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Isolation rearing reveals latent antisnake behavior in California ground squirrels (*Otospermophilus beecheyi*) searching for predatory threats

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Abstract This study of California ground squirrels (*Otospermophilus beecheyi*) investigated the long-term effects of isolation rearing on alarm-call recognition. Six wild-caught squirrels, trapped as yearlings, and six laboratory-reared squirrels were maintained in solitary cages for approximately 3 years prior to the study. Visual searching and olfactory searching were measured as squirrels emerged from their burrow-like nest box into a laboratory room after hearing repetitive playbacks of alarm calls or control sounds consisting of pulses of white-noise or ambient laboratory sounds. Before exiting completely after hearing alarm calls, both groups exhibited similar levels of visual searching that was reliably higher than after hearing the other sounds. After exiting completely, the laboratory-reared squirrels exhibited a reliably greater amount of olfactory investigation than the wild-caught squirrels. Five laboratory-reared squirrels turned around after exiting and inspected their dark nest-box opening, three of which tail flagged repeatedly and one threw substrate into the opening. Since pups recognize snakes and engage in this behavior, this latent expression of antisnake behavior illustrates its robust organizational properties in the appropriate burrow-like context irrespective of the presumed retardation of neural development known to occur in other species of rodent subjected to similar isolation rearing.

Keywords California ground squirrels · Alarm calls · Innate antisnake behavior · Isolation rearing · Nest-box exiting · Olfactory investigation · Visual searching

Introduction

The antipredator behavior of California ground squirrels (*Otospermophilus beecheyi*), a species that occupies subterranean and terrestrial habitats, has been extensively studied and described (Hanson and Coss 1997; Owings and Leger 1980; Owings and Coss 2008). Like other species (Boinski et al. 2003), their antipredator behavior is often expressed by anticipatory vigilance that can inform them about the state of their surroundings and can allow them to differentiate between dangerous and routine situations. Vigilant behavior is often provoked by conspecific alarm calls, which are extremely salient and nearly always provoke physiological arousal and behavioral responses (Owings and Virginia 1978; Owren and Rendall 1997). Alarm calls can also act as Pavlovian unconditioned stimuli (Griffin 2004; Lea et al. 2008). The evocative nature of alarm calls may be an outcome of their intensity, structure, or temporal patterning (see Morton 1977). The effectiveness of these calls could also reflect their recognizable information-related properties as signals (Rendall and Owren 2013; Stegmann 2013) or might even suggest the presence of innate recognition systems in perceivers (Davies et al. 2004).

Antipredator vocalizations have been argued to provide information about external referents, including the type and behavior of predators, the arousal level of the vocalizer, or the location of the perceived threat (Coss et al. 2007; Owings and Leger 1980; Seyfarth et al. 1980; Strusaker 1967). Such behavior has been observed in California ground squirrels (Owings and Hennessy 1984). The locomotor and vocal behavior of ground squirrels varies during the differently urgent contexts associated with the detection of raptors, mammals, and snakes (Leger and Owings 1978; Owings and Hennessy 1984; Owings and Virginia 1978).

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Avian predators can appear suddenly and swoop toward a squirrel, allowing little time for escape and eliciting the least deliberative evasive behavior (Fitch et al. 1946; Hanson and Coss 1997). Ground squirrels respond to the detection of a rapidly moving raptor by emitting whistle alarm calls and fleeing indiscriminately toward the nearest cover (Hanson and Coss 1997; Leger et al. 1979). Once detected, stealthily approaching mammalian predators can be predictable and avoided in an organized manner. Squirrels detecting a stealthily approaching mammal typically assume positions near burrows or on promontories from which they monitor the predator's movements, often emitting chatter vocalizations (Leger et al. 1979; Owings et al. 1986; Owings and Leger 1980). Ground squirrels can also hear alarm calls from within their burrows. Such alarm-call transmission within burrows has been demonstrated in other fossorial mammals (Heth et al. 1986). Groups of ground squirrels have been observed to emerge from their burrows seconds after hearing alarm calls and to then briefly scan their surroundings.

Throughout a ground squirrel's ontogeny, antipredator vocalizations are nearly universally provocative. Initially, their predictive properties appear to be unrefined; these vocalizations primarily incite arousal (Mateo 1996a, b). This activated physiological condition could be a requirement for an important course of directed learning to occur (Gould and Marler 1987). In ground squirrels, directed learning involves the maturation of perceptual processes important in the recognition of predators and in the execution of antipredator behavior (cf. Mateo 1996a, b; Palleroni et al. 2005; Seyfarth and Cheney 1980). Compared with experienced adults, immature California ground squirrels venturing above ground are chronically aroused physiologically without any obvious provocation (Hanson and Coss 2001a). As a result, they engage in higher levels of undirected environmental assessment. They also exhibit distinctly inferior antipredator tactics related to mapping escape routes on local terrains, are less discriminating than adults about the differing levels of risk posed by avian versus mammalian predators, and are less adept at executing appropriate responses to different antipredator vocalizations (Hanson and Coss 1997; Loughry and McDonough 1989; Mateo 1996a, b).

Risk assessment during burrow emergence

The behavioral properties of environmental assessment as manifested by visual searching should reflect the spatial locations from which major predators launch attacks. As with other species, squirrels searching for avian predators should rotate or elevate their heads to direct the eyes upward. Squirrels searching for mammalian predators should orient their heads laterally. Those searching for snakes

should adopt an elongate posture while keeping the eyes and noses near the ground for maximum information acquisition. If a predator is actually detected, a squirrel's head motion should be arrested and the target fixated for further assessment (cf. McAdam and Kramer 1998; Quenette 1990; Treves 2000).

Conceptually, an annulus of territory surrounding the burrow opening represents a critical region wherein there is a nearly instantaneous transition in the urgency and configuration of predatory contexts as squirrels move between the subterranean and terrestrial environments (Coss and Goldthwaite 1995; Coss and Owings 1985; Tromborg 1998). The antipredator behavior of ground squirrels can be viewed within two contexts as they emerge from burrows: a preemergent condition in which vision is more restricted and olfaction and audition predominate in environmental assessment and a postemergent condition in which, while vision predominates, all sensory modalities are employed. Preemergent squirrels must maneuver from subterranean locations where visual cues are absent to terrestrial vantage points where vision becomes effective. Postemergent squirrels can employ these vantage points to assess their surroundings in order to detect changes that might have occurred since the previous surveillance period. For ground squirrels, this type of interruption in the availability of information occurs cyclically during their nocturnal retirement into burrows.

Experimental questions and predictions

This study investigated the manner in which laboratory-reared and wild-caught California ground squirrels differed in their responses after hearing alarm calls and control sounds in a laboratory setting. Visual searching and olfactory searching were investigated when squirrels first emerged from their nest boxes into a laboratory room simulating an outdoor setting after an overnight interruption in the opportunity to engage in environmental assessment. We predicted that, after hearing alarm calls, wild-caught ground squirrels would engage in elevated levels of visual and olfactory searching upon initial emergence from their nest boxes, but would reduce this after the animal had emerged completely. Under the same conditions, laboratory-reared squirrels were predicted to be less discriminating in the response to the sound treatments and engage in lower levels of visual and olfactory searching.

Methods

The following research was approved by the University of California, Davis, Institutional Animal Care and Use Committee under protocol UCD-5486. Social isolation

after weaning is known to affect the brain development of laboratory rats within as little as 30 days, and only groups with small sample sizes have been compared employing isolation rearing for more than 1 year (cf. Greenough and Volkmar 1973; Green et al. 1983). Similar isolation rearing affecting primate brain development has also employed small sample sizes (Bryan and Riesen 1989). In our protocol, the time frame of social isolation of laboratory-reared ground squirrels was more than 3 years. Consistent with the 1991 ABS/Animal Behaviour guidelines for the use of animals in research, we subjected small numbers of ground squirrels to isolation rearing with the expectation that experimental treatments would still yield high statistical power and reliable treatment effects.

Subjects

Two groups, each composed of six California ground squirrels with different developmental histories, served as the subjects for this study. The first group of squirrels, comprised of one male and five females, was captured using Tomahawk live traps at approximately one year of age at Camp Ohlone, in the mixed oak woodlands and grasslands of Sunol Regional Wilderness, Alameda County, California. Although many older male and female adults were trapped, the unequal sex ratio of yearling females reflected their site fidelity unlike their dispersing male counterparts. To our knowledge, there is no published evidence that males and females differ substantially in their urgent responses to avian and mammalian predatory threats or visual surveillance (cf. Arenz and Leger 1997; Hanson and Coss 1997). The second group of squirrels was obtained from mothers trapped in the same location as the first group. The degree of relatedness among these females was unknown. Two females produced two litters, comprised of three males and three females. The animals were allowed to remain with their mothers until fully weaned at 60 days of age. Prior to the study, the wild-caught group was maintained for approximately 30 months in the Animal Care Facility of the Psychology Department, University of California, Davis, where the captured mothers gave birth to the laboratory-reared squirrels. The six wild-caught and six lab-born squirrels were housed in the same room and not used in any other research until this experiment. At the time of the study, the wild-caught squirrels were at least 50 months of age and the laboratory-reared squirrels at least 40 months of age. Immediately preceding the inception of research, subjects ranged in weight from 494 to 1000 g, averaging 722 g.

Rearing and maintenance conditions

All 12 subjects were maintained in wire-screened cages (39 × 56 cm floor and 25 cm height). Each cage featured

a detachable attached nest box (22.5 × 38 cm floor and 22 cm height). Squirrels were maintained continuously in these cages for periods ranging from 24 to 27 months. These cages afforded restricted visual and acoustic variability. The walls of the animal maintenance room and experimental room were uniformly cream colored, evenly distributing light from fluorescent fixtures at an intensity of 598 lux footcandles. The animals were maintained under a 12-h photoperiod extending from 07:00 to 19:00 on a daily basis. The temperature of the maintenance and experimental rooms ranged between 20° and 21° C. The subjects had views of other wire-mesh cages, housing other squirrels but could not interact with them physically. The acoustic conditions were characterized by sound pressure levels (SPL) ranging from an average minimum of 40 dB during evening hours to an average maximum of 75 dB during daytime maintenance procedures, typical values for contemporary animal care facilities (Tromborg and Coss 1995). Sound pressure levels increased with the punctate noises generated with cage cleaning and ground squirrels reacting to this procedure. The animals were provided with various preparations of either Purina or PMI rodent diet (#5001 or #5014) and water on an ad libitum basis. Occasionally, this diet was supplemented with additional nuts, fruits, vegetables, and grains.

Experimental setting

The venue for the experiment consisted of two adjacent rooms in the animal care facility, an observation room and a treatment room. These rooms had floor dimensions of 2.43 × 2.90 m and a height of 2.53 m. The observation room contained video and audio equipment, including a Sony WV-CL702 0.5 lux color video camera coupled to a FOR.A VTG-22 video field time generator, and a Panasonic VHS AG-6730 time-lapse surveillance video recorder. This room was unilluminated and equipped with a one-way mirrored window for video recording the interior of the treatment room, where the subjects were presented with acoustic treatments.

The treatment room had the same cream colored walls as the animal maintenance room, evenly distributing light from an array of three ceiling-mounted fluorescent fixtures at an average illumination intensity of 598 lux, measured at floor level with a Minolta LX-100 photometer. Lighting conformed to a 12:12 L/D cycle and was automatically timed to begin illumination at 07:00 and to terminate illumination at 19:00. Video and audio recordings were initiated simultaneously with the onset of room illumination. The floor of the room was covered with pine shavings and featured two trays in one corner to hold commercial rodent diet and water.

The treatment room was where acoustic treatments were presented to subjects. The subject's nest box, centered in the treatment room, served both as the primary refuge for the subjects and as the focal point for video recording the first time that the squirrel exited from its nest box into the designated video-recording area. Close-up video recording of behavior was accomplished by focusing the video camera through the one-way mirrored window via a 44-cm diameter convex mirror suspended from the ceiling of the treatment room over the focal area in front of the nest box.

Acoustic treatments were administered with a Tascam 202 MK II tape recorder coupled to a Teac A-20 10-band graphic equalizer and a SoundTech PL-150 power amplifier. Sound playback treatments were administered through four Realistic Optimus 7 loudspeakers (40 Hz–18 kHz) suspended from the ceiling in each corner of the treatment room 1 m above the floor. A centrally positioned Radio Shack Model 33-20-50 analog sound pressure meter provided a means for calibrating the intensity of the acoustic treatments. It also allowed the intensity of ambient (typical background) sounds in the treatment room to be monitored. This room was partially acoustically isolated from the rest of the Animal Care Facility, with extraneous sounds with frequencies between 1 and 4 kHz being attenuated by up to 20 dB. In the absence of sound treatments, the intensity of ambient noise in the room averaged 40 dB. Squirrels in their nest boxes experienced a further attenuation in sound pressure level.

Procedures

Each squirrel was transferred from the maintenance room in its detachable nest box to the treatment room, where each squirrel was allowed to habituate to the experimental setting for 2 days prior to the administration of acoustic treatments. Once in the treatment room, squirrels could enter or leave their nest boxes at any time. The administration of treatments began on the third day that each subject was held in the treatment room.

Acoustic treatments were presented on successive days and were assigned in a balanced randomized order. If the laboratory ambience condition was the first video-recorded treatment, the squirrels experienced 3 days of room habituation with no predictive consequences. Otherwise, they had 2 days to habituate. Throughout the study, lighting and video recording were all automatically initiated at 07:00. The presentation of alarm calls began shortly thereafter, with the first presentation beginning 1–5 min after the onset of video recording. Video recording and acoustic playbacks were continued until 10:00, when sound playbacks were automatically terminated. Step-lock video recordings were made at 433 ms time steps. Again, only the first episode of nest-box exiting was analyzed and no

squirrel ever exited its nest box before hearing the acoustic treatments. At the end of the fifth day, the final treatment day, the squirrel was returned in its nest box to its home cage in the maintenance room.

Acoustic treatments

The following three sound conditions were examined: (a) typical laboratory ambience of 40 dB, (b) white noise of different durations, and (c) ground squirrel antipredator vocalizations that consisted of two whistle and two chatter calls. These vocalizations were recorded in the field near Winters, California, from squirrels in wire-cage traps in response to the researcher moving toward the traps. Recording were made using a Sony model TC-D5 Pro II audio recorder coupled to a Sennheiser directional microphone (ME 80 capsule) held at distances of 1–2 m from the squirrels. The analog sound recordings were processed digitally with an Apple Macintosh computer employing the Audio Media digital sound-processing system and then edited to filter out any background noise below 500 Hz. Any spurious sounds immediately adjacent to the exemplars were also removed.

We selected for playback one juvenile three-chatter exemplar (330 ms duration), one adult four-chatter exemplar (550 ms duration), one juvenile whistle exemplar, and one adult whistle exemplar (Fig. 1). Both chatter exemplars had a fundamental frequency near 3.8 kHz, with second and third harmonics near 6.0 and 10 kHz. They were arranged into 20 clusters of 10 juvenile and 10 adult chatters, alternating with three clusters of juvenile and adult whistles in a quasi-random pattern. These clusters ranged in duration from 10 to 30 s (mean = 24 s), with each phrase element within the cluster separated by an interval ranging from 1 to 10 s. The interstimulus intervals of chatter-whistle clusters ranged from 54 to 1140 s (mean = 414 s), producing 180 min of intermittent alarm calls simulating the temporal variation of alarm calls heard by squirrels foraging in the morning (Donald Owings personal communication).

The white-noise treatment sounds were generated by a Marantz model 10 B high-frequency analyzer. This unit produced relatively pure white-noise program material with equivalent energy distributions at all frequencies between 40 Hz and 15 kHz. There were four slightly different white-noise exemplars, each approximating the duration of one of the four chatter-whistle exemplars. Individual white-noise segments had durations ranging from 150 to 500 ms (mean = 300 ms), yielding 180 min of program material. Playbacks of alarm calls and white noise were presented at sound intensities ranging from 80 to 90 db (SPL) measured at a distance of 1.5 m from the four loudspeakers. These intensities are equivalent to those

Fig. 1 Sound spectrograms of two exemplars of whistle and chatter alarm vocalizations

emitted by trapped squirrels in the field from similar distances (Hanson and Coss 2001b).

Behavioral measures

Two broad categories of investigative behaviors, direction of gaze and substrate sniffing, were examined during nest-box emergence. Both types of behavior were assessed under two different risk-related contexts: (a) before exiting, when partially exposed squirrels were theoretically less vulnerable to predation, and (b) after exiting, when fully exposed squirrels were theoretically more vulnerable to predation. Sampling of the before-exiting context started with the video frame in which the squirrel's head protruded far enough outside its nest box that the trailing edges of its ears were visible and ended with the video frame in which

the squirrel had moved forward so the base of its tail was visible. Sampling of the after-exiting context began with the next video frame up until the video frame in which the squirrel's eyes were no longer visible as it left the camera's field of view in front of its nest box (see Fig. 2).

Direction of gaze during nest-box emergence was categorized as either (1) upward, featuring a pause with the nose elevated with both eyes facing the overhead mirror, or rolling the head sideways so that one eye faced the camera, or (2) horizontal, featuring a pause in either lateral displacement or pause in the forward motion of the squirrel's head. The dark irises and conspicuous white eyerings of the squirrels provided an unambiguous index of upward head orientation (Fig. 2).

Each bout of pausing between head motions, either upward or horizontal, was treated as a unitary action for measuring the duration of gazing bouts. Using a Panasonic AG 6300 videotape recorder and Sony Trinitron 33-cm color monitor, these durations were measured from the video frame numbers in which head motion ceased until the

Fig. 2 Video still-field images of different ground squirrels exiting their nest boxes while looking upwards (a), as revealed by the upward tilt of the head, and looking horizontally (b)

video frame numbers in which head motion resumed. The duration of bouts of substrate sniffing was measured from the video frame in which the head was tilted downwards until the video frame in which the head became horizontal with the substrate. The precision of these duration measures was imposed by the 433-ms video frame interval of the step-lock video recorder, albeit within a single frame, any squirrel motion could be detected between the two interlaced field scans producing a single frame. The durations of pausing bouts for gazing and sniffing were summed for separate statistical analyses of the before- and after-exiting contexts.

Because the before- and after-exiting conditions differed in behavioral contexts and durations, these conditions were analyzed separately for the two behavioral measures. Data were analyzed using GANOVA, a general linear model statistical program (see Woodward et al. 1990) using one-factor between-subjects (wild-caught and laboratory-reared squirrels), one-factor within-subjects (three sound treatments) repeated measures analyses of variance (ANOVAs). Tests of simple effects examined group differences for each sound treatment and differences among sound treatments within each group. Because specific effects were hypothesized for alarm calls and white noise, planned comparisons were made to examine pairwise mean differences, irrespective of the statistical significance of main effects (see Winer 1962, p. 208) including comparing the mean for the chatter-whistle treatment with the average of the white-noise and ambient laboratory treatments. The reported standardized effect sizes for comparing two mean values were generated using *t* tests (see Cohen 1992).

Results

Gaze duration before exiting

Both groups of squirrels engaged in higher levels of visual searching under the alarm-call treatment than under the other two treatments (Fig. 3a). Although the main effect for groups, averaged for sound treatments, and the interaction between groups and sound treatment were not statistically significant, the main effect for sound treatment, averaged for both groups, was significant, $F_{2,20} = 7.37$; $P < 0.005$, power = 0.78. Averaged for groups, the chatter-whistle treatment was associated with significantly more gazing before exiting than the combined average of the laboratory ambience and white-noise treatments, $F_{1,10} = 8.48$; $P < 0.025$, power = 0.82). Tests of simple effects revealed that both groups differentiated the three sound treatments (laboratory reared: $F_{2,20} = 3.72$; $P < 0.05$; wild-caught: $F_{2,20} = 3.82$; $P < 0.05$). Planned comparisons for each group revealed that the chatter-

Fig. 3 Gaze duration before exiting the nest box completely (a) and after exiting completely (b). Mean and standard error values are shown. Both laboratory-reared and wild-caught ground squirrels exhibited similar patterns of gaze behavior for the three sound treatment conditions before exiting completely. Unlike the wild-caught squirrels, laboratory-reared squirrels maintained a high level of vigilance after exiting

whistle treatment generated a significantly longer duration of lateral and forward pausing and upward gaze than the laboratory ambience treatment (laboratory reared: $F_{1,10} = 5.92$; $P < 0.05$; wild caught: $F_{1,10} = 5.05$; $P < 0.05$). Mean differences for both groups showed large effect sizes (respectively, Cohen's $d = 2.56$ and 1.78). Although planned comparisons for both groups indicated that behavior under the chatter-whistle and white-noise treatments did not differ reliably, the larger means for the chatter-whistle treatment still produced large effect sizes (laboratory reared: $d = 1.37$, wild-caught: $d = 1.61$). Similarly, for the laboratory-reared and wild-caught squirrels, the difference between the gaze duration mean values for the laboratory ambience and white-noise treatments was not significant, but the higher mean values for

the white-noise treatment produced a large effect size, respectively: $d = 1.08$ and 1.31 (Fig. 3a).

Gaze duration after exiting

Both groups of squirrels engaged in higher levels of visual searching under the alarm-call treatment than under the other two sound treatments (Fig. 3b). The main effect for groups, averaged for sound treatments, and the interaction between groups and sound treatments were not significant. As with the before-exiting context, the main effect for sound treatments, averaged for both groups, was significant, $F_{2,20} = 5.23$; $P < 0.025$. Averaged for groups, the chatter-whistle treatment was associated with significantly more lateral and forward pausing and upward gaze after exiting than the combined average of the laboratory ambience and white-noise treatments, $F_{1,10} = 7.39$; $P < 0.025$, power = 0.77. However, unlike the before-exiting context, tests of simple effects showed that only the laboratory-reared squirrels differentiated the three sound treatments, $F_{2,20} = 7.31$; $P < 0.005$. On the other hand, wild-caught squirrels engaged in much less lateral and forward pausing and upward gazing prior to leaving the designated area. Planned comparisons revealed that, for the laboratory-reared squirrels, the chatter-whistle treatment yielded an appreciably higher level of total gaze with large effect sizes than the laboratory ambience and white-noise treatments, respectively, $F_{1,10} = 9.35$; $P < 0.025$, $d = 1.95$ and $F_{1,10} = 7.10$; $P < 0.025$, $d = 1.78$ (Fig. 3b).

Substrate sniffing before exiting

Both groups of squirrels engaged in very low levels of substrate sniffing under all sound treatments (Fig. 4a). There were no significant main effects for group difference averaged for sound treatments or sound treatments averaged for groups, and the interaction between groups and sound treatments was also not significant. Tests of simple effects and planned comparisons did not reveal any significant mean differences.

Substrate sniffing after exiting

There was no significant main effect for sound treatments, averaged for groups, nor was there any significant interaction between groups and sound treatments. There were also no significant simple effects comparing sound treatments for each group. Laboratory-reared squirrels engaged in higher levels of substrate sniffing under the chatter-whistle treatment than under the other two treatments (Fig. 4b). They also engaged in much higher levels of this behavior compared with wild-caught squirrels. The main effect for squirrel groups, averaged for sound treatments,

Fig. 4 Substrate sniffing before exiting the nest box completely (a) and after exiting completely (b). Both laboratory-reared and wild-caught ground squirrels exhibited similar substrate-sniffing behavior before exiting. Laboratory-reared squirrels continued to sniff the substrate after exiting, differing reliably from wild-caught squirrels under the chatter-whistle sound treatment condition

was significant, $F_{1,10} = 6.81$; $P < 0.025$ with medium power (power = 0.73). The source of this group difference is most apparent for the chatter-whistle treatment (simple effect: $F_{1,10} = 8.01$; $P = 0.025$), which exhibited high power (0.80) and a large effect size ($d = 1.79$). Planned comparisons for the laboratory-reared squirrels revealed that only the mean values for the laboratory ambience treatment and chatter-whistle treatment were reliably different, $F_{1,10} = 9.89$; $P < 0.01$, an effect characterized by high power (power = 0.87) and large effect size ($d = 1.28$).

Post hoc analysis of nest-box opening investigation

During the chatter-whistle treatment, the laboratory-reared squirrels exhibited a reliably higher level of substrate sniffing after exiting than did the wild-caught squirrels. Exclusively during this treatment, five of the six laboratory-reared squirrels turned around after exiting and investigated their dark nest-box opening (Fig. 5a). In doing

so, three squirrels flagged their tails repeatedly (Fig. 5b, c) and one threw pine-shaving substrate at the opening with its forepaws. None of the wild-caught squirrels engaged in this behavior after exiting. Log-linear analysis with maximum likelihood estimation of the frequencies of squirrels investigating their nest-box opening revealed that the interaction of squirrel groups and nest-box opening inspection was significant (Likelihood ratio $\chi^2_1 = 10.89$; $P < 0.001$, $N = 12$).

Discussion

As characterized by their possible pattern of environmental assessment during nest-box emergence, we predicted that only the wild-caught squirrels would distinguish the chatter-whistle alarm calls from the control sounds by engaging in greater visual and olfactory searching. The laboratory-reared squirrels were not expected to differentiate the three sound playbacks due to their lack of experience emerging from burrows and observing the behavior of conspecifics responding to alarm calls in nature. Counter to our predictions, both groups distinguished the alarm calls from the control sounds by exhibiting similarly elevated levels of visual searching prior to exposing their bodies completely to the open. Despite the absence of experience in nature, such investigative searching by laboratory-reared squirrels suggests that alarm calls are clearly provocative, enhancing alertness to the potential presence of predatory threats. Because of early exposure to situations in which associative learning would occur in young animals (see Heyes 1994), there are few studies in which the possibly innate meaning of conspecific alarm calls can be inferred confidently. One possible example is the flight to trees by Barbados green monkeys (*Chlorocebus sabaeus*) after they hear experimental playbacks of leopard-initiated alarm calls. These former West African vervets have experienced relaxed selection from leopards on Barbados Island for more than 350 years (Burns-Cusato et al. 2013).

In the animal maintenance room, squirrels regularly emitted alarm vocalizations toward technicians, sometimes inciting a cascade of vocalizations. In the absence of humans, squirrels rarely emitted vocalizations. Consequently, all squirrels were exposed to provocative vocalizations and associative learning could have occurred within the context of cage cleaning; for laboratory-reared squirrels, this was the only condition within which they ever experienced alarm vocalizations. Conversely, prior to capture, wild-caught squirrels presumably had heard alarm calls routinely in naturally threatening contexts. Since all subjects were regularly exposed to these antipredator vocalizations

Fig. 5 Video still-field images of a laboratory-reared ground squirrel cautiously investigating its nest-box opening after hearing alarm calls. Squirrel exits its nest box and immediately turns to inspect the dark opening (a), exhibiting a startle response 1-s later (b). While continuing to investigate its nest-box opening, the squirrel tail flags 1 min later (c)

for over 3 years without the opportunity to escape, habituation to these sounds should have been promoted, but apparently it was not.

After exiting their nest boxes the first time under the alarm-call treatment, the wild-caught squirrels exhibited a reduction in visual searching, suggesting that environmental assessment before exiting completely was sufficient to confirm the absence of potential threats. In contrast, laboratory-reared squirrels continued to maintain a high level of visual searching after exiting; they also maintained a high level of substrate sniffing that differed reliably from the wild-caught squirrels. Also unlike wild-caught squirrels, a reliably greater percentage of laboratory-reared squirrels turned around after exiting and investigated the opening of their nest box. Importantly, three laboratory-reared squirrels tail flagged repeatedly while inspecting their nest-box opening (Fig. 5c) and one threw substrate at the opening. There were no incidences of these behaviors in wild-caught squirrels.

Within and near burrows, the primary predatory threats to California ground squirrels are Northern Pacific rattlesnakes (*Crotalus oreganus*) and Pacific gopher snakes (*Pituophis melanoleucus catenifer*) (Fitch 1948). Snakes represent meaningful predatory threats, especially to pups (Coss 1991a; Fitch 1949; Owings and Coss 2008; Poran and Coss 1990). Tail flagging occurs almost exclusively after ground squirrels have detected snakes (Hennessy et al. 1981). It also occurs if squirrels are likely to encounter snakes (Barbour and Clark 2012; Hersek and Owings 1993). Thus, it is reasonable to assert that nest-box opening inspection and tail-flagging activity by the laboratory-reared squirrels characterized anticipation that a snake was lurking inside its nest box. In free-living ground squirrel pups, such anticipation probably reflects seasonal hunting by snakes, a development period in which snakes capitalize on their small body sizes and enhanced vulnerability (Owings and Coss 2008; Poran and Coss 1990). As argued by Dukes (1965), and as with this study, even one unique behavioral observation, such as substrate throwing as a probe to reveal the presence of a snake, can be informative if it promotes scientific insight.

Latent expression of antisnake behavior

For the ground squirrels that have been studied, the first expression of antisnake behavior has been recorded in preemergent pups (Coss 1991a), even during an encounter with a devenomated rattlesnake in an artificial burrow (1998 BBC film: *Squirrels Under Siege*, Bernard Walton, producer). Using a tethered rattlesnake, we have photographed antisnake behavior the first day pups emerge from natal burrows and adults cautiously sniffing burrow entrances after having seen a rattlesnake (Coss and Owings

1989, p. 34; Poran and Coss 1990, p. 225). An example of a pup cautiously sniffing the edge of its burrow before reentering it is given in Fig. 6. Laboratory-reared juveniles are hesitant to enter the dark opening of their transfer boxes following experimental exposure to a gopher snake (Coss and Biardi 1997), including one instance in which a squirrel threw substrate into this dark opening (see Coss 1993, Fig. 3).

The question remains as to why the wild-caught squirrels did not engage in any antisnake behavior after hearing the same sets of alarm calls while the laboratory-reared squirrels apparently did. This difference likely reflects the effects of isolation rearing experienced by the laboratory-reared squirrels that still permits the expression of precocious antisnake behavior that appears early in development. It is well documented in altricial laboratory rodents (Green et al. 1983; Helmeke et al. 2001) and captive-born macaques (Bryan and Riesen 1989) that sensory and social isolation associated with solitary cage rearing retards the growth of dendrites on subcortical and neocortical neurons that restricts interneural connectivity, leaving the brain in an immature state (see reviews by Andersen 2003; Black and Greenough 1998). Since the neural substrate for snake recognition is installed in pups shortly after eye opening (Coss 1991a, b) before neurons exhibit “experience-dependent” dendritic growth (Black and Greenough 1986), it is not surprising, in retrospect, that precocious antisnake behavior was expressed by laboratory-reared squirrels experiencing isolation rearing. Such persistence of antisnake behavior was also apparent in a concurrent exploratory study in which a unweaned laboratory-reared pup was hand-reared alone in a large seminatural setting with sand substrate and logs. As an adult, this squirrel exhibited all the aforementioned antisnake behaviors when presented with a

Fig. 6 Ground squirrel pup in the Coast Range foothills pauses and sniffs the opening of its natal burrow before reentering after foraging in an area with high rattlesnake density. Note the squirrel's elongate investigative posture and tail piloerection characterizing a high state of physiological arousal

live gopher snake. This is not the only example of behavioral preservation of juvenile behavior under isolation rearing. Precocious antipredator behavior by African jewel fish (*Hemichromis bimaculatus*) fry, manifested by their flight from a model with two facing eyes, is also resistant to disruption by long-term isolation rearing that retards neural growth (cf. Coss 1979; Coss and Globus 1979). Similarly, rough-and-tumble play in rat pups begins shortly after eye opening at 15 postnatal days and persists without deficit after social isolation between 15 and 24 postnatal days (Ikemoto and Panksepp 1992), the time frame in which brain development is sensitive to maternal care (Champagne and Curley 2005). Based on our current understanding of the effects of isolation rearing on the brain development of altricial laboratory rodents, the snake-directed responses of the laboratory-reared squirrels toward their dark nest-box opening are best interpreted as an artifact of the early installment of interneural connections mediating antisnake behavior that persist despite the effects of long-term isolation rearing on brain development.

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