Improving the success of reinforcement programs: effects of a soft-release on the anti-predator behaviour of captive-bred European hamsters (*Cricetus cricetus*) (#84167)

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Improving the success of reinforcement programs: effects of a soft-release on the anti-predator behaviour of captive-bred European hamsters (*Cricetus cricetus*)

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Captive breeding programs are an important pillar in biodiversity conservation, aiming to prevent the extinction of threatened species. However, the establishment of self-sustaining populations in the wild through the release of captive-bred animals is often hampered by a high mortality upon release. In this study, we investigated how a two-week training period within a large outdoor enclosure affected the anti-predator behaviour of ‘naive’ captive-bred hamsters (*Cricetus cricetus*) and how potential modifications persisted over time. During three consecutive tests, hamsters were confronted with a moving predator model (a red fox mount, *Vulpes vulpes*) and their behaviour was filmed. After the initial round of confrontation with the predator model, one group of hamsters (field group) was released into an outdoor enclosure protected from predators, while the other group (control) remained in their individual laboratory cages. After two weeks, hamsters from the field group were recaptured and individuals of both groups underwent a second confrontation test. One month after their return from the outdoor enclosure, field hamsters were subjected to a last confrontation test. Video analysis, investigating four behavioural variables, revealed that field hamsters significantly modified their behavioural response following the two weeks in the enclosure, while this was not the case for control hamsters. In addition, most behavioural modifications in field hamsters persisted over one month, while others started to revert. We suggest that an appropriate pre-release training period will enable captive-bred hamsters, to develop an adequate anti-predator behaviour that will increase their immediate survival probability upon release into the wild. These results will now need to be tested post-release but believe that such measure will be of great importance for hamster conservation programs.

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Introduction

Reinforcement or ‘restocking’ programs are widely used in biodiversity conservation to sustain or restore declining or threatened populations (Fischer and Lindenmayer, 2000; Guy, Curnoe and Banks, 2013; Bubac *et al.*, 2019). Reinforcement is defined as the intentional addition of captive-bred individuals or individuals from a stable wild population to an existing group of conspecifics, to recover endangered populations (Zlatanova, 2016). This strategy is largely used to increase population density, to compensate for low dispersion rates, to correct skewed sex-ratios or to improve the genetic status of small populations (Weeks *et al.*, 2015).

Reinforcement programs have been implemented worldwide with a variety of taxa, ranging from invertebrates to mammals (Soorae, 2018). Unfortunately, their success is often limited (Beck *et al.*, 1994; Black *et al.*, 1997; Fischer and Lindenmayer, 2000; Short, 2009). This is mainly due to the high short-term mortality of animals released during restocking programs (Griffin, Blumstein and Evans, 2000; McPhee and Silverman, 2004; Shier and Owings, 2007). Four main reasons have been proposed to explain such high post-release mortality: (1) the unfamiliarity of released animals with local conditions (Calvete and Estrada, 2004); (2) the high risk of starvation due to the inability of captive-bred animals to forage efficiently (Jule, Leaver and Lea, 2008); (3) the deficiency of their immune system (Abolins *et al.*, 2017), and (4) an alteration of the instinctive anti-predator behaviour when reared in captivity over many generations (Miller *et al.*, 1990; Fischer and Lindenmayer, 2000; Griffin *et al.*, 2000).

In a recent study, European hamsters (*Cricetus cricetus*), bred in captivity over 15 generations, showed a marked aggressive response when confronted with a mobile predator (European ferret, *Mustela putorius furo*), rather than flee and hide in an available shelter (Tissier *et al.*, 2019). Such behavioural response is not consistent with the common assumption that prey species will only show aggressive behaviour towards a predator if freezing or fleeing are not viable options (Eilam, 2005). Attacking an unknown predator is likely to be fatal for hamsters in the wild, questioning the appropriateness of such a response (Tissier et al., 2019). Non-appropriate behavioural responses to predation risk can be a major problem for reinforcement programs, especially when animals face a high predation pressure upon their release into the wild (Moseby *et al.*, 2011; La Haye *et al.*, 2020).

A number of studies have investigated possible alterations of the instinctive anti-predator behaviour in prey species as a consequence of captive breeding (Shier and Owings, 2006; Carrete and Tella, 2015; Jolly, Webb and Phillips, 2018). These studies suggest that fish, birds and mammals can improve the effectiveness of their anti-predator behaviour by undergoing a pre-release “training” (Griffin *et al.*, 2000; Guy *et al.*, 2013; Edwards *et al.*, 2021). Such training can, for example, consist of an exposure to a predator model coupled with aversive stimuli, such as alarm signals (Kleiman, 1989; McLean, Lundie-Jenkins and Jarman, 1996; Griffin *et al.*, 2000; Shier and Owings, 2007). Another pre-release training approach is the use of a ‘soft-release’: individuals experience a pre-release period inside an outdoor enclosure that mimics the environment of their future release site as closely as possible but shelters them from predation. Both methods seem to improve the ability of captive-bred individuals to recognize and avoid predators after being released and allow them to familiarize themselves with new threats (Reading, Miller and Shepherdson, 2013; Resende *et al.*, 2021). However, the success of these pre-release training programs differs between species and their efficacy has never been tested in solitary-living prey species, which lack the horizontal transmission of survival behaviour from conspecifics (Tetzlaff, Sperry and DeGregorio, 2019).

One such species is the European hamster, which, until the 1970s, was abundant across Europe and Asia (Weinhold, 2009; Surov *et al.*, 2016). However, due to habitat fragmentation, agriculture intensification, and climate change, it is now one of the most threatened mammal species in Western Europe (Weinhold, 2009; Tissier *et al.*, 2016) and has recently been classified as “critically endangered” by the IUCN (International Union for Conservation of Nature; Banaszek *et al.*, 2020).

In addition to conservation measures focusing on habitat restoration (La Haye, 2013; Tissier *et al.*, 2018, 2021), reinforcement programs have been implemented in most western-European countries in an effort to sustain and restore the most fragile hamster populations across Europe (the Netherlands: La Haye *et al.*, 2010; Belgium: Verbist, 2008; and Germany: Sander and Weinhold, 2008). In France, a reinforcement program has been in place since 2002 but its success has varied considerably (Villemey *et al.*, 2013; Chaigne *et al.*, 2015). As part of this program, captive-bred hamsters have been released at unharvested agricultural sites every spring for the past twenty years. The year of the release, to prevent attacks from terrestrial predators, these sites are protected by electric fences throughout the hamster breeding season (Villemey *et al.*, 2013). Despite this, mortality rates of released hamsters were as high as 91% within the first 4 months upon release (calculated from Wagner *et al.*, 2017) and varied greatly between years. Hence, while the reinforcement program has allowed to maintain the relict hamster population in France, it has so far failed to restore a viable, self-sustaining population (Tissier *et al.*, 2019). Given the high mortality rates following release, it would seem obvious that efforts should focus on increasing the post-release survival of captive-bred hamsters, to improve the effectiveness of this conservation measure.

In an effort to reduce post-release mortality of captive-bred hamsters, we investigated whether a pre-release training period inside a protected outdoor enclosure might elicit a more appropriate anti-predation response. To evaluate the efficacy of such training, we confronted hamsters with a predator model before and after their two-week training period inside a protected outdoor enclosure (field group) and recorded their behaviour during these tests. To study whether potential behavioural differences after such training persisted over time, we again confronted these hamsters with a predator model one month after their return to the captive indoor facility. In parallel, we investigated whether the repeated confrontation with a predator model alone could alter the behavioural responses in a group of hamsters that remained inside the captive facility throughout experimentation (control group). This experimental design allowed us to address the following questions: (1) Does a two-week training period within a large outdoor enclosure affect the anti-predation responses of 'naive' hamsters (confrontation test #1 versus #2)? (2) If anti-predation responses differ after the training, do these modifications persist over time (confrontation test #3)? And (3) Does repeated exposure to a predator model alone alter the anti-predator behaviour of hamsters (Control group, confrontation test #1 versus #2)?

We expected that (1) a two-week training period will lead to a shift in the anti-predator behaviour of hamsters between confrontation trials, leading to a more appropriate response (i.e. flee and hide rather than mounting an aggressive defense); that (2) any potential differences in anti-predator behaviour in the field group will diminish over time; finally, we expected that (3) hamsters without such training period will not show a shift in their behavioural response between confrontation tests.

Materials and methods

1. Ethical note

This study followed the EU Directive 2010/63/EU guidelines for experiments, care and use of laboratory animals. The experimental protocol was approved by an Institutional Review Board (Ethical Committee: CREMEAS) under agreement number 02015033110486252 (APAFIS#397)02. At the end of the study, hamsters were not euthanized as they were only subjected to behavioural tests without invasive treatments. Individuals, have been released into the wild the same year or the following year as part of the annual reinforcement program.

2. Animals and housing conditions

We used 27 one-year-old female European hamsters that were born and raised in our captive breeding unit (CNRS, IPHC-DEPE, Strasbourg, France). Hamsters in this unit are the descendants of wild hamsters that were caught in the region (near Blaesheim, Alsace, France) between 1996 and 2002 (Reiners *et al.*, 2014). Animals were housed individually in transparent Plexiglas cages with wire lids (420*265*180 mm, L*W*H) that contained bedding material and enrichments (wood and shredded paper). Water and food pellets (105 pellets, SAFE, 89290 Augy, France) were provided *ad libitum*. Room temperature was maintained at 20-23°C and light conditions followed the summer photoperiod (16L:8D) during experimentation. Before experimentation, hamsters were randomly assigned to two groups (control vs. field group)

3. Experimental design

To study the instinctive anti-predator responses of captive-bred hamsters and to investigate whether a pre-release training period might suffice to change their anti-predator behaviour, we developed a standardized confrontation test with a predator model (Fig. 1).

All experimental trials were conducted on the CNRS Campus (Strasbourg, France) during daylight hours (9h-17h) and were filmed with a digital video camera (Sony HDR-PJ40, Tokyo, Japan). All hamsters were raised under similar conditions prior to treatment and the tests were performed in random order. Before the confrontation tests, hamsters were allowed to familiarize with the experimental arena during two habituation sessions (~12 min each), which were separated by one week. Following these sessions, all hamsters participated in two standardized confrontation tests with a predator model, which were separated by two weeks (Tab. 1). During these two weeks, hamsters of the field group were placed inside a large outdoor enclosure (field group; N=15), while the control group (N=12) remained in their individual cages at the breeding facility (for more information on the outdoor enclosure see supplementary information). Sample size was greater in the field group to balance the potential loss of individuals during the period in the outdoor enclosure (i.e. escape/mortality from natural causes). Two weeks after the release of the field group in the enclosure, 10 of the 15 hamsters were recaptured by trap (MCL Leclercq, Wavrin, France). These 10 hamsters underwent their second confrontation test 24h after the return to the breeding unit. Similarly, control hamsters underwent their second test two weeks after their first, albeit without previous field training (Tab. 1). Finally, one month after the second confrontation test, hamsters from the field group underwent a third test. The control group could not undergo a third test as these animals had already been released as part of the annual reinforcement program.

4. Experimental protocol

4.1 The arena and the predator model

All confrontation tests were conducted within a rectangular arena, constructed from PVC boards (3x1x0.4 m, LxWxH; Fig. 1). A PVC tube (2 m long, 10 cm in diameter), perforated at 50 cm intervals, was placed in the middle of the arena. It mimicked the shape of a tunnel, providing shelter, and was accessible to hamsters throughout a trial. The efficacy of such a PVC tunnel to act as an 'anti-predation tube' (APT) had been confirmed during confrontations trials between hamsters and a mobile predator (the European ferret; Tissier et al., 2016, 2018; 2019). We used a taxidermically-mounted red-fox (*Vulpes vulpes*) and fresh fox scent to mimic the predator during confrontation trials (see supplementary information).

4.2 Confrontation tests

Hamsters were tested individually. Each test lasted 14 minutes and was divided into three phases. During an initial 5-min period, a hamster could move freely inside the PVC arena without external perturbation (phase 1). Thereafter, the fox model and associated scent were presented to the hamster for 4 min (phase 2; Fig. 1). Finally, the fox model and associated scent were removed from the arena and the hamster was left undisturbed again for 5 min (phase 3). During the confrontation with the fox model (phase 2), an experimenter was hidden behind an opaque curtain and controlled the movements of the fox model via a metal rod fixed below the tail of the fox (Fig. 1), mimicking predator attacks. The experimenter followed a strict protocol, adapted to the behaviour of the hamster (see supplementary information and Fig.S1).

4.3 Release and recapture of the field group

Within 24h of the first confrontation tests, hamsters of the field group were released inside the outdoor enclosure around sunset (see supplementary information). For two weeks, hamsters were free to explore the enclosure, to forage, to dig their own burrows and to interact with congeners and/or other small fauna. During recapture, traps were activated at sunset and hamsters that were caught during the night were returned to the laboratory at sunrise.

4.4 Behavioural recordings and statistical analysis

All confrontation tests were filmed, and video analysis was conducted using the *Behavioral Observation Research Interactive Software* (Boris, v.6.3.3-2018; Friard & Gamba, 2016). We established an ethogram, which included various behavioural variables (Tab. S1). During the confrontation tests and the video analysis, the experimenter did not know the group of each hamster tested. For video analysis, the start and end times of different behaviours included in the ethogram were marked and durations summed to establish a time budget for the different behavioural variables. We focused our analysis on the following four behavioural variables (see Tab. S1): (i) the time (% of phase duration) the hamster spent inside the shelter (APT); (ii) the time (fraction) the hamster spent exploring the arena when outside the APT; (iii) the time between introduction of the fox model into the arena and entrance of the hamster into the APT (latency); (iv) the number of hamster attacks on the fox model. The first two variables were investigated separately for each trial phase, while the last two variables only concerned phase 2.

To test (1) whether a two-week training period was sufficient to shift the anti-predator behaviour of hamsters (field group) between tests, leading to a more appropriate response (hypothesis 1) and to also test (2) whether such a shift was absent in hamsters without such training period (control group; hypothesis 3), we ran Generalized Linear Mixed Models (GLMMs) for each of the four behavioural response variables, comparing tests #1 with tests #2 (before/after treatment) of control and field hamsters (Tab. S2). Group and test number were included as fixed effects, while Hamster ID was included as a random effect to account for repeated measures. Interactions between groups and test numbers were also included (e.g.: Behavioural variable = group + test# + group*test# + hamster ID [random]). We used an ANOVA based GLMM with Tuckey-HSD for post-hoc analyses.

Similarly, to test if potential differences in anti-predator behaviour in the field group diminished over time (hypothesis 2), we ran GLMMs for each of the four behavioural response variables and tested for differences across their test numbers (#1 to #3). We used an ANOVA based GLMM with Tuckey-HSD for post-hoc analyses to compare field group tests numbers. The same procedure was also used to test for potential differences between groups during the first test round.

All analyses were conducted using R (v3.5.[illegible] R Core Team, 2021) with the RStudio interface (RStudio, Inc., v1.3.959), while figures were prepared using GraphPad prism software (v9.0.1; GraphPad Software, San Diego, CA, USA). The significance threshold was set at $p<0.05$.

Results

We conducted a total of 58 confrontation tests (control group: 24 tests; field group: 34 tests). Since not all hamsters of the field group could be recaptured, data from the missing individuals (N=5) were excluded from the analysis.

1. Behavioural differences between trials

Comparing the behavioural variables displayed during tests #1 and #2 showed overall no significant differences between groups or tests (Tab. S3). However, the interaction term between groups and test number was significant, indicating that the test comparison differed between groups (Tab. S3). Post-hoc analysis, comparing tests for each group separately, showed that most of the behavioural variables differed significantly between test #1 and test #2 in the field group, but not in the control group (Tab. 2, Figs. 2-4). For example, hamsters of the field group spent a significantly greater proportion of time hiding inside the PVC tube during and after predator confrontation following treatment (+34% and +51% during phase 2 and 3, respectively) than during the same phases in test #1 (Tab. 2, Fig. 2). Similarly, hamsters spent a significantly smaller fraction of time exploring the arena before and after predator confrontation following treatment (-

20% and -16% during phase 1 and phase 3, respectively; Tab. 2, Fig. 3) The latency period before field hamsters entered the APT was, on average, greatly reduced following treatment (87.5±95.6s vs. 3.6±4.6s before/after treatment, respectively), albeit, due to individual variation, this difference was not significant (p=0.09; Tab. 2, Fig. 4A). Finally, the number of attacks by hamsters on the fox-model decreased significantly after treatment in the field group (on average 9.6 and 3.9 attacks before/after treatment, respectively, -60%; Tab. 2, Fig. 4B).

To ensure that behavioural differences did not exist between groups before the treatment, we compared the behaviours of both groups during their first confrontation test. Our analysis did not find significant differences between groups during test #1 for the behavioural variables studied, with one exception: during the confrontation phase, hamsters of the field group spent less time hiding inside the tube than control hamsters (p=0.003; Tab. S5).

2. Persistence of behavioural changes over time

For field group hamsters, the behavioural variables during test #2 (after treatment) did not differ from those of test #3 (one month after the return to the laboratory; Tab. 3), indicating that behavioural modifications following the two weeks inside the enclosure persisted for at least one month. However, if we also include test #1 in such investigation, we find that some behavioural modifications started to revert between test #2 and #3, so that they did not differ significantly from test #1. This concerned for example the time spent inside the APT during phase 3 (Fig. 5) or the time spent exploring during phase 3 (Fig. S2). However, most behavioural modifications persisted over time.

Discussion

We found that a pre-release training period of two weeks was sufficient to elicit significant changes in the behavioural responses of captive-bred European hamsters when confronted with a predator model. Following their training period, hamsters of the field group showed a response to a predator model that is likely more appropriate when encountering a predator (i.e. hiding/fleeing rather than attacking). After training, hamsters of the field group spent more time within the APT providing shelter from the predator model, spent less time exploring the arena before and after predator confrontation, and attacked the predator model less frequently (Tab. 2, Figs. 2-4). By contrast, repeated exposure to the predator model alone, without a training period (control group), was not sufficient to elicit significant changes in hamster behaviour during trials (Tab. 2, Figs. 2-4). The behavioural modifications following treatment in the field group persisted over time (at least one month; Tab. 3) but a partial reversal was noticeable in some behavioural variables (Fig. 5, Fig. S2).

1. Behavioural modifications following field training

Following the training period inside an outdoor enclosure, field hamsters showed significant changes in their anti-predator behaviour during confrontation trials. Importantly, given our experimental design, where each hamster served as its own control, we could evaluate the treatment effects (i.e. a ‘soft-release’ versus control) on the behaviour of individuals.

During the first test-round (before treatment), we found small behavioural differences between groups. For example, hamsters of the field group spent significantly less time hiding inside the APT during and after predator exposure and also mounted a greater number of attacks on the fox model than control hamsters (Figs. 2,4). Hence, field hamsters originally displayed an anti-predator response that could be characterized as more risk-prone than that of control hamsters.

Hence, despite randomizing the individual assignment to groups, it is likely that a greater number of bold hamsters (maintaining a high activity level during predator presence; Watters and Meehan, 2007) were assigned to the field group (Fig. 4). In any case, after treatment, the behavioural response of hamsters from the field group changed significantly (Tab. 2, Figs. 2-4). After training, hamsters of the field group spent significantly more time inside the shelter (+34% and +51% during phase 2 and 3, respectively) and reduced the latency period before entering the shelter during predator model confrontation (-96%). Similarly, these hamsters also significantly decreased the time exploring the arena before and after predator confrontation (-20% and -16%, respectively) and reduced the number of attacks on the fox model (-60%) following treatment. This suggests that their "risk-prone" behavioural response during the first test shifted to a 'risk-averse' response following treatment.

The pre-release training period in the outdoor enclosure was intended to provide hamsters with the possibility to learn from a protected exposure to predation risk, rather than to expose them to a real predator-prey confrontation. To the best of our knowledge, measures to avoid predation worked effectively. All hamsters in our study had been housed in individual cages since weaning, and they never encountered conspecifics within their 'habitat'. During the training period, hamsters of the field group were exposed to a multitude of new stimuli. For the first time, they experienced natural climatic conditions, a natural soundscape, other animals, as well as intraspecific competition. They were able to express behaviours they could not engage in while inside the laboratory housing (e.g. digging a burrow, foraging, exploring a large area, etc.). Beyond the possibility to learn from the protected exposure to predation risk, living in a semi-natural environment exposed hamster to a whole range of stimuli (e.g. tactile, olfactory, sound) that differed greatly from those experienced in the laboratory. This should have enabled hamsters to develop their cognitive and behavioural capacities and to adapt their digestive and immune systems to a more natural environment (Shepherdson, 1994). The development of such capacities is strongly dependent on rearing conditions and the immediate experience before the release (Reading *et al.*, 2013; Tetzlaff *et al.*, 2019). The behavioural modifications following the 'soft-release' suggest that hamsters learned to associate certain stimuli (e.g. unknown smells or sounds) with a potential risk, triggering an appropriate reaction (e.g. seeking shelter, being more vigilant in open spaces).

One might object the above interpretation and argue that the observed behavioural modifications of field hamsters were the consequences of stress associated with recapture and return to the captive facility just before test #2. A pre-release treatment within a reinforcement program may seem counterintuitive, given the likelihood of stressful stimuli associated with such treatment (Reading *et al.*, 2013) . However, it is important to distinguish between 'chronic distress' and 'natural stress' (Reading *et al.*, 2013). The first may impact captive-bred animals and may lead to the development of abnormal behaviour (e.g. pacing in a cage, pulling out fur) because animals lack the adaptive behavioural outlet to control their situation. By contrast, 'natural stress' may occur sporadically/periodically and is necessary for the development of adaptative psychological and behavioural skills (Moodie and Chamove, 1990; Meehan and Mench, 2007; Reading *et al.*, 2013). In this context, the limited exposure to stressful stimuli inside the outdoor enclosure might have been advantageous. By contrast, captive-bred animals, especially in a research laboratory, lack sufficient stimulation from external factors (e.g. predators, congeners, natural soundscape, weather) that would enable them to develop behavioural responses more appropriate for a natural environment (Mathews *et al.*, 2005; Wells, 2009; Salvanes *et al.*, 2013). In addition, the cramped conditions of the standard breeding cages for rodents likely contribute heavily to the inferior physical, neuro-motor, psychological, and sensory conditions of captive rodents (Young, 2003). Cognitive processes are essential for mounting the appropriate behavioural response in a given situation (Curio, 1993; Griffin *et al.*, 2000). The switch from a fight to a flight response, observed during confrontation tests with field hamsters following treatment, suggests that their training period improved cognitive processes, triggering more appropriate behavioural responses to predation risk. Hence, we suggest that a training period in an outdoor enclosure, where hamsters are exposed to a variety of novel stimuli, will likely lead to an improvement of their overall condition and will be an important measure to reduce post-release mortality of hamsters.

2. Does repeated predator model confrontation alone elicit behavioural changes?

Hamsters of the control group, which remained within their standardized cages during treatment, did not display significant changes in their behavioural responses between test #1 and test #2 (Tab. 2, Figs. 2-4). Hence, repeated confrontation with the predator model and its scent alone, was insufficient to elicit a more appropriate anti-predation response (e.g. avoidance, shelter seeking). Even the multiple direct attacks by the predator model during trials, that involved physical contact and that, under natural conditions, would have resulted in death by predation, did not suffice to provoke changes in anti-predatory responses.

For survival, prey species must first detect a potential predator and then react appropriately (Lönnstedt *et al.*, 2012; Blumstein *et al.*, 2019). For this, however, they first have to be able to recognize a predator as potential danger (McLean *et al.*, 1996). All hamsters tested in this experiment reacted to the exposure and attacks of the fox model in all tests (i.e. suppression of exploration, increased use of the shelter, attacks on the fox) and, hence, likely perceived the fox model as potential danger (Tab 3, Fig. 4). However, while field hamsters also modified their behaviour during phase 1 and 3 (before/after confrontation) following treatment (e.g. increased use of shelter, reduced exploration) this was not the case for control hamsters (Figs. 2-3). Training animals to recognise their natural predators has been attempted with many species, albeit with varying success (Vilhunen, 2006; Lönnstedt *et al.*, 2012; Lopes *et al.*, 2017; reviewed in Rowell *et al.*, 2020). For some species, simple exposure to predator odours was sufficient to increase their survival during a following predator confrontation (Vilhunen, 2006). By contrast, multiple confrontations with a predator model in association with aversive stimuli were insufficient to improve the post-release survival of parrots (Lopes *et al.*, 2017). In addition, such a method, where captive animals are repeatedly exposed to an (artificial) predator model or to a risk of predation under controlled conditions might be counterproductive, as it could lead to habituation (Rowell *et al.*, 2020). For example, anti-predator behavioural responses might diminish over time, due to habituation to the threat and/or due to learning of inappropriate responses to a predation threat (Rowell *et al.*, 2020; Edwards *et al.*, 2021). Furthermore, even if a live predator is used, it might be difficult to reproduce the exact stimuli that animals experience during a predator encounter in the wild (Griffin *et al.*, 2000). In our experiment, the number of confrontation trials and exposure duration to the predator model that we used for the control group were likely insufficient to elicit any habituation. In this context, it would be interesting to investigate if and how a longer exposure and/or a greater number of trials, without a 'soft-release', affects the anti-predator response of naïve hamsters.

3. Persistence of behavioural changes over time

The behaviour of field hamsters displayed during test #2 and #3 did not differ significantly (Tab. 3), suggesting that the behavioural modifications after training persisted over time and were still present one month after their return to the captive facility. For example, during confrontation, field hamsters spent significantly more time inside the APT following training and also one month thereafter (test #2 and #3, respectively), when compared with test #1 (Fig. 5). Similarly, the number of attacks on the predator model by field hamsters was reduced following treatment and remained at such level during test #3. However, when including test #1 in our analyses, we found that some behavioural modifications of field hamsters started to revert between test #2 and #3 and did not differ significantly anymore from test #1. This was the case, for example, for the time spent inside the APT during phase 3 (Fig. 5) or the time spent exploring during phase 3 (Fig. S2). However, most behavioural modifications persisted across tests. Nevertheless, since the intensity of the behavioural modifications had started to fade one month after treatment, further reinforcements might be required for behavioural modifications to persist. The ability of animals to retain learned behaviours have been studied in a variety of animals (McLean *et al.*, 1996; Griffin *et al.*, 2000; Rowell *et al.*, 2020). Depending on the training regime and the species in question, anti-predator behaviours initiated by training have been shown to persist for up to several months, even in the absence of subsequent reinforcements (Chivers and Smith, 1994; De Azevedo and Young, 2006). Hence, our results suggest that hamsters have the capacity to retain some modifications of their behavioural response for at least one month, even in the absence of reinforcements, while others might be more susceptible to reversal.

Conclusions & perspectives for hamster conservation

Our study shows that a simple ‘soft-release’ was sufficient to elicit a shift in the behaviour of hamsters towards a more adapted anti-predation response, when confronted with a predator model. In addition, most of the observed behavioural changes were retained for at least one month. Hence, the pre-release training period of hamsters was critical to achieve behavioural modifications that will likely improve their survival when facing the risk of predation upon their release into the wild. The repeated exposure to a predator model alone was insufficient to provoke behavioural modifications (control group). Our findings have important implications for hamster reinforcement programs. We suggest that a training period (i.e. a 'soft-release'), as implemented here, should be applied before any release into the wild. However, the greater variation we observed during the last test round suggests that behavioural changes fade over time in the absence of reinforcements (Figs. 5 & S2). Consequently, a release into the wild should be implemented as soon as possible after the training period. A one-month persistence of behavioural modifications, as found here, might be sufficient to increase hamster survival chances during the most critical period following release.

Our experimental approach, testing the effectiveness of a training period inside a sheltered semi-wild enclosure to elicit a more adept anti-predator response of captive bred hamsters is only a first step. We now need to evaluate if the short-term survival of trained hamsters after their release is indeed increased, when compared with hamsters that did not undergo a pre-release training period (e.g. see Shier et al. 2006, Greggor, Price and Shier, 2019). In addition, to ensure the success of restocking programs, released hamsters do not only have to survive, they also have to reproduce and successfully wean offspring (Soorae, 2018). The latter is of particular importance for hamsters, given their short lifespan. Hence, survival and successful reproduction of captive-bred hamsters in the wild are key demographic factors to consider for meaningful conservation measures. In this context, additional studies investigating how the treatment of hamsters prior to their release into the wild affects their reproductive rate are of great importance. Restocking programs are an important instrument in biodiversity conservation and should, therefore, also consider the well-being of animals before, during, and after release (Swaisgood, 2010).

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Supplementary information includes:

Supplementary Materials and Methods

1. Outdoor enclosure
2. The arena and the predator model
3. Confrontation tests
4. Release and recapture of the field group

Supplementary Results

Table S1: Ethogram used to characterize hamster behaviour during video analyses.

Table S2: Model distribution and R package used for each response variable

Table S3: GLMM results comparing differences for behavioural variables between groups and trials (before/after training) according to test phase**.**

Table S4: GLMM results comparing differences for behavioural parameters between the three tests conducted with the field group, according to test phase.

Table S5: Model results (post-hoc tests) for behavioural variables of control and field group hamsters during test 1 (immediately following treatment)

Figure S1: Flow chart (top to bottom) indicating decision tree followed by the experimenter during phase 2 of confrontation trials, i.e. once the predator model was introduced. The diamond-shaped boxes indicate the behaviour of the hamster, while reactions of the fox model, directed by the experimenter according to hamster behaviour, are shown inside the yellow boxes.

Figure S2: Fraction of time that hamsters of the field group spent exploring the arena during phase 1 and 3 of confrontation tests (before and after predator appearance, respectively).

Individuals are indicated by black dots and significant differences between tests are indicated by asterisks.

Figure S3: Number of attacks on the fox model by hamsters of the field group (phase 2). Individuals are indicated by black dots and significant differences between tests are indicated by stars.

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Figure 1

Standardized test setup.

Representation of the PVC arena and the predator model. During the tests, a hamster was confronted with the predator model for 4 min. An experimenter controlled the movements of the fox model from behind the opaque curtain in response to hamster behaviour (see **Figure S1**)

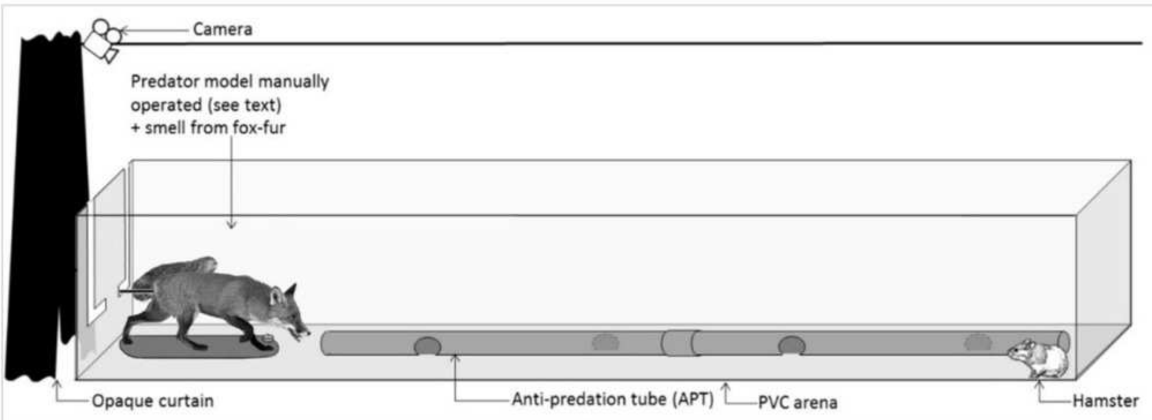
Camera
Predator model manually operated (see text) + smell from fox-fur
Opaque curtain
Anti-predation tube (APT)
PVC arena
Hamster

Figure 2

Proportion of time hamsters spent inside the PVC tube (APT) during a trial.

Values for individuals are presented (circles/squares) according to group (control/field), test number (test#1: before treatment; test#2: after treatment), and phase during a trial (phase 1-3, before, during, and after predator confrontation, respectively). Stars indicate significant differences between test numbers (*≤0.05, **≤0.01 and ***≤0.001).

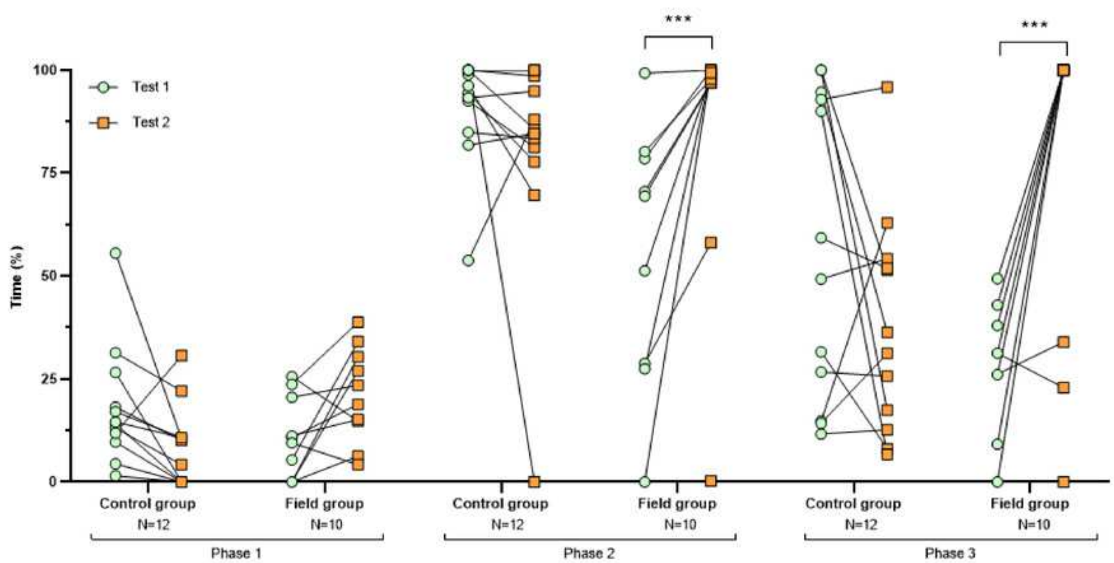

Figure 3

Proportion of time hamsters spent exploring the arena.

Values for individuals are presented (circles/squares) according to group (control/field), test number (test#1: before treatment; test #2: after treatment), and phase during a trial (phase 1-3, before, during, and after predator confrontation, respectively). Stars indicate significant differences between test numbers (*≤0.05, **≤0.01 and ***≤0.001).

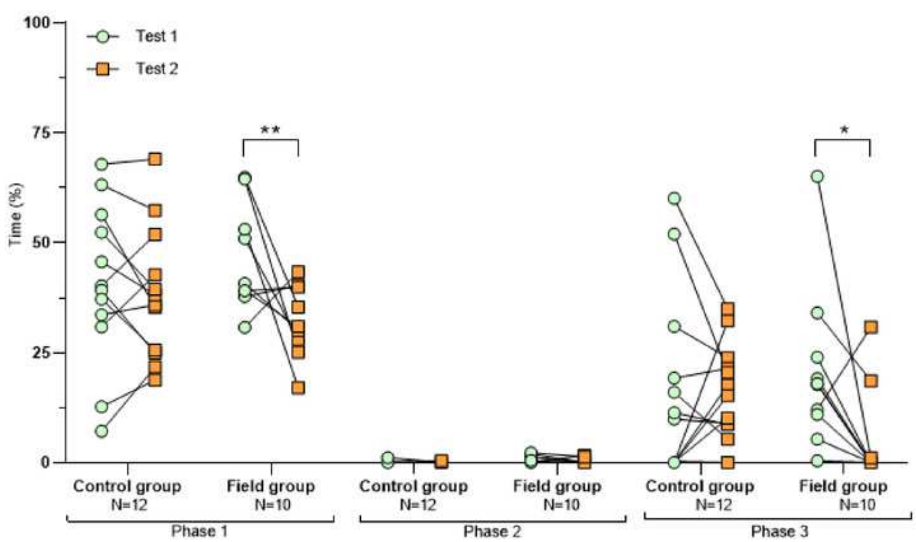

Figure 4

Latency between predator introduction and shelter seeking by hamster (A) and number of hamster attacks on the predator (B) during test phase 2.

Values for individuals are presented (circles/squares) according to group (control/field) and test number (test#1: before treatment; test#2: after treatment). Stars indicate significant differences between test.

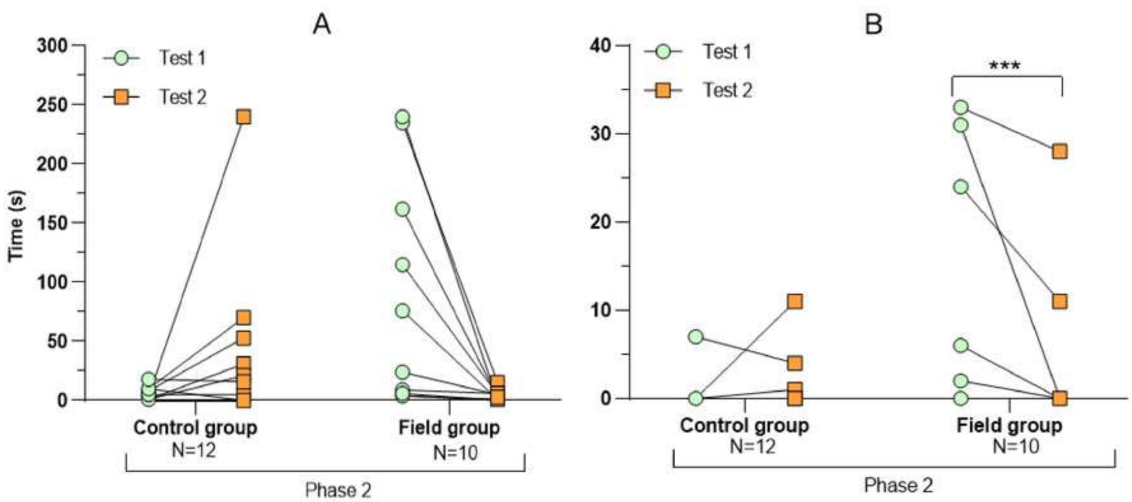

Figure 5

Proportion of time that hamsters of the field group spent inside the APT during confrontation tests.

Test #1 (pre-release), test #2 (post-recapture), and test #3 (one-month after post-recapture) according to test phase (before, during, and after predator model exposure). Individuals are indicated by black dots and significant differences between tests are indicated by stars.

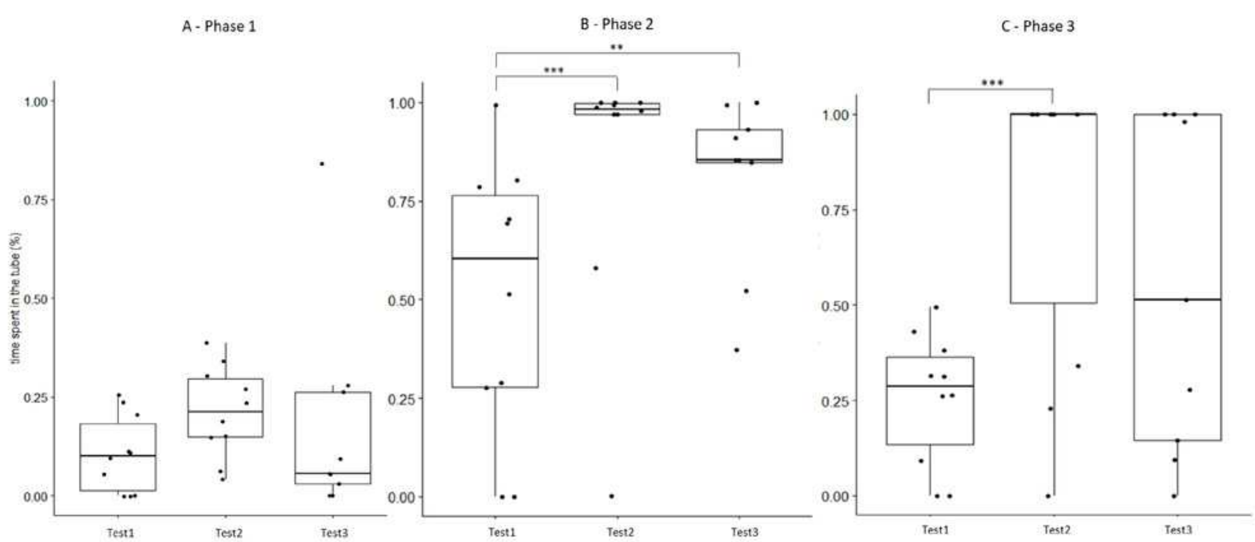

Table 1(on next page)

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Outline of experimental trials.

	Before treatment (test #1)	Treatment	24h after treatment (test #2)	1 month after treatment (test #3)
Control group (N=12)	First confrontation test	No treatment (2 weeks in the laboratory)	Second confrontation test	NA
Field group (N=10)	First confrontation test	Field training (2 weeks in the outdoor enclosure)	Second confrontation test	Third confrontation test (N=9)

Table 2(on next page)

Model results (post-hoc tests) comparing hamster behaviour of the control and field group during tests 1 and 2 (before/after treatment) according to test phase.

Bold arrows indicate the direction of a significant difference (increase/decrease), while plain arrows indicate only a (non-significant) trend and hyphens indicate no change between tests. The number of arrows indicates if the difference is less than or equal to 0.05 (one arrow), 0.01 (two arrows) or 0.001 (three arrows). When under attack (phase 2), hamsters never explored the arena, as indicated by NA.

	Variable	Phase	Estimate	*Z*	*Df*	p	Behavioural differences between test 1 and test 2
Control group	Time (%) spent inside APT	1	0.91±0.36	2.55	1	0.052	↘
		2	1.04±0.53	1.97	1	0.187	–
		3	0.82±0.47	1.74	1	0.3	↘
	Exploration (%) when outside APT	1	0.09±0.22	0.41	1	0.976	–
		2	NA	NA	NA	NA	NA
		3	0.01±0.39	0.02	1	1	–
	Latency before first entry into APT	2	0.18±0.08	2.21	1	0.095	↗
	Attacks on fox model	2	-0.83±0.54	-1.52	1	0.377	–
Field group	Time (%) spent in the APT	1	-0.82±0.36	-2.31	1	0.093	↗
		2	-2.43±0.57	-4.30	1	<0.001	↗↗↗
		3	2.33±0.59	-3.94	1	<0.001	↗↗↗
	Exploration (%) when outside APT	1	0.85±0.24	3.47	1	0.003	↘↘
		2	NA	NA	NA	NA	NA
		3	1.62±0.57	2.82	1	0.023	↘
	Latency before first entry into APT	2	-0.27± 0.12	-2.24	1	0.087	↘
	Attacks on fox model	2	0.90±0.23	3.95	1	<0.001	↘↘↘

Table 3(on next page)

Model results (post-hoc tests) for behavioural variables of field group hamsters during test 2 (immediately following treatment) and 3 (one months after treatment).

When under attack (phase 2), hamsters never explored the arena, as indicated by NA.

	Variables	Phase	Estimate	*Z*	*Df*	p
Test 2 vs. Test 3	Time (%) spent inside APT	1	-0.22±0.45	-0.48	2	0.88
		2	-0.28±0.62	-0.44	2	0.90
		3	-0.91±0.68	-1.34	2	0.37
	Exploration (%) when outside APT	1	-2.25±0.36	-0.70	2	0.76
		2	NA	NA	NA	NA
		3	0.82±0.66	1.23	2	0.43
	Latency before first entry into APT	2	-0.23±0.12	-1.92	2	0.11
	Attacks on fox model	2	-0.77±0.76	-1.02	2	0.56