JOURNAL OF Evolutionary Biology

doi:10.1111/j.1420-9101.2010.02083.x

Fossils and phylogeny uncover the evolutionary history of a unique antipredator behaviour

B. CLUCAS*†, T. J. ORD‡§¶ & D. H. OWINGS**

*College of The Environment, University of Washington, Seattle, WA, USA
†Institute of Geography, Humboldt-University, Berlin, Germany
‡Museum of Comparative Zoology and Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA, USA
§Department of Evolution and Ecology, University of California at Davis, Davis, CA, USA
¶Evolution and Ecology Research Centre, School of Biological, Earth and Environmental Sciences, University of New South Wales, Kensington, NSW, Australia
**Department of Psychology, University of California at Davis, CA, USA

Keywords:

ancestral states;
comparative phylogenetics;
correlated trait evolution;
Crotalus;
fossils;
Mustela;
Predator–prey;
Spermophilus;
Tamias.

Abstract

Recently, two squirrel species (*Spermophilus* spp.) were discovered to anoint their bodies with rattlesnake scent as a means of concealing their odour from these chemosensory predators. In this study, we tested multiple species with predator scents (rattlesnake and weasel) to determine the prevalence of scent application across the squirrel phylogeny. We reconstructed the evolutionary history of the behaviour using a phylogenetic analysis and fossil records of historic predator co-occurrence. Squirrels with historical and current rattlesnake co-occurrence all applied rattlesnake scent, whereas no relationship existed between weasel scent application and either weasel or rattlesnake co-occurrence. This was surprising because experimental tests confirmed rattlesnake and weasel scent were both effective at masking prey odour from hunting rattlesnakes (the primary predator of squirrels). Ancestral reconstructions and fossil data suggest predator scent application in squirrels is ancient in origin, arising before co-occurrences with rattlesnakes or weasels in response to some other, now extinct, chemosensory predator.

Introduction

Predation is a critical selection source faced by many animals, and prey species have often evolved specific strategies to avoid (e.g. crypsis), deter (e.g. aposematism) or escape (e.g. speed) predation. Most studies have tended to focus on the function of antipredator strategies as they exist in single species today. However, understanding how predatory avoidance mechanisms initially evolved requires both information on the historical interactions between predators and prey over evolutionary time, and experimental confirmation of the putative adaptive significance of strategies used by prey to reduce predation in contemporary settings (e.g. Berger *et al.*, 2001; see also Carbone *et al.*, 2009). Here, we present a comprehensive investigation into the evolutionary origins of an extraordinary antipredator behaviour adopted by ground squirrels presumably in response to rattlesnake predation. We use modern phylogenetic methods to integrate a comparative field study on prey behaviour with the unique perspective provided by fossil information, and finish with experimentation on predator behaviour in the laboratory.

Ground squirrels (*Spermophilus* spp.) have become a textbook example of how predator–prey dynamics can drive the evolution of elaborate prey behaviour (e.g. Alcock, 1997). These rodents are faced with myriad predators, and rattlesnakes (*Crotalus* spp.) pose an acute threat to several species (Owings & Coss, 1977). So much so that the closely related California ground squirrels (*Spermophilus beecheyi*) and rock squirrels (*Spermophilus variegatus*) have evolved an impressive arsenal of defences against rattlesnakes that includes venom resistance (Poran *et al.*, 1987), mobbing (Owings & Coss, 1977) and visual and infrared predator-deterrent

Correspondence: Barbara Clucas, College of The Environment, University of Washington, PO Box 352100, Seattle, WA 98195, USA.
Tel.: 001 206 459 0594; fax: 001 206 685 0790;
e-mail: baclucas@u.washington.edu

signalling (Rundus *et al.*, 2007). Our knowledge about this arsenal has grown recently with the discovery that these two squirrel species use this predator's scent for their own protection by chewing on rattlesnake-scented substances (e.g. shed skins) and applying it to their bodies by licking their fur (Clucas *et al.*, 2008a,b). This olfactory camouflage represents a remarkable example of opportunistic exploitation in nature, as squirrels turn the scent of danger into a protective disguise. It is not known whether this behaviour evolved specifically because of the unique predator–prey relationship between rattlesnakes and these two ground squirrel species or whether it evolved in a more distant common ancestor in some other context. For example, there is evidence that predator scent application behaviour is expressed in other rodents: Siberian chipmunks (*Eutamias sibiricus*) and Southern grasshopper mice (*Onychomys torridus*) seem to apply snake scent in a manner very similar to ground squirrels (Kobayashi & Watanabe, 1986; M. Rowe 2000, unpublished data; respectively) and the Chinese rice-field rat (*Rattus rattoides*) has been reported to apply the anal gland secretions of predator weasels (*Mustela sibirica*; Xu *et al.*, 1995; see Clucas *et al.*, 2008a for a review of prevalence and functions of scent application). If these behaviours are homologous among such distantly related species, the origin of predator scent application is potentially very ancient and not necessarily specific to snake predation *per se*, but chemosensory predators more generally.

To address these issues on the origin(s) of predator scent application in ground squirrels, we conducted a field study across multiple squirrel species testing their propensity to apply rattlesnake scent and weasel (*Mustela* spp.) scent, relative to a control scent (deer musk, *Odocoileus* spp., an equally potent scent from a nonpredator mammal that is sympatric with most ground squirrel species in North America). Using a molecular phylogeny, squirrels were selected from each monophyletic North American clade (Harrison *et al.*, 2003; Herron *et al.*, 2004) and encompassed species with varying predator regimes. Several chipmunk species were also included as outgroups. We used a correlated trait evolution analysis (Pagel, 1994; Pagel & Meade, 2006) to assess whether application of a particular predator's scent depended on the current presence of that predator. Using the fossil record we then established the historical co-occurrences of squirrels with rattlesnakes and weasels to determine the extent to which historical sympatry with predators affected present-day behaviour. Finally, we compared our fossil data of predator–prey co-occurrences with ancestor state reconstructions of rattlesnake and weasel scent application to establish the antiquity of scent application behaviour and its likely original selection source (e.g. rattlesnake, weasel or both).

We considered three hypotheses regarding the expected distribution of scent application across extant squirrel species based on alternative scenarios of how the behaviour might have evolved. (i) The Ancestral/Retained Trait Hypothesis predicts that all squirrel species should exhibit scent application because the behaviour was favoured by selection in a shared, ancient ancestor and has persisted to this day even in situations of relaxed selection (i.e. in species that currently do not overlap with snake or weasel predators). (ii) The Ancestral/Lost Trait Hypothesis predicts that only those squirrel species currently under selection from predation should exhibit scent application because the behaviour is ancestral, but ceases to be expressed after a period of relaxed selection. (iii) The Recently Evolved Trait Hypothesis predicts that scent application is a derived behaviour in species that currently co-occur with rattlesnakes or weasels and has evolved independently across distantly related squirrel species.

The aforementioned hypotheses treat rattlesnake and weasel scent application as discrete behaviours and therefore independent sources of selection. However, it is possible (and perhaps even likely) that application of scent is a general behaviour performed with any predator scent to obtain olfactory camouflage. If so, it follows that squirrels might apply one predator scent to disguise their odour from a variety of chemosensory predators. We investigate this idea in two ways. First, we tested whether scent application (with either rattlesnake, weasel or both) correlates with the presence of rattlesnakes or weasels using a correlated trait evolution analysis. We then experimentally tested the foraging behaviour of captive rattlesnakes with both rattlesnake-scented prey and weasel-scented prey to examine the adaptive significance of anointing with different predator scents. We know that rattlesnake foraging behaviour is reduced when rattlesnake scent is mixed with squirrel scent (Clucas *et al.*, 2008b). However, if weasel scent also conceals prey odour, it confirms that squirrels can obtain olfactory camouflage from one predator through the application of other predator scents.

Materials and methods

Comparative field study, the fossil record and phylogenetic analyses

Study species and locations

We included 11 squirrel species in our comparative analysis of scent application (Table S1). Six ground squirrel (*Spermophilus*) species and one antelope squirrel (*Ammospermophilus*) species were tested at two locations each, and two chipmunk species (*Neotamias*) were tested at single locations (hereafter distinct locations will be referred to as populations; Table S1). We also included a chipmunk and ground squirrel species tested with snake scent by other researchers (*Eutamias sibiricus*, Kobayashi & Watanabe, 1986; *Spermophilus spilosoma*, P. Arrowood, unpublished data).

Scent stimuli and application trials

We tested squirrels with three scent stimuli: rattlesnake (predator), weasel (predator) and deer (nonpredator). Rattlesnake scent stimuli consisted of shed skins from either sympatric rattlesnake species or the geographically closest rattlesnake species (see Table S1). Weasel and deer scent were obtained commercially. Weasel scent (*Mustela frenata* and *Mustela erminea*), which consisted of weasel anal gland secretions, was purchased from Murray's Lures. Deer scent consisted of 'Hot Musk Buck Lure' purchased from the Wildlife Research Center®. These scents can all be found naturally in the environment; snakes leave scent on shed skins and on surfaces they move and rest on; weasel anal gland secretions are found in weasel faeces as well as scent marks ('anal drags'; Brinck *et al.*, 1983); deer scent can be found as scent marks ('rubs' and 'scraps'; Alexy *et al.*, 2001). Each scent stimulus was placed on a 12.5- cm-diameter filter paper that was folded in half and the scented side was slightly raised so it was not flat on the ground; weasel and deer scents were evenly applied to filter paper with a cotton-tipped applicator, whereas rattlesnake shed skins were tied to the filter paper. Squirrels were attracted into the immediate area of the stimulus using a bait trial (half-metre diameter circle of black-oiled sunflower seeds). Subtle differences in the general appearance of stimuli did not influence the likelihood of approach (B. Clucas and T. J. Ord, personal observation). Stimuli were always handled with latex gloves and each stimulus was used only once.

Stimuli were presented at sites where either squirrels or active burrows were observed. For each species, trial sites in the same area were at least 100 m apart to ensure that individuals were not tested more than once. A portable blind was set up 20–30 m away from the presentation site and one of the three scent stimuli was then staked down at the site (with the filter paper tied to a nail). Only one test stimulus was presented at each site. Trials began when a squirrel moved within a half-metre of the stimulus and lasted 20 min.

Trials were video taped using a digital camcorder and later scored using an event recorder (JWatcher, Blumstein *et al.*, 2006) to confirm observations of scent application (defined as an individual chewing on a scent stimulus and immediately licking its body; see Clucas *et al.*, 2008a for details).

Sympatry with predators: the fossil record and present-day

The Miocene (23.8–5.3 mya) to the Pliocene (5.3–1.8 mya). We searched for historical evidence of the co-occurrence of ground squirrel, rattlesnake and weasel ancestors during the Miocene and Pliocene using the internet-based MIOMAP (Carrasco *et al.*, 2005) and literature sources (e.g. Black, 1963; Holman, 1979). These records give an estimation of when ancestors of the extant squirrel species began undergoing selection by viperid snakes and mustelids.

The Pleistocene (1.8 million to 100 000 years ago). Fossil records of extant squirrel species and their co-occurrence with extant rattlesnake and weasel species were found using the internet-based FAUNMAP (Graham *et al.*, 1996) and literature sources (e.g. Harris, 1985; Holman, 1995). We determined the proportions of fossil sites with rattlesnake and/or weasel species co-occurring to designate whether squirrel species historically co-occurred with these predators as follows: 20% and below indicated no or very rare historical presence whereas above 20% indicated historical presence. This criterion is less stringent than the one used for proportion of present-day locations (see later) because some species were found at few fossil sites; in such cases, absence or rarity of co-occurrences may have been attributed to incompleteness of the fossil record.

The present day. Present-day co-occurrence between squirrel and rattlesnake species was determined by selecting specific locations where information about snake presence was available (e.g. National Parks). For each squirrel species, we used a number of locations approximately proportional to the area of its geographical distribution (i.e. more locations for larger distributions). This method provides the most accurate assessment of co-occurrence because an overlay of distribution maps does not take into account altitudinal differences between squirrel and rattlesnake species. For example, golden-mantled ground squirrels (*Spermophilus lateralis*) occur above 3000 m in some locations whereas sympatric rattlesnake species are limited to lower elevations and, thus, are not truly syntopic (Campbell & Lamar, 2004). Proportions of locations ≥ 0.50 indicated that a squirrel species co-occurred with rattlesnakes in the majority of their distribution. Weasels are not limited by altitude as are rattlesnakes; therefore, we defined co-occurrence as when their distribution overlapped with ground squirrel distributions (which was either complete overlap or no overlap at all; Parker, 1990).

In the case of the white-tailed antelope squirrel (*Ammospermophilus leucurus*), we considered predator presence only with regard to Baja California Sur, the southern extreme of the distribution of this squirrel species (Whorley *et al.*, 2004). Whorley *et al.* (2004) found that the white-tailed antelope squirrel species is made up of two distinct mitochondrial clades – northern and southern – that were probably isolated about 2 million years ago (mya). Given that these two clades have probably experienced different predator regimes, we treated the southern clade that we tested as a separate species from the northern with regard to present predator presence.

Present-day co-occurrence of Siberian chipmunks (*E. sibiricus*) and its snake predators could not be estimated in a similar fashion as mentioned previously, as records of precise locations of these species could not be found. However, distribution maps of snake species that are known to prey on Siberian chipmunks (mamushi, *Gloydius blomhoffi* and Asian rat snakes, *Elaphe* species) and descriptions of their typical habitat types suggest significant overlap (compare Gloyd & Conant, 1990 and Schulz, 1996 with Parker, 1990).

Phylogenetic analyses

The squirrel phylogeny. Our phylogeny (Fig. S1) was based on well-resolved molecular phylogenies (mitochondrial cytochrome *b*) from Harrison *et al.*, 2003 for ground squirrel species and Piaggio & Spicer, 2001 for chipmunk species. We used divergence times from Harrison *et al.*, 2003 for the ground squirrel species and divergence times from Mercer & Roth, 2003 for the chipmunk species. We then created our phylogeny using Mesquite OSX (version 2.71; Maddison & Maddison, 2008).

Correlated trait evolution. We tested the relationship between scent application and current predator presence using the correlated trait evolution analysis for discrete variables (Pagel, 1994). We first trimmed the phylogenetic tree down to the 11 species that we had tested in the field using Mesquite (Maddison & Maddison, 2008). Two matrices were then created that represented the ‘character state’ information for predator presence (yes or no for rattlesnake and weasel) and scent application behaviour (yes or no for application of rattlesnake scent and weasel scent) for each species. The correlated trait evolution analyses were run on these matrices in BayesTraits (Pagel & Meade, 2006). We tested the relationship between the following: (i) rattlesnake presence and snake scent application; (ii) weasel presence and weasel scent application; (iii) rattlesnake presence and snake and/or weasel application; and (iv) weasel presence and snake and/or weasel application. The first two comparisons tested the specificity of the application behaviour to certain predators, whereas the latter two comparisons tested the idea that any predator scent application might conceal prey odour from a chemosensory predator.

The correlated trait evolution analysis assumes four possible states when dealing with two dichotomous variables – 0 0, 0 1, 1 0, and 1 1 – where ‘0’ indicates absence of the trait and ‘1’ indicates its presence. It then tests the likelihood that an ‘independent’ model (LI: the evolution of one trait is not dependent on the other) or a ‘dependent’ model (LD: the evolution of one trait is dependent on the other) better fits the data (Pagel, 1994; Pagel & Meade, 2006). A likelihood ratio statistic (LR) is calculated as $\mathrm{LR} = -2\log_e\ [\mathrm{LI/LD}]$, and a chi-square distribution is used to determine significance with 4 degrees of freedom (Pagel, 1994; Pagel & Meade, 2006). To control for multiple comparisons, we calculated false discovery rates (FDR; Benjamini & Hochberg, 1995) based on an initial α of 0.05 and for a total of four statistical comparisons. Corresponding FDR corrections to α thresholds are presented in the text along side *P*-values; those *P*-values below FDR thresholds are considered statistically significant.

The independent model has four parameters whereas the dependent model has eight parameters that correspond to the transitions between each of the four states (see Fig. 1). If the dependent model was a significantly better fit than the independent model, we ran restricted dependent models by setting certain transitions equal to each other based in part on the prediction that these transitions were equally likely to occur (reducing parameter number increases the power of the analysis). In these tests, we relied on AICc (Akaike Information Criterion with a correction ‘c’ for small sample sizes) to determine which (if any) of the restricted models better represented the data (Burnham & Anderson, 2004).

Ancestral state reconstructions. We conducted ancestor state reconstructions of rattlesnake scent application, weasel scent application and the application of either predator scent using parsimony and maximum likelihood methods implemented in the Mesquite package (Maddison & Maddison, 2008). Parsimony analyses assign ancestor states based on the least number of evolutionary changes to reach the observed states of extant taxa. We used an unordered model of evolution, such that the probability of gains and losses in application behaviour were equally likely (Maddison & Maddison, 2008). Parsimony reconstructions do not consider branch lengths when assigning states. Maximum likelihood analyses use a probabilistic model to assign ancestor states based on the distribution of observed states among extant taxa (regardless of the number of evolutionary changes that might occur) and the length of time a trait has had to evolve along a given branch. As with parsimony analyses, we assumed that gains or losses in the behaviour were equally likely and used the Markov k-state 1 parameter model to scale the rate of evolution based on our specified estimates of branch length (Maddison & Maddison, 2008).

The accuracy of reconstruction methods is dependent on a number of factors (e.g. whether the method used to assign states correctly approximates how evolution has actually occurred in the trait of interest). Of particular relevance to our study is taxon sampling. Ideally, data on all species should be included in the analysis, but the time and expense of testing every squirrel species in existence today make this approach impractical (if not impossible). Instead, we adopted a strategic sampling approach in which we selected species based on where they were positioned on the phylogeny and to maximize variation in whether the species tested would exhibit anointing behaviour (e.g. testing squirrel species that did and did not overlap with different predator species). It is

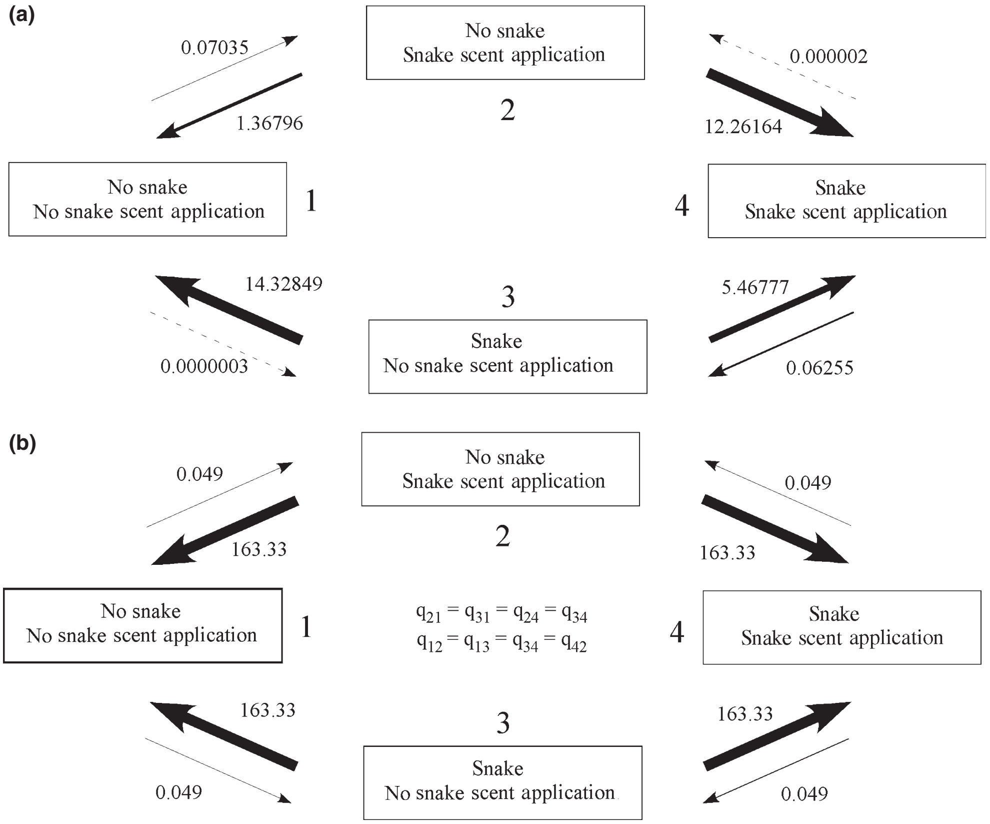

Fig. 1 (a) Results of the correlated trait evolution analysis for the dependent model of the relationship between rattlesnake presence and snake scent application behaviour. Arrow thickness indicates the magnitude of the transition rates (q_{ij}'s; numbers above arrows). The dependent model fit the data significantly better than the independent model (see Table A4a). Snake scent application is more likely to evolve in the presence of rattlesnakes (compare 3 → 4 [$q_{3,4}$] with 1 → 2 [$q_{1,2}$]) and is more likely to be lost in the absence of rattlesnakes (compare 2 → 1 [$q_{2,1}$] with 4 → 3 [$q_{4,3}$]). (b) Best restricted dependent model (see Table A5; thicker arrows represent most likely transitions and point to most stable states) in which the transitions q_{21}, q_{31}, q_{24} and q_{34} are equal to each other and q_{12}, q_{13}, q_{34} and q_{42} are equal to each other.

important to note that the accuracy of ancestor reconstructions is not simply a function of the number of species included in the analysis (Salisbury & Kim, 2001; Li *et al.*, 2008). In particular, the phylogenetic position of the taxa used can have a greater influence on the reliability of certain node reconstructions than the addition of more taxa to the analysis (Salisbury & Kim, 2001). Because of the practical constraints of the number of species that we could test in the field, we chose to optimize the accuracy of our reconstructions at the base of the phylogeny to evaluate the earliest possible occurrence of anointing behaviour. To this end, we included several basal species (species that branch directly from or close to the root node) and surveyed similar numbers of species on either side of the root node, a sampling regime that has been shown to enhance the accuracy of estimates at basal nodes (see Salisbury & Kim, 2001; Li *et al.*, 2008).

We also performed a sensitivity test to evaluate the extent our taxon sampling might influence our results. We created a hypothetical data set that covered all species in the ground squirrel and chipmunk phylogeny (see Fig. S1). To obtain a realistic distribution of how anointing behaviour might be distributed across the phylogeny, any species that co-occurred with a rattlesnake species was classified as exhibiting anointing behaviour, whereas all others were classified as lacking anointing behaviour. The data set consisted of 54 taxa, including European ground squirrels. The 'true' ancestor state was then reconstructed at the root node of the tree using parsimony and maximum likelihood. Using the same data set and phylogeny, 100 pruned trees of 11 taxa were created, in which the taxa included were selected using a random number generator with the following constraints: one taxon was always drawn from the chipmunk outgroup (clade I; see Fig. S1), three taxa were always drawn from the most basal ground squirrel lineages (clades II and III), no European or marmot taxa were drawn (clade IV) and seven taxa were drawn from the remaining species (clade V and VI). This distribution of taxon sampling loosely approximated the sampling regime of our field survey, but with the distribution of species selected across the phylogeny being roughly proportional to the number of taxa in each clade. The ancestor state at the root node was subsequently estimated for all 100 trees and compared to the true value calculated from the complete data set and phylogeny. In parsimony analyses, the correct state was resolved in 59% of reconstructions. In maximum likelihood analyses, 67% of reconstructions were within 5% of the true value (81% were within 10% of the true value). Overall, these results were encouraging because the root of the phylogeny is generally the most difficult node to resolve accurately. On a more practical note, the likelihood simulations can be used to construct 95% confidence limits reflecting reconstruction accuracy specific to taxon sampling. This confidence limit, based on assignments at the root node and using a biologically realistic simulated data set of presence and absence of anointing behaviour across the squirrel phylogeny, is ±25% (i.e. 95% of likelihood reconstructions were within 25% of the true value).

Experimental tests of the adaptive significance of predator scent application

Study species

We tested the foraging behaviour of 15 wild-caught northern Pacific rattlesnakes (five males and 10 females, 185–755 g, captured March–August 2004, each housed in separate terrariums at approximately 26–27 °C and a 12L : 12D light cycle). All rattlesnakes were likely to have interacted with or preyed on California ground squirrels. Their diet in captivity consisted of a prekilled mouse every 2 weeks. Rattlesnakes were normally fed in their terrarium but were each given the opportunity to feed on a dead mouse inside a foraging arena (see later) several times prior to the experimental trials. Subjects were not fed for 18–22 days prior to testing to increase foraging motivation.

Trials and treatment stimuli

Trials were conducted in two identical foraging arenas (Fig. S2) in separate temperature controlled rooms from July to September 2005. The arenas were each fitted with two artificial 'burrows' made of plastic rectangular boxes with Plexiglas tops whose entrances faced the arena entrance and were equidistant from the arena entrance (Fig. S2). Each rattlesnake received three different prey scent trials, (i) rattlesnake-scented, (ii) weasel-scented, and (iii) water-scented prey (see later). Prey stimuli were placed in one burrow and the second burrow remained empty. The empty burrow provided an unscented option for shelter as snakes prefer being under cover when in an arena (B. Clucas, personal observation; Theodoratus & Chiszar, 2000). The six potential treatment orders were systematically assigned so that each possible order was used two or three times across the 15 rattlesnakes. The different scent types were presented to individual rattlesnakes across the three trials always in the same foraging arena, and consecutive presentations were conducted at 3-day intervals. Trials began between 1700 and 1800 h and arena rooms were kept at approximately 27 °C with light levels at 0.05 μmol s^{-1} m^{-2} to simulate dusk (when these snakes are actively hunting for prey). The foraging arenas were sprayed down with water, wiped and allowed to dry after each trial and lined with fresh white butcher paper before the following day's trial.

Treatment stimuli were made as follows: prekilled mice were thawed to room temperature the day of the trial and treated with rattlesnake scent, weasel scent or with water (hereafter called unscented mice). Mice were scented with rattlesnake scent by placing them in plastic bag with shed rattlesnake skin and allowed to saturate for 10 min before the trial. Mice were scented with weasel scent by applying weasel anal gland secretions to the mouse with a cotton tip applicator and then allowed to saturate in a plastic bag for 10 min. Unscented mice were put into a plastic bag for 10 min after the mouse was dipped into water. Stimuli were handled at all times with latex gloves. Each individual rattlesnake was tested with scent from a different snake of similar size and same sex.

Trials were recorded onto a remote VHS system located just outside the rooms using closed circuit cameras (Sony SSC-M383; Sony Corp., Tokyo, Japan) positioned directly above each arena such that the recorded image captured the entire arena (Fig. S2). The scented mouse stimulus was placed inside a wire frame inside the burrow to prevent consumption by the snake if found and kept behind a plastic shield out of view until the snake fully entered the burrow (Fig. S2). We alternated which of the two burrows contained the mouse across trials for individual rattlesnakes. Rattlesnakes were transported via their terrarium into the arena room and transferred to a starting chamber adjoining the test arena with a snake hook. After a 10-min acclimatization period, we began the trial by remotely lifting the divider between the starting chamber and arena from outside the room.

Data collection and analysis

Trials were filmed for 20 min and the following measures were scored: (i) Latencies to encounter stimulus burrow [time elapsed from the rattlesnake's departure from the start chamber to the entry of its head into a semi-circle (radius 10 cm) in front of the burrow], (ii) Latencies to find mouse [elapsed time from stimulus burrow encounter to circumvention by the rattlesnake's head of the visual blocker in front of the mouse prey (see Fig. S2)] and (iii) Time spent within stimulus burrow (time elapsed from entry to exit). The video scorer was blind to the scent type involved.

We tested for an effect of scent type by comparing responses to burrows containing rattlesnake-scented and weasel-scented mice with those containing unscented mice using a repeated measures design. We used nonparametric statistical tests because of the violation of assumption of normality, which could not be corrected by transformations. Statistical tests were performed using SPSS (version 16.0.1, SPSS, 2007). FDR were calculated based on an initial α of 0.05 and for a total of seven statistical comparisons (an extra comparison was made for time spent in the weasel-scented vs. unscented mouse burrows because of several significant outliers). We also present effect size estimates along side P-values in the form of r-values calculated from z-scores (Field, 2005) and rely on the convention of a large effect corresponding to an r-value ≥ 0.37 (Cohen, 1969).

Results

Comparative field study, the fossil record and phylogenetic analyses

Scent application trials

Sample sizes varied among species we tested based on density of squirrels and accessibility of sites (range per scent 2–21, average 9.7 individuals; see Table S1 for details on population sample sizes). Over all species, squirrels applied rattlesnake scent (41/94) and weasel scent (18/94) but did not apply deer scent (0/80). Scent application is therefore limited to predator scents.

Sympatry with predators: the fossil record and the present

The Miocene (23.8–5.3 mya) and Pliocene (5.3–1.8 mya). Fossils of ground squirrel ancestors were found with fossils of rattlesnake ancestors as far back as approximately 15 mya in Texas, USA (Black, 1963; Holman, 1977, 1979; Carrasco *et al*., 2005), and fossils of ground squirrel ancestors were found with weasel ancestor fossils as far back as approximately 16 mya in Wyoming, USA (Black, 1963; Carrasco *et al*., 2005; see Table S2).

The Pleistocene (1.8 million to 100 000 years ago). Fossil records of extant squirrel species and their co-occurrence with extant rattlesnake and weasel species are shown in Table 1. Based on the proportions of fossil sites with rattlesnake and/or weasel species, five of seven species co-occurred with rattlesnakes historically and five of six species co-occurred with weasels historically (Table S3). Fossil records were unavailable for the remaining four squirrel species, so their historical status is unknown.

The present day. Present-day sympatry between squirrel and rattlesnake species occurred in 7 of the 11 species tested (Table S3). Seven of the nine species we tested with weasel scent co-occurred with weasel species (Table S3).

Correlated trait evolution

Rattlesnake scent application. Squirrel species with historical and current co-occurrence with rattlesnakes all expressed snake scent application (Fig. 2). The correlated trait evolution analysis showed that the dependent model of evolution was more likely than the independent model (LR = 12.95, d.f. = 4, $P = 0.011$ (FDR-$\alpha = 0.05$); Table S4a and Fig. 1a). Thus, snake scent application is more likely to evolve in the presence of rattlesnake predators. Furthermore, the state of 'application behaviour' but 'no rattlesnake predators' is evolutionarily unstable and transitions quickly to 'no scent application' and 'no rattlesnake predators' (Fig. 1a), suggesting loss of the behaviour with loss of the predator. We increased the power of the analysis by restricting the dependent model to reduce the number of parameters. The analysis of restricted models showed that a two parameter model, setting the transition of moving to 'rattlesnake predators and scent application' or 'no rattlesnake predators, no scent application' equal to each other and the remaining transitions equal to each other (moving to 'no rattlesnake predators, scent application' or 'rattlesnake predators, no scent application'), produced the best fitting model (Table S5; Fig. 1b).

For most squirrel species we tested two populations that were consistent in either co-occurring with rattlesnakes both historically and currently or neither historically nor currently. However, there were two exceptions. California ground squirrels have historically been sympatric with rattlesnakes, but certain populations in the Sacramento Valley (e.g. our study site in Davis, California) are estimated to have lost contact with rattlesnakes about 9000 years ago (Coss, 1999). The recently snake-free population in the Sacramento Valley did not apply snake scent (Fig. 2). Conversely, high-altitude Belding's ground squirrels (*Spermophilus beldingi*) historically have not been found with rattlesnakes, but some populations in south-eastern Oregon (e.g. our study site in Malheur National Wildlife Refuge) now do co-occur with rattlesnakes. The snake-present population in Oregon did not apply snake scent (Fig. 2).

Weasel scent application. Weasel scent application did not match historical or current co-occurrence with weasel species (Fig. 3). Two species that had historical and current contact with weasels did not exhibit the application behaviour (golden-mantled and California ground squirrels), and two additional species with no current contact with weasels did apply weasel scent (Baja California rock squirrels and white-tailed antelope squirrels). The correlated trait evolution analysis showed that the dependent model of evolution was not more likely than the independent model [LR = 3.989, d.f. = 4, $P = 0.407$ (FDR-$\alpha = 0.025$); Table S4b]. Thus, the evolution of weasel scent application is not significantly correlated with the presence of weasel predators.

Table 1 Pleistocene squirrel and predator fossil co-occurrence.

EPOCH Pleistocene (1.8 mya–100 000 years)			
Thousand years ago	Squirrel species	Weasel species	Rattlesnake species
~10	*Spermophilus lateralis*: CO1 *Spermophilus variegatus*: CO1 *Spermophilus spilosoma*: CO1	*Mustela erminea*: CO1 *Mustela frenata*: CO1	
~11	*Spermophilus mexicanus*: TXl		*Crotalus atrox*: TXl
~11.6	*S. variegatus*: TX2 *S. spilosoma*: TX2	*M. frenata*: TX2	
~11.9–1.5	*S. lateralis*: CO2	*M. erminea*: CO2 *M. frenata*: CO2	
~11.8–10.4	*S. lateralis*: ID2 *S. variegatus*: TX3, MXl *S. spilosoma*: MXl	*M. frenata*: ID2, TX3	*Crotalus* spp: MXl
~12–8	*S. variegatus*: AZl		*Crotalus atrox*: *AZ*1 *Crotalus scutulatus*: AZl
~13–12	*S. variegatus*: AZl		*Crotalus* spp: AZ2
~14.5–6.1	*S. lateralis*: MTl	*M. frenata*: MTl	
~15.8–14.5	*Spermophilus beldingi*: IDl	*M. erminea*: IDl	
~20–10	*S. lateralis*: CA6 *S. variegatus*: CA8	*M. frenata*: CA6, CA8	
~24–10	*S. lateralis*: WYl *S. variegatus*: WYl	*M. frenata*: WYl	
~25.5–18.1	*S. variegatus*: NM2	*M. frenata*: NM2	
~35–12.6	*S. lateralis*: NVl	*M. erminea*: NV1	*Crotalus viridis*: NV1
~35–25	*Spermophilus beecheyi*: CA7 *S. variegatus*: NMl, NM2	*M. frenata*: CA7, NMl	*C. viridis*: CA7 *Crotalus* spp: NMl, NM2
~40–10	*S. beecheyi*: CA4	*M. frenata*: CA4	*C. viridis*: CA4
~40	*S. beecheyi*: CAl	*M. frenata*: CAl	*C. viridis*: CAl
~110–10	*S. beecheyi*: CA2, CA3, CA9 *S. lateralis*: CA3, CA9	*M. frenata*: CA3, CA9	*C. viridis*: CA2, CA3
~400–10	*S. beecheyi*: CA5		*C. viridis*: CA5

AZ1: Mead *et al.*, 1984; Holman, 1995; Graham *et al.*, 1996; AZ2: Holman, 1995; Graham *et al.*, 1996; CA1: Hudson & Brattstrom, 1977; Harris, 1985; Holman, 1995; Graham *et al.*, 1996; CA2,3,4,5,6&9: Harris, 1985; Holman, 1995; Graham *et al.*, 1996; CA7: Akersten *et al.*, 1979; Harris, 1985; LaDuke, 1991; CA8: Graham *et al.*, 1996; CO1: Graham *et al.*, 1996; CO2: Harris, 1985; Graham *et al.*, 1996; ID1: Gruhn, 1961; Harris, 1985; Mead & Bell, 1994; Graham *et al.*, 1996; ID2: Guilday and Adam, 1967; Graham *et al.*, 1996; MT1: Graham *et al.*, 1996; NM1: Harris, 1985; Holman, 1995; Graham *et al.*, 1996; NM2: Harris, 1985; Graham *et al.*, 1996; NV1: Thompson & Mead, 1982; Mead *et al.*, 1982; Mead & Bell, 1994; TX1: Johnson, 1987; TX2: Logan, 1983; Harris, 1985; Graham *et al.*, 1996; TX3: Logan and Black, 1979; Harris, 1985; WY1: Harris, 1985.

General predator scent application. The correlated trait evolution analysis testing whether predator scent application is more likely to evolve in rattlesnake presence was not significant [LH independent model = −10.68, LH dependent model = −7.08, LR = 7.09, d.f. = 4, $P = 0.131$ (FDR-$\alpha = 0.038$)], nor was the analysis testing if weasel presence was correlated with predator scent application [LH independent model = −9.35, LH dependent model = −8.56, LR = 1.57, d.f. = 4, $P = 0.813$ (FDR-$\alpha = 0.013$)].

Ancestral state reconstruction

Parsimony ancestral state reconstructions of snake scent application suggest that the common ancestor of ground squirrels and chipmunks possessed the behaviour and the maximum likelihood analysis showed that this common ancestor had a 55% probability of having the behaviour (95% confidence interval, CI, accounting for uncertainty resulting from taxon sampling: 0.30–0.80; Fig. 2). The common ancestor of ground squirrels (including antelope ground squirrels) had a 68% probability of possessing the trait (95% CI: 0.43–0.93).

Parsimony ancestral state reconstructions of weasel scent application revealed that the common ancestor of ground squirrels and chipmunks was equally likely to possess or not possess the behaviour, and the maximum likelihood analysis showed that this common ancestor had a 50% probability of having the behaviour (95% CI:

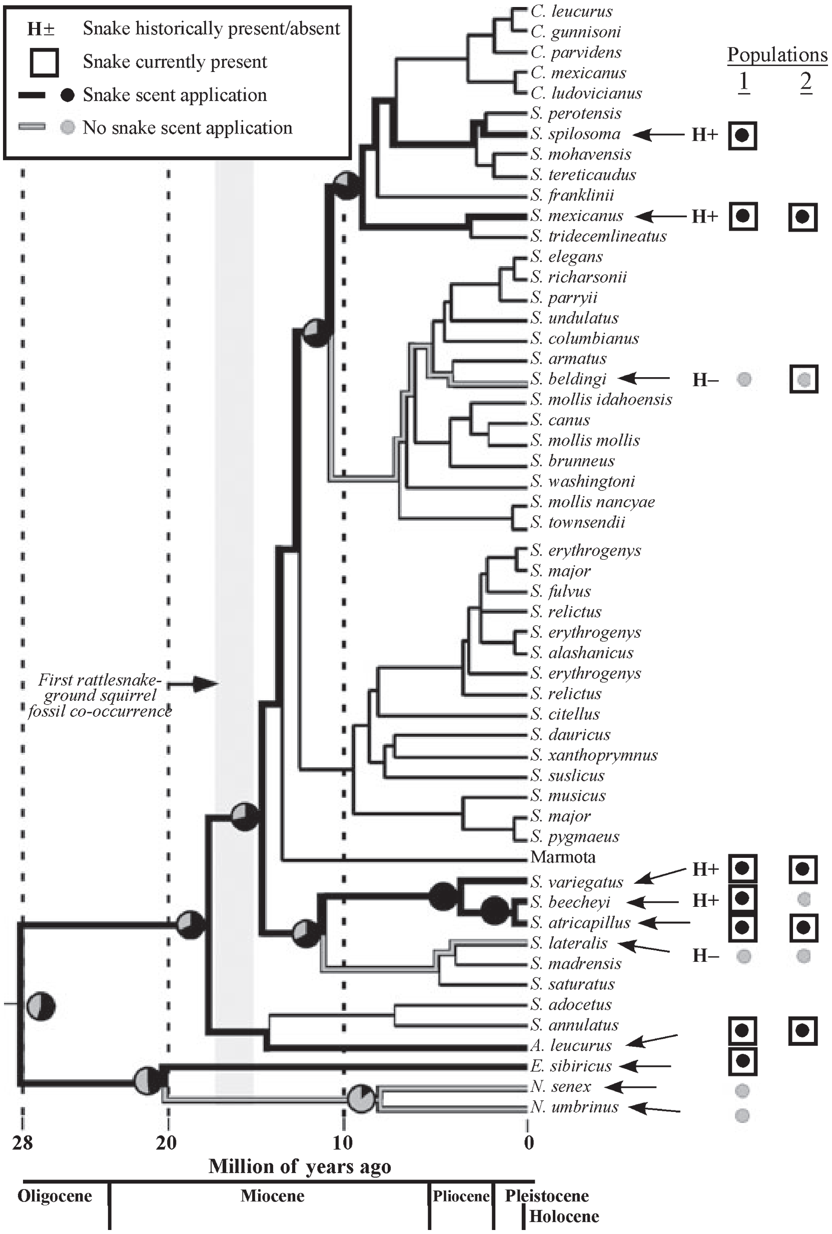

Fig. 2 Prevalence of snake scent application across squirrel species in relation to historical and current presence of rattlesnakes (denoted by H+/− and boxes, respectively). Species tested for snake scent application (indicated with arrows) were found to apply scent (black dot) if they co-occurred with rattlesnakes both historically (H+) and currently (boxes). Ancestral state reconstructions of snake scent application provide evidence of an ancient origin. Parsimony analysis reveals that the common ancestor of all species tested had the behaviour (presence reconstructed as black, absence as grey). Maximum likelihood analysis was more ambiguous, suggesting a 55% probability that the common ancestor of ground squirrel and chipmunks possessed the behaviour (black portions of pie charts represent probability of exhibiting the behaviour; likelihood scores represent raw likelihoods). However, both parsimony and likelihood analyses indicate that the common ground squirrel ancestor (excluding chipmunks) possessed the behaviour (maximum likelihood: 68%), which was further supported by fossil evidence suggesting a historical co-occurrence with rattlesnake around this time (~15 mya; indicated by grey shading).

0.25–0.75; Fig. 3). The common ancestor of ground squirrels (including antelope ground squirrels) also had a 50% probability of possessing the trait (95% CI: 0.25–0.75).

The parsimony test of ancestral state showed that the predator scent application (applying either weasel or rattlesnake scent, or both) trait is ancestral with respect to both the common ancestor of ground squirrel and chipmunks and the common ancestor of ground squirrels. Similarly, the maximum likelihood analysis indicated that predator scent application trait is ancestral (Fig. 4; probability of 0.74 (95% CI: 0.49–0.99) for ground squirrel and chipmunk ancestor, probability of 0.94 (95% CI: 0.69–1.19) for ground squirrel ancestor).

Comparison of ancestral state reconstructions and the fossil record

The fossil record estimates the first co-occurrences between rattlesnake and squirrel ancestors at approximately 15 mya, whereas the common ancestor of ground squirrels and chipmunks is estimated to have lived 28 mya (Fig. 2). Therefore, although the ancestral state reconstructions suggest that the ancestor to present-day ground squirrels and chipmunks probably possessed

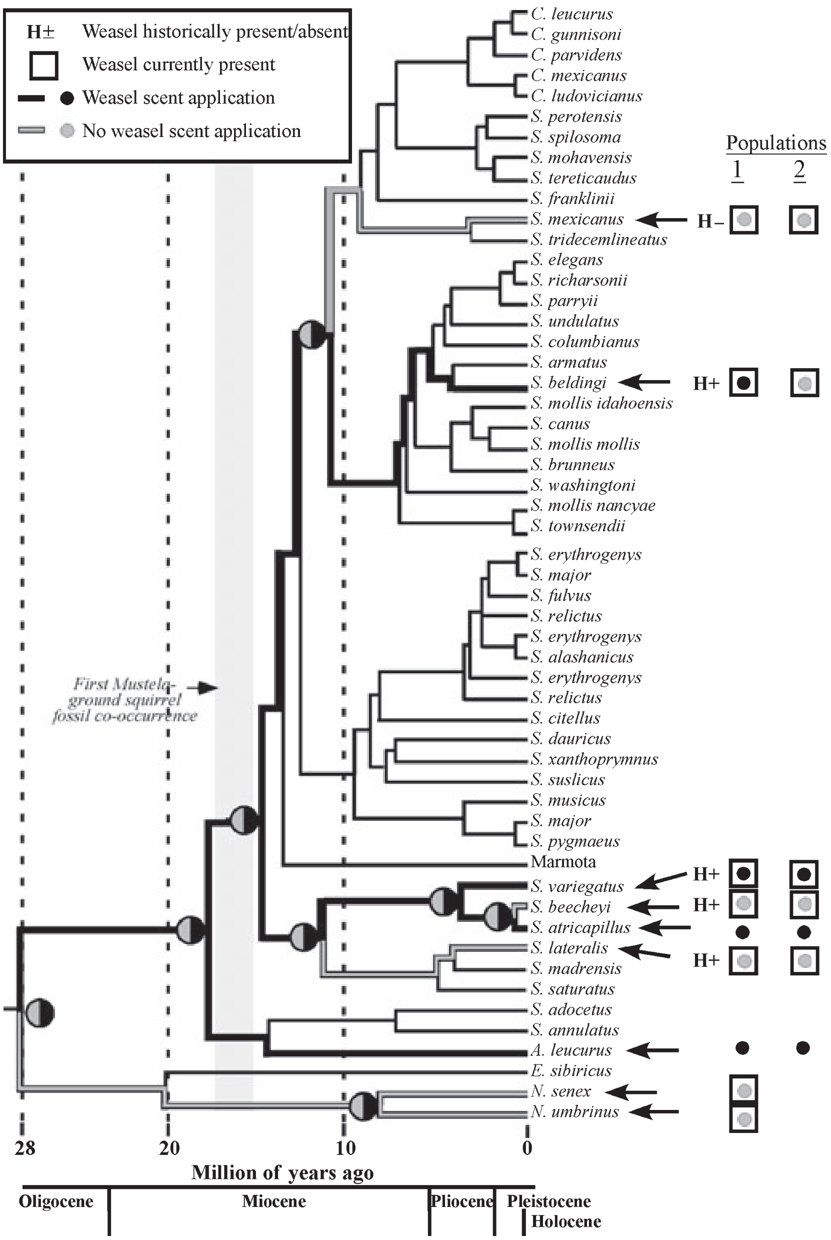

Fig. 3 Prevalence of weasel scent application across squirrel species in relation to historical and current presence of weasels (denoted by H+/− and boxes, respectively). Species tested for weasel scent application (indicated with arrows) applied scent (black dot) regardless if they co-occurred with rattlesnakes historically (H+), currently (boxes), or not. Ancestral state reconstructions of weasel scent application provide partial evidence of an ancient origin. Parsimony analysis was unable to resolve whether the common ancestor of all species tested did or did not possess the behaviour (presence reconstructed as black, absence as grey). Maximum likelihood analysis revealed similar results, suggesting a 50% probability that the common ancestor of ground squirrel and chipmunks possessed the behaviour (black portions of pie charts represent probability of exhibiting the behaviour; likelihood scores represent raw likelihoods).

some sort of predator scent application behaviour (95% CI: 0.49–0.99), it is possible that rattlesnakes were not the original source of selection. A similar scenario applies to weasel scent application, as the first co-occurrence of weasel and squirrel ancestors is estimated at about 16 mya (Fig. 3). Nevertheless, it is possible the first co-occurrence between squirrels and these predators occurred earlier with the fossil record providing only the minimum estimation of co-occurrence (see Marshall, 2008). In North America, the first squirrel fossil found is approximately 30–37 million years old (Korth, 1994) and the oldest weasel ancestor fossil found is approximately 17.5–15.8 million years old (Carrasco *et al.*, 2005) and the oldest rattlesnake ancestor fossil found is 20–24 million years old (Holman, 2000), which suggests earlier co-occurrences might have been possible.

Experimental tests of the adaptive significance of predator scent application

Effects of predator scent

Rattlesnake foraging behaviour was affected by both rattlesnake scent and weasel scent applied to mice stimuli (Fig. 5). Although latency to encounter stimulus burrows did not differ significantly between unscented mice and rattlesnake- or weasel-scented mice [Wilcoxon signed

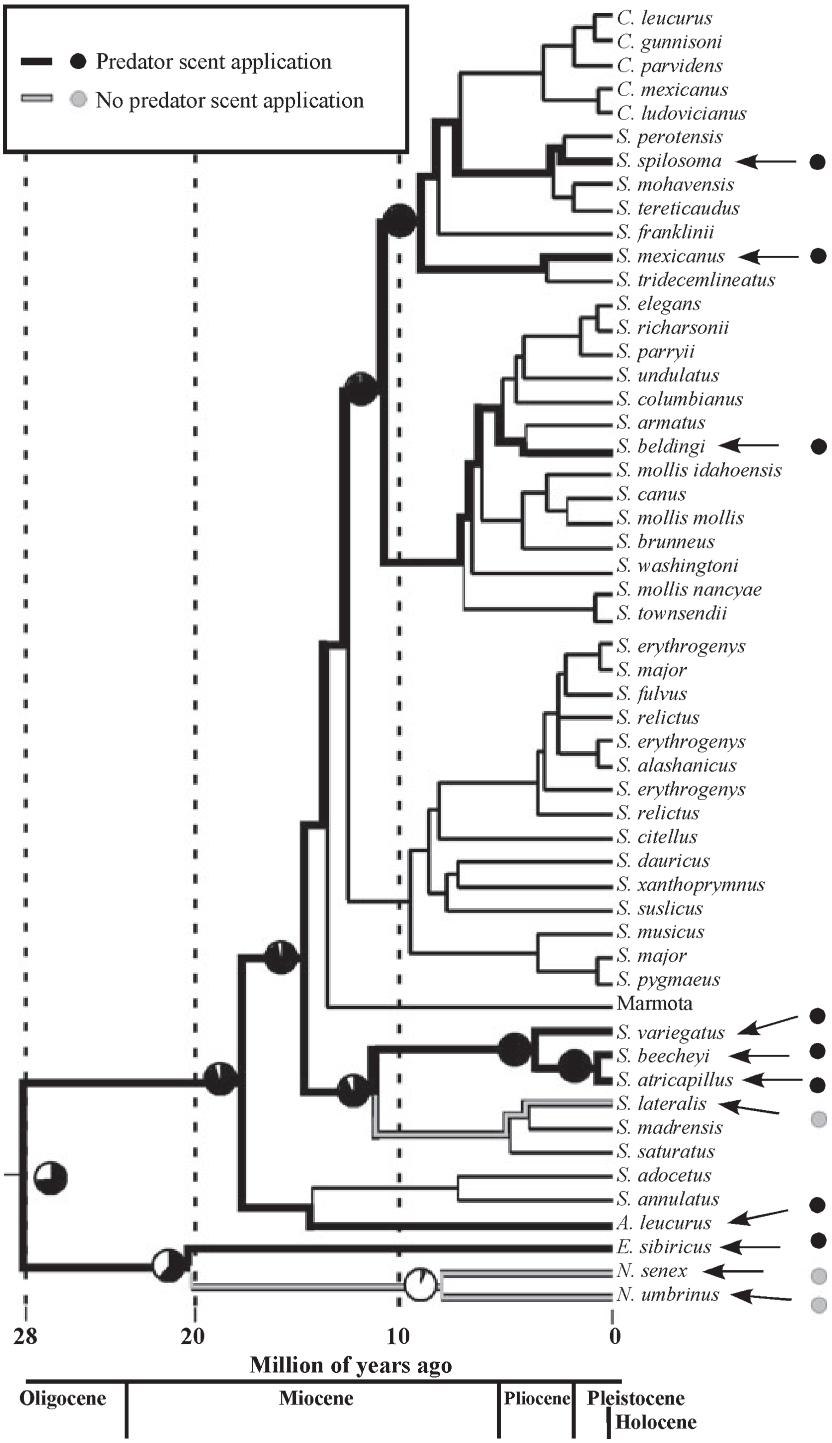

Fig. 4 Prevalence of predator scent application across squirrel species. Species that applied predator scent (rattlesnake and/or weasel) are indicated by black dots. Ancestral state reconstructions of predator scent application suggest an ancient origin of the behaviour. Parsimony analysis revealed that the common ancestor of all species tested possessed the behaviour (presence reconstructed as black, absence as grey). Maximum likelihood analysis revealed similar results, suggesting a 74% probability that the common ancestor of ground squirrel and chipmunks possessed the behaviour (black portions of pie charts represent probability of exhibiting the behaviour; likelihood scores represent raw likelihoods). The ancestor of ground squirrels (including antelope squirrels) had a 94% probability of having the behaviour and chipmunks had a 61% of possessing the behaviour.

ranks tests: unscented vs. rattlesnake: $Z = -0.114$, $P = 0.910$ (FDR-$\alpha = 0.007$), $r = -0.02$] and unscented vs. weasel: $Z = -0.454$, $P = 0.650$ (FDR-$\alpha = 0.014$–0.454), $r = -0.08$; Fig. 5a), rattlesnakes did find the unscented mouse stimulus faster than either predator scented stimulus. Latency to find the mouse was generally longer for rattlesnake- and weasel-scented mice than unscented mice [mouse vs. rattlesnake: $Z = -2.15$, $P = 0.031$ (FDR-$\alpha = 0.029$), $r = -0.39$ and mouse vs. weasel: $Z = -2.443$, $P = 0.015$ (FDR-$\alpha = 0.043$), $r = -0.45$; Fig. 5b]. Rattlesnakes also spent more time in unscented mouse burrows than rattlesnake-scented

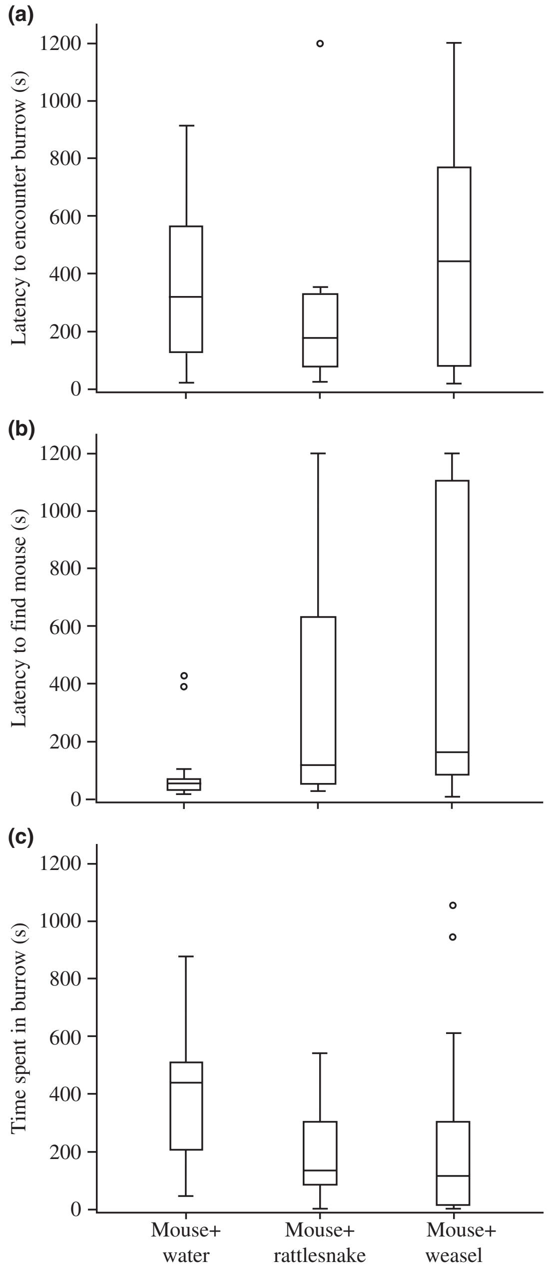

Fig. 5 Boxplots of rattlesnake foraging behaviour in relation to prey treated with different scents. (a) Latency to encounter stimulus burrow, (b) Latency to find mouse in stimulus burrow [mouse + water is significantly different from mouse + snake and mouse + weasel] and (c) Time spent in stimulus burrow [mouse + water is significantly different from mouse + snake]. See text for statistical tests. Boxes represent 50% of the data; inner lines represent median scores and whiskers are minimum and maximum values. Prominent outliers are represented by circles.

mouse burrows [$Z = -2.61$, $P = 0.009$ (FDR-$\alpha = 0.05$), $r = -0.47$; Fig. 5c]. The difference between unscented- and weasel-scented mouse burrows was not significant [$Z = -1.53$, $P = 0.12$ (FDR-$\alpha = 0.021$), $r = -0.28$]. Inspection of boxplots revealed two prominent outliers. With these data points removed, our test confirmed the difference apparent in Fig. 5c [$Z = -2.27$, $P = 0.023$ (FDR-$\alpha = 0.036$), $r = -0.44$].

Discussion

Our results suggest that predator scent application probably evolved in a common ancestor of squirrels at least 18–28 mya (Fig. 4). Its origin does not appear to be specifically related to the unique relationship between certain ground squirrels and rattlesnake species, but a more ancient predator–prey relationship with an unknown chemosensory predator. Rattlesnake and weasel scent application should therefore be viewed as a general predator scent application behaviour, rather than respective strategies targeted specifically at rattlesnakes and weasels. This was reinforced by our tests of rattlesnake foraging behaviour that showed anointing with either rattlesnake or weasel scent will mask a prey's scent. Although it remains unclear what the initial selection source might have been that lead to the evolution of scent application (and it is important to reiterate here that there is some uncertainty associated with our ancestor reconstructions and data from the fossil record), it is possible that it occurred in response to predation by a now extinct reptilian or mammalian carnivore. For example, during the Oligocene (~34–24 mya) the putative common ancestor of ground squirrels and chipmunks lived alongside snake species that were large enough to consume ground squirrels sized rodents (e.g. *Boavus* spp., Holman, 2000). Moreover, if predator scent application in rats and mice is homologous with that in squirrels (Clucas *et al.*, 2008a), based on divergence times (Adkins *et al.*, 2001) it would suggest the behaviour evolved at least 75 mya roughly 50 million years before rattlesnake and weasel ancestors fossils were first found.

In the more recent past, application with snake scent by extant squirrel species was tightly correlated with rattlesnake presence. The behaviour was maintained in squirrel species with historical and current rattlesnake predation but was lost in those species in which selection became relaxed with the absence of rattlesnakes. The lack of snake scent application in a population of California ground squirrels, a species in which rattlesnakes are historic predators but that recently lost contact (approximately 9000 years ago; Coss, 1999), suggests this behaviour can decay relatively rapidly. Such a rapid loss of an anti-snake behaviour is unique compared to other behavioural defences against rattlesnakes expressed by California ground squirrels, which have persisted under relaxed selection for tens of thousands of years (Coss *et al.*, 1993; Coss, 1999). Blumstein *et al.*, 2004 also showed a rapid loss of antipredator behaviour in an isolated population of a mammalian species (tammar wallabies, *Macropus eugenii*, free from predation for about 130 years); however, this population was on an island free of all predators. In contrast, California ground squirrels in our rattlesnake-free areas still face other chemosensory predators, namely gopher snakes (*Pituophis catenifer*). Moreover, California ground squirrels in these areas do not apply gopher snake scent whereas other populations of ground squirrels (California ground squirrels and rocks squirrels) in areas with rattlesnakes do apply gopher snake scent (B. Clucas, unpublished data). This further suggests the importance of rattlesnake presence maintaining the predator application behaviour.

Interestingly, we did not find evidence of squirrels in historically rattlesnake-free populations re-evolving scent application after rattlesnake presence was restored. A population of Belding's ground squirrels that is currently undergoing rattlesnake predation did not exhibit the behaviour with either rattlesnake or weasel scent. This is surprising because the second population of Belding's we tested did apply weasel scent and yet did not co-occur with rattlesnakes. Failure to regain a trait suggests that it may have genetic underpinnings that were irreversibly lost via mutation or genetic drift in the absence of direct selection (Lahti *et al.*, 2009). Alternatively, the length of time a population is re-exposed to a predator may have an effect on whether they exhibit appropriate antipredator behaviour. However, predation is a potent source of selection that might be expected to lead to the rapid evolution of anti-predator behaviour over short time spans. For example, moose (*Alces alces*) free of carnivore predators for 50–130 years recovered antipredator responses within one generation after the re-introduction of wolves (*Canis lupus*; Berger *et al.*, 2001) and threespine stickleback (*Gasterosteus aculeatus*) populations that have not faced predation from piscivores for 20 000 years showed heightened antipredator responses after just 25 years when contact with these predators was regained (Messler *et al.*, 2007). Considering how critical rattlesnake predation is for ground squirrels (Owings & Coss, 1977), we would expect populations that underwent recent predation loss (e.g. California ground squirrels) to quickly regain predator scent application behaviour after re-exposure. We know that the raw material of scent anointment, the motor pattern involved in applying scent, is not a limitation for the re-evolution of the behaviour. Rodents share an ancient head-to-tail grooming pattern (Berridge, 1990) from which the form of scent application is likely derived (Clucas *et al.*, 2008a), and all ground squirrels (including those that did not apply predator scent) possess this trait. It is possible that the maintenance of the behaviour comes at a cost and without rattlesnake predation as a strong re-enforcer this cost results in the behaviour being rapidly lost. The cost, for example, of being attracted to the scent of a predator may be lethal especially those for which scent application does not reduce predation risk.

Scent application with predator scent has clear adaptive significance for squirrels facing rattlesnake predation. Rattlesnakes showed reduced foraging behaviour towards rattlesnake-scented squirrel odour (Clucas *et al.*, 2008b) and took much longer to find rattlesnake- or weasel-scented prey (this study). It is not surprising that all seven species that co-occur with rattlesnakes in the majority of their distribution apply rattlesnake scent. In addition, three of six species with rattlesnake predators also applied weasel scent (even two species that do not currently co-occur with weasels). Squirrels only co-occurring with weasels did not mirror this. Indeed, out of these four species, only one population of one species (*S. beldingi*) applied any predator scent. The apparent loss of weasel scent application in five of the nine species tested is puzzling. Weasels are mustelids, a family that contains more than one genus that preys on squirrels (e.g. American badgers, *Taxidea taxus*). Under such conditions, weasel scent application should be maintained because of the presence of other mustelid predators (i.e. the multipredator hypothesis; Blumstein, 2006). It is possible that these differences between rattlesnakes and weasels as current sources of selection may reflect that mustelid species are less sensitive than snakes to conflicting odour cues when hunting prey. Testing this possibility will require an investigation into the effects of predator scent application on weasels.

Studying the evolution of behaviour by combining field experiments on behaviour, phylogenetics and palaeontology provided a unique perspective into the evolutionary history of a complex anti-predator behaviour that would otherwise have been unattainable through traditional means (see also Owen *et al.*, 2000 and Carbone *et al.*, 2009). By adopting an integrative approach, and despite uncertainties associated with some analyses, we can say that olfactory camouflage in ground squirrels goes beyond their unique predator–prey relationship with present-day rattlesnakes and that it is likely part of an older, more taxonomically widespread predator scent application behaviour in squirrels that originated under different selection sources.

Acknowledgments

Thanks to Dave Collar, Dick Coss, Annie Leonard, Michael Jennions and an anonymous reviewer for constructive comments on a previous version of the manuscript, to Aaron Rundus for help with rattlesnake care and building of the foraging arenas, and to Brian O'Meara and other participants of the 2008 University of California, Davis Applied Phylogenetics Workshop for helpful advice. B.C. was supported by a National Science Foundation Predoctoral Fellowship and an American Society of Mammalogists Grant-in-Aid. A UCMEXUS small grant to D.H.O. and B.C. also supported a portion of the fieldwork. T.J.O. was supported by the National Science Foundation (IOB-0517041). These studies

adhered to the Guidelines for the use of Animals in Research and were approved by the IACUC at the University of California, Davis (Protocol Numbers 10485, 9145 and 10734).

References

Adkins, R.M., Gelke, E.L., Rowe, D. & Honeycutt, R.L. 2001. Molecular phylogeny and divergence time estimates for major rodent groups: evidence from multiple genes. *Mol. Biol. Evol.* **18**: 777–791.

Alcock, J. 1997. *Animal Behavior: An Evolutionary Approach*, 6th edn. Sinauer Asociates, Sunderland, MA.

Alexy, K.J., Gassett, J.W., Osborn, D.A. & Miller, K.V. 2001. Remote monitoring of scraping behaviors of a wild population of white-tailed deer. *Wildlife Soc. Bull.* **29**: 873–878.

Akersten, W.A., Reynolds, R.L. & Tejada-Flores, A.E. 1979. New mammalian records from the late Pleistocene of Rancho La Brea. *Bull. So. Calif. Acad. Sci.* **78**: 141–143.

Benjamini, Y. & Hochberg, Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Series B Stat. Methodol.* **57**: 289–300.

Berger, J., Swenson, J.E. & Persson, I.-L. 2001. Recolonizing carnivores and naïve prey: conservation lessons from Pleistocene extinctions. *Science* **291**: 1036–1039.

Berridge, K.C. 1990. Comparative fine structure of action: rules of form and sequence in the grooming patterns of six rodent species. *Behaviour* **113**: 1–22.

Black, C. 1963. A review of North American tertiary Sciuridae. *Bull. Mus. Comp. Zool. Harvard Univ.* **130**: 113–248.

Blumstein, D.T. 2006. The multipredator hypothesis and the evolutionary persistence of antipredator behavior. *Ethology* **112**: 209–217.

Blumstein, D.T., Daniels, J.C. & Springett, B.P. 2004. A test of the multi-predator hypothesis: rapid loss of antipredator behavior after 130 years of isolation. *Ethology* **110**: 919–934.

Blumstein, D.T., Evans, C. & Daniels, J.C. 2006. JWatcher 1.0. Available at: http://www.jwatcher.ucla.edu/.

Brinck, C., Erlinge, S. & Sandell, M. 1983. Anal sac secretion in mustelids – a comparison. *J. Chem. Ecol.* **9**: 727–745.

Burnham, K.P. & Anderson, D.R. 2004. Multimodel inference: understanding AIC and BIC in model selection. *Sociol. Methods Res.* **33**: 261–304.

Campbell, J.A. & Lamar, W.W. 2004. *Venomous Reptiles of the Western Hemisphere*, vol **2**. Cornell University Press, New York.

Carbone, C., Maddox, T., Funston, P.J., Mills, M.G.L., Grether, G.F. & Van Valkenburgh, B. 2009. Parallels between playbacks and Pleistocene tar seeps suggest sociality in an extinct sabretooth cat Smilodon. *Biol. Lett.* **5**: 81–85.

Carrasco, M.A., Kraatz, B.P., Davis, E.B. & Barnosky, A.D. 2005. Miocene Mammal Mapping Project (MIOMAP). University of California Museum of Paleontology. Available at: http://www.ucmp.berkeley.edu/miomap/.

Clucas, B., Rowe, M.P., Owings, D.H. & Arrowood, P.C. 2008a. Snake scent application in ground squirrels (*Spermophilus* spp.): a novel form of antipredator behavior? *Anim. Behav.* **75**: 299–307.

Clucas, B., Owings, D.H. & Rowe, M.P. 2008b. Donning your enemy's cloak: ground squirrels exploit rattlesnake scent to reduce predation risk. *Proc. Biol. Sci.* **275**: 847–852.

Cohen, J. 1969. *Statistical Power Analysis for the Behavioral Sciences*. Academic Press, New York, NY.

Coss, R.G. 1999. Effects of relaxed natural selection on the evolution of behavior. In: *Geographic Variation in Behavior* (S.A. Foster & J.A. Endler, eds), pp. 180–208. Oxford University Press, New York.

Coss, R.G., Guse, K.L., Poran, N.S. & Smith, D.G. 1993. Development of antisnake defenses in California ground squirrels (*Spermophilus beecheyi*): II. Microevolutionary effects of relaxed selection from rattlesnakes. *Behaviour* **120**: 137–164.

Field, A. 2005. *Discovering Statistics Using SPSS*, 2nd edn. Sage Publications, London.

Gloyd, H.K. & Conant, R. 1990. Snakes of the Agistrodon complex – a monographic review. *Society for the Study of Amphibians and Reptiles, Contributions to Herpetology*, 6, Oxford, Ohio.

Graham, R.W., Lundelius, E.L. Jr, Graham, M.A., Schroeder, E.K., Toomey, R.S. III, Anderson, E., Barnosky, A.D., Burns, J.A., Churcher, C.S., Grayson, D.K., Guthrie, R.D., Harington, C.R., Jefferson, G.T., Martin, L.D., McDonald, H.G., Morlan, R.E., Semken, H.A. Jr, Webb, S.D., Werdelin, L. & Wilson, M.C. (FAUNMAP Working Group). 1996. Spatial response of mammals to late quaternary environmental fluctuations. *Science* **272**: 1601–1606.

Gruhn, R. 1961. The archaeology of Wilson-Butte Cave, south-central Idaho. *Occas. Pap. Ida. St. Coll. Mus.* **6**: 1–198.

Guilday, J.E. & Adam, E.K. 1967. Small mammal remains from Jaguar Cave, Lemhi County, Idaho. *Tebiwa.* **10**: 23–36.

Harris, A.H. 1985. *Late Pleistocene Vertebrate Paleoecology of the West*. University of Texas Press, Austin.

Harrison, R.G., Bogdanowicz, S.M., Hoffmann, R.S., Yensen, E. & Sherman, P.W. 2003. Phylogeny and evolutionary history of ground squirrels (Rodentia: Marmotinae). *J. Mammal. Evol.* **10**: 249–276.

Herron, R.M., Castoe, T.A. & Parkinson, C.L. 2004. Sciurid phylogeny and the paraphyly of Holartric ground squirrels (*Spermophilus*). *Mol. Phylogenet. Evol.* **31**: 1015–1030.

Holman, J.A. 1977. Amphibians and reptiles from the gulf coast Miocene of Texas. *Herpetologica* **33**: 391–403.

Holman, J.A. 1979. A review of North American tertiary snakes. *Publ. Mus. Mich. State Univ. Paleontol. Ser.* **1**: 203–260.

Holman, J.A. 1995. *Pleistocene Amphibians and Reptiles in North America*. Oxford University Press, New York.

Holman, J.A. 2000. *Fossil Snakes of North America*. Indiana University Press, Bloomington.

Hudson, D.M. & Brattstrom, B.H. 1977. A small herpetofauna from the Late Pleistocene of Newport Beach Mesa, Orange County, California. *Bull. So. Calif. Acad. Sci.* **76**: 16–20.

Johnson, E. 1987. Vertebrate remains. In: *Late Quaternary Studies on the Southern High Plains* (E. Johnson, ed.), pp. 49–89. Texas A&M Press, College Station, Texas.

Kobayashi, T. & Watanabe, M. 1986. An analysis of snake-scent application behavior in Siberian chipmunks (*Eutamias sibiricus asiaticus*). *Ethology* **72**: 40–52.

Korth, W.W. 1994. *The Tertiary Record of Rodents in North America*. Plenum Press, New York.

LaDuke, T.C. 1991. The fossil snakes of Pit 91, Rancho La Brea, California. *Contr. Sci. Nat. Hist. Mus. Los Angeles Co.* **424**: 1–28.

Lahti, D.C., Johnson, N.A., Ajie, B.C., Otto, S.P., Hendry, A.P., Blumstein, D.T., Coss, R.G., Donohue, K. & Foster, S.S. 2009. Relaxed selection in the wild. *Trends Ecol. Evol.* **24**: 487–496.

Li, G., Steel, M. & Zhang, L. 2008. More taxa are not necessarily better for the reconstruction of ancestral character states. *Syst. Biol.* **57**: 647–653.

Logan, L.E. 1983. Paleoecological implications of the mammalian fauna of Lower Sloth Cave, Guadalupe Mountains, Texas. *Natl. Speleo. Soc. Bull.* **45**: 3–11.

Logan, L.E. & Black, C.C. 1979. The Quaternary vertebrate fauna of Upper Sloth Cave Guadalupe Mountains National Park, Texas. *NPS. Proc. Trans. Ser.* **4**: 1–442.

Maddison, W.P. & Maddison, D.R. 2008. Mesquite: a modular system for evolutionary analysis. Version 2.5. Available at: http://mesquiteproject.org.

Marshall, C.R. 2008. A simple method for bracketing absolute divergence times on molecular phylogenies using multiple fossil calibration points. *Am. Nat.* **171**: 726–742.

Mead, J.I. & Bell, J.C. 1994. Late Pleistocene and Holocene herpetofaunas of the Great Basin and Colorado Plateau. In: *Natural History of the Colorado Plateau and Great Basin* (K.T. Harper, L.L. St. Clair, K.H. Thorne & W.M. Hess, eds), pp. 255–275. University Press of Colorado, Boulder, CO.

Mead, J.I., Thompson, R.S. & Van Devender, T.R. 1982. Late Wisconsinan and Holocene fauna from Smith Creek Canyon, Snake Range, Nevada. *Trans. San Diego Soc. Nat. Hist.* **20**: 1–26.

Mead, J.I., Roth, E.L., Van Devender, T.R. & Steadman, D.W. 1984. The late Wisconsinan vertebrate fauna from Deadman Cave, Southern Arizona. *Trans. San Diego Soc. Nat. Hist.* **20**: 247–276.

Mercer, J.M. & Roth, V.L. 2003. The effects of Cenozoic global change on squirrel phylogeny. *Science* **299**: 1568–1572.

Messler, A., Wund, M.A., Baker, J.A. & Foster, S.A. 2007. The effects of relaxed and reversed selection by predators on the antipredator behavior of the threespine stickleback, *Gasterosteus aculeatus*. *Ethology* **113**: 953–963.

Owen, P.R., Bell, C.J. & Mead, E.M. 2000. Fossils, diet, and conservation of black-footed ferrets (*Mustela nigripes*). *J. Mammal.* **81**: 422–433.

Owings, D.H. & Coss, R.G. 1977. Snake mobbing by California ground squirrels – adaptive variation and ontogeny. *Behaviour* **62**: 50–69.

Pagel, M. 1994. Detecting correlated evolution on phylogenies: a general method for the comparative analysis of discrete characters. *Proc. R. Soc. Lond. B Biol. Sci.* **255**: 37–45.

Pagel, M. & Meade, A. 2006. Bayesian analysis of correlated evolution of discrete characters by reversible-jump Markov chain Monte Carlo. *Am. Nat.* **167**: 808–825.

Parker, S.P., ed. 1990. *Grzimek's Encyclopedia of Mammals*, Vol. **3**, McGraw-Hill Publishing Company, New York.

Piaggio, A.J. & Spicer, G.S. 2001. Molecular phylogeny of the chipmunks inferred from mitochondrial cytochrome *b* and cytochrome oxidase II gene sequences. *Mol. Phylogenet. Evol.* **20**: 335–350.

Poran, N.S., Coss, R.G. & Benjamini, E. 1987. Resistance of California ground-squirrels (*Spermophilus beecheyi*) to the venom of the northern Pacific rattlesnake (*Crotalus viridis oreganus*) – a study of adaptive variation. *Toxicon* **25**: 767–777.

Rundus, A.S., Owings, D.H., Joshi, S.S., Chinn, E. & Giannini, N. 2007. Ground squirrels use an infrared signal to deter rattlesnake predation. *Proc. Natl Acad. Sci. USA* **104**: 14372–14376.

Salisbury, B.A. & Kim, J. 2001. Ancestral state estimation and taxon sampling density. *Syst. Biol.* **50**: 557–564.

Schulz, K.D. 1996. *A Monograph of Colubrid Snakes of the Genus Elaphe Fitzinger*. Koeltz Scientific Books, Canada.

SPSS. 2007. *SPSS for Mac OS X, Rel. 16.0.1GP, 2007.* SPSS Inc, Chicago.

Theodoratus, D.H. & Chiszar, D. 2000. Habitat selection and prey odor in the foraging behavior of western rattlesnakes (*Crotalus viridis*). *Behaviour* **137**: 119–135.

Thompson, R.S. & Mead, J.I. 1982. Late Quaternary environments and biogeography of the Great Basin. *Quat. Res.* **17**: 39–55.

Whorley, J.R., Alvarez-Castaneda, S.T. & Kenagy, G.J. 2004. Genetic structure of desert ground squirrels over a 20-degree-latitude transect from Oregon through the Baja California peninsula. *Mol. Ecol.* **13**: 2709–2720.

Xu, Z.J., Stoddart, D.M., Ding, H.B. & Zhang, J. 1995. Self-anointing behavior in the rice-field rat, *Rattus rattoides*. *J. Mammal.* **76**: 1238–1241.

Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1 Phylogeny of ground squirrels and chipmunk outgroup species.

Figure S2 Rattlesnake arena (0.63 × 0.51 × 0.80 m) (a) starting chamber, (b) foraging arena, (c) divider, and (d) video camera affixed to plexiglass arena cover.

Table S1 Species and study locations of species tested with snake, weasel and deer scent and sample sizes and proportions of individuals that applied scent for each location.

Table S2 Pliocene and Miocene co-occurrences of squirrel, rattlesnake and weasel ancestors.

Table S3 Proportions of Pleistocene (1.8 million–100 thousand year ago) fossil sites with predator fossils and proportions of current squirrel-predator co-occurrence.

Table S4a Transition parameters for the character states of rattlesnake presence (Pred) and rattlesnake scent application (SSA) and results for the independent and dependent correlated traits models (Pagel 1994) for rattlesnake presence and scent application.

Table S4b Transition parameters for the character states of weasel presence (Pred) and weasel scent application (WSA) and results for the independent and dependent correlated traits models (Pagel 1994) for weasel presence and scent application.

Table S5 Akaike information criterion (AIC) and likelihood values (LD) for dependent model selection.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

Received 26 April 2010; revised 20 June 2010; accepted 13 July 2010