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Nonlethal Effects of Predation: The Presence of Insectivorous Birds (*Parus major*) Affects The Behavior and Level of Stress in Locusts (*Schistocerca gregaria*)

Jan Kollross · Jitka Jancuchova-Laskova · Irena Kleckova · Inga Freiberga · Dalibor Kodrik · Katerina Sam

Abstract Insect exposure to their predators can affect individuals and community processes, through direct consumption or nonlethal effects. However, the links between behavioral and physiological responses and stimuli needed for the development of predator avoidance are not clear. We subjected desert locusts (*Schistocerca gregaria*) to three nonlethal treatments, using great tits (*Parus major*) as a predator. The treatments included: (1) *bird*—presence of a great tit and its calls, (2) *call*—great tit calls only, (3) *control*—absence of a great tit and its calls. In the first behavioral laboratory experiment, locusts were offered food and shelter in an open space. The duration of hiding versus feeding indicated predator responses. In the second laboratory experiment, locusts were subjected to the same three treatments, and adipokinetic hormone (AKH) levels were measured in the central nervous system (CNS) and hemolymph. In the third experiment in an outdoor aviary, birds were allowed to fly freely over locusts before we measured physiological response. During the first experiment, locusts hid for a significantly longer amount of time and fed for a significantly shorter amount of time when the bird was present and calls were played, compared to when only calls were played or compared to the control treatment. The proximity of birds to locusts in the laboratory experiment elicited a significant increase in AKH levels in the CNS and hemolymph compared to the call/control treatment. The physical proximity of the great tits affected both the behavioral and physiological responses of the locusts.

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Introduction

Insectivorous predators greatly impact insect communities resulting in cascading effects across ecosystems. (e.g., Hawlena and Schmitz 2010; Yang and Gratton 2014). Insectivorous predators can affect prey populations by direct consumption (lethal effects or "density" effects) and also through nonlethal effects, by their sole presence (trait-mediated effects

or interactions, reviewed by Bolker et al. 2003). For example, spider-intimidated grasshoppers also experienced indirectly higher mortality after they switched to eating safer but less energetically rewarding forbs instead of grasses (Beckerman et al. 1997; Schmitz 1998). Predator avoidance strategies can indirectly result in morphological changes in the prey, including a reduction in body size and fecundity (Abrams 1995; Abrams and Rowe 1996), their populations, and associated communities (Buchanan et al. 2017; Miller et al. 2020). Nonlethal effects are often defined operationally as any predator-caused factor that reduces population growth via a reduction in survival and/or developmental performance (Schmitz et al. 1997; Nelson et al. 2004; Schmitz 2007; 2009). The presence of predators can further lead to earlier maturation at the smaller size when the growth rate does not change, or slower growth rate leading to later maturation at the same or smaller size in invertebrates (Abrams 1995; Abrams and Rowe 1996).

Most existing studies focus on the lethal effect of predation; however, the nonlethal effects may have even greater consequences for insect population dynamics (Buchanan et al. 2017; Miller et al. 2020). Indeed, some of the most famous examples (e.g., the effect of wolves on moose, lynxes on hares and spiders on grasshoppers) of lethal predator–prey interactions, also impact the prey indirectly through intimidation which leads to changes in prey's traits and behavior (Lind and Cresswell 2005; Peckarsky et al. 2008; Dröge et al. 2017).

As an attempt to deter predators, insects evolved many defensive strategies, such as camouflage coloration, or physical defenses, such as toxic chemicals, hairs, spines, or tough exoskeletons. Additionally, some insects have developed defensive behaviors like body parts autotomy or dropping from the plant and feigning death as a means to avoid predation. Anti-predator defenses can be classified as primary or secondary based on the timing of the defense mechanism and as chemical, morphological, physiological, or behavioral based on the type of defense mechanism. Primary defenses act before predator attacks and secondary defenses act during or after an attack. Chemical, morphological, and behavioral defenses can be categorized as both—primary and secondary types, while physiological defenses only belong to the secondary defenses. The secondary behavioral defense includes biting kicking, jumping, flight, or autotomy

(also in crickets and locusts, Sugiura 2020). As an example of primary behavioral defenses, insects could forgo foraging, reduce activity, seek refuges, or reduce mating activity (Lind and Cresswell 2005; Peckarsky et al. 2008; Dröge et al. 2017). These primary behavioral responses seem to be uniform both for vertebrates and invertebrates, although they are more often studied in vertebrates.

While each predation event only influences a single prey per unit of time, the risk introduced by the mere presence of a predator could have more widespread effects by causing many prey individuals to significantly alter their foraging behavior (Cinel et al. 2020). Moreover, trade-offs are probably included in insect prey responding to various threats. For example, the presence of invertebrate predators can reduce the resistance of insects to pesticides (Op de Beeck et al. 2016) and the nonlethal effects of predators can thus further affect prey mortality and impact ecosystem functioning. Understanding such nonlethal effects of insectivorous predators is especially important as insects are the most dominant animal group on Earth (Stork et al. 2015), serve as prey for many different predators, and consume up to 70% of the total leaf area in some habitats. Although insects have an extraordinary diversity of anti-predator behavioral and physiological responses, predator-induced stress has not been studied extensively in insects (Cinel et al. 2020).

It can be assumed that insects activate their physiological defense systems after contact with predators, as it is common for other stressful situations (Cinel and Taylor 2019), and then change their behavior accordingly. Such responses are mostly unknown (Kodrík et al. 2015; Cinel et al. 2020) because stress responses in the insect body might be difficult to detect and quantify – both behaviorally and physiologically. Stress stimuli represented by the presence of a predator induce secretion of the stress hormones from the central nervous system (CNS), specifically from the corpora cardiaca, a small neuroendocrine gland connected with the brain. The hormones then travel to other part of the body in the hemolymph (Nässel and Zandawala 2020). In insects, the anti-stress response is regulated predominantly by adipokinetic hormones (AKHs) and octopamine (Cinel et al. 2020). AKHs act as typical stress hormones and prepare the insect for predator avoidance: they mobilize lipid, carbohydrate, and amino acid proline energy stores (Gade

et al. 1997) by stimulating catabolic reactions to gain energy. Simultaneously, AKHs inhibit synthetic reactions; thus, the mobilized energy is used to eliminate imminent contact with a predator. AKHs increase in insects depends on stress situations and signals, such as predator presence (Kodrik 2008), forced movement (running or flying, Kodrík and Socha 2005), various pathogens (Ibrahim et al. 2017, 2018; Gautam et al. 2020a, b) or toxins (Candy 2002; Kodrík et al. 2015). Additionally, as seen in Drosophila, AKHs appear to regulate even more intricate behavioral patterns (Lee and Park 2004). These authors demonstrated that AKH induces foraging behavior, which involves control of locomotory hyperactivity in starved flies just before death. Although the exact mechanism is unknown, it is believed that AKH action may involve regulation of locomotory activity, levels of energy metabolites, neuromodulation, or any combinations of these factors. In any case, it is presumed that AKH merely activates the behavioral patterns and that its continued presence is not particularly important.

Signals which trigger stress reactions in insects are rarely studied. However, it has been shown that lizard-specific vibrations and even robotic vibrations can trigger a stress response in crickets (Adamo et al. 2013), sounds emitted by bats can launch avoidance of moths (Cinel and Taylor 2019), and real but harmless spiders with manipulated mouthparts can be a stressor for grasshoppers (Schmitz et al. 1997). Yet the effect of these stimuli can differ among taxa (Cinel et al. 2020). Although birds represent one of the main predators of insects (Van Bael and Brawn 2005; Bael et al. 2008), the stress response of insects to presence of birds has not been directly studied. We expect that insects sense the presence of birds also by hearing (Fournier et al. 2013).

Hearing is important for predator location and has evolved multiple times. Insects use hearing to detect invertebrate parasitoids, predators, and terrestrial vertebrates including birds. Insects have two types of receptors- far field and near field. Near field structures are lightweight structures such as "trichoid sensilla" or antennae. When using the near field structures, they are displaced by the movement of the air and can detect close sounds < 1 kHz. Far-field receptors – so-called tympanal organs, can detect the pressure of the sound from further distances. The desert locusts (family Acrididae), have one of the most sophisticated acoustic systems (Riede 1987) and perceive sound by tympanal organs at a frequency < 1–20 kHz (Haskell 1957). Vibrations are detected by mechanoreceptors in the legs, and the scolopidial organs (Lakes-Harlan and Strauß 2014).

Insect eyes are made up of individual ommatidia, which are single photoreceptors. The ommatidia are capable of perceiving brightness and color and allow for a much wider acceptance angle than the vertebrate eye. This allows for a fast response when movement is detected (Land 1999). When light enters each ommatidium, individual photoreceptors are activated. The rate at which the ommatidia switches on and off is called the flicker frequency, which in locusts is between 40 and 90 Hz. They can respond to motion in 0.01 s (Miall 1978), but it is not known to what distance they can see an approaching predator. The color vision system in insects is trichromatic and consists of green, blue, and UV-sensitive visual fibers. Locusts can detect the pattern of polarized light by a highly specialized set of individual ommatidia—a small dorsal rim area in the compound eye (Schmeling et al. 2014; Völkel et al. 2003).

To evaluate the hormonal and behavioral predator avoidance response in desert locusts (*Schistocerca gregaria*), we exposed locusts to three different treatments (1) live birds which represented a real, life-threatening risk, (2) warning calls of the birds which represented only a potentially threatening signal, and (3) control conditions without the presence of the predator. We investigated the stress hormone levels and the behavioral response in the locusts. We conducted the experiments in indoor experimental test cages as well as in outdoor aviaries. Our hypothesis is that the presence of live birds, as well as their calls only, would cause a reduction in foraging behavior and an increased duration of hiding. We further hypothesize that the behavioral changes will be supported by elevating AKH levels in the hemolymph and brain of locusts, with the highest AKH levels observed under the call and live bird treatments.

Methods

Experimental Animals

The desert locust, *Schistocerca gregaria* occurs in Africa, the Middle East, and Asia (Topaz et al. 2012). It is polyphagous and feeds on leaves, shoots, flowers,

fruit, seeds, stems, and soft bark, and is well known to periodically form enormous destructive swarms. At least 30 bird species were observed feeding on locusts, including 10 European wintering species (Sánchez-Zapata et al. 2007).

For our experiments, the subadults (4th instar, 3–4 cm long) of gregarious form desert locusts from several genetically different clutches were purchased from a commercial supplier (Acheta.cz, Czech Republic), providing thus a genetically representative sample of individuals. After purchase, they were placed in an isolated room with natural daylight (experiments were conducted between 10 AM and 3 PM), temperatures between 23–25 °C, and 50% relative humidity. They were either kept individually in small plastic containers for 48 h (before experiment I) or together in a large cage, where food and water were provided ad libitum (before experiments II and III). None of the individuals had previous experience with any predators.

The Great tit (*Parus major*) was selected as a representative of insectivorous birds, that inhabit Europe, Northern Africa, and large areas in Asia. Although desert locusts are not typical prey of great tits in the Czech Republic, they have been reported as their prey in migratory grounds where desert locusts occur (Mullié 2009). Further, interspecific eavesdropping on birds' alarm calls, which are typically 3–5 kHz (Fallow et al. 2013), has been confirmed for many species, such as Black-Capped Chickadees (*Parus atricapillus*; Hurd 1996) superb fairy-wrens (*Malurus cyaneus*), western grebe (*Aechmophorus occidentalis*; Fallow et al. 2013) and Forster's terns (*Sterna forsteri*; Nuechterlein 1981).

The great tits were mist-netted in the proximity of the Faculty of Science, University of South Bohemia one to two days before the experiments and kept in accredited breeding areas. They were housed individually in cages (0.7×0.4×0.5 m) with a plastic bottom and three perches. The birds were provided with food (sunflower seeds, mealworms, and Insect patee for passerines by Versele-Laga) and water ad libitum daily. They were released immediately after the experiment and so they were in the breeding areas for up to three days. New birds were captured for the next trial.

All experiments were conducted in the laboratories of the Institute of Entomology, Biology Centre CAS, and in the aviaries of the Faculty of Science,

the University of South Bohemia, Ceske Budejovice, Czech Republic from May to October 2018.

Experiment I—Laboratory Behavioral Test

The laboratory experiments were conducted in a quiet experimental room with natural light conditions and in modified bird cages with perforated plastic bottom parts to enable air circulation. The cages were 0.7×0.4×0.5 m in size and were separated into two parts by 0.2 mm thick glass (Fig S1). The bottom of the cage was designed to hold locusts and the upper part of the cage to hold birds (Fig S2). Two independent experimental cages were located 2 m apart in the experimental room. The position of the cage in the room was noted and considered in the analysis (either left or right side of the room) to control for potential external factors influencing the results.

The locusts were starved for 3 days before the behavioural experiment, to keep them motivated for food searching. The shelter for the locusts (a 15×10 cm part of the egg carton) was placed at the bottom of each cage. Food (two halves of a grape, a piece of carrot, and a lettuce leaf) was placed on a Petri dish on the opposite side of the cage. There was an area approx. 0.3×0.3 m space between the shelter and the food. Immediately prior to the experiment, five locusts were placed under the shelter in the bottom part of each cage. We opted for five locusts for practical reasons, as no more than five individuals fitted into the shelter comfortably. Furthermore, as this was a behavioral experiment in which we needed to follow the behavior of individuals, we wouldn't be able to do it with certainty for more than five individuals at a time. We were not able to mark the locusts before the experiment as the additional handling would have potentially increased their stress levels.

Locusts were exposed to three different treatments (Tables S1,S2, and S3): (1) "bird treatment" – one live great tit present in the cage plus playback of a mixture of songs and warning calls of great tits (see below), (2) "call treatment"—the playback of the bird calls only, and (3) "control" – no bird and silence in the experimental room. Each treatment lasted 60 min. This time period was decided as it is the amount of time in which AKH in hemolymph can increase and peak (Candy 2002).

The playback of the bird calls was a mixed sequence of various calls from the Xeno-canto

collection (www.xeno-canto.org). Specifically, it included an alarm call (20 s), a warning call (20 s), and a song (20 s) which were repeated 60 times. The playback was played using a JB.lab R1 full-range speaker. The cages were placed on rubber pads on a table. This ensured that any vibrations were kept to a minimum in the cages. It is possible that the locusts could still detected some minor movements, and this needs to be considered when interpreting the results. The experiments were run in the following order: control, call, and bird treatment to avoid the potential effect of chemical cues released by locusts or birds that remained in the box. The new set of hungry locusts was used for every treatment; thus, the individual experiments were not affected by the potential feeding of locusts during the experiment. After the bird treatment, the experimental room was aired for at least 2 h before any more treatments were run in that room.

The locust's behavior was recorded by two cameras (Sony SHD-CX240E) positioned on a tripod above the cage for 60 min. In this way, no observer was in the room whilst the experiments were in process. The amount of time each individual locust spent either feeding or hiding in the shelter was recorded to the nearest second. We also noted whether the locusts entered the empty space between the food and the shelter (sometimes, the locust just sat next to the shelter but did not move away from it towards the food). If the locusts did not leave the shelter or did not reach the food, the time was scored as 3600 s in the shelter (i.e., 60 min). The experimental locusts were discarded after each trial.

Experiment II—Laboratory Physiological Test

In the second stress induction experiment (Table S2), we evaluated Schgr-AKH-II levels in the CNS and hemolymph of locusts under laboratory conditions. *Schistocerca gregaria* has long been known to produce two adipokinetic hormones, Locmi-AKH-I (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-Thr-NH2) (Gäde et al. 1986) and Schgr-AKH-II (pGlu-Leu-Asn-Phe-Ser-Thr-Gly-Trp-NH2 (Gäde et al. 1986). However, a small amount of a third AKH AKH (Schgr-AKH-III; pGlu-Leu-Thr-Phe-Thr-Pro-Ser-Trp-NH2) was recently discovered in *S. gregaria*; this hormone is identical to Aedae-AKH (Marchal et al. 2018). In any case, Schgr-AKH-II has been used as a

reliable indicator of stress intensity because it is one of the main *S. gregaria* stress hormones (its level in the locust body was evaluated by specific antibody—for details see below). In contrast to laboratory experiment I, the locusts were fed before the experiment ad libitum. Ten locusts were placed in the bottom of each cage with no food or shelter. This number of locusts per treatment was possible as we did not need to monitor each locust individually as we had done in the previous experiment. The locusts were moved to the experimental cages and then exposed for 60 min (which is a minimum duration recommended by Orchard and Lange 1983) to one of the three treatments. Titre of the AKH-I hormone increases mostly after the first 10–15 min and the titer of AKH-II in 15–30 min after the stressful event (Orchard and Lange 1983).

Experiment III—Outdoor Physiological Test

To simulate more natural (i.e., fresh circulating air, realistic distances of prey from birds, the higher number of communicating birds) experimental conditions, an outdoor aviary (10×15 m, Fig S3) with shrubs, small trees, and high grass was established in the campus of Biology Centre CAS (48°58′37.1"N 14°26′49.2" E). A flock of 25 young great tits was released into the aviary one week prior to the experiment. The birds were provided with food and water ad libitum in multiple locations within the aviary. The bird and the control (birds absent) treatments were conducted, with 4 perforated bottom parts of the cage covered by the glass (i.e., the lower part of the cage used in Experiment I, Fig S2) with 10 locusts in each treatment (Table S3, for the same reasons as in *Experiment II—Laboratory physiological test*). The glass prevented the direct attack of birds on the locusts. The locusts in the bird treatment were placed on the ground of the aviary. Due to the greater distance between the birds and the locusts, and the potential that the birds might not find the locusts within 60 min, we decided to run the experiment for 3 h (9–12 am). The ambient temperature was 20–25 °C. The bottom part of the cages containing the locusts were naturally shaded by the surrounding vegetation, and this prevented overheating. The control treatment cages were placed in a nearby shady area (48°58′36.7"N 14°26′45.5" E) where birds were absent.

Schgr-AKH-II Extraction from CNS and Hemolymph

Immediately after finishing experiments II and III, hemolymph was taken from the locust bodies (see below) and their heads were cut off under the Ringer saline. The brain with corpora cardiaca and corpora allata attached (i.e., the CNS) was dissected from the head and AKH was extracted from the CNS using 80% methanol. The solution was evaporated in a vacuum centrifuge and the resulting pellet was stored at -20 °C.

For the determination of the endogenous Schgr-AKH-II titer in the hemolymph by ELISA, pre-purification was undertaken as described in Goldsworthy et al. (2002). Hemolymph samples were collected from the locusts by piercing the soft cuticle between the hind leg and thorax. After piercing, the leaking hemolymph was collected in pipette tips. In total, we obtained and pooled at least 300 µl of hemolymph from each group of 10 locusts per cage and treatment and collected it into 1.5 ml Eppendorf tubes. The hemolymph was stored at -26 °C before it was extracted in 80% methanol, centrifuged and the supernatants evaporated to dryness. The dry pellets were dissolved in 0.11% trifluoroacetic acid, applied to a solid phase extraction cartridge Sep Pak C18 (Waters), and eluted with 60% acetonitrile. The eluent was analyzed on a Waters HPLC system with a fluorescence detector Waters 2475 (wavelength λ_{Ex} – 280 nm; λ_{Em} – 348 nm) using a Chromolith Performance RP-18e column (Merck), solutions A and B (A – 0.11% trifluoroacetic acid in water; B – 0.1% trifluoroacetic acid in 60% acetonitrile) and a flow rate 2 ml/min. Fractions eluting between 13.2–14.4 min were subjected to competitive ELISA. The retention time of the *S. gregaria* AKH—Schgr-AKH-II was 13.8 min under the described conditions.

ELISA Determination of Schgr-AKH-II Level

For the determination of Schgr-AKH-II level in *S. gregaria* CNS and hemolymph a common direct ELISA was used. The ELISA comprised coating of a 96-well microtiter plate (high binding Costar, Corning Incorporated, Corning, NY, USA) with the extracts of one CNS or 40 µl hemolymph equivalent. The primary rabbit antibodies used in the procedure (dilution 1:1000) were raised commercially against the Schgr-AKH-II by Moravian-Biotechnology (Brno, Czech

Republic); to exclude any possible cross-reactivity, the corresponding pre-immune serum was used as well. Swine anti-rabbit IgG labeled with horseradish peroxidase (SwAR/HRP—LabNed) (dilution 1:2000) was used as a secondary antibody and the ELISA substrate 3,3',5,5'-tetramethylbenzidine (Sigma Aldrich) was used to visualize the reaction. The absorbance values were determined in a microtiter plate reader at 450 nm.

Statistical Analysis

To determine if there was a difference in the behavioral response of the locusts under the three experimental treatments (Bird, Call, Control) linear mixed effects models were fitted to the data, using the nlme package (Pinheiro et al. 2007) in R 4.0.2 (Team 2020). Time spent in the shelter (in sec) and time spent feeding (in sec) were the response variables (of two separate models) and treatment ($N=3$) and position of the cage were predictor variables (i.e., cage identity; $N=2$ in each of the 3 trials consisting of three treatments), while the identity of locust was used as a random effect. Tukey post-hoc test from package multcomp (Hothorn et al. 2015) was used to compare all possible pairs of mean values of the time spent in the shelter and of the feeding time among the individual treatments.

To determine if there was a significant difference in the levels of the stress hormone in the hemolymph and CNS one-way ANOVA using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The effect of three (bird, call, control) and two treatments (bird, control) was tested separately for the laboratory and outside aviary experiments, respectively. The ANOVA statistics were followed by Tukey's multiple comparison test (laboratory experiments) or by Student's *t*-test (outdoor experiment) to compare the effects of treatments.

Ethical Note

This research was conducted under the ethical approval of the University of South Bohemia. The wild *P. major* individuals were captured and held under the permit CZ5031 and the permit of the city council of Ceske Budejovice, Czech Republic. The experiments on the animals were conducted under a

1415–20,424/2019–65 permit issued by the Ministry of Environment, Czech Republic.

Results

Experiment I—Laboratory Behavioral Test

Locusts spent a significantly longer time hiding when in the presence of a bird than in the call and control treatments (Fig. 1a; $F_{2,155} = 35.994$, $P < 0.0001$). In the presence of a bird, the locusts hid for approximately 2045.3 (± 115 SE) seconds. In contrast, in the call and control treatments, the locusts hid for 1102.5 (± 102 SE) and 931.4 (± 111 SE) seconds, respectively. The position of the cage in the room did not have an effect on the duration of hiding ($F_{2,155} = 1.744$, $P = 0.178$).

Similarly, the amount of time spent feeding was affected by the treatment (Fig. 1b; $F_{2,155} = 7.406$, $P = 0.0008$). The locusts fed on average 1530 (± 65 SE) seconds in the bird treatment, and 2190 (± 162 SE) and 2223 (± 168 SE) seconds in the call and control treatment, respectively. Again, the cage position did not influence feeding time ($F_{2,155} = 1.349$, $P = 0.263$).

Experiment II—Laboratory Physiological Test

The presence of the live birds and their calls (i.e., bird treatment) induced a significant increase in

Schgr-AKH-II in both the CNS (1.4 times, one-way ANOVA: $F = 5.725$, $P = 0.0056$), and hemolymph (1.3 times, one-way-ANOVA: $F = 32.12$, $P = 0.0001$) of the locusts (Fig. 2). Interestingly, application of the bird call only (call treatment) had no significant effect on either the CNS (Tukey's posttest: $P = 0.9835$) nor the hemolymph (Tukey's posttest: $P = 0.2375$).

Experiment III—Outdoor Physiological Test

In outdoor conditions, the amount of AKH was 1.3 times higher in the Bird treatment than in the control group too but the only significant change in the hormone concentration was observed in the CNS (Student's t-test: $F = 4.539$, $P = 0.0108$), not in the haemolymph of the locusts (Student's t-test: $F = 1.323$, $P = 0.1057$, Fig. 3).

Discussion

We found that the presence of the great tits drives a hormonal, as well as behavioral, response in gregarious desert locusts. The desert locusts spent twice as much time under the shelter and limited the time of feeding by 60% when a bird and its alarm call were presented to them, compared to controls. Levels of stress hormones in the CNS and hemolymph were significantly higher in the presence of the bird compared to the bird call only and control treatments. Our experiment thus provides additional information,

Fig. 1 Effect of the bird (bird + bird call), the call (bird call only), and the control (neither bird nor call) treatments on the behavior of adults *S. gregaria* locusts. Time spent in the shelter (**a**) and time spent feeding (**b**) in seconds is presented on the y axes. The significant results are marked by *** $P < 0.001$, ** $P < 0.01$, ns $P > 0.05$. Small dots represent raw values for the individual locusts and full points and whiskers show means $\pm$ SE (seconds) derived from the respective minimal models
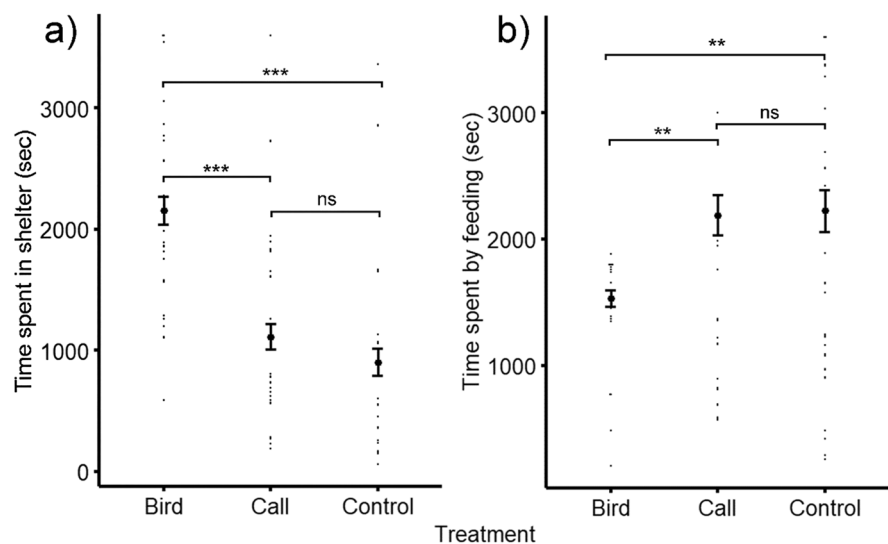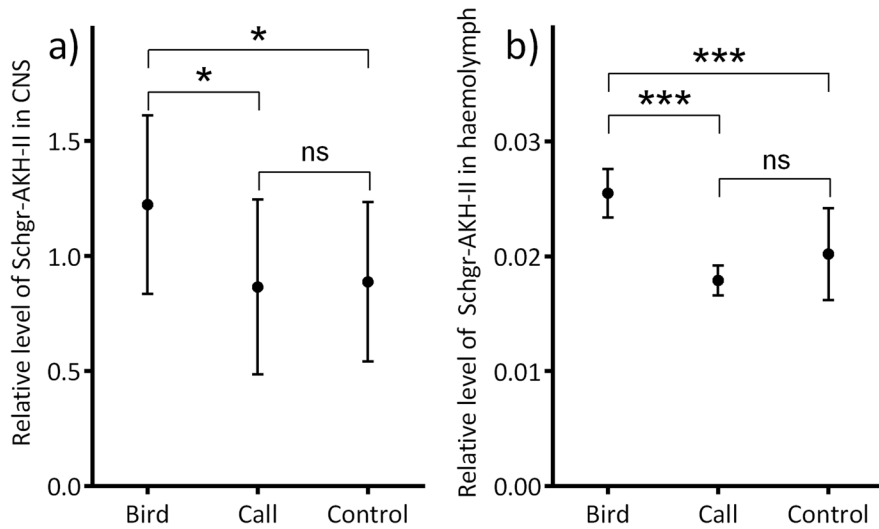
Fig. 2 The effect of treatments (Bird, Call, Control) on the Schgr-AKH-II level in the CNS **a** and the hemolymph **b** of *S. gregaria*. within the laboratory experiments. Differences between the groups were analyzed using one-way ANOVA with Tukey's post-test, and significant differences between the groups are marked by *** $P < 0.001$, * $P < 0.05$, ns $P > 0.05$.

Treatments: Bird = real great tits were present in the experimental cages and warning calls were played, Call = only warning call of great tit was played, Control = only locusts were present in the cages. The dot and whiskers show means ± SE derived from the respective minimal models
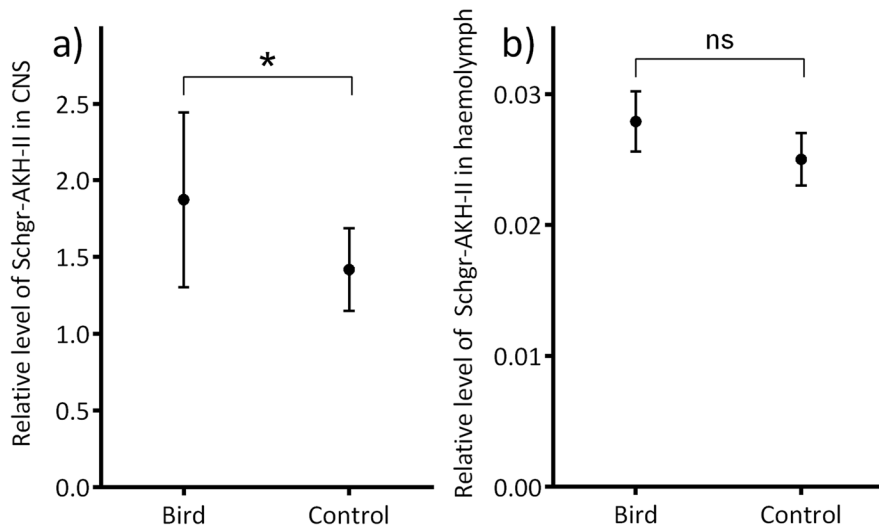

Fig. 3 The effect of treatments (Bird, Control) on the Schgr-AKH-II level in CNS **a** and hemolymph **b** of *S. gregaria* in the outdoor aviary experiments. Statistically significant differences between the groups were evaluated using the Student's t-test, and significant results are marked by * $P < 0.05$, ns $P > 0.05$.

Treatments: Bird = real great tits were present in the aviary and warning calls were played, Control = only locusts were present in the nearby shady area without birds. The dots and whiskers show means ± SE derived from the respective minimal models

based on the neurochemical analysis, on the association between the level of stress hormones and behavior in insects, although the results of the experiment

did not provide any evidence for a causal connection between AKH and behavior. It is also in line with our expectations and would mean that the stress

reaction evoked by birds might result in a lower feeding rate, slower growth, and maybe lower reproduction (Fournier et al. 2013).

Contrary to what we would expect (Minoli et al. 2012), the alarm call of great tits was not an appropriate cue to induce hormonal and behavioral change in gregarious desert locusts. The time devoted to hiding and feeding during the continuous alarm calling of great tits was not significantly different from the control treatments. There are several explanations for why this might be. Firstly, birds singing and calling are often ubiquitous in the natural environment of the locusts and so the cost of hiding permanently would be high. Secondly, the production of stress hormones is known to change the prey's behavior and prevent its predation (Adamo et al. 2013), but a permanently occurring level of stress hormones could be debilitating to the animal (Clinchy et al. 2013). Thirdly, unlike bat vocalization used to locate prey, the bird calls may not represent an immediate risk of attack (Cinel and Taylor 2019). Fourthly, it might be because desert locusts did not perceive the great tit as a potentially risky predator, but that they exhibited a stress response to silhouettes or movements due to their perception of birds as predators. This would however suggest that locusts recognize the call of bird predators but generalize the silhouette of specific birds. The additional experiments which we present in the Supplementary material (as Additional experiment IV) showed, that locusts reacted less to a warning call of kestrels than to a live bird (i.e., Great tit), while their reaction to a call of great tit and a call of kestrel did not differ. Even though kestrels might represent a more dangerous predator to locusts, the presence of an actual bird might be needed to evoke a strong stress reaction.

The final explanation may be a result of the locust's auditory capabilities. They possess a highly advanced auditory system capable of detecting a broad range of sound frequencies. Desert locusts show extreme phenotypic plasticity forming, solitary and gregarious phases, which differ extensively in behavior, and physiology including their sensory abilities, and morphology (Simpson et al. 1999; Gordon et al. 2014). In previous experiments, gregarious forms of desert locusts responded in 6 out of 12 cases to locust swarm sounds (Haskell 1957) and took evasive action after hearing ultrasound calls of hunting bats (Weber et al. 1981). Gordon et al (2014) found that

the auditory phenotypes for the two forms of locusts vary. Solitary locusts fly at night (Ould Ely et al. 2011) which might be at greater risk from predation by bats (Haskell 1957; Robert 1989) are sensitive to the higher frequencies used by bats in their echolocation calls (Gordon et al. 2014). On the other hand, gregarious locusts are more active during the day and so being able to detect birds should be important in helping them avoid predation. However, our current observation that the desert locusts did not react to bird alarm calls does not support this hypothesis. Our additional experiment which we present in Supplementary material (as Additional experiment IV) showed, that locusts reacted the same way to quiet control, as well as to the call of great tit and rattling of keys. However, their reaction to the call of kestrel was a bit stronger. This shows that they can distinguish between different types of sound but consider the call of great tit as harmless as the rattling of keys.

Existing studies have revealed that various arthropods have variable responses to different types of predator-induced stimuli. Lohrey et al. (2009) found that spiders stopped moving when a seismic or acoustic stimulus was presented, and they increased movement when they were exposed to a visual stimulus. Desert grass spider *Agelenopsis aperta* exhibited antipredator behavior to puffs of air simulating bird wing beats (Riechert and Hedrick 1990). Some crickets were shown to respond to species-specific vibrations that lizards made when walking (Adamo et al. 2013). Even the placement of a dummy predator (robotic hamster) in the terrarium resulted in changes in the behavior of crickets (Adamo et al. 2013). In another experiment, the mere presence of spiders with their mouths glued shut changed the behavior of the grasshoppers (similarly to predation treatment, where the spiders were allowed to eat them), which resulted in the grasshoppers acquiring less food and hence a decrease in their population (Schmitz et al. 1997). The indirect effects of insectivorous arthropods on arthropods have been further proved through experiments in several systems: mantids and their predation on herbivorous insects (Moran and Hurd 1997), the effect of large predatory mosquitoes on smaller mosquitoes (Chandrasegaran and Juliano 2019), the effect of Anolis lizards on Homoptera and Araneae (Spiller and Schoener 1990a; 1990b), the effect of beetle larvae on ants (Letourneau and Dyer 1998) and the effect of the sound of wasps on lepidopteran

larvae (Lee et al. 2021). Orthoptera are able to detect, through their filiform hair sensilla, the movement of air generated by predator wing beats (Gnatzy and Kämper 1990).

Birds have been rarely used as the predator in terrestrial experiments with insects, although they are one of their main predators. Signals of bird presence are visual (whole animal, part of an animal, or just its shadow), localized disturbance (e. g. vibration, movement of leaves, air movement), or different types of vocalizations. The perceived signals might be dependent on the distance of the predator to the insect or the duration of their interaction. The dimensions of our birdcage in the indoor conditions were only $0.7 \times 0.4 \times 0.5$ m, so the laboratory experiment was intense, as the locusts were in close contact with the bird, and they could see its movement. In contrast, the locusts in outdoor aviaries were not in close contact with the birds, as the birds typically moved 1—2 m away from the locusts. Pitt (1999) used 5×5 m large aviaries protecting grasshoppers from birds and observed that grasshoppers moved lower down into the vegetation, but that their mobility and feeding behavior were not affected. In a similar experiment, free-living birds were not able to access the locusts closer than 2 m, while they had free access to the locusts in the control treatment. The activity of locusts tended to be lower in the control plots; however, the pattern varied over the summer season. Similarly, to the earlier study, the locusts tended to forage deeper in the grass when birds were present (Belovsky et al. 2011).

Another crucial feature in the experiment could be the duration of exposure of the prey to the predators. Our 30-min laboratory experiment (with a predator being close) and 3 h-long outdoor experiment (with a predator) resulted in similar levels of stress hormones. We decided for the 3-h long exposition of the locust in the outdoor experiment, since we aimed to simulate more natural conditions, mimicking the continuous presence of birds and allow longer distance between birds and the locusts. We also hoped that birds will approach the locusts at least several times during the longer time, thus inducing the stress response. In general, a 30 min long exposure to predators was sufficient to detect the hormonal stress response. This would be in line with an earlier study, which found that the concentration of AKH in the hemolymph of *S. gregaria* changes and increases with

the time of exposure to the stress factor but appears as quickly as in 2 min and peaks after 60 min (Candy 2002).

The link between various components of the stress response system and behavior controlled by the nervous and endocrine systems is far from being fully understood (Storey 2004; Johnstone et al. 2012). Nevertheless, in arthropods, biogenic amines and certain neurohormones seem to play a crucial role in the control of the stress response (Kodrik 2008; Nelson et al. 2021). It is obvious that these amines are involved directly in the defense reactions, e.g., octopamine modulates anti-predator behavior in beetles (*Tribolium castaneum*; Nishi et al. 2010), and in orb-weaving spiders (Jones et al. 2011). On the other hand, biogenic amines are thought to be involved in the regulation of AKH production (Van der Horst et al. 2001) or the AKH release from the corpora cardiaca into the hemolymph (Orchard et al. 1993). There are many examples in the literature describing fluctuations in AKH levels in the insect CNS and hemolymph after a stress factor. For example, 20 min of forced running (in a horizontal laboratory shaker) resulted in a slight increase of AKH titer in the CNS of the firebug (*Pyrrhocoris apterus*), and a strong increase of the titer in the hemolymph (Kodrík and Socha 2005). A similar picture can be seen when exposing insects to various pathogens (Ibrahim et al. 2017, 2018; Gautam et al. 2020a, 2020b) or toxins including insecticides (Candy 2002; Kodrík et al. 2015). Changes in the AKH levels in the hemolymph are often faster, while in the CNS they are slower but more profound. Nevertheless, in this study, all changes in Schgr-AKH-II titer after the bird treatment were similar except for no change in Schgr-AKH-II in the hemolymph within the outdoor experiment. To explain this, we can only speculate that due to the large distance of the locusts from the birds, the stress was not strong enough, as mentioned above. The lack of the effect could be due to the 2-h delay in the measurement (in contrast to the other experiments), as the hormone levels may have declined. However, in this experiment, we wanted to simulate more natural conditions where birds would be further away but continuously present. Future experiments should focus on the dynamics of the development of stress responses in time. In conclusion, we report evidence that insects in the presence of birds decreased their foraging activity and reduced their feeding time roughly by 60%. We relate the

results of our behavioral tests to the insect's physiological stress response. Our experimental results are in line with the risk allocation hypothesis (Lima and Dill 1990; Lima and Bednekoff 1999) predicting that animals should increase their foraging effort in the low-risk environment and decrease foraging in high-risk environments. We argue that the methodological approach combining behavioral experimentation with the physiological assessment of animal responses provides crucial insight into the perception of predators by insect prey and for understanding their fitness optimization strategies. Lastly, future tests should focus on the effect of bird calls on the behavior of other insect species to expand on the knowledge we have gained from this study.

Authors Contributions Jan Kollross – conceptualization, experimental investigation, writing of the first draft, visualization; Jitka Jancuchova-Laskova- experimental investigation, writing of the first draft together with JK; Irena Kleckova – methodology, experimental investigation; Inga Freiberga— experimental investigation; Dalibor Kodrik – conceptualization, laboratory analyses of the physiological responses; Katerina Sam—supervision, conceptualization, methodology, formal analysis, resources & funding acquisition. All authors contributed equally to the editing of the manuscript.

Data Availability The data that support the findings of this study are available upon reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

Conflict of Interest None.

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