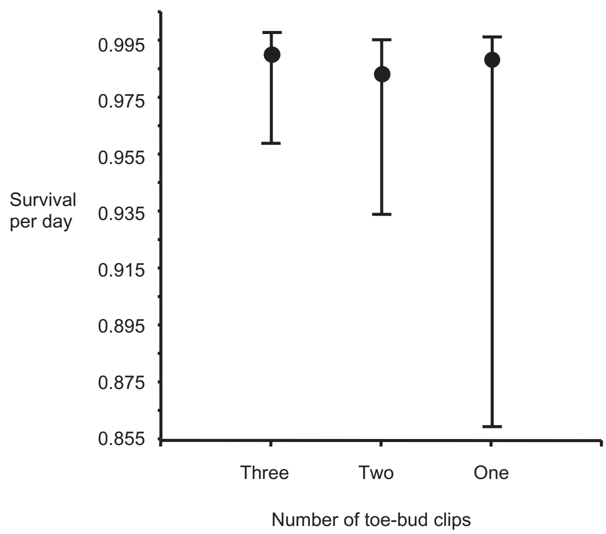

Fig. 5. Daily survival of independent antechinuses in the wild, for animals with one, two or three toe tips removed. Capture–mark–recapture modelling revealed no difference in survival among the experimental treatments. Lines show 95% confidence interval for mean survival per day.

in survival among animals with different numbers of toes marked (Fig. 5). Eighty-three per cent of female antechinuses marked with one clip were recaptured at least once after initial release into the wild, compared with 80% of those having two clips and 80% of those having three clips. The return rate in males (an underestimate of survival due to male-biased dispersal) was respectively 42%, 43% and 33%). These proportions were not significantly different for females ($\chi^2_2 = 0.07$,

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$P = 0.97$) or for males ($\chi^2_2 = 0.27$, $P = 0.87$). A likelihood ratio test showed that a model with the lowest AIC value (survival difference between groups and no time dependence in capture probabilities) was no better in explaining the trapping data than a model with the second lowest AIC (no survival difference between groups and no time dependence in capture probabilities) ($\chi^2_2 = 0.482$, $P = 0.7859$).

DISCUSSION

Growth did not decrease with increasing numbers of toes clipped in our experiments. There was no apparent decrease in the body length growth trajectory in the days following marking of juvenile antechinuses, regardless of the number of toes clipped. The 9-week time scale of this experiment would have been appropriate to detect any growth retardation, as nestlings were growing more than 1 mm in body length each day on average during this stage.

We found no evidence that weight gain in nestling antechinuses decreased over time with increasing numbers of toes clipped, in fact nestlings marked on three toes grew at a significantly faster rate than those marked on two toes. Our finding that there was no apparent negative effect of toe clipping on growth is consistent with studies comparing toe clipping with other methods of marking small mammals in the wild. Korn (1987) found that toe clipping did not cause weight loss, but stress from trapping did cause easily detectable weight loss in small rodents on a time scale of days and weeks. Wood and Slade (1990) compared toe clipping with ear tagging in a large sample of voles including juveniles, and also found no effect on growth over 2 years. Experimental studies of juvenile reptiles and amphibian metamorphs have also found no effect of toe clipping on growth rate (Jennings *et al.* 1991; Ott & Scott 1999).

The statistically significant lower growth rate in nestlings marked on two toes in our study is puzzling, because all mothers had access to the same amount and type of food, housing and space. Young in each litter were chosen haphazardly during marking, so it is unlikely that young receiving two toe-bud clips were smaller or thinner than those receiving one or three clips. A partial explanation is likely to be that the number of toe buds clipped was correlated with the timing of conception, which can affect offspring growth rate and survival probability in antechinuses (Selwood 1983; Fisher *et al.* 2005). A long time between mating and ovulation is associated with fast growth, high survival, and probably higher genetic quality, because embryos conceived at non-optimal times in the cycle are more likely to be defective (Selwood 1983). We marked all offspring when they reached 46 days of age, so the last to be marked were born later, therefore they had longer mating-conception intervals. Because we started with the simplest combinations in order to minimize the number of young marked on three toes, young marked later were more likely to have three toe buds clipped (one clip: 33.0 ± 0.17 days, two clips: 32.7 ± 0.08, three clips: 37.1 ± 0.13). Including maternal identity in the statistical analysis did not remove the confounding effect of timing, because 10 litters consisted entirely of young with three toes clipped. The offspring quality explanation is supported by the slightly, non-significantly, but consistently lower survival of offspring with two toe clips compared with offspring with three toe clips. However, conception timing does not explain why young marked on one toe also grew faster than young marked on two toes (although not as fast as those with three toes clipped). Whatever the cause, our large sample size allowed us to detect a difference of very small magnitude (Fig. 3), so it may not be biologically significant.

There was no difference in adult female body weight associated with different numbers of toes marked. However, we caught only 16 adult females from the experiment at the end of the field study in June 2005, so our ability to detect differences in adult body mass between treatments is limited.

There was no evidence from our models that losing more toes reduced survival of brown antechinuses in the short or long term. We are certain that toe-bud clipping of juveniles caused no lethal infections. None of the 236 young offspring showed signs of infection or inflammation and none died until a week later (when two offspring died for unknown reasons). In some species of rodents, toe-clipping juveniles can apparently cause mothers to neglect offspring (Leclercq & Rozenfeld 2001). If toe-bud clipping had caused maternal rejection or reduced the amount of milk received by juvenile antechinuses, offspring would have lost weight after marking. This did not happen. Offspring deaths at around 70 days old were linked to genetic quality of the parents and apparently not disease or malnutrition (Fisher *et al.* 2006).

The American Society of Mammalogists recommends that toe clipping should only be used to mark small species and juveniles, it should not be applied to specialized or fleshy toes, to the forefeet of species that dig, or to primary digits of mammals that climb (Gannon *et al.* 2007). The implication is that toe loss might hinder normal movement in these types of mammals. Experiments have shown that toe clipping does not increase the probability that owls will catch ground-dwelling voles (Ambrose 1972). Several authors have hypothesized that any toe loss might affect small arboreal animals more than terrestrial ones (Paulissen & Meyer 2000; McCarthy & Parris 2004; Bloch & Irschick 2005), but agility is not necessarily affected by toe clipping in climbing lizards (Paulissen

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& Meyer 2000). We did not clip primary digits in our study. If the lack of other toe-tips or nails hindered agility, hunting, or climbing ability of independent antechinuses in the wild, we predicted a negative correlation between the number of toes marked and survival of independent animals. There was no statistically significant difference in survival of independent antechinuses among experimental groups. Our results agree with previous field research that has found no apparent effect of toe clipping on survival in the field in other small mammals such as voles, mice and naked mole-rats (Pavone & Boonstra 1985; Wood & Slade 1990; Braude & Ciszek 1998). In prairie voles, toe clipping reduced mobility and therefore lowered capture probability in the long term, even though survival was not affected (Wood & Slade 1990). Antechinuses with more toes marked did not have lower capture probabilities in our experiments.

We could not assess physiological stress and pain in this study. There is no way to take blood to measure stress hormones in such tiny creatures without harm. However, we suggest that pain is not problematic. The toe-bud clipping procedure is very brief, the area of the body cut is so small that it is hardly visible without magnification, and the young are extremely altricial at the time of marking with important features undeveloped; their eyes and ears are closed, they have no fur, movements are uncoordinated and they cannot yet walk or vocalize. Recent guidelines published by the Australian National Health & Medical Research Council Animal Welfare Committee allow toe clipping without anaesthesia for hairless neonatal mice up to 2 weeks old (National Health & Medical Research Council Animal Welfare Committee 2007). This is acceptable to animal ethics committees because in the words of the National Health & Medical Research Council, 'developmental elements required for pain perception are relatively undeveloped' at this stage. Presumably, memory and perception of pain in the near-embryonic offspring of small marsupial pouch young is even less than in neonatal mice.

We conclude that toe-bud clipping has little if any effect on survival and growth of nestling antechinuses when up to three toe buds are removed. It is also unlikely to be harmful to the young of other altricial small mammals with comparably small, unspecialized toes. We cannot extrapolate our results to marking more than three toes, but our marking scheme is more than adequate for most field studies, in which the number of young marked is likely to be limited by the time it takes to process breeding females in traps, and adults can be microchipped.

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