Title: Urban fox squirrels exhibit habituation to humans but respond to stimuli from natural predators

Running header: Fox squirrel behavior in urban areas

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Abstract

Animals in urban areas that experience frequent exposure to humans often behave differently than those in less urban areas, such as less vigilance or anti-predator behavior. These behavioral shifts may be an adaptive response to urbanization and caused by habituation to humans. A possible negative consequence is cross-habituation to natural predators where urban animals exhibit reduced anti-predator behavior in the presence of humans but also to their natural predators. We tested the hypothesis that habituation to humans in urban populations of fox squirrels (*Sciurus niger*) causes cross-habituation to stimuli from two possible predators (hawks and domestic dogs). We exposed squirrels in multiple urban and less urban sites to acoustic playbacks of a control stimulus (non-predatory bird calls), a natural predator (hawk), and dogs and recorded their vigilance and three different anti-predator behaviors when a human approached them while either broadcasting one of these three playbacks or no playbacks at all. In trials with no playbacks, urban squirrels exhibited reduced vigilance and anti-predator behavior compared to those in less urban areas but there was little evidence that urbanization altered the correlations among the different behaviors we quantified. Urban squirrels exhibited increased vigilance and anti-predator behavior when exposed to a human paired with hawk playbacks compared to the control playbacks. This indicates that urban squirrels did perceive and assess risk to the natural predator appropriately despite exhibiting habituation to humans. There is currently little evidence that habituation to humans causes animals to lose their fear of natural predators.

Keywords: Anti-predator behavior, cross-habituation, first alert distance, flight initiation distance, stimulus generalization, urbanization

Introduction

Urbanization has a multitude of effects, not only for humans, but for animals as well. Anthropogenic factors can shape the natural world and affect animals through habitat fragmentation, changes in temperature or precipitation, and various types of pollution (chemical, noise, light), among other changes (Gaston, 2010; Johnson & Munshi-South, 2017). These are often drastic alterations to natural environments, yet, some animals seem to thrive in urban areas by adapting to the novel environmental conditions. Behavior plays an important role in enabling role in enabling animals to persist through environmental change (Baldwin, 1896; Bartholomew, 1964; West Eberhard, 1989; Price et al., 2003; Snell-Rood, 2013) and it seems to play a major role in facilitating the ability of animals to cope with new challenges that they face in urban environments (Ditchkoff et al., 2006; Tuomainen and Candolin, 2011; Lowry et al., 2013; Ryan and Partan, 2014).

One of the most widely documented behavioral adjustments of animals in urban areas occurs in risk-taking behavior, which can include animal personality traits that approximate the willingness of an animal to take risks such as boldness, neophobia, aggression, or exploratory behavior (Réale et al., 2007). From song sparrows (*Melospiza melodia*) in North Carolina to striped mice (*Apodemus agrarius*) in Germany, individuals in urban areas exhibit increased boldness or exploratory behavior compared to those in nearby rural populations (Evans et al., 2010; Dammhahn et al., 2020). Differences in anti-predator behavior between urban and rural populations have been a particular focus about the behavioral mechanisms animals may use to cope with urbanization. This is likely the result of the accessibility of one assay, the flight initiation distance (FID), that is often viewed as a proxy to measure anti-predator behavior (Cooke, 1980; Ydenberg & Dill, 1986; Lima and Dill, 1990). FID is the distance at which an

59 animal flees from an approaching human. Individuals with shorter FIDs are considered to be
60 bolder than individuals with longer FIDs since they demonstrate reduced fear of the "predator".
61 FID is also thought to reflect the risk assessment an individual animal as FID is the escape
62 decision an animal makes while assessing its surrounding environment and deciding whether it is
63 more advantageous to flee or remain in place (Ydenberg & Dill, 1986; Cooper & Frederick,
64 2007). For example, animals exhibit flexibility depending upon the initial distance of the
65 predator, such as a foraging animal delaying the decision to flee (a lower FID) until the predator
66 is closer (Dill and Houtman, 1989; Blumstein, 2003). Other studies that have measured FID also
67 suggest it reflects attention to risk, such as FID being lower when the distance to refuge is
68 decreased (Dill & Houtman, 1989; Stankowich and Blumstein, 2005) or being affected by
69 intruder group size (Geist et al., 2005; McGowan et al., 2014), and predator lethality or size
70 (Stankowich and Blumstein, 2005; Cooper & Frederick, 2010).

71 Substantial evidence supports the hypothesis that animals in more urban environments
72 exhibit less anti-predator behavior, as reflected by a lower FID. For example, a meta-analysis of
73 180 bird species, 16 lizard species, and 16 mammal species, Samia et al. (2015) showed that
74 populations of these bird, lizard, and mammal species that experienced elevated levels of human
75 disturbance exhibited lower FID. This could be because anti-predator behaviors carry energetic
76 or time costs as vigilance and escape behaviors take time away for resource acquisition
77 (Ydenberg and Dill, 1986; Cooper and Frederick, 2007; Møller, 2012). Animals are expected to
78 behave in a way that optimizes the acquisition of resources (food, mates, etc.) relative to the risks
79 required to obtain those resources (Lima and Dill, 1990; Brown, 1999). Humans in urban
80 environments are usually non-threatening and urban environments may be characterized by
81 either exhibiting reductions in the density of non-human predators, especially larger predators, or

82 prey experience reductions in predation rates even if the number of predators is unchanged
83 (Fischer et al., 2012; Eötvös et al., 2018). Thus, animals in urban environments may optimize
84 resource acquisition by exhibiting lower levels of anti-predator behavior in urban environments,
85 although we note that native mesopredators and invasive predators (domestic cats and dogs)
86 could actually be more abundant in urban areas (Crooks, 2002; Ordeñana et al., 2010; Eötvös et
87 al., 2018).

88 Although the consistency of these observations across populations of the same species or
89 among species suggest that a reduction in anti-predator in urban environments is adaptive
90 (Møller, 2008; Carrette et al., 2016), there may be potential costs for urban animals due to the
91 phenomenon of cross-habituation. Hinde (1954) showed that behavioral responses of captive
92 birds to a simulated predator declines with the number of times it is presented with that stimulus
93 (i.e., habituation). However, if these birds that were habituated to this stimulus are then presented
94 with a *different* stimulus but in the same referential category (e.g., a second simulated predator),
95 they exhibited an attenuated response to this second stimulus compared to a group of naïve birds.
96 This suggests that animals that are habituated to one stimulus can also exhibit a reduced response
97 to a second but similar stimulus (cross-habituated; see also discussion in Curio, 1993). In
98 experimental psychology, this is known as "stimulus generalization" where an animal habituated
99 to one stimulus exhibits and attenuated response to a second stimulus from the same or different
100 sensory modality (Guttman and Kalish, 1956; Thompson and Spencer, Rankin et al., 2009).
101 Related concepts occur in the context of behavioral syndromes where individuals that exhibit
102 high levels of a behavior in one context also exhibit it in another context even though it may not
103 be adaptive (Sih et al., 2004). For example, fish with high levels of boldness behavior in a
104 courtship context may acquire more mates or food (e.g., Godin and Dugatkin, 1996) but may

105 have reduced survival if they also exhibit higher levels of boldness in the vicinity of a predator
106 (Dugatkin, 1992; but see Godin and Davis, 1995). The latter being an example of "behavioral
107 spillover" due to the inflexibility of boldness within individuals across different contexts
108 (Arnqvist and Henriksson, 1997; Sih et al., 2004).

109 Animals in urban environments should experience greater exposure to humans or their
110 stimuli and likely view humans as predators even if they habituate to their presence (Frid and
111 Dil, 2002). For example, even if they habituate behaviorally, they can still show a physiological
112 stress response to humans (Fowler, 1999; Ellenberg et al., 2006). Although animals in urban
113 areas may exhibit increases in boldness or reductions in anti-predator behavior due to habituation
114 to humans (McCleery, 2009; Rodriguez-Prieto et al., 2009; Vincze et al., 2016; Uchida et al.,
115 2019), it is less certain if the frequent exposure to humans causes urban animals to *cross-*
116 *habituate* to other threatening stimuli. Do animals in urban environments exhibit cross-
117 habituation where they exhibit a reduction in FID or increased boldness when faced with a
118 natural predator? If such cross-habituation occurs and causes reductions in anti-predator behavior
119 in urban environments, it could conceivably have important impacts wildlife populations by
120 increasing their vulnerability to either native or non-native predators (Geffroy et al., 2015). For
121 example, short-nesting birds in areas with high levels of human activity (outdoor recreation, dog
122 walking) exhibit less anti-predator behavior when faced with a human observer approaching their
123 nests and chick mortality in those areas is higher compared to areas with less human activity
124 perhaps due to increased predation by dogs (Baudains and Lloyd, 2007; although see other
125 examples in Nisbet, 2000 that do not support this conclusion). Unfortunately, studies addressing
126 cross-habituation due to frequent exposure to humans are rare and its importance is currently
127 being debated (Fitzgerald and Stronza, 2016). For example, seals and squirrels in areas with

128 higher human activity exhibit less of a behavioral response when natural predators were
129 observed visiting the area (Olson and Acevedo-Gutiérrez, 2017) or due to acoustic playbacks of
130 a natural predator (McCleery, 2009). This suggests cross-habituation where frequent exposure to
131 human activity reduces their response to other stimuli from natural predators. Other studies do
132 not support these findings as they show the response of animals in more urban areas to a stimulus
133 from a natural predator is not attenuated compared to those in more rural locations (Labra and
134 Leonard, 1999; Coleman et al., 2008; Seress et al., 2011; Bokony et al., 2012; Cavalli et al.,
135 2016; Weaver et al., 2018; Vincze et al., 2019).

136 One way to examine if cross-habituation to natural predators due to human activity
137 occurs in nature is to quantify the vigilance and anti-predator behavior of individuals in response
138 to a human by themselves (a typical FID protocol) or a human paired with a benign or
139 threatening (such as a natural predator) stimulus. If animals do not exhibit enhanced vigilance or
140 increased FID to a human observer that is paired to some stimulus from a natural predator
141 compared to if they were approached by a human without such a stimulus, this supports the
142 hypothesis that animals do not view the situation as risky due to the presence of a human (i.e.,
143 they exhibit cross-habituation). We call this the "cross-habituation due to human stimuli"
144 hypothesis given the long precedent of studies in experimental psychology examining cross-
145 habituation (Hinde, 1954), although we note that this type of cross-habituation has also been
146 termed "human-mediated behavioral spillover" (Geffroy et al., 2015) or "transfer habituation"
147 (Coleman et al., 2008; McCleery, 2009) and examines similar phenomenon as the "threat-
148 specific risk-taking" and "general risk-taking" hypotheses, with the latter predicting that urban
149 animals take more risks in the presence of both humans and non-human natural predators
150 (Vincze et al., 2019).

151 Here, we tested the cross-habituation due to human stimuli hypothesis in fox squirrels
152 (*Sciurus niger*) in urban and less urban areas by quantifying their vigilance and multiple
153 components of their anti-predator behavior when they were presented with a human observer
154 with a control acoustic playback (common non-threatening bird), a human observer paired with
155 the playback of a natural predator (hawk), or a human observer paired with a playback of an
156 invasive predator (dog). We used vocalizations from red-tailed hawks (*Buteo jamaicensis*) as the
157 stimulus from a natural predator as they are common in both urban and suburban areas in our
158 study area, have been observed to kill squirrels (personal observations), and previous studies in
159 fox squirrels and other tree squirrels illustrate that hawk playbacks can elicit an increase in anti-
160 predator behavior (McCleery, 2009; Lilly et al., 2019). We used vocalizations from domestic
161 dogs for the stimulus from an invasive predator that is characteristic of urban environments, and
162 vocalizations from black-capped chickadee (*Poecile atricapillus*) as a control stimulus because
163 they are common in our study areas and are not known to be harmful to fox squirrels. We
164 predicted that squirrels in the urban areas but not those in the less urban areas would exhibit no
165 change in vigilance and anti-predator behavior when they were exposed to the human+dog or
166 human+hawk stimuli compared to the human+control playback. This would support the
167 hypothesis that reductions in boldness or anti-predator behavior in urban environments are due to
168 cross-habituation to the presence of humans (at least partially) that causes them to exhibit less
169 vigilance and anti-predator when faced with natural predators.

170 In a separate set of experiments conducted with squirrels in the same urban and less
171 urban areas, we also conducted standard FID trials (without any playbacks) to test a second
172 hypothesis. If 1) squirrels in urban areas showed reduced anti-predator behavior (reduced FID) in
173 a standard FID trial (without any acoustic stimulus) compared to those in less urban areas

(experiment 2) *and* 2) both squirrels in urban and less urban areas exhibited a similar level of vigilance and anti-predator behavior when exposed to the human+dog or human+natural predator stimuli compared to the human+control playback (experiment 1), this would support the hypothesis that although they exhibit habituation to humans (1), they do *not* exhibit cross-habituation to other stimuli from non-human predators (2).

We used fox squirrels as a model because they are ubiquitous in urban environments in the midwestern United States but they can also be found in suburban and rural areas (McCleery, 2008, 2009). Arboreal tree squirrels are also common in urban areas worldwide but continue to experience predation from natural predators, although one study suggested it was quite rare compared to rural areas (McCleery et al., 2008). Squirrels in urban areas may also experience predation from domestic cats and dogs (Koprowski, 1994; Wauters et al., 1997; Loss et al., 2013; Jokimäki et al., 2017). Despite their strong presence in urban areas, most studies on the effects of urbanization on animal behavior focus on birds (Samia et al., 2015; Geoffroy et al., 2020) and how urbanization affects animal behavior may differ between animals that can escape a predator (or human) through flight versus non-volant taxa that cannot do so. Only one study has used fox squirrels to explore how behavior is influenced by urbanization (McCleery, 2009).

For experiment 1 (FID trials with playbacks), squirrels were located in their natural habitat, one of three playbacks was broadcast, and the human observer walked towards the squirrel while we recorded the following four aspects of their vigilance and anti-predator behavior. First, we recorded the distance to which the observer could get to before they exhibited vigilance behavior towards the observer ("first alert distance" or FAD, similar to Fernández-Juricic and Schroeder, 2003; Blumstein et al., 2005). Second, we recorded how close the observer could get to them before they ran away (FID). Third, was the probability that the

197 squirrel escape by running up a tree. Fourth, the latency following the trial it took them to
198 resume their typical behavior (foraging or traveling off tree). In the second experiment (FID
199 trials without playbacks), we collected the same behavioral data but the trials were conducted
200 without playbacks.

201 Finally, using data from experiment 2 (FID trials with no playbacks), we assessed
202 whether the degree of phenotypic correlation among FID or FAD and probability to climb a tree
203 and latency to resume typical behavior following the trial differed between urban and less urban
204 squirrels. We did this because previous studies suggest that phenotypic correlations among
205 different vigilance or anti-predator behaviors (i.e., the behavioral syndrome) may differ between
206 urban and rural populations (Evans et al., 2010; Scales et al., 2011; Carrette and Tella, 2017;
207 Lapiedra et al., 2017). For example, the sign and magnitude of the phenotypic correlation
208 between anti-predator behavior towards humans and other animal personality traits (exploration,
209 aggression, boldness) may differ between urban and rural populations. This could be because
210 selection against anti-predator behavior in urban environments produces a correlated response in
211 other risk-taking behaviors. If there was a strong phenotypic correlation between two behaviors
212 in squirrels from less urban sites compared to those in urban sites, this would support the
213 hypothesis that squirrels in urban areas exhibit greater behavioral flexibility.

214

215 **Materials and Methods**

216 *Study sites and subjects*

217 We conducted both experiments on natural populations of adult fox squirrels from six
218 sites in and around Ann Arbor, Michigan (Table 1). Adult squirrels and sex were visually

219 identified by size (small juvenile squirrels were excluded) and anatomy (males were identified
220 by presence of testes), respectively. Sites were chosen to represent an urban-rural gradient based
221 on human population density (Center for International Earth Science Information Network, 2018,
222 Table 1). Urban sites included Prospect Park as well as two locations on the University of
223 Michigan's (UM) main campus, including North and Central Campus. Prospect Park is a 9.5-
224 acre park located near downtown Ypsilanti, Michigan with regular daily visitors and about 13
225 km away from UM central campus. North and Central Campus are both areas of the UM's main
226 campus, but ~3-4 km away from one another. Less urban sites included Nichols Arboretum,
227 County Farm Park, and Saginaw Forest. Nichols Arboretum is owned and operated by UM and is
228 located ~1 km away UM Central Campus. County Farm Park is owned and operated by
229 Washtenaw County and is about 4.5 km away from UM Central Campus. Saginaw Forest is also
230 owned and operated by UM and is located approximately 7 km from UM Central Campus. At all
231 research sites, dogs are allowed but hunting is not allowed. Approval to conduct this research at
232 each site was obtained from UM (Central Campus, Nichols Arboretum, North Campus, Saginaw
233 Forest), Washtenaw County Parks & Recreation (County Farm Park), and the City of Ypsilanti
234 (Prospect Park). All procedures were non-invasive and involved behavioral observation or short-
235 term exposure to playbacks of acoustic stimuli. All experiments followed the guidelines set by
236 the Animal Behavior Society/Association for the Study of Animal Behaviour (Anonymous,
237 2012) and were approved by the UM Institutional Animal Care and Use Committee (protocol #
238 PRO00009076).

239 Given that habituation to humans may cause animals in urban areas to exhibit less anti-
240 predator behavior (McCleery, 2009; Rodriguez-Prieto et al., 2009; Vincze et al., 2016; Uchida et
241 al., 2019), we focused on human presence as the major factor difference between our study sites

(which should also reflect general urbanization). Sites were classified as "urban" based upon having a human population density >1000 persons per km^2 whereas the less urban sites had anywhere from 25-250 persons per km^2 (Saginaw) to 250-1000 persons per km^2 (County Farm Park, Nichols Arboretum). To help better assess any differences in human and dog presence at our sites, we recorded dog and human presence while we were visiting sites conducting our behavioral observations. We counted the total number of individual dogs observed but only counted the number of humans up to 50. If human presence exceeded 50 different people, then a rough estimate of 50, 75, or 100 was recorded. Human presence was also recorded as 100 for all numbers estimated to be greater than 100. These are imperfect measures of human presence given the difficulty of counting humans while simultaneously collecting behavioral data on squirrels yet they can be used as an index of human activity. Overall, they support the classification of these sites as urban and less urban with the former being locations where animals should have higher exposure to humans. Although human and dog presence varied among the different sites (Table 1), the number of humans observed averaged over all the urban sites (n = 81 observations, mean ± SE = 61.81 ± 4.46 humans) was significantly higher than those observed averaged over all the less urban sites (4.41 ± 0.56: Mann-Whitney-Wilcoxon Test, W = 1104, $p < 0.0001$). However, we did observed significantly fewer dogs at the urban sites (0.36 ± 0.10) compared to the less urban sites (0.93 ± 0.13, Mann-Whitney-Wilcoxon Test, W = 4856.5, $p < 0.0001$).

In experiment 1, squirrels (n = 94 trials, 54 females and 40 males) were approached by a human observer with one of three different playback treatments (control, hawk or dog playbacks). There were three distinct playback treatments (described in detail below): control playback (n = 26 total trials on 17 females and 9 on males), dog playbacks (n= 40 total trials on

265 24 females and 16 males), and hawk playbacks (n= 28 total trials on 13 females and 15 males).
266 In total, these trials were conducted at the three less urban sites (n = 51 trials: 15 with control
267 playback, 20 with dog playbacks, and 16 with hawk playbacks) and the three urban sites (n = 43
268 trials: 11 with control playback, 20 with dog playbacks, and 12 with hawk playbacks). In
269 experiment 2, squirrels (n = 77 trials, 39 females and 38 males) were approached by a human
270 observer without playback or any other stimuli, creating one anthropogenic stimulus of one
271 human approaching the squirrel (described below). These trials were conducted at the three less
272 urban sites (n = 39 trials: 19 females, 20 males) and the three urban sites (n = 38 trials: 20
273 females, 18 males).

274 Similar to most studies that measure anti-predator using FID, trials were conducted on
275 unmarked squirrels at each site. Squirrels were located by walking around each site and trials
276 were started when squirrels were observed. Focal individuals were selected randomly, however,
277 only squirrels that were feeding or foraging on the ground were included in this experiment.
278 Because it was not possible to mark squirrels individually, it is possible that the same squirrel
279 was observed on different days, although we visited different areas of each study site to try and
280 reduce this possibility. However, it is very unlikely that the same squirrel was observed multiple
281 times on the same day, because after each trial was completed, the observer traveled
282 approximately 20 meters away from the previous location and started a trial with a different
283 squirrel. Additionally, sites were only revisited after at least three days since the previous visit to
284 reduce the possibility of a squirrel becoming habituated to the trials should it be sampled again.
285 If squirrels at these sites were habituating to our protocols, we would expect that their behavioral
286 responses would decline with trial number or date when the trial was conducted. The fact that

none of our behavioral variables were associated with date of when the trial was conducted (Tables 2-3) supports our assumption that squirrels were not habituating to our protocols.

In total, we observed fox squirrels for 52.36 hours over 30 different days. A single observer (AK) conducted all trials. Sites were visited between 800 and 1900 h EST and data were collected from October 2019 through January 2020. Trials with no playbacks were conducted from 25 October 2019 to 16 November 2019 (from 812 to 1810 h) whereas trials using playbacks (playback trials) were conducted from 18 November 2019 to 27 January 2020 (from 802 to 1609 h). Both the order in which sites were visited and the order in which playback versions across playback treatments (see below) were randomized. This was done to ensure that there was no impact of the order on the results. No two sites were visited on the same day. All trials with no playbacks were conducted prior to the playback trials in this study. This was due to personnel limitations and prevents us from directly comparing trials with and without playbacks given that squirrel behavior likely changes seasonally in Michigan from October to January (when the trials were conducted) due to food caching behavior in autumn but not winter. Trials were not conducted when it was raining or snowing (it was either cloudy, partly cloudy, partly sunny, or sunny during the trials). Results from two one-way ANOVAs showed that the time of day for playback trials did not vary among the three different treatment groups ($F_{2,91} = 0.44$, $p = 0.65$) and that the time of day when baseline trials were conducted did not vary among squirrels at the urban or less urban sites (t-test: $t_{75.4} = 0.21$, $p = 0.83$). Air temperature varied during all the trials varied from -6.1° to 10.5°C (mean = 2.7° C).

Quantifying behavioral responses of squirrels

We measured the behavioral responses of squirrels to humans or humans plus different playbacks using protocols developed for tree squirrels (Dill and Houtman, 1989; Gustafson and

310 VanDruff, 1990; McCleery, 2009). At the beginning of each trial, a marker was placed at the
311 starting position of the observer and trial data were recorded (GPS location, time of day,
312 temperature, general weather conditions, and squirrel sex). The squirrel was approached by a
313 single observer (AK) at a slow and steady pace in a direct line to the squirrel (Fig. S1).
314 Additional markers were placed when a squirrel displayed the first alert and when they fled. The
315 FAD was defined as the distance between the observer and the squirrel when it first stopped
316 moving (froze) and looked at the observer with one or both eyes. FID was recorded as the
317 distance between the squirrel's initial position and the observer location when it actively fled
318 (stopped feeding and foraging and moved rapidly away from observer). A marker was also
319 placed at an estimate of the squirrel's initial position to the place where they fled to if refuge was
320 not taken in a tree, and the distance between this marker and the squirrel's initial position was
321 recorded as "flight distance". We recorded this because some studies have noted that FID is
322 variable depending on intruder starting distances and distance to a refuge (Dill & Houtman,
323 1989; Blumstein, 2003). Consequently, the distance between the observer and the focal squirrel
324 at the start of the trial (starting positions, hereafter referred to as "initial distance") and the
325 distance between the focal squirrel's initial position and the nearest tree were also measured
326 ("distance to nearest tree"). If the focal animal took refuge in a tree other than the one nearest,
327 distance between the squirrel's initial position and its refuge tree of choice ("distance to the
328 chosen tree") was also recorded. If an individual took refuge in a tree, a laser rangefinder was
329 used to measure how high they climbed, and this distance was recorded (same as vertical escape
330 distance in Uchida et al., 2017). Lastly, latency to resume behavior was recorded ("latency").
331 This was measured with a stopwatch to determine how long it took for the animal to cease
332 alert/vigilance behavior and resume typical activity (foraging or traveling off tree). Out of all the

333 trials, nearly all squirrels ceased alert behavior and resumed typical behavior within a couple
334 minutes (n = 171 trials, mean ± SE = 130.5 ± 10.3 s), but there was one individual squirrel that
335 remained alert for longer than ten minutes and we recorded its latency as ten minutes. Distances
336 were measured with a tape measure and presented in meters.

337 *Playback trials*

338 Playback trials were conducted using the same protocol shown above, with the addition
339 of an acoustic stimulus being broadcasted while the observer approached the squirrels. The
340 control stimulus consisted of recordings of black-capped chickadee calls. Black-capped
341 chickadees were chosen because they are not known to be predators of fox squirrels and fox
342 squirrels are not known to be predators of them (Korschgen, 1981; Koprowski, 1994).
343 Additionally, a previous study in another species of tree squirrel that lives in similar habitats as
344 fox squirrels showed that individuals exhibited a significantly reduced response to black-capped
345 chickadee playbacks compared to calls of other anthropogenic noises (car alarm, buzzer) and
346 playbacks of red-tailed hawks (Bohls and Koehnle, 2017). Because of this, we expected that
347 black-capped chickadee recordings would represent a neutral vocalization for fox squirrels and
348 they can act as control to ensure that any differences in squirrel behavior across playback
349 treatments are attributable to the vocalization information of the playback rather than an added
350 exposure to noise. To simulate the threat of a terrestrial predator, recordings of domestic dogs
351 barking were broadcasted. Domestic dogs were chosen not only because they are terrestrial
352 predators of fox squirrels (Koprowski, 1994; Wauters et al., 1997), but also because they have a
353 strong association with urbanization. Other species of tree squirrels that live in the same habitats
354 as fox squirrels also adjust their risk-taking behavior according to the abundance of domestic
355 dogs (Bowers and Breland, 1996; Cooper et al., 2008). Because of this, fox squirrels may have

356 varying degrees of exposure to these predators depending on their degree of urbanization,
357 although we noted above that using our observations, dog presence at our less urban sites
358 actually tended to be higher than at the urban sites. For the avian predator, recordings of red-
359 tailed hawk calls were broadcasted to the focal individual. Red-tailed hawks were chosen since
360 they are year-round predators of fox squirrels in Michigan (Koprowski, 1994; personal
361 observations) and other studies illustrate that tree squirrels respond to hawk playbacks with
362 increased anti-predator behavior (McCleery, 2009; Lilly et al., 2019). Playbacks of the
363 recordings were broadcast to individuals at the start of the trial and when the observer began the
364 approach and suspended when the squirrel took flight. Vocalizations were broadcast through a
365 JAMBOX Red Dot speaker (Jawbone, San Francisco, CA) connected to an Apple iPhone 6s
366 (Mountain View, CA) with a constant volume set for the speaker and phone. Given how the trials
367 were conducted in real time (not video recorded), the single observer (AK) was not blind to the
368 playback treatments or locations of where the experiments took place.

369 All vocalization recordings were found online (Control A: Place, 2015; Control B: Floyd,
370 2017a; Control C: Floyd, 2017b; Dog A: Simion, 2016; Dog B: Simion, 2018; Dog C: Simion,
371 2017; Hawk A: Chartier, 2008; Hawk B: Addison, 2017; Hawk C: Wilson, 2010). To reduce the
372 chance that responses to the playbacks were specific to a recorded individual, each playback
373 treatment had three separate recordings. For example, the hawk playback treatment consisted of
374 three recorded versions of red-tailed hawks taken from three separate birds/recordings, and
375 subsequently labeled Hawk A, Hawk B, and Hawk C. This was performed to ensure that squirrel
376 responses to playbacks of red-tailed hawks were not specific to the call qualities of one hawk.
377 The same was done for dog vocalizations and the control playback.

378 *Statistical analyses*

We analyzed the data from experiment 1 (playback trials) and experiment 2 (no playback trials) because the two experiments were not conducted synchronously and seasonal changes from Fall to Winter in Michigan may alter squirrel behavior. We used three separate linear mixed-effects models (LMMs) for data from the playback trials to examine the effects of the acoustic playback manipulations on FAD, FID, probability of escaping the observer by climbing up a tree, and latency to resume activity following the trial. Although the linear distance a squirrel climbed up a tree (from base of tree to location of squirrel) has been used in other studies of tree squirrels (e.g., Uchida et al., 2017), the distance a squirrel climbed up a tree in our study exhibited a Poisson distribution where many squirrels did not climb up a tree at all and a few climbed up very high. Consequently, we used a generalized linear mixed-effect model (GLMM, binomial errors) with a binary response variable (escaped by climbing a tree or did not climb a tree) to assess the probability that squirrels climbed a tree to escape during the trial. Models included site category of data collection (urban, less urban), playback treatment (dog, hawk, control), distance to the nearest tree, sex, Julian date of the trial, and initial distance of the observer as fixed effects. Distance to the nearest tree was included not only because previous studies show it can impact anti-predator behavior (measured using FID: Dill & Houtman, 1989; Blumstein, 2003) but also because it helps control for any differences in vegetation among the different study sites, which could also impact their behavior. An interaction between playback treatment and site category was included to examine if the effects of the playbacks on squirrel behavior differed between squirrels at the urban and less urban sites, which allowed us to test whether cross-habituation occurs in squirrels in urban sites. Because we had repeated samples from the same site, we also included a random intercept for site. We assessed the statistical

401 significance of these variables using post-hoc Tukey's Honest Significant Differences that were
402 corrected for multiple comparisons.

403 Data from the trials where squirrels were only approached by a human observer and not
404 exposed to playbacks (experiment 2) were analyzed in a similar manner. Three separate LMMs
405 were used to examine the effects of urbanization on FAD, FID, and latency to resume activity
406 following the trial. A GLMM (binomial errors) was used to examine the effects of urbanization
407 on the probability that squirrels climbed a tree to escape during the trial. Models included site
408 category of data collection (urban, less urban), distance to the nearest tree, sex, Julian date of the
409 trial, and initial distance of the observer as fixed effects. Similar to the above, we also included a
410 random intercept for site.

411 To assess the degree of association between FAD or FID and the two other behavioral
412 variables (probability of climbing a tree, latency to resume typical behavior), we ran two separate
413 LMMs (one for FAD and the other for FID). Each model included fixed effects for site category,
414 probability of climbing a tree (yes/no), latency to resume typical behavior, and interactions
415 between site category and each of the two behavioral variables. We also included a random
416 intercept term for site identity. We also report the Pearson' or point-biserial (escaping up tree
417 was binary variable) correlations between these variables. We did not run a similar model
418 looking at the association between FAD and FID given that they are clearly not independent of
419 one another (for all squirrels [n = 171] across all treatments in this study: Pearson $r = 0.88$, 95%
420 CI = 0.84-0.91).

421 Continuous predictor variables were standardized to a mean of 0 and SD of 1. We
422 confirmed model diagnostics visually and all models met the appropriate assumptions (normality
423 of residuals, constant variance, no high leverage observations). Latency to resume behavior was

log+1 transformed (base e) to improve homoscedasticity and normality. There were also no predictor variables that were found to be colinear as all variance inflation factors (VIFs) were less than 3.68 (Zuur et al., 2010), though the higher VIF were due to interaction terms and most not in interactions were <1.5. All analyses were conducted in R version 4.02 (R Core Team, 2020) with lme4 (version 1.1.23, Bates et al., 2015) and p-values estimated using lmerTest (version 3.1.2, Kuznetsova et al., 2017). Tukey's post-hoc tests were used to evaluate if the responses to the playback treatments differed between squirrels in urban and less urban areas using emmeans (1.5.2-1: Lenth, 2020) and *p* values from these analyses were adjusted for multiple comparisons. Mean and SE are presented below.

Results

Do urban squirrels exhibit cross-habituation to stimuli from natural predators?

The effects of the playbacks on FAD depending upon whether the squirrels were located at the urban or less urban sites (Table 2), with urban squirrels exhibiting the highest FAD when exposed to hawk playbacks and squirrels at less urban sites exhibiting higher overall FAD and no response to the playback treatments (Fig. 1A). Average FAD for urban squirrels exposed to the hawk vocalizations (n = 12 trials, 17.17 ± 1.33 m) was 37% longer than squirrels who were exposed to the control playback (n = 11, 12.56 ± 0.94 m, Tukey's $p = 0.0016$) and 20% longer than those exposed to the dog playbacks (n = 20, 14.30 ± 0.83 m, Tukey's $p = 0.0025$, Fig. 1A). By contrast, the FAD of squirrels at the less urban sites were just higher overall (Fig. 1A) and the FAD of those who were exposed to the hawk playbacks (n = 16, 14.62 ± 1.14 m) did not differ from those exposed to the control playback (n = 15, 14.46 ± 0.88 m, Tukey's $p = 0.95$) or dog

446 playbacks (n = 20, 14.85 ± 1.05 m, Tukey's $p = 0.64$, Fig. 1A). There were no significant
447 differences in FAD for squirrels exposed to dog playbacks and those exposed to the control
448 playback for squirrels at urban sites (Tukey's $p = 0.83$) or those at less urban sites (Tukey's $p =$
449 0.84).

450 Similar to FAD, the effects of the playbacks on FID also depended upon whether the
451 squirrels were located at the urban or less urban sites (Table 2), with urban squirrels exhibiting
452 the highest FID when exposed to hawk playbacks and squirrels at less urban sites exhibiting the
453 highest FID in response to the dog playbacks (Fig. 1B). Average FID for urban squirrels exposed
454 to the hawk vocalizations (n = 12 trials, 14.95 ± 0.98 m) was 44.3% longer than squirrels who
455 were exposed to the control playback (n = 11, 10.37 ± 3.13 m, Tukey's $p = 0.0006$) and 29.9%
456 longer than those exposed to the dog playbacks (n = 20, 11.86 ± 2.65 m, Tukey's $p = 0.0005$,
457 Fig. 1A). In squirrels at urban sites, there was no difference in FID between those exposed to the
458 control playback and those exposed to the dog vocalizations (Tukey's $p = 0.90$). In squirrels at
459 less urban sites, FID for those exposed to the dog playbacks (13.60 ± 0.98 m,) was 12.2% higher
460 than those exposed to the control playback (12.12 ± 0.88 m, Tukey's $p = 0.049$), but was similar
461 to those exposed to the FID of those exposed to hawk playbacks (13.11 ± 0.96 m, Tukey's $p =$
462 0.66, Fig. 1B). Unlike urban squirrels, the FID of those at the less urban sites who were exposed
463 to hawk playbacks was similar compared to those exposed to the control playback (Tukey's $p =$
464 0.34).

465 The playback treatments had no significant effects on the probability that squirrels in
466 urban or less urban sites exhibited an escape response by climbing a tree (Table 2, Fig. 1C).
467 Urban squirrels exposed to the hawk playbacks were most likely to exhibit an escape response by
468 climbing a tree (75.0% of trials) compared to those exposed to the control playback (36.4%,

Tukey's $p = 0.30$) or dog playbacks (30%, Tukey's $p = 0.096$), but these differences were not significant (Table 2, Fig. 1C). There was also no difference in the probability of urban squirrels climbing a tree when they were exposed dog playbacks compared to the control playback (Tukey's $p = 0.89$). Compared to the control playback (53.3%), squirrels in less urban sites were more likely to climb a tree if they were exposed to hawk playbacks (75%, Tukey's $p = 0.34$) or dog playbacks (70%, Tukey's $p = 0.34$), but once again these differences were not significant. There was no difference in the probability of climbing a tree for squirrels at less urban sites exposed to the hawk playbacks and those exposed to dog playbacks (Tukey's $p = 0.99$).

Squirrels at both the urban and less urban sites exhibited the same response when it comes to their latency to resume their typical behavior following the trial (hawk > dog > control, Table 2, Fig. 1D). Squirrels at urban sites that were exposed to the hawk playbacks took 436% longer to resume their pre-trial behavior (213.33 ± 28.9 s) compared to those who were exposed to the control playback (39.82 ± 7.70 s, Tukey's $p < 0.0001$) and 147% longer than those exposed to dog playbacks (86.25 ± 13.30 s, Tukey's $p = 0.011$). Squirrels at the urban sites also took 114% longer to resume typical behavior if they were exposed to dog playbacks compared to those exposed to the control playback (Tukey's $p < 0.001$). Similarly, squirrels at the less urban sites that were exposed to the hawk playbacks took 218% longer to resume their pre-trial behavior (291.25 ± 35.45 s) compared to those who were exposed to the control playback (91.47 ± 25.45 s, Tukey's $p < 0.0001$) and 55.7% longer than those exposed to dog playbacks (187.05 ± 21.38 s, Tukey's $p = 0.045$, Fig. 1D). Squirrels at the less urban sites also took 95.6% longer to resume typical behavior if they were exposed to dog playbacks compared to those exposed to the control playback (Tukey's $p < 0.001$).

There was no effect of sex or Julian date of trial, on FAD, FID, probability of climbing a tree, or latency in these trials (Table 2). There was no effect of the initial distance that a squirrel was from a tree when the trial started on FAD, FID, or probability to climb a tree, but, surprisingly, squirrels were less likely to climb a tree if they were closer to one when the trial started (Table 2). Trials that started with the human observer a greater distance away from the squirrel had higher FAD, FID, and latency to resume typical behavior, but not the probability to climb a tree (Table 2).

Do squirrels in urban areas exhibit habituation to humans?

Overall, urban squirrels (n = 38) exhibited habituation to humans as they allowed a human observer to get closer to them before they exhibited vigilance (FAD) or fled (FID) and tended to be less likely to climb a tree during the trial and more quickly return to typical behavior following the trial compared to those in less urban sites (n = 39, Table 3, Fig. 2). Although the average FAD for squirrels observed at the urban site (10.09 ± 0.74 m) was 43.8% shorter than for those at the less urban sites (14.51 ± 0.69 m), this difference were not significant ($p = 0.10$, Table 3, Fig. 2A). FID in the squirrels at the urban site (6.36 ± 0.52 m) was 97.2% shorter compared to those at the less urban sites (12.54 ± 0.62 m, $p = 0.043$, Table 3, Fig. 2B). Squirrels at the urban sites were less likely to climb a tree while the observer approached (34.2% of trials) compared to those at the less urban sites (48.6%), although this difference was not significant ($p = 0.093$, Table 3, Fig. 2C). Latency to resume behavior following the trial was shorter for urban squirrels (43.9 ± 14.4 s) compared to those at the less urban sites (157.51 ± 25.35 s), but this difference was not significant ($p = 0.11$, Table 3, Fig. 2D). Trials where the observer started the trial at a longer initial distance to the squirrel had significantly higher FAD and FID but not probability of climbing a tree or latency to resume behavior following the trial (Table 3). There were no

514 significant effects of sex, Julian date, or distance to the nearest tree on FAD, FID, probability of
515 climbing a tree, or latency (Table 3).

516 *Does urbanization impact associations between behaviors?*

517 Squirrels at urban sites (n = 38) that exhibited a higher FAD also exhibited a significantly
518 higher latency to resume typical behavior following the trial (r = 0.59, 95% CI = 0.33-0.77, $t_{22.9}$
519 = 3.07, p = 0.005) but this association between these two variables was significantly reduced in
520 squirrels at the less urban sites (n = 39, r = 0.09, 95% CI = -0.23-0.39), as indicated by the nearly
521 significant interaction between site category and latency ($t_{33.1}$ = -2.01, p = 0.053, Table 4, Fig.
522 3A). Similarly, squirrels at urban sites that exhibited a higher FID also tended to exhibit a higher
523 latency to resume typical behavior following the trial (r = 0.53, 95% CI = 0.26-0.73, $t_{68.2}$ = 1.86,
524 p = 0.066), but the association between these two behaviors was slightly lessened in squirrels at
525 less urban sites (r = 0.18, 95% CI = -0.14-0.47), although this was not significant ($t_{70.5}$ = -1.17, p
526 = 0.24, Table 4, Fig. 3B). In squirrels at both urban (r = 0.46, 95% CI = 0.17-0.68) and less
527 urban sites (r = 0.39, 95% CI = 0.09-0.63), squirrels that were more likely to escape the human
528 observer by climbing a tree also had a longer latency to resume typical behavior following the
529 trial ($t_{72.9}$ = 2.08, p = 0.041) and this did not depend upon whether the squirrels were at urban or
530 less urban sites ($t_{72.3}$ = 0.05, p = 0.96).

531

532 **Discussion**

533 The main goal of this study was to test the hypothesis that urban squirrels exhibit cross-
534 habituation to stimuli from natural predators due to repeated exposure to humans that causes
535 habituation to humans. Overall, squirrels at both urban and less urban sites responded to the

536 playbacks in such a way that indicates our experimental approach can be used for short-term
537 elevations of predation risk (similar to Coleman et al., 2008; McCleery, 2009; Bohls and
538 Koehnle, 2017; Lilly et al., 2019). However, the behavioral response depended upon which of
539 the four behaviors we measured: there were strong effects on FID and latency to resume typical
540 behavior following the trial in squirrels at both urban and less urban sites but little effect of the
541 playbacks on the probability of squirrels climbing a tree and no effect on FAD in squirrels in less
542 urban sites. There was little evidence of cross-habituation in urban squirrels as they still
543 exhibited increased vigilance (FAD) and anti-predator behavior (FID, latency to resume typical
544 behavior after the trial) when exposed to the playbacks of a natural predator (hawk) compared to
545 the control playback or the dog playbacks. These results indicate that urban squirrels do still pay
546 attention to predation risk and can discriminate and respond accordingly by becoming vigilant
547 and fleeing when the human is at a greater distance if the human is also paired with hawk
548 playbacks. In contrast, squirrels at less urban sites did not exhibit differences in FAD when
549 exposed to the playbacks and FID was highest when they were exposed to dog playbacks. Our
550 former result for FAD is similar to Coleman et al. (2008) who showed that Gunther's dik-diks
551 (*Madoqua guentheri*) in areas located a greater distance from human settlement did not exhibit
552 any change in vigilance behavior when presented with playbacks from a potential predator
553 versus the control playback (bird vocalizations) whereas dik-diks located closer to human
554 settlements did exhibit higher vigilance when exposed to the predator playbacks compared to the
555 control playbacks. Squirrels at both urban and less urban sites responded similarly to the
556 playbacks in terms of the latency to resume typical behavior after the trial, being highest when
557 they were exposed to hawk vocalizations and lowest when exposed to the control playback.

558 Overall, our results reject the hypothesis that urban squirrels exhibit cross-habituation due
559 to frequent exposure to humans that causes them to habituate to humans. Our results are similar
560 to those presented elsewhere, such as Coleman et al. (2008) mentioned above. For example,
561 Cooper et al. (2008) showed that gray squirrels (*Sciurus carolinensis*) in urban areas exhibited a
562 lower FAD when exposed to a human with a leashed dog versus just a human, whereas the cross-
563 habituation due to human stimuli hypothesis would predict that squirrels in urban areas would
564 exhibit a similar FAD when exposed to humans or humans with a dog. Previous studies (see also
565 Labra and Leonard, 1999; Seress et al., 2011; Bokony et al., 2012; Cavalli et al., 2016; Weaver et
566 al., 2018; Vincze et al. 2019) together with our results support that animals in urban habitats or
567 those frequently exposed to humans, even if habituated to humans, still exhibit increases in anti-
568 predator behavior in response to a non-human predator.

569 Other studies have reached a different conclusion and suggested that animals in urban
570 habitats exhibit reductions in both the anti-predator behavior exhibited in response to a human or
571 natural predator, perhaps due to cross-habituation due to frequent exposure to humans
572 (McCleery, 2009; Carette and Tella, 2017; Olson and Acevedo-Gutiérrez, 2017). For example,
573 McCleery (2009) showed that fox squirrels in urban areas exhibited a lesser increase in anti-
574 predator behavior when presented with hawk or coyote (*Canis latrans*) vocalizations whereas
575 those in rural areas significantly increased their anti-predator behavior when exposed to these
576 vocalizations. These results were used to make the inference that fox squirrels in urban areas
577 exhibited cross-habituation. Other studies using similar approaches have also found a similar
578 result. For instance, Carette and Tella (2017) showed that burrowing owls (*Athene cunicularia*)
579 in urban areas exhibited both a reduced FID when a human approached them and reduced anti-
580 predator behavior (latency to approach model predator placed near their nest) when faced with a

model of a natural predator, suggesting reduced overall anti-predator behavior in urban animals perhaps due to cross-habituation due to frequent exposure to humans. However, we think there is a difference between our study and previous studies on this topic that may explain these differences. We argue that in order to conclusively test the cross-habituation due to human stimuli hypothesis, one needs to 1) compare the behavioral responses within a population (e.g., behavioral response of urban population in the FID trial [human only or human plus control stimulus] vs. response to predator stimulus) and 2) present the human and predator stimuli *together*. That is the same behavior (vigilance, FID, other anti-predator behaviors) should be examined in response to a human plus a control stimulus (acoustic stimulus such as in our study or novel object for a visual stimulus) and a human plus the stimulus of a natural predator and the responses to these stimuli should be compared within a population rather than across. Olson and Acevedo-Gutiérrez (2017) and Carrette and Tella (2017) did not do either of these whereas McCleery (2009) did compare the behavioral response to the different stimuli within each population (urban, suburban, rural) but did not necessarily distinguish the behavioral response of the squirrels to a human by itself or paired with a control stimulus (acoustic in their case) versus the response to hawk or coyote vocalizations. Instead, McCleery (2009) observed squirrels for 2 min for pre-trial observations (where they only stimulus was the human observer) and then observed their initial behavioral response 20 s after a playback of hawk or coyote vocalizations followed by 2 min of post-playback observations. We argue here that our results present the most appropriate test of the cross-habituation to human stimuli hypothesis and reject it.

The effects of the dog playbacks on squirrel behavior in our study were surprising for a few reasons. Feral or non-feral domestic cats and dogs may have lethal and sublethal effects on wildlife (Miller et al., 2001; Banks and Bryant, 2007; Loss et al., 2013) including tree squirrels

604 (Koprowski, 1994; Wauters et al., 1997; Tumlison, 2012; Jokimäki et al., 2017). Previous studies
605 in tree squirrels show that they exhibit increased anti-predator behavior (vigilance or FID) to the
606 physical presence of a dog with a human handler (Gustafson and VanDruff, 1990; Cooper et al.,
607 2008) or behave in such a way (measured using giving up density: Brown, 1988) in areas with
608 high levels of dogs and cats that suggests that they perceive a higher predation risk in such areas
609 (i.e., giving up density was higher in study areas where cats and dogs are present: Bowers and
610 Breland, 1996). We therefore expected that squirrels would respond to the dog playbacks in
611 similar way to how they responded to the hawk playbacks. Instead, we found that squirrels at
612 both sites did not differ in their FAD when exposed to dog playbacks compared to the control
613 playbacks but they did exhibit a significantly longer latency to resume typical activity following
614 dog playbacks compared to the control playbacks. Additionally, squirrels in less urban sites but
615 not those in urban sites had a significantly higher FID when exposed to dog playbacks compared
616 to the control playback. This differs from previous studies in gray squirrels and burrowing owls
617 that showed that FAD and/or FID were increased in squirrels or owls in urban areas when they
618 were presented with a human plus dog compared to just a human (Gustafson and VanDruff,
619 1990; Cooper et al., 2008; Cavalli et al., 2016). Unlike studies in gray squirrels (Gustafson and
620 VanDruff, 1990; Cooper et al., 2008), we found a higher FID in response to the dog only in
621 squirrels from less urban areas but not those from urban areas. One interpretation of our results is
622 that squirrels in urban sites in our study were habituated to dog vocalizations, whereas squirrels
623 in less urban areas viewed the sounds of dogs paired with humans as threatening. Although our
624 "snapshot" estimates of dog abundance recorded at all the sites showed that dog presence was
625 *higher* at the less urban sites (see above), it seems likely that cumulative exposure to dogs is
626 actually higher for squirrels in urban sites due to the higher overall human presence. This

627 suggests that squirrels in urban environments exhibit selective habituation where the response to
628 humans or stimuli from their commensals (dogs) is attenuated but the increased response to
629 natural predators is maintained despite this habituation.

630 Our study operated under the assumption that squirrels in less urban areas experienced
631 less exposure to humans than those in urban areas, thereby causing the latter to habituate to
632 human stimuli. This assumption was supported by human population density data (Table 1) and
633 also our own data collection efforts while we visited sites, with the latter showing a substantially
634 higher presence of humans at our urban sites (see above). Our results from the trials conducted
635 without playbacks also support our assumption that squirrels in urban areas were habituated to
636 human presence whereas those in less urban areas were not. Similar to most other studies in
637 terrestrial animals (Samia et al., 2015) and in studies specifically in tree squirrels (McCleery,
638 2009; Engelhardt and Weladji, 2011; Sarno et al., 2015; Uchida et al., 2020), we found that
639 squirrels in urban areas exhibited a shorter FAD and FID, lower probability to climb a tree to
640 escape the human observer, and a shorter latency to resume typical behavior following the trial,
641 although only FID was significantly different. Focusing only our trials without playbacks, our
642 results are similar to Uchida et al. (2019) as they suggest that the reduction in FID that we
643 observed in urban squirrels is due to both reduced vigilance and habituation to humans given that
644 we found a lower FAD (vigilance) and FID (habituation) in squirrels in the urban areas.
645 Although only the difference in FID between squirrels in urban and less urban sites was
646 statistically significant, the congruency of our results with previous studies strongly supports this
647 assumption that squirrels at our urban sites were more habituated to humans. This is somewhat
648 surprising given that these are presumably sympatric populations of squirrels with large amount
649 of gene flow between them with the linear distance between some urban and less urban sites

being ~1 km. Unless selection favoring reductions in anti-predator behavior is extremely strong in urban areas or features of urban landscapes strongly impede gene flow (Johnson and Munshi-South, 2017), it seems likely that these behavioral differences are driven by plasticity given that the likely exchange of individuals between suburban and urban sites prevents local genetic adaptation to these different sites (see discussion in Sol et al., 2013). It is also possible that these behavioral differences are due to personality-dependent colonization of urban habitats, such as squirrels with higher boldness or tolerance for humans or reduced vigilance and anti-predator behavior settling in urban areas (Carrete and Tella, 2010; Sprau and Dingemanse, 2017), but we cannot distinguish among these possibilities at this time.

Behavior has long been appreciated to be one of the first adaptive responses to changing environmental conditions (Baldwin, 1896; Bartholomew, 1964; West Eberhard, 1989), yet recent studies have emphasized how strong and and/or inflexible correlations between behaviors (behavioral syndromes: Sih et al., 2004) may act as an evolutionary constraint by preventing an optimal behavior from being produced (Dochtermann and Dingemanse, 2013). This could be due to some shared pleiotropic mechanism that yokes two behaviors together, such as genetic pleiotropy (van Oers et al., 2005) or hormone levels causing simultaneous changes in two behaviors (Dantzer and Swanson, 2017). Basic quantitative genetics would predict that if there is a shared mechanism, selection on one behavioral trait may pull the others along (Lande and Arnold, 1983; Bell, 2005; Dochtermann and Roff, 2010). In the case of animals adapting to urban environments, it's possible that selection favoring individuals with reduced anti-predator behavior (as this may increase resource acquisition) causes some correlated shift in other behaviors, such as boldness, neophobia, aggression, among other behavioral traits. There was relatively little evidence that the correlations among the different behaviors we recorded differed

673 between squirrels in urban and less urban sites. We found that the association between latency to
674 resume typical behavior following a trial and FAD differed between urban and less urban
675 populations. Squirrels in urban but not less urban sites that allowed humans to get closer to them
676 before exhibiting first vigilance (FAD) also took a significantly shorter amount of time to resume
677 typical behavior following the end of the trial. However, there was no significant deviance in the
678 association between FID and latency or probability to escape by climbing a tree and latency in
679 squirrels from urban and less urban sites. As such, our results somewhat differ from previous
680 studies (Evans et al., 2010; Scales et al., 2011; Bokony et al., 2012; Myers and Hyman, 2016;
681 Carrete and Tella, 2017) that indicate that urban environments may favor shifts in the behavioral
682 syndrome by enhancing or lessening the associations between two different behaviors. However,
683 we note that these are merely phenotypic correlations and we did not address the repeatability or
684 heritability of our behavioral measures given that our animals were not marked uniquely,
685 although phenotypic correlations may approximate genetic correlations (Dochtermann, 2011).
686 Moreover, whether or not these shifts in the behavioral syndrome are adaptive or evidence of
687 some type of constraint where selection favoring a reduction in vigilance or anti-predator
688 behavior causes a correlated response in other behaviors needs to be viewed with caution. For
689 example, these changes could be caused by some shared environmental factor that induces
690 simultaneous shifts in both behaviors (i.e,. environmental co-variance: Mitchell-Olds and Shaw,
691 1987; Kruuk et al., 2003). Finally, it should not be surprising that the large environmental
692 differences between urban and less urban populations would cause a rapid reorganization of the
693 magnitude or even sign of the phenotypic correlations among different behaviors. For example,
694 abrupt shifts in the environment can rapidly change the genetic variance-covariance matrix of
695 highly heritable phenotypes like morphological traits (Eroukhmanoff and Svensson, 2011;

696 Kimmel et al., 2012) and among-population comparisons show that the nature of phenotypic
697 correlations among behaviors is not static (Bell, 2005; Dingemanse et al., 2007). Unfortunately,
698 at this point we cannot infer whether the shift in phenotypic correlations we documented here
699 (for FAD and latency) is adaptive, evidence of a constraint or correlated response to selection, or
700 merely due to environmental co-variance.

701 Overall, our results provide insight into how urbanization may shape the behavioral
702 characteristics of non-volant animals. As most studies on this topic are in birds (Samia et al.,
703 2015), which can escape from humans using flight, it is important to consider if the same
704 patterns are found in terrestrial mammals. Our results and others discussed above confirm these
705 patterns and show that non-volant animals in urban environments exhibit less vigilance and anti-
706 predator behavior and also highlight that urbanization may alter the association among different
707 behaviors. Finally, our results reject the cross-habituation due to human stimuli hypothesis as
708 squirrels in urban environments that were habituated to humans still exhibited a strong
709 behavioral response to acoustic stimuli from a natural predator (hawks). Although we do not
710 wish to imply that habituation to humans is cost-free in terms of not causing a reduction in anti-
711 predator behavior to natural predators, most studies on this topic fail to find evidence that
712 animals habituated to humans also exhibit cross-habituation to stimuli from natural predator.
713 Given that urbanization is unlikely to slow at any time soon, increased effort is needed to
714 determine if habituation to humans carries such heavy costs and whether it could even be
715 beneficial for animals that cannot avoid it due to seasonal food pulses coinciding with a large
716 influx of tourists (Wheat and Wilmers, 2016). Other studies suggest the costs of human
717 habituation may be more nuanced, such as habituation to humans reducing the latency to return
718 to the nest following a disturbance in nesting shorebirds, but potentially causing increased chick

mortality due to the presence of dogs that often are paired with human stimuli (Baudains and Lloyd, 2007). Clearly more work is needed on this subject, especially on a greater number of species including species that are not "urban exploiters" like tree squirrels, but the existing evidence rejects the proposal by Geffroy et al. (2015) that there is a cost to human habituation in terms of lowering the anti-predator behavior of animals to other natural predators.

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Data Availability Statement

All data will be archived at Dryad before final acceptance and all regulations set by *(insert journal title)* will be followed.

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Table 1. Overview of data collection sites. Behavioral data from fox squirrels were collected at six sites around Ann Arbor, Michigan, USA located in areas that were classified as urban or less urban. Urban sites were in areas with human population densities of greater than 1000 persons per square km whereas less urban sites were in areas with population densities of 250 – 1000 persons per square km (County Farm Park, Nichols Arboretum) or fewer than 250 persons per square km (Saginaw Forest). Population density estimates obtained from the Center for International Earth Information Network of Columbia University (2018). Total days of observation and hours over these days are provided. When we visited the sites, human and dog presence was quantified (see methods) with mean and 1 standard deviation (SD) shown below. UM refers to the University of Michigan.

Site Name	GPS Location of Site	Site Category	Population Density (persons/ sq km)	Human Presence		Dog Presence		Total Trials	Total Observation Days	Total Observation Hours
				Mean Count/Trial	SD	Mean Count/Trial	SD			
Central Campus	N 42°16.622' W083°44.293'	Urban	> 1000	97.7	7.3	0.39	0.61	33	6	9.68
North Campus	N 42°17.753' W083°42.620'	Urban	> 1000	68.0	11.4	0	0	25	4	6.18
Prospect Park	N 42°14.825' W083°36.093'	Urban	> 1000	3.6	10.2	0.69	1.55	23	4	6.08
County Farm Park	N 42°15.430' W083°42.518'	Less urban	250 - 1000	1.6	1.3	0.41	0.50	32	5	8.73
Nichols Arboretum	N 42°16.940' W083°43.410'	Less urban	250 - 1000	11.9	2.3	2.32	1.19	28	5	9.07
Saginaw Forest	N 42°16.235' W083°48.465'	Less urban	25 - 250	0.37	0.50	0.20	0.41	30	6	12.62

Table 2. Effects of acoustic playbacks (control, hawk, dog) on first alert distance (FAD), flight initiation distance (FID), probability of escaping human observer by climbing a tree, and latency to resume behavior following the trial for fox squirrels observed at urban or less urban sites. Reference (intercept) was "less urban" for site, "control playback" for treatment, and "female" for sex. A random effect for site identity was included in the model or FAD ($\sigma^2 = 6.5$), FID ($\sigma^2 = 4.9$), probability of climbing a tree ($\sigma^2 = 0.07$), and latency ($\sigma^2 = 0.37$). Latency was ln+1 transformed. Results are from 94 trials from six study sites.

Response Variable	Variable	*b*	SE	*t* or *z*	*P*
First alert distance (FAD)	Intercept (Less urban, Female, Control)	14.94	0.89	16.71	<0.0001
	Site (Urban)	-2.32	1.33	-1.74	0.11
	Dog playbacks	0.51	0.90	0.57	0.57
	Hawk playbacks	-0.29	0.98	-0.30	0.76
	Distance to tree	0.36	0.29	1.23	0.22
	Sex (Male)	0.29	0.58	0.51	0.61
	Julian Date	-0.10	0.28	-0.34	0.73
	Initial Distance	3.20	0.29	10.84	<0.0001
	Site (Urban) x Dog playbacks	0.07	1.35	0.05	0.96
	Site (Urban) x Hawk playbacks	4.29	1.49	2.86	0.005
Flight initiation distance (FID)	Intercept (Less urban, Female, Control)	12.3	0.95	7.31	<0.0001
	Site (Urban)	-1.64	1.39	-1.18	0.27
	Dog playbacks	1.89	0.79	2.40	0.018
	Hawk playbacks	1.21	0.85	1.42	0.16
	Distance to tree	0.08	0.26	0.30	0.76
	Sex (Male)	0.01	0.50	0.01	0.99
	Julian Date	-0.39	0.25	-1.59	0.11
	Initial Distance	2.99	0.26	11.51	<0.0001
	Site (Urban) x Dog playbacks	-1.52	1.18	-1.29	0.20
	Site (Urban) x Hawk playbacks	2.53	1.30	1.94	0.056
Probability of climbing a tree	Intercept (Less urban, Female, Control)	-0.20	0.62	-0.33	0.74
	Site (Urban)	-0.61	0.92	-0.66	0.51
	Dog playbacks	1.14	0.82	1.39	0.16

	Hawk playbacks	1.26	0.90	1.40	0.16
	Distance to tree	-0.64	0.27	-2.34	0.02
	Sex (Male)	0.61	0.55	1.10	0.27
	Julian Date	-0.26	0.27	-0.97	0.33
	Initial Distance	0.01	0.27	0.04	0.96
	Site (Urban) x Dog playbacks	-1.52	1.20	-1.27	0.20
	Site (Urban) x Hawk playbacks	0.18	1.34	0.14	0.89
Latency to resume behavior	Intercept (Less urban, Female, Control)	3.83	0.39	9.69	0.0001
	Site (Urban)	-0.60	0.57	-1.05	0.33
	Dog playbacks	1.14	0.23	4.91	<0.0001
	Hawk playbacks	1.71	0.25	6.76	<0.0001
	Distance to tree	0.03	0.07	0.39	0.70
	Sex (Male)	0.24	0.15	1.61	0.11
	Julian Date	0.07	0.07	-0.91	0.36
	Initial Distance	0.06	0.08	0.82	0.41
	Site (Urban) x Dog playbacks	0.03	0.35	0.10	0.92
	Site (Urban) x Hawk playbacks	0.22	0.39	0.57	0.57

Table 3. Differences between fox squirrels at urban and less urban sites that were not exposed to any playbacks for first alert distance (FAD), flight initiation distance (FID), probability of escaping the human observer by climbing a tree during the trial, and latency to resume behavior following the trial. A random effect for site identity was included in the model or FAD (σ^2 = 6.9), FID (σ^2 = 6.24), probability of climbing a tree (σ^2 = 1.23), and latency (σ^2 = 1.22). Latency was ln+1 transformed. Results are from 77 trials from six sites.

Response Variable	Variable	*b*	SE	*t* or *z*	*P*
First alert distance (FAD)	Intercept (Less urban, Female)	14.11	1.63	8.67	0.0007
	Site (Urban)	-4.79	2.26	-2.11	0.10
	Distance to tree	-0.37	0.38	-0.98	0.33
	Sex (Male)	1.17	0.66	1.77	0.08
	Julian Date	0.15	0.40	0.39	0.70
	Initial Distance	2.63	0.35	7.51	<0.0001
Flight initiation distance (FID)	Intercept (Less urban, Female)	12.16	1.54	7.89	0.0009
	Site (Urban)	-6.22	2.14	-2.90	0.043
	Distance to tree	-0.56	0.35	-1.59	0.12
	Sex (Male)	0.85	0.61	1.38	0.17
	Julian Date	0.01	0.37	0.03	0.97
	Initial Distance	1.38	0.32	4.24	<0.0001
Probability of climbing a tree	Intercept (Less urban, Female)	0.71	0.79	0.89	0.37
	Site (Urban)	-1.89	1.12	-1.68	0.09
	Distance to tree	-0.09	0.32	-0.27	0.78
	Sex (Male)	0.17	0.55	0.31	0.76
	Julian Date	0.47	0.35	1.34	0.18
	Initial Distance	0.23	0.30	0.76	0.45
Latency to resume behavior	Intercept (Less urban, Female)	4.48	0.67	6.70	0.002
	Site (Urban)	-1.87	0.93	1.99	0.11
	Distance to tree	-0.07	0.13	-0.57	0.57
	Sex (Male)	0.06	0.23	0.25	0.80
	Julian Date	0.07	0.14	0.52	0.61

Initial Distance	0.002	0.12	0.02	0.98

Table 4. Associations between latency to resume behavior following the trial ("Latency") and first alert distance (FAD), flight initiation distance (FID), and probability of escaping the human observer by climbing a tree during the trial ("Went up tree?") in fox squirrels at urban and less urban sites that were not exposed to any playbacks. A random effect for site identity was included in the model or FAD ($\sigma^2 = 0.02$), FID ($\sigma^2 = 0.08$), and probability of climbing a tree ($\sigma^2 = 1.03$). FAD, FID, and latency were ln+1 transformed. Results are from 77 trials from six sites.

Response Variable	Variable	*b*	SE	*t* or *z*	*P*
First alert distance (FAD)	Intercept (Urban, Female)	2.42	0.10	24.2	0.0004
	Site (Less urban)	0.27	0.14	1.91	0.16
	Latency	0.25	0.08	3.07	0.005
	Went up tree?	-0.10	0.07	-1.39	0.17
	Site (Less urban) x Latency	-0.23	0.12	-2.01	0.053
	Site (Less urban) x Went up tree?	0.13	0.09	1.41	0.16
Flight initiation distance (FID)	Intercept (Urban, Female)	1.94	0.18	11.0	0.0005
	Site (Less urban)	0.63	0.25	2.54	0.067
	Latency	0.16	0.08	1.86	0.067
	Went up tree?	-0.08	0.07	-1.18	0.24
	Site (Less urban) x Latency	-0.14	0.12	-1.17	0.24
	Site (Less urban) x Went up tree?	0.06	0.09	0.70	0.48
Latency to resume behavior	Intercept (Urban, Female)	2.77	0.61	4.56	0.01
	Site (Less urban)	1.61	0.86	1.88	0.13
	Went up tree?	0.43	0.21	2.08	0.041
	Site (Less urban) x Went up tree?	0.01	0.26	0.05	0.96

Figures

Figure 1. Effects of human observer approaching a squirrel while broadcasting one of three playback treatments (control playback, hawk or dog vocalizations) on A) first alert distance (FAD), B) flight initiation distance (FID), C) probability of escaping up a tree during the trial, and D) latency to resume typical behavior following the trial. Trials were conducted at less urban (n = 15 control, 20 dog, 16 hawk) and urban (n = 11 control, 20 dog, 12 hawk) sites. Results shown in Table 2. Upper and lower hinges correspond to first and third quartile, respectively. Upper/lower whiskers extend from the hinge to the highest/lowest value that is within 1.5x the interquartile range. Solid horizontal line shows median.

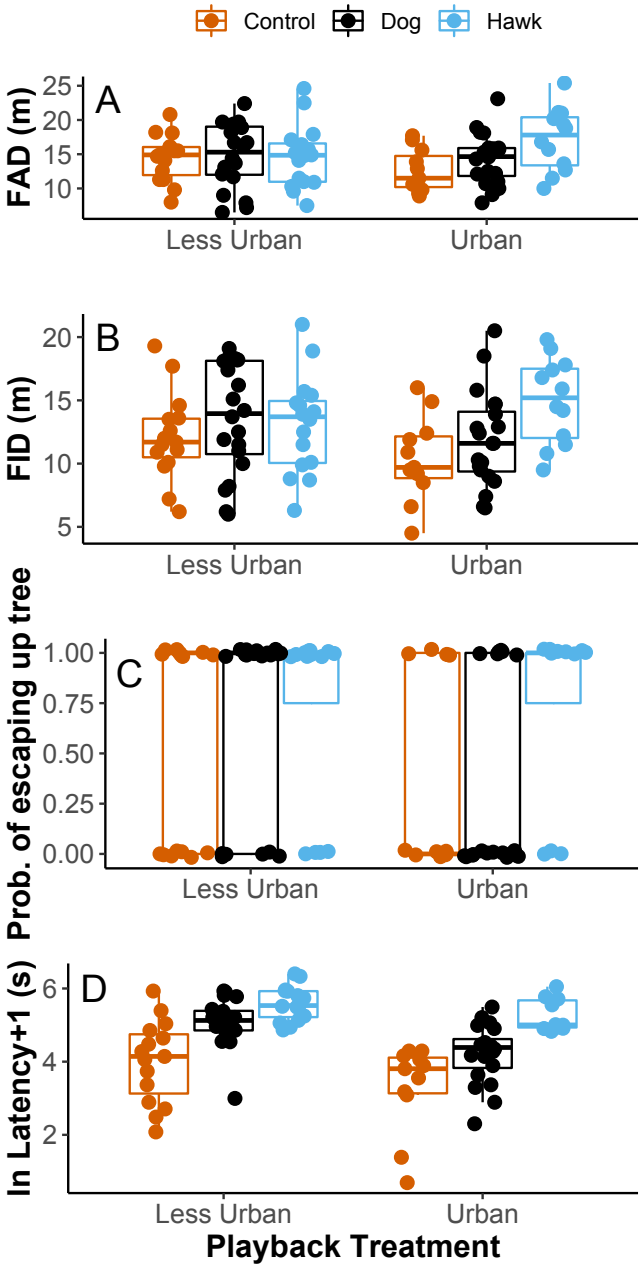


Figure 2. Variation in A) vigilance (first alert distance: FAD), B) anti-predator behavior (flight initiation distance: FID), C) probability of escaping up a tree during the trial, and D) latency to resume behavior following the trial among fox squirrels at urban (n = 38 trials) and less urban (n = 39) sites in trials where squirrels were not exposed to any playbacks. Squirrels in urban areas had significantly shorter FID compared to those in the less urban sites, but there were no other significant differences (Table 3). Upper and lower hinges correspond to first and third quartile, respectively. Upper/lower whiskers extend from the hinge to the highest/lowest value that is within 1.5x the interquartile range. Solid horizontal line shows median.

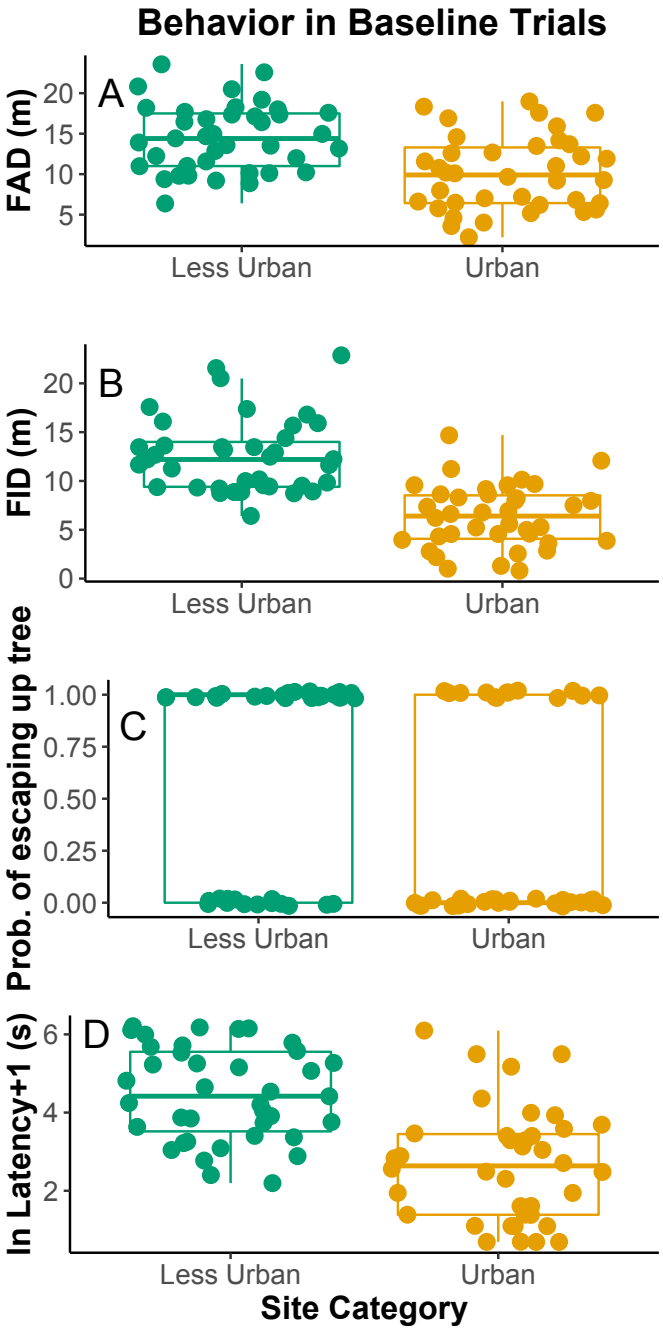
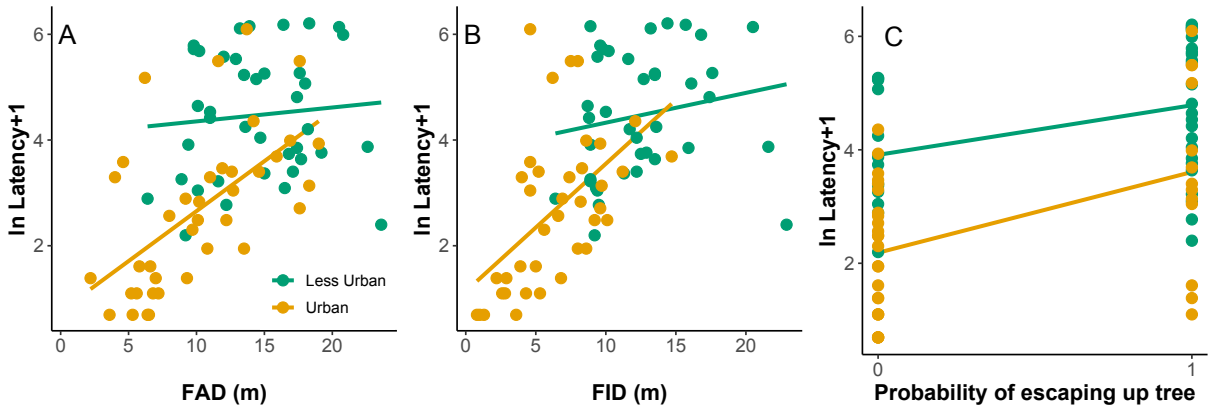


Figure 3. Associations between the latency of fox squirrels in urban and less urban study areas to resume typical behavior after a disturbance trial with A) first alert distance (FAD), B) flight initiation distance (FID), and C) probability of escaping the human observer by climbing up a tree. FAD and latency to resume typical behavior were significantly positively associated in urban squirrels but not those in less urban areas. In squirrels in both urban and less urban areas, those that had a higher FID or climbed a tree also took longer to resume typical behavior. differed between urban and less urban populations of squirrels. Note that latency is ln+1 transformed.



Supplementary Materials

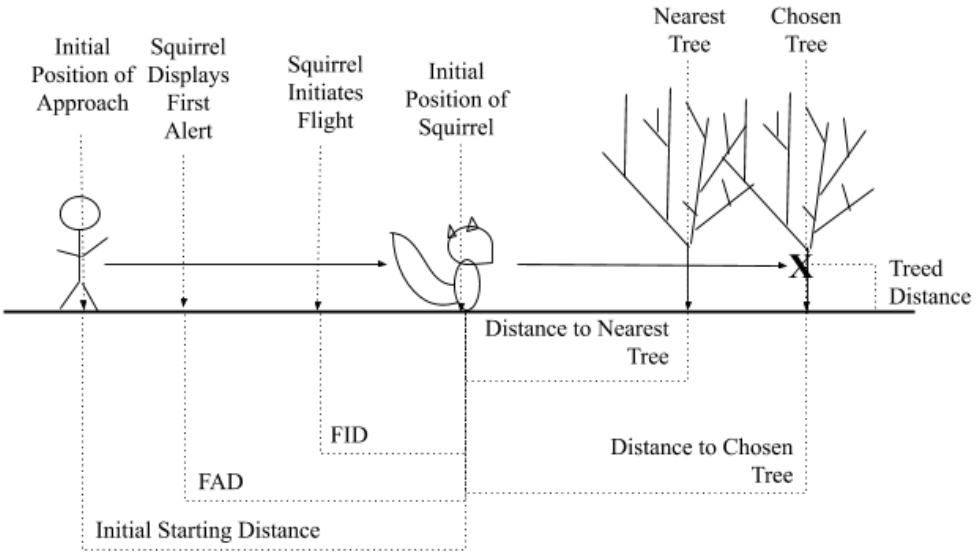



Figure S1. Overview of trial protocol. A marker was placed at the initial position of the observer when they initially identified a squirrel for a trial. The distance between observer initial position and the squirrel's initial position is the initial distance. From this position, the observer walked at a slow, steady, and constant pace in a direct line towards the squirrel. The observer then dropped a marker to represent FAD when the squirrel first displayed "alert" behavior (freezing with eye contact). An additional marker was dropped when the squirrel fled from its initial position to represent FID. Another marker was placed at an estimate of the squirrel's initial position. In playback manipulation trials, acoustic stimuli were broadcast when the observer started approaching the squirrel and stopped playing the stimuli when the squirrel fled. Distance from the squirrel's initial position to the nearest tree was recorded along with how high in the tree the squirrel took refuge. If the squirrel took refuge in a tree other than the one nearest, then distance to the chosen tree was recorded. Once the squirrel took refuge latency to resume normal activity (foraging or traveling off tree) was recorded. Trials ended when squirrels resumed normal activity or if latency exceeded 10 min.