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

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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 rattle-snake 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

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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 rattle-snake 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.
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 squirls 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 non-predator 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 (non-predator). 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).

Sympathy 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 estimate 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 $LR = -2\log_e \left[ \frac{L_I}{L_D} \right]$, 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 $\alpha$ of 0.05 and for a total of four statistical comparisons. Corresponding FDR corrections to $\alpha$ 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
It is 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.

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 ($q_{ij}$; 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 $3 \rightarrow 4$ [$q_{34}$] with $1 \rightarrow 2$ [$q_{12}$]) and is more likely to be lost in the absence of rattlesnakes (compare $2 \rightarrow 1$ [$q_{21}$] with $4 \rightarrow 3$ [$q_{43}$]). (b) Best restricted dependent model (see Table A5; thicker arrows represent most likely transitions and point to most stable states) in which the transitions $q_{21}, q_{12}, q_{34}$ and $q_{43}$ are equal to each other and $q_{13}, q_{14}$ and $q_{42}$ are equal to each other.
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 antointing 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 squirrel. Their diet in captivity consisted of a prekilled squirrel and kept behind a plastic shield out of view until the scented mouse was placed inside a wire frame inside the burrow to prevent consumption by the snake if found within 25% of the true value).

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 before 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 \( z \) 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 \( \geq 0.37 \) (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 sympathy 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- \( z = 0.05 \); 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- \( z = 0.025 \); Table S4b). Thus, the evolution of weasel scent application is not significantly correlated with the presence of weasel predators.
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 \([LH \text{ independent model} = -10.68, LH \text{ dependent model} = -7.08, LR = 7.09, d.f. = 4, P = 0.131 (FDR-\alpha = 0.038)]\), nor was the analysis testing if weasel presence was correlated with predator scent application \([LH \text{ independent model} = -9.35, LH \text{ dependent model} = -8.56, LR = 1.57, d.f. = 4, P = 0.813 (FDR-\alpha = 0.013)]\).

Ancestral state reconstruction

Parsimony ancestral state reconstructions of weasel scent application revealed 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 suggested 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: 0.30–0.80).

Table 1 Pleistocene squirrel and predator fossil co-occurrence.

<table>
<thead>
<tr>
<th>Thousand years ago</th>
<th>Squirrel species</th>
<th>Weasel species</th>
<th>Rattlesnake species</th>
</tr>
</thead>
<tbody>
<tr>
<td>~10</td>
<td>Spermophilus lateralis: CO1</td>
<td>Mustela erminea: CO1</td>
<td>Crotalus atrox: TX1</td>
</tr>
<tr>
<td>~11</td>
<td>Spermophilus variegatus: CO1</td>
<td>Mustela frenata: CO1</td>
<td></td>
</tr>
<tr>
<td>~11.6</td>
<td>S. variegatus: TX2</td>
<td>M. frenata: TX2</td>
<td></td>
</tr>
<tr>
<td>~11.9–1.5</td>
<td>S. lateralis: CO2</td>
<td>M. erminea: CO2</td>
<td></td>
</tr>
<tr>
<td>~11.8–10.4</td>
<td>S. lateralis: ID2</td>
<td>M. frenata: ID2, TX3</td>
<td>Crotalus spp: MXI</td>
</tr>
<tr>
<td>~12–8</td>
<td>S. variegatus: AZ1</td>
<td></td>
<td>Crotalus atrox: AZ1</td>
</tr>
<tr>
<td>~13–12</td>
<td>S. variegatus: AZ2</td>
<td></td>
<td>Crotalus scutulatus: AZ1</td>
</tr>
<tr>
<td>~14.5–6.1</td>
<td>S. lateralis: MT1</td>
<td>M. frenata: MT1</td>
<td></td>
</tr>
<tr>
<td>~15.9–14.5</td>
<td>Spermophilus beldingi: ID1</td>
<td>M. erminea: ID1</td>
<td></td>
</tr>
<tr>
<td>~20–10</td>
<td>S. lateralis: CA8</td>
<td>M. frenata: CA8, CA9</td>
<td></td>
</tr>
<tr>
<td>~24–10</td>
<td>S. lateralis: WY1</td>
<td>M. frenata: WY1</td>
<td></td>
</tr>
<tr>
<td>~25.5–18.1</td>
<td>S. variegatus: NM2</td>
<td>M. frenata: NM2</td>
<td>Crotalus viridis: NV1</td>
</tr>
<tr>
<td>~35–12.6</td>
<td>S. lateralis: NV1</td>
<td>M. erminea: NV1</td>
<td>C. vitidis: CA7</td>
</tr>
<tr>
<td>~35–25</td>
<td>Spermophilus beecheyi: CA7</td>
<td>M. frenata: CA7, NM1</td>
<td>C. vitidis: NM1, NM2</td>
</tr>
<tr>
<td>~40–10</td>
<td>S. beecheyi: CA4</td>
<td>M. frenata: CA4</td>
<td>C. vitidis: CA4</td>
</tr>
<tr>
<td>~40</td>
<td>S. beecheyi: CAI</td>
<td>M. frenata: CAI</td>
<td>C. vitidis: CAI</td>
</tr>
<tr>
<td>~110–10</td>
<td>S. beecheyi: CA2, CA3, CA9</td>
<td>M. frenata: CA3, CA9</td>
<td>C. vitidis: CA2, CA3</td>
</tr>
<tr>
<td>~400–10</td>
<td>S. beecheyi: CA5</td>
<td></td>
<td>C. vitidis: CA5</td>
</tr>
</tbody>
</table>

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

**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 (\(\sim 34–24\) 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.

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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**


Evolutionary history of an antipredator behaviour

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.

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